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Hans U. Lutz *Editor*

Naturally Occurring Antibodies (NAbs)

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Volume 750
NATURALLY OCCURRING ANTIBODIES (NAbs)
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Naturally Occurring Antibodies (NAbs)

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DEDICATION

Dedicated to my wife, Ivanka Lutz, for supporting my efforts in editing this book

PREFACE

Welcome to a subject that is new for some and barely understood by others: **naturally occurring antibodies**. The term "naturally occurring (auto)antibodies" (NAbs) stands for physiological antibodies, or autoantibodies generated in healthy humans and/or vertebrates, in contrast to those that are induced by exogenous antigens. In the years passed, some authors in the field used instead the term "natural (auto)antibodies". Though "induced" and "naturally occurring" antibodies differ in many aspects from each other, both are "natural" in the sense that they are produced by nature. Therefore, other authors have coined the term "(auto)antibodies produced constitutively", implying that such (auto)antibodies are produced without stimulation by exogenous antigen molecules. For simplicity, we use here the term "naturally occurring (auto)antibodies", abbreviated as NAbs.

The existence of NAbs in healthy beings contrasted with the immunologic dogma, formulated in 1959 by Burnet, according to whom "auto-reactive cell clones do not exist in healthy beings, because they are deleted in ontogeny." During the next 30 years or so, a few hundred scientists in different countries nevertheless dared to continue to investigate NAbs and their unique properties, so to say in the backyard of immunology. These scientists learned that upon investigation NAbs have close to germline immunoglobulin chain sequences and their production does not require induction by exogenous antigens. It is certain that NAbs have developed over millions of years. NAbs are produced spontaneously and some can be upregulated by the presence of autoantigens (rather than induced).

In order to give the reader an idea of the fascinating potential of NAbs, this volume illustrates first the functional properties of NAbs. Authors from pioneering groups report in their chapters on the tissue homeostatic, tissue regenerating and regulatory properties of NAbs and NAbs in pooled human IgG (Chapters 1-4, 6-7 discuss these findings). They found for the first time that many NAbs are involved in the clearance of senescent, apoptotic and oxidatively damaged cells, as well as tumor cells. Another group of researchers (Chapter 5) has been interested in how NAbs exert their effects and has found that some NAbs gain functionality not only by binding to their antigens but by modifying bound antigens enzymatically. Others (Chapter 8) discovered NAbs that have a protective effect against viral and bacterial infections (first line defense), or that have a disease-inhibiting potential in so far uncurable diseases, as it is emerging for multiple sclerosis and Alzheimer's disease (Chapters 4 and 7).

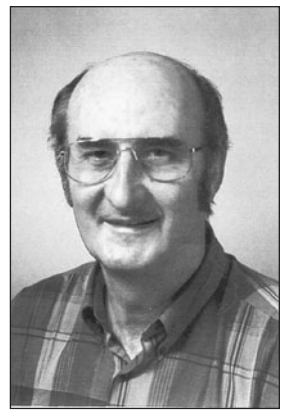
Scientists interested in the regulation and modulation of components of the immune system found a whole variety of NAbs to cytokines with regulatory and protective functions and NAbs that modulate, e.g., dendritic cells, regulatory T cells, B cells and granulocytes (Chapters 9-12). Two chapters (13,14) report that NAbs with beneficial roles can become initiators of disease following the destruction of biological compartments or following proteolytic modification of NAbs.

Thorough studies of the unique properties of NAbs (Chapters 15-17) have increased our understanding of their stepwise generation in ontogeny and the phenomenon of polyspecificity. Many studies on NAbs have been carried out on pooled human IgG and later on IVIG (immunoglobulin for intravenous application) prepared from human IgG. Considering the large plasma pools and initial difficulties in preparing IVIG that does not induce adverse effects upon infusion into recipients, this volume ends with a historical chapter on how pooled human plasma was fractionated and the IgG component pretreated for a safe intravenous application (Chapter 18). The first positive effects of immunoglobulin molecules in IVIG were observed in 1981 in treating patients with an iatrogenic IgG deficiency and secondary ITP. This result called for similar IVIG treatments of other autoimmune diseases, as reported later in numerous publications. The clinical success combined with the low adverse event profile of IVIG therapies stimulated the interest in NAbs, the potential mediators of the beneficial effects of IVIG.

My thanks go to the contributing authors for the effort and enthusiasm they have devoted to their subject and to this task. Along with my coauthors we thank Landes Bioscience for having offered the opportunity to compose this work and provide the first open platform on NAbs. We hope to have triggered your interest.

Hans U. Lutz
Zurich, Switzerland

ABOUT THE EDITOR...



HANS U. LUTZ, born May 5, 1942 is a Swiss citizen. He completed his studies in microbiology and biochemistry with a PhD at the University of Zurich in 1971 and received "summa cum laude" for the exam and his dissertation on photophosphorylation in chromatophores of *Rhodospirillum rubrum* in the group of Prof. R. Bachofen. During his postdoctoral period he got familiar with the characterization of artificial membranes (Prof. P. Läuger, University of Konstanze, Germany) and with membrane biochemistry at the Worcester Foundation for Experimental Biology, Shrewsbury, USA, in collaborating with Prof. J. Palek and G. Fairbanks. His interest in red blood cell (RBC) membrane biochemistry was the basis for the discovery that ATP-depletion of RBC results in the release of hemoglobin-filled vesicles that lack the cytoskeleton. In returning to Switzerland in 1976 he joined the Institute of Biochemistry at the Swiss Federal Institute of Technology (ETH), where he eventually had a tenure position until his retirement, some teaching duties in biochemistry and immunology (complement), for some years extra responsibilities in the research commission of the ETH, the European group for red cell membrane research, the Board of the European complement network, and throughout the time he had the freedom of an investigator to get grant money and bright PhD students. He developed his own research field related to RBC aging. In the late 70s and early 80s he isolated and described the first IgG naturally occurring antibodies (NAbs) to red blood cell membrane proteins (to spectrin and to band 3 protein) and presented evidence for their role in tissue homeostasis. The low affinity IgG anti-band 3 NAbs directed to the anion transport protein bind bivalently to oligomerized band 3 protein as generated

during erythrocyte aging and oxidative damage. In trying to understand how such low titer, low affinity NAbs can effectively opsonize erythrocytes, he found with his group that immune-complexed anti-band 3 NAbs preferentially capture dimeric C3b because these NAbs have a rare affinity for C3 within the Fab portion. Indeed, artificially generated $C3b_2$-IgG complexes stimulated complement amplification 750 times better than C3b. In extending these findings his group discovered that dimeric C3b deposits best to any immune complex formed from $F(ab')_2$ because the lack of the Fc portion facilitates deposition of dimeric C3b, but dimeric C3b deposits only if the $F(ab')_2$-IC is rigidified by bound IgG anti-hinge NAbs. Thus, anti-hinge NAbs that normally downregulate antibody production by B cells (Terness et al) contribute to initiation of a systemic inflammatory reaction when proteases released from neutrophils and pathogens (primarily elastase) cleave IgG molecules into $F(ab')_2$ fragments. He could verify with his group and clinicians that at the onset of a systemic inflammatory response in sepsis stimulation of complement amplification (factor Bb) is proportional to $F(ab')_2$ production and both parameters depend linearly on liberated elastase.

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SECTION I

NAbs IN TISSUE REPAIR AND TISSUE HOMEOSTASIS

CHAPTER 1

NATURALLY OCCURRING IgM ANTIBODIES TO OXIDATION-SPECIFIC EPITOPES

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Abstract: Naturally occurring antibodies (NAbs) have specificity for both microbial and self antigens, which allows them to act in the first line defense against invading pathogens, as well as in tissue homeostasis by mediating the clearance of cellular debris. This latter recognition of self by NAbs was often thought to reflect the polyreactivity of low affinity antibodies. The finding that oxidation-specific epitopes are dominant targets of naturally occurring IgM antibodies shed light on this and provided novel insights into the understanding of the house keeping functions of NAbs. Oxidation-specific epitopes represent stress-induced or altered self structures that are generated as a consequence of lipid peroxidation during many physiological and pathological situations. Importantly, the same structures have been found in the membranes of dying cells. Only oxidized lipids and dying cells—but not native membrane lipids or viable cells—are recognized by this set of NAbs. Thus, oxidation-specific epitopes represent ideal marks that identify biological waste for its clearance and the neutralization of its pro-inflammatory properties. Furthermore, this binding property of NAbs has also important implications for various chronic inflammatory diseases, including atherosclerosis.

INTRODUCTION

House Keeping Functions of Naturally Occurring Antibodies (NAbs)

NAbs are typically characterized by variable regions that are encoded by germline V_H and V_L genes with no or very few mutations. Therefore, they exhibit a restricted and stable repertoire of binding specificities that is thought to be a product of natural

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selection. This provides NAbs with broad reactivity for phylogenetically conserved structures of nucleic acids, (glyco)proteins, and (phospho)lipids,[1,2] which allows them to bind microbial antigens and mediate important non-redundant functions in the first line defense against invading pathogens, such as bacteria and viruses.[3] In addition to their capacity to bind microbial structures, NAbs also recognize self structures and therefore have frequently been considered to be autoreactive.[4] However, in many cases these "self structures" represent altered or stress-induced neo-self structures that are generated during various biological processes. This subtle binding property allows NAbs to selectively recognize and identify body waste, most prominently dying cells, which contain a variety of eat-me tags that are bound by NAbs.[5] Thereby, NAbs mediate critical homeostatic house-keeping functions by facilitating the clearance of damaged cells and cellular debris. This is important because excessive accumulation of cellular debris activates adaptive immune responses against self-constituents and triggers autoimmunity, such as systemic lupus erythematosus. Indeed, lupus-prone lymphoproliferative (lpr) mice develop more severe autoimmunity when bred with mice deficient in serum IgM (sIgM), which represent to large extent IgM NAbs.[6] Moreover, an increased accumulation of body waste may also lead to direct activation of inflammatory reactions that could trigger and/or propagate a variety of pathologies. In fact, a number of structurally altered self-components are recognized as "danger signals" by innate immune receptors.[7] Thus, in addition to their role in antimicrobial host defense, NAbs have another important and non-redundant function in mediating house keeping functions.

OXIDATION-SPECIFIC EPITOPES AS "DANGER ASSOCIATED MOLECULAR PATTERNS"

In recent years it has become increasingly clear that innate immune responses have also the capacity to recognize and respond to the presence of endogenous molecular structures, which in analogy to microbial pathogen associated molecular patterns (PAMPs) are commonly termed danger associated molecular patterns (DAMPs). There is growing evidence that oxidized lipids and the derivatives thereof represent prominent examples of such endogenous "danger signals.[8]" Both cellular membranes and lipoprotein particles contain various lipid species that can easily become oxidized by multiple mechanisms under physiological and pathological conditions, in which lipid peroxidation occurs. As a consequence highly reactive lipid products are generated, which in turn can covalently attach to and modify autologous molecules such as proteins, lipids, and even DNA. These newly formed structures represent antigenic neo-self epitopes that have been termed "oxidation-specific" epitopes, and have been shown to be recognized by a whole array of innate immune responses (recently reviewed in ref. 8). Accordingly, many products of lipid peroxidation have also been shown to possess robust pro-inflammatory properties in vitro and in vivo. In fact, common oxidation products such as malondialdehyde (MDA)—a classical biomarker of increased oxidative stress—have been documented in tissues of various inflammatory conditions. These include atherosclerosis, acute lung injury, renal and liver diseases, as well as multiple sclerosis and Alzheimer's disease among others (for references see ref. 9).

Of greatest importance, however, is the finding that cells undergoing apoptosis—but not viable cells—also accumulate oxidation-specific epitopes in their membranes.[10,11]

Thus, oxidation-specific epitopes are perfect tags for the discrimination of dying cells from viable cells and the identification of biological waste by NAbs.[12]

OXIDATION-SPECIFIC EPITOPES ARE BOUND BY SPECIFIC ANTIBODIES

The discovery that lipid-peroxidation leads to the generation of novel structures that are recognized by specific antibodies arose from the serendipitous observation by Witztum et al. that even very subtle modifications such as non-enzymatic glycation of autologous low density lipoprotein (LDL) resulted in its rapid clearance when injected intravenously into diabetic subjects.[13] Subsequently, it was shown that this was due to immune recognition of modified LDL and that a variety of modifications can generate neo-self determinants that render LDL immunogenic.[14] Interestingly, active immunization with differently modified antigens induced antibodies that were shown to recognize the same modification also when it was present on a different protein backbone than the immunizing agent, indicating that the induced responses were hapten-specific. In analogy to these findings, Witztum and colleagues later demonstrated that breakdown products that are formed during the oxidation of LDL could generate similar immunogenic modifications on oxidized LDL (OxLDL).[15-17] OxLDL has a central role in the pathogenesis of atherosclerosis.[18] It is generated in the process of atherosclerotic lesion formation, where it has been shown to accumulate in the vascular wall. Multiple enzymatic and non-enzymatic oxidative processes have been identified that promote the oxidation of several constituents of LDL, such as phospholipids.[19] For example, the major phospholipid of LDL, phosphatidylcholine, contains an oxidation-prone polyunsaturated fatty acid in the sn-2 position, which upon oxidation among others gives rise to highly reactive breakdown products such as malondialdehyde (MDA) with its many complex condensation products, and 4-hydroxynonenal (4-HNE).[20] These reactive aldehydes can form covalent adducts with ε-amino groups of lysine residues in ApoB (the major protein of LDL) or any other protein, as well as amino groups of phospholipids, such as phosphatidylethanolamine, and give rise to newly formed adducts. Moreover, the residual oxidized phospholipid backbone 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphatidylcholine (POVPC) with the truncated fatty acid in position 2 can also form adducts, which in this case consist of the entire oxidized phospholipids. Because any phospholipid containing a polyunsaturated fatty acid is susceptible to oxidation, generation of these antigenic neo-epitopes can also occur on cellular membranes, e.g. of cells undergoing apoptosis. Immunization with oxidatively modified LDL was found to give rise to specific IgG and IgM antibodies, and these modifications have also been shown to be recognized by antibodies in a hapten-specific manner.[15,16] Moreover, using model antigens such as Cu^{2+}-oxidized LDL and MDA-modified LDL (MDA-LDL), the presence of autoantibodies with specificity for oxidation-epitopes could be documented in the sera of animal models of atherosclerosis and humans.[15,21] Interestingly, IgM Abs against oxidation-specific epitopes are present in the sera of naïve, non-immunized mice and their titers are greatly increased in atherosclerotic $LDLR^{-/-}$ or $ApoE^{-/-}$ mice.[21,22] Moreover, we have shown that even completely germ-free mice have IgM antibodies to these same epitopes in their serum, suggesting that most of these IgM Abs are in fact IgM NAbs.[23]

OXIDATION-SPECIFIC EPITOPES ARE PROMINENT TARGETS OF IgM NAbs

The fact that atherosclerotic mice have high titers of antibodies against OxLDL enabled Dr. Witztum and colleagues to clone a large set of monoclonal antibodies (mAbs) with specificity for OxLDL from the spleens of non-immunized ApoE$^{-/-}$ mice that had received an atherogenic diet.[24] Surprisingly, all mAbs—termed EO—were found to be of the IgM class. The subsequent characterization of a prototypic clone with specificity for Cu^{2+}-oxidized LDL, called EO6, revealed that it specifically recognized the phosphocholine-headgroup (PC) of oxidized, but not native phosphatidylcholine.[25] EO6 was also found to bind the surface of apoptotic, but not viable cells,[10] which was consistent with the later mass spectrometrical demonstration of an increased content of oxidized phospholipids in membranes of apoptotic cells.[11] A detailed analysis of the variable region of EO6 then revealed that both the V_H and V_L sequence were 100% germline-encoded.[26] Moreover, the same germline gene usage has been reported for a prototypic NAb, called T15,[27] which has been studied extensively for its capacity to bind PC covalently linked to (lipo)teichoic acid of the capsular polysaccharide of *S. pneumonia*. In fact, T15 is known to provide optimal protection from pneumococcal infections in mice.[28] The fact that T15/EO6 IgM mAbs bind both microbial antigens as well as neo-self epitopes elegantly demonstrates the dual reactivity that has been discussed as a property of many if not most IgM NAbs.[29]

In addition to PC of oxidized phospholipids a whole range of other lipid peroxidation-derived structures are generated; many of which could serve as oxidation-specific epitopes for NAbs. Indeed, other germline encoded IgM Abs against OxLDL could be isolated, using an analogous approach to the isolation of EO hybridomas. For example, LRO1 has been cloned from the spleens of cholesterol-fed LDLR$^{-/-}$ mice, and it was found to specifically bind an epitope of oxidized—but not native—cardiolipin.[30] Similar to EO6, LRO1 was also shown to bind apoptotic cells. Moreover, we found that sera of germ-free mice contain IgM NAbs against yet other oxidation-epitopes, including MDA-derived and 4-HNE-derived adducts, suggesting the existence of IgM NAbs with these specificities as well.[23] Of note, the antibody titers to some but not all of these epitopes increased when germ-free mice were colonized with commensal gut bacteria, which further supports the notion of a dual reactivity for microbial antigens and oxidation-epitopes as equivalent self-structures, and demonstrates the capacity to upregulate IgM NAbs with such specificities.

All these insights and the examples of EO6 and LRO1 suggested that oxidation-specific epitopes in general could represent targets for a whole set of IgM NAbs. Thus, many more clones with reactivity for other oxidation-derived structures may exist. We therefore formally tested this hypothesis by studying the repertoire of IgM Abs derived from B-1 cells, which are the major source of IgM NAbs in mice.[23] To do this, B-1 cells were isolated from naïve mice and cultured with various toll-like receptor agonists and IL-5 to induce plasma cell differentiation and IgM secretion. These in vitro stimulations resulted in the production of IgM Abs against Cu^{2+}-oxidized LDL, 4-HNE-modified LDL, and most prominently MDA-LDL, among others. Interestingly, the levels of IgM that specifically bound MDA-LDL were substantially higher than those against α1,3-Dextran, which is a prototypic microbial B-1 cell antigen. In a subsequent approach we selectively reconstituted RAG1$^{-/-}$ mice with B-1 cells from naïve donors to generate a mouse model that only expresses B-1 cell derived IgM NAbs. Plasma from these mice contained primarily IgM

NAbs, which included antibodies against a whole panel of oxidation-specific epitopes. Moreover, antigen absorption studies with plasma of reconstituted mice showed that as much as 30% of all plasma IgM had specificity for various oxidation-epitopes. Among these IgM NAbs the titers to MDA-type adducts were the most prominent ones, which is consistent with the data obtained from in vitro stimulated B-1 cells. In fact, ELISpot analyses of the spleens of these as well as naïve wild type mice revealed that on average 12% of all IgM-secreting cells (ISC) had specificity for MDA-LDL. The existence of an IgM NAb with specificity for the highly abundant oxidation-epitope MDA was further confirmed when we obtained hybridomas using splenocytes of B-1 cell reconstituted mice and cloned a set of monoclonal Abs against MDA-LDL. One exemplary clone, termed NA17, was analyzed in more detail and was found to have complete germline gene usage of the V_H rearrangement and only one nucleotide insertion (C) at the splice site of the V_L and J_L germline gene segments. Additional MDA-specific IgM clones with complete germline gene usage in their variable regions have been identified since then (K. Hartvigsen and J.L. Witztum, personal communication). Therefore, oxidation-specific epitopes are prominent targets of IgM NAbs in mice. To demonstrate that this observation is not only true for the murine system, we characterized the specificity of IgM Abs in umbilical cord blood.[23] Unlike IgG, IgM antibodies in human cord blood are exclusively derived from the infant and thus a good surrogate for NAbs.[31] In support of our murine data, IgM Abs in human umbilical cord blood contained specificities for MDA-LDL and Cu^{2+}-oxidized LDL. Moreover, when these specific titers were corrected for the reduced total IgM levels in cord blood compared with matched maternal blood samples, it could be shown that oxidation-epitope-specific IgM antibodies were actually enriched in the umbilical cord blood samples.

Therefore, multiple lines of evidence show that oxidation-epitopes are major targets of IgM NAbs in mice and humans. It is unlikely that this newly found property of NAbs simply reflects the polyreactivity that has been attributed to them,[32] because studies with monoclonal IgM NAbs show great specificity for one, but not the other oxidation-specific epitope tested. For example, the PC-specific IgM T15/EO6 does not bind MDA-epitopes, while the MDA-specific NA17 does not bind PC-epitopes (Fig. 1). Both epitopes can be found on the surface of apoptotic cells[10] (Fig. 2). It seems that the prominent representation of IgM with specificities for oxidation-epitopes reflects the ubiquitous presence of these structures, which in some cases have been shown to possess robust pro-inflammatory activities. Thus, there is a great demand for mechanisms that prevent and/or protect from their accumulation in the organism. The pentameric nature of IgM molecules should further improve this activity in vivo, as oxidation-specific epitopes are typically repetitive in nature.

FUNCTIONAL ROLE OF IgM NAbs WITH SPECIFICITY FOR OXIDATION-EPITOPES

Given the fact that oxidation-specific epitopes are prominently found on the surface of dying cells, it can be assumed that NAbs specific for these structures mediate important house keeping functions through the recognition of dying cells and cellular debris. Indeed, monoclonal IgM Abs, such as EO6, EO14, LRO1, and NA17 that were all cloned for their ability to bind epitopes of OxLDL, also strongly stain the surface of apoptotic cells.[10,23,30] Similarily, IgM antibodies in human umbilical cord blood bind the

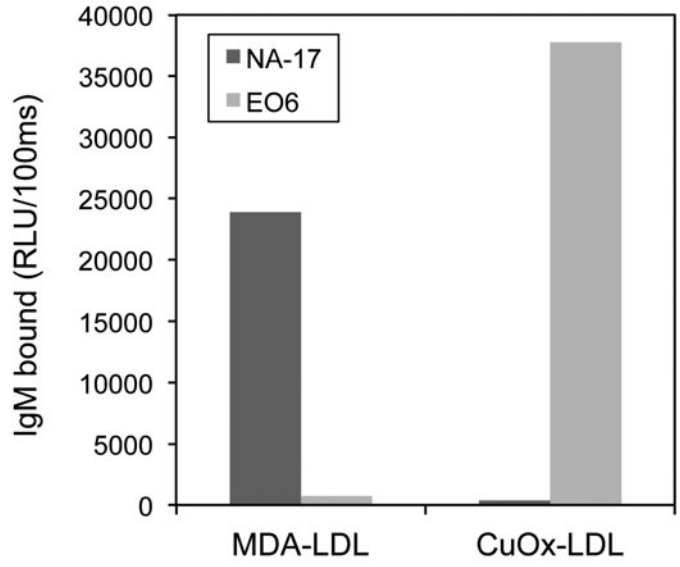

Figure 1. IgM NAbs against oxidation-specific epitopes have defined specificities. Monoclonal antibodies, NA-17 and EO6, having germline configuration have been studied for their binding to LDL modified by either MDA or $Cu2^{+}$-mediated oxidation. Shown is an ELISA of IgM binding to the coated antigens MDA-LDL and CuOx-LDL, which is enriched in PC-containing oxidized phospholipids. NA-17 binds MDA-LDL, but not CuOx-LDL. In contrast, EO6 binds CuOx-LDL, but not MDA-LDL. However, both mAbs derived from IgM NAbs bind apoptotic cell, which carry many different oxidation-sepcific epitopes (see refs. 23 and 26).

surface of apoptotic cells and approximately 50% of this binding could be competed by soluble MDA-BSA,[23] suggesting that the binding of IgM NAbs is to a large extent mediated by the recognition of MDA-epitopes. This notion is further supported by the fact that immunization of mice with syngeneic apoptotic cells resulted in high IgM titers against epitopes of OxLDL, and that a majority of the induced antibodies that bound apoptotic cells had specificity for OxLDL.[11] Thus, oxidation-epitopes are major neo-determinants on apoptotic cells.

A non-redundant role of IgM in the clearance of apoptotic cells has been established by the observation that mice deficient in secreted IgM (sIgM), which lack NAbs, show an increased propensity to develop autoimmune disease when crossed with lupus prone mice.[6] In line with this, it could be specifically shown that purified preparations of $T15^{id+}$ IgM were able to normalize the impaired in vivo clearance of apoptotic cells in sIgM-deficient and B-cell deficient mice, respectively.[33,34] In an analogous experiment we could show that the MDA-specific naturally occurring IgM NA17 also significantly increased the macrophage-mediated in vivo clearance of apoptotic cells that were injected intraperitoneally into $RAG1^{-/-}$ mice.[23] In contrast, equal amounts of the KLH-specific control IgM, which does not bind apoptotic cells, did not have an effect. Thus, NAbs specific for oxidation-epitopes provide an important homeostatic house keeping function by promoting the C1q-dependent clearance of dying cells by macrophages (see also Chapter 2 by Elkon and Silverman).

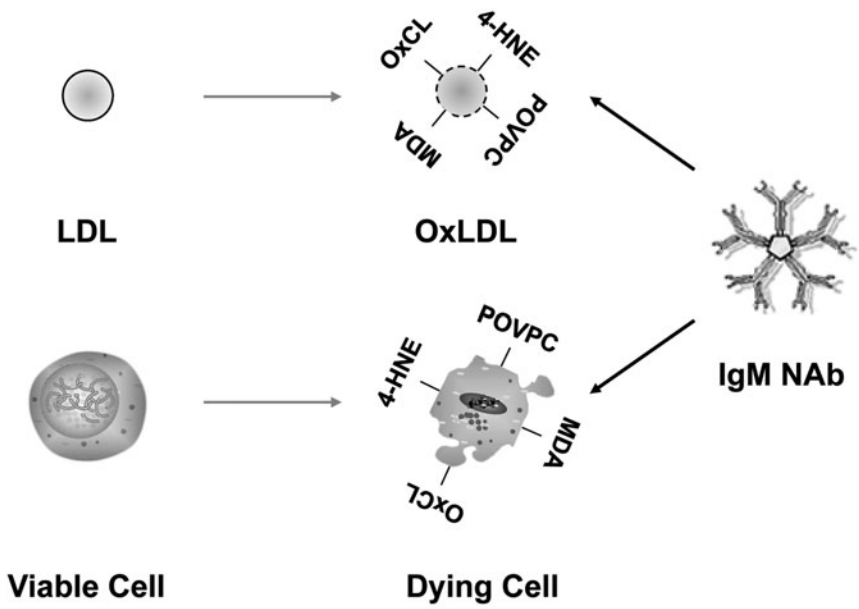

Figure 2. IgM NAbs bind OxLDL and dying cells via the same oxidation-specific epitopes. Oxidation-specific epitopes are found on the surface of OxLDL and dying cells, but not viable cells and native LDL, respectively. MDA = Malondialdehyde-derived adducts; POVPC = 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphatidylcholine; 4-HNE = 4-hydroxynonenal-derived adducts; OxCL = Oxidized cardiolipin.

On the other hand, IgM NAbs against epitopes of OxLDL have also been shown to inhibit the uptake of apoptotic cells by macrophages in the presence of heat inactivated serum,[10] suggesting inhibition of complement-independent uptake mechanisms. Therefore, this subset of IgM NAbs could potentially prevent the recognition of apoptotic cells by other cellular receptors that induce inflammatory responses. Indeed, in addition to their role in the complement-dependent clearance and removal of apoptotic cells, NAbs with specificity for oxidation-epitopes also possess an important function by preventing inflammatory reactions induced by the same oxidized lipids that they recognize. This has been demonstrated by assessing the capacity of apoptotic cells and their blebs, which carry oxidation-specific epitopes, to induce endothelial cell activation as determined by monocyte adhesion.[11,35] This pro-inflammatory activity of apoptotic cells was fully abolished in the presence of T15/EO6 IgM antibodies, while T15/EO6 had no effect on the LPS-induced activation of endothelial cells. In addition, T15/EO6 IgM also blocked the activity of BSA-conjugated oxidized phosphatidylcholine to induce the secretion of IL-6 by macrophages in vitro.[36] Thus, the recognition of oxidation-specific epitopes does not only allow NAbs to identify cellular debris for clearance, but it also allows NAbs to directly prevent inflammatory reactions that could result from an excessive accumulation of dying cells. Both functions of this set of NAbs are likely to be critical in vivo, especially under pathological situations of increased oxidative stress. Additional anti-inflammatory activities of $T15^{id+}$ IgM are discussed elsewhere in this book (see also Chapter 2 by Elkon and Silverman).

NATURALLY OCCURRING IgM ANTIBODIES IN ATHEROSCLEROSIS

Increased oxidative stress is associated with many diseases, and therefore the body's need to respond to the consequences thereof is of great importance. Atherosclerosis is a prototypic disease in which increased oxidative stress and lipid peroxidation are critical pathogenic components. It is a chronic inflammatory disease of the vessel walls, the underlying cause for heart attacks and a majority of strokes. It is now well established that in addition to dyslipidemia, inflammatory processes are equally involved in its pathogenesis.[37] Moreover, both innate and adaptive immunity have been found to profoundly modulate atherogenesis.[38,39] In this regard, Lewis and colleagues demonstrated recently a significant role for IgM antibodies in murine atherosclerosis.[40] They could show that atherosclerosis-prone LDLR$^{-/-}$ mice have significantly accelerated plaque formation when they were also deficient for secreted IgM. Because most IgM in uninfected mice are NAbs,[1] these data suggest a protective role for IgM NAbs in atherosclerosis.

Our observation that more than 30% of all naturally occurring IgM antibodies are directed against oxidation-epitopes, suggests that a major part of the protective effect is mediated by this specific subset of NAbs, because products of lipid peroxidation are key mediators of the atherosclerotic disease process. Indeed, all oxidation-specific epitopes that are recognized by NAbs have also been documented inside atherosclerotic lesions. One specific example is PC of oxidized phospholipids, which is recognized by the natural IgM T15/EO6.[26] We could show earlier that immunization of LDLR$^{-/-}$ mice with heat-killed PC-containing pneumococcal extracts (R36a) gave rise to robust IgM titers against OxLDL, reflecting the molecular mimicry between PC of *S. pneumonia* and the PC of oxidized phospholipids of OxLDL.[41] Further characterization of these thymus independent Type 2 (TI-2) antibody responses, which typically occur as a result of multivalent cross-linking of the B-cell receptor in the absence of cognate T-cell help,[42] revealed that the increased anti-OxLDL titers were predominantly of the T15/EO6 clonotype. Importantly, this boosting of T15/EO6 plasma levels significantly decreased atherosclerotic lesion formation in LDLR$^{-/-}$ mice when fed an atherogenic diet. In a subsequent study Faria-Neto et al. confirmed the atheroprotective role of T15/EO6 IgM by demonstrating that administration of purified T15^{id+} IgM significantly delayed lesion formation in a vein graft atherosclerosis model.[43] Therefore, the naturally occurring IgM T15/EO6 mediates a beneficial effect in the chronic inflammatory disease of atherosclerosis. The protective mechanism of T15/EO6 is likely mediated by its recognition of OxLDL and apopotic cells, both of which accumulate in atherosclerotic lesions. For example, it could be shown that purified T15/EO6 as well as plasma from *R36a*-immunized mice have the capacity to block the binding and uptake of OxLDL by macrophages.[25,41] This in turn results in decreased scavenger receptor mediated foam cell formation in vivo and limits the pro-inflammatory activities of OxLDL. Moreover, because the accumulation and impaired clearance of apoptotic cells has been implicated in the development of atherosclerotic lesions,[44] T15/EO6 IgM may also limit atherogenesis by aiding the clearance of apoptotic cells that contain oxidized phospholipids in their membranes. Whether NAbs could also promote the clearance of circulating OxLDL is still under debate. In this regard Reardon et al. did not observe any difference in the clearance rate of injected LDL or OxLDL between immune-competent ApoE$^{-/-}$ mice and immune-deficient ApoE$^{-/-}$RAG$^{-/-}$ mice, which lack antibodies.[45]

NATURALLY OCCURRING IgM ANTIBODIES AS BIOMARKERS OF CARDIOVASCULAR DISEASE

The initial demonstration of autoantibodies to OxLDL in humans[15,46] has elicited a whole array of epidemiological studies that aimed at identifying a clinical diagnostic benefit of measuring anti-OxLDL antibody titers to predict cardiovascular disease (CVD). Indeed, a number of studies could demonstrate a significant correlation of autoantibody titers to OxLDL with surrogate markers of CVD or even clinical events.[47] However, other studies did not find anti-OxLDL antibodies to be independent predictors of CVD. Only recently stronger emphasis was put on the analyses of different isotypes in the antibody response to OxLDL in humans, and these studies revealed that serum levels of IgM—but not IgG antibodies- to epitopes of OxLDL show an inverse association with markers of CVD, such as intima-media thickness of carotid arteries[48] or stenosis of coronary arteries,[49] respectively. Because a large part of IgM in uninfected individuals are considered NAbs, these insights suggest a potential role of NAbs in human CVD as well. Clearly, the simple measurement of antibody titers to models of OxLDL, which carries multiple different epitopes, does not identify them as true NAbs in the sense of germline encoded antibodies. Nevertheless, in analogy to naturally occurring T15/EO6 IgM, IgM antibodies with specificity for PC exist in humans as well. For instance, we could show a significant correlation between IgM antibodies to Cu^{2+}-oxidized LDL and PC-containing capsular polysaccharides in patients with known pneumococcal pneumonia.[41] Moreover, significant IgM titers to PC-BSA conjugates have been documented in selected patient populations with increased CVD risk, and low anti-PC IgM levels were suggested to be an independent risk factor.[50-52] It remains to be shown that these specific antibodies really represent NAbs. Nonetheless, the emerging evidence suggesting a beneficial role for NAbs in human atherosclerosis is intriguing. To fully establish this, future studies need to focus on identifying methods that allow the selective determination of IgM NAb levels with specificity for oxidation-epitopes in humans.

CONCLUSION

In summary, the finding that oxidation-specific epitopes are dominant targets of IgM NAbs identified a novel mechanism by which NAbs distinguish body waste from healthy tissues. Moreover, the characterization of oxidation-epitopes as stress-induced self structures provides important insights into the non redundant house-keeping functions of NAbs. The ubiquitous presence of oxidation-epitopes on the surface of dying cells and in various inflammatory conditions identifies them as common targets in many different physiological and pathological situations. Likely, the continuous generation of these epitopes throughout life has contributed to the positive selection of NAbs with respective specificities. NAbs with specificity for oxidation-epitopes therefore may represent a generalized defense mechanism against the consequences of oxidative stress, including chronic inflammatory diseases such as atherosclerosis.

The exact mechanisms by which this set of NAbs mediates its beneficial functions in vivo will clearly be a major focus of future studies. It is also not known whether the entire repertoire of oxidation-epitope specific IgM is needed for optimal house keeping functions, or whether isolated NAbs with selected specificities alone can mediate protective effects. A better insight into these questions will lay the foundation for the development of novel NAb-based therapies for atherosclerosis and other chronic inflammatory diseases.

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CHAPTER 2

NATURALLY OCCURRING AUTOANTIBODIES TO APOPTOTIC CELLS

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Abstract: Subsets of IgM naturally occurring autoantibodies (NAbs) bind to the cell surface membranes of dying cells. The antibodies predominantly have specificities against lipid antigens or oxidized lipids. Chief among these lipid antigens are phosphorylcholine (PC) and malondialdehyde (MDA). Antibodies to negatively charged phospholipids such as phosphatidylserine (PS) have been described and there is controversy as to whether these antibodies are related to anticardiolipin antibodies observed in disease states. IgM NAbs that bind to apoptotic cells recruit classical complement pathway components and facilitate phagocytosis by both macrophages and dendritic cells, and may block inflammatory pathways. Under these circumstances, pathologic immune responses to self (autoimmunity) are avoided, whereas mice lacking serum IgM develop a lupus-like disease with associated IgG autoantibody responses. Based on these observations, IgM anti-PC NAbs were found to attenuate inflammation in mouse models of arthritis. IgM NAbs antibodies therefore appear to play pivotal roles in the dampening inflammation and maintenance of tolerance.

INTRODUCTION

Naturally Occurring Autoantibodies: Nature and Specificities

Numerous studies in the past have suggested that healthy individuals have naturally occurring autoantibodies (NAbs) that recognize self-antigens in their bodies.[1] In mice, reactivity to self appears to be more prominent with IgM antibodies produced by $CD5^+$ B-1a lymphocytes that are a distinct population of mature B lymphocytes. B-1 cells are

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

most abundant in the peritoneal and pleural cavities and are believed to be self-replenishing, while they primarily traffic to the spleen to produce the IgM that contributes more than 50% of the circulating IgM.[1-4] While B-1 cells have also been shown to contribute to defense against infection,[5,6] recent studies indicate that some B-1 cell derived IgM NAbs enhance the efficiency of removal of senescent, damaged as well as dead and dying cells. Rapid removal of these cells by macrophages prevents the release of self-antigens that are potential ligands for pro-inflammatory innate immune receptors, such as Toll-like receptors (TLRs). In addition, macrophage interactions with apoptotic cells can positively suppress inflammatory responses (reviewed in ref. 7), and suppression has also been demonstrated with IgM NAbs that form complexes with apoptotic cells.[8] The best characterized type of IgM NAbs with homeostatic properties are those that recognize the small phosphorylcholine (PC) head group in cell-membrane associated phospholipids, that is exposed on the surface of cells undergoing apoptotic cell death.

There is also another set of NAbs that recognizes a different type of cell membrane-associated determinant associated with damaged red cells. Within the repertoire of B-1 cells, one of the most characteristic differences from the repertoires of other types of B cells are the clonal expansions of VH11 and VH12-expressing B-1 cells that can represent 5–15% of the pool.[9] This type of B-1 cell IgM NAbs specifically recognize mouse red blood cells treated with bromelain.[9-11] This distinct determinant appears to also become expressed on aging sheep red cells, which in the past reports appeared to have cross-reactivity with phosphatidyl choline containing liposomes.[12] However, the PC-specific NAbs mentioned above have little or no reactivity with red cells of most species, whether young or senescent. This may be explained, in part, by the special biology of mature red cells, which lack mitochondria and the machinery required to initiate the intrinsic programmed cell death pathway that is associated with high levels of oxidative damage (see below). While healthy humans also generally express NAbs to PC (see Chapter 1 by Binder) and senescent red cells (see Chapter 6 by Lutz), lupus patients can also develop antibodies against cardiolipin and β2 glycoprotein I, as well as to single stranded (ss) DNA,[13-15] which may become exposed or deposited on dead and dying cells and therefore may play secondary roles for the immune recognition of dead and dying cells. In this chapter, we will focus on NAbs that directly bind to apoptotic cells, discuss anticardiolipin antibodies as well as the possible roles of these antibodies in health and disease.

CELL SURFACE MEMBRANE CHANGES DURING CELL DEATH

Three common pathways of cell death are now well recognized: apoptosis, necrosis and autophagy. Apoptosis is the 'physiological' form of cell death that occurs as cells approach the end of their normal lifespan and this form of cell death is also observed during homeostatic processes such as thymic negative selection or cell number contraction that occurs during resolution of inflammation (reviewed in ref. 16). Although some of the changes in the cell membrane discussed below may briefly be observed following necrotic or autophagic cell death, for the purposes of this discussion we will focus on cell surface alterations that occur during apoptosis.

During cell death, multiple alterations to cell membranes occur. The early stages are associated with the exposure on the cell surface of phosphatidylserine (PS), which in health is sequestered to the inner leaflet of the membrane. Apoptosis-associated PS exposure

is caused by the reduced function of a translocase and possibly by activation of a lipid scramblase.[17] Depending on the precise mode of cell death and the speed with which the cell is subsequently phagocytosed, PS may become oxidized by reactive oxygen species (ROS) generated by the mitochondria during apoptosis.[18,19] ROS modify polyunsaturated lipids to generate the highly reactive aldehyde, malondialdehyde (MDA) on the cell surface. Another very important change affects the abundant neutral phospholipid, phosphatidylcholine, that, in contrast to PS, is normally concentrated in the outer leaflet of the cell membrane. Kim et al.[20] have shown that during apoptosis, caspase-activated iPLA2 (Ca^{2+} independent phospholipase A2) is responsible for the generation of lyso-PC (PC that lacks a fatty acid in the sn-2 position). This modified lipid is present on the cell surface in an altered conformation as compared with its parent molecule, but may also diffuse away from the cell membrane and serve as a chemoattractant for macrophages ('find me' signal).[21,22] While PS may become diffusely displayed on the surface of dying cells, during the activation of some types of healthy cells, including lymphocytes, there can be transient expression of localized membrane PS patches, which may facilitate certain types of cell-cell interactions. In addition, in healthy cells the negatively charged phospholipid, cardiolipin, is normally limited to the inner leaflet of mitochondria, where it interacts with cytochrome C. Following cell injury and especially after oxidation, it may redistribute to the outer mitochondrial membrane.

ANTIBODIES TO CELL SURFACE ANTIGENS THAT BECOME EXPOSED ON APOPTOTIC CELLS

As mentioned above, numerous alterations to the surface of dying cells have been observed and many of these changes involve lipids. Lipid modification leads to the generation of neo-epitopes that are recognized by antibodies, as discussed below. These modified phospholipid residues can also be specifically recognized by a range of cellular receptors as well as by soluble factors that act as bridging molecules for the recognition and clearance of dead and dying cells. These serum opsonins (bridging proteins) include collectins such as C1q, mannose binding lectin (MBL), Annexin I, Growth Arrest Specific-6 (GAS6), Protein S, β2 glycoprotein I (β2-GPI) and milk fat globule epidermal growth factor 8 (MFGE-8), among others (reviewed in ref. 23). While the binding of bridging proteins is essential for apoptotic clearance and the maintenance of homeostasis, binding of antibodies to apoptotic cells may further facilitate the clearance of dying cells by amplifying the efficiency of the recruitment by certain opsonins (see below).

Antibodies to PC, Malondialdehyde (MDA) and Lipoproteins (see Fig. 1 for Lipid Structures)

Healthy humans and non-autoimmune strains of mice commonly express IgM NAbs to PC and to the oxidation-associated neo-determinant, malondialdehyde.[5,24] All immunocompetent strains of mice produce structurally related anti-PC NAbs that share T15 idiotypic markers, that predominantly, or solely, arise in the B-1 B-cell compartment discussed above. T15-related B cells dominate the immune responses to PC-antigens and are required for effective defense from infection by *Streptococcus pneumonia*e, as PC is an immunodominant determinant in the cell wall polysaccharide shared by all pneumococcal strains.[25]

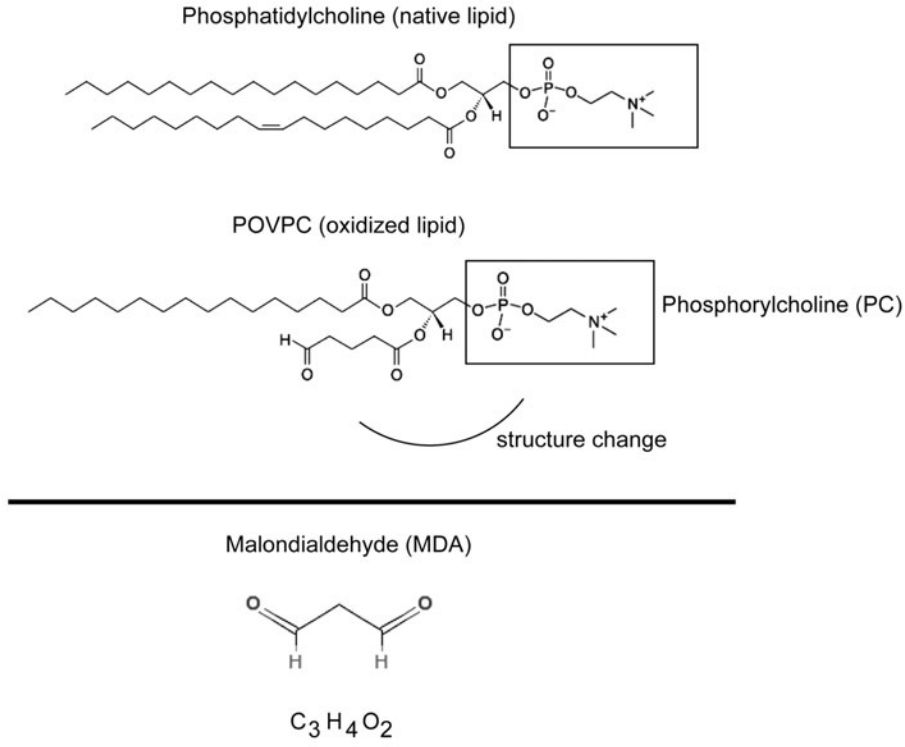

Figure 1. Phospholipid related antigens involved in NAb responses to apoptotic cells. Phosphorylcholine (PC) is the head group in neutral phospholipids that is inaccessible in the native phosphatidyl choline (PtC) on the surface of healthy cells. In contrast, on apoptotic cells PC is thought to become accessible to recognition by NAbs through the release of the sn-2 fatty acid by phospholipase A2 and/or oxidative processes that induce a conformational change. Oxidation generates 1-palmitoyl-2-(5-oxovaleroyl)-sn glycero-3-phosphocholine (POVPC) as shown. Malondialdehyde (MDA) is a reactive aldehyde that is formed by interactions of reactive oxygen species with lipids. MDA can become expressed on the surface of cells dying by apoptosis.

The prototypic T15 antibody expresses canonical VH and Vk gene re-arrangements of the VHS107.1 gene paired with a Vk22Jk5 light re-arrangement. The VHS107.1 germline gene appears to have been selected during the evolution of the murine immune system for its capacity to enable PC-antigen binding, as mice with targeted deletion of the VHS107.1 gene are functionally impaired in the recognition of microbial or neo-self apoptosis-associated antigens.[8,26,27] Yet wild-type mice raised under germ-free conditions display the same developmental program with high representation of anti-PC B cells and antibodies during the early neonatal period as mice raised under standard conditions.[28] This suggests that microbial ligands are not required for the clonal selection of these B cells during early development. Due to the ubiquity of apoptotic cells, especially during development, and the known resistance of B-1 cells to self-ligand mediated negative selection, it has been postulated that B cells expressing these anti-PC NAbs are in fact positively selected by modified apoptotic cell membrane-associated ligands during the early development of the B-cell compartment, probably in the fetal liver.

Low density lipoproteins (LDL) are complex macromolecules containing both phospholipids and a number of specialized proteins involved in lipoprotein transport and regulation. Witztum and colleagues found that in hyperlipidemic mice (secondary to deficiency of Apoliporotein E or low density lipoprotein receptor), there was a spontaneous and progressive expansion of NAb responses that recognized determinants on oxidatively modified LDL, but not reduced native LDL.[29] During early disease, these antibodies were predominantly IgM but later, IgG became more prevalent. When B-cell hybridomas were generated from unstimulated splenocytes from Apo-E deficient mice, serologic surveys demonstrated that ~30% of wells contained IgM antibodies that bound to oxidized LDL, but not native LDL or MDA-modified LDL.[29] Later studies of fractionated atherosclerotic plaques, which contained the apoptotic cells and oxidized LDL-stuffed macrophages, demonstrated that many of the OxLDL-reactive monoclonal IgM recognized the modified lipid, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC). Further immunogenetic characterization of these B-cell hybridomas showed that POVPC recognition was restricted to clones that expressed antibody gene re-arrangements identical to those of the classical T15 clone.[30] In fact, a number of these hybridomas, typified by the EO6 clone, expressed identical re-arrangements without a single point mutation, and their antibody products bound PC and expressed the T15 idiotype.[30]

The biologic relevance of this NAb response was evaluated in studies demonstrating that the EO6/T15 monoclonal antibody bound to apoptotic porcine aortic endothelial cells but not to healthy cells,[31] and that this binding was dose-dependently inhibited by PC chloride or by PC-albumin conjugate, which suggests that the determinants recognized on apoptotic cells are PC-containing determinants and/or use a similar antigenic binding site on this prototypic antibody.[30,32] This murine antibody therefore appears similar to those described by Kim et al.,[20] in which IgM NAbs were shown to recognize lyso-phosphatidyl choline and related neo-antigens on apoptotic cells. Therefore, there is a type of human and murine IgM NAbs that bind to oxidized phosphorylcholine (PC)-containing phospholipids and also POVPC protein adducts, but not to native low density lipoprotein (LDL) or non-oxidized phosphatidylcholine such as in 1-palmitoyl-2-arachidonyl-sn-glyceroyl-3-phosphorylcholine (PAPC).[30,32]

PC determinants may also be immunodominant during in vivo responses in non-autoimmune mice. Chen et al. showed that following intravenous infusion of syngeneic apoptotic thymocytes more than 25% of induced IgM-secreting cells in the spleen recognized PC antigens, and these apoptotic cell infusions induced antibodies that also expressed the T15 idiotype.[8] In addition, there was also a comparable high frequency of NAb-secreting B cells that instead recognized MDA. In earlier studies Mevorach et al.[33] showed that infusion of apoptotic cells also enhanced the production of antibodies to single stranded DNA and to cardiolipin.

Antibodies to Cardiolipin

Both PS and CL are negatively charged phospholipids. It is therefore possible that PS exposure on apoptotic cells contributes to the stimulation of anti-CL and/or that anti-CL bind to PS on dying cells. Despite the fact that neither of these possibilities have been clearly proven, we briefly discuss aCL. Anti-CL antibodies were first described in infectious diseases, initially syphilis, and more recently, tuberculosis and HIV-1. These antibodies are a diagnostic criterion for the primary anti-phospholipid syndrome (PAPS) and are detected in approximately a third of patients with systemic lupus erythematosus

(SLE). If cardiolipin becomes exposed on a cell surface or apoptotic bleb, it forms a complex with the 50 kDa serum glycoprotein, β2-GPI (also termed apolipoprotein H), that is present at high levels in the circulation (~200 μg/ml).[34] Importantly, antibodies that specifically recognize the complex of CL and β2-GPI do not commonly arise during infections and are more specific for SLE and PAPS.

In contrast to NAbs of the IgM isotype, IgG anti-CL and IgG anti-β2-GPI are considered disease markers[35] and these may potentially be directly "pathogenic" (associated with the clinical complications of fetal loss, thrombosis and thrombocytopenia). In SLE patients, IgG anti-β2-GPI antibodies are predominantly of the IgG2 subclass,[36] which may suggest that their pathogenic features involve the blocking of the functional properties of β2-GPI by an IgG autoantibody. As human IgG2 antibodies have limited capacity to form immune complexes that trigger activating FcγR, the predominant expression of the IgG2 subclass among anti-β2-GPI antibodies likely indicates that FcγR mediated pathways are not central to the pathogenesis of these responses. While it remains to be shown precisely how these antibodies are associated with clinical complications, murine models have implicated complement activation.[37] It should also be mentioned that although IgM anti-β2-GPI has been added as a criteria for the diagnosis of the Anti-Phospholipid syndrome, this association has been challenged, and recent studies have shown an association with protection from lupus nephritis.[38]

BIOLOGICAL FUNCTIONS OF NAbs TO APOPTOTIC CELLS

The proposed functions of NAbs binding to apoptotic cells were initially controversial as some studies suggested that these antibodies block apoptotic cell phagocytosis,[31] while others suggested that phagocytosis was facilitated through activation of the classical pathway of complement.[20] Most recent studies strongly support the role of anti-PC NAbs in promotion of apoptotic cell clearance following recruitment of C1q and/or mannose binding lectin (MBL).[8] Furthermore, phagocytosis of NAb-opsonized apoptotic cells was also shown to promote the anti-inflammatory properties of apoptotic cells (see below).

Binding of NAbs to Apoptotic Cell Recruits Complement

Previous studies revealed that complement opsonization is required for efficient phagocytosis of dying cells in vitro[39,40] and that uptake of these opsonized cells is associated with expression of the anti-inflammatory cytokine, TGF-β.[40] The fact that C1q binds to apoptotic cells in vitro,[41] that apoptotic cells accumulate in the kidneys of mice deficient in C1q,[42] and that complement activation on apoptotic cells promotes phagocytosis of these cells by macrophages in vitro, suggested a pivotal role for complement in the non-inflammatory clearance of dying cells. Since mice deficient in the secreted form of IgM NAbs developed IgG autoantibodies and a lupus-like disease,[43,44] Kim et al.[20] proposed that IgM deficiency may contribute to the pathogenesis of lupus because IgM NAbs are necessary for complement recruitment and the efficient clearance of apoptotic cells. In support of this hypothesis, it was observed in these in vitro studies that, in the absence of IgM, much less C3 and C3 breakdown products were deposited on apoptotic cells. As C3 recruitment is a downstream consequence of recruitment of the initiating recognition molecule, C1q, this suggested that in the absence of IgM there was much less complement recruitment to apoptotic cells. Quartier et al.[45] and Ogden

et al.[46] later provided additional support for these findings in studies that used sera from genetically manipulated mice with complement deficiencies. Using the prototypic T15 monoclonal IgM NAb from the EO6 hybridoma, Chen et al.[8] directly demonstrated that IgM NAb mediated C1q recruitment onto apoptotic cells. In sum, these studies demonstrate the contribution of IgM NAbs which may form immune complexes with exposed cellular neo-determinants resulting in conformational changes in the IgM constant region that facilitates the recruitment of C1q[47] which can result in activation of the classical complement cascade.

Chen et al. observed that IgM anti-PC NAbs also enhanced MBL deposition on IgM or on the apoptotic cell membrane itself.[8] MBL is a multimeric collectin immune recognition protein that initiates the lectin pathway of complement activation that can result in downstream C3 deposition.

Under normal physiologic conditions, C1q or MBL binding can initiate the catalytic complement cascade resulting in substantial deposition of C3b on the cell surface membrane. C3b/C3bi is a potent ligand for the abundant receptors on macrophage cell surface, CR3 and CR4, that enhance ingestion of opsonized cells.[39] Moreover, complement activation can proceed and lead to membrane attack complex formation, which results in lysis (post apoptotic necrosis). The lysis-induced release of intracellular constituents can stimulate an inflammatory immune responses. An important question then arises: if NAbs can recruit complement onto the surface of apoptotic cells, why do apoptotic cells not commonly lyse? There are at least 2 reasons. First, in contrast to bacterial cells, mammalian cells express complement regulatory proteins CD55 (DAF) and CD46 (MCP) that function to deactivate complement and prevent bystander activation. Second, under normal circumstances, the flagging by IgM of apoptotic cells with high levels of C1q, MBL and C3b/C3bi is likely to result in clearance that is so efficient and rapid that cell corpses are removed without substantial release of their contents, which thus prevents an inflammatory response (Fig. 2).

NAbs That Bind Apoptotic Cells Facilitate Phagocytosis by Macrophages

As mentioned above, in the absence of IgM, there was a significant relative defect in clearance of apoptotic cells by macrophages both in vitro and in vivo.[45,46] The efficiency of apoptotic cell clearance by macrophages was restored following addback of sera with IgM, and complement was required for this effect. These studies therefore strongly validate the role of NAbs in the clearance of apoptotic cells through activation of complement.

Binding of NAbs to Apoptotic Cell Facilitates Phagocytosis by Dendritic Cells

In many settings, macrophages are primarily responsible for the clearance of apoptotic cells, a process that is essential to prevent the release of pro-inflammatory factors and autoantigens that can select pathogenic B- and T-cell clones. There are also subsets of dendritic cells (DCs) that share the phagocytic properties of activated macrophages, especially at early stages of differentiation and/or those expressing CD103 and high levels of CD11c. Phagocytosis of apoptotic cells by such DCs is part of a process of constant steady-state sampling and presentation of self-Ags, which in vivo experiments suggest actively reinforces immune tolerance.[48] Yet DCs also serve as sentinel immune cells and when induced to full maturity, lose the phagocytic capacity, upregulate costimulatory molecules and chemokine receptors, migrate to draining lymph nodes, and become potent

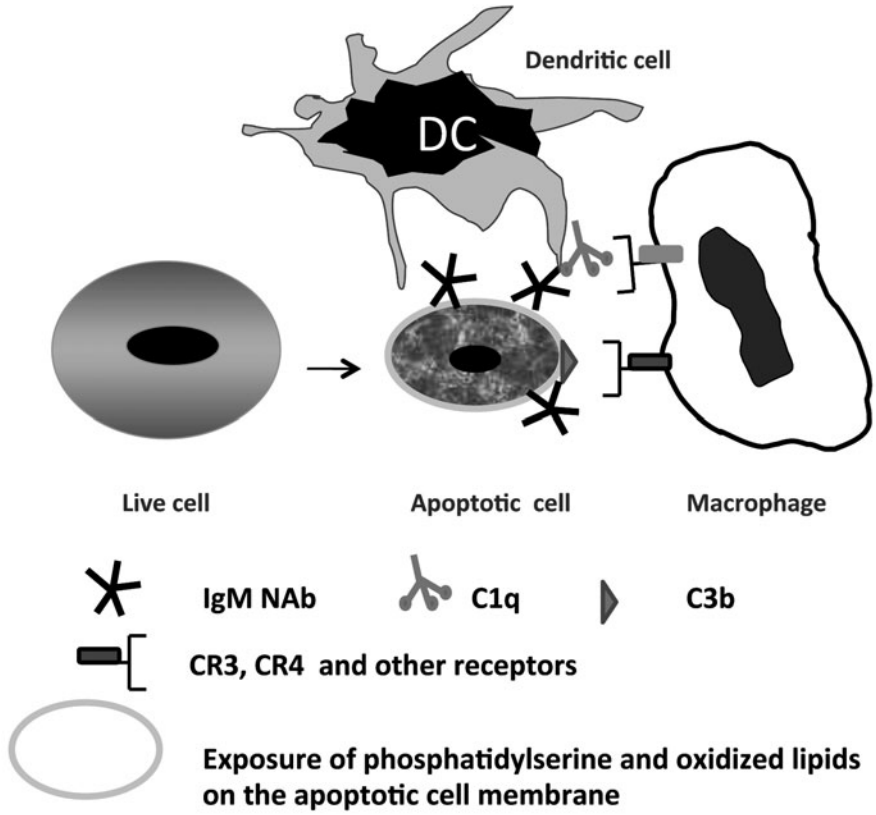

Figure 2. Role of IgM NAbs in the clearance of apoptotic cells. When cells die, a series of biochemical pathways are initiated which result in numerous biochemical alterations to membrane lipids on the cell surface membrane. Among these, IgMNAbs recognize phosphatidylserine (PS), phosphorylcholine (PC) andmalondialdehyde (MDA) (see Fig. 1). Once bound to the cell surface neoantigens, IgMs recruit C1q, which in turn may directly bind to receptors on phagocytes and also activate the classical complement pathway. The classical complement proteins, particularly C1q and C3b, opsonize the dying cells for ingestion by macrophages and dendritic cells (DCs). C3b is recognized by the CR3 and CR4 (CD11b) receptors whereas C1q receptors remain controversial.[53] Phagocytosisof apoptotic cells ingested by macrophages and DC prevent inflammatory responses and help produce tolerance to self. Note that other ligands and receptors are also involved in the phagocytosis of apoptotic cells, as discussed in the text and the differential expression of receptors and macrophages has not formally been compared. See reference 16 for a fuller discussion of these topics.

antigen presenting cells (APCs). When DCs are fully activated they can also be high-level producers of a range of cytokines and chemokines.

NAbs, such as the prototypic monoclonal T15 IgM (from the E06 hybridoma), not only enhanced the in vivo clearance of apoptotic cells by peritoneal macrophages, but also similarly enhanced the in vitro ingestion of apoptotic cells by DCs.[8] Notably, T15 IgM mediated apoptotic cell engulfment by DCs was almost entirely C1q- and/or MBL-dependent. Moreover, circulating NAbs to PC and MDA related determinants in murine neonatal and adult sera had similar properties for enhancing apoptotic cell phagocytosis by DCs.[8] It should be noted that as phagocytosis of apoptotic cells by DCs

occurs in serum-free media supplemented with either MBL or C1q, and in serum-free cultures supplemented with sera from C4-deficient and C3-deficient mice,[27] it suggests that IgM NAb-mediated enhancement of DC phagocytosis of apoptotic cells does not have an absolute requirement for C4, C3, iC3b nor for activation of the downstream complement cascade.[8] Conversely, while it was reported that complement can be directly deposited onto apoptotic cells,[20] in replicate cultures only small amounts of complement were deposited on apoptotic cells in the absence of IgM.[8]

IgM Anti-PC Antibodies Block Inflammatory Responses of Macrophages and DCs

Inflammation is a protective host response to foreign challenge or tissue injury that is ultimately beneficial as it leads to the restoration of tissue structure and function. The resolution of an inflammatory response is therefore essential for homeostasis and involves numerous pathways. Not only the clearance of apoptotic cells, but the exposure to the membranes of apoptotic cells have been recognized as important mechanisms for the control and resolution of inflammation in vivo (reviewed in ref. 7).

Recent in vitro studies have shown that the IgM anti-PC antibodies to apoptotic cells blocked inflammatory cytokine (IL-6, IL-12p70, IL-17, TNFa) responses by bone marrow derived DCs to Toll-like receptors (TLR) (agonist in parenthesis): TLR3 (poly (I:C), TLR4 (LPS), TLR7(imiquimod) and TLR9 (CpG DNA).[27] IgM antibodies also suppressed IFN related genes, including IP-10[27] and IFN-β1 and IRF-4 (unpublished). However, we were surprised to find that the IgM antibodies did not induce bone marrow-derived DCs to produce IL-10 or TGF-β, factors implicated in the suppression of inflammatory responses in other settings. During these incubations a certain proportion of cells died with the characteristic features of apoptosis, and complexes spontaneously formed with the IgM anti-PC. Hence, the anti-inflammatory effects demonstrated were likely the result of the influence of IgM-apoptotic cell complexes. In addition, it should be noted that C1q can, even when bound to IgG containing immune complexes, lead to anti-inflammatory effects especially to Type 1 interferons.[49]

To confirm the biologic relevance of these findings, C57BL/6 mice received infusions of the T15 IgM or isotype control and then were challenged with LPS or poly(I:C) in vivo. The T15 IgMNAb also inhibited these in vivo responses as evidenced by suppression of blood levels of IL-6, IL-12p70, IL-17, TNFα and the chemokines, MIP-1a, MCP-1, KC (analogous to human IL-8) and IP-10, which have all been implicated in human autoimmune disease.[27] In mice treated with this anti-PC IgM, challenge with LPS also appeared to result in lower levels of activation markers on splenic macrophages and DCs, which included CD40, CD86 and MHC II, though this could also have reflected changes in the cellular trafficking of these cells.[27] These findings provided the first demonstration that IgM NAbs to apoptotic cells can regulate inflammatory responses from innate immune cells in vivo.

REGULATORY NAbs MAY BLOCK DEVELOPMENT OF INFLAMMATORY AUTOIMMUNE DISEASE

Apoptotic cells have previously been shown to attenuate collagen-induced arthritis (CIA).[50] Specifically evaluating the role of IgM, Chen et al.[27] reported that pretreatment

with the T15 anti-PC NAb markedly reduced clinical disease activity, synovial leukocytic infiltrates, and bone and joint damage in CIA. Notably, there were no differences in IgG anti-collagen Type II (CII) levels induced by collagen immunization in the different treatment groups, which suggested that T15-NAb was primarily inhibiting the end-organ inflammatory response and not the autoimmune responses from B cells.

To further define the role of the adaptive immune system in this process, studies were also performed in the model of arthritis induced by passive transfer of anti-CII IgG, in which the innate immune system dominates pathogenic pathways. Pretreatment with the IgM T15-NAb significantly diminished joint swelling.[27] Taken together, these findings indicate that NAbs to apoptotic cell membrane determinants, such as PC, can convey immunoregulatory properties in models of arthritis by acting to blunt pro-inflammatory effector mechanisms mediated by the recruitment of IgG-autoantibody immune complexes. These findings confirmed the in vivo anti-inflammatory properties of regulatory NAbs to apoptotic cells and suggested that this is mediated by inhibition of both TLR responses as well as IgG-immune complex mediated inflammatory responses.[27]

CONCLUSION: UNANSWERED QUESTIONS AND FUTURE APPLICATIONS

Recent findings with IgM NAbs raise a number of questions. A better understanding of their genesis as well as their role in clearance of dying cells in the spleen and sites of inflammation is necessary. Specifically, we need to understand how IgM NAbs fit in with the plethora of other molecules implicated in the clearance of apoptotic cells. Furthermore, we need to better characterize the nature of the cell surface interactions that are involved in phagocytosis (see also ref. 51). Are anti-CL antibodies generated in response to modified lipids on mitochondrial or even cell surface membranes? Could modulation of IgM/IgG ratios influence in vivo function and clinical outcomes?

Results from other studies examining the representation of human IgM antibodies to PC in different patient populations have suggested that higher levels may reflect protective properties while low levels may predispose to comorbid conditions.[52] It remains to be determined whether human IgM NAbs have comparable functional properties related to cellular immune responses. Future investigations are therefore needed to understand the potential roles of regulatory NAbs in health, and how such factors may protect or modulate the pathogenesis of inflammatory and autoimmune diseases.

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CHAPTER 3

NATURALLY OCCURRING ANTIBODIES DIRECTED AGAINST CARBOHYDRATE TUMOR ANTIGENS

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Abstract: Healthy persons carry within their pool of circulating antibodies immunoglobulins preferentially of IgM isotype, which are directed against a variety of tumor-associated antigens. In closer scrutiny of their nature, some of these antibodies could be defined as naturally occurring antibodies due to the germline configuration of the variable immunoglobulin region. The majority of these immunoglobulins recognize carbohydrate antigens which can be classified as oncofetal antigens. Many of these IgM antibodies present in the peripheral blood circulation can bind to tumor cells and of these a minor portion are also able to destroy tumor cells by several mechanisms, as for instance complement-mediated cytolysis or apoptosis. It was postulated that anti-carbohydrate antibodies are part of an anti-tumor immune response, while their presence in the peripheral blood of healthy donors is still waiting for a plausible explanation. It may be that recognition of defined epitopes, including carbohydrate sequences, by naturally occurring antibodies constitutes the humoral arm of an anti-tumor immune response as part of the often postulated tumor surveillance. The cytotoxic capacity of these antibodies inspired several research groups and pharmaceutical companies to design novel strategies of immunoglobulin-based anti-tumor immunotherapy.

INTRODUCTION

Several research groups have reported that the peripheral blood of healthy persons contains antibodies which recognize carbohydrate structures. High throughput screening by means of glycan-array technology revealed that these antibodies react with a range of distinct carbohydrates consisting for example of blood groups (apart from the ABO

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

system, Le^x (CD15), sulfated Le^x (CD15u), Le^y (CD174), I-antigen, P1, P^k and more complex N- and O-linked oligosaccharides).[1-5] The respective antigens may occur as a carbohydrate moiety of glycoproteins or glycosphingolipids either as soluble molecules or as integral glycoconjugates of the cell membrane. Some of the glycan epitopes recognized by antibodies are present under healthy conditions; some are distinctly associated with malignant processes. A variety of human anti-carbohydrate antibodies act against antigens which occur frequently in bacteria or non-human mammalians and are like those against the α-gal epitope (Galα1–3Galβ1–4GlcNAc)[6,7] produced in considerable concentrations (according to the literature, the amount of these antibodies comprises approximately 1% of immunoglobulins). Another example are circulating antibodies against terminal N-glycolylneuraminic acid-containing sialoglycans.[8] N-glycolylneuraminic acid (Neu5Gc) is a variant of neuraminic acid which is not produced in humans. For antibodies against Neu5Gc the major antigen source seems to be food of mammalian origin.

There is considerable evidence that many tumor-associated antigens consist of carbohydrate structures and further that carbohydrate-specific naturally occurring antibodies (NAbs) can recognize these antigens. Many issues with regard to these carbohydrate-specific NAbs are still unsolved and open a wide field of fundamental, immunological research.

For most of the anti-carbohydrate antibodies in peripheral blood we do not know whether they belong to the class of NAbs or to those antibodies produced after contact with an exogenous antigen. Certainly, both types of antibodies are present. Another question is: why do we produce such a great variety of carbohydrate-directed antibodies recognizing foreign and autoantigens? We will summarize our current knowledge and discuss prominent hypotheses concerning these issues. Anti-carbohydrate antibodies in the blood of healthy persons may not only bind to tumor cells but a small percentage are also able to destroy them. Are these cytotoxic antibodies part of the immune system responsible for tumor surveillance? We have some evidence that the cellular branch of the immune system, for instance represented by natural killer (NK) cells, is responsible for monitoring and destroying tumor cells, especially in the early phase of tumor growth.[9] On the side of humoral immunity some experimental results indicate a role of NAbs in tumor defense.[10,11] Nevertheless, it is not known whether anti-carbohydrate NAbs have a function in tumor surveillance.

CHARACTERIZATION AND ORIGIN OF ANTI-CARBOHYDRATE ANTIBODIES

A majority of anti-carbohydrate antibodies seems to be derived from the pool of NAbs produced already during fetal life, which do not need a stimulus by an exogenous antigen. Evidence for this is based on the genetic analysis of immunoglobulin structure of autoantibodies including those against carbohydrates. Nonmutated germline variable region configuration of genes encoding heavy and light chains is a feature of NAbs.[12] For those NAbs isolated from cancer patients it was found that light chains are primarily and in contrast to most circulating antibodies of the lambda type. More than 80% of these antibodies were encoded by V_H genes of the V_{H3} family and within this family especially of the germline genes *DP47* and *DP49*.[13] These NAbs are produced over a long period if not lifelong. The mechanisms of this continuous antibody production are not yet clear.

In particular our knowledge about half-lives and replenishment of these antibodies is still very limited.

On the other hand a certain amount of anti-carbohydrate antibodies is produced during early stages of childhood when the gastrointestinal tract is colonized by bacteria. The presence of these anti-carbohydrate antibodies reflects the large variety of bacterial oligosaccharides the young organisms are confronted with.

Competition between invading pathogenic microorganisms and the host defense system against these attacks may also have been the driving force to create the diversity of surface oligosaccharides on both sides—pathogens and host—and for the pathogens to continuously invent new mechanisms of mimicry to escape recognition by the immune system.[14] The complexity of carbohydrate structures of our body may have further arisen "in part from our need to both evade pathogenic relationships and to co-evolve symbiotic relationships with our non-pathogenic resident microbes.[15]" For this interdependent process Gordon coined the term "glycan legislation.[16]" Similarily, apart from antibodies, the host defense system has also developed glycan recognition proteins (lectins) during evolution which function both in the control of innate and adaptive immune responses. There are many lectins like those of the selectin and siglec family and the galectins, which function for example in antigen presentation and lymphocyte activation.[17-19]

During breast feeding another rich source of carbohydrates is offered to the young organism. We can find over 200 different oligosaccharides in mothers' milk.[20] When these carbohydrates reach the infant's gastrointestinal tract they can exert beneficial protective effects against bacterial attacks. Bacteria use oligosaccharide structures of the host by means of their fimbriae as attachment sites.[21] Oligosacharides derived of the mothers' milk can serve as soluble, anti-adhesive competitors with the cell surface glycans and thus may prevent infection at an early stage. Do oligosaccharides of the mother's milk stimulate anti-carbohydrate antibody production? To my knowledge the relationship between breast feeding and production of anti-carbohydrate antibodies has not yet been investigated.

B lymphocytes producing these antibodies belong to the splenic marginal zone B lymphocytes and the B1 type of the peritoneal and pleural cavities.[22] They are described as a B-cell subset already active during fetal life. Earlier, these B lymphocytes were characterized by the expression of CD5; now human B1 cells of umbilical cord and adult peripheral blood were closer defined in that they additionally express a distinct pattern of surface markers ($CD20^+$, $CD27^+$, $CD43^+$, $CD70^-$).[23] Although the majority of these B1 cells are CD5 positive, there exist also $CD5^+$, $CD27^+$ and $CD43^-$ B lymphocytes which belong to the subgroup of B memory cells. Therefore a closer definition of B1 cells by several CD markers is useful and also reflects the complexity within the differentiation of B lymphocytes. B1 cells can be further separated according to their function into B1a and B1b cells. While B1a cells produce NAbs, which are stimulated by so-called danger signals via non-specific Toll-like receptor activation as part of the innate immune system, B1b cells and those B cells of the splenic marginal zone seem to be responsible for the production of anti-carbohydrate NAbs. In contrast to the marginal zone B cells which are mainly responsible for the production of plasma cells, the B1b have the capability both to proliferate and produce plasma cells and thus may be the source of memory cells securing the long lasting production of anti-carbohydrate antibodies.[24] Interestingly and opposite to the dogma that only B2 cells undergo affinity maturation, there may also occur somatic hypermutations in peritoneal B1 cells mainly of IgA and much less of IgM antibodies of B1b cells.[25] Whether the population of long-living B1a lymphocytes, responsible for

the production of circulating NAbs against bacterial pathogens and autoantigens, also produce anti-carbohydrate antibodies is not yet known.[26]

One may also find anti-carbohydrate antibodies of the IgG isotype with somatic mutations in the V genes, which is consistent with an antigen-driven process. Although NAbs of IgG isotype reactive against tumor antigens have been identified, to our knowledge NAbs described to react with a carbohydrate epitope belong exclusively to the IgM class.[27]

Although many of the anti-carbohydrate antibodies of peripheral blood may originate from the pool of NAbs, we cannot exclude, as we have seen, the presence of those produced by an antigen-driven process. Thus, as a cautionary remark, we cannot assume that all anti-carbohydrate antibodies in peripheral blood, even those of IgM isotype, are of the same origin. This also applies to anti-carbohydrate antibodies in IVIG preparations, which contain a small percentage of IgM or IgA immunoglobulins.

Most NAbs are polyreactive antibodies, which means that the antigen-binding pocket of these antibodies is more flexible and thus can bind to several antigens albeit in different intensities. Polyreactive antibodies are a predominant part of the newborn's antibody repertoire.[28] It is estimated that about 20% of B lymphocytes in the peripheral blood produce polyreactive antibodies; most of these immunoglobulins belong to the IgM class.[29] The polyreactive nature of NAbs may explain why anti-carbohydrate antibodies can recognize both bacterial antigens and autoantigens provided these carbohydrates have similar structural characteristics, as for example charged side groups at a given carbon atom or a typical conformational pattern of hydrobobic and hydrophilic sites within the molecule. The antigen-binding pocket of polyreactive antibodies seems to be relatively flexible and multiple three-dimensional conformations may exist before antigen contact. It can also be that changes within the antigen-binding pocket of the antibody take place during antigen contact.[29] However, the high specificity of anti-carbohydrate antibodies recognizing even small anomeric differences in glycosidic bonds of sugar remains to be explained in more depth. Using a carbohydrate microarray carrying specific carbohydrates in varied densities, significant differences in the binding capacity of serum antibodies were observed. This may point to the presence of subpopulations of serum antibodies with different affinities to distinct carbohydrate epitopes.[30]

Why do antibodies recognize carbohydrate autoantigens and how did these antibodies evolve? For some glycans a close structural relationship between autoantigens and, e.g., bacterial antigens has been described and led to the "molecular mimicry hypothesis.[31]" For instance, the ganglioside GM1 has some structural similarity to the *Campylobacter jejuni* lipopolysaccharide, carrying a GM1-like glycan. The Guillain-Barré syndrome develops in a significant portion of patients subsequent to an infection with Campylobacter, and in patients suffering from the Guillain-Barré syndrome high titers of anti-GM1 antibodies of different isotypes are found.[31] Another hint to the link between bacterial infections and anti-carbohydrate autoantibodies is given by the better survival of patients suffering from gastric cancer and *Helicobacter pylori* infection at the same time. This connection seems to be correlated with higher titers of the Thomsen-Friedenreich carbohydrate antigen.[32]

The production of xenoreactive, anti-carbohydrate antibodies as observed for the lifelong production of α-Gal antibodies can be explained by continuous stimulation by carbohydrate antigens of gastrointestinal bacteria of the microflora.[7] Antibodies against sialoglycans containing a terminal N-glycolylneuraminic acid (Neu5Gc) may be produced due to dietary habits, i.e., consumption of red meat or milk.[33] In further consequence of this hypothesis strict vegetarians should possess less or no anti-Neu5Gc antibodies. To my knowledge this has not yet been verified in a respective epidemiological study. Notably

and in contrast to the majority of naturally occurring anti-carbohydrate antibodies these xenoreactive immunoglobulins are to the largest extent of IgG isotype. Regardless of the antibodies´ origin it seems that certain features of glycan structures such as the terminal sugar and the configuration of the glycosidic bonds are decisive for antibody recognition.

Several central questions have to be answered concerning the relation between serum titers of anti-carbohydrate antibodies and tumor diagnosis and therapy: are distinct antibodies related to the outcome and progression of tumors? In particular, is the production of these antibodies against tumor-associated antigens initiated by the presence of a growing tumor mass or are these antibodies already present in healthy persons, thus contributing to a tumor surveillance system? From a practical perspective: are these antibodies only of diagnostic value or do they have a direct tumor cytotoxic capacity? In order to elaborate on these issues we should first have a closer look on the pecularities of tumor-associated glycosylation.

TUMOR-ASSOCIATED CARBOHYDRATE ANTIGENS AS TARGET STRUCTURES FOR ANTI-TUMOR ANTIBODIES

Malignant cells differ from their normal counterparts in their varied expression of distinct macromolecular structures which provide an advantage for tumor survival in relation to their interaction with the environment, as for instance suspended growth control, enhanced metastatic potential or escape from immune surveillance. These tumor-associated structures can be classified into several categories: (i) tumor-specific mutated oncogenes, (ii) oncoviral antigens, (iii) abnormal expression of gene products, preferentially those involved in growth regulation, (iv) reappearance of early differentiation antigens ("oncofetal antigens") and (v) abnormal posttranslational modifications, as for instance changes in the glycosylation of distinct structures. Changes, especially those summarized under (iv) and (v), are to a large extent caused by alterations in cell surface expressed glycans. Most cell surface proteins are glycosylated and in addition, a variety of glycosphingolipids are expressed in different composition on the cell surface depending on differentiation stage and tissue. Due to changes in the transcription of glycosyltransferases during tumorigenesis, oligosaccharide structures of glycosylated macromolecules on tumor cells occur in different amounts and composition.[34] There are examples for elongated and also for shortened oligosaccharide sequences on defined glycoproteins of tumor cell surfaces (Fig. 1).[35] For instance, the Muc-1 glycoprotein on tumor cells is characterized by its shortened O-linked oligosaccharide chains, which can be defined as the Tn (T-nouvelle structure, CD175) and the Thomsen-Friedenreich antigen (CD176).[36-38] Another example is the CD55 (decay acceleration factor, DAF) which on stomach carcinoma cells is expressed with a tumor-specific carbohydrate epitope recognized by an antibody.[39]

Expression of glycans such as the Thomsen-Friedenreich antigen is closely related to the malignancy process of tumors as stage-specific oncofetal antigens (Fig. 2).[40-42] In colorectal cancers the staining intensity with monoclonal antibodies (mAbs) against CD176 is increased in liver metastasis of this tumor class.[43] Moreover, patients with CD176-positive primary tumors have a much higher risk to develop liver metastases and importantly, normal mucosa was always found to be negative for this antigen.[43] The nonsialylated CD176 is rarely found in normal human cells. For instance all hematopoietic cells express its α2,3 sialylated and thus negatively charged form.[44] An exception are certain T-cell subsets including thymocytes expressing in a limited differentiation or

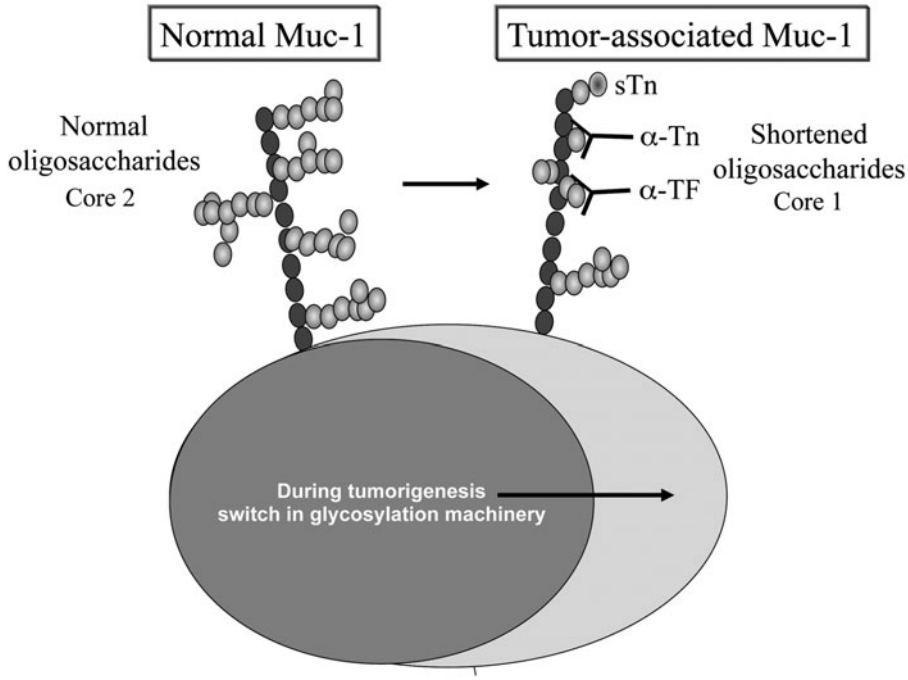
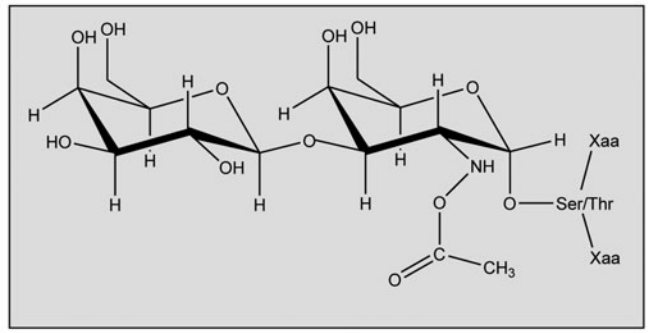


Figure 1. Changes of glycoprotein glycosylation during tumorigenesis. During tumorigenesis alterations in the glycosylation machinery, as for example a switch in glycosyltransferases, occurs so that instead of mucins with complex O-linked oligosaccharide chains those with much shorter oligosaccharides (Tn/CD175, TF/CD176) are expressed on the cell surface. Tn (T nouvelle)/CD175 consist of a single sugar, N-acetylgalactosamine, α1- O-linked to serine or threonine. The Thomsen-Friedenreich (TF, CD176) glycan structure is defined as the O-linked disaccharide Galβ1–3GalNAc which can either occur as TFα (α1-linked to the protein, core-1) or as TFβ (β-1 attached to the protein backbone). The Tα form is predominantly carried by glycoproteins of the mucin type, whereas the TFβ form is exclusively part of glycosphingolipids (Type 4 histo-blood group antigens). As a consequence of this tumor-specific change, antibodies against CD175 or CD176 can now bind to the tumor-associated mucin.

activation phase the nonsialylated CD176 antigen. T cells containing nonsialylated CD176 for a longer period are eliminated, possibly by apoptosis.[45-48] The key enzyme for the transition to the sialylated state of CD176 is the α2,3 sialyltransferase (ST3Gal1).[47] In a few cases leukemia cells express nonsialylated CD176. Indeed, these cells could be destroyed in vitro by treatment with a CD176-specific monoclonal antibody. In the same line desialylation of CD176 by exogenous sialidase and subsequent monoclonal antibody treatment had the same effect.[44] Therefore it seems that in the normal healthy tissue and maybe in some tumors unmasked CD176 is a "danger signal." The question remains why colorectal tumor cells apparently have a selective advantage in expressing higher amounts of unmasked CD176? It was proposed that expression of CD176 favors adhesion to the asialoglycoprotein receptor on liver cells.[49] With regard to the prognostic value of CD176 expression in breast cancer there exists a controversial opinion. It was reported that CD176-positive breast cancers had a significantly better prognosis than CD176-negative tumors.[50] One has to consider that the routes of dissemination in breast cancer are different compared with those of colorectal cancer in that breast cancer cells

The Thomsen-Friedenreich antigen (CD176)



Structure of the Thomsen Friedenreich antigen
Galβ1-3GalNAcα1-R

- The TF disaccharide (core-1 structure) is exposed on cancer cells due to truncated glycosylation of cell surface glycoproteins.
- It is strongly expressed on human carcinomas (primary colon carcinoma are to 60% positive for CD176, liver metastases to 91%).
- It is an independent prognostic marker in colorectal cancer (patients with TF+ primary tumors have a significantly higher risk to develop metastases than patients with TF- tumors (57% vs 14%).
- In tumor patients the anti-CD176 titer is strongly reduced.
- Soluble TF is released by tumor cells into the blood – this may cause a consumption of anti-TF antibodies.

Figure 2. Structure and expression of the Thomsen-Friedenreich antigen /CD176.

primarily metastasize to lung, pleura and bones.[50] An important aspect is that healthy persons contain in their serum a considerable concentration of anti-CD176 antibodies whereas patients suffering from colorectal cancer have much lower serum titers of anti-CD176 antibodies.[51] These antibodies were primarily of IgM and much less of IgG isotype. Antibodies against the Tn antigen (CD175), which also shows an increased expression on some cancer types, were also detected. Thus anti-CD176 antibodies may be part of a humoral tumor surveillance system. The anti-tumor effect of CD176 antibodies was further corroborated in an in vivo experimental system in which liver metastases of the colon cell line Colo 26 were prevented by treatment with a CD176 monoclonal antibody.[52]

Also TF structures have been successfully applied as vaccines and CD176 monoclonal antibodies as anti-tumor therapeutic reagents.[53]

Another example is the serological tumor marker CA19–9, which consists of a glycan attached to mucins expressed on breast, ovarian and pancreatic tumors (Table 1).[54] In some tumors like melanoma, sarcoma and neuroblastoma the quantity of gangliosides GM2,

Table 1. Carbohydrate markers of various human tumors

Tumor	Carbohydrate Antigens
Neuroblastoma	GD1a, GD3 (CD60a), GD2, GM1
Melanoma	GM1, GM2, GM3, GD2, GD3 (CD60a), 9-O-acetylated GD3 (CD60b), 7-O-acetylated GD3 (CD60c)
Sarcoma	GM2, GM3, GD1a, GD2, GT1b, polysialylated glycans (NCAM/CD56)
Breast carcinoma	GM2, sialyl Tn (CD715s), Thomsen-Friedenreich (TF/CD176), Lewis^{y} (CD174), sialyl Lewis^{a} (CA 19–9), sialyl Lewis^{x} (CD15s)
Colon carcinoma	GM2, CD175s, CD176, sialyl Lewis^{a}, CD174
Pancreatic carcinoma	GM2, GD1b, sialyl Lewis^{a}, CD15s

The carbohydrate markers listed here are a collection of the most common glycan markers on these tumors based on findings described in the literature. Certainly there are many more variations in the tumor cell glycosylation, as for instance those of prominent mucin glycoproteins. Strikingly, many glycan tumor markers are found in the class of gangliosides and blood group antigens.

GD2 and GD3 is very high (Table 1).[55] A prominent marker for neuroblastoma cells is the ganglioside GD2. Consequently, anti-GD2 monoclonal antibodies have been applied in anti-neuroblastoma therapeutic trials.[56] In some of these trials an improved outcome in patients with high-risk neuroblastoma was observed as compared with standard therapies. Anti-GD2 antibodies of healthy donors have cytotoxic capacity against neuroblastoma cells. The cytotoxic capacity of anti-GD2 antibodies derived from peripheral blood was demonstrated when purified GD2 added to the test system abolished the cytotoxic effect.[57] Apparently, the titers of these anti-carbohydrate antibodies in neuroblastoma patients were negligible.[58,59] A successful treatment of neuroblastoma with these antibodies of healthy donors was performed in a nude rat model bearing solid human neuroblastoma tumors[57,60] and further in a small clinical study of nine neuroblastoma children patients suffering from late stage neuroblastoma.[61] Neuroblastoma patients had undergone several different treatments like chemotherapy, surgery, bone marrow transplantations and application of anti-GD2 monoclonal antibodies before they were included in this study. Several treatments with plasma containing high titers of anti-neuroblastoma cytotoxic antibodies led to a considerable life prolongation in one patient and curation from the tumor in another patient.[61] Large-scale screening of sera from healthy donors revealed that a considerably small percentage of approximately 1–2% donors in the Western world have high titers of cytotoxic antibodies against several human tumors like neuroblastoma and melanoma (Schwartz-Albiez, unpublished results). Although only few donors carry cytotoxic anti-tumor antibodies of IgM isotype in their peripheral blood, almost all healthy persons have anti-tumor binding antibodies. Interestingly, IgG of peripheral blood only rarely had tumor-binding capacity (Schwartz-Albiez, unpublished results). Also, notably, the cytotoxic activity was only found in the IgM and not in the IgG pool of serum antibodies.[62] In our experience the amount of bound IgM to tumor cells did not positively correlate with the anti-tumor cytotoxic effect of the respective IgM fraction. For instance, some IgM samples with comparatively low IgM binding capacity showed a higher cytotoxicity than other samples with higher IgM binding to melanoma or neuroblastoma target cells. In conclusion, the group of cytotoxic IgM within the pool of peripheral blood IgM may vary from person to person. Further, a

cytotoxic antibody has to attach to a specific antigen which then, after antibody contact, may render a cytotoxic signal as pointed out in more detail below.

Possibly, the history of an individual's infections may influence the titer and the diversity of cytotoxic antibodies. In a small comparative study it was shown that the age distribution of high titer anti-tumor cytotoxic IgM antibodies in an African population was significantly broader compared with that of Europeans.[63] This phenomenon is not easy to interpret and requires more sophisticated research for its explanation. In general, Africans are confronted with more and maybe more virulent pathogens than Europeans; correspondingly their antibody repertoire should also be much wider. Whether the plasma cells producing these IgMs are derived from unmutated B-cell memory cells of a T-cell dependent (TD) pathway receiving an antigen rechallenge or are derived from T-cell independent Type 2 (TI2) lymphocytes is not clear. At this point we do not know whether plasma cells producing NAbs can be challenged by external antigen stimuli. In conclusion, by some unknown mechanism the frequency and variety of crossreactive, cytotoxic IgMs seemed to be boosted by infections.

Although genetic predispositions may play a role in the outgrowth of neuroblastoma, an observed correlation between the incidence of neuroblastoma and the standard of living (implying also a different history of infections) may contribute to the generation of these tumors. Indication for this correlation was a significantly lower rate of neuroblastomas in some African countries in comparison to Western countries.[64,65] Therefore it would be a rewarding task to investigate the functional impact of cytotoxic IgM antibody production on neuroblastoma incidence since this would shed more light on the role of humoral tumor surveillance.

CD174 (Lewis^{y}) antibodies are also present in the blood of healthy persons. CD174 is, like CD176, a marker for malignancy in colorectal tumors.[66] At the same time CD174 is also expressed on activated vascular endothelium of inflammatory tissue in close proximity to tumors.[66] Tumors, for example colorectal cancer, are often accompanied by strong inflammatory processes in the surrounding tissue. Inflammatory disturbances in the neighboring vasculature may help tumor cells to intra- and extravasate during metastasis formation. Possibly in this context CD174 is a homing signal for invading tumor cells.

In order to exploit the anti-tumor recognition of NAbs, Vollmers et al. have isolated and cloned antibodies derived from cancer patients.[67] One of these antibodies, SC-1, derived from a patient with stomach cancer, detects a tumor-specific N-linked oligosaccharide of CD55, the decay accelerating factor (DAF).[68,69] Another example is the antibody PAM-1 which reacts with a glycan structure of CFR-1, the cysteine-rich fibroblast growth factor receptor, present on human carcinomas.[70] Further, SAM-6 binds to a carbohydrate moiety of the heat shock protein (HSP) GRP78.[71] While these antibodies were isolated from cancer patients their presence in healthy persons has to our knowledge not been studied. For some of these NAb-derived monoclonal antibodies an apoptotic function was described as outlined in more detail below.

From the literature available it seems that titers of antibodies against antigens like CD176, CD174 of GD2 and GD3 are higher in healthy persons than in cancer patients. Few reports describe the opposite situation. For example, Indian patients suffering from childhood acute lymphoblastic leukemia had significantly higher antibody titers against a 9-O-acetylated sialoglycan than normal persons.[72,73]

Thus, due to changes in the glycome machinery of tumor cells one may say that these oncofetal antigens appear at the wrong time and place, when compared with normal ontogeny. Without exaggeration more tumor-associated changes are found in the glycan

than in the protein moiety of surface structures. Therefore they are not only potential targets for new tumor diagnostic markers but also for immunotherapeutic anti-tumor strategies.

MECHANISMS OF CELL LYSIS

Naturally occurring anti-tumor antibodies of the IgM class exert in principle 2 direct mechanisms of cytotoxicity: complement-dependent cell lysis[74] or apoptosis.[75,76]

Clinical and experimental data support a potential role of complement in the control of neoplastic cells. In cancer patients, endogenous complement activation has been demonstrated with subsequent deposition of complement components on tumor tissue.[77] Certain monoclonal antibodies applied in immunotherapy of cancer were also shown to deposit complement on malignant cells.[78,79] After screening of sera from healthy donors for their cytotoxicity on human tumor target cells we found that the major mechanism of cytotoxicity in an in vitro assay system was complement-dependent lysis (Schwartz-Albiez, unpublished results). Binding of distinct IgM antibodies from the pool of immunoglobulins to the tumor cells is the important step, followed by complement lysis along the classical complement lysis pathway.

Tumor cells are, however, able to protect themselves against a complement-mediated cell lysis as outlined in more detail in the next paragraph.

For some NAbs an apoptotic tumor lysis has been reported. For instance, the monoclonal antibodies PAM-1, SAM-6 and SC-1 directed against tumor-associated carbohydrates and derived from NAbs induce apoptosis in carcinoma. Antibody PAM-1 derived from a patient with stomach cancer identifies a variant of the cysteine-rich fibroblast growth factor receptor 1 (CFR-1) and inhibits cell growth and induces apoptosis.[80] SAM-6 binds to a cell surface receptor and to oxidized low-density lipoprotein (LDL).[71] The antibody/LDL complex is internalized, lipid depots are formed and cytochrome c is released from mitochondria followed by activation of caspases. The antibody SC-1 reacts with a glycosylation variant of the decay accelerating factor (DAF/CD55).[69] After binding of SC-1 the surface expression of CD55 variant is enhanced followed by internalization of the antigen/antibody complex and activation of caspase-6. Interestingly the wild type CD55, also present on the surface of the tumor cells, is not affected. In this context it has to be noted that CD55 is an important inhibitory regulator of complement-dependent cytotoxicity. Although not characterized for their epitope specificity, immunoglobulins of IgM isotype from IVIG preparations were described to lyse human leukemia cell lines via apoptosis.[76]

The CD95/FAS receptor, also named APO-1, is a prominent death receptor on the cell surface; its activation by binding of the FAS ligand or CD95-specific monoclonal antibodies leads to apoptosis. It seems that sialylation of CD95 regulates its sensitivity toward apoptotic signaling. Treatment of leukemic cells with *Vibrio cholerae* sialidase prior to application of CD95-specific antibody rendered the cell more susceptible to apoptotic signaling.[81] Looking from a different angle at the same phenomenon, monoclonal CD176 antibodies reacting with the nonsialylated Thomsen-Friedenreich oligosaccharide had in vitro a cytotoxic, apoptotic effect on certain human tumor cell lines carrying the unmasked nonsialylated CD176 on their surfaces.[44] The CD176 glycan structure may be part of the carbohydrate moiety of CD95 and given its sterical accessibility, CD176 antibodies may also induce CD95-mediated apoptosis.[82] These findings are in accordance with previous observations that binding of lectins (or antibodies) to unmasked CD176 on thymocytes can induce apoptosis as mentioned earlier in this chapter.

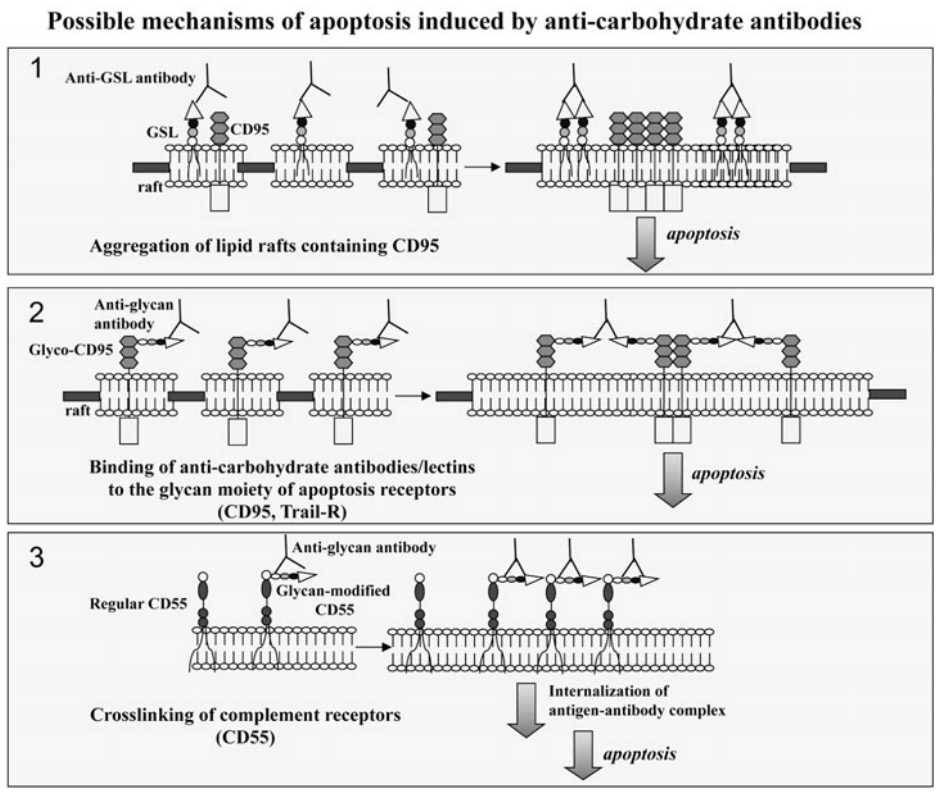


Figure 3. Possible mechanisms of apoptosis induced by anti-carbohydrate antibodies. Several modes of how anti-carbohydrate antibodies can induce apoptosis are illustrated: 1) Antibodies directed against surface-expressed glycosphingolipids (GSL) may crosslink these GSL so that in the fluid membrane they are pulled together modifying at the same time the raft formation on the cell surface. A consequence of these lateral shifts apoptosis receptors like CD95 molecules become aggregated which further entails inside the cell the clustering of apoptosis relevant protein complexes that induce apoptosis. 2) Binding of antibodies to a specific oligosaccharide structure of CD176 on CD95 (or the Trail receptor 4) may lead to concentrated assembly of CD95 on the cell surface with the consecutive intracellular consequences as described under (1). 3) In tumor cells the GPI-linked decay accelerating factor CD55 occurs in a glycan modified version. Antibodies against this tumor-associated CD55 variant lead to enhanced expression of this particular CD55, further to the internalization of the antigen/antibody complexes and finally to apoptosis.

Interestingly this effect could not be observed with CD175 (Tn) antibodies. Although sera of healthy donors contain both CD175- and CD176-specific antibodies, only those against CD176 seem to have a cytotoxic effect.

Also, certain galectins are able to induce apoptosis of target cells when bound to carbohydrates such as CD176.[47] The exact apoptotic mechanisms induced by cell surface glycan interactions with either carbohydrate-directed antibodies or lectins are not explored in detail. Several modes of action may be possible: (i) binding to certain membrane glycolipids which aggregate lipid rafts containing CD95 (clusters of apoptotic signaling molecule enriched rafts, CASMER),[83,84] (ii) binding to the glycan moiety of distinct receptors involved in apoptosis induction as for instance to CD95/

CD176[81,82] or (iii) crosslinking of complement receptors on the cell surface (Fig. 3).[13] Induction of raft formation and its possible functional consequences is dependent on the respective composition of the rafts. Therefore not only apoptosis but also activation may result from raft formation or reorganization. In human lymphocytes complexation of rafts by antibodies against the surface-expressed 9-O-acetylated or 7-O-acetylated ganglioside GD3 (CD60b, CD60c) entails cell activation.[85] A link between anti-carbohydrate IgM NAbs and natural killer (NK) cells' cell-mediated ADCC can be imagined at this point by accumulation of C3-derived complement components at the surface of target cells during IgM-mediated complement lysis and subsequent triggering of the CR3 (CD11b/CD18) receptor on NK cells by accumulated C3b and iC3b.

PROTECTION MECHANISMS OF TUMOR CELLS

Tumors may have several mechanisms to escape immunosurveillance. These mechanisms include avoidance of cell death, autonomy from growth factors and ignorance of growth-inhibitory signals.[86] Among these, complement resistance represents a hindrance for the efficiency of antibody-dependent tumoricidal effects. In order to generate a protective microenvironment, tumor cells secrete several soluble complement inhibitors and express on their surface ecto-proteases which degrade complement proteins or ecto-protein kinases which impair the functional activity of certain complement components.[87,88]

Like normal cells, neoplastic cells are protected from autologous complement attack by several cell-surface complement inhibitors, such as CD35 (complement receptor Type 1, CR1), CD55 (decay accelerating factor, DAF), CD46 (membrane cofactor protein, MCP), and CD59 (Protectin).[89] These regulators control the cascade reaction at the level of C3 activation (CD35, CD46, CD55) or prevent the insertion of the membrane attack complex (CD59).

It is evident from numerous studies that almost all tumors studied express at least one of the membrane complement regulatory proteins (mCRP), like CD46, CD55 and CD59.[88,90] CD35 has only been identified on follicular dendritic cell tumors and malignant endometrial tissue. Increased mCRP expression on cancer cells compared with the corresponding normal tissue may be the consequence of a selective force, which is driven by multiple events of complement attack during neoplastic transformation.[90]

The importance of complement resistance is underlined by various experiments leading to neutralisation of mCRPs, which sensitizes tumor cells to complement attack. This has been demonstrated by (a) the use of blocking antibodies against CD55, CD46 and CD59[91]; (b) the removal of CD55 and CD59 by phosphatidylinositol-phospholipase C (PI-PLC)[92] and (c) knock down of mCRP expression by antisense strategies.[93]

Expression of CD55 and CD59 on melanoma cells seems to be correlated with their resistance against complement-mediated cell lysis. Contrary to this observation, neuroblastoma cells are sensitive to antibody-mediated complement lysis despite their high expression of CD55/CD59.

THERAPEUTIC APPROACHES

The presence of elevated levels of certain glycans in human tumors has inspired many groups to either induce an anti-carbohydrate response in tumor patients by vaccines consisting of selected oligosaccharides, mimotopes resembling oligosaccharide or by induction of an anti-idiotypic response against tumor-associated carbohydrate antigens. For treatment of tumor patients with naturally occurring anti-carbohydrate antibodies 2 strategies have been followed up so far:

a. Isolation of B lymphocytes from tumor patients producing these antibodies and production of monoclonal antibodies thereof for further treatment.[67] This strategy has been used for generation of mAbs like SAM-6 and PAM-1 as described above.
b. Screening of sera/plasma from healthy donors for their cytotoxic capacity and subsequent application of highly cytotoxic plasma for tumor treatment. This strategy, using pooled anti-neuroblastoma cytotoxic plasma, had been prescribed for the treatment of children patients suffering from neuroblastoma.[61]

Isolation of anti-carbohydrate tumor-directed IgM antibodies from pooled immunoglobulin fractions requires a sophisticated separation procedure, in particular when these preparations shall be produced at industrial large scale for tumor treatment in a standardized form.

CONCLUSION AND PERSPECTIVES

Evidence has been mounted which shows that naturally occurring IgM antibodies directed against carbohydrate epitopes are able to kill distinct human tumor cells both in vivo and in vitro. Interestingly these antibodies are found in much larger quantities in healthy persons than in tumor patients. This observation may point to a function in tumor surveillance which, however, could not be proven directly. Malignant tumors, as for instance melanomas, exert escape mechanisms which can protect them against a complement-mediated antibody attack. In order to design successful strategies for therapeutic application of NAbs in cancer treatment, efforts have to be intensified to understand the molecular basis of anti-carbohydrate antibody tumor cytotoxicity and to design methods to isolate these antibodies or at least exploit their killing function for further therapeutic application. Despite these difficulties which have to be overcome, naturally occurring anti-tumor cytotoxic antibodies either in their native form or biotechnologically modified may represent a new generation of immunotherapeutic tumor drugs.

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CHAPTER 4

NATURALLY OCCURRING ANTIBODIES AS THERAPEUTICS FOR NEUROLOGIC DISEASE:

Can Human Monoclonal IgMs Replace the Limited Resource IVIG?

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Abstract: Naturally occurring autoantibodies (NAbs) are common in normal humans. The majority of NAbs are IgMs, but a small proportion are IgGs. Therefore a certain portion of pooled whole human IgG (IVIG) can be considered NAbs. While the applications of IVIG to modulate human disease have increased dramatically, the use of IgMs as drugs has lagged. In fact, much of the contaminating IgM component of IVIG is disposed of as waste. However, a number of model studies, including those targeting Alzheimer and multiple sclerosis (MS) suggest that IgMs may better modulate disease at much lower doses than IVIG. Our own studies in a model of MS show that polyclonal human IgM promotes better remyelination than IVIG and that monoclonal IgMs promote greater remyelination than monoclonal IgGs containing identical variable region sequences. We propose that this difference is due to the ability of IgM to cross link cell surface antigens better than IgGs and induce signals in nervous system cells. Monoclonal antibodies (mAbs) that promote remyelination induce a transient Ca^{2+} influx in myelin forming cells, whereas IgGs with identical variable sequences do not. MAbs that promote remyelination were identified in human serum and in EBV-immortalized human B-cell lines obtained from normal adults, fetal cord blood, and rheumatoid arthritis and MS patients. Therefore therapeutic mAbs are present and common in normal circulation. All therapeutic mAbs were IgMs and bound to nervous system cells, however, the tissue binding patterns suggest that binding any one of multiple antigens induces repair. An expression vector was constructed that can manufacture gram quantities of recombinant monoclonal human IgM. Therefore the technology exists to determine

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whether human monoclonal NAbs can modulate human disease. IVIG can modulate neurologic disease, but using IVIG to treat these chronic diseases is unsustainable. A long-term solution is to identify the functional component of IVIG and test whether a recombinant human monoclonal can replicate its efficacy.

INTRODUCTION

Naturally occurring autoantibodies (NAbs) are part of the human immunoglobulin repertoire.[1,2] NAbs are frequently derived from germline immunoglobulin genes, often react with self antigens, are of relatively low affinity, are polyreactive and are typically IgMs. In contrast, conventional antibodies contain somatic mutations, react with non-self foreign antigens, and are usually high affinity IgGs. NAbs provide an important host defense mechanism against invading pathogens. Accumulating evidence also supports the concept that NAbs are natural systemic surveillance molecules, signaling damaged or stressed cells or eliminating toxic cellular debris.

Intravenous immunoglobulin (IVIG) is a preparation of human polyclonal immunoglobulin G (IgG) obtained by pooling thousands of units of plasma from normal healthy blood donors. IVIG typically contains more than 95% unmodified IgG, and trace amounts of immunoglobulin A (IgA) or immunoglobulin M (IgM). IVIG is typically prepared using a cold ethanol fractionation process followed by ultrafiltration and ion exchange chromatography and packaged as sterile lyophilized powder or in solution. Because some IgGs are NAbs and IVIG contains a broad spectrum of antibodies from across the population, a proportion of IVIG contains NAbs.

The use of IVIG began in the 1980s as treatment for antibody deficiencies, autoimmune, and inflammatory disorders, such as immune thrombocytopenic purpura, and has now expanded to a number of conditions that impact the nervous system.[3] The proposed mechanism of action of IVIG involves interactions with many aspects of the immune system, including altering immune cells, interaction with Fc receptors, inhibition of complement, induction of apoptosis, and interference with cytokines and anti-idiotypic responses. The result of treatment with IVIG is often referred to as restoring homeostasis.

Homeostatic regulation is also a trait associated with NAbs. The use of other isotype antibodies isolated from human plasma, including IgM, seriously lags behind the published uses of IVIG. In fact, much of the plasma IgM and IgA removed from IVIG is disposed of as waste. We believe there is convincing evidence for the potential use of polyclonal and monoclonal human IgM as disease modifying drugs.

CURRENT USE OF IVIG IN NEUROLOGIC DISEASE

IVIG is now the drug of choice for the treatment of Guillian-Barré syndrome, chronic inflammatory demyelinating polyneuropathy and multifocal motor neuropathy. The use of IVIG in multiple sclerosis (MS) is less definitive. Initial positive clinical trials in MS have been followed by a number of trials that failed to demonstrate efficacy. Over the same time span a number of disease modifying drugs were marketed for MS, decreasing the focus on IVIG. MS is a common neurological disease affecting approximately 400,000 people in the USA. After trauma, it is the most common cause of severe disability in young adults.[4] MS is an inflammatory demyelinating disease with progressive axon loss

and disability. The illness commonly begins with clearly defined acute attacks which resolve (relapsing-remitting MS; RRMS). Many patients will ultimately develop worsening neurological deficits over the first 15 years of their disease (secondary-progressive MS; SPMS), but approximately 10–15% of MS patients will develop a continuously progressive disease without periods of exacerbation (primary progressive MS; PPMS). Therefore early treatment during the early period of RRMS has been the design of most clinical trials with the hope to improve disease course.

Several randomized, double-blind studies in RRMS have shown a beneficial effect of IVIG in MS, including a reduction in the relapse rate and CNS lesion activity as detected by magnetic resonance imaging.[5-7] More recent studies did not find a beneficial effect of IVIG in RRMS,[8] or in secondary progressive MS.[9] In the first study patients received 0.2 or 0.4 g⁄kg of IVIG every 4 weeks for a year. At that point there were no differences from placebo in the proportion of relapse-free patients nor in the extent of lesions observed by magnetic resonance imaging (MRI) which was followed weekly. However, there were notable differences between the later negative trials and the earlier positive ones. Among these, later studies of IVIG in RRMS often used smaller dosages and, patients were treated at different stages in disease. As alternative drugs for MS expanded, patients in later trials were often using prescribed medications prior to being treated with IVIG. The therapeutic dose of IVIG is typically 2 g per kg of body weight administered as 5 daily doses of 400 mg per kg—clearly a large and expensive dose of drug, but at present there is little data supporting a minimum effective dose or an established dosing regimen for MS. For example, one positive trial treated patients monthly for two years with IVIG to observe a reduction in disability and relapse rate in RRMS.[5] In addition, positive trials treated MS patients at the time of exacerbation. If MS patients were treated at a time in disease progression, when extensive axon loss had occurred, IVIG could not reverse these deficits. The possible importance of length of treatment may also be a factor in the outcome of IVIG trials for optic neuritis.

IVIG TO TREAT OPTIC NEURITIS

Visual problems, including decreased color vision, visual field deficits and visual acuity loss, are common in MS[10] with optic neuritis occurring in up to 65% of patients. IVIG treatment has been tested in patients with optic neuritis who failed to recover visual acuity after six months,[11] and in patients during the acute phase of optic neuritis.[12] Both trials treated patients over the course of three months. In the first study patients had moderately advanced deficits, often for several years. Neither study showed a beneficial effect on long-term visual acuity. The Noseworthy study[13] reported that although there was no improvement in visual acuity, the stated endpoint of the trial, an improvement in visual field measurements nearly approached significance. A recent trial in optic neuritis by Tselis[14] which did report a recovery of visual acuity, initiated IVIG treatment early and continued its use for six months. Another difference in this third trial was that IVIG was administered after patients failed to respond to high-dose intravenous methylprednisolone. These studies indicate that dosage and timing of IVIG therapy are important variables. Therefore, failure to demonstrate clinically significant improvements in optic neuritis by using IVIG may reflect arbitrary choices in treatment regimens. If treatment is begun after severe or irreversible axonal loss or is not sustained for a sufficient period, potential benefits could be missed.

LESSONS FROM NAbs TO PROMOTE CNS REMYELINATION

The observation that natural antibodies could promote CNS repair was first described in the mouse model of TMEV-induced demyelination. Theiler's murine encephalomyelitis virus (TMEV)–mediated disease results in spinal cord lesion pathology nearly identical to that observed in human progressive multiple sclerosis (MS) with progressive neurologic deficits.[15] Spinal cord demyelination begins at 21 days post virus infection and plateaus at 90 days post infection. After this point, the disease involves primarily axonal injury and the accumulating dropout of large caliber axons, accounting for progressive clinical deficits.[16-18] This model provides a platform for testing reagents designed to promote remyelination as well as to protect axons.

To test the hypothesis that virus-induced autoimmunity could be exacerbated by a subsequent autoimmune response to CNS antigens, mice with chronic TMEV-induced demyelination were immunized with complete spinal cord homogenate in incomplete Freund's adjuvant. However, when the spinal cords of these mice were examined five weeks later, rather than more extensive demyelination, significant remyelination was observed.[19] Passive-transfer studies in which antisera or purified immunoglobulins from uninfected mice immunized with myelin antigens were transferred into mice with chronic TMEV-mediated spinal cord demyelination also demonstrated extensive remyelination after five weeks compared with mice that received control antisera.[20] An identical spinal cord immunization protocol performed in mice with spinal cord injury described a greater number of regenerating new axons through the injury site,[21,22] suggesting that this autoreactive polyclonal response can affect a number of aspects of CNS repair.

To determine whether a monoclonal antibody could also promote remyelination, hundreds of supernatants from mouse monoclonal clones directed against spinal cord homogenate were tested in groups of 10 in TMEV-infected mice for the ability to improve spinal remyelination. If a group of supernatants was positive, the individual clones were then tested for the ability to improve remyelination. This resulted in identifying the first remyelination-promoting mAb, an IgM designated spinal cord homogenate 94.03 (SCH 94.03).[23] As little as 10 μg of SCH94.03 injected intraperitoneally into TMEV-infected mice, induced four fold greater remyelinated lesion area than isotype control treated mice. Upon characterizing SCH94.03, it bound to the surface of living oligodendrocytes.[24] At that point we hypothesized that monoclonal IgM-mediated remyelination required recognition of oligodendrocyte plasma membrane antigens. Several well-characterized mouse IgMs which recognize glycolipids on the surface of living oligodendrocytes (A2B5, O1, O4, HNK-1) and myelin in live slices of CNS[25] were tested in the TMEV model and all promoted remyelination.[26] In contrast, a mouse IgG directed against ganglioside GD3 (R24) did not promote remyelination, providing the first evidence that the IgM isotype may be necessary for in vivo efficacy. These repair-promoting monoclonals had some characteristics of NAbs. They were all of the IgM isotype, and were polyreactive with low affinity with a range of structurally unrelated, self and non-self antigens, specifically cytoskeleton, nuclear proteins and DNA.[24,27,28]

HUMAN ANTIBODIES THAT PROMOTE REMYELINATION

Armed with the observation that NAbs of the IgM isotype bind to oligodendrocytes and induced remyelination we asked whether similar NAbs were present in the human

population and if so, could these antibodies promote remyelination? We employed two strategies to identify potential therapeutic human antibodies.

The first strategy employed the use of the Mayo Clinic serum bank, a collection of over 140,000 samples archived over 50 years. The archive was searched for samples from patients with monoclonal gammopathies; diseases such as lymphoma, Waldenstrom's syndrome, myeloma and monoclonal gammopathy of unknown significance, which result in an immortalized B-cell clone and a high level of serum monoclonal protein. Samples with more than 10 μg/ml of monoclonal protein were screened for binding to slices of live mouse cerebellum and to the surface of primary mixed glial cells in culture.[29] Six of 52 serum-derived human IgMs (sHIgM) and 0 of 50 serum-derived human IgGs (sHIgG) bound in these assays. The histories of the patients synthesizing these positive samples were examined to ensure that there were no neurologic- or antibody-associated diseases. Monoclonal antibodies were purified and then tested for the ability to promote remyelination. A single 500 μg peripheral dose of either of two human IgMs (sHIgM22 and sHIgM46) promoted significant remyelination in chronically TMEV-infected mice.[29] Oligodendrocytes isolated from human tissue from patients undergoing temporal lobectomy for epilepsy demonstrated that both human IgMs bound to the surface of human oligodendrocytes in vitro. This provided evidence that these IgMs may be effective in promoting remyelination in humans. We concluded that reparative NAbs do exist in the human population.

Our second strategy to obtain human monoclonal antibodies employed the use of Epstein Barr virus to immortalize human peripheral B-cell lymphocytes.[30] Cells isolated from blood samples obtained from fetal umbilical cord, patients with multiple sclerosis, patients with rheumatoid arthritis and normal adults, were transformed with EBV. Clones were selected and expanded and the supernatants assayed for monoclonal protein. Our experience was similar to that reported by Brown and Miller[30] in that the majority of B-cell clones secreted a monoclonal IgM (60 of 96). Clones that produced at least 2 ug/ml of mAb were screened further. Eleven antibodies were identified that bound strongly to structures in unfixed slices of mouse cerebellum and sufficient mAb was isolated from the supernatant of eight cell lines to test in vivo at a dose of 500 μg per mouse for the ability to promote remyelination.

Four of eight human IgMs produced by EBV-immortalized cell lines that bound to live slices of mouse cerebellum promoted significant remyelination (Table 1) in the TMEV model of spinal cord demyelination ($p > 0.05$) when compared with saline-treated mice. Two were from cells isolated from MS patients, and one each from cells from a normal adult and fetal umbilical cord blood. From this result we concluded that B cells producing CNS-reactive mAbs are common in the human immune repertoire regardless of age or state of health. The fact that so many monoclonal IgMs can promote remyelination[26] emphasizes that the exact antigen bound by the IgM is not critical. What is important is that the antigen be on the membrane of oligodendrocytes and myelin and that the IgM induce a signal to the cell. The underlying mechanism of remyelination is likely common.

It is possible to use one of these cell lines to generate a remyelination promoting IgM to test in humans. However, the presence of EBV in the process of creating a certified production cell line for a molecule that may be used in a clinical trial creates a tremendous amount of additional manufacturing and safety testing when submitting to the Food and Drug Administration. To avoid this hurdle in drug development we chose to sequence the variable regions of several IgMs that promote remyelination and to construct an expression vector capable of receiving any human heavy and light chain variable region sequence to generate large quantities of pure recombinant mAb in a virus free system.

Table 1. Human monoclonal IgMs isolated from EBV immortalized B-cell lines promote CNS remyelination

mAb	# of Mice	Area of White Matter (mm^2)	Area of Myelin Pathology (mm^2)	Area of CNS-Type Remyelination (mm^2)	Area of CNS-Type Remyelination (%)
AKJR4	4	8.7 ± 0.8	1.1 ± 0.2	0.05 ± 0.03	4.2 ± 2.0
AKJR8	6	10.0 ± 0.7	1.0 ± 0.1	0.12 ± 0.02	13.3 ± 2.2
CB2BG8	12	10.2 ± 0.5	1.2 ± 0.2	0.26 ± 0.04	23.5 ± 3.1 *
CB2GE7	5	8.9 ± 1.3	1.2 ± 0.4	0.14 ± 0.03	14.3 ± 4.1
MSI10E10	5	9.3 ± 1.4	1.9 ± 0.6	0.18 ± 0.04	11.3 ± 3.7
MSI11G4	5	8.4 ± 0.6	1.1 ± 0.3	0.24 ± 0.07	22.8 ± 6.1 *
MSI19D10	13	8.1 ± 0.3	1.1 ± 0.1	0.27 ± 0.05	24.4 ± 2.9 *
NA8FE7	5	8.5 ± 0.8	0.9 ± 0.2	0.14 ± 0.04	16.5 ± 3.6 *
Saline	12	9.9 ± 0.4	1.1 ± 0.1	0.07 ± 0.01	8.3 ± 1.4

Eight human monoclonal IgMs that bound to live slices of live mouse cerebellum were tested for the ability to induce remyelination—four were positive. This result indicates that NAbs that can promote CNS repair are common in the human Ig profile. SJL mice with chronic TMEV-mediated spinal cord demyelination received 0.5 mg of purified human IgM intraperitoneally and the extent of repair was assessed five weeks later by histologic examination of spinal cord lesions.[29] Antibody nomenclature indicates the origin of peripheral B cells used for EBV immortalization: AKJR, rheumatoid arthritis patients, CB, fetal umbilical cord blood, MSI, multiple sclerosis patients, NA, normal adult. Values represent the mean ± standard error of the mean. Statistics by t-test of the percentage of area of CNS-type remyelination per area of white matter pathology in mice treated with human IgMs as compared with those treated with PBS. Only animals with areas of white matter pathology ≥ 5% were included in statistical analysis. * indicates $p < 0.05$.

DEVELOPMENT OF A RECOMBINANT EXPRESSION VECTOR FOR HUMAN IgMs

Using the expression vector described[31] and derivations of it we have constructed cell lines that synthesize pure recombinant human IgMs with a mouse or human J chain as part of the final pentameric molecule. The first recombinant human IgM produced using this vector was called rHIgM22. This antibody promoted spinal cord remyelination in the TMEV model equal to or better than the serum-derived form.[31] The recombinant IgM, rHIgM22 binds strongly to the surface of live oligodendrocytes and myelin and accelerates CNS remyelination in a toxin-induced model of MS as well.[32] Spinal cord remyelination by rHIgM22 is induced after a single low dose of 25 μg/kg,[33] similar to the low doses required for efficacy of the mouse IgM, SCH94.03. It is remarkable that a single peripheral injection of the IgM promotes maximal tissue repair within five weeks in a model of chronic progressive MS that normally presents with little spontaneous repair over the lifespan of the animals. Spinal cord myelin repair by rHIgM22 was also assayed non-invasively using T1 and T2 weighted MRI.[34] Every one of 13 mice treated with rHIgM22 showed a remarkable decrease in overall lesion volume load in the spinal cord after five weeks as detected by MRI after just a single dose of human IgM.

It has been generally accepted that IgMs, with a molecular weight of close to 1 million, are too large to cross the blood-brain barrier (BBB) and enter the CNS. However, evidence is accumulating that some IgMs can cross the BBB.[35,36] We have shown that[35] S-methionine labeled SCH94.03 administered intraperitoneally accumulated in the brain and spinal cord of normal and TMEV-infected mice—to approximately 0.4% of the administered[35] S dose. We also tracked rHIgM22 in vivo in TMEV-infected mice by MRI using a 7-Tesla magnet.[34] rHIgM22 entered the brain and accumulated at demyelinated lesions. In mice that were not infected with the virus or did not have demyelination, rHIgM22 did not accumulate in the CNS nor did a control human IgM that does not bind to myelin or oligodendrocytes. These data support the concept that some IgMs can cross the BBB with low efficiency, enter the CNS, and accumulate within injured regions of the CNS. It should be stressed that very little drug is required in the CNS to produce a therapeutic effect. Only 0.02% of a peripheral dose of morphine enters the brain, yet this is sufficient for analgesia. For most CNS therapeutics on the market, less than 0.2% of a peripheral dose enters the CNS. If an antibody of interest does not enter the CNS sufficiently well pumps can now reliably deliver small volumes directly to the sub arachnoid space and the cerebral spinal fluid.

EVIDENCE THAT THE IgM ISOTYPE IS SUPERIOR TO IgG IN REPAIR OF THE NERVOUS SYSTEM

There is now strong evidence supporting the hypothesis that the monoclonal IgMs that promote remyelination do so by direct signaling of specific nervous system cells rather than by a homeostatic mechanism attributed to the larger multiple doses of IVIG.[37,38] IVIG and polyclonal human IgM were tested for the ability to promote remyelination in the TMEV model.[29] Both were effective, but polyclonal IgM and two human monoclonal IgMs were far superior. In the lysolecithin model of demyelination a single dose of human polyclonal IgM accelerated remyelination similar to the monoclonal IgMs, while IVIG was no different than the saline-treated control group.[32] Recognition of the appropriate tissues or cells appears to be an important defining characteristic even of polyclonal Ig preparations. Polyclonal human IgM binds well to the surface of live oligodendrocytes, whereas IVIG does not. The concept to treat patients with chronic optic neuritis with IVIG[11] was driven by the observation that IVIG improved the extent of spinal cord remyelination in our mouse model of MS.[29] Our clinical and experimental data suggest that polyclonal human IgM, monoclonal human IgMs that bind to oligodendrocytes or a subfraction of IVIG with an affinity for myelin and/or oligodendrocytes may be better reagents for trial in chronic optic neuritis.

Based on the observation that polyclonal IgM bound to oligodendrocytes, whereas IVIG did not, we tested whether the monoclonal IgMs that promoted remyelination remained effective as fragments. Monomers or $F(ab')_2$ fragments of the human monoclonal sHIgM22 did not bind to oligodendrocytes with sufficient affinity to be detected by flow cytometry or immunocytochemistry.[39] An IgG_4 form of rHIgM22 did not bind detectably to the surface of oligodendrocytes. Pre-clustering the IgG_4 form with a secondary antibody increases oligodendrocyte binding to detectable levels, but still without the intensity of the IgM form (unpublished). A natural IgG_1 switch variant was isolated from cells synthesizing the mouse IgM, SCH94.03.[39] The switch variant was characterized by sequence analysis of protein and the cDNA isolated from a cloned cell

line. The variable region of the heavy and light chains of the IgM and IgG_1 antibodies were identical differing only in the heavy chain constant region used for assembly into a pentameric or monomeric form. The IgG_1 form did not bind to live cerebellar slices or to the surface of live oligodendrocytes. When tested in vivo for the ability to promote remyelination SCH94.03 IgG_1 did induce remyelination (22.7% of spinal cord quadrants, $p = 0.025$) compared with the endogenous level of repair (20.1%), but far from the level of repair induced by SCH94.03 IgM (41.0% $p < 0.001$). A comparison indicated that all three treatment groups were different from each other again supporting the concept that the IgM is more effective than the IgG form.

We concluded that the individual variable region binding sites of the reparative IgMs have a low affinity for their target, but that the avidity of the pentameric IgM structure with 10 potential binding sites facilitates the biologic activity. Avidity describes the combined strength of multiple interactions. Avidity is distinct from affinity, which describes the strength of a single interaction. Avidity describes a synergistic increase in binding strength rather than only the sum of each individual interaction. An IgM with multiple binding sites can simultaneously interact with several of the same antigens. When an IgM is bound to a fluid target on a cell membrane any individual variable region binding interaction may be broken, but with many binding interactions occurring simultaneously, the loss of a single site prevents the loss of the overall multivalent structure.[40]

Ca^{2+} SIGNALING IS A COMMON LINK IN IgMs THAT PROMOTE REMYELINATION

To establish a connection between an antibody's ability to bind to oligodendrocytes and a direct signaling event in those cells, we studied Ca^{2+} influx. Transient elevation of Ca^{2+} in cells is associated with cell survival, whereas persistent elevation of Ca^{2+} is associated with cell death. The remyelination-promoting antibodies SCH94.03, SCH79.08, O4, sHIgM22, sHIgM46, and CB2BG8 all induced Ca^{2+} responses in oligodendrocytes (OL) (Table 2). Isotype control antibodies CH12, AKJR4, sHIgM12, sHIgM14, and sHIgM47 which do not promote remyelination, do not evoke Ca^{2+}responses. These data demonstrate a strong relationship between antibody-mediated Ca^{2+} signaling and promotion of remyelination ($p = 0.015$, Fisher exact). We also showed that signaling in oligodendrocytes is mediated by ligand-gated ion channel glutamate receptors[37] and that Ca^{2+} flows in from external sources. Disrupting oligodendrocyte lipid microdomains (lipid rafts) by the detergent B-methyl cyclodextrin prevents Ca^{2+} signaling[41] suggesting that the Ca^{2+} channels are functionally linked within microdomains. The IgG_1 switch variant of SCH94.03 was not able to induce Ca^{2+} signals in oligodendrocytes.[39] However, pre-treating oligodendrocytes with the IgG_1 form could block Ca^{2+} flux induced by the IgM, indicating that the IgG_1 can bind to the target cells sufficiently well to prevent the IgM from accessing enough antigen on the membrane to allow signaling. The IgG_1 form of SCH94.03 did not block the ability of rHIgM22 to induce Ca^{2+} flux indicating that the IgG_1 is not blocking overall antibody binding and signaling, but is interfering only with the ability of SCH94.03 IgM to broadly access the plasma membrane. These studies again stress the concept that the absolute oligodendrocyte surface antigen of an IgM is not important in the ability to promote remyelination. What is important is the ability to bind well enough to signal the target cells.

Table 2. IgM induced Ca^{2+} response correlates with the ability to promote remyelination

Antibody	Species	Remyelination	OL Surface Binding	% Responding OLs
O4	mouse	yes	yes	21*
SCH94.03	mouse	yes	yes	27*
SCH79.08	mouse	yes	no	25*
sHIgM22	human	yes	yes	10*
sHIgM46	human	yes	yes	11*
CB2BG8	human	yes	yes	22*
AKJR8	human	yes	no	36*
CH12	mouse	no	no	0
sHIgM12	human	no	no	0
sHIgM14	human	no	yes	0
sHIgM47	human	no	yes	0
AKJR4	human	no	no	0

The ability of 10 μg/ml of IgM to induce Ca^{2+} flux in mixed primary glial cultures was performed as described.[37] Between 60 and 125 individual OLs were examined for each treatment group. An IgM's ability to bind to the surface of OLs as determined by immunocytochemistry did not correlate with the ability to promote remyelination in the TMEV model of spinal cord demyelination. However, an IgM's ability to induce a Ca^{2+} response did correlate with the ability to promote remyelination. Human IgMs were compared with sHIgM12, and mouse IgMs were compared with CH12, a human and mouse IgM, respectively, that do not bind to mature OLs or promote remyelination in vivo. Comparisons of induced Ca^{2+} flux in OLs using Z-test revealed a significant difference *($p < 0.002$). All other comparisons did not reach $p < 0.05$.

NATURALLY OCCURRING AUTOANTIBODIES IN THE TREATMENT OF ALZHEIMER DISEASE

IVIG contains NAbs directed against β amyloid (Aβ) and IVIG may be effective in attenuating cognitive impairment in Alzheimer disease.[42,43] Endogenous autoantibodies, of both IgG and IgM isotypes, directed against Aβ are common in normal humans[44,45] suggesting a role in the normal homeostasis of Aβ. It is well established that anti-Aβ antibodies delivered to the brains of mice that overexpress Aβ can reverse histologic and cognitive impairment[46,47] and these observations underlie the Aβ immunization strategies in human trials. Monoclonal antibodies against Aβ, primarily high affinity IgGs, are under development as drugs after the failure of Aβ protein immunization trials in humans, but there is also a growing interest in anti-Aβ IgMs. Lindhagen-Persson[48] found that naturally occurring human anti-Aβ IgMs bound strongly to oligomeric Aβ. The authors suggest that IgM avidity can be exploited to target oligomeric Aβ in vivo and that the normal role of human anti-Aβ IgMs may be protective. A study by Banks et al.[36] evaluated the use of human monoclonal IgMs directed against Aβ to improve a transgenic mouse model of Alzheimer disease. Antibodies were delivered by intracerebral injection or intravenously. The IgM that accumulated in the brains of normal and diseased mice improved both memory and learning deficits in this model.

CONCLUSION

IVIG use has expanded greatly since 1980 and its efficacy is proven in several neurologic diseases. However, the use of IVIG to treat long-term chronic diseases such as multiple sclerosis and Alzheimer is not realistic. IVIG in these diseases may be replaced with monoclonal antibody therapy if the appropriate targets can be identified. Focusing the use of IVIG for diseases which actually require all of the Ig components will eliminate the occasional shortages of IVIG.

The serum of healthy humans contains naturally occurring autoantibodies directed against the surface of oligodendrocytes, neurons and Aβ. Targeting these molecules with mAbs, especially monoclonal IgMs, may modulate disease. One recombinant human monoclonal antibody, rHIgM22, derived from the NAb repertoire binds to lipid rafts on oligodendrocytes, induces calcium influx through ligand-gated ion channels, induces the phosphorylation of specific signaling molecules and induces in vivo remyelination. Long-term treatment of primary oligodendrocytes in culture with rHIgM22 prevented apoptotic signaling and inhibited oligodendrocyte differentiation by Lyn.[38] In contrast to current therapies for multiple sclerosis aimed at modulating inflammation, remyelination-promoting IgMs are designed to induce tissue repair by acting within the CNS at sites of damage on the cells responsible for myelin synthesis. We propose that those monoclonal IgMs that promote remyelination do so by initiating a cascade of events, beginning with binding to the oligodendrocyte plasma membrane, likely to a sphingolipid. The pentameric IgM rearranges membrane microdomains and the associated signaling molecules, whereby calcium influx initiates the activation of signal transduction molecules. Activated signal transduction molecules lead to changes at the level of protein transcription that alters oligodendrocyte survival and ultimately the extent of new myelin that protects axons from further damage.

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CHAPTER 5

NATURE AND NURTURE OF CATALYTIC ANTIBODIES

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Abstract: Immunoglobulins (antibodies) frequently express constitutive functions. Two such functions are nucleophilic catalysis and the reversible binding to B-cell superantigens. Constitutive or "naturally-occurring" antibodies are produced spontaneously from germline genetic information. The antibody structural elements mediating the constitutive functions have originated over millions of years of phylogenic evolution, contrasting with antigen-driven, somatic sequence diversification of the complementarity determining regions (CDR) that underlies the better-known high affinity antigen binding function of antibodies. Often, the framework regions (FRs) play a dominant role in antibody constitutive functions. Catalytic antibody subsets with promiscuous, autoantigen-directed and microbe-directed specificities have been identified. Mucosal antibodies may be specialized to express high-level catalytic activity against microbes transmitted by the mucosal route, exemplified by constitutive production of IgA class antibodies in mucosal secretions that catalyze the cleavage of HIV gp120. Catalytic specificity can be gained by constitutive noncovalent superantigen binding at the FRs and by adaptive development of noncovalent classical antigen or superantigen binding, respectively, at the CDRs and FRs. Growing evidence suggests important functional roles for catalytic antibodies in homeostasis, autoimmune disease and protection against infection. Adaptive antibody responses to microbial superantigens are proscribed under physiological circumstances. Covalent electrophilic immunogen binding to constitutively expressed nucleophilic sites in B-cell receptors bypasses the restriction on adaptive antibody production, and simultaneous occupancy of the CDR binding site by a stimulatory antigenic epitope can also overcome the downregulatory effect of superantigen binding at the FRs. These concepts may be useful for developing novel vaccines that capitalize and improve on constitutive antibody functions for protection against microbes.

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

INTRODUCTION

Structural stability of proteins is vital for maintaining higher-order life functions, and keeping proteins free of somatic mutations within the life-time of an individual is a hallmark of the disease-free state. The adaptive immune system is an exception. Immune molecules are specialized to mutate adaptively by somatic means, exemplified by the six complementarity determining regions (CDRs) of the immunoglobulin light and heavy chain variable domains (V_L and V_H domains). Within days to weeks, the CDRs acquire the ability to bind target antigens specifically with high affinity, a crucial property for defense against microbial infections.

The inherited antibody repertoire is usually conceived as a plastic starting point for adaptive development of specific, high affinity antigen binding sites formed by the CDRs. In humans, the inherited repertoire of germline genes that ultimately gives rise to the antibody V domains consists of about 50 light and heavy chain V_L and V_H region genes each, tens of diversity (D) genes, and tens of joining (J) genes. The lowest extant organisms in the phylogenetic tree of life expressing recognizable immunoglobulin molecules are the jawed fish (Fig. 1).[1] The ability to mutate antibodies had already

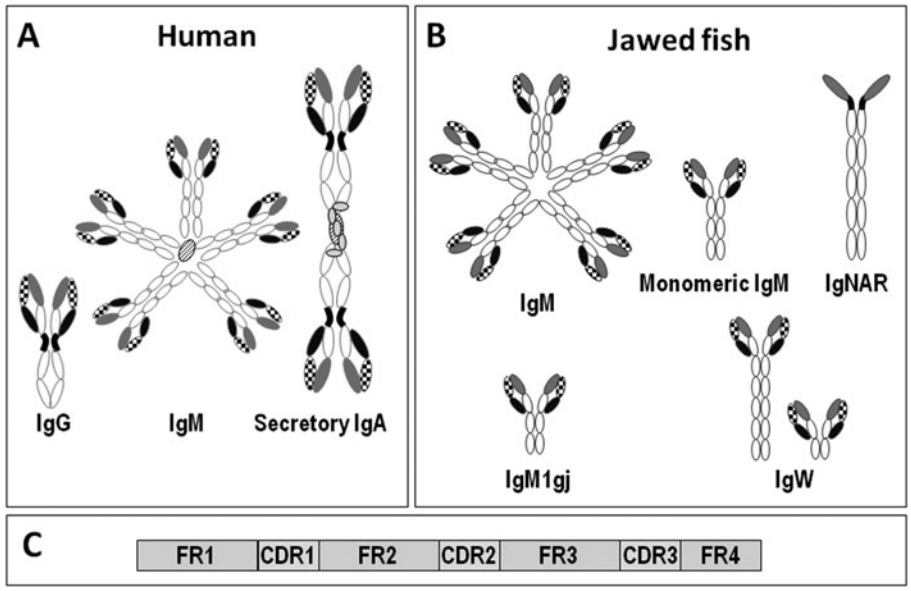

Figure 1. A) Domain organization of human IgM, IgG and secretory IgA. B) Domain organization of immunoglobulin isoforms in cartilaginous fish. C) Organization of complementarity determining regions (CDRs) and framework regions (FRs) of a typical variable (V) domain. Gray and white, respectively, heavy chain V and constant domains; Stippled and black, respectively, light chain V and constant domains. In fish (panel B), IgW refers to an isoform containing two forms of the heavy chain, a long seven domain form and a short 3 domain form. IgNAR (NAR, novel antigen receptor) is a heavy chain homodimer free of light chains. IgM1gj (gj, germline-joined) contains a V_H domain joined without somatic diversification at the V-D-J junctions. The monomeric IgM form is found in blood. Three L chain isotypes that cannot be classified as either κ- or λ-like have been identified in cartilaginous fish. Summarized from reference 1. In panel (C), binding of B-cell superantigens and traditional antigens is dominated, respectively, by the FRs and CDRs. A color version of this figure is available online at www.landesbioscience.com/curie.

appeared at this stage, albeit at a rudimentary level compared with higher organisms. No extant organism with immutable immunoglobulins has been identified. The missing link of a less sophisticated pre-form suggests an abrupt evolutionary advantage due to the development of somatically mutable immunoglobulins. This inspired John Marchalonis and coworkers to suggest the "big-bang" theory of adaptive immunity.[2] The concept of adaptive immunity mediated by the V domains is a central tenet of modern immunology, and it has been applied to great advantage in biotechnology, witnessed, for example, by the current > $15 billion/year market for therapy with monoclonal antibodies displaying specific, high affinity antigen binding activities acquired by adaptive means.

Interspersed between the V domain CDRs are the framework regions (FRs). Like the CDRs, the FRs undergo random sequence diversification by somatic hypermutation. However, classical antigens generally fail to select the FR mutations, as their primary contact amino acids are located in the CDRs. Thus, it is often stated that the FRs merely form a scaffold enabling the CDRs to fulfill their adaptive function in the somatic immune response. This view is no longer tenable given structural and functional evidence concerning subsets of antibodies with catalytic activity and reversible binding activity for microbial superantigens. These antibody subsets express constitutive V domain functions expressed without the requirement for antigen-driven somatic selection, often entailing a central participation of the FRs. There is growing realization of the beneficial and harmful effects of these functions in homeostasis, microbial infection and autoimmune disease (Table 1). FR and CDR encoded constitutive antibody functions have presumably improved over the course of phylogenic evolution under the influence of discrete selection pressures. CDR loop sequences, for instance, could undergo improvement if they enjoy superior conformational flexibility enabling adaptive development of binding to diverse epitopes in concert with random structural diversification. A more refined appreciation of the constitutive V domain functions may help explain long-standing immunological uncertainties and could offer innovative solutions for diseases that have remained intractable by conventional vaccine and immune therapy approaches, e.g., HIV infection, *Staphyloccus aureus* infection and Alzheimer disease.

CONSTITUTIVE OR "NATURALLY-OCCURRING" ANTIBODIES

There is no precise meaning of the terms "naturally-occurring" antibodies and "natural antibodies." The terms were coined to explain observations of antibody reactivities that can be differentiated functionally or structurally from those of antibodies induced by stimulation of the immune system with a known antigen. The "natural" and "induced" antibody subsets are both products of nature. Further, it is possible that certain "natural" antibodies" are actually "induced," e.g., their production may depend on autoantigen-driven adaptive selection of B cells. Therefore, the authors prefer to avoid these terms except when it is demonstrably clear that the antibodies are synthesized free of the influence of antigen. The first antibodies classified as "naturally-occurring" were polyreactive antibodies with the ability to bind two or more antigenic epitopes.[3,4] Polyreactive antibodies often recognize epitopes with no discernible structural resemblance. Thus, it is difficult to explain their binding pattern on the basis of cross-reactivity. Despite modest to low affinity, polyreactive antibodies are important for immunological defense because of their high concentrations in biological fluids. Indeed, large proportions of antibodies found in blood and mucosal secretions are thought to express polyreactive binding activity.

Table 1. Antibody subset expression and functions in various immune states. The classical antibody subset is composed of antibodies that develop high affinity antigen binding activity by traditional B-cell clonal selection. Constitutive antibodies included in the table are the catalytic, superantigen-binding and polyreactive antibody subsets.

Immune Phenotype	Constitutive Antibodies	Classical Antibodies
Homeostasis	• Polyreactive autoantigen binding • Promiscuous catalytic clearance of autoantigens • Catalytic mucosal defense against microbial superantigens	• No high affinity antibody synthesis
Autoimmune reactions	• Diminished catalytic clearance of small autoantigen peptides • Pathogenic, specific catalytic destruction of autoantigens • Beneficial, specific catalytic destruction of toxic autoantigens (e.g., amyloid) • Beneficial and harmful catalytic activation of zymogens	• Pathogenic autoantigen binding • Beneficial toxic autoantigen binding (e.g., amyloid)
Response to microbial infection	• Diminished catalytic clearance of small microbial peptides • Downregulated superantigen binding • Very slow adaptive amplification of superantigen catalytic cleavage • Adaptive catalytic response to CDR-binding antigens?	• Beneficial microbe binding
Response to vaccination	• Adaptive covalent vaccine-induced covalent and catalytic activity • Covalent vaccine-induced bypass of forbidden adaptive response to superantigens	• Beneficial microbe binding

Polyreactive antibodies are produced constitutively without stimulation by an exogenous antigen molecule. The clonal selection theory holds that antigen binding to the B-cell receptor (BCR; antibody complexed to signal transducing proteins expressed on the B-cell surface) drives B-cell clonal proliferation and adaptive selection of mutant BCRs expressing the greatest antigen binding affinity. The constitutively-expressed repertoire is generated by pairing of discrete V_L/V_H domains that are in turn produced from about 150 distinct V, D and J germline genes by V-(D)-J gene recombination, a step entailing extensive removal and addition of nucleotides defining the structure of CDR3 of each subunit. Structural diversity attributable to the V-(D)-J gene recombination and combinatorial pairing is constitutive or innate in the sense that it is produced randomly prior to arrival of the antigen on the scene. This innate, constitutive repertoire is enormous, estimated to be ~10^{13} B-cell clones expressing antibodies with unique structures.

The central point in resolving etymological debates about constitutive vs. somatically adapted antibody subsets concerns the role of antigen. Polyreactive antigen binding by constitutively produced antibodies depends at least in part on CDR3 of the V_H domain.[3] Therefore, V-D-J somatic diversification must contribute to acquisition of the polyreactive binding activity. Germ-free animals produce polyreactive antibodies, indicating that stimulation with exogenous antigens is not essential. A role for self-antigen in driving polyreactive antibody formation can be conceived, as these antibodies often bind self-antigens.[3,4] On the other hand, mitogen and cytokine stimulation alone is sufficient to drive differentiation of the constitutive B-cell clones into polyreactive antibody-producing plasma cells.[5] Also, the polyreactive BCR may be capable of autonomous positive signaling in a pre-BCR manner with no requirement of antigen signaling.[6]

BEYOND BINDING: CATALYTIC ANTIBODIES

The field of catalytic antibodies emerged from an interesting nurture vs. nature conundrum that lasted more than a decade. The idea that catalysts can be applied for efficient chemical transformation of biological and industrial molecules inspired chemical engineers to conduct immunizations with tetrahedral transition state analogs (TSAs) of ester substrates. The resultant antibodies were advertised as "nurtured" molecules with a tailored ability to catalyze ester hydrolysis attributable to noncovalent stabilization of the negatively charged transition state of the esterase reaction.[7] Later studies identified protease antibodies[8] and nuclease antibodies[9] that were generated by *bona fide* natural mechanisms with no reliance on immunization with artificial antigens. Mechanistic studies suggested that some antibodies raised by immunization with the TSAs actually use the same nucleophilic mechanism for chemical catalysis as naturally-occurring catalytic antibodies.[10] Moreover, it turned out that the immunogens thought to be chemically inert TSAs actually express electrophilic reactivity sufficient to bind covalently to the constitutive nucleophilic sites of antibodies.[11] This suggested that the TSAs stimulate adaptive improvement of the constitutive nucleophilic site. Clearly, nature played no small role in what was then thought to be the "nurtured" esterase function of the antibodies. A better example of nurtured catalysis may be the induction of a lactamase antibody by immunization with an anti-idiotypic antibody to a β lactamase enzyme.[12] In this molecular imprinting strategy, the anti-idiotypic antibody is assumed to express a surface complementary to the enzyme active site, thereby inducing an antibody with enzymatic activity.

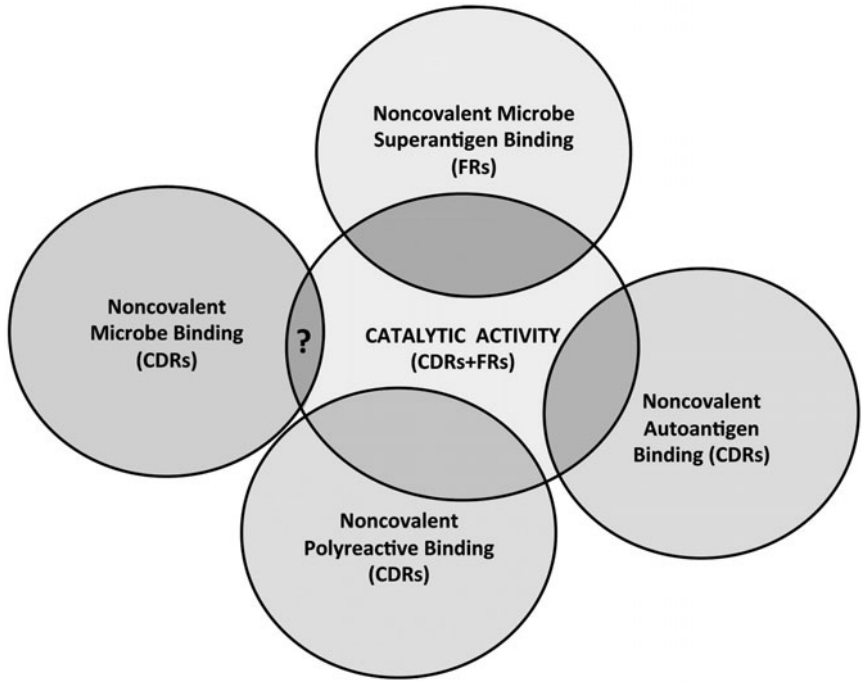

Figure 2. Antibody subsets with distinct functions and structural genesis. Note overlapping regions of the catalytic and other antibody subsets that impart to the catalytic antibodies their functional characteristics in homeostasis, autoimmune disease and microbial infections. The ? symbol denotes current lack of example antibodies that can effectively combine the catalytic function with noncovalent CDR-based recognition of microbial antigens. In contrast, numerous examples of autoantigen-specific catalytic antibodies are available. Assuming that autoantigen noncovalent binding occurs largely at the CDRs, it may be concluded that expression of the catalytic function in the CDR-driven autoimmune response is feasible. Both catalysis and ability to bind microbial superantigens noncovalently at the FRs are germline V gene functions expressed by constitutively-produced antibodies. Initial examples of combined expression of these functions are available. A color version of this figure is available online at www.landesbioscience.com/curie.

Kohler and Paul summarized the evidence for antibody interactions with ligands and substrates outside the classical antigen binding sites formed by the CDRs, including the interactions enabling chemical catalysis.[13] The immune genesis and functional roles of various catalytic antibody subsets is distinct from the classical antigen-binding antibodies (Fig. 2). As for classical antibodies, the initial step in catalysis by antibodies is noncovalent antigen binding (Fig. 3; noncovalent immune complex). Thereafter, a nucleophilic catalytic site in the spatial vicinity of the antigen binding site catalyzes the chemical conversion of the polypeptide antigen (Fig. 3; covalent immune complex 1 and 2).[14] The catalytic amino acids are located in part in the FRs and in part in the CDRs.[15-17] Catalysts turn over repeatedly to cleave multiple antigen molecules, and catalytic antibodies neutralize antigens more potently than conventional antibodies. Certain antibodies utilize the nucleophilic sites to form covalent immune complexes without proceeding to completion of the catalytic cycle. Such antibodies express enhanced antigen neutralizing activity due to their "infinite affinity" for the antigen. A review of catalytic antibodies and their relationship with other major antibody subsets follows.

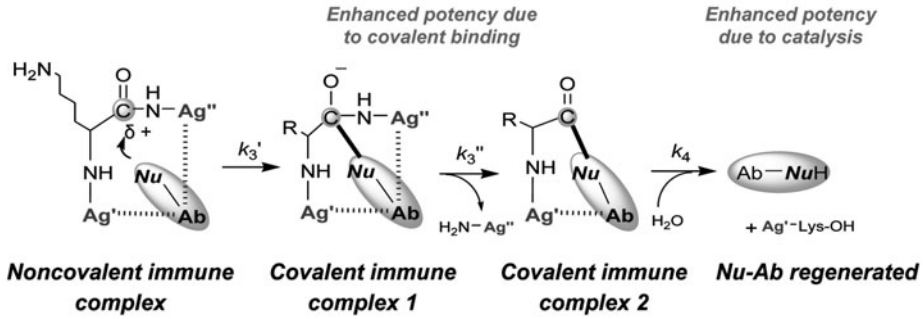

Figure 3. Mechanism of antigen-specific proteolysis by antibodies. Specificity is derived from noncovalent epitope–paratope binding. The antibody nucleophile attacks the weakly electrophilic peptide bond carbonyl. Covalent immune complex 1 is a resonant stable complex prior to expulsion of the C-terminal antigen fragment. Covalent immune complex 2 is an acyl-Ab complex. Ag' and Ag'' are components of the epitope recognized by the Ab. Ag'Lys-OH is the N-terminal antigen fragment and NH_2-Ag'' is the C-terminal antigen fragment. Covalent antigen binding alone is sufficient to enhance neutralizing potency compared with reversibly-binding antibodies, as the covalent reaction results in non-dissociable immune complexes. If catalysis occurs (that is, if active Ab is regenerated), this enhances potency further by reuse of a single catalyst molecule to permanently inactivate multiple target antigen molecules.

PROMISCUOUS CATALYTIC ANTIBODIES

Healthy humans and animals express catalytic antibodies that catalyze the hydrolysis of small amide and peptide substrates.[18-20] Other than requiring a positively charged Arg/Lys on the N-terminal side of the cleavage site, this catalytic antibody subset is promiscuous, displaying very low noncovalent binding affinity (Km values in the high micromolar range) and little or no dependence on the structure of the 'microantigen' substrate. Clearly, these antibodies fulfill their catalytic function without dependence on the classical adaptive development of noncovalent antigen recognition forces. The catalytic function itself is germline encoded. Each of the amino acids constituting the nucleophilic, catalytic triad of an antibody V_L domain are encoded by its germline V gene counterpart.[16] The adaptively-matured V_L contained certain somatic mutations remote from the nucleophilic triad. There was no loss of catalytic activity after reversion of the remote, somatically-derived amino acids to their germline counterparts.[16] Evidently, catalysis is a constitutive property acquired over phylogenic antibody evolution, not a de novo function developed by antigen-driven affinity maturation.

Studies on polyclonal antibodies isolated from blood provide initial insight to the fate of the catalytic function over the course of adaptive B-cell differentiation. IgMs, the first antibody class produced by B cells, expressed the promiscuous catalytic activity at high levels, whereas class-switched IgG antibodies were poorly catalytic.[19] Monomeric IgM also expressed superior catalysis compared with IgG, ruling out avidity effects as the cause of differing catalytic rates seen for the two antibody classes. IgMs and IgGs are distinguished by their differing constant domain architecture. The integrity of catalytic sites is dependent on precise, sub-Ångstrom-level spatial positioning of amino functional groups that is highly susceptible to changes in backbone movements, even movements caused by structural changes remote from the catalytic site. The feasibility of Ångstrom-level movements of V domain amino acid side chains upon attachment to the constant domains has been documented.[21] Therefore, a loss of catalytic site integrity due

to μ→γ constant domain replacement cannot be ruled out. Alternatively, as IgM→IgG class-switching occurs concomitantly with increasing accumulation of CDR mutations by the somatic hypermutation process, loss of the catalytic function activity may derive from remote CDR mutations.

Interestingly, blood-borne IgA antibodies express the promiscuous catalytic activity at levels even superior to IgM antibodies.[22] Improved activity of the class-switched, mature IgA antibodies is inconsistent with arguments of promiscuous catalysis as a vestigial property that could have been important at an early stage in antibody phylogeny but is functionally inconsequential in higher organisms. Patients with septic shock who die express low levels of promiscuous catalytic antibodies compared with patients who survive.[20] Patients with autoimmune disease also express lower levels of promiscuous catalytic antibodies than healthy individuals without disease.[18] A homeostatic function for promiscuous catalytic antibodies appears likely. Metabolic clearance of undesired proteins by antibodies with polyreactive antigen binding activity has been suggested, e.g., clearance of toxic microbial proteins and autoantigens that have outlived their utility.

Small peptides but not large proteins are cleaved rapidly by antibodies. IgA, IgM and IgG concentrations in blood are 2–10 mg/ml, compared with the nanogram/ml levels of classical protease enzymes. Promiscuous catalysis is readily detected at antibody concentrations magnitudes of orders lower than the physiological antibody concentrations. A strong case is available, thus, for metabolic clearance of small peptides by catalytic antibodies as a homeostatic function.

Consistent with observations that the free antibody light chain subunits express catalytic activities greater than intact antibodies,[23] robust peptide bond cleaving activities have been described for monoclonal light chains from multiple myeloma patients.[24,25] Some catalytic light chains induce cell death through an apoptotic pathway, an effect with potential significance for renal light chain accumulation and kidney failure in multiple myeloma patients.[26]

CATALYTIC AUTOANTIBODIES

The catalytic activity of antibodies was discovered as an autoantigen-specific function. Catalytic autoantibodies specific for vasoactive intestinal peptide (VIP),[8] DNA,[9] RNA,[27] thyroglobulin,[28] prothrombin,[29,30] Factor VIII,[31] Factor IX,[32] myelin basic protein,[33] and amyloid β peptide[34] have been reported. Catalytic antibodies may exert beneficial or deleterious effects depending on the biological context (see details below). Unlike the promiscuous catalysts in the preceding paragraph, these autoantibodies display catalytic specificity for individual autoantigens. Molecular interactions conferring specificity to the catalytic reaction can be understood from the split-site model,[35] in which noncovalent antigen binding and the subsequent catalytic cleavage step occur at two distinct but coordinated subsites (Fig. 4A,B). For the autoantigenic peptide VIP, mutations at certain antibody CDR residues resulted in loss of noncovalent binding activity without loss of catalytic rate constants.[15] Conversely, mutations at catalytic residues did not cause loss of binding affinity. Monoclonal antibodies with proteolytic activity cleave their specific substrate at multiple peptide bonds. Consistent with the split-site reaction model, the catalytic site can be oriented in register with alternate peptide bonds of the antigen in the noncovalently associated immune complex, resulting in cleavage at different bonds in the ensuing catalytic step of the reaction (Fig. 4C).

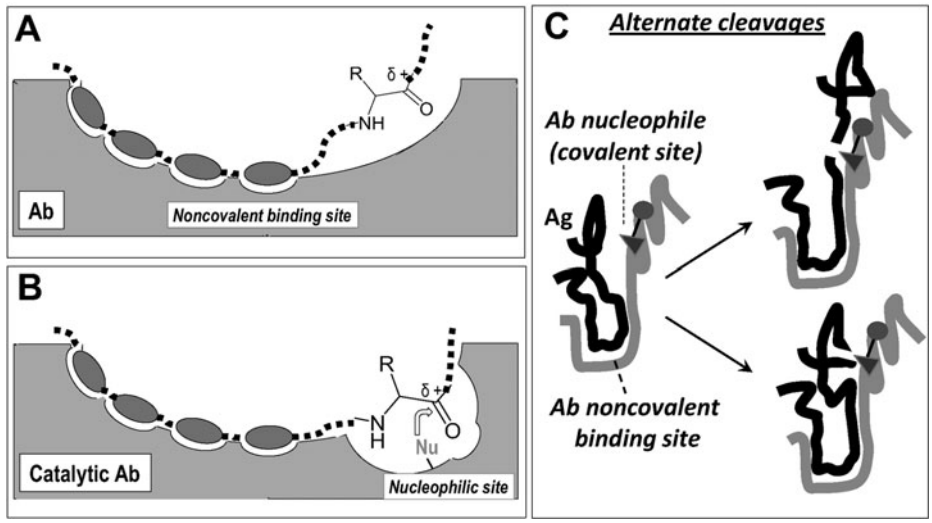

Figure 4. Split-site catalytic antibody model. A) Noncovalent epitope–paratope binding. Epitope component shown as a beaded structure. B) Noncovalent epitope–paratope binding coordinated with placement of a peptide bond of the antigen in register with the nucleophilic subsite (mostly localized in FR), initiating peptide bond hydrolysis. C) Two-step antigen recognition enabling cleavage at alternate peptide bonds. Intramolecular interactions impart nucleophilicity to an antibody residue (triangle activated by general base). Initial noncovalent binding rigidifies the peptide epitope but not the remote scissile peptide bond region. In the second step of the reaction, the nucleophile can be placed in register with alternate peptide bonds, resulting in cleavage at alternate sites by the antibody. Ab, antibody; Ag, antigen). A color version of this figure is available online at www.landesbioscience.com/curie.

Most proteolytic reactions produce functionally inactive products, e.g., the Factor VIII products obtained by antibody digestion are unable to mediate the cofactor function of intact FVIII in blood coagulation.[36] However, autoantibody-catalyzed cleavage of the precursor proteins prothrombin[29,30] and Factor IX[32] can generate the opposite result, that is, production of enzymatically active thrombin-like and Factor IXa-like products with the ability to promote coagulation. Catalytic autoantibodies were originally described as pathogenic mediators, analogous to the traditional conception of reversibly binding antigen-specific autoantibodies as mediators of Ehrlich's *horror autotoxicus* theory of autoimmunity. The Factor IX-activating catalytic autoantibodies found in acquired hemophilia patients, in contrast, are proposed to exert a beneficial pro-coagulant effect. Hemophilia in these patients is thought to be mediated at least in part by the anti-coagulant effect of FVIII binding autoantibodies. The procoagulant FIX-activating autoantibodies may be beneficial by counteracting the anti-coagulant effect of anti-FVIII autoantibodies.

The story of amyloid β peptide is also of interest. Accumulation of amyloid β aggregates in the brain is the hallmark of Alzheimer disease and amyloid β oligomer toxicity may underlie progressive neurodegeneration in this disease. Overproduction of amyloid β peptide with advancing age is devoid of any known physiological function. Catalytic autoantibodies that hydrolyze amyloid β peptide are proposed to be beneficial by virtue of their ability to impede formation of amyloid aggregates, dissolve the aggregates and block the toxic effects of the peptide oligomers.[34] Given that self-antigens can fulfill both

biologically essential and toxic functions, catalytic autoimmune reactions can be conceived to be functionally harmful when directed against biologically essential self-antigens and beneficial when directed against toxic self-antigens.

Unlike the non-covalent binding function, stimulation of the immune system by exogenous antigens generally fails to induce rapid improvement of catalysis by IgG antibodies. At the terminal step of the catalytic cycle, BCRs will release the antigen fragments produced by the proteolytic reaction. Adaptive improvement of catalysis, therefore, militates against the B-cell clonal selection theory, which holds that prolonged BCR occupancy by antigen is necessary for immune selection. Autoantibodies, on the other hand, can frequently express antigen-specific catalytic activity, and the frequent expression of catalysis by autoantibodies demands an explanation.

The answer may lie in electrophilic autoantigens that bind B cells and induce adaptive improvement of the germline encoded catalytic function. Engineered antigens containing artificial electrophiles are capable of inducing proteolytic antibodies by binding covalently to nucleophilic BCR sites.[14,19] This indicated that the nucleophilic reactivity underlying catalysis is selectable over the course of B-cell differentiation. Autoimmune diseases are often associated with increased post-translational generation of autoantigen adducts with lipid peroxidation metabolites and advanced glycation end products.[37] Such adducts contain reactive electrophiles capable of stimulating adaptive immune selection of catalytic antibody nucleophilicity. Yet another possibility is that under the abnormal B-cell regulatory pathways found in autoimmune disease, peptide bond cleavage by catalytic BCRs is itself a selectable event. Productive use of the energy liberated upon noncovalent antigen-BCR binding (binding energy) is central to initiation of signal transduction processes that eventually culminate in B-cell division and antibody synthesis. The process entails transduction of the binding energy into a remote conformational change that activates various stimulatory metabolic pathways. The cleavage reaction is a highly exothermic process. BCR signal transduction permitting productive use of the "catalytic energy" can be conceived, that is, the energy liberated from the catalytic reaction may cause a conformational change in the BCR that drives production of second messengers that in turn stimulate B-cell division. In view of accumulating evidence for autoantigen-specific catalytic autoantibodies, further study of non-traditional B-cell stimulatory mechanisms is warranted.

CATALYTIC ANTIBODIES IN DEFENSE AGAINST MICROBES

Do catalytic antibodies help the immune system fulfill its *raison d'être*, protection against microbes? As noted above, despite its promiscuous nature, the constitutive catalytic function of antibodies could potentially be useful in clearing small microbial peptides. Antibodies with DNase and RNase activity found in milk are proposed to hydrolyze viral and bacterial nucleic acids, although antibody access to internal microbial constituents was not shown.[38] Cleavage of protease-activated-receptor-2 by secretory IgA in milk induces a signal transducing pathway in intestinal epithelial cells that results in expression of anti-microbial β-defensins, which are known to help reduce neonatal infections.[39]

In addition, there is a strong connection between constitutive production of the subset of antibodies with catalytic activity and the subset that recognizes microbial B-cell superantigens by noncovalent means. Superantigens are defined as molecules recognized by antibodies with no requirement for adaptive B-cell differentiation or prior

exposure to the superantigen. Reversible antibody binding to the superantigenic sites of *Staphylococcus aureus* Protein A,[40] HIV gp120[41] and *Peptostreptococcus magnus*[42] is dominated by noncovalent interactions at antibody FRs, and certain CDR residues can also be involved. Like catalysis, superantigen binding is a constitutive antibody function derived from germline-encoded V domain elements. Discussion of the impact of reversibly binding and catalytic antibodies to a superantigenic epitope of HIV follows. The potential generality of catalytic antibody mediated defense against other microbes expressing B-cell superantigens remains to be investigated.

The immunodominant epitopes expressed by the HIV coat protein gp120 are highly mutable. However, the gp120 determinant that binds the primary HIV receptor on host cells, cell surface CD4, is essential for infection and is maintained in mostly constant form. The CD4 binding site (CD4BS) displays little structural variability in HIV-1 strains found across the world. This is one of the few immune vulnerabilities of HIV. Despite exposure of the CD4BS on the HIV surface, the immune system fails to mount a sufficiently protective antibody response to the CD4BS. The CD4BS core ($CD4BS^{core}$) is composed of residues 421–433. It overlaps the B-cell superantigen determinant of the protein.[43,44] Superantigens bind specifically to constitutively produced antibodies expressed as BCRs on the surface of B lymphocytes. Unlike the stimulatory binding of traditional antigens to the CDRs, superantigen binding at the FRs causes downregulation of B-cell differentiation, premature cell death and failure to mount an adaptive antibody response. We suggested that the constitutive ability of antibodies to bind superantigens was originally developed by Darwinian evolution processes over millions of years as a defense against primordial microbes.[45] The superantigenic $CD4BS^{core}$ epitope may be HIV's answer. Development of a CD4BS with superantigenic character leaves HIV open to constitutive immunity, but it also minimizes virus neutralization by downregulating the adaptive antibody response.

A subset of the antibodies to the superantigenic $CD4BS^{core}$ proceed to catalyze the breakdown of peptide bonds, destroying gp120 permanently.[46] The catalytic sites are present in antibodies produced without requirement for prior HIV infection.[46,47] Sexual transmission of HIV generally occurs through the rectal and vaginal mucosal surfaces. Only a minority of sexual intercourse events with an infected individual causes virus transmission. Remarkably, secretory IgA class antibodies found at mucosal surfaces of non-infected humans rapidly catalyze the cleavage of gp120 and neutralize HIV in tissue culture.[47] Blood-borne IgA and IgM antibodies display lower catalytic proficiencies. A single catalytic antibody molecule is reused to cleave thousands of gp120 molecules. The neutralization potency of catalytic antibodies, therefore, is superior to traditional antibodies that bind the antigen reversibly on a 1:1 basis. It may be hypothesized that the catalytic secretory IgAs represent a constitutive defense against mucosal HIV transmission.

The $CD4BS^{core}$ appears to be the proverbial Achilles heel of the virus. Potent HIV neutralizing antibodies have been isolated from non-infected patients with lupus,[48] an autoimmune disease that is associated with resistance to HIV infection[49] and increased catalytic antibody production.[50] Catalytic antibody light chains to the $CD4BS^{core}$ have been reported.[51] It is not clear whether an autonomous BCR signaling event or a discrete antigen drives amplification of the antibodies. No structural homology is evident between the $CD4BS^{core}$ and human protein sequences available in the databanks. However, we found partial $CD4BS^{core}$ sequence identity with certain human endogenous retroviral sequences (HERVs).[45] HERVs are evolutionary remnants of retroviral integration into the

host genome. About 2–10% of the human genome is composed of HERVs, and HERV expression is amplified in lupus patients.[52]

An explicit example of adaptive production of antibodies to the superantigenic CD4BS^{core} is provided by patients who survived HIV infection over a prolonged period of about two decades.[53] IgA class antibodies specific for the CD4BS^{core} identified in the blood of the survivors neutralized heterologous viral strains potently. The IgAs contained an antibody subset that binds gp120 non-covalently and another antibody subset that proceeds to hydrolyze gp120. It appears that adaptive production of the antibodies to the CD4BS^{core} is proscribed, but in the rare circumstances that adaptive anti-CD4BS^{core} antibodies are generated, they neutralize HIV strains from across the world with exceptional potency. The immune response to microbial infection is a stochastic process relying on occurrence of immunologically favored, high probability B lymphocyte differentiation events. Low probability events are manifested with passage of time. Understanding how B cells from HIV survivors bypass physiological restriction on adaptive production of antibodies to the superantigenic CD4BS^{core} could furnish insight to design a vaccine that induces similar antibodies. The potential bypass mechanisms are: (a) The B cells may slowly produce antibodies that bind the CD4BS via their CDRs with no utilization of the pre-existing CD4BS binding site programmed genetically into the FRs; and (b) Cellular downregulation due to CD4BS binding to the antibody FR site may be effectively counteracted by a favorable differentiation signal generated upon simultaneous engagement of another epitope on the same gp120 molecule by the CDRs.

In addition to gp120, two other HIV proteins essential for virus infection are cleaved by catalytic antibodies from HIV infected patients, reverse transcriptase and integrase.[54,55] Sufficient details of patient clinical history and antibody specificity have not been provided, making it difficult to assess whether the catalytic antibodies are produced via the classical adaptive response pathway. Also, the authors reported enrichment of the catalytic antibody subset in the Protein A binding fraction, raising questions whether Protein A binding to discrete V domain FRs responsible for superantigen epitope recognition was a contributory factor in identifying the catalysts. There is no evidence for the expression of superantigenic epitopes by HIV integrase or reverse transcriptase, but this is not an inconceivable feature of these proteins. Note that a rapid adaptive catalytic antibody response to microbial antigens akin to the classical adaptive binding response is feasible only if the catalytic event itself is immunologically selectable via productive use of the catalytic energy for driving B-cell maturation.

CATALYTIC ANTIBODIES AS SECOND GENERATION THERAPEUTICS

Reversibly binding monoclonal antibodies and polyclonal IgG from healthy humans have emerged as a paradigm for therapy of cancer, neurological disease and autoimmune disease. Yet cost and limited efficacy are substantial concerns. Catalytic antibodies hold potential for improved efficacy, and in some instances, lesser side effects. According to Thomas Kuhn, for a new paradigm candidate to be accepted by a scientific community, "First, the new candidate must seem to resolve some outstanding and generally recognized problem that can be met in no other way. Second, the new paradigm must promise to preserve a relatively large part of the concrete problem solving activity that has accrued to science through its predecessors." With the exceptions noted

below, the current therapeutic antibody paradigm derives from the notion of antibodies as "magic bullets" that target defined antigens with minimal collateral damage to other antigens. A high level specificity for individual antigens, therefore, is an important property for minimizing catalytic antibody side effects. Maximizing efficacy will depend on the rapidity of catalysis. Any future paradigm of catalytic antibodies as second generation therapeutics will depend on identifying antibody preparations with sufficient specificity and turnover rate.

Catalytic IVIG?

Intravenously administered formulations of pooled human IgG (IVIG) are largely composed of constitutively produced antibodies. IVIG provides incremental benefit in patients with certain autoimmune diseases and immunodeficiency states. In addition, the use of IVIG and its IgM-containing counterpart IVIGM for treatment and prevention of bacterial infection has been debated. The manufacturing procedure includes use of the Cohn-Oncley cold ethanol fractionation method developed in the 1940s. While V domain binding functions are usually maintained, IVIG is essentially devoid of catalytic activity,[22] presumably because the catalytic sites are disrupted by stringent solvent treatments. A commercial IVIGM preparation also displayed minimal catalytic activity. Moreover, IVIG is generally formulated using only IgG class antibodies, which express catalytic activities orders of magnitude lower than native IgM and IgA antibodies. As the promiscuous catalytic function of endogenous antibodies is likely to fulfill a homeostatic role, it is reasonable to examine the potential benefits of catalytic IVIG formulations composed of IgM/IgA class antibodies purified under non-denaturing conditions.

Alzheimer Immunotherapy

No therapy for a sustained improvement of cognition is available for Alzheimer's disease. In amyloid β overexpressing transgenic mice, small amounts of intravenously infused monoclonal antibodies that bind amyloid β peptide enter the brain, clear brain deposits of the peptide and improve cognition.[56] However, human trials have raised concern about limited therapeutic efficacy and side effects. A monoclonal IgG to the amyloid β peptide modestly reduced the risk of cognitive decline in patients negative for the ApoE4 allele, a genetic trait associated with delayed onset of Alzheimer disease.[57] Microglia, the brain's resident macrophages, fulfill the beneficial function of clearing antibody-amyloid β immune complexes by uptake through their Fc receptors. However, microglial activation by this pathway holds the risk of inflammatory mediator release, potentially exacerbating the already inflamed state of the Alzheimer brain. Immune complex deposition in the vascular walls can cause cerebral microbleeds. Provided that the antibodies cleave amyloid β peptide with sufficient specificity, catalytic antibodies hold many of the advantages and none of the disadvantages of reversibly binding antibodies. Because catalysts do not form long-lived immune complexes, the risks of vascular immune complex deposition and microglial release of inflammatory mediators occurring as a result of Fc-receptor mediated immune complex uptake is minimized. High turnover catalytic V_L domains to amyloid β with serine protease character have been isolated using phage display library methods[21] and a single chain catalytic Fv is also available[58] for further development.

Infectious Disease

Uda and coworkers have developed a strategy to isolate high turnover catalytic subunits from monoclonal antibodies. They apply molecular modeling to identify potential catalytic triads in the antibodies raised to ordinary polypeptide antigens, prepare the individual heavy and light chains, and screen for catalytic cleavage of the polypeptide antigen. The strategy was successful in preparing catalytic light chains capable of rapid cleavage of *Helicobacter pylori* urease. This bacterium is the etiologic agent in several gastroduodenal diseases. Oral administration of the light chain reduced the number of *H. pylori* in the stomachs of mice.[59]

HIV immunotherapy is plagued by the problems of poor potency and emergence of antibody-resistant viral mutants. Can catalytic antibodies be used for passive HIV immunotherapy? The answer depends on the epitope specificity and neutralizing potency of the catalysts. Targeting the $CD4BS^{core}$ minimizes the opportunity for developing antibody-resistant strains, as mutations in the $CD4BS^{core}$ are predicted to result in loss of viral infectivity. Indeed, anti-$CD4BS^{core}$ antibodies from long-term survivors of HIV infection neutralized the autologous HIV strain potently, arguing against emergence of resistant strains despite the selective pressure imposed by the antibodies over prolonged durations. Catalytic monoclonal antibodies to the $CD4BS^{core}$ raised by immunization with an electrophilic peptide mimetic neutralize diverse HIV strains in tissue culture, supporting their potential therapeutic utility.[60,61]

HIV infected individuals produce catalytic antibodies to the viral integrase and reverse transcriptase enzymes. Intracellular expression of catalytic antibodies to these proteins holds potential for early blockade of viral propagation via interference with copying viral RNA into proviral DNA and DNA integration into the host genome. Gene therapy protocols for intracellular antibody expression[62] can be conceived for persistent delivery of catalytic anti-HIV antibodies. Reactivation of HIV infection can occur due to integration of the viral genome into host DNA. Drugs that deplete proviral DNA reservoirs are under investigation to address the problem of HIV latency.[63] Catalytic antibodies combined with a proviral DNA-depleting drug may be suitable for consideration as an alternative therapy for the infection.

CATALYTIC AND COVALENT VACCINATION

In the case of HIV, a clear path to a vaccine that induces broadly neutralizing antibodies can be foreseen if the following milestones can be reached: (a) Reproduction of the correct $CD4BS^{core}$ conformation in the vaccine candidate, and (b) Rapid adaptive production of neutralizing anti-$CD4BS^{core}$ antibodies upon administration of the vaccine candidate. Preclinical studies of the 'covalent vaccination' strategy are promising.[60,61] Central points are:

- The covalent binding of B cells by an electrophilic polypeptide containing the $CD4BS^{core}$, the vaccine candidate, induces production of broadly neutralizing antibodies. The polypeptide is activated chemically by linking lysine side chains to the strongly electrophilic phosphonate diester group. Naturally-occurring nucleophilic sites are found ubiquitously in BCRs.[19,64] Noncovalent $CD4BS^{core}$ epitope binding to BCRs positions the electrophilic group within covalent binding

distance of nucleophilic groups. Like the catalytic reaction discussed above, covalent bonding between the electrophile and nucleophile liberates a very large amount of energy. Alone, noncovalent CD4BS^{core} binding to BCRs does not stimulate antibody synthesis. As administration of covalently reactive CD4BS^{core} containing antigens to experimental animals induced neutralizing anti-CD4BS^{core} antibodies, it appears that the binding energy released from the covalent antigen-BCR reaction is used productively to induce a remote BCR conformational change that stimulates signal transduction processes permitting differentiation of B cells into plasma cells producing class-switched neutralizing antibodies.

- A unique feature of the strategy is recruitment and clonal expansion of the small subset of B cells capable of producing antibodies with constitutive, pre-existing specificity directed to the CD4BS^{core}. The induced antibodies neutralized diverse HIV strains, a long-sought objective in HIV vaccine research (see example of a neutralizing antibody in Fig. 5A). The CD4BS^{core} binds at a site located mainly in the FRs. Neutralizing antibody production occurs without dependence on typical adaptive mutational processes occurring in the CDRs. However, adaptive improvement of the antibodies due to FR mutations is feasible, indicated by evidence from immunization of animals with electrophilic gp120 and an electrophilic CD4BS^{core} peptide mimetic.[60,61] Robust neutralization of diverse HIV strains by the antibodies in tissue culture was evident. The antibodies displayed specific recognition of the CD4BS^{core}, confirming mimicry of the native CD4BS^{core} by the vaccine candidates.

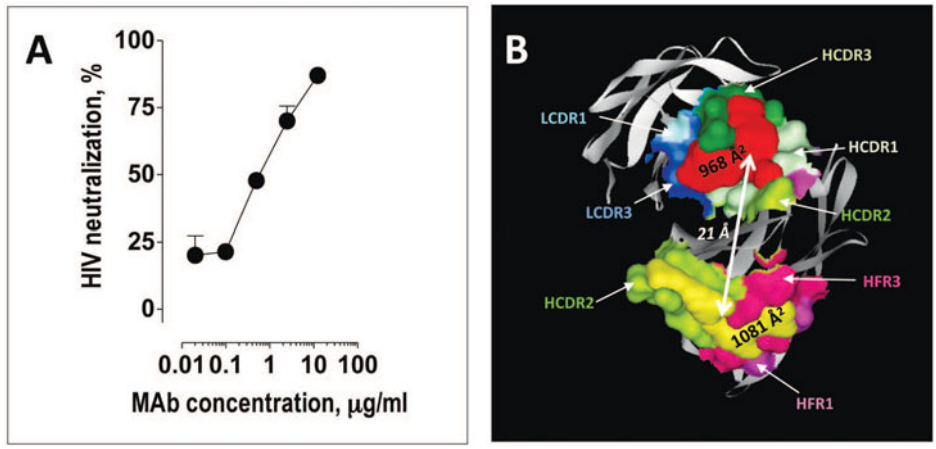

Figure 5. Binary epitope reactivity of neutralizing antibodies raised by immunization with electrophilic gp120. A) Representative HIV neutralization data for monoclonal antibody 3A5. Clade C, CCR5-dependent HIV, strain ZA009. Host cells, peripheral blood mononuclear cells. B) Fab YZ23 structure solved by crystallography (2.5Å resolution, PDB 3CLE) showing the classical antigen binding cavity formed by the heavy and light chain CDRs (HCDR3, HCDR1, a small HCDR2 segment and LCDR3) filled with a red object and a second cavity dominated by heavy chain FR1 and FR3 (HFR1, FFR3) filled with a yellow object. A segment of heavy chain CDR2 (HCDR2) distant from the classical antigen binding cavity helps form the second cavity. The CDR-dominated and FR-dominated cavity bind, respectively, gp120 residues 301–311 and the CD4BScore. Double headed arrow indicates inter-cavity centroid to centroid distance. Cavity surface areas are indicated within red and yellow objects. Neutralization data and the crystal structure are from reference 60.

- Immunization with full-length electrophilic gp120 revealed another novel mechanism for overcoming the physiological hurdle in producing anti-CD4BS^{core} antibodies.[60] Neutralizing antibodies to electrophilic gp120 displayed binary epitope reactivity, that is, the ability to bind the CD4BS^{core} at the antibody FRs and a second spatially distant epitope composed of gp120 residues 301–311 at the antigen binding cavity formed by the CDRs (Fig. 5B). The binary reactivity suggests that simultaneous stimulatory binding of the second immunogenic epitope at the CDRs compensates for the downregulatory CD4BS^{core} binding at the framework regions.
- In addition to inducing reversibly-binding antibodies, covalent binding of the electrophilic vaccine candidate selects BCRs with the greatest nucleophilic reactivity.[14,65] In turn, the improved nucleophilic reactivity enhances antibody inactivation of HIV as follows. First, specific pairing of the antibody nucleophile with the weakly electrophilic carbonyls of gp120 forms stable immune complexes with covalent character (Fig. 3). Binary epitope-reactive antibodies were induced by immunization with the electrophilic analog of full-length gp120 and similar antibodies with single epitope reactivity were induced by immunization with an electrophilic analog, a synthetic gp120 peptide corresponding to an immunodominant epitope located in the third variable domain of gp120.[14,65] As the covalent bond is very strong, the antibody-HIV complexes did not dissociate, increasing the HIV neutralization potency. Second, the subset of antibodies, containing combining sites that support water attack on the covalent gp120-antibody complex, is capable of catalyzing gp120 cleavage. A subset of catalytic antibodies capable of rapidly catalyzing the cleavage of gp120 was obtained by immunization with the electrophilic CD4BS^{core} peptide.[61]

Enzyme active sites have evolved to undergo precise active site conformational readjustments that fulfill the changing requirements of individual steps in the catalytic reaction cycle, that is, initial noncovalent binding, nucleophilic attack and transition state binding, water attack on the covalent acyl-antibody complex and product release (Fig. 3). Note that inducing efficient antibody catalysis is more feasible in case of superantigen epitope targeting,-as catalytic antibodies to the superantigens are produced constitutively. For such targets, the electrophilic antigen analog must merely amplify the pre-existing, constitutive subset of catalytic antibody producing B cells. For non-superantigenic targets, de novo induction of the catalytic function is required, a more onerous hurdle. Monoclonal anti-gp120 IgGs with slow catalytic activity have been identified following immunization with an electrophilic analog of full-length gp120[14] and a gp120 pentapeptide without evident superantigenic character.[66] Successful vaccination requires induction of robust polyclonal antibody responses in biological fluids. The monoclonal antibodies with low-level catalytic activity do not justify projections of vaccine-relevant antibody catalysis. Indeed, monoclonal antibodies capable of slow, antigen-specific proteolytic activity are induced even by routine immunization with polypeptides devoid of exogenously-introduced electrophiles.[67,68]

Despite caveats concerning induction of efficient de novo catalysis, the electrophilic antigen analogs offer indisputable value by virtue of their ability to induce adaptively strengthened antibody nucleophilic activity resulting in covalent recognition of the target antigen.[65,69] This applies regardless of the superantigenic character of the target. Side-by-side immunizations using an electrophilic antigen analog and the control antigen

devoid of exogenous electrophiles revealed the superiority of the former immunogen, evident from enhanced antigen neutralization by polyclonal antibodies attributable to covalent immune complexation.[65]

CONCLUSION

The field of antibody catalysis has a secure place alongside other constitutive antibody functions in helping develop novel concepts that offer insight to immune homeostasis, autoimmune reactions and protection against infection. The field is also increasingly closer to realizing its utilitarian potential for developing novel therapies and vaccine strategies directed at intractable diseases.

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CONFLICT STATEMENT

Sudhir Paul, Stephanie Planque and Yasuhiro Nishiyama have a financial interest in patents covering the covalent immunization and catalytic antibody areas. Sudhir Paul and Richard Massey have a financial interest in Covalent Bioscience Inc., and Sudhir Paul is a scientific advisor for the company. Carl V. Hanson declares no competing financial interest.

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CHAPTER 6

NATURALLY OCCURRING AUTOANTIBODIES IN MEDIATING CLEARANCE OF SENESCENT RED BLOOD CELLS

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Abstract: Germline-encoded naturally occurring autoantibodies (NAbs) developed about 400 to 450 million years ago to provide specificity for clearance of body waste in animals with 3 germ layers. Such NAbs became a necessity to selectively clear aged red blood cells (RBC) surviving 60 to 120 d in higher vertebrates. IgG NAbs to senescent RBC are directed to the most abundant integral membrane protein, the anion-transport protein or band 3 protein, but only bind firmly upon its oligomerization, which facilitates bivalent binding. The main constituent of RBC, the oxygen-carrying hemoglobin, is susceptible to oxidative damage. Oxidized hemoglobin forms hemichromes (a form of aggregates) that bind to the cytoplasmic portion of band 3 protein, induces their clustering on the cytoplasmic, as well as the exoplasmic side and thereby provides the prerequisites for the low affinity IgG anti-band 3 NAbs to bind bivalently. Bound anti-band 3 NAbs overcome their low numbers per RBC by stimulating complement amplification. An affinity for C3 outside the antigen binding region is responsible for a preferential formation of $C3b_2$-IgG complexes from anti-band 3 NAbs. These complexes first bind oligomeric properdin, which enhances their affinity for factor B in assembling an alternative C3 convertase.

INTRODUCTION TO TISSUE HOMEOSTASIS

Selective clearance of senescent, apoptotic or of tumor cells, as well as of altered, damaged proteins must have been a major challenge in primitive vertebrates that had for the first time developed a third germ layer. While these animals inherited a primitive complement system and a whole variety of defensins and lectins to fight pathogens, they lacked germline encoded molecules that provided specificity for altered self.

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

Tissues of mesodermal origin could not get rid of their dead or aberrant cells by release into either the entodermal tract or to the surroundings. Correspondingly, the most primitive vertebrates, jawless fish (Agnatha), have acquired a C3-like protein that can be activated via a lectin pathway, but they lack true immunoglobulins.[1] Instead, they have a multitude of humoral effector molecules comprised of leucine-rich repeats, capable of rearranging themselves.[2] Cartilaginous fishes, like sharks, can opsonize pathogens via the lectin pathway. For tissue homeostatic tasks they have, for the first time in evolution, germline-encoded immunoglobulin-like molecules (IgW, "Ig new antigen receptor," and IgM).[3] Existing sharks cannot mount an adaptive immune response. Instead, they upregulate preexisting naturally occurring antibodies (NAbs).[3] Thus, it is likely that germline-encoded immunoglobulins developed about 400 to 450 million years ago to provide specificity for altered self in clearance of body waste. The development of such immunoglobulins required differentiation between altered self and self rather than between self and non-self. Thus, the developing immune system was not based on eliminating self-reactive clones, but on creation of a tuned system taking advantage of accessibility, avidity and patterns as is currently studied in quite some detail.[4,5]

MEANS TO CLEAR SENESCENT RED BLOOD CELLS

Humans have about 5 L of blood containing 4.5 x 10^9 red blood cells/mL. Human red blood cells (RBC) have a lifespan of 120 d. Thus, each of us has to clear daily 20 to 25 g of terminally aged erythrocytes. If a human being were not able to clear senescent RBCs, but were able to mummify and store them somewhere in the body- like a tree its tracheae in form of wood - this person would have gained 430 kg by the age of 60 y. Hence, based on this simple calculation clearance of senescent RBCs is an absolute necessity and has to be secured by redundant means.

RBCs age primarily as a consequence of oxidative damage to which they are exposed while performing their role as oxygen carriers. Oxidative damage to proteins, enzymes and lipids results in loss of functional competencc. This impairment cannot be compensated for by de novo protein synthesis, because RBC from higher vertebrates are anucleated and lack the endoplasmic protein synthesis. Damage eventually results in a loss of membrane, ions and an increased cell density. Within 120 ± 4 d RBC have reached the point to be cleared, though several RBC functions remain almost normal. As outlined before, aging RBCs increase in cell density, a property which allows to isolate RBCs of different cell age, most efficiently on self-forming Percoll density gradients,[6] following the removal of leucocytes.[7] The membrane proteins of density-separated RBC reveal on SDS PAGE characteristic changes, the most obvious one is a shift in the content of stainable protein in band 4.1a and 4.1b.[8] The older an RBC the more stainable protein migrates in band 4.1a than 4.1b. The ratio of the protein content in the 2 bands appears as a reliable age parameter for human RBCs, but not for dog RBCs where this ratio increases from the youngest to the middle aged, but not further to the oldest RBC.[9]

Senescent RBC are cleared by phagocytosis, which is dependent on opsonization by autologous IgG. Senescent RBC carry significantly more autologous IgG and thus more IgG naturally occurring autoantibodies (NAbs) than young RBC, and are far more efficiently phagocytosed by macrophages than young RBC, as first firmly established by Kay[10] and confirmed by several groups for human and mouse RBCs.[11-14]

The selective opsonization of senescent RBCs by IgG NAbs had a tough stand, because several groups claimed over the years that desialylation of certain glycoproteins is the hallmark of senescent RBC and results in the loss of negative charges and the exposure of penultimate galactosyl residues that are recognized directly[15-17] or by certain NAbs.[11] Indeed, a neuraminidase treatment accelerated in vivo clearance of RBC in rats and rabbits.[16,17] RBCs can loose individual sugar residues by enzymatic processes or membrane in form of vesicles. A uniform loss of glycoconjugates from aging RBCs, indicative of vesicle release, has been established experimentally by determining glycoconjugates and the total sialic acid content per glycophorin in density-fractionated RBC.[18] Both sets of results strongly suggest that clearance of senescent RBCs is not mediated by recognition of desialylated sites, because the majority of sialic acid molecules is lost along with other glycoconjugates in the form of vesicles.

The selective clearance of senescent RBC by an IgG NAb-dependent phagocytosis was further challenged by findings on apoptotic cells that are cleared when exoplasmically exposed phosphatidylserine (PS) binds to the scavenger receptor on phagocytes.[19] PS is normally located in the inner leaflet of any plasma membrane and the ATP-requiring amino-phospholipid translocase maintains this disequilibrium.[20,21] In accordance with data on apoptotic cells, some authors claimed that aging RBCs from mice[22,23] and healthy humans[24] are recognized and engulfed by phagocytes by virtue of PS being exposed on the outer leaflet of the plasma membrane. It is correct that an experimentally induced exposure of PS on the exoplasmic leaflet of the membrane results in a PS-dependent clearance of RBC by binding to scavenger receptor on phagocytes.[25] Nevertheless, an exoplasmic PS exposure is neither linked to cellular senescence[26] nor to oxidative damage by a variety of agents,[27] but either to energy depletion, a cell age-dependent loss of aminophospholipid translocase activity,[28] or to an induced entry of calcium ions in the process also called eryptosis.[29] Correspondingly, exoplasmic PS exposure can occur independently of cellular aging, as in neocytolysis of young RBC[30] or can accompany an IgG NAb-mediated RBC clearance. This is the case in several forms of anemia, e.g., β-thalassemia[31] and sickle cell disease,[32] and in chronic hemodialysis patients, where β-2-microglobulin is increased and appears to stimulate exoplasmic PS exposure.[33]

In contrast to this, the binding of IgG NAbs to RBC and the selective phagocytosis of RBC carrying bound IgG is unique for in vivo aged RBCs, irrespective of whether the senescent RBCs are collected (a) as the most dense cells from a RBC population, or (b) as biotinylated RBCs by capturing them on avidin-coated magnetic beads from blood of a dog which was biotinylated more than 100 d earlier,[34] or (c) as a whole RBC population taken from an animal in which erythropoiesis was dampened by prolonged hypertransfusion.[13]

THE SPECIFICITY OF NAbs INVOLVED IN CLEARANCE OF SENESCENT RED BLOOD CELLS

The specificity of the IgG NAbs that bind to senescent RBCs and mediate their phagocytosis has been a controversial issue over the years. We found that human IgG contains several types of IgG NAbs that bind to RBC proteins on immunoblots, above all anti-spectrin and anti-band 3 NAbs.[35] In the late 70ties we performed immunoprecipitations with second antibody on Triton extracts of ^{125}I-iodinated young and old RBC, which were either supplemented with human IgG or not. Precipitates from old RBC revealed label

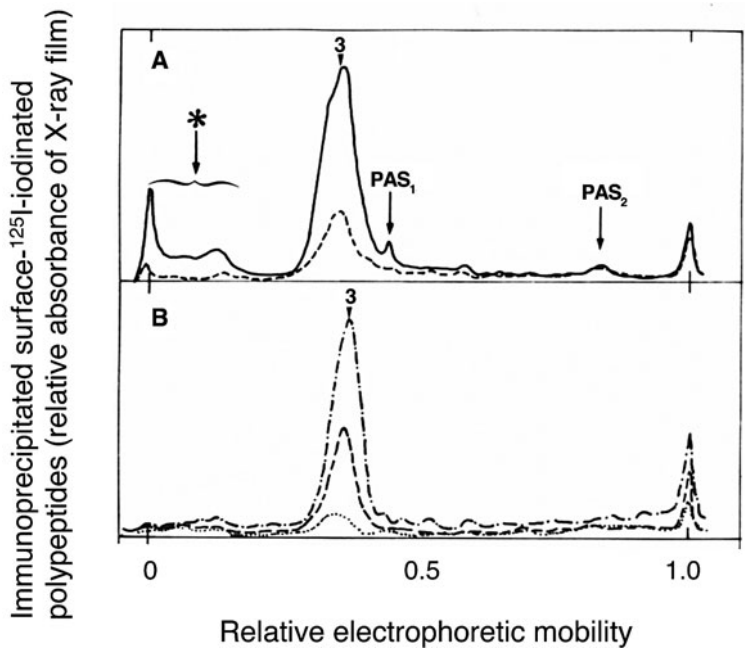

Figure 1. Qualitative analysis of immunoprecipitates from non-ionic detergent extracts of membranes of young and old human RBC by SDS PAGE and autoradiography. A) Immunoprecipitates obtained with second antibody alone from extracts of ^{125}I-iodinated young (------) and extracts of old red blood cells (——). The creatine ratio was 4.9. The number 3 refers to band 3 protein and the asterisk indicates labeled high molecular weight material. B) Immunoprecipitates obtained from extracts of young cells without any addition (…….), with second antibody alone (------), or with both 0.2 mg/mL of autologous IgG and second antibody (-·-·-·). A,B) Reprinted from: Lutz et al. Biomedica Biochimica Acta 1983; 42:117–121;[37] ©1983 with permission of Wiley-VCH Verlag GmbH & Co.

in band 3 and in 2 high MW regions possibly comprising bound IgG and band 3 dimer (Fig.. 1).[36] The components at 100 and 200 kDa shared the peptide composition with that of ^{125}I-iodinated band 3. Precipitates from young RBC contained a small amount of label in band 3, which was significantly increased by supplementing the extracts with autologous IgG (Fig. 1).[36,37] These findings, and the fact that binding of autologous IgG to blotted band 3 of young and old RBC was the same, allowed the following conclusion: human IgG may contain low affinity IgG anti-band 3 NAbs that bind best to oligomerized or aggregated rather than to monomeric band 3 protein on aged RBC and on immunoblots where the densely packed band 3 mimicked oligomerization. These unexpected findings triggered our interest in the topology of band 3, which we studied in quite some detail by using bivalent, aminogroup-specific cross-linkers on unmodified RBC and on RBC to which we had introduced additional free amino-groups into the sugar side chains of glycoproteins.[38] Less than 2% of band 3 was cross-linkable on RBC of any cell age, independent of whether amino-group supplemented or not. In contrast to this, 20% of band 3 protein were cross-linkable on spectrin-free vesicles, which lack the cytoskeleton. Correspondingly, human IgG binding was 14 times higher to spectrin-free vesicles than to ATP-maintaining RBC.[39] Hence, the cell-age specific antigen with band 3 properties appeared to behave as a monomer when probed with an 11 Angstrom long cross-linker

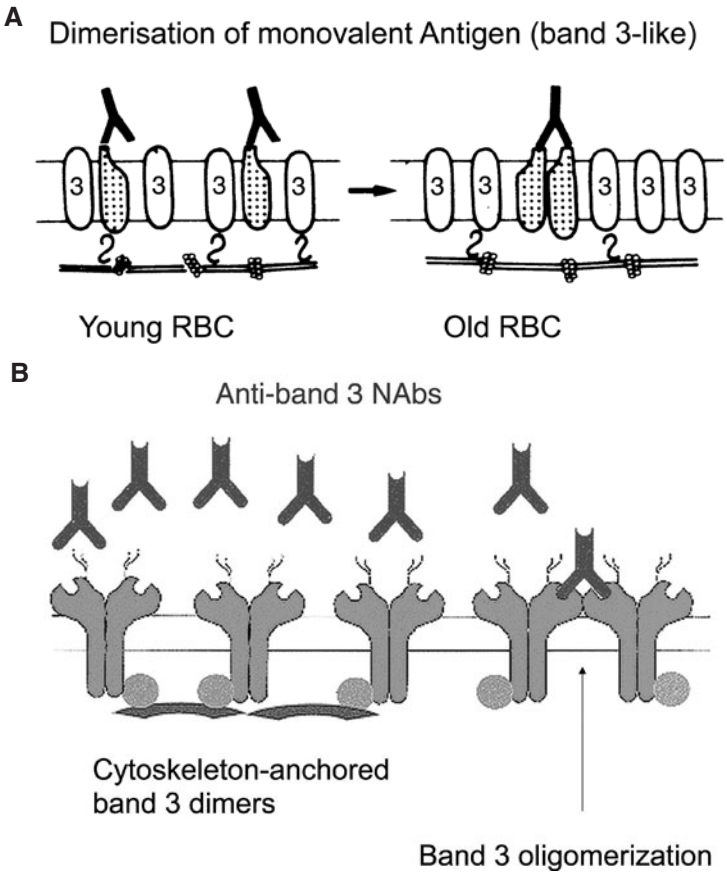

Figure 2. How a cell age specific antigen becomes exposed and allows firm, bivalent binding of anti-band 3 NAbs. A) The cell age specific antigen is monomeric, but largely immobilized by interactions with the cytoskeleton. Detachment from the cytoskeleton allows the monomeric protein to form dimers that bind the low affinity NAb bivalently. Reprinted from: Lutz et al. Swiss Medical Weekly 1981; 111:1507-1517;[36] ©1981 with permission of Swiss Medical Weekly. B) Studies of band 3 topology suggest that band 3 exists primarily as cytoskeleton-anchored band 3 dimers, which as such cannot bind anti-band 3 bivalently. By detachment from the cytoskeleton, band 3 tetramers or oligomers are formed which facilitate bivalent binding of anti-band 3 NAbs. Anti-band 3 NAbs bind to an antigenic peptide region on band 3 protein, but do not bind to band 3 carbohydrate (fine lines on band 3).

or IgG, and as such did not allow anti-band 3 NAbs to bind bivalently. These findings led to a scheme, showing that a low affinity IgG NAb selectively binds to senescent RBC, on which a small portion of this cell-age specific antigen with band 3 properties is oligomerized (Fig. 2A). Data from others later showed that band 3 protein exists primarily as a V-shaped dimer,[40] which as such evidently did not allow a bivalent binding of anti-band 3 NAbs.[38] These findings were partly published in "remote" journals.[36,37] Why and how did this come about? We submitted the data on IgG binding to young and old RBC for publication to a well known American journal in 1981. Acceptance was declined with the argument that we had not yet shown that human plasma contains IgG anti-band 3 NAbs. Nevertheless, Kaspar Winterhalter from our Dept at the ETH, who

was at that time a member of the organizers of the meeting of the Swiss Hematology Society, considered our findings highly relevant and invited me for a main presentation, followed by an article in Schw. Med. Wschr.[36] Though this paper, published in 1981, is in German, it contains a detailed abstract in English and Figure 2A. Several overseas colleagues had read our paper on the purification of IgG anti-spectrin NAbs,[35] where the existence of anti-band 3 NAbs was evident from blots. Several had seen the article in Schw. Med Wschr.[36] and in BBA,[39] but Low et al. referred only once[41] among their many papers on this subject to reference 36 as the first publication suggesting that an IgG NAb to band 3 protein bound to oligomerized band 3 protein on aging RBCs.

Low's paper[41] triggered a wide interest in the phenomenon. The authors mimicked the oxidative process of aging by treating RBC with phenylhydrazine, a treatment that results in generation of hemichromes, which evidently formed band 3 clusters that attracted exoplasmically added autologous IgG. The effect of phenylhydrazine was indeed due to band 3 clustering, because an analogous treatment of RBC with acridine orange that oligomerizes band 3 protein without hemichrome generation also greatly increased IgG binding to RBC. One of his collaborators, Drenckhahn, then showed for the first time on old RBC from healthy donors and from those with an unstable hemoglobin that precipitates of hemoglobin (Heinz bodies) are associated with clusters of band 3 protein and clusters of surface-bound IgG, which they could identify as IgG anti-band 3 NAbs.[42] The Low group continued to work on these aspects, and directly demonstrated that Heinz bodies (hemichromes) induce clustering of band 3 in sickle RBC, identified these aggregates as the major sites of autologous IgG binding (IgG being enriched 250-fold at the sites of these aggregates as compared with regions where band 3 was not aggregated),[43] isolated such complexes from dense human RBC,[44] and showed that clustering agents not only stimulated autologous IgG binding, but also deposition of C3 and RBC phagocytosis.[45] While most of this happened in the 80s, we published the paper we had been asked for, namely the first paper on the isolation and characterization of IgG anti-band 3 NAbs from IgG of individuals and from IVIG.[46] Purified IgG anti-band 3 NAbs bound to domains within the 55 kDa chymotryptic fragment (this fragment had earlier been referred to a 65 kDa fragment) rather than to the carbohydrate localized in the 38 kDa fragment. The antigenic sites were equally present on blots from membranes of young and old RBCs, implying that conformational and/or topologic rather than enzymatic alterations may reveal the antigenic regions on old RBC. The binding specificity of purified anti-band 3 NAbs[46] and the results from studying protein topology on RBC[38,40] allowed drawing of a more detailed model on how anti-band 3 NAbs selectively bind to oxidatively stressed RBC (Fig. 2B). Work from Beppu et al. confirmed our findings.[47] These authors repeated anti-band 3 NAbs purification and showed that binding of anti-band 3 NAbs to RBC was similarly dependent on moderate oxidation. It was inhibitable by purified band 3 protein and α tocopherol prevented the lipid oxidation and the subsequent IgG binding.[47]

THE FINE SPECIFICITY OF ANTI-BAND 3 NAbs

Beppu et al. then used endo-β-galactosidase- or neuraminidase-treated band 3 or similarly treated oligosaccharides thereof to interfere with IgG binding to oxidatively stressed RBC, as measured by binding of labeled anti-human IgG.[48] Both types of treatments destroyed the inhibitory potency of either band 3 or its oligosaccharides by 79 to 99%. The results suggested that the antigenic sites of band 3 are located in sialylated

poly-N-acetyllatosaminyl carbohydrate side chains of band 3 protein. Since these findings completely contradicted our results,[46] we reinvestigated whether the enzymes endo-β-galactosidase, neuraminidase, and endoglycosidase H destroyed the antigenic sites on human RBC. RBC were enzyme-treated, membranes prepared, membrane proteins electrophoretically spread and blotted, and blots probed with purified and ^{125}I-iodinated anti-band 3 NAbs. There was no change (100–103%) in anti-band 3 binding to band 3 protein in these blotted membranes.[49] Moreover, anti-band 3 binding was exclusively to the 55 kDa chymotryptic fragment of band 3, while the carbohydrate-containing 38 kDa fragment was free of bound anti-band 3 NAb.

Beppu et al. did not give up and subsequently published a paper, entitled "Involvement of sialylated poly-N-acteyllactosaminyl sugar chains of band 3 in anti-band 3 autoantibody binding."[50] Young and senescent RBC were probed for their bound autologous IgG by adding ^{125}I-iodinated anti-human IgG antibody. After incubation the RBC were treated with either glycosidases or glycosidase-treated oligosaccharides. Upon washing RBC-associated radioactivity was determined. A treatment of cells with endo-β-galactosidase lowered the cell bound, labeled anti-IgG by one third. This rebuttal in which the carbohydrate-mediated binding of anti-band 3 had decreased from originally 79–99 to about 33% motivated the authors to the following sentence: "We cannot exclude the possibility that other components on senescent erythrocytes are also recognized by natural antibodies distinct from those shown here."

We did not argue and were busy investigating other aspects. Now, in having to review this topic from quite a distance, I think I need to give an explanation why the 2 groups arrived at these opposing positions. Beppu's group continued to work on this subject and published a whole series of papers on how the sialylated poly-N-acetyllactosaminyl sugar chains as found in the 38 kDa fragment of band 3 protein may mediate cell-cell interactions. One paper, however, revealed the pitfall. Ando et al. tried to prove that the antigenic sites on oxidatively treated or senescent RBC are sialylated poly-N-acetyllactosaminyl residues of band 3 protein.[51] For this purpose the authors studied binding of anti-band 3 NAbs not only to blotted band 3 protein, but also to blotted lactoferrin, an 80 kDa iron-binding protein that also contains sialylated poly-N-acetyllactosaminyl sugar chains. Binding of their anti-band 3 NAbs to band 3 and to lactoferrin was very similar and binding to either one was similarly reduced by pretreatment with glycosidase F, endo-β-galactosidase or neurmainidase. By absorption studies on column-bound antigens the authors found that about 67% of anti-band 3 NAbs bound equally to the carbohydrate portion of band 3 and to lactoferrin. This is one type of interpretation. **Another explanation of the same finding is that 67% of the antibodies in the anti-band 3 NAb preparation of Ando et al. were anti-lactoferrin antibodies.** Why? In checking the methodological details band 3 protein was of comparable quality in our experiments and theirs. Not so the starting material from which they purified anti-band 3 NAbs from. We used Sandoglobulin, an IVIG preparation that did not contain anti-lactoferrin antibodies (brand E in Table 3 in ref. 52). **Ando et al., however, used plasma from a blood donor with blood group AB from which they affinity-purified anti-band 3 as well as anti-lactoferrin** (see page 640 in ref. 51). Thus, antibodies to the sialylated poly-N-acetyllactosamine structure from lactoferrin were captured by the carbohydrate portion of immobilized band 3 protein, such that the eluted anti-band 3 antibodies not only contained IgG antibodies to the band 3 peptide, but also anti-carbohydrate antibodies, originating from anti-lactoferrin antibodies in the starting material. The authors could and should have checked whether a sequential purification of both types of antibodies from the same starting material yielded the same

results as a parallel purification. Ando et al. were evidently not aware of the fact that anti-lactoferrin antibodies are found in low concentrations with low affinities in milk and plasma of healthy controls (see ref. 53) and are considered a type of NAb,[52] despite their similarities with ANCA type antibodies that occur primarily in sclerosing cholangitis and Crohns' disease.[53] Thus, anti-band 3 NAbs bind to a peptide antigen localized in the 55 kDa chymotryptic fragment as initially reported,[46,49] but not to a carbohydrate-containing portion in the 38 kDa chymotryptic fragment of band 3 protein.

Attempts to further localize the binding sites of these NAbs within band 3 protein have been made by Kay, who passed detergent extracts from young and old RBC over a column containing IgG eluted from senescent RBC.[54] Using this method she could recover a polypeptide of 62 kDa of unknown origin, which when added to stored RBC fully prevented their serum-dependent phagocytosis. This fragment was most likely a proteolytic breakdown product of band 3, as we suspected.[36] Kay et al. followed this aspect and showed that the phagocytosis-inducing ability of IgG eluted from senescent RBC was fully absorbed by purified band 3, strongly indicating that band 3 protein or an enzymatically altered band 3 protein may serve as senescent cell antigen.[55] A year later Kay reported that the senescent cell antigen is localized on both chymotryptic fragments (55 and 38 kDa), but neither on the carbohydrate of the 38 kDa fragment nor in the 41 kDa tryptic fragment localized in the cytoplasm.[56] In 1988, when we had already shown that purified anti-band 3 NAbs bind to oxidatively stressed RBC and induce a complement dependent phagocytosis,[57] Kay et al. did not find evidence for the concept that aggregation of band 3 plays a role in generating the senescent cell antigen, and continued to suggest that degradation of band 3 is a critical event in IgG binding to aging RBC.[58] Kay and her group then performed immunoblots on peptides across band 3 protein and incubated the blotted peptides with IgG eluted from senescent RBC.[59] The antigenic peptides were from exoplasmic portions of the 55 kDa chymotryptic fragment (538–554) and the 38 kDa chymotryptic fragment (788–827), but did not include peptides carrying carbohydrates.[59,60] The findings differed from ours[49] in so far as anti-band 3 NAbs appeared to bind not only to a peptide portion within the 55 kDa, but also to a peptide portion within the 38 kDa fragment (788–827). Since this amino acid stretch is part of the most C-terminal exoplasmic loop, which interacts with loop 2 (containing amino acids 538–554) of the next band 3 protein in the process of forming band 3 aggregates on senescent RBC,[59] it may be possible that this region is sterically inaccessible to anti-band 3 NAbs on the partially structured chymotryptic 38 kDa fragment and intact band 3 protein, but becomes accessible when offered in form of short peptides. While this explanation may appear constructed, the opposite situation also exists and is well known for band 3 protein, namely that the Wright^b antigen (a Diego blood group) in position 658 (Glut) only becomes accessible when band 3 protein interacts with glycophorin A (for a review see ref. 61).

FUNCTIONAL PROPERTIES OF NAbs AND HOW A LOW AFFINITY IgG NAb, LIKE ANTI-BAND 3, STIMULATES COMPLEMENT AMPLIFICATION

Tisssue homeostatic IgG NAbs directed to cytoplasmic proteins, like anti-spectrin,[35] anti-actin[62] and many others that have relatively high affinities for their antigens are compatible with life, because such NAbs encounter their antigens in case of danger, when cells lyse. Even IgM NAbs, with their 10 binding arms that provide an extreme avidity,

can be compatible with life when they are directed to unique, exoplasmic neoepitopes whose exposure calls for an immediate and efficient clearance.[63,64] Tissue homeostatic IgG NAbs, like anti-band 3 NAbs that gradually accumulate on aging RBC, differ in many respects from the above mentioned ones. In comparison to IgM NAbs, IgG NAbs have a lower chance to cross-react with related targets, but their bivalent binding provides only a minimal gain in avidity and they are less effective activators of the classical complement pathway than IgM NAbs. If such IgG NAbs bind to an antigen that becomes exposed by either enzymatic or topologic alterations of antigen-precursors, a narrow specificity is mandatory to prevent an inadvertent binding to the precursor. This requirement becomes a major problem in the case of a very abundant protein, like band 3 protein that exists in 10^6 copies per RBC. IgG anti-band 3 NAbs fulfill all these requirements with the disadvantage that they have a low affinity (5–7 x 10^6 l/mol), exist at low concentrations[46] and poorly fix complement via the classical pathway.

The functional properties of purified anti-band 3 NAbs were studied by using an in vitro phagocytosis assay on RBC oxidatively damaged by diamide, as developed by the group of Arese.[65] In a joint work we studied phagocytosis of diamide-treated RBC as a function of purified NAbs or diamide. Increasing concentrations of diamide induced the formation of SS-bonded band 3 oligomers.[57] The main findings were that phagocytosis of diamide-treated RBC required anti-band 3 NAbs and complement activation.[57] In fact, a 100-fold excess of anti-band 3 NAbs was required to overcome the need of complement, and most surprisingly, anti-band 3 NAbs appeared to stimulate complement deposition best under conditions favoring activation of the alternative complement pathway. This potency appeared to correlate with generation of covalent adducts of C3b with IgG as verified by 2 different approaches.[14] These findings established that purified IgG anti-band 3 NAbs bind to band 3 protein and induce C3b deposition, provided that band 3 is aggregated. In fact, IgG anti-band 3 NAbs induced a C3b deposition that exceeded the number of bound NAbs by 2 orders of magnitude in the presence of Mg^{2+} and EGTA.[14] Hence, anti-band 3 NAbs stimulated complement amplification (or activated the alternative complement pathway) and this was evidently accompanied by the formation of covalent complexes between C3b and IgG.[14]

These findings triggered our interest in the mode of how IgG antibodies can stimulate complement amplification. Anti-band 3 NAbs are not the only IgG antibodies capable of activating the alternative complement pathway (AP). The phenomenon has been known for quite some time and was first described in the early 70s[66] for guinea pig IgGs and a group of IgGs with a weak affinity for bacteria (pneumococci, *E. coli*).[67] These IgG molecules that were considered "natural antibodies" or even their $F(ab')_2$ fragments appeared as having an affinity for some common surface antigens of these bacteria.[68] These antibodies activated AP, because they stimulated lysis of bacteria in the presence of Mg^{2+} and EGTA. Likewise, primary antibodies against viruses (mumps, herpes simplex, influenza and measles)[69] stimulated lysis of virus-infected cells via the AP as IgG and $F(ab')_2$ fragments by a similarly unknown mechanism. In 1981 Gadd and Reid offered for the first time an explanation on how such low affinity IgG antibodies and their $F(ab')_2$ fragments may activate AP, namely by generating covalently linked complexes between C3b and IgG, which prolong the half life of the C3 convertase.[70] The $C3b_n$-IgG complexes had apparent MWs of 360 and 580 kD, reminiscent of C3b-IgG and $C3b_2$-IgG complexes similar to those that we had observed years later on senescent but not young RBC.[14] The availability of purified anti-band 3 NAbs[46] allowed the investigation of why these NAbs were so effective in stimulating complement amplification. IgG anti-band 3 NAbs and nascent C3b preferentially formed

$C3b_n$-IgG complexes not only while forming an immune complex with band 3, but even when activated in vitro by trypsin.[71] Whole IgG depleted of anti-band 3 or affinity purified anti-spectrin NAbs did not generate such complexes with C3b.

Nascent C3b forms predominantly $C3b_2$-IgG complexes with anti-band 3 NAbs in vitro, and even at a 10^5 fold excess of other IgGs on in vivo aged RBC. The 2 C3b molecules are sequentially ester-bonded to one IgG heavy chain (Fig. 1 in ref. 71). The reason for the preferential formation of $C3b_2$-IgG complexes is a unique affinity of anti-band 3 NAbs for C3. The C3 binding domain is not located in the antigen binding region of anti-band 3 NAbs, but somewhere along the Fab portion, because binding of anti-band 3 NAbs to band 3 protein could not be inhibited by a huge excess of C3, whether studied in the presence or absence of physiological IgG concentrations.[72] As originally suggested by Gadd and Reid, $C3b_2$-IgG complexes are long lived C3 convertase precursors. More importantly the dimeric C3b in these complexes renders them the most powerful C3 convertase precursors by introducing a detour in the assembly of the C3 convertase: instead of binding factor B, the dimeric C3b first interacts with oligomeric properdin, which upon having bound strongly increases the affinity of these complexes for factor B.[73] These results clarify how IgG anti-band 3 NAbs can induce a C3b deposition that is 2 orders of magnitudes higher than that of bound anti-band 3 in the presence of Mg^{2+} and EGTA. It is likely, but has not been investigated, that the same mechanism is also responsible for stimulation of complement amplification by the many other NAbs known to stimulate AP.[74,75]

In identifying the C3b-containing complexes that are generated when anti-band 3 NAbs stimulate AP activation, we have named the larger complex $C3b_2$-IgG and the smaller one C3b-IgG. It turned out that the assignment "C3b-IgG" was wrong in our as well as all other earlier discussions of the subject, because a detailed analysis of these complexes by 2-dimensional SDS PAGE with cleavage of the ester bonds between the dimensions revealed that aside of $C3b_2$-IgG complexes only dimeric C3b rather than C3b-IgG complexes are formed, which release exclusively C3b and no IgG heavy chain upon cleavage of the ester bond.[76] Thus, $C3b_2$-IgG complexes, rather than C3b-IgG complexes, demonstrate a reduced rate of inactivation by factors H and I,[77] have an enhanced bactericidal activity[78] and markedly stimulate phagocytosis.[79] Several pieces of evidence further suggest that the 2 C3b molecules within $C3b_2$-IgG complexes are sequentially attached to one IgG heavy chain.[76] Interestingly, the dimeric character of these complexes is maintained following inactivation of C3b, such that $iC3b_2$-IgG and $C3dg_2$-IgG complexes retain their dimeric character which allows their bivalent and therefore efficient binding to the corresponding receptors.[76]

ANTI-BAND 3 AUTOANTIBODIES OR ANTI-BAND 3 NAbs IN ANEMIA

The clearance of senescent RBC from splenectomized patients with the unstable hemoglobin "Köln" proceeds by the same mechanism as that of senescent normal RBC as outlined before. Hemichromes induced clusters of band 3 proteins that attracted high amounts of plasma IgG anti-band 3 NAbs.[42] The phenomenon was also studied on RBC from patients with β-thalassemia intermedia[80,81] and from patients with congenital dyserythropoietic anemia Type II (CDA-II).[82] In thalassemia the 2 hemoglobin polypeptides are synthesized to different extents. In β-thalassemia an excess of unpaired α-hemoglobin chains is formed, which undergoes oxidation and induces band 3 protein clusters that attract exoplasmically an amount of IgG, which was 20-fold higher than in controls.[80] This

high extent of opsonization was most likely not due to the presence of induced pathologic autoantibodies, but the result of anti-band 3 NAbs that were upregulated. A similar mechanism involving anti-band 3 NAbs may be responsible for the accelerated clearance of RBC in congenital dyserythropoietic anemia Type II. In this anemia, band 3 clusters are formed preferentially because band 3 protein contains abnormally short carbohydrate chains, which in their full length would have inhibited oligomerization.[82] Note that band 3 oligomers on CDA-II RBC bind anti-band 3 NAbs despite their band 3 proteins lacking the sialylated N-acetyl-lactosamine carbohydrate in the 38 kDa chymotryptic fragment—yet another argument against the claim that anti-band 3 NAbs recognize carbohydrate in this fragment.[50] Analogous conclusions were drawn from studying mouse RBC infected with Plasmodium chabaudi.[83] Ternynck et al. found that the concentrations of IgG autoantibodies to cytoskeletal proteins and to band 3 protein increased within days following infection, persisted for more than 20 d beyond infection, remained polyspecific and were not preceded by an IgM peak of antibodies as during an induced immune response.

WHAT ALTERATIONS ARE REQUIRED FOR ANTI-BAND 3 NAbs TO BIND TO BAND 3 CLUSTERS

Oxidative damage is the prerequisite for band 3 oligomerzation, but the effect of SS bond-inducing diamide is dependent on cellular glutathione with the result that prolonged incubations reverse its effect, but not in cells with limited glutathione as in RBC from glucose-6-phosphate dehydrogenase deficient patients.[84] In contrast to this, a treatment of RBC with a non-penetrating bivalent crosslinker, bis(sulfosuccinimidyl)suberate (BS^3), for 5 min at 37°C increased IgG anti-band 3 NAb binding without altering the cellular redox state.[85] The BS^3 treatment induced interdimeric cross-links comprising 30 to 40% of all band 3 protein and thereby stimulated binding of IgG NAbs. This binding of IgG NAbs was, however, significantly higher when determined at 0° than at 37°C,[85] most likely because the BS^3 treatment induced band 3 oligomers, which were covalently stabilized only in form of inter-dimeric complexes that remained diffusible at 37°C and allowed higher oligomers to dissociate at 37°C. Hence, it may well be possible that additional alterations on the cytoplasmic side of band 3 protein are required for the formation of stable band 3 clusters. Turrini's group has indeed described that those band 3 proteins that form large band 3 clusters were primarily phosphorylated by Syk kinase and that inhibitors of this type of phosphorylation lowered band 3 clustering and IgG NAb binding.[84] Nevertheless, it is not established whether the use of Syk kinase-specific inhibitors selectively impaired binding of IgG anti-band 3 NAbs, because these studies were performed with human serum IgG that contains aside of anti-band 3 also anti-spectrin NAbs.[35] While the primary role of IgG anti-spectin NAbs is to opsonize hemolyzing RBCs,[86] anti-spectrin NAbs also bind to in vivo aged and oxidatively damaged RBC and stimulate their clearance in homozygous β-thalassemia[87] and in rats.[88] In support of these findings, the BS^3 treatment of RBC stimulated binding of both anti-band 3 and that of anti-spectrin NAbs, despite the BS^3 treatment of RBC, did not expose spectrin, as verified by surface iodination.[85] Thus, anti-spectrin NAbs that are somewhat more poly-reactive than anti-band 3 NAbs may bind to band 3-associated proteins and may even enhance anti-band 3 NAb binding, as verified experimentally.[85] Future work will have to clarify the extent by which Syk-dependent band 3 phosphorylation modifies binding of IgG anti-band 3 as well as that of IgG anti-spectrin NAbs.

CONCLUSION AND OUTLOOK

Knowing more about the properties and roles of IgG NAbs will further our understanding of tissue homeostatis and how low affinity antibodies overcome their weakness and gain the potency to stimulate complement amplification. A precise location of the C3 binding site within anti-band 3 NAbs will help to design antibodies to other targets capable of stimulating complement amplification. It is unlikely to manipulate the system of NAbs by eradicating one or the other. Far more probable is suppressing or upregulating the effect of one or the other type of NAbs on the basis of the interplay between them. Thus, we ought to study how NAbs interact and how interdependent the role of a particular NAb is. Such knowledge may provide the prerequisites of how to interfere with "negative" effects of NAbs. One example is a pair of NAbs comprising anti-C3 and anti-idiotypic NAbs, which lowers the complement receptor dependent in vivo phagocytosis of RBC from patients with a positive direct antiglobulin test.[89] Elevated concentrations of this pair of NAbs interact with RBC-bound C3b or even $C3b_2$-IgG complexes and lower the complement receptor-mediated phagocytosis 2–3 fold.

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CHAPTER 7

NATURALLY OCCURRING AUTOANTIBODIES AGAINST β-AMYLOID

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Abstract: Naturally occurring antibodies (NAbs) have been described for more than 30 years. Recently, NAbs against β-Amyloid and against other proteins involved in neurodegenerative disorders have been detected in humans. Based on the current evidence, it is hypothesized that anti-Aβ NAbs can inhibit the fibrillation and toxicity of β-aymloid, can improve cognition in a transgenic mouse model and interfere with oligomers of Aβ. Different functions of these NAbs have been described in the current literature. Based on the results of the diverse studies a Phase-III study using IVIG has been initiated in patients with AD. The results will show whether the application of NAbs will change the fate of the disease. This chapter summarizes our current knowledge on NAbs against Aβ.

INTRODUCTION

Alzheimer disease (AD) is the most common neurodegenerative disorder and is like other neurodegenerative disorders characterized by the aggregation and deposition of particular proteins. Cortical atrophy, neuronal loss, region-specific amyloid deposition, neuritic plaques and neurofibrillary tangles are the key neuropathological features in the AD brain. Neurofibrillary tangles consist of intracellularly formed hyperphosphorylated tau proteins. Tau is a highly soluble microtubule-associated protein and its main function is to modulate the stability of axonal microtubules.[1] Hyperphosphorylation of the tau protein, however, can result in self-assembly of tangles of paired helical filaments and straight filaments, and consecutively in disintegration and structural dysfunction of the protein. Furthermore, the formation of insoluble β-amyloid (Aβ) peptides may lead to extracellular plaque deposition in the brain. Aβ, a peptide of 38–43 amino acids, arises

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from a larger precursor protein, the amyloid precursor protein (APP), through enzymatic cleavage by secretases and is deposited as plaques in the diseased brain.[2] Although, evidence exist on processing of Aβ, only little is known about the intracellular processing, metabolism and the fate of Aβ following secretase cleavage.

Recently, physiological autoantibodies (naturally occurring autoantibodies, NAbs) against Aβ (anti-Aβ NAbs) have been described to be present in humans.[3,4] Antibodies directed against Aβ and tau were described in patients with AD as early as 1993.[5,6] However, their role in the pathophysiology of AD was unclear and has not been investigated in further detail. Since the description by Du et al., a number of articles have attempted to further characterize those NAbs.[3] Istrin and colleagues[7] demonstrated that preformed fibrils can be dissolved in the presence of anti-Aβ NAbs, and that IVIG increases the cellular tolerance to Aβ, enhances microglial migration toward Aβ deposits and mediates phagocytosis of Aβ in primary microglia in vitro. IVIG-treated microglia exhibited a ramified resting morphology, but also high levels of the activation marker CD45, indicating an immunomodulatory effect of IVIG on microglial cells. Another mechanism had been shown by Weksler's group, who identified mono- and polyclonal IgM NAbs that hydrolyse Aβ.[8] Hydrolysis of peripheral Aβ by IgMs may induce increased Aβ release from the brain. Catalytic IgMs were found to be increased in AD patients, presumably reflecting a protective autoimmune response. In a histopathological study, Kellner et al. have shown that the majority of neuritic plaques are decorated by IgG and that Aβ-immunized AD patients may develop a reduced plaque burden and an increase in phagocytic microglia.[9] While those mechanisms require extraneuronal interaction of antigen and antibody, there is evidence that anti-Aβ antibodies can also act by clearance of intraneuronal Aβ. However, this has not yet been demonstrated for anti-Aβ NAbs.[10]

The aim of this chapter is to compile the current evidence on physiology, biochemistry and characterization of naturally occurring autoantibodies against Aβ.

ISOLATION OF ANTI-Aβ NAbs

IgG anti-Aβ NAbs have been isolated from blood of healthy persons and AD patients, as well as from commercially available IVIG. Several different isolation procedures using affinity chromatography have been described, however, the different isolation procedures have not been compared concerning their efficacy and their ability to recognize particular epitopes. The original affinity purification protocol by Du and colleagues used CNBr-activated sepharose complexed to $A\beta_{1-40}$.[3] To reduce the risk of steric hindrance and also to permit the interaction of individual and naturally folded Aβ molecules on the column, we later generated an improved affinity purification approach by introducing cysteine to the N-terminal end of $A\beta_{1-40/42}$. The exposed sulfhydryl group of the Cys-Aβ peptide was then covalently immobilized to the iodoacetyl group at the end of a long spacer molecule, linked to the chromatographic matrix. The subsequent characterization of the antibody fraction eluted from these affinity columns provided convincing evidence that the anti-Aβ NAbs preferentially recognize the C-terminal end of $A\beta_{1-40/42}$. In addition, this study showed that anti-Aβ NAbs bind best to Aβ oligomers but not to the monomeric or fibrillar forms of Aβ.[11] This finding was confirmed by the study from Szabo and coworkers

who used a different experimental approach, applying thiophilic chromatography and using chaotropic salts to dislodge weakly bound antibodies without significantly reducing the binding of specific anti-Aβ NAbs.[12] Using these methods, the study showed that human blood contains polyvalent IgG NAbs that bind to Aβ with relatively low avidity and specificity, as well as IgG NAbs that bind to linear and conformational epitopes on amyloid monomers and aggregates with moderate to high avidity.

ORIGIN OF NATURALLY OCCURRING AUTOANTIBODIES

NAbs are effectors of the innate immune system and are produced without prior contact to the specific antigen they recognize.[13] The majority of these NAbs are polyclonal, thus binding to several different antigens and displaying predominately the IgM isotype, even though IgG and IgA isotypes also exist.[14] Current evidence exists that B cells producing NAbs are part of a distinct B cell subset, the so called B1 cell pool. The different B cell subsets vary in terms of location, migration ability and dependency on T-cell help for being activated. The knowledge about the function and origin of B1 B cells stems mainly from studies in rodents and the knowledge in humans is limited.[13,15] B1 cells account for approximately 5% of the whole B cell pool in mice, depending on the strain.[16]

B1 cells can further be divided—in relation to their function—into a B1a pool and a B1b pool[17,18] in contrast to circulating and follicular B cells, conventionally termed B2 cells, which are produced in the bone marrow from progenitor cells and require T-cell activation for clonal expansion.

Until recently, it was understood that B1 cells are mainly present in coelomic cavities[19] and not in lymph follicles, thus not undergoing affinity maturation.[20] However, as shown by Ansel[26] B1 cells are capable of circulating into the periphery, which is in line with the recent evidence by Griffin et al.,[20] who characterized the surface markers of human B cells in the human peripheral blood. Interestingly, CD5 has long been used to characterize B1 cells,[21] however, recent data from Griffin et al. demonstrated in B1 cells from umbilical cord blood as well as from human peripheral blood that they display a $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ pattern.[18] Furthermore, they were able to show that B1 cells not only occur in the peritoneum, but also in the peripheral blood of healthy human donors.

The origin of these B1 cells is still unclear. Two different models have been described, and both are summarized by Dorshkind and Montecino.[19] In brief, the selection model assumes a common progenitor cell for both B1 and B2 cells, whereby selection to one or the other group takes place at the level of surface IgM^{+} cells.[22] In contrast, the lineage model hypothesizes that both B1 and B2 cells have different hematopoietic progenitors and represent different lineages. The basis for this model is provided by the observation that irradiation of fetal tissue leads to the production mainly of B1 cells after cell recovery. In contrast, irradiation of bone marrow from adults mainly generates B2 cells.[23]

Currently, no data is available on the subpopulation of B cells from which anti-Aβ NAbs stem from. It may well be that using the above surface markers to identify B1 cells in humans we will soon be able to increase our knowledge about these cells in humans.

EPITOPE

Aβ is characterized by a number of peptides with different length encompassing amino acids (AA) 1-38/42. Mainly, three regions can be recognized by the currently available antibodies, which characterize 3 epitopes: the N-terminal epitope (~AA1–10), the mid-terminal epitope (~AA17–32) and the C-terminal sequence (~AA32–42). N-terminally directed antibodies bind to aggregated Aβ in vitro as well as to cerebral and vascular deposits and unprocessed APP. The binding ability is primarily a result of the three-dimensional structure of Aβ, in which the N-terminal region is easily accessible even in higher aggregated states of Aβ. Antibodies against the N-terminal epitope may bind not only to plaques, but also to Aβ deposited in the vasculature and may carry the potential risk of cerebral microhemorrhages. Mid-terminal directed antibodies apparently bind to Aβ, but seem to have differential recognition patterns for oligomeric epitopes and less often recognize aggregated Aβ, plaques and vascular amyloid. However, these antibodies do not bind to Aβ deposited in plaques, but have been shown to promote clearance by the peripheral sink mechanism, as has been shown for the antibody m266. The third group recognizes the AA32–42 epitope. These antibodies bind to APP and are able to neutralize toxic effects of Aβ. Unfortunately, such antibodies have been less well studied than the other two groups and only one clinical study is currently focusing on an antibody directed against the C-terminus. Recent studies characterizing the anti-Aβ NAbs epitopes have shown that they preferably bind to the C-terminal sequence.[11]

The β amyloid protein (Aβ) is believed to play a central role in AD and like several other proteins associated with neurodegeneration, has the ability to form a diverse set of different assemblies starting from dimers all the way up to aggregates or fibrils.[24] Historically, Aβ fibrils, similar to those present in Aβ plaques, have been considered primarily responsible for neuronal dysfunction and death. However, recent data suggest that nonfibrillar, water-soluble assemblies of Aβ may also be important for disease development.[25-27] To date, it is unclear if anti-Aβ NAbs recognize monomeric or oligomeric Aβ or fibrils. First evidence has been provided that anti-Aβ NAbs recognize conformational epitopes present on oligomers and fibrils. These are not present on Aβ monomers.[28]

GLYCOSYLATION

IgG antibodies are glycoproteins to which a sugar chain is covalently linked to asparagine 297 of the Fc region. This sugar chain plays an essential role in the interaction with immune effectors such as the Fc receptor[29] and complement C1q.[30] The removal of this sugar chain reduces the interaction with the Fc receptor and complement C1q without preventing the binding to the antigen.[31] Furthermore, differences in the glycosylation of the Fc domain may affect the Fc-mediated effector-function.[32] It was shown that deglycosylation does not affect the binding affinity of the antibody to the FcRn and the half-life in vivo, but the pro-inflammatory processes in terms of reduced microglia activation. Different glycoforms can influence the antibody-dependent cellular cytotoxicity and the complement-mediated cytotoxicity. Deglycosylated antibodies against Aβ can sequester Aβ without inducing a neuroinflammatory response.[33] Furthermore, phagocytosis of Aβ was promoted with a consecutively increased cytokine release in primary microglial cultures.[34,35] To our knowledge neither the glycoprotein sidechain nor the sequence have been investigated in further detail on anti-Aβ NAbs.

ASSAYS USED TO DETECT AUTOANTIBODIES AGAINST Aβ

In the initial description, Du et al. found a significantly decreased anti-Aβ NAb titer in the CSF of patients with Alzheimer disease, suggesting that lowered levels of Aβ-specific antibodies may be a factor that contributes to the pathogenesis of AD.[3] These results have been confirmed by several groups.[4,36-41] In contrast, increased titers[42-44] or no difference between AD patients and healthy controls have been reported as well.[45,46] Different methods of measurement may be one explanation for the conflicting data. The peptides that served as antigens differed in the various assays published. In some assays monomeric Aβ, in others oligomeric or fibrillar Aβ, and in even others just certain epitope regions (Aβ25–35) were employed. Furthermore, as there are no human antibodies available that could serve as an adequate reference antibody in ELISA, quantification is difficult. The autoantibodies are of polyclonal origin and, thus, differing in epitope specificity, affinity and isotype. Therefore, a comparison between the overall levels is not feasible, especially since it is not known which of the antibodies exert a protective role in the homeostasis of Aβ.[12] Moreover, the groups of AD patients and controls investigated in these different studies are quite heterogeneous. Recently a validated assay was developed by Glabe and coworkers[47] and the company DRG (www.drg-diagnostics.de**).**

METABOLISM OF ANTI-Aβ NAbs

The fate of anti-Aβ NAbs has only been addressed by a few studies. The half-life of anti-Aβ NAbs (IgG) in humans has been calculated to be around 11 days (personal communication M. Weksler) following infusion of IVIG. Bacher et al. investigated the fate of ^{111}Indium-conjugated, affinity-purified human NAbs in the transgenic APP23 mice model.[48] In this study, blood clearance half-lives were around 20–30 hours for anti-Aβ NAbs, compared with 29 hours for the commercially available antibodies 4G8 and 27 hours for 6E10 and 50 hours for the CD20 antibody Rituximab, which was used as a reference antibody. Anti-Aβ NAbs were excreted in the urinary tract, nevertheless, liver and kidney uptake of anti-Aβ NAbs increased over time. The brain-to-blood radioactivity ratio for anti-Aβ NAbs at later time points (> 48 hours) was unexpectedly high compared with the other antibodies investigated. In the brain the distribution varied, with highest values found in the hippocampus, indicating a cerebral accumulation of human anti-Aβ NAbs in the APP23 model.

CLINICAL STUDIES IN ALZHEIMER DISEASE

The observation that IVIG, purified from the plasma of presumably healthy donors, contains naturally occurring anti-Aβ IgG antibodies[7,49,50] and that the sera of patients with AD may have reduced concentrations of these IgGs as compared with age-matched controls,[4,36] has provided the rationale for exploring the therapeutic use of IVIG in these individuals. Several small pilot trials have been initiated for the treatment of AD. A detailed review of the current clinical trials using IVIG has been published recently.[51]

The first description of the use of IVIG in patients with AD was published in 1998/2000 by Kountouris et al.[52] The study included 16 AD patients for a duration of 12 months. Eight patients received 140 g of peracetam (a NSAIDs) for one year only and 8 patients received a complementary monthly dose of IVIG of 0.2 g/kg body weight. The treatment demonstrated "a significant improvement in the group of patients, obtaining the additional treatment with IVIG." Unfortunately, the clinical study was only published as an abstract and thus no information on the rationale, clinical data and detailed results are available. Currently, only data from a total of 37 AD patients following IVIG treatment have been published.[53,54] The primary outcome in the studies was a change in the concentration of Aβ in the blood and CSF. Secondary objectives were the concentration of anti-Aβ NAbs, cognitive function, activities of daily living (ADL) and other AD relevant outcomes. In the available studies a decrease of Aβ in the CSF and an increase in the blood was found. In one study an amelioration of cognitive functions was seen using the Mini Mental Status Examination (MMSE). However, due to the small number of patients in all the studies, an effect of IVIG on cognition must be seen with caution. Side effects in those pilot studies were expectedly low.

A dose-finding study involving 56 AD patients, who were treated with Octagam 10% (IVIG) for 6 months with different doses and dosing intervals (0.1 g/kg/2wks; 0.25 g/kg/2wks; 0.4 g/kg/2wks; 0.2 g/kg/4wks; 0.5 g/kg/4wks; 0.8 g/kg/4wks; Placebo/2wks; Placebo/4wks) has been completed recently and data should be available in the first quarter 2012.[55] A large Phase III study involving more than 360 AD patients with two doses (0.2 g/kg every 2 wks and 0.4g/kg every 4 wks) is currently underway and results should be available in 2012/2013.[56] The study aims at AD-relevant outcomes including cognitive scores such as Alzheimer Disease Assessment Scale (ADAS-cog) and the clinical global impression scale (CGI).

Further evidence for the effect of IVIG was presented in a study in which anonymized medical claims data was used in a retrospective case-controlled analysis to evaluate the incidence of AD and related disorders in an IVIG treated vs. untreated population.[57] 847 IVIG-treated patients were compared with 84,700 untreated controls matched on age, gender, and other risk factors. The proportion of patients diagnosed with dementia was 2.0 percent among treated cases and 4.2 percent among untreated controls ($p < 0.002$). The relative risk reported was 0.58 for treated patients vs. controls ($p = 0.024$), "indicating a 42 percent lower incidence rate of dementia in patients treated with IVIG."

CONCLUSION

The concept of an innate system for disposing misfolded proteins is interesting and opens a new path in the investigation of neurodegenerative disorders. Further preclinical and clinical research is needed to delineate in greater detail the pathways by which naturally occurring autoantibodies act in the different pathological processes associated with neurodegenerative disorders. These studies are underway in a large number of laboratories and in 2013 the results of a large Phase III clinical trial should be available that may provide evidence that IVIG is an effective treatment for the terrible handicaps associated with Alzheimer disease.

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CHAPTER 8

MULTI-FACETED ROLE OF NATURALLY OCCURRING AUTOANTIBODIES IN FIGHTING PATHOGENS

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Abstract: Naturally occurring antibodies (NAbs) play a vital role in the first line of defense against bacterial and viral infections. Most studies in mice and man have attributed this role to NAbs of the IgM isotype. However, there is also a significant amount of data on the anti-infectious function of NAbs of the IgG isotype. Most of these observations are derived from studies using a privileged source of NAbs, the pooled human IgG for intravenous application, IVIG. In addition to its use as a replacement in humoral immunodeficiencies, IVIG is extensively used in autoimmune and inflammatory diseases. The properties of NAbs, the principal components of IVIG, are considered crucial for their immune-regulatory properties, owing to their ability to recognize self-antigens and even autoantibodies. By virtue of these specificities for several cellular antigens, including exposed proteins that act as receptors for a variety of pathogens, certain NAbs in IVIG have a therapeutic role in preventing or modulating infections. We summarize in this chapter several examples that highlight the importance of NAbs in the control of certain bacterial and viral infections.

INTRODUCTION

Normal human serum contains antibodies of the immunoglobulin G (IgG), IgM and IgA isotypes that are produced in the absence of any prior immunization and represent primarily naturally occurring autoantibodies (NAbs).[1] Several lines of evidence suggest

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that NAbs are produced by positively selected auto-reactive B lymphocytes.[2] Several physiological functions have been proposed for NAbs including their role in maintaining homeostasis and in defense against infections.[3] In this chapter, we summarize several lines of arguments and examples that highlight the importance of NAbs in the control of certain bacterial and viral infections. Most of these examples originate from the use of IVIG, which is a privileged source of naturally occurring antibodies, as IVIG contains essentially IgG obtained from pools of plasma from several thousands of healthy blood donors.

NAbs IN THE TREATMENT OF BACTERIAL INFECTIONS

The Role of NAbs in the Treatment of *Clostridium difficile* Infection

Clostridium difficile (CD) is the most common cause of nosocomial infectious diarrhea. The high virulence, the frequent relapses and the high prevalence require the development of more effective treatments against CD infections (CDI).[4] Currently, IVIG is among the available treatments administered in cases of severe CDI. It was shown in a retrospective analysis of IVIG-treated patients with five of them with recurrent relapse, who were treated with IVIG at 300 to 500 mg/kg, one to six doses per day. One patient showed a complete response, three patients showed a partial resolution of symptoms and one died without an apparent therapeutic response.[5] A retrospective analysis was conducted by McPherson et al.[6] on 264 patients with CDI, including 14 patients under IVIG treatment. Nine of 14 patients had a complete response, four patients did not show improvement and one patient had a partial response from two doses but died two months later after a recurrence. No complications were attributable to the IVIG in this study. The authors conclude that intravenous immunoglobulin may be effective without side effects for severe, refractory, or recurrent Clostridium difficile diarrhea after conventional treatment failed.[6] Hassett et al.[7] reported on a 49-year-old woman deficient for IgG1 with nine episodes of CDI associated with colitis in two years, who was successfully treated with IVIG in combination with a probiotic agent. This treatment combined oral vancomycin, metronidazole and rifampicin with 1 g per day of *Saccharomyces boulardii* probiotic in combination with 30 g of IVIG every 2 weeks to compensate the immune deficiency. The authors conclude that combining the standard therapy with IVIG appears to be more effective in this immunocompromised patient, than standard care alone. All these studies suggest that IVIG may be beneficial in cases of CDI recurrence, but definitive recommendations are not possible with the currently available data. Other studies and other controlled trials should be conducted to clarify the benefits of IVIG in the treatment of infection in CDI. Current guidelines suggest that IVIG may be considered to prevent multiple recurrences in patients with severe CDI.[8]

NAbs IN THE CONTROL OF VIRAL INFECTION

Intravenous (IV), intramuscular (IM) and virus-specific immunoglobulins (Ig) play an important role in immunotherapies against viral infections. The standard IM Ig is used in some clinical cases of hepatitis A prevention and measles. Hyperimmune globulins against the varicella zoster virus, Hepatitis B and rabies have proven efficacy in post-exposure prophylaxis. Hyperimmune globulins against cytomegalovirus (CMV) are indicated in

prevention of primary CMV infection and effectively reduced CMV infections after liver transplantation. Hyperimmune globulins against respiratory syncytial virus (RSV) have been developed and are used to prevent RSV infection in children with high risk. IVIG has demonstrated its therapeutic benefit in the prevention of disease associated with CMV and graft rejection in allogeneic bone marrow transplantation. In addition, IVIG in combination with ganciclovir is an effective treatment against the disease associated with CMV infection in bone marrow transplant and that of solid organs.

Intravenous Immunoglobulins in the Treatment of Dengue Illness

Infection with dengue virus (DV) has a high mortality/morbidity in the world and threatens about 20 million people annually in tropical and subtropical countries.[9] About 250,000–500,000 infected people develop severe dengue illness each year inducing approximately 24 000 deaths. The infection with dengue virus results principally in two syndromes: dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) associated with thrombocytopenia, altered homeostasis, and damage to the liver indicated by increase in aspartate aminotransferase and alanine aminotransferase activity.[9-11]

The high numbers of IgG molecules associated with platelets (PAIgG) in idiopathic thrombocytopenic purpura (ITP) suggested that these antibodies are involved in the induction of thrombocytopenia.[12,13] Several studies similarly suggested that production of PAIgG in patients with secondary infection by DV may be involved in thrombocytopenia by inducing lysis or phagocytosis.[12] The high levels of PAIgG in patients with secondary infection by DV were inversely correlated with platelet counts.[14-16] In fact, Ascher et al.[17] showed an effective clinical response of IVIG in a patient with an acute secondary dengue infection. Ostranoff et al.[18] reported that five patients in Brazil with dengue virus infection and severe thrombocytopenia who were treated with IVIG (500 mg/kg daily infusions for 5 days) improved their platelet counts and clinical signs. However, the only randomized trial published to date evaluating the effect of IVIG on thrombocytopenia associated with dengue infection has shown disappointing results.[19] In this study, patients (mostly children) with acute infection and thrombocytopenia (20,000–80,000/mm^2) were randomized to receive either IVIG (0.4g/kg/day for 3 days, n = 15) or placebo (n = 16). Despite the small number of patients in this study, the results show that IVIG seems to have no effect on platelet counts, which suggests that many factors other than platelet clearance by macrophages via Fc-γ receptors are responsible for thrombocytopenia associated with dengue infection. Another explanation for the lack of efficiency of IVIG in reversing thrombocytopenia could emerge from the observation that during a dengue epidemic in Taiwan in 1998/1999, the auto-reactive antibodies to platelets were of IgM and not IgG isotype.[20] Generally, inefficiency of IVIG in the treatment of severe thrombocytopenia associated with secondary infection by DV suggests that the clearance of platelets by macrophages via a Fc receptor-dependent pathway is not a primary mechanism of thrombocytopenia in secondary dengue virus infection. In addition to the possible effects attributed to the interactions between IVIG and Fc-γ receptors, these immunoglobulins may interfere with the process of infection and/or the cascades inducing cytokine production.[21,22] Indeed, it was shown that IVIG selectively triggers the production of receptor antagonist of IL-1 (IL-1ra).[22]

Other immune mechanisms have also been proposed to be responsible for the clearance of platelets, since the levels of complement activation correlated with disease severity.[23-26] Activation of the complement system can be either (1) protective by limiting

viral replication, increasing the virus elimination and inhibiting the antibody-dependent enhancement (ADE); or (2) deleterious following an exacerbated inflammatory response.[27] Bokisch et al. reported on an accelerated complement consumption and a marked reduction in plasma complement components during shock in patients with dengue shock syndrome (DSS).[28] This led to the proposal that complement activation plays an important role in disease pathogenesis[28] and Avirutnan et al. proposed that the major nonstructural DV protein, NS1, is involved in complement activation.[29] A prospective study measuring viral load, the concentration of the viral NS1 protein, and serum complement-activation products in 163 samples from patients infected with DV and 16 patients with other febrile illnesses concluded that NS1 protein in serum is implicated in complement activation. The concentration of viral NS1 protein in plasma and that of the terminal complement complex, SC5b-9, correlate with progression and severity of the disease.[29] Recently, in contrast with this first report, the same author associated with another group reported that viral NS1, which accumulates in blood and is displayed on the surface of infected cells, is implicated in immune evasion.[30] Indeed, the NS1 protein inhibits the classical and lectin complement activation pathways by interacting directly with C4. This interaction reduced the C4b production and thus decreased the enzymatic activity of the C3 convertase (C4b2a). Although the NS1 binds to C4b, it does not degrade C4b and does not block the formation of the C3 convertase nor accelerate the decay of C3 and C5 convertases. Instead, NS1 facilitates cleavage of the C4 protein by recruiting and activating protease C1s. NS1 allows degradation of C4 and C4b and thereby protects DV from lysis by complement.[30]

Circulating high levels of NS1 in the presence of pre-existing heterologous non-neutralizing antibodies may mediate complement activation and trigger plasma leakage that with hemorrhagic syndrome are the clinical features associated with dengue infection.[31] Other studies showed that the endothelial cell binding activity was inhibited by pretreatment with DV NS1 protein. The antibodies against NS1 produced after dengue virus infection may, at least in part, account for the cross-reactivity of patient sera with endothelial cells. Furthermore, dengue patient sera induced endothelial cell apoptosis via a caspase-dependent pathway that was also inhibited by NS1 pretreatment. In addition to apoptosis, patient sera caused cell lysis in the presence of complement, and sera from patients with shock showed higher percentages of cytotoxicity than those from dengue fever patients.[32]

In conclusion and despite benefits in ITP, the studies published so far do not show benefit with IVIG in preventing or treating thrombocytopenia in dengue and therefore no treatment by IVIG is recommended at this time. However, the possible beneficial effects of IVIG on vascular leakage and resultant dengue shock syndrome, one of the main complications and cause of death, remains to be clinically verified. Further studies are warranted to assess the effect of such treatment on mortality associated with the DV infection.

Antibody Therapy for the Prevention of Severe Respiratory Syncytial Virus Infection

Respiratory syncytial virus (RSV) is a ubiquitous enveloped RNA paramyxovirus. RSV is the leading cause of bronchiolitis and viral pneumonia in infants and young children. This is a virus whose only known reservoir are human beings and whose transmission is through direct contact with infected secretions. The absence of an effective vaccine against RSV has led to testing the efficacy of preparations enriched in naturally occurring

antibodies against RSV. One named RSV-IGIV (RespiGam, MedImmune, Gaithersburg, MD) was prepared by harvesting RSV-specific immunoglobulins from pooled human plasma. This polyclonal RSV hyperimmune globulin was made from healthy human blood that had a high level of immunoglobulins against RSV without the donors having been immunized. This preparation was approved by the Federal Drug Administration (FDA) in January 1996 and indicated for treatment of infants and children under 24 mo with CLD or a history of preterm birth (< 35 weeks of gestation). The success of RSV-IGIV validated the immunoprophylaxis approach for RSV prevention and led to the development of Synagis (palivizumab; MedImmune, Gaithersburg, MD), a humanized monoclonal antibody (mAb) that binds to the RSV F protein. Palivizumab is a potent anti-RSV mAb that is about 50-fold more potent than RSV-IGIV. In mid 1998 the FDA approved the use of palivizumab. This antibody reduced the severe RSV infection of the lower respiratory tract in infants and children at high risk. Both preparations, palivizumab and RSV-IGIV, decreased the incidence of hospitalization due to RSV infections and admissions to intensive care and their effects appear to be qualitatively similar.[33]

Intravenous Immunoglobulin in Immunoprophylaxis against Cytomegalovirus Infection

Cytomegalovirus (CMV) is a ubiquitous herpes virus that infects 60–100% of humans.[34,35] A primary infection occurs most often during the first 2 decades of life. In an immunocompetent person, infection is often asymptomatic or may manifest with a mild febrile illness like mononucleosis. The incidence of CMV infection is highest during the first three months after liver transplantation, particularly in the first six weeks. After pediatric liver transplantation, CMV infection is responsible for a morbidity with an incidence of 40% and a mortality rate reported as 19%. Improved diagnostic tests, immunosuppression and the treatment of CMV infection led to a significant reduction in morbidity/mortality after surgery related to this virus.[36,37] Three main approaches are known as preventive strategies against CMV infection in children: single ganciclovir, ganciclovir and immunoglobulins or more immunoglobulin alone.[38] Cytogam®, Cytomegalovirus Immune Globulin Intravenous (Human) (CMV-IGIV) is a preparation of immunoglobulins G (IgG) containing a standard amount of antibodies against CMV. These immunoglobulins are purified from human plasma of adults selected for their high titers of antibodies against the CMV and are stabilized with 5% sucrose and 1% human albumin.[39] However, treatment by IVIG in the prevention of disease related to CMV infection is limited to pediatric recipients of liver transplantation.[40] In case of solid organ transplantation in adults, the prevention strategy against CMV is mainly based on antiviral treatment with ganciclovir (intravenously for 4 to 12 weeks). However, previous studies have also demonstrated the benefits of IVIG in the case of CMV infection in adult liver transplant recipients.[41]

Intravenous Immunoglobulins and HIV Infection

Naturally Occurring Antibodies against HIV Receptors and Co-Receptors

IVIG preparations have been shown to contain NAbs against several cell surface molecules, including the cluster of differentiation CD4, the β-chemokine (CC) receptor CCR5 (CD195) and the dendritic cell-specific intercellular adhesion molecule-3-grabbing

non-integrin molecule (DC-SIGN, CD209) of which the latter is involved in the binding of HIV-1 to dendritic cells (DCs) and in the transmission of HIV from dendritic cells to T lymphocytes.[42-45] In this chapter, we summarize some in vitro HIV-1 neutralization tests using antibodies to CCR5 and DC-SIGN, purified from IVIG preparations.

NAbs against CCR5 in HIV Infection

The role of NAbs in immune regulation has been documented by studies showing the beneficial effect of IVIG therapies in patients with autoimmune diseases. Several mechanisms of action have been reported mainly depending of the presence of NAbs against a multitude of receptors and molecules of interest in regulating the immune response in these IgG preparations. For example, it was reported that IVIG contains NAbs that recognize the cytokine receptors, CD4, CD5, integrins and selectin molecules, and the death receptor FAS (CD95).[43] In addition, IVIG contains NAbs directed against chemokine receptors CXCR4 (CD197) and especially CCR5 (CD195) that are described as HIV coreceptors for X4 and R5 strains, respectively.[46,47] Viruses using CCR5, also called R5-tropic strains, are found in the early stages of infection by HIV and thus described as responsible for mucosal transmission.[48,49] The importance of CCR5 in HIV transmission was demonstrated by the fact that in certain humans, the CCR5 gene has a deletion (Delta 32). Homozygous carriers of this mutation are resistant to infection by R5-tropic HIV strains.[50-52]

The CCR5 molecule is thus considered a potent target for therapeutic strategies aimed to block the entry of HIV.[53] Therefore, NAbs to CCR5, previously found in some HIV-exposed, but uninfected patients may play a protective role against HIV infection. Resistance to the HIV infection is also linked to the existence of antibodies against HLA class I, CD4 and other surface receptors.[54-56] Therefore, the presence of naturally occurring anti-CCR5 antibodies at the sites of viral transmission could be effective in preventing attachment and penetration of HIV. In this context, we have shown that anti-CCR5 NAbs purified from IVIG preparations were able to inhibit the infection of macrophages and $CD4^+$ T lymphocytes by R5 strains of HIV.[44] The anti-CCR5 IgG NAb was purified by affinity chromatography on a synthetic peptide corresponding to a 20-mer motif from the N-terminus of CCR5. The latter region is involved in the interaction with the viral glycoprotein gp120. The purified IgG NAbs were able to recognize immobilized CCR5 protein in an ELISA assay as well as the expressed protein on human macrophages and $CD4^+$ T lymphocytes. The corresponding $F(ab)_2$ fragments interacted specifically with the binding site for the natural ligand of CCR5, the Chemokine ligand 5 (CCL5), also called RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted). Tested in vitro, anti-CCR5 NAbs isolated from IVIG were able to block infection by HIV R5 strains of cells susceptible to infection by these viral strains. No inhibitory effect was obtained on cells susceptible to infection with X4 tropic strains. The degree of inhibition was comparable to that observed with a CCR5-blocking monoclonal antibody (clone 2D7) and RANTES.[41] On the other hand, the presence of anti-CCR5 NAbs has been documented in sera from individuals homozygous for the CCR5 deletion (CCR5delta32), who had been repeatedly exposed to HIV. The anti-CCR5 NAbs compete with the radiolabeled ligand RANTES and blocked its binding to the CCR5 receptor and thus inhibited the infection of mononuclear cells by R5 strains, but not X4 strains.[57] The chimeric immunogenic peptides containing CCR5 incorporated into the capsid protein of Flock House virus makes it possible to maintain a highly immunogenic conformation of

peptides. Administered to mice via systemic or mucosal pathways induces the production of anti-CCR5 from IgG and IgA isotypes in serum and vaginal secretions. In analogy with seronegative individuals exposed to HIV, these mice produce anti-CCR5 autoantibodies that significantly reduced expression levels of CCR5 on $CD4^+$ T cells. In vitro studies have shown that these mouse antibodies were able to (1) bind specifically to human and mice CD4, (2) reduce the expression of CCR5 on the $CD4^+$ T-cell surfaces, (3) inhibit chemotaxis induced by MIP-1, another ligand of CCR5 and finally (4) block infection by R5 HIV strains. These data suggest that strategies designed to generate anti-CCR5 antibodies at the genital mucosa might be possible and allow induction of protective mucosal immunity against HIV transmission.[58]

Genital mucosa is the primary site of HIV transmission. R5-tropic strains are preferentially transmitted by the mucosal route. In view of the importance of CCR5 as a portal of entry, anti-CCR5 NAbs may block this HIV co-receptor in addition to modulate its surface expression on target cells and thus inhibit the infectivity of R5 strains of HIV-1.[59,60] After crossing the epithelial barrier, the virus spreads rapidly by contact between dendritic cells and $CD4^+$ T cells that promote HIV replication at an early stage of infection.[61-63] Therefore, blocking CCR5 with anti-CCR5 NAbs could inhibit the spread of R5-tropic virus in vivo. CCR5 and CXCR4 co-receptors of strains R5 and X4 tropic HIV-1, respectively, represent a potential target to block viral entry and infection of lymphocytes and macrophages in vivo. A number of monoclonal antibodies have been developed trying to block the interaction of the virus with the co-receptors.[64]

As described previously, IVIG contains anti-CD4 NAbs that block HIV entry.[65] Thus, the presence of anti-CD4 and anti-CCR5 NAbs that both block HIV infection allows the speculation that other NAbs directed against additional HIV coreceptors such as CXCR4, CCR3, CCR2b and DC-SIGN may exist.

NAbs against DC-SIGN in HIV Infection

Immature dendritic cells (iDCs) present in mucosal tissue are among the first targets of HIV.[66,67] The viral particles are captured by iDCs, then transported to the lymph nodes, where the virus is transmitted efficiently to $CD4^+$ T lymphocytes. The interaction of viruses with dendritic cells involves cell surface molecules such as a C-type lectin receptor, DC-SIGN.[68] This molecule also allows the internalization of infectious particles and their recycling to the plasma membrane prior to transmission to $CD4^+$ T lymphocytes.[69-71] DC-SIGN (CD209), a C-type lectin, plays a crucial role in binding of HIV to the dendritic cells surface and thus the transmission of HIV to target cells. At mucosal surfaces, dendritic cells are among the first cell targets that come into contact with the virus during sexual transmission. Surface molecules such as C-type lectins facilitate the attachment of the virus at the DC surface, followed by virus internalization. The virus that remains infectious will then be transported to the lymph nodes and transmitted to $CD4^+$ T cells taking advantage of the contact between dendritic cells and lymphocytes during antigen presentation. DC-SIGN is a mannose-binding C-type lectin expressed by DCs in the mucosal tissue, including the rectum, uterus and cervix.[72,74] DC-SIGN is organized as an ectodomain with seven complete and one incomplete copy of a 23-residue motif and a calcium-dependent lectin domain.[75] Induced anti-DC-SIGN antibodies are able to inhibit the binding of viral gp120 to target cells and to block HIV transmission.[70] The main site to which the

viral ligand gp120 binds is located at the top of the C-type lectin domain of DC-SIGN and includes two Ca^{2+} binding sites.[74,75]

IVIG preparations contain NAbs against the carbohydrate recognition domain (CRD) of DC-SIGN. These anti-DC-SIGN NAbs inhibit HIV transmission *in trans* from DC to autologous $CD4^+$ T cells.[45] The NAbs were purified by affinity chromatography by using a synthetic peptide of the CRD domain, including the Ca^{2+}-binding site 2, which is involved in HIV gp120 binding and Ca^{2+}-binding site 1, which stabilizes the gp120 binding. Moreover, pre-incubation of virus with peptides derived from the CRD motif prevents binding of HIV to DC-SIGN-containing cells in a dose-dependent manner.[45] Thus, blocking the interaction of gp120 with DC-SIGN may be effective in prophylaxis or therapeutic intervention. Identification of the epitopes of the blocking antibodies against DC-SIGN provides potential targets for vaccine strategies aimed at eliciting mucosal antibodies that inhibit gp120/DC-SIGN interactions. In summary, these observations suggest that affinity purified anti-CCR5 and anti-DC-SIGN NAbs from IVIG could be exploited therapeutically (see Fig. 1).

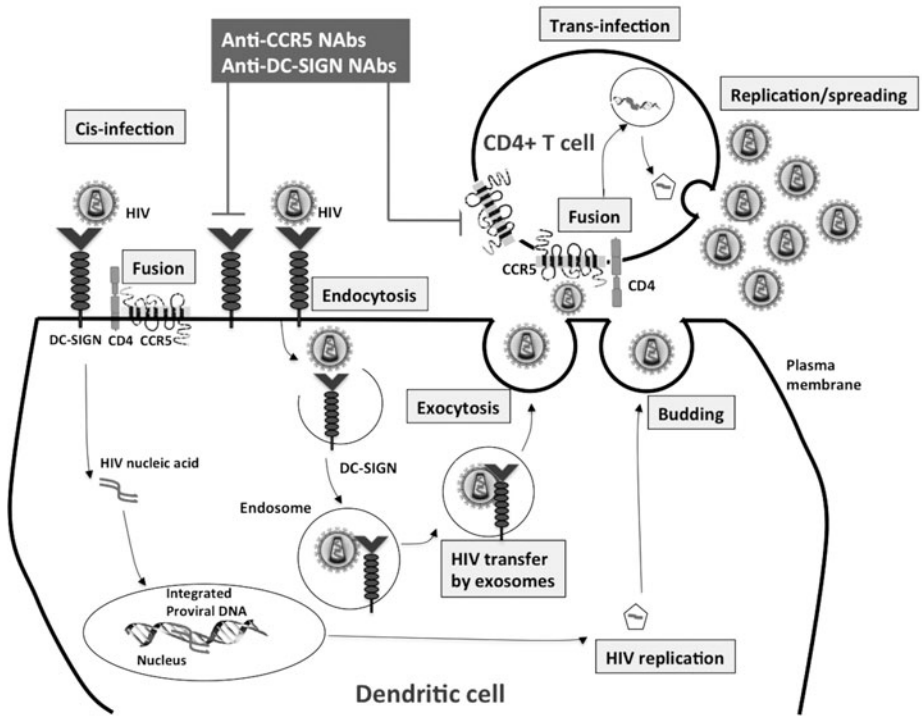

Figure 1. Involvement of DC-SIGN molecule in HIV infection. DC-SIGN is involved in both possible pathways of infection, namely the "cis- and trans-infection." The "cis-infection" comprises dendritic cell infection and/or internalization of infectious viral particles followed by provirus replication and exocytosis of conserved infectious particles. During the antigen presentation through the immunological synapse formation, DC transmit the virus to $CD4^+$ T lymphocytes. The importance of DC-SIGN and CCR5 in these mechanisms makes them potential targets of therapeutic strategies using NAbs that bind to these receptors and thereby may block HIV virus infection.

IVIG Treatment in HIV-Infected Patients

Several lines of evidence suggest the existence of other immunopathogenic factors during HIV infection such as increased TNF-α activity.[76] The TNF-α activity may enhance HIV-1 replication and may be implicated in development of immunodeficiency clinical manifestations in HIV-1-infected patients.[77] In the same way, a clear association between activation of the TNF system in vivo and the progression of clinical disease in HIV-1-seropositive patients was reported.[78,79] These elements underline that one possible therapeutic strategies would be to reduce the activity of the TNF system by IVIG adminstration.[80,81] In vivo, it has been suggested that IVIG infusion in patients with primary hypogammaglobulinemia induces increased plasma levels of soluble TNF receptors (sTNFR),[82] which in turn may block various biologic effects mediated by TNF-α.[83,84]

The action of TNF-α is mediated through binding to specific cell surface receptors (p55- and p75-TNFR), and the level of expression of these membrane-bound TNFR is evidently also of importance for the activity of TNF-α.[85] The in vivo down-modulation of TNF-α activity by IVIG administration has been reported in an experimental autoimmune encephalitis model.[81] These studies may provide pointers suggesting potential beneficial effects of IVIG in HIV-1-infected patients. However, placebo-controlled studies are needed to study the possible therapeutic role of IVIG as an immunomodulating agent in combination with antiretroviral therapy in HIV-1 infection. The effects of IVIG administration (400 mg per kilogram every 28 d) were compared with those of placebo (0.1% albumin) on $CD4^+$ lymphocyte count variation in HIV-infected children. $CD4^+$ counts were measured at entry and every 12 weeks. Analysis showed that the $CD4^+$ count declines by 13.5 cells/mm3 per month in IVIG as compared with the placebo recipients, suggesting beneficial effects of IVIG on the immunodeficiency in HIV-1-infected patients.[86]

The outlook for HIV-1-infected individuals has improved since the introduction of highly active antiretroviral therapy (HAART) in mid-1990s.[87,88] However, the use of HAART is limited by toxicity, high costs, the need for strict adherence to the medication, and the risk of development of viral resistance against the antiretroviral drugs. HAART suppresses viral replication and indirectly prevents the loss of CD4 and thereby decreases the antigenic activation of the immune system. An alternative therapeutic strategy would be to reduce directly the activation status of the immune system without affecting viral replication. Several immunosuppressive agents have been used for this purpose, such as hydroxurea[89] thalidomide,[90] and cyclosporine A,[91] but have not given interesting results for different reasons.

In another study, six HAART-naive adults with chronic HIV-1 infection were included in an open label and single center study to evaluate the effect of IVIG on the state of chronic immune activation and the plasma viral load (pVL). Patients were treated with IVIG (at a dosage of 400 mg/kg body weight, at week 0 and 4) which was well tolerated. Treatment with IVIG resulted in a small and temporary decrease in T-cell activation.[92] Unfortunately, the limited number of patients and the lack of a control group do not allow recommendations on the use of IVIG as an immunomodulatory agent in inhibiting immune activation. Even though the IVIG effects observed in this study were relatively small as compared with the HAART effects, the immunomodulating therapy by IVIG administration to the chronic HIV-infected patients could contribute to a prolonged asymptomatic phase. Additional trials should be performed to evaluate whether an immunomodulating therapy can indeed be effective in reducing immune activation and $CD4^+$ T-cell decline, thus delaying clinical progression with or without HAART.

In HIV-1-infected patients, studies have indicated beneficial effects of IVIG treatment on certain HIV-1 related complications, such as recurrent bacterial infections and idiopathic thrombocytopenic purpura[93] since evidence was obtained that IVIG decreased the number of serious bacterial infections in HIV-infected children before the availability of zidovudine.[94] The therapeutic management with IVIG has been tested in HIV-positive children. Indeed, in randomized double-blind clinical trials on children with AIDS who received zidovudine as therapy, the effect of IVIG was compared with that of placebo and revealed a decreased risk of bacterial infections in the IVIG group. This effect is only possible in patients not receiving prophylactic treatment against opportunistic infections, trimethoprim-sulfa-methoxazole.[95] Unfortunately, none of the recent studies has evaluated the IVIG therapy in children or adults with AIDS who received the HAART treatment. Although some evidence has been obtained for a prophylactic IVIG treatment in HIV-infected patients to decrease the frequency of serious infections, these data remain controversial as these studies did not take into account the concomitant trimethoprim-sulfa-methoxazole prophylactic treatment.[96] Thus, at the present time, the prophylactic use of IVIG to prevent the occurrence of recurrent bacterial infections in HIV-infected adults cannot be recommended.

CONCLUSION

In conclusion, all cited examples illustrate the importance of NAbs in the control of infection. In addition to the well-established role as a first line defense, the naturally occurring antibodies have proven of value in limiting a wide range of bacterial and viral infections in therapeutic settings. Extensive studies on the mechanisms of action of IVIG in autoimmune and inflammatory conditions for the past two decades have revealed that the beneficial effect underlying the anti-infectious properties of NAbs may extend well beyond their ability to neutralize toxins, immobilize microorganisms, neutralize bacterial/viral activity, agglutinate, precipitate and activate complement. Indeed, NAbs may exert anti-infectious roles by their ability to prevent the binding of pathogens to their receptors by virtue of their anti-receptor specificities and by interacting with the cellular compartment of the immune system to enhance the immunostimulatory adjuvant effect. Further research will help in defining the methods of therapeutic exploitation of the anti-infectious properties of NAbs.

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SECTION II

NAbs MODULATING AND REGULATING CELLS OF THE IMMUNE SYSTEM

CHAPTER 9

IMMUNOREGULATION BY NATURALLY OCCURRING AND DISEASE-ASSOCIATED AUTOANTIBODIES:

Binding to Cytokines and Their Role in Regulation of T-Cell Responses

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Abstract: The role of naturally occurring autoantibodies (NAbs) in homeostasis and in disease manifestations is poorly understood. In the present chapter, we review how NAbs may interfere with the cytokine network and how NAbs, through formation of complement-activating immune complexes with soluble self-antigens, may promote the uptake and presentation of self-molecules by antigen-presenting cells. Both naturally occurring and disease-associated autoantibodies against a variety of cytokines have been reported, including NAbs against interleukin (IL)-1α, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, interferon (IFN)-α, IFN-β, IFN-γ, macrophage chemotactic protein-1 and IL-21. NAbs against a variety of other self-antigens have also been reported, and using thyroglobulin as an example we discuss how NAbs are capable of promoting uptake of immune complexes via complement receptors and Fc-receptors on antigen-presenting cells and thereby regulate T-cell activity. Knowledge of the influence of NAbs against cytokines on immune homeostasis is likely to have wide-ranging implications both in understanding pathogenesis and in treatment of many immunoinflammatory disorders, including a number of autoimmune and autoinflammatory diseases.

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

INTRODUCTION

Naturally occurring antibodies play an essential role in our defense against invading microorganisms by directly neutralizing viruses and bacteria, by activating the complement system and by enhancement of phagocytosis, as described elsewhere in this book and in reference 1. In contrast, naturally occurring autoantibodies (NAbs) are characterized by binding to self-molecules, and their primary function may involve clearance of senescent cells and metabolic waste products, but they also appear to play important roles in immunoinflammatory processes.[2-6] In the present chapter, we shall focus on the immunomodulatory effects that self-reactive NAbs may have as a result of interactions with cytokines or formation of complement-activating immune complexes (ICs) with other soluble self-antigens and targeting these self-antigens to B cells and presumably other antigen-presenting cells (APCs).

NAbs have been shown to bind a variety of self-molecules, ranging from cytokines and other plasma proteins to tissue-specific antigens, structural proteins, metabolic enzymes, heat shock proteins and DNA (Tables 1 and 2). Some NAbs, including those reacting with various cytokines and thyroglobulin (TG), are relatively specific, while others, e.g., IgM anti-DNA antibodies of all species, tend to be polyreactive.[7] NAbs exist as IgM, IgA and IgG isotypes. The occurrence of the IgG isotype among NAbs is indicative of T-cell involvement in shaping of the autoreactive B-cell repertoire.[8-10] IgM NAbs are already synthesized in newborns, and different babies produce IgM NAbs to a similar set of self-molecules.[10]

AUTOANTIBODIES AGAINST CYTOKINES

Several reports have documented the presence of autoantibodies against cytokines both in healthy individuals (NAbs) and in patients with various immunoinflammatory disorders (disease-associated autoantibodies, DAbs) (Table 1). In most cases, the physiological and/or pathological significance of these antibodies is unclear. However, recent evidence suggests that certain anti-cytokine NAbs have significant, if not decisive, pathogenic roles in rare disorders (reviewed in refs. 9, 11, 12).

Naturally Occurring Autoantibodies against Cytokines

The biological roles of NAbs against cytokines are poorly understood. Fab fragments of some of these NAbs bind in a saturable and highly specific manner to their respective cytokine. as for example in the cases of NAbs to IL-1α, IL-6 and GM-CSF.[9] In some cases, these antibodies are found at levels at or above 50 nM, for example in healthy blood donors and apparently without untoward effects.[13]

Why and how high-affinity NAbs to some cytokines and not to others are induced in healthy individuals is unknown. Also obscure is whether these in vitro neutralizing antibodies neutralize their respective cytokines in vivo too, or whether they exhibit carrier- or cytokine-regulatory, or even cytokine-protective functions.[5]

Table 1. Selected cytokines to which naturally occurring autoantibodies have been reported[1]

NAbs against Cytokine	DAbs against Cytokine	Disease Association
Type 1 interferons	*Type 1 interferons*	
IFN-α/β	IFN-α/β/ω	Viral infections, autoimmune and neoplastic diseases, chronic graft-vs.-host disease, thymoma, myasthenia gravis, autoimmune polyendocrinopathy syndrome Type I
Type 2 interferons	*Type 2 interferons*	
IFN-γ	IFN-γ	Viral and mycobacterial infections, multiple sclerosis, Guillain Barré syndrome
Interleukins	*Interleukins*	
IL-1α	IL-1α	Rheumatoid arthritis, juvenile chronic arthritis, thymoma, myasthenia gravis
IL-2 IL-3	IL-2	inflammatory bowel diseases
IL-6	IL-6	Rheumatoid, arthritis, systemic sclerosis, alcoholic cirrhosis, Type 2 diabetes
IL-10	IL-10	Inflammatory bowel diseases
	IL-12p35 and IL-12p40	Thymoma, myasthenia gravis
	IL-17A, IL-17F	Thymoma, myasthenia gravis, autoimmune polyendocrinopathy syndrome Type I
IL-21		
	IL-22	Autoimmune polyendocrinopathy syndrome Type I
Growth factors	*Growth factors*	
GM-CSF	GM-CSF	Pulmonary alveolar proteinosis
G-CSF	G-CSF	Felty's syndrome (rheumatoid arthritis), systemic lupus erythematosus
TGF-β NGF	TGF-β	Inflammatory bowel diseases
Other cytokines	*Other cytokines*	
LIF TNF-α		
Chemokines	*Chemokines*	
IL-8	IL-8	Acute lung injury/acute respiratory distress syndrome
MCP-1		
	MIP-α/β	HIV infection

[1]Abbreviations: NAbs: naturally occurring autoantibodies; DAbs: disease-associated autoantibodies; IFN: interferon; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; G-CSF: granulocyte colony-stimulating factor; TGF: transforming growth factor; NGF: nerve growth factor; LIF: leukemia inhibitory factor; TNF: tumor necrosis factor; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein.

Table 2. Selected self-antigens to which naturally occurring autoantibodies have been reported[1]

Acetylcholinesterase[10]	HLA class I[96]
Actin[8,76,87-89]	Hsp40[10]
Albumin[10,87-89]	Hsp47[10]
Anion transport protein (band 3)[3]	Hsp60 peptides[10]
Annexin[10]	Hsp90[97]
Beta-galactosidase[90]	IgG[91]
Beta2-crystallin[10]	Insulin[90]
Beta2-microglobulin[10]	Keratin[8,10,88]
Cardiolipin[91]	Laminin[94]
Catalase[10]	LDL[10]
CD4[76]	MOG[10]
Chorionic gonadotropin[10]	Myelin basic protein[65,89]
Collagen[88,92]	Myoglobulin[8,76,87-89,94]
Cytochrome c[88]	Myosin[10,89,94]
dsDNA[8,10,76,89,93,94]	Prolactin[89]
Factor II[10]	Protease[10]
Factor VIII[95]	Pyruvate dehydrogenase[91]
Factor X[10]	Spectrin[93]
Fetuin[87]	ssDNA[10,91]
Fibrin[10]	T-cell receptors[98]
Fibrinogen[10]	Thyroglobulin[10,58,76,77,79,80,87-89,94,99]
GAD[10]	Thyroid peroxidase[84,89,100]
Galectin 1 and -3[10]	Transferrin[8,76,87,88]
Geisolin[10]	Tubulin[8,87-89,93]
Hemoglobin-α [10]	Ubiquitin[10]

[1]The table is not complete with respect to reported antigens and references.

NAbs against Type 1 and Type 2 Interferons

Low levels of IgG and IgM capable of neutralizing IFN-α, IFN-β and IFN-γ have been detected in blood of apparently healthy individuals (as reviewed in refs. 14, 15). IgG NAbs against IFN-α are especially frequent, and are easily detectable in pharmaceutical preparations of normal human IgG (IVIG). NAbs against IFN-α have been found in approximately 10% of healthy Caucasians, but the exact frequency is likely to be higher, as these NAbs are often difficult to detect in plasma because they are complexed with native IFN-α.[16] IgG NAbs against IFN-α are of high avidity and specific, as they do not cross-bind IFN-β or other cytokines. NAbs to IFN-α have been demonstrated in vivo in bioactive form after treatment with IVIG.[17] As these NAbs neutralize IFN-α, IVIG therapy suppresses both antiviral and other effects of endogenous IFN-α, which may explain some of the many therapeutic effects of IVIG.[5,14,17,18]

NAbs against Interleukin-1α

NAbs to interleukin (IL)-1α were first found by direct binding of IgG from normal individuals to radiolabeled, human, recombinant IL-1α and by IgG-mediated competitive

interference with IL-1α binding to cellular IL-1 receptors.[19] Subsequently, human IVIG, cord blood and sera of patients with various immunoinflammatory disorders were found to contain high-avidity autoantibodies that bind to and neutralize IL-1α both in vitro and in vivo.[5,9,17,18,20-22] They bind IL-1α in a saturable fashion and through the Fab fragments of the IgG isotypes IgG1, IgG2 and IgG4. The occurrence of detectable anti-IL-1α IgG in sera of healthy individuals vary with age and sex with male preponderance and markedly increased frequency with age (up to 75% positives among elderly males).[16,19,22]

A human anti-IL-1α autoantibody has been cloned.[23] It is an IgG4/κ monoclonal antibody which reacts with IL-1α, but not with IL-1β, the IL-1-receptor antagonist (IL-1Ra) or several other cytokines. It binds with high affinity (K_d »10^{-10} M), and the presence of somatic mutations in the variable regions suggests antigen-driven affinity maturation.

As IL-1α from antigen presenting cells is an important co-activator of T cells, particularly in its membrane-bound form, it is important to note that anti-IL-1α NAbs not only neutralize IL-1α in lysates of human blood monocytes, but also membrane-associated IL-1-α activity.[21]

NAbs against Interleukin-6

IgG NAbs to IL-6 were first reported in sera of normal individuals.[24] Since then, the presence of NAbs to IL-6 and similar antibodies in patients with immunoinflammatory and fibrotic diseases has been confirmed (reviewed in refs. 9, 11, 14, 15). High-affinity IgG NAbs to IL-6 have been detected in up to 15% of normal Danish blood donors with 1% having titers ranging from 64 to greater than 10,000 and 0.1% having exceedingly high titers.[13] The anti-IL-6 NAbs bind to IL-6 through their Fab fragments and they effectively inhibit binding of IL-6 to IL-6 receptors and, hence, neutralize the bioactivity of IL-6. NAb-positive donors with high antibody titers have no overt signs of pathology even though they are likely to be functionally IL-6-deficient. NAbs to IL-6 are detectable in IVIG and are found in bioactive form, binding IL-6 in the circulation following IVIG administration.[17]

NAbs against Interleukin-10

IgG NAbs against IL-10 have been reported in normal sera and in preparations of pooled normal human IgG.[15,25] Indeed, 0.4% of healthy Danish blood donors present with these NAbs at such high concentrations and avidity that these blood donors are functionally IL-10-deficient.[26] These NAbs are of the IgG isotype and of polyclonal origin. They prevent IL-10 from binding to its receptor thereby neutralizing IL-10 bioactivity. Anti-IL-10 NAbs are highly specific in that they fail to bind viral forms of IL-10 and other members of the human IL-10 family including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29.

NAbs against Granulocyte-Macrophage Colony-Stimulating Factor

Using a direct radioligand binding assay, high-affinity NAbs to granulocyte-macrophage colony-stimulating factor (GM-CSF) were reported at very high titers in 4 of 1,238 (0.3%) apparently healthy blood donors.[15] Later, all of 72 tested, apparently

healthy Japanese individuals were shown to possess low levels of these NAbs such that more than 99% of GM-CSF were bound and neutralized by these antibodies.[27] Anti-GM-CSF NAbs have also been found in human IVIG.[9,18]

NAbs against XXC- and CC Chemokines

NAbs to various chemokines have also been reported. For example, IL-8- and macrophage chemotactic protein (MCP)-1-containing IgG-immune complexes have been demonstrated in sera from healthy individuals, where the chemokines themselves are usually not detected. It is hypothesized that circulating IgG NAbs to chemokines may play a role as a sink for the spill-over of chemokines produced in local tissues.[28]

NAbs against Other Cytokines

IgG NAbs to other cytokines, for example IL-2, IL-3, granulocyte colony-stimulating factor (G-CSF), nerve growth factor (NGF), leukemia inhibitory factor (LIF), tumor necrosis factor (TNF)-α, and soluble TNF receptors have also been reported in normal and diseased individuals, while IgE antibodies to IL-4, TNF-α, TNF-β and various chemokines have been reported in sera of AIDS patients. Some of both isotypes, however, cannot be regarded as NAbs in that they bind the relevant mediator(s) with low avidities and in some cases bind only to cytokines denatured by adsorption to nitrocellulose membranes or plastic surfaces (using ELISA-technologies).

Disease-Associated Autoantibodies against Cytokines (DAbs)

Anti-cytokine DAbs have been reported in a wide range of pathological disorders, suggesting that pathogenetically obscure diseases may eventually be at least partly explained by the presence of anti-cytokine DAbs (reviewed in refs. 9, 11, 12).

DAbs against Type 1 and Type 2 Interferons

Anti-Type 1 interferon (IFN) DAbs, preferentially of IgG type, were first reported in patients with varicella-zoster and hepatitis virus infections, in patients with autoimmune and neoplastic diseases, and in a patient with chronic graft-vs.-host disease (reviewed in refs. l 5, 14, 15, 29, 30). Later reports have documented DAbs that bind to other IFN species, usually in patients with various infectious diseases or severe immunodeficiencies.

Neutralizing DAbs against IFN-α/β/ω have been demonstrated primarily in patients with thymoma and/or myasthenia gravis.[31] However, patients with thymic malignancy/ myasthenia gravis have also been reported to express DAbs to a variety of other cytokines including, IL-1α, IL-12 p35 and IL-12p40, and IL-17A.[31]

High-titer DAbs against IFN-α2 and IFN-ω have been reported in 100% of European patients with autoimmune polyendocrinopathy syndrome Type I (APS-I).[32] This disease is a result of mutations in the autoimmune regulator gene (AIRE), which impairs thymic self-tolerance induction in developing T cells. The ensuing autoimmunity particularly targets ectodermal and endocrine tissues, but chronic candidiasis is a frequent and early manifestation. Although the underlying immunodeficiency of APS-1 is unclear,

neutralizing anti-IFN DAbs and, most recently, anti-IL-17A and anti-IL-22 DAbs appear to be implicated directly in the pathogenesis, as they appear before development of candidiasis in all informative cases.[33,34]

Anti-IFN-γ DAbs have been reported in patients with viral infections and in cerebrospinal fluids from patients with multiple sclerosis and Guillain Barré syndrome (reviewed in ref. 15). Circulating DAbs to IFN-γ have also been positively correlated with the severity of both tuberculous and nontuberculous mycobacterial infections (reviewed in ref. 11). It is characteristic that patients with extremely high antibody titers had rapidly progressive disease and severe immunodeficiency, most likely due to DAb-induced blockade of the crucial macrophage-activating effect of IFN-γ.

DAbs against Interleukin-1α

Although IL-1α is secreted from cells producing the cytokine, the dominant form of IL-1α appears to be the cell-associated precursor form that is found both intracellularly and on the surface of many cell types, including keratinocytes and 'professional' APCs such as macrophages and B cells. On these cells IL-1α is thought to be involved as a juxtacrine co-activator of T cells.[9] IL-1α is usually absent in the circulation, if present then only at low concentrations. During infection and inflammation, however, substantial amounts of IL-1α may be found in the blood, most likely released from dying cells.

Cell-associated IL-1α is biologically active, and its biological activities are neutralized by antibodies to IL-1α, including IgG anti-IL-1α NAbs, but not by antibodies against IL-1β.[21]

The prevalence of DAbs to IL-1α in immunoinflammatory disorders vary considerably. For example, anti-IL-1α DAbs have been found more commonly and at higher levels in patients with non-destructive forms of arthritis.[35] Progression of joint destruction in patients with rheumatoid arthritis was negatively associated with the occurrence of circulating IL-1α DAbs, but patients who seroconverted more than two years after the onset of RA showed the most aggressive development of joint erosion. Interestingly, transgenic mice overexpressing the membrane form of human IL-1α in macrophage-like and fibroblast-like synoviocytes develop severe arthritis, correlating with the degree of membrane expression of IL-1α, but not circulating IL-1α.[36] Taken together, this suggests that IL-1α and/or the lack of IL-1α DAbs play a role in the erosive processes of rheumatoid arthritis. Along with DAbs to other cytokines, anti-IL-1α DAbs have also been demonstrated in patients with juvenile chronic arthritis, thymoma and myasthenia gravis.[22,31]

DAbs against Interleukin-6

There is an increased prevalence of high-avidity IgG DAbs to IL-6 in patients with rheumatoid arthritis and systemic sclerosis, and the presence of these antibodies signals a poor survival in patients with alcoholic cirrhosis, possibly because of an increased risk for recurrent infections.[9,37,38] A 2.5-fold increase in anti-IL-6 DAb-positivity has recently been reported in Type 2 diabetic patients, and mice vaccinated with IL-6 develop obesity and impaired glucose tolerance.[39] These data suggest that an autoimmune reaction against IL-6 may be involved in a subset of Type 2 diabetics.

DAbs against Granulocyte-Macrophage Colony-Stimulating Factor

Anti-GM-CSF DAbs are of clinical interest not only because of GM-CSF's growth-potentiating effect on macrophages and granulocytes, but also because GM-CSF appears to be a central mediator affecting bronchial epithelial cells, possibly through its marked effect on eosinophils, both as a chemoattractant, growth promoter and stimulator. In accordance with results obtained in GM-CSF knockout mice, anti-GM-CSF DAbs have been associated with pulmonary alveolar proteinosis (PAP), a rare disease in which surfactant lipids and proteins accumulate in pulmonary alveolar macrophages and alveoli, resulting in respiratory insufficiency and failure.[40] Recently, isolated anti-GMCSF DAbs from a patient with PAP were shown to reproduce the pathologic manifestations of the human disease in previously healthy primates.[41] These findings may have therapeutic implications for the potential use of GM-CSF not only to treat PAP, but also other immunoinflammatory respiratory disorders such as asthma.

DAbs against Granulocyte Colony-Stimulating Factor

IgG DAbs to granulocyte colony-stimulating factor (G-CSF) have been demonstrated in Felty's syndrome, a relatively rare complication in rheumatoid arthritis, and in some patients suffering from systemic lupus erythematosus (SLE) with accompanying neutropenia.[42] IgM antibodies were found in 6 neutropenic and 3 normocytic SLE patients. Interestingly, anti-G-CSF antibodies were associated with an exaggerated serum level of G-CSF and a low neutrophil count. This may suggest that exposure to high levels of intrinsic G-CSF (not known whether bioinactive) may trigger the production of G-CSF DAbs and, further, that these DAbs may have a carrier function in vivo thus slowing the elimination of G-CSF from the circulation.[5]

DAbs against Other Cytokines

Using radioimmune and radioreceptor assays, DAbs against macrophage-inhibitory protein (MIP)-1α and MIP-1β have been demonstrated in about 1% of patients, suffering from HIV infection.[43] These antibodies specifically inhibited receptor binding of both chemokines; there was no association between the presence of antibodies and disease stage, or HIV progression rate.

IgG DAbs against IL-2, IL-10 and transforming growth factor-β (TGF-β) have recently been demonstrated at relatively high concentrations in up to 33% of patients with inflammatory bowel diseases using ELISA and Western blot assays.[44]

Neutralizing DAbs against IL-12 p35 and IL-12p40 have been demonstrated primarily in patients with thymoma and/or myasthenia gravis.[31] Indeed, patients with thymic malignancy/myasthenia gravis express DAbs to a variety of other cytokines including IFN-α/β, IFN-ω, IL-1α and IL-17A.[31,45] Interestingly, among these patients those with opportunistic infections possess multiple anti-cytokine DAbs, suggesting that these antibodies may be important in the pathogenesis of infections in patients with thymic malignancy.

Most patients with autoimmune polyendocrine syndrome Type I suffer from chronic mucocutaneous candidiasis, and this immunodeficiency was recently associated with high titers of DAbs against IL-17A, IL-17F, and/or IL-22.[46] The DAbs against IL-17A, IL-17F

and IL-22 neutralized these cytokines, but not a host of other cytokines. As these DAbs were not found in healthy controls nor in 102 patients with other immunoinflammatory disorders, DAbs against IL-17A, IL-17F and IL-22 may have a causative relationship with the development of chronic mucocutaneous candidiasis in patients with this polyendocrine syndrome.

Anti-IL-8 DAbs, often complexed with endogenous IL-8, have been shown to be an important prognostic indicator for the development and outcome of acute lung injury/acute respiratory distress syndrome (ARDS).[47] The IL-8/anti-IL-8 complexes purified from lung edema fluids activate and trigger chemotaxis of neutrophils and regulate neutrophil apoptosis via IgG receptor FcγRIIa. These ICs promote an inflammatory phenotype of human umbilical vein endothelial cells, and they upregulate the expression of intercellular adhesion molecule (ICAM)-1 on the cell surface. Lung tissues from patients with ARDS also express high levels of ICAM-1. Hence, IL-8/anti-IL-8 complexes may contribute to pathogenesis of lung inflammation by inducing activation of endothelial cells through engagement of IgG receptors.

Therapy-Induced Autoantibodies to Cytokines (TAbs)

Anti-cytokine TAbs may develop in response to prolonged therapies with natural and recombinant-derived human cytokines. It is unclear whether preexisting anti-cytokine NAbs play a role in this regard, but they are probably not without importance, considering the high avidity and high titers of some of these NAbs (discussed above). In general, however, TAbs develop over time as a result of repeated 'inoculations' with cytokines. They may wax and wane, change in affinity, give rise to side-effects, and/or influence the primary intended therapy.

The development of neutralizing TAbs was first noted in scattered patients undergoing therapies with human fibroblast-derived IFN-β and human recombinant IFN-α.[48-51] This finding received little attention at that time, but the increased use of recombinant human cytokines and cytokine constructs for therapeutic purposes have sharpened the awareness of the clinical importance of anti-cytokine TAbs.[52-54] Thus, induction of TAbs has now been reported in patients treated with several human recombinant cytokines and growth factors including IFN-α, IFN-β, IFN-γ, IL-2, GM-CSF, often resulting in therapeutic failure and in rare cases even in serious side-effects (reviewed in refs. 9, 15, 55).

IMMUNOREGULATORY NAbs AGAINST NON-CYTOKINE SELF-ANTIGENS

While the above-mentioned anti-cytokine NAbs are immunoregulatory by nature, there is evidence to suggest that NAbs against other self-antigens may also be immunoregulatory, as we shall describe in the following.

NAbs against a broad variety of self-antigens has been demonstrated (Table 2), but their physiological functions, if any, has not been fully elucidated. While some of these NAbs have been suggested to play a role in the clearance of senescent cells and metabolic waste products, their role in maintenance (or breakage) of tolerance toward self remains obscure.[2-4]

One mechanism by which NAbs against soluble self-antigens may play a regulatory role is by targeting self-antigens to APCs and thereby affecting self-antigen presentation

to T cells. It is likely that NAbs of IgG isotype are capable of directing captured self-antigens to Fcγ-receptor expressing APCs.[56] Similarly, NAbs of IgM or IgA isotype may direct self-antigens to B cells and macrophages, expressing the Fcα/μ receptor.[57] All these events may promote presentation of self-antigens to autoreactive T cells.

One of the self-antigens against which NAbs have been most frequently described is thyroglobulin (TG). In a serum-free environment, a subset comprising 2–4% of normal, circulating B cells is capable of binding TG (this subset presumably represents B cells with polyreactive surface immunoglobulins), while TG binds to the entire B-cell population in the presence of autologous serum.[58] The binding can be substantially inhibited by immunoabsorption of TG-reactive autoantibodies from serum or by heat inactivation of serum complement, suggesting that formation of complement-activating ICs promoted the binding of TG to the B cells. In accordance with this hypothesis, blockade of complement receptors 1 (CR1/CD35) and 2 (CR2/CD21) reduced the binding of TG to B cells by > 90%, and addition of TG to preparations of normal mononuclear cells suspended in sera from healthy individuals lead to increased deposition of C3-fragments on B cells.[58,59] In accordance with these findings using TG as model self-antigen, Thornton et al. showed that normal serum contains NAbs with reactivity against the primary antigen keyhole limpet hemocyanin, and that ICs formed with these NAbs are capable of fixing fragments of complement component 3 (C3) and bind to B cells via CR1 and CR2 (Fig. 1B).[60,61] They further demonstrated that expression of the T-cell co-stimulatory molecule CD80 by B cells required a secondary ligation of IC-associated IgG to FcγRII.[60]

It is widely accepted that B cells bearing specific antigen-receptors efficiently take up and present antigens to T cells (Fig. 1A).[62] As outlined, non-specific B cells may also engage in antigen presentation provided for the antigen in question is incorporated in complement-opsonized ICs and thereby targeted to CR1, CR2 and FcγRII (Fig. 1B).[60,61,63,64] This recruitment of non-specific B cells increases the number of antigen-presenting B cells from a few antigen-specific ones to the entire B-cell population. However, only B cells bearing specific antigen receptors are eventually stimulated by T-helper (Th) cells for antibody production.[61] Thornton et al. also showed that iC3b-containing TG/NAb complexes were also taken up by neutrophils, via complement receptors CR1 and CR3 (CD11b/CD18), indicating that the formation of ICs with NAbs may also be similarly important for the uptake of self-antigens by myeloid cells (of relevance for dendritic cells).[60] Taken together, these findings suggest that antigen presentation is strongly promoted by incorporation of antigens into complement-activating ICs with NAbs. Correspondingly, the presentation of TG on B cells is proportional to the anti-TG NAb content of the surrounding serum, and is thus considerably higher in the presence of sera from patients with Hashimoto's thyroiditis (HT) or Graves' disease (GD) with a high content of anti-TG, as compared with sera from healthy individuals.

In analogous studies using myelin basic protein (MBP) as self-antigen, we found that sera from patients with multiple sclerosis (MS) and sera from healthy individuals contained approximately equal concentrations of MBP-reactive IgM.[65] Upon addition of MBP to normal mononuclear cells suspended in patient or control sera, MBP co-deposited with IgM, IgG and C3-fragments on monocytes. While the deposition of IgM and C3 was approximately similar in patient and control sera, the IgG deposition was increased 9-fold in the presence of MS sera, presumably due to the presence of circulating high-affinity IgG DAbs in the patients. Notably,

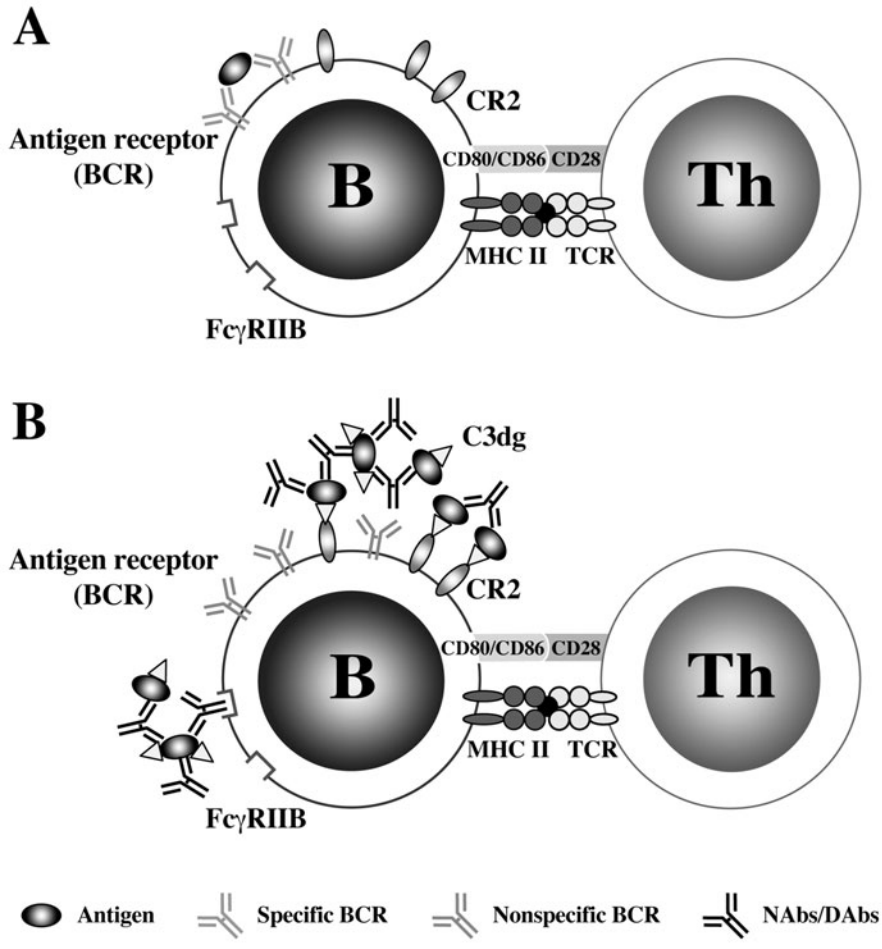

Figure 1. Antigen-presentation by antigen-specific and -nonspecific B cells. B cells may serve as antigen-presenting cells by two routes: A) Antigen-specific B cells take up minute amounts of antigen by virtue of the B-cell antigen receptor (BCR), degrade the antigen into peptides, which are incorporated into major histocompatibility complex class II molecules (MHC II), presented on the cell surface and recognized by T-cell receptors (TCR) of T helper (Th) cells. This, together with co-stimulatory signals mediated through the CD80/CD86–CD28 pathway and others, activates the Th cells. B) Antigen-nonspecific B cells, however, may also contribute to presentation of a given antigen, provided that the antigen is found in complement-activating immune complexes (ICs) generated by preexisting NAbs and/or DAbs. Attached to the ICs, the final degradation product of complement component 3 (C3dg) may bind to complement receptor 2 (CR2) on B cells, which then take up antigen, process it, and present antigen peptides on MHC class II molecules. C3-opsonized NAb/DAb-containing ICs may also be taken up by Fcγ receptor IIB (FcγRIIB). This latter type of interaction stimulates the CD80-dependent binding to Th cells.

the deposition of C3 fragments as well as that of IgM and MBP on monocytes was abrogated by disruption of the tertiary structure of MBP by boiling, as would be expected if complex formation depends upon the interaction of antibodies with conformational epitopes on MBP.

REGULATION OF T-CELL RESPONSES BY NAbs AND COMPLEMENT

In the presence of untreated serum, even $CD4^+$ Th cells from healthy individuals respond to a challenge with TG at high concentrations (≥ 10 μg/ml), although TG induces increased responses by Th cells (and B cells) from patients with autoimmune thyroid disease.[58,66] Inactivation of serum complement, immunoabsorption of TG-reactive NAbs or disruption of the tertiary structure of TG by boiling.significantly inhibits the TG-induced Th cell proliferation and production of T-cell cytokines such as IL-2 and IL-5. This suggests that Th cell responses to self-antigens are strongly influenced by formation of ICs between the self-antigens and NAbs. Presumably, NAbs target self-antigens to APCs, as has been shown for polyclonal antibodies to foreign antigens, as well as for DAbs to the thyroid self-antigen, thyroid peroxidase (TPO).[67-69]

Moreover, recruitment of T cells to the site of infection may also be influenced by NAbs and complement. Askenase and Tsuji demonstrated that T-cell-dependent contact sensitivity responses to hapten and subsequent rises in local IFN-γ levels were absent in pan B-cell- and antibody-deficient mice, but could be restored by adoptive transfer of purified normal peritoneal B-1 cells, or by i.v. injection of antigen-specific IgM monoclonal antibodies.[70] They concluded that the contact sensitivity response was initiated by the formation of complement-activating ICs between hapten and naturally occurring IgM antibodies, followed by complement activation and C5a-mediated release of vasoactive substances by mast cells, facilitating the recruitment of T cells.

It remains to be clarified whether IC-formation with NAbs contributes to maintenance of tolerance, or whether it promotes breakage of tolerance. In mononuclear cell cultures from healthy individuals, grown in media containing autologous serum (30% v/v), TG induces immediate production of TNF-α and IL-10 by mononuclear cells, followed by an almost exclusive production of IL-10, a regulatory cytokine with a protective role in autoimmune diseases.[71,72] A subset of T cells with a CD45RO memory phenotype seems to orchestrate this IL-10 production, suggesting that the TG-driven T-cell response in healthy individuals is protective and contributes to the maintenance of tolerance.[72] By comparison, the foreign antigen tetanus toxoid induces no IL-10 production, but instead a mixed pro-inflammatory Th1/Th2-response (IL-2, IFN-γ, IL-4 and IL-5) under similar conditions.[72] An absolute requirement for an intact tertiary structure of TG and MBP for induction of an IL-10 release by mononuclear cells supports a role for NAbs in maintaining tolerance.[65,73]

DIFFERENCES BETWEEN AUTOANTIBODIES IN HEALTH AND DISEASE

NAbs and DAbs against cytokines and other self-antigens as discussed here, differ from one another in terms of isotype distribution and epitope recognition patterns. For example, in autoimmune thyroid disease most of the anti-TG activity is associated with IgG, with only ~1% in the form of IgM, whereas TG-reactive NAbs are predominantly of IgM isotype. Nevertheless, as much as 0.3% of IgG in IVIG preparations for intravenous use are reactive with TG or cytokines such as IL-1α and IL-6.[9,13,74,75] There is also evidence to suggest that the antibody recognition patterns of NAbs differ from those of DAbs. For example, the latter are more restricted in idiotypes and less polyreactive than NAbs.[76] A certain idiotype, T44 Id, is associated with autoimmune thyroid disease and recognizes one of at least six epitopic clusters on human TG, designated region II.[77,78] DAbs derived from sera of patients with Hashimoto's thyreoiditis and Graves' disease

recognize primarily region II and occasionally another region (region IV).[79] By contrast, NAbs frequently react with region V and rarely with region II.[80] Thus, recognition of region V may reflect the normal homeostatic recognition of TG.

Interestingly, the reactivity against particular epitopes commonly recognized by both NAbs and DAbs seems to change with aging, without affecting the total IgG anti-TG autoreactivity.[80] We have recently demonstrated that NAbs to thyroid peroxidase (TPO) show a quantitatively different recognition pattern than DAbs from patients with HT. Anti-TPO NAbs recognize an immunodominant region involving two conformational, overlapping epitopes on TPO, referred to as immunodominant regions A (IDR-A) and –B (IDR-B).[81,82] In HT, approximately 50% of anti-TPO DAbs are directed to the IDR-B epitope, while DAbs against the IDR-A and non-A/non-B regions are approximately equally distributed.[83] TPO-reactive NAbs, on the other hand, contain a significantly lower proportion of antibodies to IDR-A.[84] Interestingly, the propensity to produce autoantibodies directed against the IDR-A epitope of TPO seems to be inherited. We recently demonstrated that HT patients and their healthy, monozygotic co-twins had higher proportions of IDR-A-reactive anti-TPO antibodies (medians 19% and 18%, respectively) than healthy ordinary siblings to HT patients (9%) and euthyroid controls with no family history of HT (0%).[85] These data confirmed the findings by Jaume et al. based on family studies that IDR-recognition patterns were genetically transmitted.[86] In other words, the propensity to produce certain DAb reactivities may be inherited. Further studies are required to determine whether this applies to DAbs in general.

CONCLUSION

We have reviewed the immonoregulatory role of NAbs with special focus on autoantibodies against cytokines and other soluble self-antigens. Based on numerous publications, we and others believe that NAbs against cytokines and other self-molecules may in many cases contribute to homeostasis, and that DAbs may contribute to disease manifestations, in some instances perhaps as causative pathogenetic factors. These diseases likely include both autoimmune and autoinflammatory conditions. Moreover, TAbs may neutralize the effect of a number of "biologic" drugs and give rise to side-effects

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CHAPTER 10

MODULATION OF DENDRITIC CELLS AND REGULATORY T CELLS BY NATURALLY OCCURRING ANTIBODIES

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Abstract: Most studies on the effects of naturally occurring autoantibodies (NAbs) on immune cells have been performed in the context of research on the immunomodulatory effects of intravenous immunoglobulin (IVIG). Among others, IVIG inhibits the differentiation, maturation and functions of dendritic cells (DC), thereby suppressing T-cell activation. In addition, IVIG stimulates expansion and suppressive function of regulatory T cells (Treg) carrying the antigens CD4, CD25 and Foxp3. Current data on the immunomodulatory effects of IVIG on DC and Treg are summarized, and possible molecular interactions between NAbs and DC or Treg that mediate these effects are discussed.

INTRODUCTION

Our current knowledge about the effects of NAbs on immune cells is largely derived from studies of the immunomodulatory effects of IVIG. IVIG-formulations consist of human IgG purified from a pool of plasma derived from several thousands of healthy blood donors. IVIG consists largely of IgG, with a few percents of IgA and in some preparations trace amounts of IgM. The IgG-molecules in IVIG-formulations are representative of the NAb repertoire in the human population and the donors' exposure to pathogens as is evident from the content of induced Abs.

Initially, IVIG was used as a substitution therapy in immunoglobulin deficiencies. However, since the demonstration in 1981 that IVIG ameliorates immune thrombocytopenic purpura (ITP),[1] IVIG is increasingly being used for the treatment of a wide range of

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autoimmune and inflammatory diseases.[2] In addition to antibody-mediated diseases, IVIG is also effective in treatment of several disorders caused by derailment of cellular immunity, like Kawasaki disease, multiple sclerosis,[3,4] graft-vs.-host disease in recipients of allogeneic hematopoietic stem cell transplants,[5] and allogeneic organ transplant rejection.[6-9] Studies exploring the mechanisms of action by which IVIG suppresses cellular immune responses show inhibitory effects on Antigen-Presenting Cells (APC), among which the effects on DC have been most extensively investigated, and stimulatory effects on naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg). These aspects are reviewed here.

NAbs AND DENDRITIC CELLS

Stimulatory Effects on Dendritic Cells (DC)

Most of the available studies show that IVIG inhibits the differentiation and function of DC. Nevertheless, one report has described the purification of NAbs from IVIG which stimulate DC-functions.[10] The authors observed that DC differentiation from monocytes of patients with X-linked agammaglobulinemia is severely impaired, when studied ex vivo in the presence of GM-CSF and autologous plasma. Differentiation of patients' monocytes to DC could be restored by reconstitution of the IgG-content of the autologous plasma by adding IVIG up to physiological levels.[10-12] Since DC differentiation was also restored by addition of anti-CD40 mAb, the authors hypothesized that IVIG might contain NAbs against CD40. They could indeed isolate IgG NAbs reactive with CD40 from IVIG. These anti-CD40 NAbs represented less than 1% of total IgG within the IVIG formulation, and stimulated in vitro differentiation and maturation of human DC from monocytes (Fig. 1, examples 1 and 2).[10] Expression of MHC class II and co-stimulatory molecules was upregulated by addition of anti-CD40 NAbs. In contrast to anti-CD40 monoclonal antibodies (mAbs), which strongly stimulate production of the pro-inflammatory cytokine IL-12 by monocyte-derived DC (mo-DC), the anti-CD40 NAbs stimulated IL-10, but not IL-12 production.[10] The authors showed that these anti-CD40 NAbs do not activate the NF-κB signaling pathway within mo-DC, but induce enhanced levels of the "cAMP-response element-binding protein 1" (CREB-1), which is involved in transcriptional activation of IL-10.[10] Patients with primary immuno-deficiencies, like X-linked agammaglobulinemia and common variable immunodeficiency, are highly susceptible to bacterial infections, which is at least partly due to their low serum IgG concentrations. These studies suggest that restoration of DC differentiation and function by anti-CD40 NAbs present in IVIG is another factor contributing to remission from pathological manifestations.[10,11]

Inhibitory Effects on Dendritic Cells

In contrast to the study discussed above, all other studies show that IVIG at concentrations corresponding to the physiological IgG concentration in human plasma (12–14 mg/ml) inhibits DC *differentiation* from monocytes and DC *maturation*. First, IVIG suppresses GM-CSF-driven *differentiation* of human monocytes to DC.[13] Similarly, IVIG inhibits in vitro *differentiation* of DC from monocytes under the influence of IFN-α present in serum of SLE-patients.[14] DC exhibit two different phases during their life cycle: *Immature* DC are specialized in recognition and uptake of pathogens, and processing of antigens to deliver their peptides for presentation by MHC molecules on

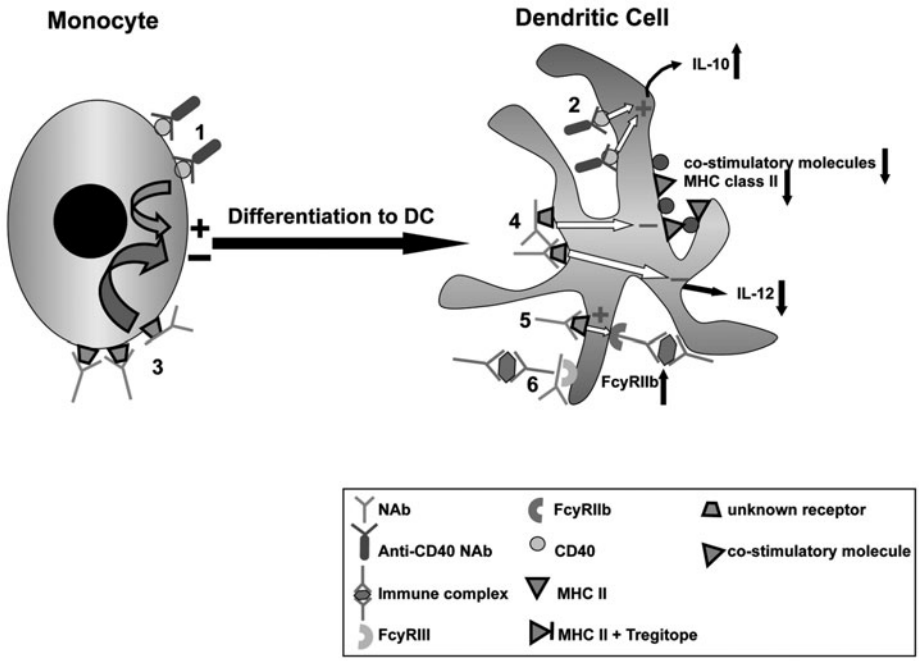

Figure 1. Effects of NAbs on differentiation and functions of dendritic cells. 1) Anti-CD40 NAbs stimulate the differentiation of monocytes to DC. 2) Anti-CD40 NAbs stimulate production of anti-inflammatory IL-10 by DC. 3) Other NAbs inhibit differentiation of monocytes to DC by binding to (an) unknown receptor(s). NAbs may bind either via their $F(ab')_2$ part (by specific recognition of the receptor as an antigen) or via their Fc-part to such receptor(s). 4) NAbs inhibit upregulation of MHC class II molecules and co-stimulatory molecules on DC, and suppress production of pro-inflammatory IL-12 by binding to (an) unknown receptor(s), resulting in decreased T-cell stimulatory capacity. 5) NAbs upregulate expression of the inhibitory Fcγreceptor IIb on DC by binding to (an) unknown receptor(s), resulting in inhibition of DC-functions upon binding of immune complexes. 6) NAbs inhibit binding of immune complexes to activating FcγRIII, thereby suppressing the presentation of antigen contained in immune complexes to T cells.

their surface. After pathogen recognition, they mature to *mature* DC, which possess potent T-cell stimulatory capacity because of enhanced expression of MHC molecules, co-stimulatory molecules, and production of T-cell stimulatory cytokines. IVIG inhibits the *differentiation* of DC from monocytes (Fig. 1, example 3), as well as their *maturation*, resulting in reduced expression of MHC class II and co-stimulatory molecules like CD80 and CD86 (Fig. 1, example 4).[13] In addition, IVIG inhibits LPS-induced production of the pro-inflammatory cytokine IL-12, but stimulates secretion of the anti-inflammatory cytokine IL-10 by human mo-DC.[13] As a consequence, mo-DC differentiated or matured in the presence of IVIG are impaired in their T-cell stimulatory capacity. Some, but not all, of the inhibitory effects of IVIG on DC-maturation have been confirmed in cultures with primary DC isolated from human blood using anti-CD1c mAb, an antigen expressed on the major subset of circulating DC.[8,15] However, in cultures of primary blood-derived DC IVIG was found to suppress production of both IL-10 and IL-12.[8,15]

While protein antigens are presented to T cells by MHC-molecules, lipid and glycolipid antigens are recognized by TCR in the context of CD1 molecules. Like MHC class II,

the expression of CD1a, CD1b and CD1c on mo-DC is downregulated by IVIG, but expression of CD1d is upregulated. As a result, DC differentiated from monocytes in the presence of IVIG are poor activators of T cells that recognize antigens in the context of CD1a, CD1b or CD1c, but potent stimulators of invariant $CD3^+CD56^+$ Natural Killer-T cells (NKT) cells that are restricted by CD1d.[16]

In contrast to the inhibitory effects of IVIG on *human* DC, IVIG has no effect on the expression of MHC class II or co-stimulatory molecules on DC generated by GM-CSF from mouse bone marrow.[17] The discrepant results observed between human and mouse DC may be due to interspecies differences between DC surface molecules recognized by human NAbs present in IVIG. The limited data that are available suggest that suppression of human DC function by IVIG occurs at least partially via antigen-specific interactions. Both IgG Fc-parts and $F(ab')_2$ fragments prepared from IVIG bind to the surface of human mo-DC.[13] While the effects of Fc-parts on DC maturation and function have not been studied, $F(ab')_2$-fragments are able to inhibit LPS-induced phosphorylation of "extracellular signal-regulated kinase" (ERK1/2), an intracellular signaling molecule that mediates the inflammatory response induced by Toll-Like Receptor (TLR)-ligation, in mo-DC.[18] In addition, Fc-fragments have no effect on CD1d-expression on mo-DC.[16] It is far from clear via which DC surface molecules NAbs exert their suppressive effect on DC differentiation and functions. In the following paragraphs I will discuss a few DC-surface molecules that may be involved in mediating the inhibitory effects of NAbs on human DC:

1. DC-SIGN

DC-SIGN is a C-type lectin, which mainly binds mannosylated and fucosylated stuctures. It is expressed on human mo-DC and macrophages, and binds to the HIV envelope protein gp120. DC-SIGN is involved in DC-mediated transmission of HIV from mucosal surfaces to T cells in lymph nodes.[19] Interestingly, NAbs directed against the carbohydrate recognition domain of DC-SIGN have been isolated from IVIG. These NAbs inhibited transmission of HIV to T lymphocytes.[20] In addition, DC-SIGN is involved in modulation of DC maturation and cytokine production by pathogens. Ligation of DC-SIGN with a specific mAb inhibits DC-maturation.[21] While ligation of DC-SIGN by mannose-expressing pathogens stimulates pro-inflammatory cytokine secretion by mo-DC, fucose-expressing pathogens or synthetic fucose-containing ligands inhibit LPS-induced production of IL-6 and IL-12 but stimulate the secretion of anti-inflammatory IL-10 by mo-DC.[22]

In the mouse system the anti-inflammatory effects of human IVIG in several models of autoantibody-induced disorders were found to be mediated by IgG molecules that contain two sialic acid molecules bound via an α2,6-linkage to the biantennary, single glycan chain that is attached to the heavy chain within its Fc-region, and could be recapitulated by fully sialylated recombinant IgG Fc molecules.[23,24] These sialylated IgG Fc molecules are recognized by the C-type lectin SIGN-R1,[25] which is expressed on macrophages in the splenic marginal zone of mice and on endothelial cells in the liver and in lymph nodes.[26] It was shown that this interaction is indispensable for the anti-inflammatory effects of IVIG in these mouse models.[25]

The human equivalent of SIGN-R1 is L-SIGN, which is expressed on endothelial cells in human liver, lymph nodes and placenta, but not on macrophages or DC.[27] Compared with L-SIGN, human DC-SIGN has less homology to murine SIGN-R1, but its expression

on macrophages and DC and its function in modulation of DC-function by carbohydrate structures suggest that sialylated IgG Fc molecules might exert anti-inflammatory effects in humans via binding to DC-SIGN. Indeed, human DC-SIGN expressed on CHO cells has been shown to bind sialylated human Fc-parts.[25] Recently, by using SIGN-R1-deficient mice in which a transgene of human DC-SIGN was expressed, it was shown that human DC-SIGN could substitute for mouse SIGN-R1 in mediating protection from autoantibody-triggered arthritis by IVIG. In contrast, human L-SIGN did not.[28] Importantly, it has to be realized that the protective effects of sialylated Fc-parts have only been shown in mouse models of antibody-triggered inflammation, and not in T-cell driven inflammatory responses. In addition, the role of sialylated Fc and DC-SIGN in the anti-inflammatory effects of IVIG in humans remains unclear. The following observations with human cells argue against it: (1) α2,6–sialylated IgG Fc portions are dispensable for the inhibition of TLR-mediated activation of human mo-DC[18]; (2) IVIG inhibits cytokine production of primary human blood DC that do not express DC-SIGN.[8,15]

2. Inhibitory FcγRIIb

In several experimental mouse models of autoantibody-induced inflammatory disorders, the inhibitory FcγRIIb was found to be indispensible for the anti-inflammatory effects of IVIG.[29-33] IVIG treatment enhances FcγRIIb expression on effector macrophages in mouse spleen,[29] or at the site of inflammation.[31] This raises the threshold concentration of (pathogenic) immune complexes necessary to trigger activation of these effector macrophages. The net result of this pathway is attenuation of the autoantibody-mediated inflammation. Human DC also express FcγRIIb, and ligation of this receptor by a specific antibody results in inhibition of their maturation and functions.[34-36] A recent study shows that FcγRIIb is upregulated on circulating monocytes and B cells during IVIG-therapy in humans,[37] suggesting that its expression may also be upregulated on macrophages and DC in humans in vivo (Fig. 1, example 5). If this were true, the mechanism by which IVIG enhances expression of this receptor is probably indirect, since addition of IVIG to human mo-DC in vitro does not result in enhanced FcγRIIb-expression.[36] This would be in line with the current model for FcγRIIb induction on macrophages in mice, which proposes that 2,6-sialylated IgG molecules bind to SIGN-R1 on regulatory macrophages, thereby stimulating them to secrete a soluble mediator that induces FcγRIIb-expression on macrophages at sites of inflammation.[38]

3. Activatory FcγRIII

In two different autoimmune disease mouse models, the anti-inflammatory effects of IVIG could be recapitulated by administration of IgG-antigen immune complexes.[32] Surprisingly, the anti-inflammatory effects of IVIG or those of immune-complexes in these models are mediated by stimulation of DC via the activating FcγRIII. Adoptive transfer of IVIG-treated DC could ameliorate immune thrombocytopenic purpura (ITP) in mice, and this effect could be recapitulated by transfer of DC, on which FcγRIII was cross-linked with a specific mAb prior to transfer.[33] How stimulation of DC via an activating receptor, like FcγRIII, ameliorates inflammation is unclear. The results seem to be compatible with the "two-step" model discussed above, in which a regulatory cell type (in this case DC) senses IVIG or immune-complexes, and subsequently suppresses the inflammatory response of effector macrophages. A

recent study, however, challenges this concept, and provides evidence that interaction of IVIG with DC does not inhibit the phagocytic activity of macrophages, but targets platelets, leading to reduced binding of platelets to phagocytes.[39] Although treatment of inflammation by stimulating an activating FcγRIII seems paradoxical, more examples of inhibitory effects of activating FcγRIII signaling are available. In another ITP mouse model it was observed that IVIG suppresses expression of the IFN-γ receptor on macrophages via binding to FcγRIII.[40]

Besides inhibition of DC maturation and cytokine production, NAbs inhibit presentation of peptides derived from IgG-containing immune complexes by DC to T cells. By inhibiting the uptake of immune complexes via activating FcγRIII, IVIG suppresses the generation of antigen-specific T-cell responses in mice immunized with a protein antigen (Fig. 1, example 6).[17] This effect is mediated by the Fc-parts of IgG and requires a high dose of IVIG, consistent with the low affinity of monomeric compared with complexed IgG for this type of FcγR.[41]

Finally, IVIG formulations contain variable amounts of dimeric IgG, depending on the formulation (lyophilized or liquid), the IgG concentration of liquid IVIG, the pH of the IgG solution, the storage temperature, and the size of the donor pool providing the starting material. Idiotype/anti-idiotype interaction between antibodies from distinct donors contribute to the formation of IgG-dimers in therapeutic IVIG-formulations.[42,43] IgG-dimers and multimers present in IVIG stimulate killing of human DC by NK cells by "bridge-formation" between FcγR on DC and NK cells. NK cells are activated via their FcγRIII, and kill DC (Fig. 2).[44] These effects are not observed with monomeric IgG, the form which is present in plasma. However, NAbs in plasma can exert similar effects if complexed to (auto-)antigens.

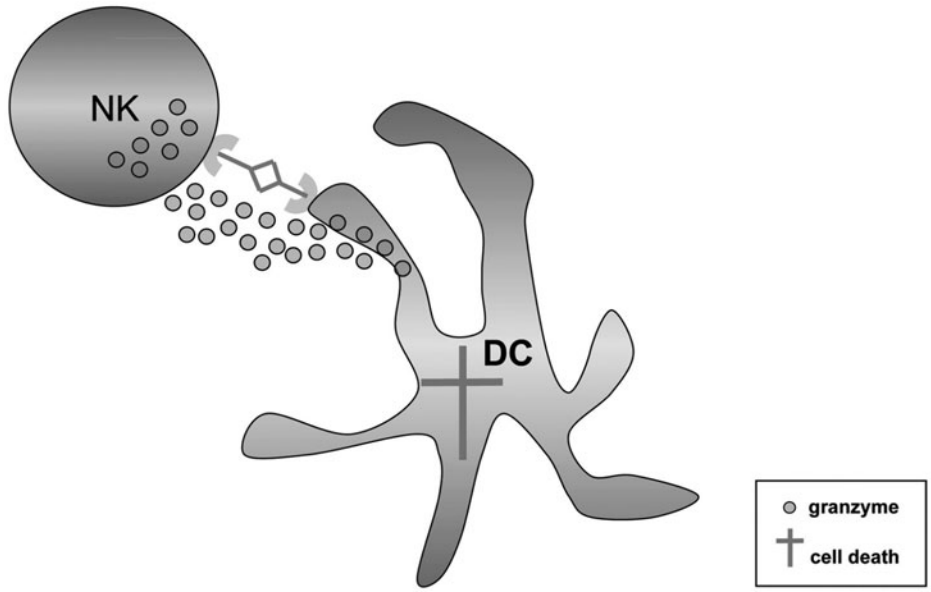

Figure 2. Effects of NAbs on the interaction between DC and NK cells. Immune complexes stimulate killing of DC by NK cells via "bridge-formation" between FcγR on the two cell types.

NAbs AND T CELLS

The proliferation and cytokine production of human T cells after mitogenic or allogeneic stimulation in vitro are effectively suppressed by therapeutic formulations of IVIG.[45-47] The cornerstone of many clinical immunosuppressive regiments are calcineurin inhibitors, like Cyclosporin or Tacrolimus, because of their potency to inhibit T-cell responses. Notably, when added at a concentration comparable to that in human plasma, IVIG inhibits proliferation and cytokine production by human T cells as effectively as calcineurin inhibitors do.[15] Inhibition of T-cell activation by IVIG is at least partly due to the suppressive effects of IVIG on APC, like DC, as described above. There is, however, some evidence that IVIG also suppresses highly purified human T cells in the absence of APC or other accessory cells, demonstrating that NAbs can act directly on human T cells.[46,48] Interestingly, the latter study showed that IgG purified from a single donor suppresses T-cell activation, indicating that the inhibitory effect is not related to possible conformational changes of IgG during processing, neither to the pooling of IgG from different individuals as in IVIG formulations, but is an intrinsic property of native IgG molecules.[48] In addition, there is increasing evidence that IVIG suppresses T-cell responses by stimulation of regulatory T cells (Treg).

The molecular interactions mediating the direct effects of IVIG on conventional T cells are still unclear. They are probably caused by NAbs that are auto-reactive, and bind to their targets by low-affinity interactions. Indeed, it has been shown that the NAb repertoire includes antibodies directed toward T-cell surface signaling molecules, such a TCR-β chains[49] and CD4.[50] It has been found that low-affinity interactions of IVIG with TCR can result in altered or inhibitory signaling.[51-53] In contrast to the direct effects of IVIG on human T cells, highly purified mouse $CD4^+$ T cells are not suppressed by IVIG,[17] suggesting that NAbs affecting human T cells recognize surface molecules that are not conserved between man and mice.

NAbs AND REGULATORY T CELLS

Increasing evidence shows that NAbs can stimulate expansion and the suppressive function of naturally occurring T regulatory cells (Tregs). Several types of Treg have been described, but the most thoroughly studied ones are the naturally occurring $CD4^+CD25^+Foxp3^+$ Treg. The known effect of NAbs on Treg are summarized in Figure 3. Addition of a physiological concentration of IVIG (6 mg/ml) to human $CD4^+$ T cells cultured in the presence of IL-2 results in upregulation of intracellular expression of Foxp3, the key transcription factor controlling Treg development and function. In addition, the suppressive effects of Treg on cytokine production by conventional $CD4^+CD25^-$ T cells were enhanced when IVIG was added to the co-cultures.[54] In an experimental autoimmune encephalomyelitis (EAE) model in mice, protection from EAE by an IVIG treatment was associated with increased numbers of $CD4^+CD25^+Foxp3^+$ Treg in spleen, lymph nodes and blood.[55] The critical involvement of Treg in prevention of EAE was shown in experiments in which Treg were depleted from the animals and in adoptive transfer experiments. Indeed, transfer of Treg from IVIG-treated mice suppressed symptoms of EAE more effectively than transfer of Treg from mice not treated with IVIG. Moreover, Treg isolated from mice treated with IVIG suppressed proliferation of conventional T cells in vitro more effectively compared with Treg from control mice. To differentiate

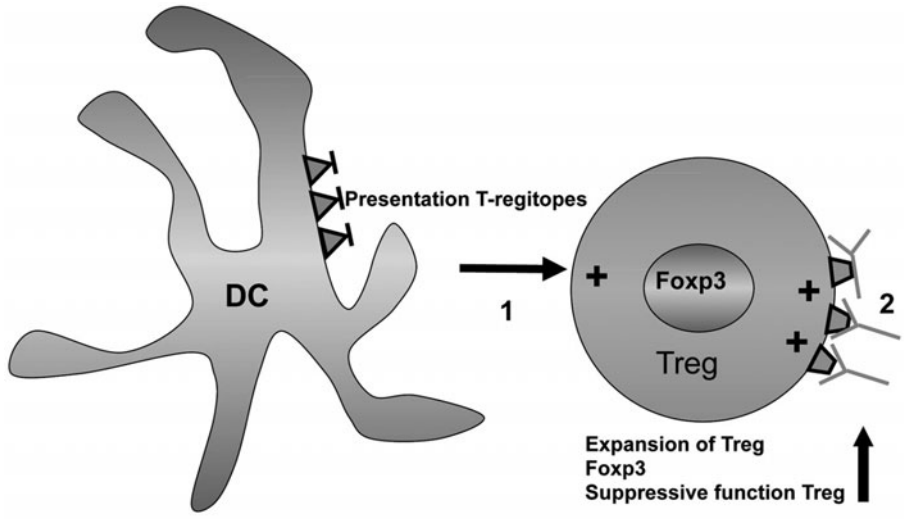

Figure 3. Effects of NAbs on regulatory T cells. NAbs stimulate expansion, foxp3-expression and suppressive function of Treg by: 1) Presentation of specific peptides derived from their Fc-parts (T-regitopes) to Treg. 2) Binding to (an) unknown receptor(s). Details concerning the symbols are explained in Figure 1.

between expansion of pre-existing Treg and de novo conversion of conventional T cells to Treg, adoptive transfer experiments with TCR-transgenic T cells specific for an influenza peptide, followed by immunization with the peptide, were performed. The results showed that IVIG-therapy stimulates expansion of pre-existing Treg, but not de novo induction of Treg from conventional T cells. Similarly, IVIG stimulated proliferation of Treg, but not of conventional $CD4^+CD25^-$ T cells, in vitro.[55] Consistent with these results, it was found that Treg are critically involved in prevention of T helper cell-mediated allogeneic skin graft rejection by IVIG. This was established in a model in which adoptive transfer of syngeneic wild-type $CD4^+$ T cells into T-cell deficient mice resulted in allogeneic skin graft rejection. Intravenous administration of IVIG prevented allograft rejection, but the protective effect of IVIG was completely lost upon selective depletion of Treg from the transferred $CD4^+$ T cells.[56] Together, these studies show that particular NAbs can stimulate expansion and suppressive function of pre-existing $CD4^+Foxp3^+$ Treg in vitro and in two different animal models.

These findings are supported by in vivo findings on humans. Patients with Kawasaki Disease and Guillain-Barré syndrome have significantly lower numbers of circulating Tregs as compared with healthy individuals, or individuals with infections. After IVIG-therapy Treg numbers increase,[57-59] indicating that NAbs stimulate expansion of Treg in humans in vivo. However, whether an IVIG-therapy enhances the suppressive capacity of human Treg in vivo is as yet unknown.

Modulation of Treg by NAbs probably represents among others one of the modes of action involved in the therapeutic effect of IVIG in disorders caused by (hyper-) activation of T cells. However, the molecular mechanisms by which NAbs stimulate Treg expansion and function are still enigmatic. IgG molecules present in IVIG bind to both human and mouse Treg,[55,56] suggesting that the effects are mediated by binding of

(certain) IgG-molecules to molecules expressed on the cell surface of Treg. In support of this concept, we observed that upon culturing Treg with IVIG specifically those Treg are activated that have IVIG bound to their surface.[56] Do IgG molecules bind to FcγR on Treg? Resting T cells do not express FcγR, but upon activation a subset of *mouse* Treg expresses FcγR. In contrast, conventional murine $CD4^+CD25^-$ T cells do not express FcγR upon activation. However, upon incubation with IVIG, both FcγR-positive Treg and FcγR-negative Treg from mice bind IgG molecules. Moreover, *human* Treg do not express any known FcγR, also not after activation, while they are able to bind IgG-molecules.[56] Together, these data suggest that NAbs may activate Treg by binding to one or more surface molecules, but that FcγR are most likely not involved in this process.

Another interesting hypothesis is that the Fc-part of IgG contains special epitopes that can be presented by MHC class II molecules, and are specifically recognized by the TCR of Treg. Indeed, two peptides derived from a part of the Fc-region that is conserved in all human IgG allotypes, were found to enhance expression of CD25, Foxp3 and CTLA-4 on human $CD4^+$ T cells in vitro.[60] Co-incubation of these so-called "Tregitopes" with antigen led to a decreased T-cell response to the antigen. Selective depletion of $CD4^+CD25^+$ Treg from the cultures demonstrated that the suppressive effect was indeed due to Treg. Administration of a murine homolog of the Fc-region "Tregitope" resulted in suppression of T-cell reactivity and antibody response to a known immunogen in mice.[60] Interestingly, these "Tregitopes" are promiscuous in MHC-class II binding, indicating that they can be recognized in the context of different MHC class II alleles.

The intracellular signaling pathway involved in stimulation of Treg expansion and suppressive capacity by NAbs is unknown. We observed that in vitro addition of IVIG to Treg enhances their suppressive capacity and stimulates phosphorylation of ZAP-70, an intracellular signaling molecule involved in activation of T cells.[56] Although stimulation of ZAP-70 phosphorylation by CD4 mAb also enhances the suppressive capacity of Treg,[61] it is not yet known whether ZAP-70 signaling is similarly involved in the upregulation of the suppressive capacity of IVIG.

CONCLUSION

In contrast to immunosuppressive drugs, IVIG therapy has no serious adverse effects, even when given life-long at a regular basis. Therefore, IVIG may be considered as prophylaxis or treatment for a wide range of T-cell mediated immune diseases, including organ transplant rejection. However, expansion of its use is hampered by the high costs of IVIG therapy. In addition, there is the danger of an upcoming global shortage of human plasma due to increased demand.[62] Therefore, optimization of its use, or even replacement by recombinant products, will become inevitable. For this purpose, elucidation of the molecular interactions between NAbs within IVIG and DC or Treg is required, as well as the identification of the active components within IVIG that modulate the functions of immune cells. Encouraging results have been obtained by the identification of the minor fraction of IgG molecules within human IVIG with fully sialylated Fc-portions as the active component that protects against disorders caused by autoantibodies in experimental mouse models. Fully sialylated recombinant IgG1 Fc demonstrated comparable anti-inflammatory activity to that obtained with a 300-fold higher dose of IVIG in a mouse arthritis model.[24] However, as argued above, it is unclear whether fully sialylated Fc-fragments are responsible for the beneficial effects of IVIG observed in human diseases.

Furthermore, there is no evidence that IgG molecules with fully sialylated Fc portions are able to modulate T-cell mediated immune disorders. Studies on the molecular interactions between NAbs and human DC and Treg are required to identify the components within IVIG that are responsible for the effects of IVIG-treatment on immunological disorders in humans. Separation of IVIG into two products, an immunosuppressive fraction to be used for immunosuppressive therapy, and the non-immunosuppressive residual IgG for substitution therapy in IgG-deficiencies, will economize the use of IVIG. Such studies are also important for our understanding of how NAbs regulate T-cell mediated immune responses in pathological conditions.

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CHAPTER 11

CONTROL OF B CELLS EXPRESSING NATURALLY OCCURRING AUTOANTIBODIES

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Abstract: Naturally occurring autoantibodies (NAbs) are typically polyreactive, bind with low affinity to a discrete set of autoantigens and are encoded by variable region genes in germline configuration. They differ from disease-associated autoantibodies (autoAb), which are mostly monoreactive, somatically mutated and of high affinities. Structure-function studies have shown that polyreactivity of NAbs relies on the somatically generated complementarity determining region, CDR3, of the heavy chain. This finding suggested that NAb-producing B cells were positively selected from the pre-immune B-cell repertoire. The biological significance of this selection remains, however, unclear. Data originating mainly from transgenic mice have shown that mature NAb-producing B cells are frequently ignorant toward their antigen, possibly due to their low affinity, though active tolerance mechanisms are not excluded. An important issue is whether NAb-producing B cells constitute the pool from which pathologic autoAb emerge after autoantigen-driven maturation. We summarize results obtained in mouse models, showing that some infectious agents are able to induce an autoantigen-driven activation of certain NAb-producing B cells. However direct proof that selection by autoantigen may lead to somatic hypermutation are still lacking. Other data tend to suggest that pathologic autoAbs may derive from non-autoimmune B cells that have diversified by somatic hypermutation of their variable region genes.

INTRODUCTION

Naturally occurring, autoreactive B cells are thought to be physically eliminated or rendered functionally silent through different mechanisms of tolerance. However, since the original finding from G. Dighiero and S. Avrameas, a large body of evidence has shown that healthy individuals have many autoreactive B cells in their naïve repertoire (reviewed

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

in refs. 1-3). Although this issue has remained controversial for long, recent elegant studies from Nussenzweig's group have shown that autoreactivity decreases with each step of B-cell development, but is not eliminated.[4] The biological significance of this apparent paradox to the clonal tolerance theory remains mysterious, but it now is clear that self-reactivity is physiologic and should be distinguished from autoimmunity. Although NAbs constitute a significant part of serum immunoglobulins, most NAb-expressing B cells do not secrete their (auto)Abs and seem to coexist peacefully with their autoantigen further deepening the paradox. In this chapter we mainly focus on two issues: (1) the structure-function relationships between NAbs and autoantigens, emphasizing that they were the first to indicate that NAb-producing B cells may be positively selected in the normal repertoire; (2) the functional state of NAb-producing B cells in vivo and some mechanisms that may lead to their activation. We also ask whether NAb-producing B cells constitute the pool from which pathologic (autoimmune disease-associated) autoAb can emerge.

STRUCTURE-ACTIVITY RELATIONSHIPS AMONG NAbs

Differences between NAbs and Disease-Associated autoAbs

In the current thinking, although data are sometimes contradictory, NAbs are mostly of the IgM isotype, are polyreactive, bind with low affinity to a discrete population of self antigens, and their V genes are in germline configuration. By contrast, autoimmune disease-associated autoAbs are often class-switched, are monoreactive with high affinity and are somatically mutated with patterns suggesting (auto)antigen selection (see for instance refs. 5–7). One should add an often overlooked differential characteristic: in healthy individuals serum concentrations of NAbs are quite low, often contrasting with the high frequencies of NAb-expressing B cells in the repertoire, while it is rather the opposite for disease-associated autoAb. This, for instance, has been long known for rheumatoid factors (RF: autoAb directed against the Fc piece of IgG): 0.2% of peripheral blood B cells can be activated to produce IgM RF, whereas IgM RFs are barely detectable in the serum of healthy individuals.[8] This paradox remains to be explained. The issue of the isotype is probably the weakest one, since it has been established that NAbs can also be IgG and IgA.[2] This is an important point because it shows that NAb-expressing B cells are able upon certain circumstances to undergo class-switching. Other features such as V-gene usage and producing B-cell sub-populations (B-1 or regular follicular B cells) are no longer considered distinctive, at least in humans.

Structural Basis of NAb Polyreactivity

The most popular distinctive feature of NAbs is their polyreactivity and respective repertoires (the latter being reviewed elsewhere in this book). We must, however, keep in mind that these characteristics have not been studied as extensively in autoAb. A significant breakthrough in the knowledge was the demonstration that specificity and polyreactivity of NAbs in humans were highly dependent of the somatically generated heavy chain CDR3 (HCDR3).[9] This was later confirmed in different models of autoAb by mutagenesis or HCDR3 swapping experiments.[10-13] This result is of significant importance because it provides circumstantial evidence that NAbs may be positively selected in the early B-cell ontogeny. This hypothesis was later sustained by a significant body of work summarized

hereafter. In mice NAbs are mostly produced by B-1 ($CD5^+$) B cells. The positive role of self-antigens in selecting B-1 cells was clearly demonstrated by using transgenic mice for a naturally generated autoreactive B-cell receptor (BCR) that recognizes Thy-1 (CD90), a glycoprotein expressed at the membrane of T cells. In fact, Hayakawa and colleagues showed that anti-Thy-1 specific B cells did not accumulate in Thy-1-deficient animals,[14] while the presence of the self-antigen promoted B-1 cell accumulation and thus NAb secretion. A positive role for self-antigen in early B-cell development has also been demonstrated for Fcγ, ssDNA, thyroglobulin and phosphorylcholine.[15-17]

Recently Köhler and colleagues presented evidence that the pre-B-cell receptor and the BCR of NAbs-expressing B cells are functionally equivalent and that they share structural elements involved in multispecific recognition of self-antigens.[13] The pre-B-cell receptor (pre-BCR) is expressed by pre-B cells and is composed of a functionally rearranged heavy (H) chain associated to an invariant surrogate light chain. The surrogate light chain has a structure similar to a kappa light chain and results from the assembly of two proteins, VpreB and λ5. The expression of the pre-BCR is mandatory for further B-cell development. There has been a long controversy whether the pre-BCR must interact with (auto)antigens or whether the surrogate light chain induces ligand-independent pre-BCR crosslinking and subsequent autonomous signaling which is required for positive selection of B cells expressing a functional H chain. In the study by Köhler and colleagues it is shown that most pre-BCR are self-polyreactive. Polyreactivity is dependent on the λ5 part of the surrogate light chain and/or on the HCDR3 of the heavy chain.[18] The λ5 region is essential for pre-BCR function and is characterized by carrying multiple arginine residues.[18] It is also known that polyreactive NAbs often have long HCDR3, enriched in positively charged amino acids (i.e. arginine).[11,12,19,20] These residues play a role in polyreactivity and particularly in the binding to negatively charged molecules such as nucleic acids. The same group shows by in vitro chain swapping experiments that the HCDR3 of a polyreactive Ab (but not that of a monoreactive one) can functionally replace the λ5 chain; and reciprocally, the λ5 region can transfer polyreactivity, when replacing the HCDR3 of a non-polyreactive Ab. Most importantly they show in vivo that expressing a polyreactive pre-BCR or BCR confers a strong selective advantage during early B-cell development. Taken together these findings most readily explain the enrichment of the naïve repertoire with polyreactive, autoreactive B cells. During early B-cell development the polyreactive potential may well lead to ligand-independent direct interactions between pre-BCR (or BCR) complexes, ie. binding to one another independently of antigens. Alternatively polyreactivity might explain why pre-BCR structures interact with galectin-1 or heparan sulfates furthering B-cell differentiation.[20,21]

The biological significance of positive selection of B cells by autoantigens during the early phases of B-cell development remains intriguing. A large body of evidence indicates that, from a phylogenetic point of view, the adaptive immune system has evolved to components that are less and less dependent on self recognition for their development and functions. In this view, B-cell positive selection by autoantigens in NAb formation might be an evolutionary "byproduct" (ie. a consequence of a useless B-cell positive selection) of B-cell development. In reality, NAbs play important roles in tissue homeostasis (see elsewhere in this book) and in the early defense against pathogens.[22] Positive selection of NAbs could confer a biological advantage, because the selecting antigens, including surface carbohydrates and nucleic acids, may provide them the potential to cross-react with generic molecular patterns frequently expressed by microbes such as PAMPs (Pathogen-associated molecular patterns)

REGULATION OF AUTOIMMUNITY

Many NAb-Expressing B Cells Are Autoantigen Ignorant

The naïve pre-immune repertoire is enriched in NAb-expressing B cells because: (1) NAb-expressing B cells are positively selected during early B-cell ontogeny; (2) NAb-expressing B cells are essentially low affinity cells, they escape tolerance mechanisms, most probably because they fall below the threshold for its induction.[23]

Healthy individuals maintain relatively constant levels of circulating IgM, IgA and IgG of which significant proportions are NAbs.[3] However for each set of autoantigens, the concentrations of NAbs are low compared with that of NAb-expressing B cells. This has been clearly demonstrated for rheumatoid factor (RF)-expressing B cells, which represent up to 2/1000 of naïve B cells, whereas RFs are barely detectable in the serum of healthy humans.[8] This is somewhat surprising given that RF-specific B cells are permanently in contact with high concentrations of IgG in different forms (soluble, aggregated, membrane-bound). These observations have led to several questions: (1) Why do B cells with the capacity to produce NAbs coexist peacefully with their autoantigen? (2) Can these B cells be activated to produce NAbs in vivo or are they anergic? (3) Do NAb-expressing B cells (ie. with the potential to produce NAbs) participate in autoimmunity or in other words what are the links between natural self-reactivity and pathologic autoimmunity?

Although B cells with the capacity to produce NAbs are frequent in the repertoire, their follow up and their behavior are extremely difficult to analyze not only in humans, but also in mice. Most of the knowledge on autoAb-producing B cells in general has come from the analysis of transgenic mice (reviewed in ref. 24). The classic transgenic (Tg) models, expressing a single, selected, auto-reactive specificity simplify the analysis of auto-reactive B cells, because they create animals in which the vast majority of the B lymphocytes express the desired self-specificity. The informations obtained from these mice were completed by data where autoreactive B cells were rendered able to switch and to introduce somatic hypermutations (SHM). These "knock-in" Tg mice, containing the rearranged Tg in the immunoglobulin locus after homologous recombination, were developed in order to reduce the frequency of auto-reactive B cells, and to study the peripheral B-cell tolerance mechanisms. These strategies were successful and led to numerous conclusions on the auto-reactive B-cell fate both in the bone marrow and in the periphery. However, very few models focused on transgenic NAbs. The most detailed studies were performed with low affinity RF.

The first model was reported by Shlomchik and colleagues[25] and was of importance because it was possible to control for the presence of self antigens. This classic Tg mice were designed with the heavy chain and light chain of a low affinity, monoreactive RF (AM14), originating from a MRL/lpr mouse, a strain which spontaneously develops a lupus-like disease with high levels of antinuclear and RF autoantibodies. It is therefore not a classical NAb, but its affinity for IgG is in the range of those with RF activity. This RF is specific for the "a" allotype of mouse IgG2a, and does not recognize the "b" allotype. Thus, the Tg mice express a monoreactive IgM-RF specific for IgG2a of the "a" allotype, allowing for the analysis of the RF-expressing B-cell fate in the absence (genetic background with the "b" allotype of the IgG2a), or the presence of the self antigen (genetic background with the "a" allotype of the IgG2a). In the absence of the self-antigen, IgM-RF-producing B cells represent the majority of the

peripheral B cells, but this is also true in the presence of the self-antigen. In the latter case, the Tg RF-expressing B cells are not deleted, do not edit a new receptor and are not anergic. Serum levels of RFs are even higher than in control mice without self antigen. These RF-expressing B cells seem ignorant toward their self antigen. We also created Tg lines expressing a chimeric low affinity RF with murine constant regions and human variable regions. Precisely, this RF (designated Smi) is a prototype of a multireactive NAb, reacting with a low affinity with thyroglobulin, myoglobin, ssDNA and IgG ($K_D = 10^{-6}$M).[26] Since "Smi RF" does not bind murine IgG, autoreactivity was introduced by crossing Tg mice with knock-in mice, expressing the constant region of the human gamma chain of IgG1 (designated cIgG). The offsprings, designated Smi x cIgG, produce a normal repertoire of chimeric cIgG (murine V regions and human C region). In these mice, RF-expressing B cells are not deleted in the bone marrow, they circulate and populate normally the B zones of peripheral lymphoid organs. They do not edit their receptor and do not present classical features of anergic B cells. Interestingly, akin to Shlomchik's model, these NAb-expressing B cells appear to be autoantigen-ignorant both in vivo and in vitro, since they cannot be activated with human IgG (even if aggregated) and myoglobin. However, they are not anergic, because they can be activated by B-cell receptor-dependent anti-IgM and B-cell receptor-independent means like LPS. In addition, there is no BCR downmodulation in Smi x cIgG mice and no abnormal expression of membrane activation markers. This state has been designated as "clonal ignorance" or "clonal indifference," is independent of CD32 expression by B cells (our unpublished data) and has been reproduced in different animal models.[27,28] This phenotype may be related to the low affinity of the Tg autoAb Smi. However, although the affinity of Smi for its autoantigens is below the threshold to induce tolerance mechanisms (deletion, receptor editing or anergy) and to permit the activation of follicular mature B cells, it is high enough to signal through the BCR at certain steps of B-cell development during which the cells are known to be more sensitive to BCR stimuli. Indeed, the constitutive presence of cIgG in Smi x cIgG mice promotes the increase of the RF-expressing Tg B cells.[15,29] These data support the signal strength model of B-cell development which implies that, in addition to antigenic specificity, B cell positive selection could determine in which subset the B-cell receptor-selected clone will reside (B-1, follicular and marginal zone B cells).[30] In addition, the window of affinities allowing them to escape the tolerance mechanisms controlling autoreactive B cells is probably larger than generally thought, since we obtained similar results with mice expressing a RF with slightly higher affinity (Hul, $K_D = 5x10^{-8}$M).[31]

Can NAb-Expressing B Cells Be Activated In Vivo?

In otherwise healthy humans significant levels of autoantibodies, including RF, anti-nuclear antibodies and anti-phospholipid antibodies, have recurrently been reported during the active phases of infectious states, usually with no clinical consequences.[32] Similar findings have been described in mouse infectious diseases.[33] These autoAbs usually have the features of NAbs or are NAbs (e.g., see other chapters in this volume). NAb-expressing B cells could be activated because they cross react with infectious agents or because they participate in the hypergammaglobulinemia that occurs during many infectious diseases. As summarized below the mechanisms of how a hypergammaglobulinemia is induced are far from obvious.

Mechanisms of Polyclonal B-Cell Activation and Induction of Hypergammaglobulinemia in Infections

It is current knowledge that substantial, nonspecific B-cell activation accompanies viral and bacterial infections and possibly also immune responses against inert antigens.[34] Only a small fraction of the newly synthesized immunoglobulins is specific for the eliciting antigen. This phenomenon is thought to contribute to the long-term maintenance of memory B cells in the absence of the antigen.[35] Significant hypergammaglobulinemia can be detected especially during persistent infections. This is well documented in humans during, for instance, subacute endocarditis, tuberculosis, HIV or HCV viral infections; clinical situations during which NAbs are more frequently detected in serum than in healthy donors. This phenomenon also occurs in mice infected with lymphocytic choriomeningitis virus (LCMV), vaccinia virus, vesicular stomatitis virus and *Borrelia burgdorferi*.[31,34,36] Earlier notions emphasized the role of cytokines produced by either activated antigen-presenting cells, T cells activated by microbial peptides or by specific B cells stimulated through their BCR. However, recent studies on LCMV-infected mice have shown that the mechanisms of hypergammaglobulinemia were probably more complex, involving virus-specific T cells and cognate T-cell – B-cell interactions independently of BCR specificity.[34] In this model, LCMV-induced hypergammaglobulinemia is polyclonal, mostly of IgG isotype and only a minor proportion of the activated cells produce virus-specific antibodies. Hypergammaglobulinemia requires: (1) virus-specific T-cell help, since it is not induced in T-cell-deficient mice reconstituted with ovalbumin-specific T cells; (2) CD40-CD40L interactions; (3) expression of MHC class II on B cells. It was further shown that B-cell proliferation accompanies this type of hypergammaglobulinemia.[37] Important for autoimmunity, clonal cell division and cognate T-cell help might permit isotype switching and somatic hypermutations. Hunzicker et al. found indeed that a virus-induced hypergammaglobulinemia was accompanied by an isotype shift from IgM to IgG.[34] However, the predominance of IgG may have another explanation, since switched memory human B cells divide extensively in response to bystander T-cell help, whereas IgM memory B cells and naïve B cells are less responsive or unresponsive.[35]

The second type of a polyclonal stimulus that can trigger B-cell activation in the absence of antigen is represented by pathogen-associated molecular pattern (PAMPs) receptors, of which the best characterized being Toll-like receptors (TLRs).[38] TLRs are expressed on immune cells, including B lymphocytes with some variations between species. TLR ligands are B-cell mitogens in vitro and in vivo. Interestingly, Jellison and colleagues showed that MyD88, the main adaptor of TLR signaling, was not required within the B cells for LCMV-induced polyclonal B-cell activation.[37] Hypergammaglobulinemia also occurs after murine infection with *Borrelia burgdorferi (B. burgdorferi)*.[31,39] In many inbred mouse strains *B. burgdorferi* induces a chronic, systemic infection. Infection results in a significant increase (at least 3- to 4- fold) of the total number of lymph node B cells and in hypergammaglobulinemia, mostly IgM, a small fraction of which being directed against *B. burgdorferi*. Activation of B cells through TLRs certainly plays an important role. Lipoproteins including OspA/B (outer surface proteins A/B) possess potent B-cell mitogenic properties, capable of stimulating polyclonal proliferation and Ig production in vitro and involve TLRs, particularly TLR2.[40,41] We have shown that the proliferation of purified B cells induced by *B. burgdorferi* is completely abolished when *MyD88* is inactivated.[31] In vivo, the participation of other cells, expressing TLRs and/or other Pattern Recognition Receptors, is likely but, notably, the process is not dependent on CD4 T cells, since their blockade does not reduce the production of immunoglobulin.

Do NAb-Expressing B Cells Participate in Polyclonal Activation during Infections?

LCMV-induced hypergammaglobulinemia has been shown to involve autoreactivity, including anti-DNA, anti-thyroglobulin, and anti-insulin autoantibody formation.[34] Basically, this may not be surprising if we take for granted that autoreactive B cells present in normal individuals are essentially "natural" low affinity B cells (NAb-expressing B cells) that have escaped tolerance mechanisms because they fall below the threshold for its induction and that are autoantigen-ignorant at the mature state. Then, polyclonal activation could occur similar to that of other B cells and would result in the production of more of these autoantibodies with no requirement for the presence of autoantigen and with no clinical consequences. The induction of an autoimmune disease would necessitate that this process is accompanied by a mechanism of autoantigen-driven affinity maturation. Indeed, most of autoantibodies from patients with autoimmune diseases are isotype-switched and somatically mutated generally with a pattern, suggesting an autoantigen-driven mechanism. Another nonexclusive possibility would be that some NAb-expressing B cells may have sufficient affinity to bind to autoantigens and to receive some type of signal through the BCR. Under normal circumstances such cells would remain ignorant, because a second signal is lacking or because they are kept silent by active immunological ignorance mechanisms. RF again represents an interesting model to address these issues, since they are one of the most frequent autoantibodies detected during infectious diseases in humans. Under normal conditions RF-expressing B cells seem to ignore their autoantigen and do not secrete RF. The Smi x cIgG and Hul x cIgG mice reproduce this physiological situation.[15,26,31] We have demonstrated recently that a chronic infection with *B. burgdorferi* is able to break this state of immunological ignorance: Tg RF-expressing B cells proliferate like other B cells and RFs contribute to the resulting hypergammaglobulinemia.[31] In vitro experiments showed that this autoantigen-specific step is induced by *B. burgdorferi*-IgG immune complexes, but not by anti-*B. burgdorferi*-IgG or by *B. burgdorferi* alone. In addition these experiments show that this autoantigen-specific step involves concurrent stimulations of the BCR and a MyD88-dependent, most probably TLR pathway. Previous studies from Roosnek and Lanzavecchia have shown in vitro that RF-expressing B cells can capture exogenous antigens in immune complex form, process them and present antigenic peptides to specific CD4 T cells.[42] Taken together the data are suggestive of the model of tolerance breach depicted in Figure 1. However this Tg model does not allow us to determine whether these mechanisms can lead to class switch and somatic hypermutations.

Several other data further support that autoreactive B-cell tolerance can be broken by concurrent BCR and TLR signaling and extend this mechanism to B cells expressing other types of autoantibodies. Leadbetter et al. have shown that mammalian chromatin-containing IgG immune complexes induce the proliferation of RF-expressing B cells in vitro.[43] Notably they have provided strong evidence that this mechanism results from the sequential engagement of the BCR and TLR9 and requires intact TLR signaling.[44] The clinical relevance of these data may be questioned since, in patients with autoimmune diseases, RF can be detected in the absence of anti-DNA/chromatin or anti-RNA autoantibodies and vice versa. However, recent in vitro evidence supports that chromatin- and RNA-specific B cells can be directly activated by their autoantigen in a similar TLR-dependent fashion at least as measured by cellular proliferation.[45] In addition, Berland et al. using a knock-in mouse model (564Igi), recently demonstrated that B cells expressing a RNA-specific BCR can be activated in vivo to class switch

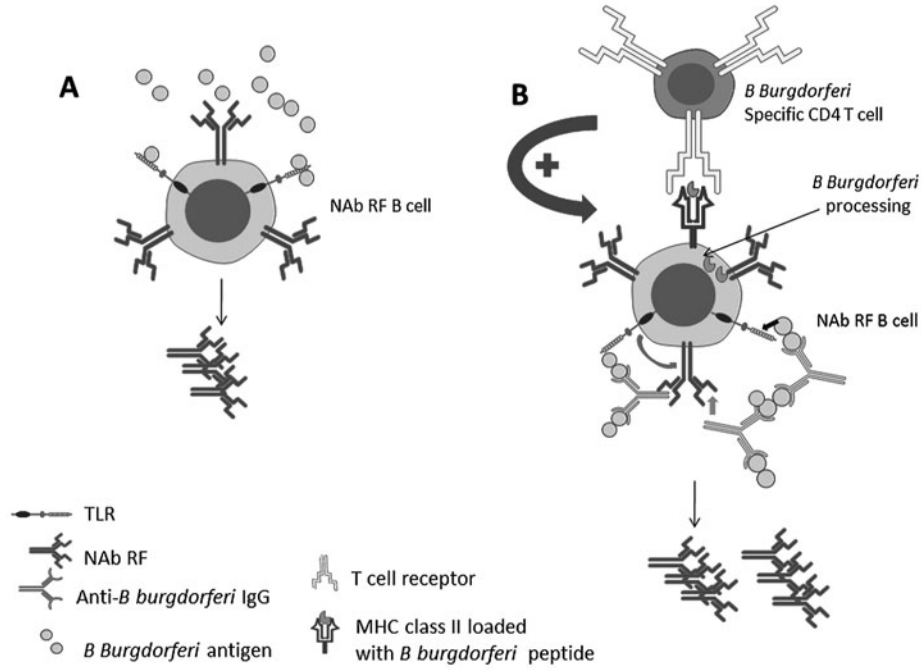

Figure 1. Possible mechanisms of RF-specific B-cell activation during *B. burgdorferi* infection. A) In mice transgenic for Rheumatoid Factor NAbs (Smi or Hul, designated NAb RF) NAb RF-expressing B cells are stimulated by *B. burgdorferi* through TLR (Toll Like Receptor) interaction and produce low levels of RF-specific NAbs. Such activation does not occur if *MyD88* is invalidated in B cells. B) In NAb x cIgG animals the presence of anti-*B. burgdorferi* chimeric IgG (with human constant region recognized by the RF Smi and Hul) leads to the formation of *B. burgdorferi*–containing immune complexes which are able to cosignal through TLR (small black or purple arrow) and RF B-cell receptors (small light gray or blue arrow shows recognition of IgG constant region by NAb RF Smi or Hul). The curved medium gray (or green) arrow shows a potential cross-link between the B-cell receptor and the TLR induced by the immune complex. The immune complexes are internalized and *B. burgdorferi* antigens are processed into antigenic peptides. Then $CD4^+$ T cells which are specific for B burgdorferi peptides cooperate with NAb RF B cells to increase NAb RF production (the large dark gray or red arrow symbolizes T-cell help leading to increased NAb RF production). (Adapted from ref. 31). A color version of this figure is available online at http://www.landesbioscience.com/curie.

and produce autoantibodies in a TLR7-dependent fashion.[46] Since normal individuals harbor many NAb-expressing B cells that are directed to nucleic acids, it is reasonable to conceive that these cells may be activated by exposure to DNA/RNA containing materials from infectious agents. Pisetsky and colleagues have indeed shown that bacterial DNA induces production of cross-reactive anti-dsDNA autoantibodies in pre-autoimmune NZBxNZW mice.[47]

The next question is whether any NAb-expressing B cell can be activated by this mechanism provided that the autoantigen is accessible to the BCR. An important result from the Smi and Hul RF models is that normal human immunoglobulins (i.e., devoid of anti-*B. burgdorferi* antibody*)* are unable to increase the activation of RF induced by *B. burgdorferi*. This suggests that a physical interaction or a cross-link between the TLR and the BCR may be required. For anti-nucleic acid producing B cells, such interaction

could occur at the membrane of the TLR7/9-associated subcellular compartment after internalization of the BCR complexed with RNA/DNA fragments. If a cross-link between TLR and the BCR proved to be required, it could together with the high frequencies of RF and anti-nuclear antibodies expressing B cells in the naïve repertoire, account for the fact that these autoantibodies are the most frequently produced ones during infectious diseases. In this view, Rui et al. have demonstrated in the anti-hen-egg lysozyme (HEL) model that stimulation with the antigen is synergistic with CpG DNA through TLR9 signaling and may require charge-based interactions between HEL and CpG DNA.[48]

To our knowledge it has not been proven that an experimental infection can induce anti-chromatin/RNA production by BCR/TLR synergy. In our RF mice models infected by *B. burgdorferi* the autoantigen-dependent step in RF production requires CD4 T-cell help and CD40-CD40L interaction. While it is clear that RF-expressing B cells can present non-self antigens to T cells during an infection, the mechanisms by which anti-nucleic acid-specific B cells could receive such help are less obvious. The scenario proposed by R Zinkernagel in LCMV-infected mice in which B cells can process antigens independently of their BCR specificity, is however a possibility. However it is noteworthy that the infection of NZBxNZW lupus-prone mice by *B. burgdorferi* does not increase anti-nuclear autoantibodies production and does not modify the course of the lupus disease (our unpublished results).

Do Disease-Associated autoAbs Originate from the NAb-Expressing B Cell Pool?

As mentioned before, the main difference between NAbs and disease-associated autoAbs is that the latter have undergone somatic hypermutations. Somatic mutations generate variants of higher affinity for the autoAg but also of lower "multireactivity." Some authors have argued that the loss of multireactivity may be at least as important as the increase of affinity for an autoAb to become pathogenic.[3] According to this hypothesis, NAbs would be harmless because their multireactivity would favor interactions between themselves and possibly other serum components with a "natural neutralisation" as a consequence. Somatic mutations generating variants of lower polyreactivity would isolate the clones and expose their pathogenic potential. Possibly illustrating this hypothesis, we recently described a monoclonal antiphospholipid autoAb, CIC15, originating from a patient with recurrent fetal losses.[7] The transfer of CIC15 to pregnant mice reproduced the human disease. CIC15 binds to cardiolipin and is annexin V dependent. This autoAb is somatically mutated. To clarify the role of the somatic mutations, they were reverted to the germline configuration. The resulting "germline" Ab reacted with multiple self-antigens characteristic of NAbs, partially lost its reactivity to cardiolipin and was no longer dependent on annexin V and, more importantly, was no longer pathogenic. These results illustrate that the antigen-driven somatic hypermutations of NAb-expressing B cells can destroy polyreactivity and be responsible for pathogenicity. Given current knowledge on antigen-driven maturations of B cells, it is difficult to imagine how multireactivity may be counterselected for. It may not be a true counterselection, instead somatic mutations in the V regions may have a high probability to destroy polyreactivity.

Other data suggest that disease-associated autoAbs may not derive from the NAb-expressing B-cell pool, but rather by somatic hypermutations from nonautoimmune mature B cells, responding to antigens in the periphery. To demonstrate that pathologic autoAbs originate from NAbs requires the identification of somatic mutations. This is

often problematic, because of V gene polymorphism, especially in humans, but most of all by the imprecision of V_H to D and D to J_H rearrangements and by the presence of untemplated nucleotides in the HCDR3 region that are added by terminal deoxynucleotidyl transferase (Tdt). Yet the HCDR3 region plays an important role in antigen binding in general and in NAb specificity in particular. Recently Wysocky and colleagues took advantage of an autoimmune prone mouse model that develops an SLE-like disease and in which all somatic mutations within antinuclear autoAb variable regions, including HCDR3, can be unequivocally identified.[49] Mutation reversion analyses demonstrated that most antinuclear autoAbs arose from nonautoimmune B cells that had diversified immunoglobulin genes by somatic hypermutation. These data emphasize the importance of yet ill defined self-tolerance mechanisms that operate at the postmutational state of B-cell ontogeny (reviewed in ref. 50).

CONCLUSION

From the pool of B-cell precursors in the genetically diverse pre-immune repertoire, NAb-expressing B cells are positively selected. The mechanisms enabling this selection from pre-B to fully mature B cells remain unclear. It is also not clear whether all B cells need to be selected to develop into mature B cells. This would imply that all naive newly generated B cells would be autoreactive. The fact that, for example, transgenic B cells knock-in for antibodies with no detectable self-reactivity develop normally, argues against this hypothesis. However, it is very difficult to be sure that a given antibody does not bind with low affinity to some autoantigen. In the periphery NAb-expressing B cells seem ignorant toward their antigen, probably because their affinities fall below the threshold necessary for activation. However, active, regulatory mechanisms may play a role; for instance the role of regulatory T cells has not been studied in detail. In addition, some NAb-expressing B cells may have sufficient affinity to undergo an autoantigen-driven activation provided for that a second and even a third signal is available. In our model the autoantigen-dependent activation of RF-expressing B cells needs TLR signaling and T-cell help. Thus, many NAb-expressing B cells would be unable to undergo such activation, because of the lack of T-cell help. RF-expressing B cells are, however, particular, because they can present antigen captured as an immune-complex. On the other hand, the presence of memory B cells, expressing somatically mutated NAbs in apparently healthy individuals, suggests that other types of NAb-expressing B cells can undergo full activation and even somatic hypermutations.[51] The critical question whether disease-associated autoAbs may derive from the NAb-expressing B-cell pool is still unresolved and needs further investigations.

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CHAPTER 12

GRANULOCYTE DEATH REGULATION BY NATURALLY OCCURRING AUTOANTIBODIES

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Abstract: Programmed cell death (PCD) plays a central role in the regulation of granulocytes that are key effector cells of the innate immune system. Granulocytes are produced in high amounts in the bone marrow. A safe elimination of granulocytes by cell death (apoptosis) is essential to maintain the numbers of these cells balanced. In many acute and chronic inflammatory diseases, delayed apoptosis is one mechanism that contributes to accumulation of neutrophil and eosinophil granulocytes at the site of inflammation. On the other hand, a safe elimination of granulocytes by cell death is required to avoid unwanted tissue damage for instance by secretion of toxic products from these cells. Recent evidence shows that humans produce an array of naturally occurring autoantibodies (NAbs) with the capacity to regulate granulocyte death, including agonistic and antagonistic NAbs that bind to the receptors Fas, Siglec-8, and Siglec-9. Together with other factors, these various NAbs exhibit different properties in terms of the form of cell death they induce, the molecular signaling pathways they engage, as well as the efficacy or potency by which they induce cell death. Moreover, several regulatory mechanisms seem to exist that control their biological activity. Novel insights support the concept of granulocyte death regulation by NAbs, which might have important implications for our understanding of the pathogenesis and treatment of inflammatory diseases, including many autoimmune and allergic disorders.

INTRODUCTION

Regulation of immune cells by programmed cell death is a key process in the maintenance of immune homeostasis, the control of immune responses, and the

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resolution phase of inflammtion.[1-4] As effector cells of the innate immune system, neutrophil and eosinophil granulocytes are involved in a broad range of acute and chronic inflammatory diseases. Unspecific secretion of toxic products and phagocytosis are key functions of these cells and a tight regulation of their lifespan is required to avoid uncontrolled tissue damage.[2,3] Granulocytes are short-living cells that die in the absence of inflammation, within a few days in a process called spontaneous apoptosis. At the site of inflammation, granulocyte apoptosis is delayed by the presence of survival factors including cytokines, a process that supports the often massive accumulation of granulocytes. Certain granulocyte survival factors have the capacity to block caspase-dependent, pro-apoptotic death pathways, as shown for the cytokines granulocyte/macrophage colony-stimulating factor (GM-CSF) or interleukin-5 (IL-5).[2,3,5] On the other hand, granulocytes express death receptors of the tumor necrosis factor (TNF)/nerve growth factor (NGF) family, including Fas, with the capacity to accelerate the apoptosis of neutrophils and eosinophils.[2,3,5]

Recent evidence shows that members of the novel receptor family of sialic acid binding immunoglobulin (Ig)-like lectins (Siglecs) are also empowered to transmit death signals into granulocytes.[5] Siglec-8 is expressed on eosinophils, whereas neutrophils express the structurally related family member Siglec-9.[6] Interestingly, Siglec-8- and Siglec-9-mediated granulocyte death is dramatically enhanced in the presence of cytokines.[7,8] By recruitment of alternative caspase-independent death pathways,[8] Siglecs have the capacity to transmit death signals despite an anti-apoptotic microenvironment, such as at the site of inflammation.[5] Therefore, Siglecs are also viewed as "safeguards" to protect from overwhelming immune responses by granulocytes.[5,9]

Evidence is accumulating that human intravenous immunoglobulin (IVIG) preparations, derived from the pooled plasma of thousands of healthy blood donors, contain functional NAbs to Fas, Siglec-8 and Siglec-9 receptors, that trigger granulocyte death at clinically relevant concentrations.[10,11] IVIG is increasingly being used as a high-dose therapy for the treatment of inflammatory diseases for licensed indications and off-label applications.[12-14] The mode of its anti-inflammatory action is complex and experimental evidence suggests that, among other factors, death regulation of immune cells by NAbs is involved.[10,12,13,15-17] These mechanisms may act in a mutually nonexclusive fashion depending on the pathogenesis of the disease, and may include both F(ab)- and Fc-related processes.[10,12-14] IVIG-induced programmed cell death of granulocytes appears to be F(ab)- and not Fc-mediated, as revealed by experiments using $F(ab)_2$ fragments of IVIG, Fcγ receptor blocking experiments and other controls.[16,18]

In 1998 Viard et al. identified blocking antibodies directed against Fas within IVIG with the capacity to inhibit Fas-mediated keratinocyte apoptosis in toxic epidermal necrolysis (TEN), also known as Lyell's syndrome.[19] Soon thereafter it became clear that IVIG not only contains blocking, but also agonistic anti-Fas antibodies with the capacity to induce caspase-dependent apoptosis in human neutrophils, monocytes and lymphocytes.[20-22] Both, the protection of tissue cells by blocking anti-Fas antibodies, and the elimination of immune cells by agonistic pro-apoptotic antibodies might contribute to the anti-inflammatory effect of high-dose IVIG treatment. The concurrent presence of agonistic and antagonistic anti-Fas antibodies in IVIG has been confirmed and it has been shown that the resulting effect on granulocyte death is concentration-dependent.[20] The balance between Fas-stimulating and Fas-blocking antibodies has recently been shown to vary among different IVIG preparations.[23]

In 2006 we identified NAbs directed against Siglec-9, soon followed by the discovery of anti-Siglec-8 NAbs, showing that regulation of granulocyte death by NAbs is not restricted to Fas, but may include antibodies to several other death-promoting receptors.[16,18] Are these diverse antibodies as regulatory means redundant or complementary? Indeed, there are relevant differences between the systems involving Fas and Siglecs. For instance, as opposed to Fas-mediated apoptosis, Siglec-triggered death effects on granulocytes are cytokine-dependent and dramatically increased in inflammatory, primed granulocytes, as compared with unstimulated, quiescent cells.[8,24] Experimental evidence further suggests that Siglecs have the capacity to turn anti-apoptotic survival factors into killers by recruitment of alternative pathways of programmed cell death.[5] Under inflammatory conditions, Siglec-specific NAbs might therefore play a key role in the regulation of granulocyte death. Compared with the classical death receptor TNF/NGF family, considerably less is known about the novel family of Siglecs, which shall therefore be briefly introduced in the next section.

SIGLECS

Siglecs form a family of cell surface receptors that is broadly expressed on immune cells and that characteristically binds to sialic acid-containing carbohydrate structures (sialoglycans).[6,25-28] Interest in Siglecs has grown over the recent years as experimental evidence has accumulated showing that these receptors play an important role in the regulation of the immune system. Siglecs mediate predominantly inhibitory signals, with the exception of a few members that transmit activating signals.[6] Besides induction of cell death, Siglecs have been shown to regulate processes such as cell expansion, cytokine production, cellular activation and cell adhesion processes (reviewed in ref. 6).

Given their carbohydrate-binding characteristics and their subsumption to the immunoglobulin superfamily, Siglecs are classified as I-type lectins. Siglecs are single-pass transmembrane receptors that comprise an extracellular region consisting of a sialic acid-binding N-terminal variable (V)-set immunoglobulin (Ig) domain followed by a series of C2-set Ig domains (1–16), a transmembrane region and a cytosolic tail with tyrosine residues located within characteristic signaling domains. Like other inhibitory immune receptor families such as killer cell immunoglobulin-like receptors (KIRs), most but not all Siglecs possess an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM). In several members of the so-called CD33-related Siglec (CD33rSiglec) subfamily this ITIM is followed by a more distal ITIM-like signaling domain, as depicted for Siglec-8 and Siglec-9 in Figure 1. Upon engagement of the receptor, ITIMs are tyrosine phosphorylated and recruit inhibitory phosphatases, such as Src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), SHP-2 and SH2-containing inositol phosphatase (SHIP), eventually resulting in inhibitory or death signals depending on the cell type.[5,6,26,27,29,30] In Siglecs, increased tyrosine phophorylation of these motifs has also been observed within a few minutes after cytokine stimulation.[8,31] These phosphorylation events might contribute to the cytokine-dependency of Siglecs-mediated cell death.[5]

Siglecs are encoded as a large gene cluster on chromosome 19 in humans, which includes the evolutionary conserved members sialoadhesin, CD22, CD33 and myelin-associated glycoprotein, as well as the CD33rSiglec subfamily. The CD33rSiglec

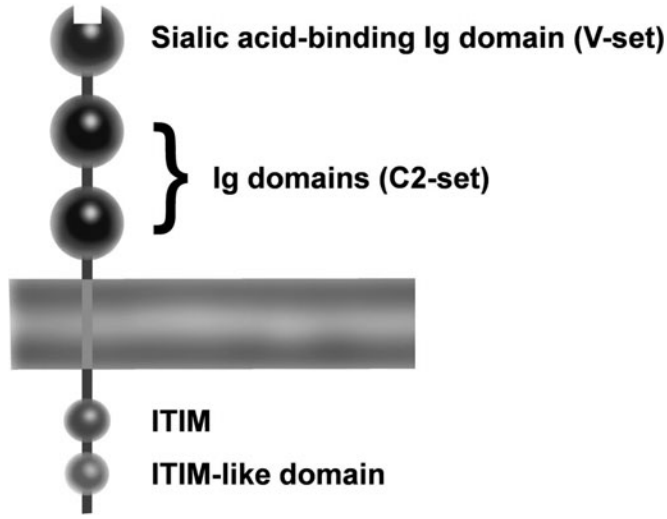

Figure 1. Domains arrangement in Siglec-8 and Siglec-9. The extracellular region consists of one sialic acid-binding, N-terminal variable (V)-set immunoglobulin (Ig) domain followed by 2 C2-set Ig domains. The number of C2-set Ig domains may vary in other Siglec family members (1–16). The intracellular regions of Siglec-8 and Siglec-9 contain a proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) followed by a more distal ITIM-like signaling domain. Upon tyrosine phosphorylation these tyrosine-based motifs recruit inhibitory phosphatases, which may result either in inhibitory or death signals depending on the cell type. Art by Aldona von Gunten.

subfamily consists predominantly of inhibitory leukocyte receptors including Siglec-8 and Siglec-9.[6] CD33rSiglecs differ significantly between different species, which is reflected by nomenclature, such that for instance CD33rSiglecs in humans are numbered, but lettered in the mouse.[6] Based on a recent comparative genomic analysis of species including primates, rodents, dog, cow, marsupials, amphibians and fish, it has been proposed that CD33rSiglecs expanded in mammals through a major inverse duplication event involving a smaller primordial sub-cluster of Siglec genes over 180 million years ago.[32] While rodents appear to have undergone a loss of CD33rSiglec genes after the inverse duplication, CD33rSiglecs expanded in primates and several are pseudogenes with signaling motifs consistent with activating receptors.[32] It has been proposed that the occurrence of evolutionary changes in their sialic acid ligands, i.e., sialobiology, might have resulted accordingly in rapid adjustments of Siglec receptors.[33] Interestingly, pathogens have acquired various strategies to decorate their surface by sialic acid structures resembling those of vertebrates,[34] and by this means, might evade immune responses through molecular mimicry, eventually by engagement of Siglecs.[26,27,35] The diversification of CD33rSiglecs within mammals may therefore reflect the ongoing evolutionary arms race between host and pathogen.[26,35,36] Provided the widely differing CD33rSiglec repertoires between mammals, investigations in vivo using genetically modified animals will require careful and sophisticated strategies.[5,10]

NEUTROPHIL DEATH INDUCED BY NATURAL ANTI-SIGLEC-9 AUTOANTIBODIES

Siglec-9 is highly expressed on human neutrophils and monocytes, and at lower levels on subpopulations of lymphocytes.[6] In a study using purified human bone marrow cells, we found that neutrophils gain Siglec-9 late in differentiation, not before the myelocyte stage but before CD16 expression.[8] Siglecs can be masked by binding in cis to endogenous ligands on the same cell surface, as shown for a significant proportion of Siglec-9 receptors on monocytes.[8] In contrast, on neutrophils the majority of Siglec-9 receptors appears to be unmasked,[8] free to bind in trans to ligands on other cells or on structures of the surrounding tissue environment. Functionally, ligation of Siglec-9 by monoclonal antibodies has been shown to result in inhibition of cellular activities in a variety of different cell types, and in cell death of neutrophils.[8,37-39] The recruitment of the inhibitory phosphatases, SHP-1 and SHP-2, upon tyrosine phosphorylation of intracellular signaling domains of the receptor, might be implicated in both inhibitory and death promoting functions of Siglec-9.[5,8,31,37,38]

In analogy to the classical death receptor Fas, ligation of Siglec-9 on human primary neutrophils by a monoclonal antibody induces caspase-dependent apoptosis.[8,40] In striking contrast to Fas, however, neutrophil death induced by Siglec-9 is cytokine-dependent, with granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon-α (IFN-α), and IFN-γ, dramatically enhancing the rate of death. Similarly, Siglec-9 mediated death is increased in human neutrophils, isolated and studied ex vivo from inflammatory diseases, such as sepsis or rheumatoid arthritis (Fig. 2A).[8,40] Light and transmission electron microscopic analysis revealed that unstimulated neutrophils undergoing Siglec-9-mediated death show classical features of apoptosis (Fig. 2B).[8] In contrast, GM-CSF-primed inflammatory neutrophils undergoing Siglec-9-mediated death exhibited multiple vacuole-like structures, an observation that prompted us to refer to this kind of death as autophagy-like cell death.[8,17] Siglec-9 in the absence of cytokines triggers classical apoptotic pathways, involving cleavage and activation of caspases, similar to Fas-mediated apoptosis.[8] In contrast, autophagy-like cell death in the presence of cytokines turned out to involve caspase-independent molecular pathways that include reactive oxygen species.[8] Taken together, the cytokine microenvironment appears to greatly influence Siglec-9-mediated neutrophil death, since in inflammatory cells alternative non-apoptotic death pathways are recruited.[5]

IVIG accelerates the spontaneous apoptosis of neutrophils in vitro at concentrations reached systemically upon IVIG high-dose therapy.[18,20] It has been shown that IVIG-mediated cell death is dramatically enhanced among neutrophils primed with cytokines (GM-CSF, IFN-γ) as compared with unstimulated cells.[18] IVIG induced an autophagy-like morphology in neutrophils, reminiscent of cells treated with monoclonal autoantibodies to Siglec-9 (see above). This finding suggested the presence of anti-Siglec-9 NAbs in IVIG. Indeed, the presence of functional anti-Siglec-9 NAbs in IVIG was confirmed by experiments using surface plasmon resonance (Fig. 3A).[18] In flow cytometric experiments, purified anti-Siglec-9 NAbs derived from IVIG exhibited a similar surface staining pattern on neutrophils as a commercial monoclonal anti-Siglec-9 Ab (Fig. 3B).[18]

Treating human neutrophils with IVIG: what impact has the removal of anti-Siglec-9 NAbs on neutrophil viability? In the absence of cytokines treatment of normal neutrophils

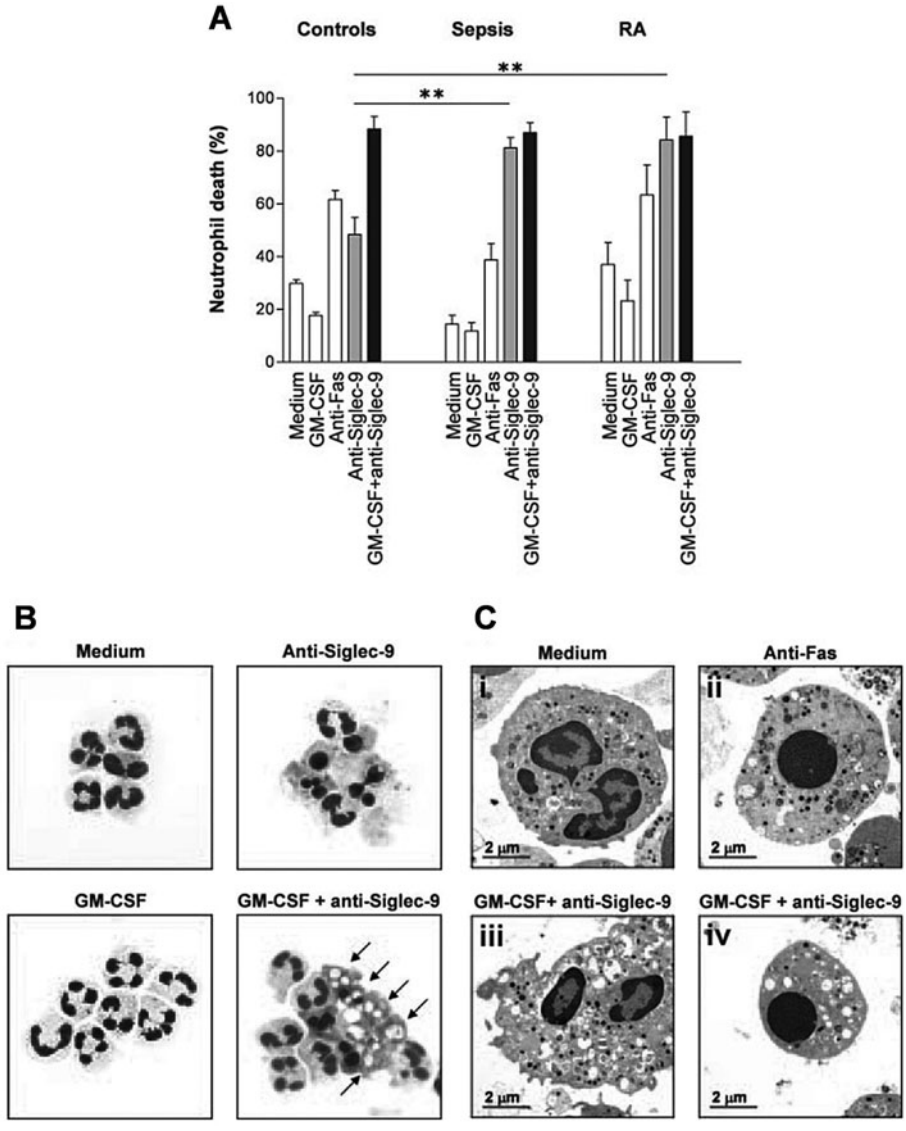

Figure 2. Inflammatory neutrophils demonstrate increased Siglec-9–mediated cell death that has morphologic characteristics of apoptosis and autophagy-like cell death. A) Blood neutrophils from healthy controls and from patients with sepsis, as well as joint fluid neutrophils from rheumatic arthritis patients were incubated with anti-Siglec-9 mAb in the absence or presence of 25 ng/ml GM-CSF. Cell death was assessed after 24 h **$p < 0.01$. B) Light microscopy: anti–Siglec-9 cross-linking alone induced neutrophil apoptosis (reduced cell volume and nuclear condensation compared). GM-CSF followed by Siglec-9 engagement resulted in both apoptotic and aberrant death characterized by cytoplasmic vacuolization (arrows). C) Transmission electron microscopy: panel i demonstrates a normal viable neutrophil; panel ii shows an apoptotic neutrophil with condensation of the nucleus and cell shrinkage. Panels iii and iv demonstrate the aberrant morphology of GM-CSF–primed and Siglec-9–stimulated neutrophils. Approximately 25% of these cells showed condensation of the nucleus and cell shrinkage (iv). The plasma membrane appeared to be intact. The cells in panels B and C were from 15-h cultures. Reproduced with permission from von Gunten S et al. Blood 2005; 106:1423-31.[8] ©2005 The American Society of Hematology.

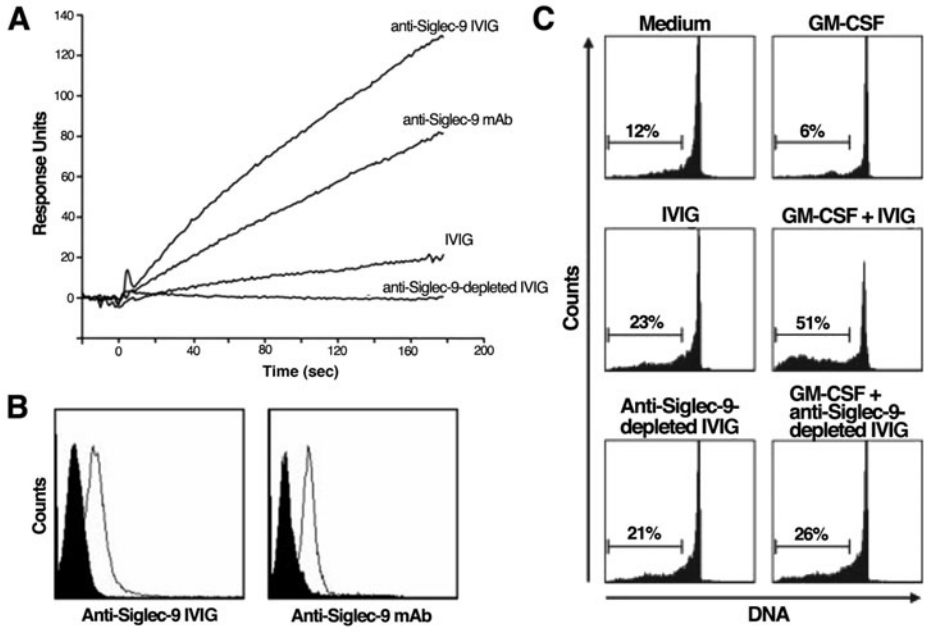

Figure 3. IVIG contains NAbs against human Siglec-9 that mediate cytokine-dependent neutrophil death. A) Surface plasma resonance (SPR): Sensorgrams of the binding activity of different fractions of IVIG, affinity purified anti-Siglec-9 from IVIG (anti–Siglec-9 IVIG), and a control anti–Siglec-9 mAb to immobilized recombinant Siglec-9/Fc fusion protein, confirming the existence of autoantibodies against Siglec-9 in IVIG. B) Flow cytometry: Purified human anti–Siglec-9 NAbs from IVIG and mouse anti–Siglec-9 mAb similarly bound to the cell surfaces of freshly isolated neutrophils (black peak: control staining; white peak: anti–Siglec-9 staining). C) DNA fragmentation assay. Both IVIG and anti–Siglec-9–depleted IVIG induced DNA fragmentation in the absence of GM-CSF. In the presence of GM-CSF, IVIG treatment of neutrophils resulted in enhanced DNA fragmentation that was not seen with anti–Siglec-9–depleted IVIG. Reproduced from von Gunten S et al. Blood 2006; 108:4255-9.[18]

with IVIG or anti-Siglec-9-depleted IVIG results in programmed cell death, by both preparations (Fig. 3C).[18] This is expected considering that IVIG contains agonistic anti-Fas NAbs and eventually other redundant pro-apoptotic antibodies that may cause neutrophil death in the absence of anti-Siglec-9 NAbs. In contrast, the situation is different for inflammatory neutrophils. In GM-CSF-primed cells the heavily accelerated IVIG-induced death response observed with total IVIG is almost completely lost, if anti-Siglec-9-depleted IVIG is used.[18] These findings suggest that upon IVIG treatment anti-Siglec-9 NAbs are responsible for the increased and cytokine-dependent death response of inflammatory neutrophils as compared with unstimulated neutrophils. The increased responsiveness of inflammatory neutrophils to Siglec-9-mediated death might provide a feedback mechanism triggered by anti-Siglec-9-NAbs under inflammatory conditions. These findings also suggest that in disease, Siglec-9-dependent effects of IVIG may vary according to the stage of the disease and patient characteristics (Fig. 4). The increased Siglec-9-associated neutrophil cytotoxicity of IVIG in a cytokine-rich environment might contribute to the anti-inflammatory effects of IVIG in the broad range of inflammatory diseases involving neutrophils.

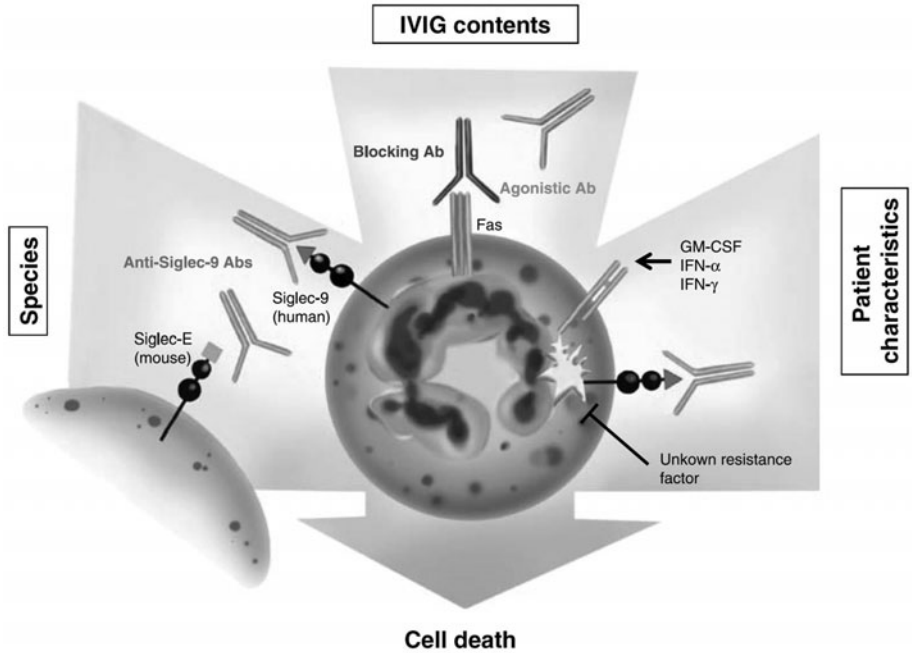

Figure 4. Schematic representation of proposed factors influencing IVIG-mediated cell death of a neutrophilic granulocyte as a target cell. The efficacy and/or potency of the cytotoxic effects of IVIG depend on the preparation being used and on characteristics of the treated patient or species (experimental models). Engagement of Fas or Siglec-9 by agonistic autoantibodies contained in IVIG induces cell death in neutrophils. Batch-to-batch variations in titer and ratio of agonistic vs. antagonistic anti-Fas autoantibodies result in differential effects on Fas, which are concentration dependent. The systemic or local cytokine profile of the patient may facilitate IVIG-induced neutrophil death, because Siglec-9-mediated cell death is enhanced in the presence of cytokines due to priming effects. For yet unknown reasons, transient resistance may block Siglec-9 death pathways in a subset of patients, depending on the stage of disease. Siglec-E, but not Siglec-9, is expressed in mouse neutrophils. With kind permission from Springer Science+Business Media from von Gunten S et al. J Clin Immunol 2010; 30(Suppl 1):S24-30.[10]

EOSINOPHIL DEATH INDUCED BY NATURAL ANTI-SIGLEC-8 AUTOANTIBODIES: A ROLE IN ALLERGIC DISEASE?

Siglec-8 is highly expressed on human eosinophilic granulocytes and mast cells, and weakly on basophils.[6,9] Asthma and other forms of allergic inflammation involve selective tissue accumulation and activation of eosinophils, basophils and mast cells.[41] Marked, unexplained blood and tissue eosinophilia is the hallmark of the hypereosinophilic syndrome (HES), which is associated with a variety of clinical manifestations, including idiopathic hypereosinophilic syndrome (IHES), Churg-Strauss syndrome (CSS), and eosinophil-associated gastrointestinal disease (EGID).[42-46] The regulation of eosinophils, basophils or mast cells by cell inhibition or death has important implications for the pathogenesis and treatment of these diseases.[9,41-44,47,48] Recent insights on Siglec-8 biology suggest a potential role of anti-Siglec-8 NAbs in the regulation of these cells.[11]

In eosinophils, ligation of Siglec-8 by monoclonal antibodies results in caspase-, mitochondrial-, and reactive oxygen species-dependent apoptosis.[7,24] In analogy to

Siglec-9 on neutrophils, Siglec-8 mediated eosinophil death is significantly increased by cytokines, including GM-CSF and interleukin (IL)-5.[24] Whereas little is known about Siglec-8 function on basophils, it has become clear that in mast cells Siglec-8 counteracts stimulatory signals, resulting in inhibition of the release of inflammatory mediators.[49] In contrast to eosinophils, Siglec-8 engagement failed to induce human mast cell apoptosis.[49] Targeting of Siglec-8 is currently discussed as a novel treatment strategy for the treatment of asthma, allergies, HES and related disorders, where overproduction and overactivity of eosinophils and mast cells is occurring.[9,48] This notion is supported by results from in vivo models of allergic asthma and hypereosinophilia on Siglec-F,[50-54] which is a closely related functional paralog of human Siglec-8 in mice.[48]

IVIG contains NAbs specific for Siglec-8.[16] The functionality of them has been tested in death assays using primary human eosinophils.[16] Eosinophils, like neutrophils, undergo cell death upon treatment with IVIG in vitro, which is enhanced in the presence of cytokines such as GM-CSF, IL-5, IFN-γ, TNF-α, and leptin, but not IL-1b and macrophage migration inhibitory factor (MIF) (Fig. 5A).[16] Inflammatory eosinophils from HES patients, presumably exposed to IL-5 in vivo,[55,56] exhibit an increased susceptibility for IVIG-mediated eosinophil death ex vivo.[16] As reported for neutrophils, IVIG-mediated eosinophil death is the result of specific F(ab)-dependent effects of PCD-inducing antibodies within pooled IgG and does not depend on the Fc-portion, as shown in experiments using $F(ab')_2$ fragments of IVIG and Fcγ receptor blocking assays.[16]

SIMILARITIES AND DIFFERENCES BETWEEN GRANULOCYTE DEATH-INDUCING NATURALLY OCCURRING AUTOANTIBODIES

Why do humans produce NAbs against divergent death receptors, if the common outcome is death? Current knowledge provides several answers. For instance, one obvious explanation is that the heterogeneity of these antibodies might provide a means for targeting specific cell types, while other cells are exempt, as distinct death receptors exhibit divergent tissue expression. In contrast to Fas, which is widely expressed on cellular surfaces of many tissues, the expression pattern of Siglec-8 and Siglec-9 is narrow and restricted to a defined set of specific immune effector cells. Second, unequal forms of PCD may be triggered by different death receptors upon ligation with NAbs. As discussed, under certain conditions Siglecs recruit alternative caspase-independent cell death pathways in granulocytes that exhibit an autophagy-like morphology, as opposed to Fas, which induces classical and caspase-dependent apoptosis.[5] Third, the sensitivity of cells for death signals triggered by different death receptors is variable. Both the efficacy and potency of agonistic Siglec-specific, but not Fas-directed, NAbs is significantly enhanced in inflammatory granulocytes as opposed to unstimulated cells. This is in line with the hypothesis that the raison d'être of Siglec-mediated death regulation of granulocytes is in inflammatory disease, providing a feedback regulation mechanism to control granulocyte immune responses.[5] Fourth, a divergent range of receptor-associated biological functions has been described for Siglecs and Fas: In addition to cell death regulation, reported functions of Siglecs include the regulation of cell proliferation and cellular activation, cytokine secretion and cell adhesion.[6,26] NAbs to Siglecs might therefore be implicated in the regulation of several of these cellular processes, depending on the cell type. Taken together, the role of NAbs to Fas and Siglecs might differ considerably and depending on the circumstances these antibodies might act mutually exclusive or in concert.

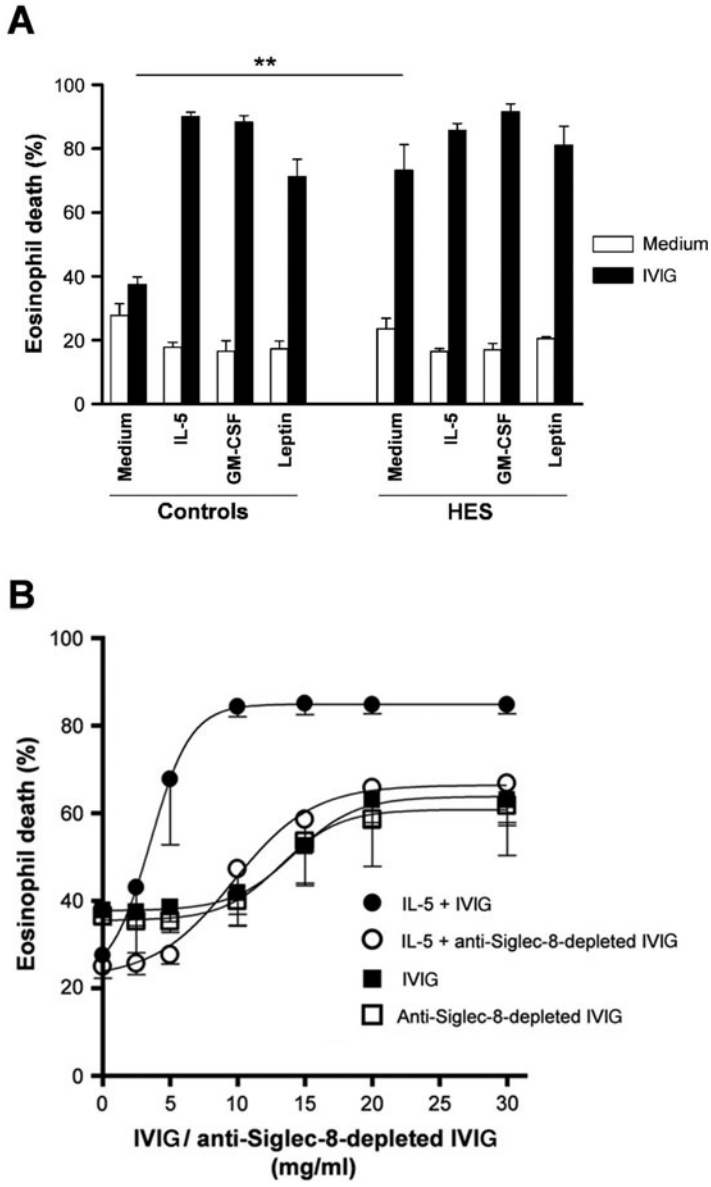

Figure 5. A) Eosinophils from patients with hypereosinophilic syndrome (HES) demonstrate increased IVIG-mediated cell death susceptibility. Although spontaneous death of HES eosinophils was delayed, IVIG-mediated death was significantly enhanced compared with eosinophils from healthy individuals. In vitro priming with IL-5, GM-CSF, or leptin had no additional effect on these inflammatory eosinophils, which suggests that blood eosinophils from patients with HES were primed in vivo. **$p < 0.01$. B) Concentration-effect curves of total IVIG and anti–Siglec-8–depleted IVIG. respectively, in 24-h eosinophil cultures. In the absence of IL-5 pretreatment, both death efficacy and potency of anti–Siglec-8–depleted IVIG was similar to total IVIG. Depletion of anti–Siglec-8 NAbs, however, resulted in loss of IL-5-mediated increase of death efficacy and potency of IVIG. Results of 24-h cultures are shown in all panels. Reproduced from von Gunten S et al. J Allergy Clin Immunol 2007; 119:1005-11.[16]

REGULATION OF GRANULOCYTE DEATH-INDUCING NATURALLY OCCURRING AUTOANTIBODIES

The elimination of granulocytes by cell death is one important immunoregulatory process. We now understand that a variety of NAbs exists that act as agonists on granulocyte death receptors, including Fas, Siglec-8 or Siglec-9. Such autoantibodies are thought to contribute to the anti-inflammatory action of IVIG.[10-13,15] On the other hand, regulatory mechanisms must exist that control the activity of these potentially harmful NAbs. Based on recent experimental evidence, the following potential regulatory mechanisms for the control of death-inducing NAbs are currently being discussed:

Antagonistic Antibodies

As discussed above agonistic and antagonistic autoantibodies to Fas have been detected in human IVIG that compete for binding to the receptor (Fig. 4). The resulting death effect is concentration-dependent and depends on titer and ratio of agonistic vs. antagonistic anti-Fas autoantibodies.[20]

Anti-Idiotypic Antibodies

We have experimental evidence that humans produce anti-idiotypic (Id) NAbs that bind to anti-Siglec-9 NAbs.[57] Anti-idiotypes are antibodies that are capable of interacting with variable regions of other antibody molecules to form Id-anti-Id complexes. Id-anti-Id complexes are thought to contribute to dimer formation in IVIG.[58] Since both dimeric and monomeric IVIG contain anti-Fas autoantibodies,[57] it is possible that a proportion of these antibodies are bound by anti-idiotypes. Anti-idiotypic regulation[59] might present one means to control the accessibility of NAbs against death receptors on granulocytes.

Soluble Receptors

Soluble forms of the Fas receptor exist that protect Fas-bearing sensitive cells from apoptosis induced by agonistic antibodies or by the Fas ligand.[60,61] Soluble Fas molecules could act as scavengers for anti-Fas NAbs and block the interaction of these antibodies with the Fas receptor (Fig. 4).

Sensitivity of the Target Cell

Due to cytokine-mediated priming effects inflammatory granulocytes exhibit an enhanced sensitivity to Siglec-8- and Siglec-9-triggered cell death compared with non-inflammatory, unstimulated cells. Similarly, cytokine-exposed granulocytes undergo increased cell death induced by IVIG, a phenomenon that has been associated with anti-Siglec NAbs in these preparations.[16,18] Septic shock patients exhibit divergent Siglec-9-mediated neutrophil death responses, ranging from increased susceptibility to yet unexplained transient resistance.[40] The sensitivity of granulocytes for functional Siglec-specific NAbs may depend on patient characteristics, such as the individual cytokine profile (systemically in the blood or locally at the site of inflammation), severity and stage of disease (Fig. 4).[10,11]

ANTI-Fas AND ANTI-SIGLEC NATURALLY OCCURRING AUTOANTIBODIES IN INTRAVENOUS IMMUNOGLOBULIN: CLINICAL IMPLICATIONS

Experimental evidence suggests that the modulation of granulocyte death by NAbs to Fas and Siglecs could contribute to the anti-inflammatory effects of IVIG.[10,11,20] Inflammatory granulocytes exhibit an increased susceptibility for IVIG-mediated death, an effect that is abolished upon depletion or blocking of anti-Siglec-8 and anti-Siglec-9 NAbs in commercial IVIG preparations. We have hypothesized that the increased susceptibility of activated neutrophils and eosinophils toward these NAbs with the recruitment of caspase-independent death pathways might override the anti-apoptotic effects of the local cytokine microenvironment.[11,17] The resulting IVIG-mediated clearance of granulocytes from the site of inflammation would be welcome in the treatment of disorders associated with hyperactivity of or with pathologically increased numbers of granulocytes. For instance in Churg-Strauss syndrome, a disorder associated with (hyper)eosinophilia, IVIG treatments have been shown to reduce eosinophil numbers in the blood,[62] and allowed the reduction of systemic steroid doses.[63] On the other hand, the occurrence of granulocyte death-inducing NAbs in IVIG could lead to unwanted side-effects, since the reduction of granulocyte numbers could be detrimental in certain patients,[11] including sepsis[64] and neonatal neutropenia.[65] The occurrence of obscure IVIG-induced neutropenia has been reported in various disorders,[66-70] and it remains to be shown if this phenomenon is linked to neutrophil death-inducing NAbs.[18] Depending on the application, the enrichment or depletion of anti-Fas and anti-Siglec antibodies in IVIG preparations might have beneficial effects on the outcome of the treated patient.

CONCLUSION

The recent discovery of functional naturally occurring autoantibodies specific for death receptors suggests a role of these antibodies in the regulation of tissue homeostasis and immune responses.[10-12,17] Since regulation by cell death is central for the control of granulocytes, such death receptor specific NAbs might play an important role in diseases associated with neutrophil or eosinophil responses, including many autoimmune and allergic diseases. The concept that cellular innate immunity is at least partially controlled by NAbs is intriguing.

Future studies are required to further elucidate the regulation of granulocyte death by NAbs. In terms of in vivo studies it is urgent to consider that Siglecs exhibit significant species-specific differences in terms of function, structure, or expression pattern.[6,26] For instance, NAbs to human Siglec-9 would not recognize its paralog Siglec-E in mice (Fig. 4). In vivo investigations, using xenogenic (human) immunoglobulins in animal models will require careful and sophisticated strategies in terms of potential loss-of-function or gain-of-function effects, and it is implicit that experimental evidence needs to be confirmed in a human system.[10] Many open questions remain to be addressed: Can NAbs to Fas or Siglecs become pathogenic? Could failure of control mechanisms or dysregulated activity of these NAbs be involved in the pathogenesis of autoimmune diseases? Currently, most studies have been performed using pooled sera as a source of such antibodies, whereas data on single sera are missing. In future studies it would be interesting to determine individual titers of anti-Fas or anti-Siglec NAbs in health and disease, as these might vary depending on factors such as age, gender, genetics and stage of prevailing disease.

Granulocyte death regulation by NAbs seems to provide a natural "safe guard" mechanism to control neutrophil- and eosinophil-associated immune responses. Future studies will show if the pharmacological exploitation of this natural regulatory mechanism can be achieved, for instance with monoclonal antibodies in analogy to NAbs, or in form of synthetic molecules that imitate natural ligands of NAb target receptors. A better understanding of granulocyte regulation by NAbs might provide novel insights into the pathogenesis of autoimmune and allergic diseases. Furthermore, it will lead to a better understanding of currently used therapeutics such as IVIG, and eventually pave the way to novel treatment strategies for the therapy of granulocyte-associated chronic inflammatory conditions.

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SECTION III

NAbs CAN INDUCE DISEASES WHEN COMPARTMENT BORDERS BREAK OR PROTEASES DEGRADE IgG

CHAPTER 13

NATURALLY OCCURRING AUTOANTIBODIES MEDIATE ISCHEMIA/REPERFUSION-INDUCED TISSUE INJURY

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Abstract: Ischemia and reperfusion events within the heart and brain and similar trauma-induced ischemia/reperfusion events lead to significant morbidity and mortality. In the past two decades, an excessive innate immune response has been identified as the mediator of reperfusion-induced tissue damage. Recent evidence indicates that naturally occurring autoantibody (NAb) activation of complement is a major mechanism of injury due to ischemia/reperfusion. This chapter focuses on the antigens exposed by ischemia and recognized by NAbs, the mechanism of complement activation by damaging NAbs and the protective role of IVIG in ischemia/reperfusion-induced pathology.

INTRODUCTION

Ischemia, or interruption of blood flow to an organ, results in hypoxia, which induces cellular death and tissue damage. Paradoxically, this damage is significantly enhanced during reperfusion or return of blood flow. Ischemia/reperfusion (IR) events occur in multiple clinical conditions including myocardial infarction, stroke, hemorrhagic shock and organ transplantation. Thus, IR-induced pathology affects many different organs and results in high mortality rates as, for example, myocardial and cerebral IR-induced tissue damage are among the top five leading causes of death in the United States. In addition, the mortality rate for intestinal IR remains at ~70%. Thus, understanding the mechanisms leading to IR-induced damage is currently an area of intense study.

Although the precise mechanisms of reperfusion-induced injury are not known, complement activation and antibodies, specifically naturally occurring autoantibodies

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(NAbs), play a prominent role. In this chapter we will examine the role of NAbs in IR-induced injury in the context of multiple organs. NAbs and the IR-induced damage and inflammation will be briefly introduced and the specific antigens recognized by the NAbs will be discussed. In addition, the specific complement activation pathways initiated by NAbs in rodent models of IR will be examined. Finally, we will discuss therapeutic use of pooled human antibodies which include multiple NAbs.

ISCHEMIA/REPERFUSION-INDUCED TISSUE DAMAGE AND INFLAMMATION

Ischemia/reperfusion induces an acute response that results in significant tissue damage and inflammation. Clinical events such as myocardial infarction, cerebral ischemic stroke and intestinal ischemia result in cellular damage that is significantly magnified by return of blood flow or reperfusion. Similarly, surgical manipulations to decrease blood flow, tourniquet use during hemorrhage and organ transplants result in reperfusion-induced tissue injury when blood is returned to the tissue. During the ischemic period, multiple cell types undergo mitochondrial changes in pH which increase the cellular sodium and calcium concentrations.[1] The activation of sodium and calcium transporters results in a decrease in ATP production.[2] In addition, ischemia activates multiple enzymatic pathways which lead to increased production of reactive oxygen species and programmed cell death. Within the innate immune response, hypoxia induces signaling through NF-κB and subsequent pro-inflammatory cytokine production (reviewed in refs. 3,4). In the absence of reperfusion the cellular changes will lead to cell death.[5] Paradoxically, re-oxygenation of the tissue enhances the cellular damage and activates an excessive and damaging innate immune response (reviewed in ref. 6). The innate immune response to IR is multifactorial and includes a neutrophil[7,8] and mast cell response,[9] a secretory response of cytokines and eicosanoids and complement activation (reviewed in ref. 10).

NATURALLY OCCURRING ANTIBODIES AND ISCHEMIA/REPERFUSION

NAbs are produced in the absence of exogenous stimulation and are generally IgM or IgG3. These unconventional antibodies frequently have a low affinity for multiple self and nonself antigens including bacterial and viral antigens and apoptotic cells.[11-13] Although most NAbs are believed to be produced by long lived, peritoneal, B-1 B cells, evidence exists that some splenic B cells also produce natural antibodies.[14,15]

Rag-1$^{-/-}$ mice are antibody deficient and resistant to IR-induced tissue damage. Studies by the Carroll group showed that reconstitution of *Rag-1*$^{-/-}$ mice with IgM obtained from normal mouse sera restored injury indicating that NAbs are required for IR-induced heart, intestine and skeletal muscle injury.[16-18] In contrast, kidney IR appears to be antibody independent despite a requirement for B cells.[19,20] Based on the requirement for NAbs, it was hypothesized that cells subjected to hypoxic conditions express cryptic or newly exposed antigens on the plasma membrane which are recognized by low affinity binding of NAbs.[16,21,22]

Interestingly, not all NAbs reconstituted IR-induced injury in the *Rag-1*$^{-/-}$ mice. For example, NAbs from complement receptor 2 (CD21/CD35; CR2) deficient mice

were not sufficient to restore injury in the *Rag-1*$^{-/-}$ mouse.[23,24] Recent data indicate that similar to peritoneal B-1 B cells, CR2^{hi} marginal zone B cells produce the NAb repertoire necessary to induce tissue damage in response to IR.[25] Together, these data suggest that CR2 is required for the production of the appropriate antibody repertoire.

As a B-cell coreceptor, CR2 (CD21) binds fragments of complement component C3 and enhances antibody production (reviewed in ref. 21). Further evidence indicated that the injury inducing NAbs were more prevalent in autoimmune mice suggesting that production of autoreactive NAbs was upregulated.[26] Since CR2 is required for generation of the "pathogenic antibodies," it was likely that the CR2 ligands are also required. Previous studies indicated that *C3*$^{-/-}$ mice were also resistant to IR-induced tissue damage.[17] However, it was not clear if C3 was required for complement activation or for binding CR2 and initiating production of autoreactive NAbs. Recent data indicate that adoptive transfer of splenic B cells (either marginal zone or follicular B cells) or administering serum from CR2 sufficient, *C3*$^{-/-}$ mice to the antibody-deficient *Rag-1*$^{-/-}$ mice induced wildtype levels of damage in response to IR.[25] Thus, these data indicate that although CR2 is critical, the C3 ligands are not required for production of pathogenic, autoreactive antibodies.[25]

ANTIGENS RECOGNIZED BY NAbs IN RODENT MODELS OF IR

As NAbs are critical to intestinal ischemia/reperfusion induced tissue damage, identification of the specific NAb and therefore, the ischemia-exposed antigens recognized by the NAbs has been an area of intense study during the last two decades. Antibody deficient mice (*Rag*-1$^{-/-}$ or *Rag-2*$^{-/-}$) resist intestinal IR-induced damage unless injected with antibodies which recognize the antigens exposed during ischemia.[16,27] Thus, injection of specific monoclonal antibodies (mAb) which induce tissue damage and inflammation were useful tools in the identification of ischemia-liberated antigens. Multiple antigens have been identified using this method.

NONMUSCLE MYOSIN TYPE II A AND C AND GLYCOGEN PHOSPHORYLASE

Initial studies generated hybridomas from peritoneal, B-1 B cells and used pooled supernatants to screen for specific clones secreting damaging IgM. Using this method, Zhang et al. identified an IgM producing clone, CM22, which induced significant mucosal damage following intestinal IR when injected into an antibody deficient, IR-resistant *Rag-1*$^{-/-}$ mouse.[28] In addition, deposition of the IgM and C3 colocalized within the injured villi. Importantly, the sequence of the Ig variable region indicated > 96% similarity with the germline sequence suggesting the clone produces an IgM NAb.[28] Further studies indicated that the clone recognized nonmuscle myosin heavy chain (NMHC) Type II A and C in the murine model of intestinal IR.[29] In addition, a 12 amino acid peptide sequence from the mouse NMHC II-C isoform prevented injury and intestinal permeability when administered prior to intestinal reperfusion.[29] The same peptide also inhibited injury and deposition of IgM and C3 in a murine model of skeletal muscle ischemia/reperfusion.[28,30] Further studies indicated that the NMHC-specific antibody also bound glycogen phosphorylase in skeletal muscle IR.

This polyclonal activity is typical of NAbs. Finally, consistent with the intestinal and skeletal muscle studies, blockade of the same monoclonal Ab attenuated damage due to myocardial infarction.[31] Administration of a synthetic peptide, mimicking the conserved NMHC II-C epitope, significantly lowered NAb binding.[30] The decreased NAb binding correlated with decreased complement activation, inflammation and tissue damage. Thus, NMHC II-C expression is recognized by NAbs and may provide a therapeutic target for treatment of reperfusion-induced injury.

NAb RECOGNITION OF ANNEXIN IV

Another group fused peritoneal lymph node and splenic B cells with a myeloma to create hybridomas.[32] Based on the hypothesis that a single cell suspension of intestinal epithelial cells (IEC) would express antigens similar to those expressed during intestinal IR, a second damage-inducing mAb was identified.[32] The hybridoma (B4) secreted an IgM NAb which recognized the phospholipid binding protein, annexin IV, on IEC but did not recognize freshly isolated splenocytes or thymocytes.[32] In addition, after transfer of the mAb, intestinal damage and inflammation ensued in the normally IR-resistant *Rag-1*$^{-/-}$ mice. Furthermore, annexin IV was recognized by IgM from healthy humans indicating that annexin IV may be recognized by NAbs in humans similar to mice.[32]

Interestingly, the above antigens exposed by ischemia are normally intracellular antigens which are translocated to the cellular membrane presumably during the ischemic period as phospholipid changes occur. Additional antigens exposed by ischemia and recognized by NAbs include either negatively charged phospholipids from the inner leaflet of the plasma membrane or lyso-phospholipids. Some NAbs require the presence of a serum cofactor such as β2-glycoprotein I or annexin proteins. At least one NAb, T15/EO6, activates complement after recognition of lyso-phosphatidylcholine[33] suggesting that phospholipid and phospholipid-binding proteins may initiate complement activation resulting in exposure of nonmuscle myosin or annexin IV in the cellular membrane. It is also possible that the flipping of these molecules allows their recognition by NAbs.

ANTI-PHOSPHOLIPID AND BETA 2 GLYCOPROTEIN I RECOGNITION BY ANTIBODIES

Within 15–30 min postreperfusion, metabolism of the cellular lipids resulted in significant increases in the intestinal lyso-phospholipid and free arachidonic acid production.[34] Corresponding to the total concentration of the parent lipids, phosphatidylcholine and phosphatidylethanolamine, decreased in the intestinal tissue subjected to IR.[34] In addition, the phospholipid binding protein, β2-glycoprotein I, binds the ischemic membranes in a similar time frame.[35]

To investigate the role of NAbs directed to antigens that are exposed by ischemia in the mouse IR model, additional studies were performed. Monoclonal antibodies recognizing β2-glycoprotein I and cardiolipin, a negatively charged phospholipid, were derived from unimmunized mice which spontaneously develop degenerative cardiovascular disease.[36] Injection of a mixture of anti-β2-glycoprotein I and anti-phospholipid mAb resulted in

IR-induced mucosal injury in antibody deficient, *Rag-1*$^{-/-}$ mice.[35] However, neither mAb alone was sufficient to induce injury in response to IR.[35] Surprisingly, each antibody individually induced injury in the *CR2* deficient mouse.[35] In the latter mouse model, the anti-phospholipid mAb restored complement activation in response to IR, while the anti-β2 glycoprotein I mAb restored eicosanoid production and cellular infiltration into the local and remote organs which were damaged.[35] Together these data suggest that each mAb induces a specific response and the combination is required for IR-induced mucosal injury.

Recent studies indicated that peptides which compete with β2-glycoprotein I for binding to the altered phospholipid bilayer attenuate intestinal IR-induced tissue damage in wildtype mice.[37] These data suggest that β2-glycoprotein I recognized changes are instrumental for IR-induced damage. It is not clear if β2-glycoprotein I and NAbs recognize phospholipid oxidation changes in the lipid moiety or the head groups.[33,38,39] However, damaging NAbs appear to recognize the changes in the lipids as men with low anti-phosphorylcholine (IgM isotype) levels are more susceptible to atherosclerosis and ischemic stroke when compared with men with higher IgM levels.[40] Although these data suggest that the NAb to the head group are protective, it is possible that a difference exists between the mouse model and humans due to the sequestration of the natural IgM in the atherosclerotic plaques.

OTHER POSSIBLE ANTIGENS AND NAb ANTIGEN SUMMARY

An additional antigen suggested to be recognized by mAb during reperfusion is the protein-RNA complex, U1-ribonucleoprotein (U1-RNP).[41] Injection of *Rag-1*$^{-/-}$ mice with anti-U1-RNP containing sera from autoimmune patients restored IR-induced injury. However, it is unknown if the antibodies were NAb-like or autoimmune antibodies. In addition, the studies did not clearly show that the anti-U1-RNP specific antibodies induced the damage.

The fact that multiple NAbs induce damage in response to ischemia/reperfusion suggests a similarity of the antigens. Although the sequence and location of each of the antigens discussed above are distinct, it is possible to suggest at least one similarity, namely their binding to lipids. Despite acting as an intracellular calcium binding protein, which modulates NF-κB signaling, annexin IV binds to phosphatidylserine and phosphatidic acid under acidic pH such as in response to IR.[42] β2-glycoprotein I also recognizes phosphatidylserine on apoptotic cells.[43] Finally, NMHC also binds phosphatidylserine[44] and is cleaved during apoptosis to facilitate exposure on the cell surface. Thus, the NAbs appear to bind proteins associated with phosphatidylserine when expressed on the cell surface.[45] Together, these data suggest that ischemia induces an immediate change in phosphatidylserine from the inner leaflet to the outer leaflet (Fig. 1A,B). It is possible that lysis of some cells during the ischemic period may liberate the intracellular antigens and initiate the cascade. Another possibility is that the flipping or scrambling of the lipid bilayer would expose annexin IV and NMHCII A/C on the cell surface. In addition, the serum protein, β2-glycoprotein I, binds to the phosphatidylserine (Fig. 1C). Thus, it will be critical for future studies to understand the changes within the membrane which expose antigens in response to ischemia which are recognized by NAbs and subsequently activate complement.

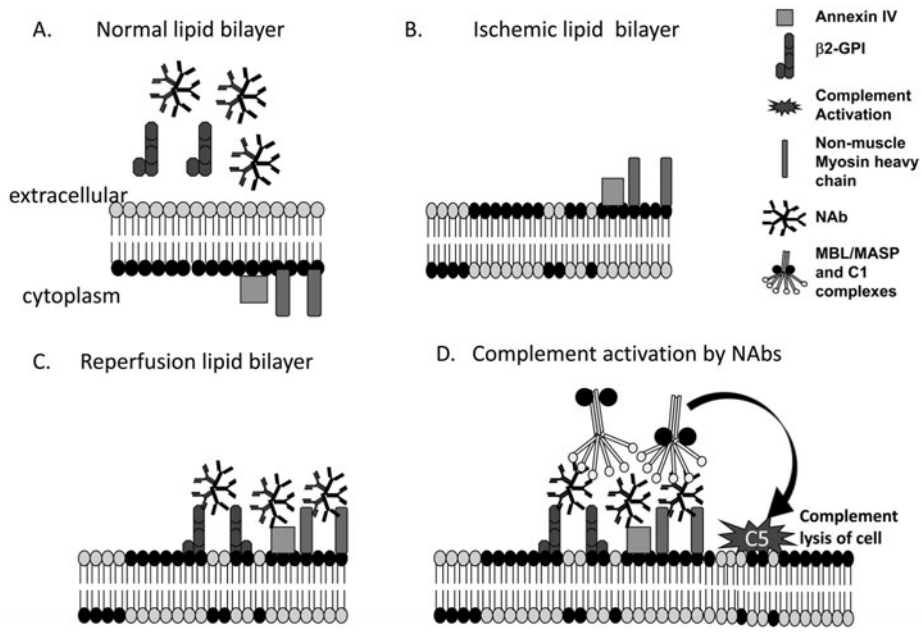

Figure 1. Ischemia induces lipid bilayer re-arrangement and exposes multiple antigens which are recognized by complement activating NAbs. A) Under normal conditions, the lipid bilayer maintains a polarity which prevents extracellular annexin IV and nonmuscle myosin expression and prevents β2-GPI binding. B) Ischemic conditions stress the cells and alter the lipid bilayer allowing expression of phospholipids from the inner leaflet. Flipping of the lipids is also associated with extracellular expression of annexin IV and nonmuscle myosin heavy chain. C) Upon reperfusion, NAbs recognize one or more of the multiple ischemia-exposed antigens, including annexin IV, β2-GPI, nonmuscle myosin heavy chain and/or inner leaflet phospholipids. D) The MBL and C1q recognize bound NAbs, resulting in initiation of the lectin and classical complement pathways. The complement cascade continues resulting in tissue damage.

COMPLEMENT ACTIVATION IN RESPONSE TO NAb BINDING TO TISSUE

The complement system consists of more than 30 proteins that form a cascade of proteolytically cleaved products. Complement activation is initiated by one of 3 pathways, classical, lectin and alternative, which converge at complement component C3. Activation of C3 results in release of C3a and deposition of C3b which forms the C5 convertase. The C5 convertase cleaves C5 producing C5a and C5b. As the initiator of the membrane attack complex, C5b deposits on the cell membrane and assembles C6, C7, C8 and multiple C9 components to form the lytic complex. In addition to generating convertases, the classical pathway produces the byproducts, C4a, C3a and C5a which are also immunologically active as anaphylatoxins.

Despite a similar outcome, the complement cascades are initiated by different mechanisms. Via the Fc region, antigen-antibody complexes initiate the classical pathway by formation of the C1q complex which cleaves C2 and C4 and forms the C3 convertase, C2aC4b. The C3 convertase splits C3 generating C3b which binds C2aC4b and becomes C2aC4bC3b, the C5 convertase. The C5 convertase initiates the membrane attack complex.

Recognition of carbohydrates by the mannose binding lectin (MBL) initiates the lectin pathway that cleaves C2 and C4 forming the same C3 and C5 convertases as in the classical pathway. C3b released by either the classical or lectin pathways may stimulate complement amplification via the alternative complement pathway, whereby additional and distinct C3 and C5 convertases are generated.

Although complement activation effectively kills pathogens, inappropriate or excessive complement activation may cause tissue destruction. Depletion of C3 with cobra venom factor or the use of *C3*$^{-/-}$ mice indicated that complement was required for IR-induced tissue damage.[46] A recent study also indicated that rodent myocardial infarction was attenuated by treating with a recombinant, humanized cobra venom factor, suggesting a possible therapeutic.[47] In response to intestinal, myocardial or skeletal muscle IR, all three complement initiation pathways appear to play a role[48-50] while only the alternative pathway is critical in kidney IR.[51]

MECHANISM OF COMPLEMENT ACTIVATION BY NAbs

Early studies indicated a requirement of complement activation for IR-induced tissue damage.[52-54] As NAbs were also required, initial studies suggested that the classical complement initiation pathway mediated the damage.[16,17] In addition, treating mice with a C1q esterase inhibitor attenuated myocardial,[55,56] intestinal,[57] skeletal muscle,[58] and cerebral[59,60] IR-induced damage. Although C1 inhibitor attenuated intestinal damage, C1Inh inhibits both the classical and lectin pathways.[61,62] Therefore, additional studies have also examined IR-induced injury in *C1q*$^{-/-}$ and mannose binding lectin (MBL) deficient mice. In response to cerebral,[63] myocardial,[64] kidney,[65] skeletal muscle[50] or intestinal[48] IR-induced injury, *C1q*$^{-/-}$ mice sustained local injury and C3 deposition within the damaged tissue despite protection from systemic lung damage.[50] In vitro binding assays indicate that IgM associates with MBL.[66,67] Subsequent intestinal IR studies illustrated that IgM and MBL colocalized in the intestine of *Rag-1*$^{-/-}$ mice after injection with a NMHC-specific NAb.[49] Importantly, *MBL*$^{-/-}$ mice were protected from skeletal,[50] intestinal,[49] and myocardial[68] IR. Correlating with the rodent studies, patients with MBL deficiency sustain less systemic inflammation and multiple organ failure after cardiac surgery than patients with normal levels of MBL.[69] In addition, low MBL levels in kidney, liver and pancreas transplant recipients correlates with increased survival (reviewed in ref. 70). These data suggest that complement activation is via the lectin pathway rather than the classical pathway. Interestingly, factor B, a component of the alternative pathway, also appears to be critical in response to IR within the kidney[71] but not the intestine.[48] However, the alternative pathway does play a role to some extent. As a soluble glycoprotein, factor H (fH) inhibits the alternative pathway by inhibiting C3 activation. Recent studies linked fH with CR2 to target the inhibitor to regions of C3b deposition. Treatment of mice with the targeted inhibitor, CR2-fH, attenuated both myocardial[72] and intestinal[71] IR-induced damage. Together these data suggest that the mechanism of IR-induced tissue damage may differ by organ and that the alternative pathway may be critical for amplification of the quantity of C3b produced.

Thus, in many organs, IR-induced injury appears to require both NAbs and complement activation by the lectin pathway. These data suggested the model shown in Figure 1C,D

with ischemic endothelial cells expressing multiple newly exposed antigens which are recognized by NAbs which induce the lectin pathway of complement activation.[73] This hypothesis was recently confirmed by elegant studies using a myocardial IR model with genetically deficient mice which lacked either IgM or MBL or were deficient in both IgM and MBL. These studies clearly showed that both IgM and MBL were essential for complement-mediated tissue damage in response to IR.[68] Subsequent studies isolated the NAb-complement complex and found MBL and C1q present.[74] Similar to previous results, factor B was not present in the antibody-antigen complex.[74] Thus, during reperfusion, NAbs recognize multiple newly exposed antigens expressed on the ischemic tissue resulting in local and remote organ damage which is mediated by the classical and lectin pathways of complement activation.

MULTIPLE HUMAN NAbs IN IVIG APPEAR PROTECTIVE

Antibodies in the form of intravenous immunoglobulin (IVIG) are used therapeutically to treat both inflammatory and autoimmune diseases. IVIG is a preparation of purified human antibodies obtained from plasma of more than one thousand healthy donors. Most IVIG preparations are > 99% pure IgG while other preparations contain low levels of IgA and/or IgM. By containing a wide spectrum of IgG antibodies including NAbs and anti-idiotypic antibodies, IVIG modulates the immune response, although the exact mechanisms are not clear. Initial studies (with unknown IgM or IgA contamination) suggested the mechanism of IVIG protection included binding of active C3b on the cell surface to prevent membrane attack complex formation and subsequent tissue damage.[75-77] Subsequently, C3b dimers complexed with IgG ($C3b_2$-IgG complexes) were identified as potent precursors of alternative C3 convertases, which stimulate complement amplification. Using both in vitro and in vivo studies, IVIG (IgG only) attenuated $C3b_2$-IgG complex production by stimulating their inactivation.[78,79] Thus, high dose IVIG decreased overall complement activation by attenuating amplification by the alternative pathway.[79] Despite activating the classical or lectin pathway activation, IVIG (unknown contaminating IgA or IgM) also downregulates production of C1q, the initiating molecule of the classical pathway.[80] Finally, by preventing activation of the membrane attack complex, IVIG also prevented C5a activity.[81] Thus, understanding the mechanism by which IVIG alters complement activity continues to progress.

In experimental and clinical models, IVIG also appears to modulate the immune response to IR. Administration of human IVIG prevented rat intestinal IR-induced local and remote organ damage and eicosanoid production, despite significant neutrophil infiltration.[82] As neither complement nor antibody was deposited in intestinal tissues, it is likely that the pooled NAbs administered prior to ischemia sequestered C3, degraded $C3b_2$-IgG complexes and/or blocked antibody binding with the presence of anti-idiotypic antibodies. Importantly, in a mouse model of ischemic stroke, low dose IVIG (containing less than 0.05 mg/ml IgA and trace amounts of IgM) decreases the infarct size by ~50% when administered either prior to ischemia or 3 hours after beginning reperfusion.[83] A recent review of data suggested that IVIG be evaluated as a treatment for human stroke patients.[84] Thus, a bolus of IVIG may provide protection to intestinal and brain IR.

CONCLUSION AND FUTURE DIRECTIONS

Myocardial, cerebral IR as well as trauma-induced skeletal muscle and intestinal IR are major causes of morbidity and mortality in the world today. Understanding the mechanism of sterile inflammation which ensues in response to IR is critical to decreasing pathology. NAbs clearly play a role in complement-mediated, IR-induced tissue damage of the skeletal muscle, intestine, heart and brain. Recent progress identified specific antigens which may be expressed due to alterations of the phospholipid bilayer. In addition, the mechanism of complement activation has been explored in rodent models of tissue injury. Although significant advances have identified the role of NAbs and their antigens in rodent models of IR, translation of these findings to larger animals and humans remains to be investigated. It is unknown if the peptides from either NMHC or β2-GPI which are protective in rodents will provide a similar therapeutic effect in humans. Understanding the mechanism by which NAbs are recognized by the lectin and the classical complement pathways may provide insight of additional therapeutic markers. Finally, as the NAb-containing preparation of IVIG is protective in the same rodent models, understanding the specific mechanism of protection of IVIG remains a critical need. Thus, by linking the innate and adaptive immune responses, NAbs provide a unique mechanism within IR-induced tissue damage.

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CHAPTER 14

HOW IMMUNE COMPLEXES FROM CERTAIN IgG NAbs AND ANY F(ab')$_2$ CAN MEDIATE EXCESSIVE COMPLEMENT ACTIVATION

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Abstract: In sepsis death follows an excessive inflammatory response involving cytokines and complement that is activated primarily via the amplifying C3/C5 convertase. Excessive stimulation of complement amplification requires IgG-containing or F(ab')$_2$-containing immune complexes (IC) that capture dimeric C3b on one of their heavy chains or heavy chain fragments. The ability of IgG-IC to capture dimeric C3b by the Fab portion is dependent on an affinity for C3 within the Fab portion, but outside the antigen-binding region. This property is rare among IgG NAbs. In contrast to this, the lack of the Fc portion renders the Fab regions of any F(ab')$_2$-IC accessible to nascent C3b, but dimeric C3b deposits only if F(ab')$_2$-IC form secondary IC with anti-hinge NAbs that rigidify the complex and thereby promote deposition of dimeric C3b. Both types of complexes, C3b$_2$-IgG-IC and C3b$_2$-F(ab')$_2$-IC/anti-hinge NAbs, are potent precursors of alternative C3 convertases and stimulate complement amplification along with properdin up to 750 times more effectively than C3b and properdin. F(ab')$_2$ fragments are not normally generated, but are formed from NAbs by enzymes from pathogens and neutrophils in sepsis. Unlike IgG-IC F(ab')$_2$-IC are not cleared by Fc-receptor dependent processes and circulate long enough to form secondary IC with anti-hinge NAbs that rigidify the complexes such that they capture dimeric C3b and gain the potency to stimulate complement amplification.

INTRODUCTION

NAbs are a priori beneficial, but some of them can, under very unique conditions, mediate excessive systemic complement activation and thereby promote disease without

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

having mutated to auto-aggressive antibodies. This phenomenon occurs in diseases that do not exert an evolutionary pressure like infarction or SIRS in sepsis and trauma. For example, the acute inflammatory response as in infarction and ischemia/reperfusion involves NAbs as was first observed by Carroll's group in ischemic skeletal muscle.[1] Ischemia is accompanied by hypoxia and pH changes, which result in sudden exposure of normally hidden autoantigens. The liberated autoantigens get in contact with IgM and IgG NAbs that are present in the locally available plasma. NAbs against many of the exposed proteins exist in plasma to clear such components, when liberated in a homeostatic process. During ischemia the release of such components is, however, massive, such that the large number of liberated autoantigens get more and more in contact with NAbs upon being locally replenished during reperfusion. Immune complexes formed by these NAbs and liberated autoantigens activate complement not only by initiating the classical complement pathway, but to a significant extent by stimulating complement amplification via the alternative complement pathway, since damage to intestinal cells was only 1/3 in factor D knockouts subjected to ischemia/reperfusion.[2] The phenomenon is reviewed in Chapter 13 by S.D. Fleming in this volume.

In contrast to the situation in ischemia, excessive complement activation during SIRS appears to be stimulated primarily by immune complexes generated from certain IgG NAbs and any $F(ab')_2$ fragments.[3] SIRS can develop from severe sepsis, but also in patients with trauma having no infections. Excessive complement activation in SIRS can induce paralysis of neutrophils, the cytokine storm of macrophages, the release of tissue factor,[4] and death by destructive processes. As long as nascent C3b deposits to immune complexes that initiate complement activation via the classical complement pathway, the number of excessively produced nascent C3b is limited. If, however, the immune complexes stimulate complement amplification, millions of nascent C3b molecules are generated, many of which bind covalently not only to opsonized pathogens, but also to nearby self components without being opsonized. This destructive process accompanied by proinflammatory cytokines will eventually result in binding, entrapment and phagocytosis of self structures and thereby exerts a devastating role in an organism. Until recently it has remained unclear how immune complexes stimulate excessive complement activation via the alternative complement pathway, but the phenomenon as such is known for 30 to 40 years.[5] The mechanism will be reviewed in this chapter.

STIMULATION OF COMPLEMENT AMPLIFICATION BY $C3b_2$-IgG CONTAINING IMMUNE COMPLEXES

Complement amplification is normally stimulated by C3b newly generated by one of the three complement pathways. A newly generated C3b molecule nucleates an alternative C3 convertase (C3bBb) that is stabilized by properdin (C3bBbP) and catalyzes the cleavage of additional C3 molecules to C3a and C3b. This process is eventually limited by factor H and I, which together will inactivate C3b to iC3b. This control is overrun if the number of generated C3b molecules is ten to hundred times higher, implying that the precursor of such superactive C3 convertases must be far more stable than monovalently bound C3b and that the generated precursor or enzyme has to be far more effective than a C3bBb complex. One such effective C3 convertase precursor is formed on IgG immune complexes (IgG-IC) that captured dimeric C3b by one of the heavy chains. Dimeric C3b deposits to immune complexed IgG antibodies that have an affinity for C3

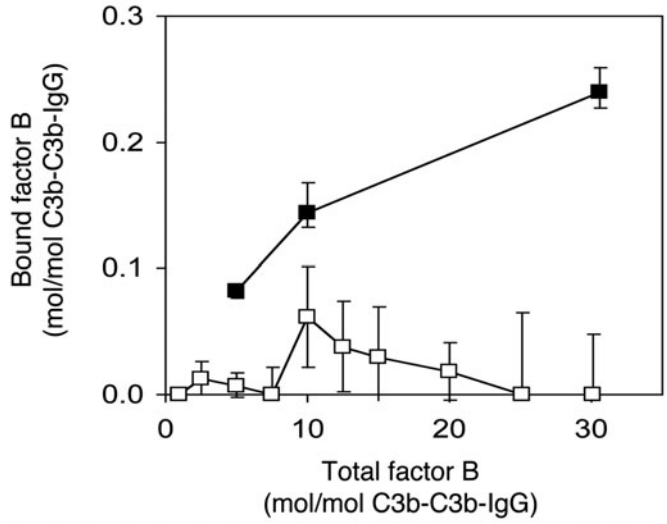

Figure 1. Binding of factor B to $C3b_2$-IgG complexes before or after preincubation with properdin. Labeled factor B (10–290 nM) was incubated with 1.93 nM $C3b_2$-IgG complexes without properdin (open squares) or with 147 nM properdin (closed squares) in veronal buffered saline at pH 7.4 with 5 mM $MgCl_2$. Results are from triplicates and are given as means +/- SD. Reproduced with permission, from Jelezarova et al. Biochem J 2000; 349:217–223;[8] ©2000 the Biochemical Society.

outside the antigen-binding sites.[6] An immune complexed IgG NAb having this affinity for C3 preferentially forms $C3b_2$-IgG complexes[7] and these complexes are far more effective C3 converase precursors than C3b. The reason for their high efficiency is that the dimeric C3b within this complex has a longer half life than $C3b^7$ and the dimeric C3b provides an increased affinity for properdin that exists in oligomeric form in plasma. As a consequence a $C3b_2$-IgG containing immune complex first binds oligomeric properdin which in its bound state greatly increases the affinity for factor B (Fig. 1).[8] The prolonged half life of $C3b_2$-IgG complexes and the stimulation by an increased affinity for factor B, render such complexes about 750 times more effective in generating C3 convertases than monovalently bound C3b. Such IgG molecules that stimulate complement amplification appear to be rare, but have not been searched for systematically. They appear to recruit primarily from IgG NAbs[9,10] and remain active in Mg^{2+} EGTA- treated human serum.[11] While Valim and Lachmann suggested that the IgG2 subclass predominantly activated the alternative complement pathway,[12] Banda et al. claim that IgG molecules capable of initiating the murine alternative complement pathway require N-glycans bound to Asp227 within the Fc portion of the heavy chains.[13] In none of the two cases it has been investigated how complement amplification is stimulated and whether this stimulation was in any way related to the formation of C3b-containing IgG-IC. In contrast to this, generation of $C3b_2$-IgG complexes in the course of an alternative complement pathway stimulation is known for 30 years. In fact, Gadd and Reid were the first to notice that IgG-IC that stimulate complement amplification, capture C3b molecules by one of their heavy chains.[11] While their finding suggested the presence of either monomeric or dimeric C3b on one heavy chain, we could demonstrate by 2-dimensional SDS PAGE with a hydroxylamine treatment between the dimensions that C3b-carrying IgG-IC contain

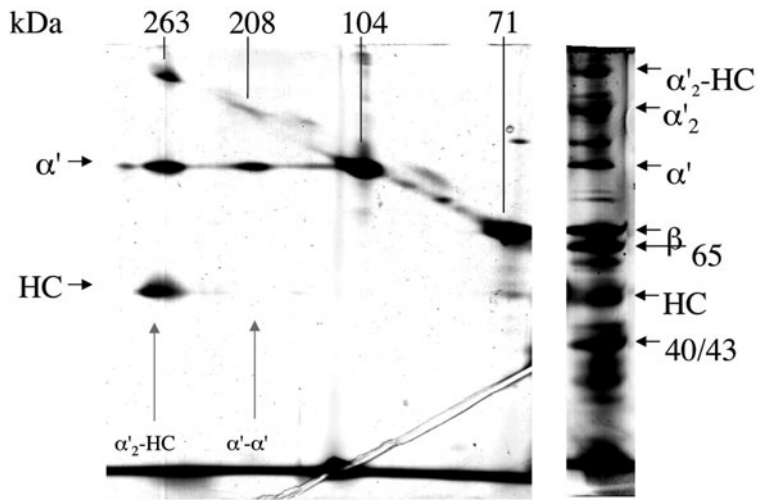

Figure 2. Two-dimensional SDS PAGE of $C3b_2$-IgG complexes. Purified $C3b_2$-IgG complexes were reduced, alkylated and run on a 6% polyacrylamide gel. Gel strips were cut from top of the separating gel to the β band of C3b, treated with hydroxylamine as described and loaded on an 8% gel for the second dimension. A silver stained gel is shown. The calculated MW of complexes are given in kDa: 263 for $\alpha C3_2$-HC, 208 for $\alpha C3_2$. This figure was originally published as Figure 3B, by Jelezarova E, et al. J Biol Chem 2003; 278:51806–51812;[14] ©2003 the American Society for Biochemistry and Molecular Biology.

exclusively ester-bonded dimeric C3b on one heavy chain, but are contaminated by free C3b dimers (Fig. 2).[14] These findings are in favor of the mechanism that we have suggested for the stimulation of complement amplification by IgG-IC. Long known additional findings support the mechanism further, namely that not only IgG-IC, but also $F(ab')_2$-IC stimulate complement amplification at least to the same extent[9-11,15] with up to 56% of C3 being activated in 100% plasma,[16] by generating $C3b_2$-$F(ab')_2$-IC.[11,15] These findings demonstrate that the ability of IgG-IC to stimulate complement amplification differed from their ability to activate the classical pathway, because stimulation of complement amplification did not require the Fc portion. The ability of almost any type of $F(ab')_2$-IC to stimulate complement amplification appeared obvious, because the lack of the Fc portion may have increased the accessibility for nascent C3b to deposit to the CH1 domain of $F(ab')_2$-IC complexes.

ANTI-HINGE NAbs RIGIDIFY $F(ab')_2$-IC TO CAPTURE DIMERIC C3b AND TO STIMULATE COMPLEMENT AMPLIFICATION

Several groups have demonstrated that not only IgG-IC, but also $F(ab')_2$-IC stimulate complement amplification to similar extents.[11,16-19] There is, however, an important difference already discovered in 1971 by Kevin Reid,[16] namely that $F(ab')_2$-IC require aside of the complement proteins an unknown, non-complement serum factor to stimulate complement amplification. In our attempt to search for this serum factor we hypothesized that "IgG anti-hinge NAbs" that had been studied more recently in quite some details

by the group of Terness (for reviews see refs. 20, 21), may represent this serum factor. Anti-hinge NAbs have even earlier been studied and named "pepsin agglutinators," because they agglutinated IgG upon its treatment with pepsin that generates $F(ab')_2$ and Fc.[22,23] IgG anti-hinge antibodies are germline-encoded NAbs that bind to a conformational hinge region epitope that becomes accessible exclusively upon cleavage of IgG1 by pepsin.[24] Anti-hinge NAbs have an important role in the regulation of the B-cell antibody production. Their plasma concentration increases slightly when B cells start producing antibodies and eventually anti-hinge NAbs stop B-cell antibody production by binding to the hinge region of antigen-carrying B-cell receptor and Fc receptor of the antibody-producing B cell.[21] Thus, anti-hinge NAbs have an important regulatory function within the innate and adaptive immune system.

Despite having this immune regulatory role, the very same IgG NAbs can exert damaging effects. In studying the damaging role of anti-hinge NAbs, $C3b_2$-$F(ab')_2$-IC formation and C3 activation induced by these complexes was studied with whole serum, with serum absorbed on $F(ab')_2$, and with serum absorbed on $F(ab')_2$ to which purified anti-hinge NAbs were added. Anti-hinge NAbs had the expected effect and restored complex formation and C3 activation in serum absorbed on $F(ab')_2$, although at a 10 time higher concentration than expected, presumably because the purity of the material was not yet 100%.[25] Interestingly, $F(ab')_2$-IC added to serum generated complexes that carried two and even three C3b molecules on one of the shortened heavy chains. Hence, our data strongly suggest that anti-hinge NAbs represent Reid's serum factor which rigidifies $F(ab')_2$-IC and thereby facilitates covalent binding of dimeric C3b to the Fd region of one arm of an immune-complexed $F(ab')_2$ in generating a potent C3 convertase precursor (Fig. 3).

We have also verified this hypothesis on $F(ab')_2$-IC from affinity-purified anti-spectrin NAbs. Since these NAbs lack a CH1-mediated affinity for C3, they stimulate complement amplification as $F(ab')_2$-IC, but not as IgG-IC in normal human serum. Absorption of

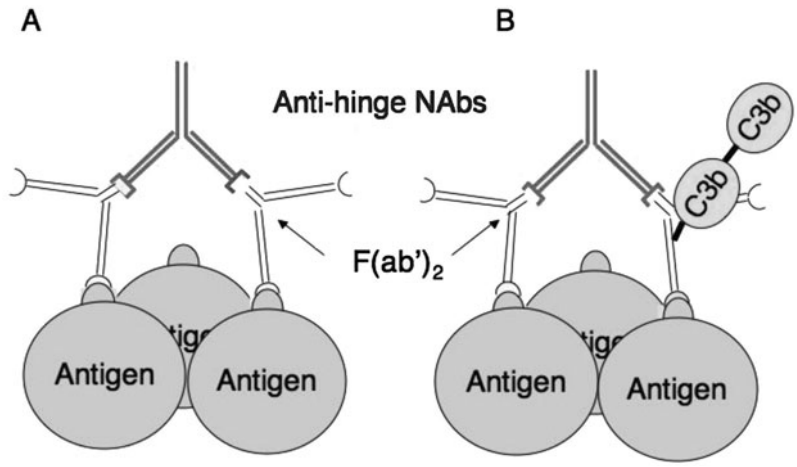

Figure 3. Generation of potent C3 convertase precursors. A. Anti-hinge NAbs form secondary IC with $F(ab')_2$-IC. (B). $F(ab')_2$-IC rigidified by anti-hinge NAbs allow nascent C3b to deposit as ester-linked C3b dimer onto the shortened and rigidified heavy chain of a $F(ab')_2$. Reproduced with permission from Fumia et al. Mol Immunol 2008; 45:2951–2961.[25] ©2008 Elsevier.

normal serum on immobilized $F(ab')_2$ removed anti-hinge NAbs and abrogated the ability of $F(ab')_2$-IC to stimulate complement amplification, while supplementation with purified anti-hinge NAbs restored stimulation of complement amplification.[25] Thus, anti-hinge NAbs represent the serum factor that is required to rigidify $F(ab')_2$-IC such that nascent C3b can deposit as a dimer to one Fab portion of $F(ab')_2$-IC. This finding may further explain why the efficacy by which $F(ab')_2$-IC stimulate complement amplification is high with human serum, but decreases with serum from more primitive species that are known to have lower concentrations of anti-hinge NAbs.[26]

$F(ab')_2$-CONTAINING IMMUNE COMPLEXES FORM SECONDARY IC WITH ANTI-HINGE NAbs IN PLASMA OF SEPTIC PATIENTS

$F(ab')_2$ fragments from IgG molecules may be dangerous, because they can form immune complexes with autoantigens and eventually potent alternative complement pathway C3 convertases. The reason is that $F(ab')_2$-IC persist long enough in plasma to form secondary immune complexes with IgG anti-hinge NAbs, because $F(ab')_2$-IC, unlike IgG-IC are not cleared via Fc-receptor carrying phagocytes. Eventually, anti-hinge NAbs bind to $F(ab')_2$-IC and stabilize antigen-bound $F(ab')_2$ to the point that the rigidified $F(ab')_2$ captures dimeric C3b on one Fab and thereby forms an effective C3 convertase precursor.

The most specific enzyme that generates $F(ab')_2$ fragments from IgG molecules, pepsin, is restricted to the gastrointestinal tract in higher organisms. However, a number of other proteases that can also cleave IgG into $F(ab')_2$ and $F(ab')_2$-like fragments (in which only one heavy chain is cleaved) can occur in blood during inflammation and bacterial infections. These $F(ab')_2$-generating proteases originate from endogenous sources, like neutrophils (elastase),[27-29] from pathogens like Staphylococci[30] (glutamyl endopeptidase), from Streptococci (streptococcal immunoglobulin-degrading enzyme, IdeS),[31] and many others.

It was our goal to investigate whether $F(ab')_2$ fragments are generated during severe sepsis and whether these fragments upon complexing autoantigens form secondary $F(ab')_2$-IC with anti-hinge NAbs and give rise to excessive complement activation. Nine patients in intensive care with several types of bacterial infection, elevated CRP values and neutrophil numbers have been studied for elastase, generation of $F(ab')_2$ fragments, factor Bb concentration, and formation of secondary immune complexes comprised of $F(ab')_2$-IC and anti-hinge NAbs. Both the concentration of $F(ab')_2$ and that of the activated complement factor B (Bb) increased linearly with the total concentration of elastase in plasma (Fig. 4).[25] These results provide suggestive evidence for the role of elastase in generating $F(ab')_2$ from IgG and the ability of secondary IC to act as potent precursors of alternative C3 convertases. Gelfiltrations on plasma proteins from these patients revealed that the total concentration of $F(ab')_2$ was about three μg/ml plasma, but less than 10% of this material migrated with its MW of about 100 kDa.[3] The majority of $F(ab')_2$ (1.7 +/–0.4 μg/ml, n = 9) was recovered in pool A with MWs in the range from 200 to 800 kDa and 0.4+/–0.1 μg/ml migrated in pool B with MWs of about 150 kDa. In contrast to this, none of the pools from controls contained measurable concentrations of $F(ab')_2$. Hence, these results do not only confirm the formation of $F(ab')_2$ during severe sepsis, but illustrate the formation of secondary immune complexes, of which the largest one (pool A) contained aside of 1.7 μg/ml $F(ab')_2$, unknown antigens, and 0.9 μg/ml anti-hinge NAbs.[25] At first it appears that a total concentration of $F(ab')_2$ of 3μg/ml is minute, but

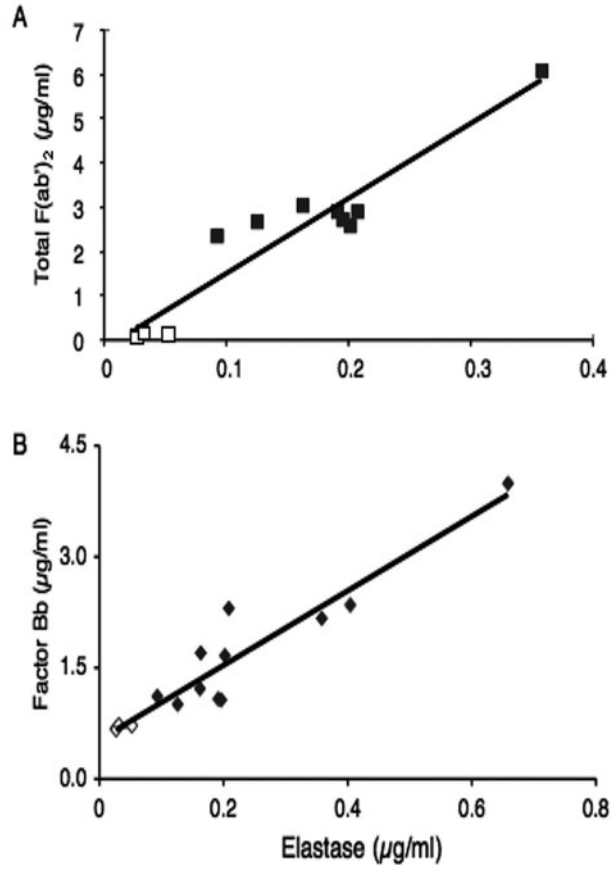

Figure 4. The plasma concentration of $F(ab')_2$ and that of activated factor B (Bb) correlated with that of elastase in plasma from septic patients. The total concentration of $F(ab')_2$ in plasma (A), data from patients number 4–12) and that of activated factor B (B), data from patients number 1–12). Values from control plasma are shown by open symbols. Reproduced with permission from Fumia et al. Mol Immunol 2008; 45:2951–2961.[25] ©2008 Elsevier.

if all the $F(ab')_2$-IC in pool A formed pairwise secondary immune complexes, of which all captured dimeric C3b and each of them allowed assembly of C3 convertases, these convertases would have generated theoretically 600 μg/ml C3b. This estimate would mean activation of 50% of C3, a huge portion, but not unrealistic for immune complexes that stimulate complement amplification.

HOW TO PREVENT $F(ab')_2$-IC FROM STIMULATING COMPLEMENT AMPLIFICATION?

Theoretically there are mainly two routes by which the massive complement amplification induced by $F(ab')_2$-IC could be stopped. One calls for inhibitors of elastase and similar types of proteases, another route prevents $F(ab')_2$-IC from being rigidified by

anti-hinge NAbs. The latter goal is reached by injecting a sufficient amount of irrelevant $F(ab')_2$ fragments that bind to anti-hinge NAbs and thereby prevent them from rigidifying $F(ab')_2$-IC. This approach has been taken by Dr. Dietrich 45 years ago in form of Gamma Venin, a $F(ab')_2$ preparation from pooled whole human IgG.[32] It turned out to be quite effective in nine patients with severe sepsis. His explanation of why the treatment was effective, was, however, inappropriate on the basis of what was known. He argued that the many types of $F(ab')_2$ helped clearing pathogens, although efficient clearance normally requires immune complex formation by IgG antibodies and their recognition by phagocytic receptors via the Fc portion of these antibody molecules. Therefore the immunologists may have concluded that IgG should have been used instead of $F(ab')_2$ and they convinced the companies to isolate and pool human IgG for intravenous application (IVIG).

In the meantime the argument of Dietrich has turned out to be partially correct, because work from Yano et al.[26] and Brezski et al.[33] indeed suggested that anti-hinge NAbs can aid in clearance of targets that have bound antigen-specific $F(ab')_2$. This type of cooperation of anti-hinge NAbs with $F(ab')_2$ is, however, highly dependent on the relative concentrations of total $F(ab')_2$, antigen-specific $F(ab')_2$ and anti-hinge NAbs. For example, anti-hinge NAbs that existed in varying concentrations in nonhuman primates induced a rapid clearance of about 75% of platelets purposely tagged with a monoclonal anti-platelet $F(ab')_2$ in 5 of 30 animals.[26] On the other hand, an injection of $F(ab')_2$ from pooled human IgG in amounts that can complex all available anti-hinge NAbs, as performed by Dietrich, must have prevented anti-hinge NAbs from binding to newly generated $F(ab')_2$-IC and thereby exerted a beneficial effect in patients with a severe sepsis.

Intact IgG (IVIG) as a replacement of $F(ab')_2$ from pooled human IgG cannot exert an analogous effect, because the binding site for anti-hinge NAbs is not accessible in intact IgG. Expectedly, IVIG failed to cure patients with severe sepsis irrespective of the brand.[34] Nevertheless, high concentrations of IVIG stimulate factor I and H dependent inactivation of $C3b_2$-containing complexes by a factor of two,[35] a value evidently too small to cure from severe sepsis. IVIG has since been used successfully in an increasing number of autoimmune diseases, where it suppresses autoaggressive antibody formation by several means.[36] Hence, it may be appropriate to repeat isolation of $F(ab')_2$ from pooled whole human IgG (Gamma Venin) and to study its efficacy in treating patients with sepsis and SIRS.

The other route in treating patients with massive complement overreaction calls for inhibitors of elastase and similar proteases capable of generating $F(ab')_2$ from IgG. This approach has been used successfully in disease models using animals.[37-39] Recently elastase inhibitors have also been applied to humans. In patients with acute respiratory distress syndrome (ARDS/SIRS) sivelestat had beneficial effects on pulmonary functions.[40] Among patients with multi organ failure sivelestat reduced hospital mortality from 33 to 6 percent.[41] Togo et al. studied the effect of sivelestat on survival of patients who developed gastrointerstinal septic ARDS and found that sivelestat was most effective if patients were well oxygenated.[42] In all cases treatment with the elastase inhibitor reduced the inflammatory process, but none of the authors has so far related the benefit of such a treatment to the inhibition of $F(ab')_2$ generation. In general they referred to the reduced degradation of elastin and matrix proteins resulting in reduced lung injury[38] and its inhibiting effect on NF-kappaB.[43] Thus, there is ample room for clinical trials in which effective drugs like sivelestat are applied and the patients studied for IgG cleavage and the parameters of complement amplification.

CONCLUSION

The results summarized have revealed that in sepsis and SIRS proteases from neutrophils and pathogens can generate $F(ab')_2$ fragments from IgG NAbs and preformed Abs. In case these $F(ab')_2$ fragments form immune complexes (IC) with autoantigens the $F(ab')_2$-IC have a high probability to form secondary IC with anti-hinge NAbs. Binding of anti-hinge NAbs to $F(ab')_2$-IC rigidifies these complexes and thereby enables them to capture C3b dimers. Bound C3b dimers are precursors of alternative C3 convertases, being about 750 times more effective than bound C3b. The extent to which the generated C3 convertases stimulate excessive complement activation via the amplification loop is difficult to predict. Excessive complement activation is dependent on the concentration of anti-hinge NAb-stabilized $F(ab')_2$-IC and thus is a function of antigen-specific $F(ab')_2$, the concentration of immune complexes, and that of anti-hinge NAbs. The process is inhibitable by irrelevant $F(ab')_2$ fragments that bind to anti-hinge NAbs and prevent it from stabilizing $F(ab')_2$-IC. The findings can explain why $F(ab')_2$ from whole human IgG, but not IgG itself (IVIG), was effective in stopping severe sepsis.

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SECTION IV

UNIQUE PROPERTIES OF NAbs

CHAPTER 15

THE NATURAL AUTOANTIBODY REPERTOIRE IN NEWBORNS AND ADULTS: A Current Overview

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Abstract: Antibody networks have been studied in the past based on the connectivity between idiotypes and anti-idiotypes—antibodies that bind one another. Here we call attention to a different network of antibodies, antibodies connected by their reactivities to sets of antigens—the antigen-reactivity network. The recent development of antigen microarray chip technology for detecting global patterns of antibody reactivities makes it possible to study the immune system quantitatively using network analysis tools. Here, we review the analyses of IgM and IgG autoantibody reactivities of sera of mothers and their offspring (umbilical cords) to 300 defined self-antigens; the autoantibody reactivities present in cord blood represent the natural autoimmune repertories with which healthy humans begin life and the mothers' reactivities reflect the development of the repertoires in healthy young adults. Comparing the cord and maternal reactivities using several analytic tools led to the following conclusions: (1) The IgG repertoires showed a high correlation between each mother and her newborn; the IgM repertoires of all the cords were very similar and each cord differed from its mother's IgM repertoire. Thus, different humans are born with very similar IgM autoantibodies produced in utero and with unique IgG autoantibodies found in their individual mothers. (2) Autoantibody repertoires appear to be structured into sets of reactivities that are organized into cliques—reactivities to particular antigens are correlated. (3) Autoantibody repertoires are organized as networks of reactivities in which certain key antigen reactivities dominate the network—the dominant antigen reactivities manifest a "causal" relationship to sets of other correlated reactivities. Thus, repertoires of autoantibodies in healthy subjects, the immunological homunculus, are structured in hierarchies of antigen reactivities.

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INTRODUCTION

The immune system is a key player in daily body maintenance and defense and its proper functionality is vital both to the survival of the individual and to its well-being. The immune system is composed of complex networks of molecules, cells and organs that act together to maintain and repair the body and protect it.[1-6] The immune system is dynamic, a constantly evolving network whose complexity is comparable to that of the central nervous system.

Every exposure to an antigen, be it an invader (bacteria, virus) or a self component alters the immune state and the antibody repertoire. Antibodies binding to molecules of the body itself—autoantibodies—are associated with the pathologic inflammatory processes that cause autoimmune diseases. However, autoantibodies in healthy individuals, in contrast to pathogenic autoantibodies, are thought to function in body maintenance and healing. It appears that naturally occurring autoantibodies (NAbs) and auto-reactive T cells in healthy individuals are directed to a selected, limited set of self-molecules. The autoimmune repertoire serves the immune system as an internal representation of the body, and has been termed the immunological homunculus.[1,2] Natural autoimmune T cells and B cells and autoantibodies may provide an early immune response to pathogens, expressing molecules that are cross-reactive with particular self-antigens. An example is the response to bacterial heat shock proteins and to other molecules that are highly conserved. Natural autoimmunity has also been proposed to prevent pathogenic autoimmunity by generating regulatory circuits or by blocking access by potentially pathogenic agents to key self-antigens.[7]

To characterize the immunological homunculus network (IHN), we used informatics tools to study patterns of antibody reactivity to hundreds of self-molecules (of our design) arrayed on glass slides—an antigen chip.[8-11] This immune microarray (Fig. 1) consists of various antigens covalently linked to the surface of a glass slide. A drop of blood serum (or any other body fluid) is tested for antibody reactivity by measuring antibody binding to each antigen spot using fluorescence labeling. Note that the binding of antibodies to a spotted antigen cannot tell us about the stimulus that induced the antibodies, and it cannot define the affinity or the specificity of any particular antibody or collective of antibodies. Indeed, a positive antigen-binding signal probably reflects a polyclonal mixture of antibodies binding to a variety of structural epitopes exposed by each spotted antigen.

Results using the antigen chip suggest that the particular self-reactivities comprising the IHN could serve as a set of biomarkers that help the immune system to initiate and regulate the inflammatory processes that maintain the body.[7] A full description of the immunological homunculus network would require information about an individual's T-cell antigen specificities (repertoire) and frequencies of T-cell functional types (Th1, Th2, Th3, CTL, Treg and so forth), their B-cell repertoire and B-cell types, autoantibody repertoire and antibody isotypes, and innate immune cells (macrophages, dendritic cells, neutrophils and so forth). Most of this information is not accessible—in fact, much of it is not characterized in detail but known only in general terms. Nevertheless, antibodies are precisely measurable and the pattern of one's global repertoire of autoantibodies in blood and body fluids is accessible. The autoantibody homunculus, at least, can be consulted. Moreover, microarray technology combined with advanced system-level analysis methods has opened new opportunities for approaching the vast information stored in antibody repertoires. Therefore, it has been proposed that the global pattern of autoantibodies can reveal various states of the immune network and provide some insights about the body state of the individual.[7,10]

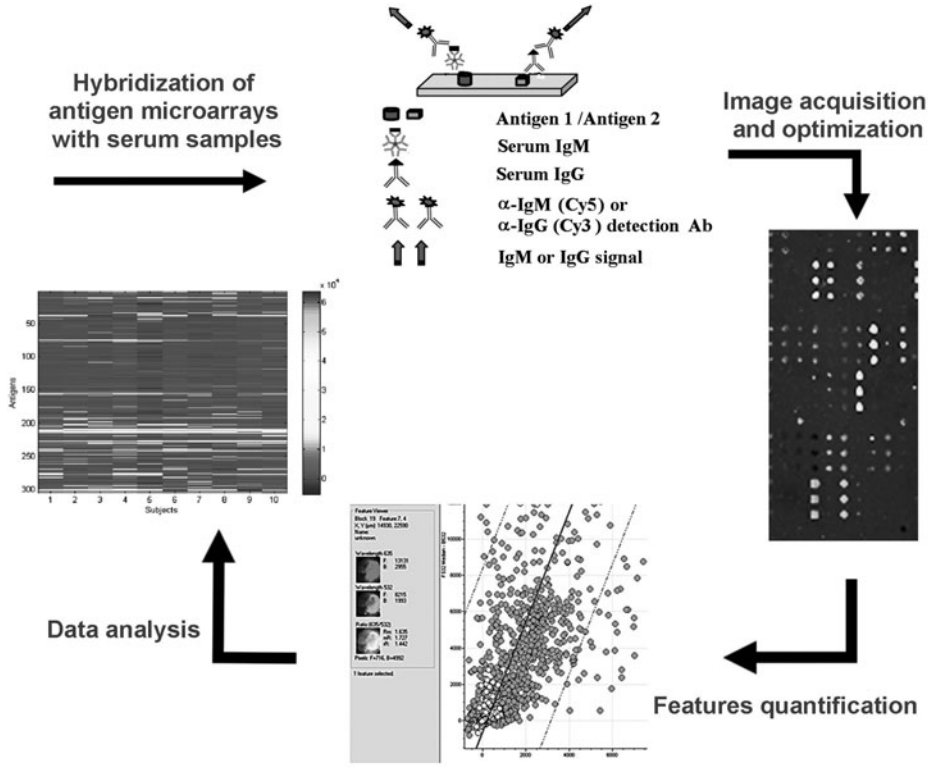

Figure 1. Schematic flow of the antigen chip from production to analysis. Step 1—Hybridization of antigen microarrays with serum samples: The tested body fluid (serum in this case) is hybridized on the antigen microarray to react with the chosen antigens. Then, secondary antibodies labeled with fluorescent dyes (Cy3 and Cy5) are added to assess the amount of antibody reactivity to each antigen. Step 2—Image acquisition and optimization: After incubating, washing and drying, the microarray is ready for reading and the fluorescence intensities are scanned. Step 3—Feature quantification: The scanned fluorescent images are translated to quantitative antibody reactivities based on the spot fluorescence level. The process includes optimization of the background reading and removal of problematic spots. Step 4—Data analysis: This step includes illuminating information hidden in the data using appropriate algorithms, to be explained in the text.

THE MATERNAL AND NEWBORN AUTOANTIBODY REPERTOIRES

The immune system expresses both the genetic endowment of the individual and the life experience of the individual. During pregnancy, the immune system has a very special role in the mother–offspring dyad by providing not only defense against infectious agents, but also IgG antibodies of the mother, which are actively transferred to the fetus. Immediately after birth, the immune system deals with post-natal adaptation to life in a continuous and dynamic process. Like the central nervous system, the immune system is *self-organizing*: it begins with genetically coded, primary instructions, to which it adds information retrieved from the individual's experience with the environment in

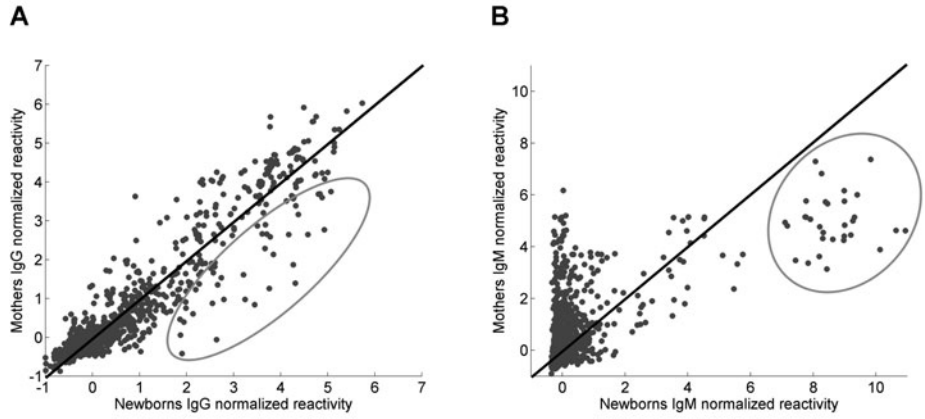

Figure 2. The normalized antibody reactivities of newborns (x axis) and mothers (y axis) as scatter plots. For the IgG scatter plot (A), most of the antigens exhibit similar reactivities of newborns and mothers, where only a small fraction of the antigens shows significant deviations from this behavior. We note the relatively small but significant group of antigens marked by an ellipse indicating antigens with higher reactivities in the newborns. For the IgM scatter plot (B), 2 types of antigen populations can be detected, the vast majority of antigens are characterized by relatively low reactivities of the newborns compared with their mothers, but a second group (marked by an ellipse) is characterized by high levels of reactivities, where the newborns' reactivities are somewhat higher than the mothers'.

health and disease. Just as each person develops a unique brain, each person develops an individualized immune system.[2]

To characterize the development of natural autoantibodies from birth to adulthood, we re-examined the antibody binding of 10 pairs of mothers (blood sample) and their newborns (umbilical cord sample) to 300 (mostly self) antigens previously reported by Merbl et al.[12] Figure 2 depicts the normalized antibody reactivities of the newborns (x axis) and their mothers (y axis) as scatter plots, such that each point in the graph represents a specific antigen reactivity in the maternal-newborn plan.

For the IgG isotype (Fig. 2A), we observed basically similar reactivity levels of the mothers and their newborns, reflected by the fact that the points form approximately a symmetric distribution along the diagonal. This can be explained by the fact that most of the fetal IgG antibodies originate from the mother, actively transported across the placenta.[13] Note, however, that there is a relatively small but significant group of antigen reactivities marked by the gray ellipse indicating higher reactivities in the newborns. Several factors might explain this phenomenon: selective transfer of certain IgG antibodies, the accumulation of such antibodies in the fetus, dissociation of maternal IgG-IgM antibody complexes during active transfer across the placenta and active production by the fetus of IgG antibodies to these antigens. Indeed, it has been suggested by Akilesh et al.[14] that, in addition to the role of the placental FcR (FcRn) in transferring maternal IgG to the fetus, the placenta also protects bound IgG from catabolism and maintains high IgG serum levels. Therefore, an accumulation of certain IgG antibodies could take place if these antibodies are transferred more than others and the rate of antibody catabolism differs between the fetus and the mother.

ANTIGEN-REACTIVITY CORRELATIONS

For many autoantibodies of the IgM isotype (Fig. 2B), the levels of the mothers are generally higher than those of their newborns; but certain antibody-reactivities manifest higher reactivity in the newborns. In contrast to IgG, IgM antibodies do not cross the placenta, so IgM autoantibodies in cord blood must have been produced by the developing fetus before birth.[12,15] Therefore, it is not surprising that some fetal reactivities differ from those of the mother.

Recently, we extended the study reported by Merbl et al.[12] by a different analysis of additional data from five females, who were not pregnant, and from eight newborns at birth and at day seven.[15] The analysis was performed from the perspective of the immune system as a complex functional network of antibodies by correlating their reactivities among themselves. We analyzed the correlations of antigen reactivities and of subjects to detect relationships between particular antigen-reactivities in the populations of mothers and newborns in both their IgG and IgM repertoires (Fig. 3).

The IgG subject correlation matrix in Figure 3A depicts a high correlation (light gray or white) between each mother and her offspring (red squares), again, indicating the maternal source of the IgG fetal repertoire. The high correlation values between each newborn at day 1 and again at day 7 (green squares) indicate conservation of the IgG autoantibody repertoire. Note that there are low correlation values between each of the adults and each of the infants and between the groups, indicating the individual nature of the IgG repertoire.

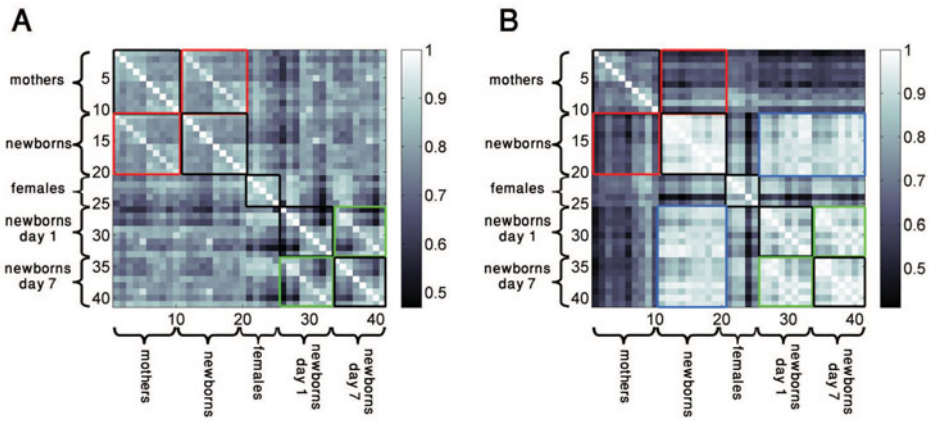

Figure 3. The subject correlation matrices for the IgG (A) and IgM (B) isotypes, color coded from low correlation (dark gray) to high correlation (white). The matrices are ordered according to the mothers (1–10), and their newborns (11–20), five females (21–25) and the additional newborns at birth (26–33) and at day 7 (34–41). The white diagonal lines are formed by the absolute correlations between each subject in both the x and y axes. A) The IgG correlation matrix: note that there are additional white diagonals in the red squares; this signifies the high correlation between each mother and her newborn; the additional diagonals in the green squares signify the high correlation between each newborn at day 1 and day 7. B) The IgM correlation matrix: note the strong correlation between all the newborns (blue squares) and the very weak correlation between each mother and her newborn (red squares). The high correlation values inside the green squares indicate that the IgM autoantibody repertoires changed very little in the first 7 d of life. We are now studying whether antibodies to bacteria develop in the first few weeks after birth and the effect of bacterial colonization of the gut on the development of the autoantibody repertoire.

The IgM subject correlation matrix (Fig. 3B) shows very weak correlation values (dark gray and black) between each mother and her newborn, with strong correlations between all the newborns (blue square) indicating the universal (common) nature of the congenital IgM repertoire.

MODULAR ORGANIZATION AND ANTIGEN CLIQUES

The antigen correlation matrices described above were analyzed using the functional holography (FH) method of Baruchi et al.,[16] originally devised for analyzing recorded brain activity, and also shown to be useful in the analysis of gene-expression data. In a recent work, this method was used to identify expression relations between genes and gene network motifs.[17] By using this approach on the antigen reactivity data, we were able to unveil new information about functional relations between self-antigens—modular organization of the autoantibody network and the formation of self-antigen cliques.

The modular organization of IgM antibodies is shown in Figure 4 for the 10 mothers and added women (A) and for the newborns (B). It was found that the mothers and the added women exhibit modular organization of their IgM repertoires (Fig. 4A) into antigen cliques—distinct subgroups of highly correlated antigen reactivities. In contrast to the IgM of the mothers, the IgM repertoires of the newborns were not organized into separate antigen cliques (Fig. 4B). The presence of IgM cliques of reactivity in the mothers and their lack in the newborns suggest that humans develop coordinated sets of IgM autoantibody reactivities during healthy post-natal development. The IgG reactivities of the newborns are more organized than their IgM reactivities (data not shown), as expected from the transfer of maternal IgG to the fetus.

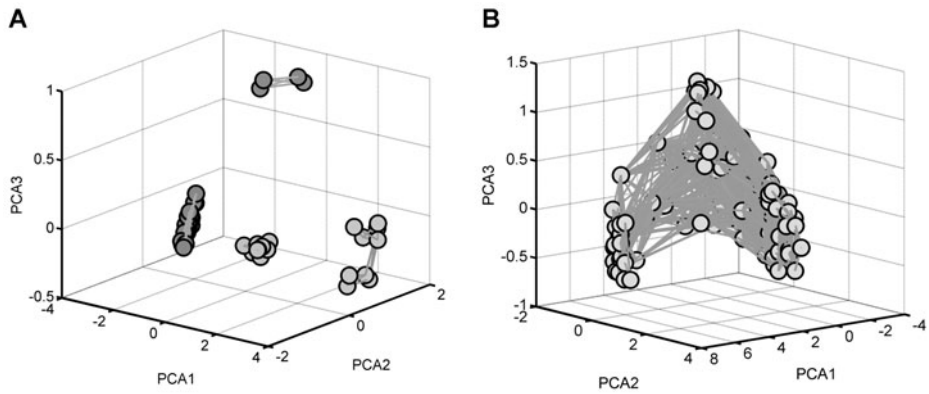

Figure 4. IgM antigen cliques. We present the antigen-reactivity correlation information in a 3-dimensional principal component analysis (PCA) space, whose axes are the three leading principal vectors computed by the PCA algorithm. Each antigen is placed in this space according to its three "eigenvalues" for the three leading principal vectors. Note that antigens that manifest high normalized correlations will be placed in close vicinity in the PCA space. A) The antigen network of the 45 antigens that compose the strong cliques in the maternal data set. B) The antigen network for the large cluster of 150 antigens identified for the newborns. In the presentation of the antigen networks, nodes (antigens) with high correlations (< 0.85) are shown linked by the orange lines. A color version of this image is available online at www.landesbioscience.com/curie.

IMMUNE NETWORK ARCHITECTURE AND IMMUNE TREES

Natural antibody networks have been studied in the past based on the connectivity between idiotypes and anti-idiotypes—antibodies that bind one another.[18-20] More recently, we extended our analysis of autoantibody reactivities[15] by applying graph and network theory analysis methods.[21-23] This approach calls attention to a different network of antibodies, autoantibodies associated by their reactivities to sets of self-antigens. In this immune network, the nodes (circles) represent the antigen reactivities and the links between the nodes (often called edges) represent the relationships between the autoantibody reactivities calculated for each group of subjects. In other words, an immune network for a given group of subjects corresponds to the network of similarities between antigen reactivities within that group of subjects.

In the complete network of antigen correlations every node is linked to all other nodes. However, most of the links are not significant as they correspond to very weak correlations. Therefore, the complete graph contains a high level of non-significant information that may mask the essential motifs. To extract the relevant information, it is possible to generate a condensed representation of the complete network by using various methods such as the Minimal Spanning Tree (MST) methodology,[24-27] which we employed here. The MST is a widely used sub-graph of the complete network which is constructed using a special algorithm that enables us to extract the most relevant information from the full network.[28] The idea of the MST algorithm is to select the subset of more informative links (about the hierarchical structure of the system) and reduce the complete all-to-all network (that contains $N(N\text{-}1)$ links) to a representative sub-graph (that contains only $N\text{-}1$ links). Hence, generating the maximum information immune networks (or immune trees) by the MST, makes it possible to investigate essential organizational motifs, such as the network topological organization. Moreover, similar to neuronal and gene networks, the immune system can exhibit activated and inhibited reactivities, such that both positive and negative antigen correlations contain important information. While the typical construction of the correlation-based MST has a bias toward strong positive correlations, we analyzed here the absolute value of the antigen-antigen correlations, in order to give equal importance to both strong positive and negative correlations.[29] We thus constructed the immune MST for each group of subjects (mothers/newborns), each isotype (IgG/IgM) and integrated isotype tree for each group[29] (Fig. 5).

We assessed and compared the topological organization of the IgG and IgM immune trees of the mothers and newborns. Next, the networks of the two subject groups, mothers and newborns, were compared by employing the widely used divergence rate measure.[30] The analysis revealed a high topological similarity between the newborns' and mothers' IgG networks and significant topological differences between the newborns' and the maternal IgM networks. These results indicate partial conservation of the IgG immune network topology from birth to adulthood, and significant reorganization of the IgM immune networks during the healthy development of the immune system, also shown above using other types of analysis.

Our previous work[29] uncovered previously unrecognized features of natural autoantibody networks. It was shown that the repertoires of mother and newborn manifest generally different network architectures: the composite tree of IgG and IgM reactivities shows that the nodes of IgG and IgM reactivities are largely overlapping in the mothers, but are mostly distinct in the newborns; it is quite possible that the overlap between the isotypes in the mothers results from adaptive immune responses to particular antigens.

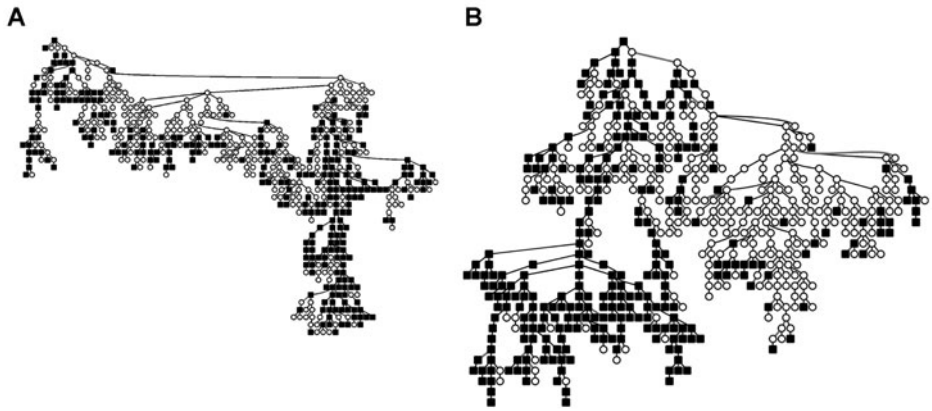

Figure 5. Hierarchical organization of the "integrated similarity immune trees" for the IgM and IgG isotypes. A) The Minimum Spanning Tree (MST) of the maternal data set, and B) the MST of the newborns. The black squares and white circles (nodes) represent the IgG and IgM isotypes respectively. We present the negative correlations between two nodes by the use of red or black lines, reflecting the fact that the organization of these immune networks can exhibit activated and inhibited reactivity responses. Note that many clusters or sub-trees are composed of a single isotype in the newborns, but less so for the mothers, where the sub-trees of IgG and IgM appear to overlap to a great extent.

Both negative and positive relations between antigen reactivities participate in connectivity throughout the MST network, reflecting the fact that the organization of these immune networks can exhibit activated as well as inhibited reactivity response.

This methodology is unique in its description of the network-tree architecture of the natural autoantibody repertoires in healthy mothers and newborns; the causal mechanisms responsible for this network architecture and for the differences between mothers and newborns need to be investigated.

ANTIGEN DEPENDENCY NETWORKS AND INFLUENTIAL ANTIGEN-REACTIVITIES

In this section we introduce a system-level analysis of antigen-dependency networks as a step toward the inference of causal relations between antigens.[31] The analysis is based on the measure of "partial correlations," which are becoming ever more widely used to investigate complex systems. Examples range from studies of biological systems such as gene networks[30,32] to financial systems.[33,34]

In simple words, the partial (or residual) correlation is a measure of the effect (or contribution) of a given antigen-reactivity, say *j*, on the correlations between another pair of antigen-reactivities, say *i* and *k*. This partial correlation approach enables one to define the hypothetical influence of antigen-reactivity *j*, as the sum of the influence of that antigen-reactivity (*j)* on all other antigen-reactivities *i*.[31] In this construction of "antigen dependency networks," the nodes represent the antigens spotted on the chip and the arrows between the nodes indicate the directionality of the influence—which antigen-reactivity influences which other antigen-reactivities; the approach defines a kind of causal influence of one reactivity on other reactivities. It's as if immunization to

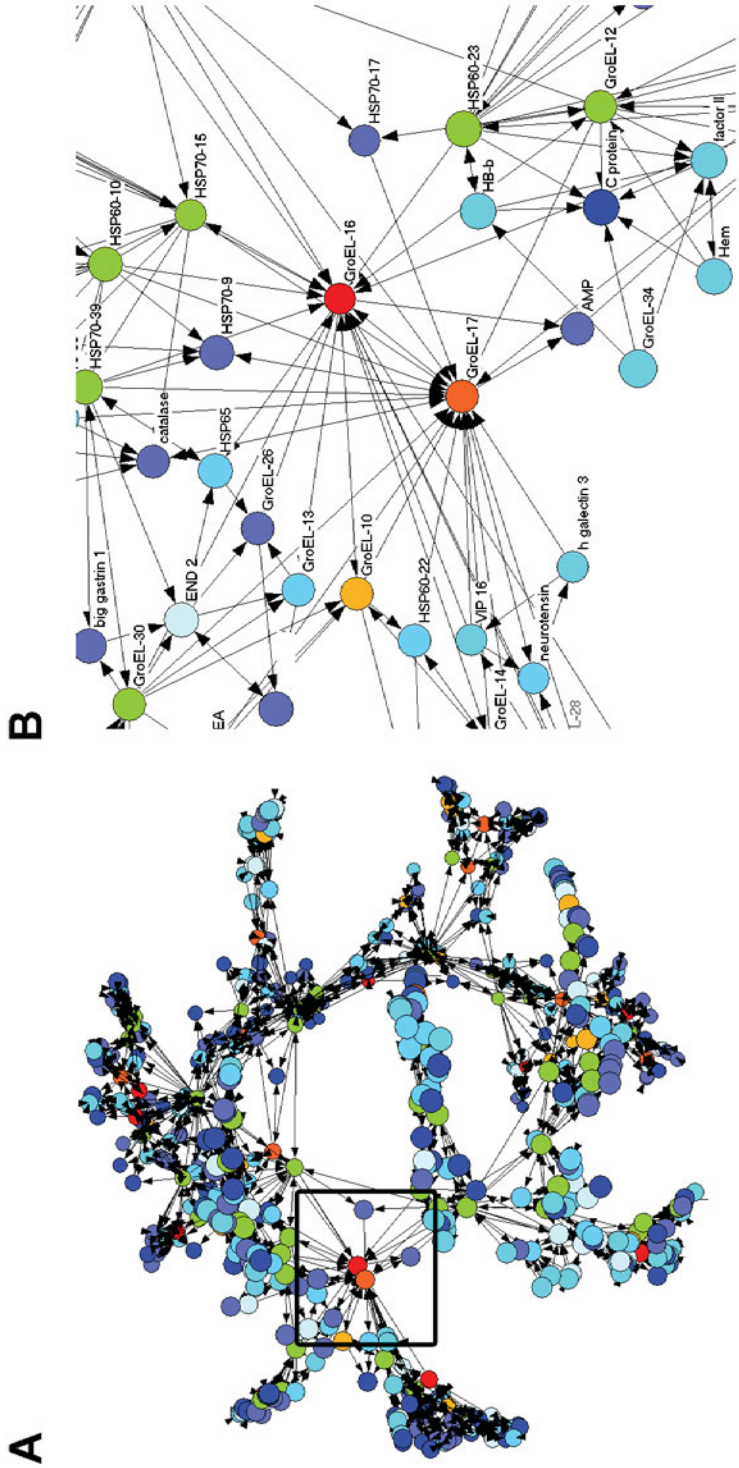

Figure 6. Figure legend on following page.

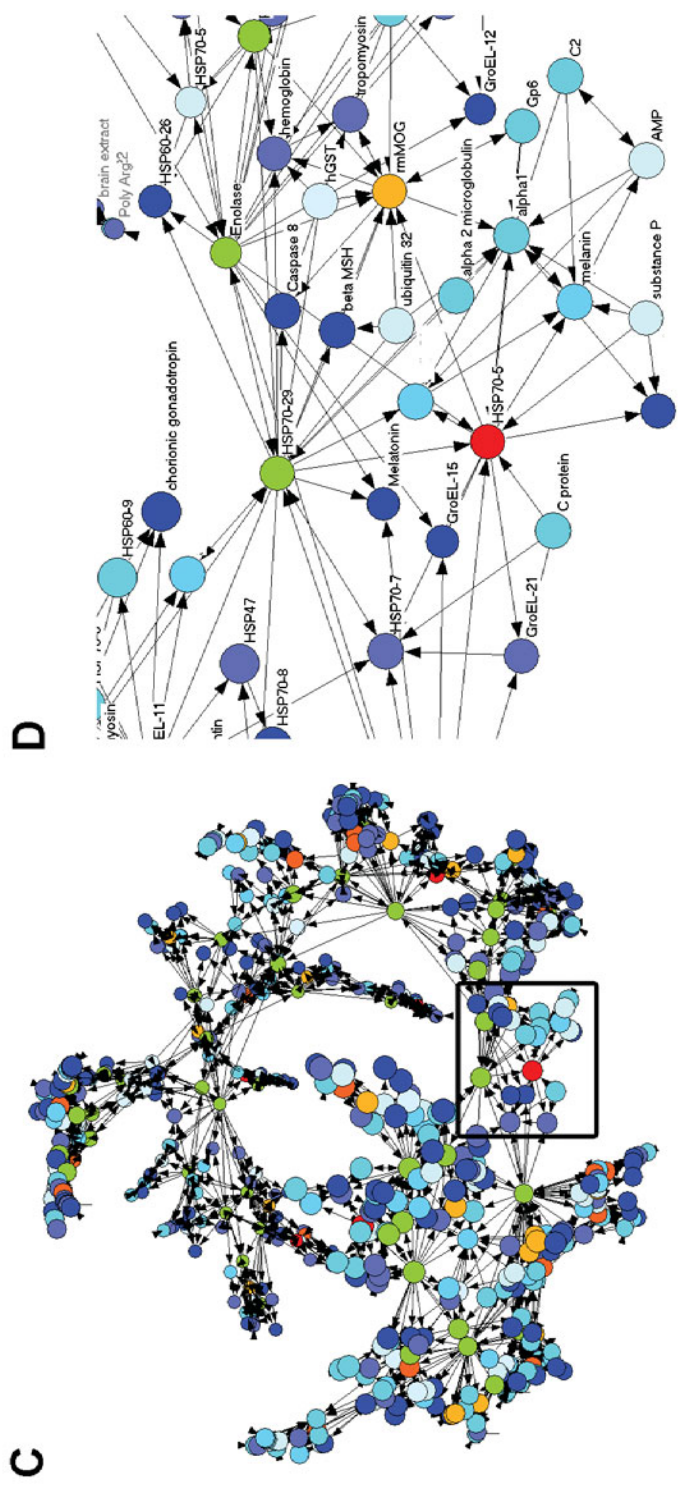

Figure 6, continued from previous page. A hybrid presentation of the dependency network for the combined IgG and IgM isotypes. The selected layout, Fruchterman-Reingold 3D, visualizes the differences between the maternal (A) and the newborn (C) networks. The black squares indicate the area that was zoomed in for A in B and for C in D. The colors indicate the strength of the system level influence (SLI) of each antigen on the correlations between all other antigen pairs, ranging from the most affecting antigens (dark red) to the least affecting antigens (dark blue). The arrows indicate the directionality of the influence, in other words, which is the antigen that positively affects the correlations of which other antigen. Note the wide dispersal of highly affecting antigens in the networks.

one particular antigen induces an immune reaction to other antigens. The colors indicate the strength of the system-level influence (SLI) of each antigen on the correlations between all other antigen pairs, from the most affecting antigens (dark red) to the least affecting antigen (dark blue) in the network. In other words, a dependency network for a given group of subjects corresponds to the network of dependencies between antigen reactivities within that group of subjects. A concrete example can be seen in the case where autoimmunization to one myelin antigen epitope in multiple sclerosis patients might lead to "spreading" to other antigen reactivities in the course of myelin damage.[35] Influential antigen-reactivities act as "drivers" for additional reactivities to form a network.

We constructed the "antigen dependency networks" for the groups of mothers and newborns.[31] We first constructed the IgG, IgM and combined IgG and IgM "networks of antigen dependencies" for the two groups (Fig. 6). Next, the networks of the two subject groups were compared by employing two technical measures that were developed in the context of network theory and are widely used—the divergence rate[30] and modularity score.[4] The first method was used to compare the position of each antigen-reactivity and its connections. The second method was used to assess the differences in the modular organization between the two networks—maternal and newborn. We found a higher modularity for the maternal IgG network. We found that the most influential "driver" antigens in the mothers and newborns are composed of both IgG and IgM isotypes, which points to a role for the maternally transferred IgG in influencing the newborn's network. Thus, the analysis of antigen dependency networks enabled us to unveil driver autoantibody reactivities in the maturation of the immune system. Which are these driver self-antigens?

The analysis of the system-level influence (SLI) revealed that about 10–15% of the antigen reactivities are drivers (colored in red and orange in Fig. 6); these antigen-reactivities manifest significantly higher driver influences than the other antigen reactivities. These driver reactivities tend to be evenly spread in the dependency networks. We found that the driver antigen reactivities of the IgG networks are prominently composed of epitopes of Heat Shock Proteins (HSPs) (~50% of the top 20 most influential antigens (antigens with high SLI scores) are HSP60 related and ~20% are HSP70 related). It is true that the array of antigens spotted on the chip contains many HSP peptide epitopes (~30% of all the spots). However, the enrichment of highly-ranked HSP molecules in the IgG networks and the absence of HSP epitopes as drivers in the IgM networks suggest that the dominance of these HSP eptiopes for the IgG repertoires is not merely an artifact. Note that HSP60 appears to function as a biomarker of inflammation and stress for the immune system,[7,36] which suits the position of HSPs as drivers in the maternal and newborn networks.

DISCUSSION

The results presented here support the concept of the immunological homunculus[1,37-39]—the idea that the healthy immune system includes autoreactivity to a selected set of particular self-molecules. Indeed, the homunculus idea triggered the development of our antigen microarray chip.[10] The fact that the primary autoantibody homunculus arises during the uterine life of the fetus, an environment normally free of foreign antigens, suggests that self-molecules are likely to serve as the immunogenic stimulus inducing IgM and IgA autoantibodies. The exact mechanism of this stimulus is still unknown, but it seems that the development of B cells, like that of T cells, may involve positive selection for self-reactivity.[40]

As we have shown here, the B-cell arm of the immune system has evolved to produce IgM autoantibodies to certain self-molecules even before birth; hence it is reasonable to conclude that these autoantibodies may provide potential advantages that offset the occasional autoimmune disease in post-natal life associated with their target autoreactivities.[1,41] It is conceivable that NAbs, in recognizing specific body molecules, help the immune system gather and integrate essential information about the state of the body and thus provide potential health benefits.[2] In any case, the inclusion of some major disease–associated self antigens in the innate autoantibody repertoire suggests that autoimmune disease could arise through a lapse in the regulation of natural, otherwise benign, autoimmunity.[42]

For the IgM autoantibodies, which are not transferred from mother to fetus, we found that all the newborns shared a universal innate immune profile, in agreement with the concept of the immunological homunculus—the immune system's internal image of key body molecules.[42] On the other hand, the maternal IgM and IgG autoantibody repertoires are highly diverse, implying that healthy development of the autoimmune state from birth to adulthood arises from immunological learning according to one's personal life experience. In other words, it seems that the immunological homunculus is not static but responds with personal immune experience. At present, we do not know whether a healthy maturation of the autoantibody repertoire is induced by immune experience with foreign antigens cross-reactive with self and/or by autoimmune contact with sets of self-antigens during normal body maintenance.[2,3] Study of the autoantibody repertoires developing in germ-free and antigen-free animals would shed light on this question. Moreover, it would be important to study whether particular types of autoimmune repertoire organization are potential seeds of the later development of clinical autoimmune disease.[9,10]

Analyzing the maternal IgM and IgG autoantibody correlations, we found that immune state diversity goes hand in hand with the development of a modular organization, reflected by the formation of antigen reactivity cliques or functional immune groups. The dominance of HSP epitopes[43] as "drivers" of other autoantibody reactivities is another indication of the intrinsic organization of natural autoimmunity.

The observation of an organized network of autoantibodies in mothers and newborns challenges the Clonal-Selection Theory (CST) of adaptive immunity, which has dominated immunological thinking for the past half-century.[44] The classical CST[2,45] discourse emphasized the functional independence of individual immune cells and so a network of antibody reactivities was outside of CST expectations. Moreover, according to the CST, it was inconceivable that the healthy immune system recognizes components of the body, since any recognition of self-molecules was thought to produce an autoimmune disease. Thus, the CST postulates that the immune repertoire, during development, must be purged of lymphocytes bearing receptors that could bind self-molecules. The self, as viewed by the CST, goes unnoticed and autoimmunity is forbidden. The present results do not fit the worldview of the CST. The present findings indicate that the healthy immune system, even before birth as the self-reactive IgM in the umbilical cord illustrates, recognizes self-molecules and, moreover, does so in a highly ordered architecture of reactivities. However, larger data sets are needed to verify the existence of autoantibody cliques and the complete identification of the antigen reactivities of each clique.

In general, the results presented here are consistent with the concept of the Immunological Homunculus, the idea that healthy immune repertoires contain certain T cells and B cells that have been positively selected to respond to key body molecules to form a functional "internal image" of the body.[2,3,7,36] This homunculus theory is

based on the regularity of immune self-recognition, consistently observed in healthy individuals. In practice, autoreactivity is not the aberration proposed by the CST, but is actually structured within the functional architecture of the immune system. The dominant position of particular antigen-reactivities, such as HSP epitopes would seem to reflect the biases of selected targets of self-recognition.[32] Note that both the CST and the anti-idiotypic network paradigms are based on individual differences between the immune repertoires developed by individual subjects; the immunological homunculus idea, in contrast, highlights the existence of antigen reactivities shared by individuals within a population. The relative uniformity of IgM autoantibody repertoires in newborns as a group[12,15] fits the homunculus idea: The demonstration of antigen dependency networks with dominant driver reactivities provides an additional way to view the homunculus.

The prevalence of dominant NAbs to body molecules, such as HSPs, suggests that such autoantibodies might provide some advantage to the organism. In fact, it has been suggested that NAbs might function to prevent autoimmune disease[46] or serve as immune biomarkers of the body state.[7,36] The persistence of dominant driver reactivities from newborns to mothers might account for the reports that IgM repertoires show little change from birth.[47-49] These other studies[49-51] were done using crude tissue blots of undefined self-molecules, while the antigen microarray technology used here apparently made it possible to detect the changes, despite the persisting drivers, in fine specificity of the autoimmune repertoire occurring subsequent to birth.

At present, we can view the newborn and maternal repertoires as snapshots of the dynamic evolution of the autoantibody repertoire during healthy maturation. It is reasonable to consider that the mothers at their own births arrived outfitted with the common IgM autoantibody repertoire we found shared by all the newborns.[12,15] Thus, we might reason that the state of the maternal repertoire probably reflects its physiological evolution from the newborn state. To test this hypothesis, we are presently undertaking a longitudinal study of the evolution of the antibody repertoires of individual humans from birth to an array of antigens including both self and foreign molecules.

Niels Jerne proposed that, in addition to the recognition of foreign antigens, lymphocytes and antibodies also respond to the unique antigen receptors—idiotopes—of other lymphocytes.[19,20] The interactions between idiotopes create an anti-idiotypic regulatory network. The Jerne idiotypic network theory views the immune system as a dynamical network of continuously interacting cells and antibodies,[50-52] even in the absence of external antigens.[53] Hence, in contrast to the CST concept of clonal independence, Jerne proposed that the immune response to a foreign antigen takes place when the entry of an antigen into the system perturbs the internal homeostasis of interacting idiotopes.[19,20,54] The organized autoantigen-reactivity networks expressed at the level of the newborn population, however, adds a level of organization to the concept of the individualized anti-idiotypic networks proposed by Jerne.

CONCLUSION

The discovery and analysis of immune system network architecture shown here and elsewhere[55] serve as an introduction to basic questions in systems immunology: What mechanisms are responsible for driver antigen-reactivities; what is the dynamic function of the relatively large number of most influential antigens that serve as drivers;

and how is the architecture of the immune network modified by vaccinations, infections, neoplasia, autoimmune diseases and other perturbations of immune homeostasis? The antigen microarray provides one tool to study these questions.

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CHAPTER 16

ANTIBODY POLYSPECIFICITY: What Does It Matter?

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Abstract: Polyspecificity (polyreactivity) is currently considered an intrinsic property of a subset of antibodies, primarily of naturally occurring autoantibodies. Polyspecificity is no longer viewed as a biologically irrelevant stickiness. Furthermore, the capacity to bind defined sets of unrelated antigens finds its structural explanation. What is most intriguing, the elucidation of the role of polyspecificity may promote a better understanding of specific recognition as a function of the entire immune system. The early events of immune recognition depend on polyspecific binding. Thus, the completeness of the naïve repertoires of antigen receptors is ensured. The process of immunologically-relevant antigen recognition that is initiated goes beyond simple molecular interaction with the antigenic determinants. It involves cellular cooperation and culminates in antibody response maturation. Recent findings also pave the way for the clinical application of posttranslationally induced polyspecificity.

INTRODUCTION

The community of immunologists has had some difficulty in accepting the existence of polyspecific antibodies. The problem originates from an immunological paradigm. Some 80 years ago, the convincing experiments of Karl Landsteiner showed that antibodies could engage antigens in a strikingly monospecific manner.[1] Ever since, antibodies have embodied the analytical power of the immune system associated with affinity and specificity. These properties even made them an indispensable tool in immunology and related fields.

There are no universally accepted criteria for defining an antibody as being polyspecific, but detection of comparable and significant reactivity to more than one structurally

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unrelated self- or foreign antigens is sufficient to classify an antibody as polyspecific. While none of the different panels of antigens, used traditionally for testing, provide sufficient breadth to rule out possible polyspecificity, arrays of $n \times 10^4$ antigens may reach the exhaustive level that strictly defines an individual antibody as monospecific. Nevertheless, a considerable portion of antibodies exhibit polyspecificity even when tested on a small panel of self- and non-self antigens.

The ability of a single immunoglobulin molecule to bind to several unrelated antigens was formally proven by studying monoclonal immunoglobulins (i) isolated from patients with B-cell malignancies, (ii) produced by the hybridoma technology as well as (iii) by Epstein-Barr virus transfected B lymphocytes.[2-4] It became obvious at this stage that polyspecific antibodies could not only be of IgM, but also of IgG and IgA isotypes.[5] Furthermore, the spectrum of antigens, bound by a single antibody, could be modified by physical and chemical agents—"induced polyspecificity," which is not a laboratory artifact, but can be observed in vivo too.[6]

BIOPHYSICAL MECHANISMS OF POLYSPECIFIC ANTIGEN BINDING

Antibody binding is often used synonymously with antibody recognition. Strictly speaking, binding relates only to the molecular interaction while recognition relates to its functional relevance and happens at a systemic level.[7] Considering molecular interactions, polyspecificity involves the capacity of a single binding site to bind different ligands. For instance, using a polyreactive IgE antibody, James and Tawfik showed that it binds different antigens through a set of specific hydrogen bonds.[8] Thus, in contrast to non-specific stickiness, such antibodies do bind specifically to a set of ligands.

The structural basis of polyspecificity relates to properties of the variable domains, mostly of the heavy chain.[9,10] Polyspecificity can be transferred to a monoreactive antibody by grafting only the CDR3 of the heavy Ig chain from a polyspecific one.[11] These findings are supported by other studies using site-directed mutagenesis of the CDRH3 region of the polyspecific IgMκ antibody SMI, which is derived from a patient with chronic lymphocytic leukemia. Some of these amino acid replacements alone are sufficient to render the antibody monospecific.[12,13] Some mutations in the CDRH3 do not affect SMI polyspecificity, while others increased it even further. These findings are not unexpected, as CDR3 is known for its central role in antigen binding. Interestingly, all attempts to find any differences between the structure, the length or the amino acid composition of HCDR3 from monospecific and from polyspecific antibodies have been so far unsuccessful.[14] Furthermore, these results suggest that a single amino acid replacement away from the binding site could completely abolish polyspecificity.[2] Thus, polyspecificity does not depend only on restricted features of the variable loops but also on the overall structure of the whole variable domain. Several mechanisms have been proposed to explain this phenomenon.[14,15] At present, a conformational plasticity of the paratope is the favored one.[16-19] A variable degree of conformational changes in the antigen-binding sites has been observed in the very early X-ray diffraction analyses of antigen-antibody complexes. Naturally occurring antibodies (NAbs) with variable regions in germline configuration were found to undergo the most extensive conformational changes.[17]

The same group compared the bound- and free Fab fragment structures of the esterolytic antibody 48G7. Interestingly, the binding to the antigen was not accompanied by structural changes, i.e., the 48G7 paratope was pre-organized in an optimal binding

conformation. In complex with the same antigen, the respective germline antibody underwent significant conformational adjustments converging with the conformation of 48G7 in its bound state. Thus, the role of the somatic mutations in this germline antibody was to rigidify the paratope and fix the most appropriate conformation for the bound state. Further studies have demonstrated that the maturation of 48G7 optimized not only the geometry of the antigen-binding site, but also the short-range electrostatics of the immunoglobulin molecule.[17,20] In another system, the conformational changes in the germline progenitor and in the mature form of the 28B4 antibody, which catalyzes the oxidation of p-nitrotoluene-methyl sulfide, were similar to those in 48G7.[21] In addition, the affinity maturation of 28B4 was accompanied by replacements in amino acids that established direct contacts with the antigen. In these as well as in other studies the maturation of the antibody was accompanied by a decrease in the flexibility of the antigen-binding sites.

The X-ray structural analysis of proteins is an indirect qualitative method that sheds light on the final states of the interacting molecules. Jimenez et al. have performed quantitative analyses of the molecular flexibility changes of antibodies in the process of affinity maturation. Using spectroscopy, they followed the response of an anti-fluorescein antibody molecule to mechanical stress induced by photo excitation of the bound antigen (a fluorescein molecule).[22] These investigations quantitatively measured the decrease of the molecular flexibility of antigen-binding sites that paralleled the accumulation of somatic mutations. Furthermore, thermodynamic analyses of a set of monoclonal germline and induced antibodies revealed that the maturation of immune repertoires was accompanied by a decrease in the negative value of the association entropy (increased order).[16] The entropy changes depend to a great extent on the molecular flexibility of the interacting partners. The results obtained demonstrated that the maturation of the antibodies was accompanied by restrictions in the conformational freedom of their paratopes. The high negative value of association entropy had an unfavorable contribution to the free energy of interaction of germline antibodies. This means that high flexibility of the paratope results in high activation energy of the binding reaction or low reaction rate and high temperature dependence. Finally, the thermodynamic analyses confirmed that germline antibodies with flexible paratopes were poly specific.

Induced fit- and conformational selection are the main modes of flexible binding.[23-25] The induced fit concept evolved from the previously described "lock and key" enzyme/substrate binding, stating that the complementarity is a result of the interaction.[26] Although thermodynamically reasonable, this mode may often be too slow for biologically meaningful interactions, since the formation of any unstable complex is rate-limiting.[24] An alternative hypothesis states that instead of inducing a flexible change in the ligands the binding depends rather on the selection among pre-existing diverse conformations. Compared with the former model, the latter ensures much higher reaction rates. Indeed, a growing body of evidence supports the second hypothesis.[24,27]

On the other hand, a set of flexible antigens could bind to the same antibody and, thus, render it functionally polyspecific.[28] This may be the case with some carbohydrates and peptides. Carbohydrate and peptide ligands are intrinsically flexible and this probably allows for cross-reactivity with antibodies that do not need to have flexible paratopes. As a practical application of this phenomenon, a single peptide could mimic multiple carbohydrate epitopes facilitating vaccine development.[29] At the other extreme, identical rigid binding footprints on different antigens are the basis for "public" epitopes. Antibodies, binding to different molecules through "public" epitopes, would be characterized as

polyspecific. Such epitopes are also often found on carbohydrates. For instance, E5, an antibody to Schistosoma mansoni with specificity for the Lewis X antigen, is also a HIV-1 virus-neutralizing antibody. Actually, the ligand does not have to express the exact chemical moiety the recognized epitope is part of. A constellation of atoms that provides the same or a very similar footprint, consisting, for instance, of a set of hydrogen donors or acceptors with the right directionality, would suffice. Such a footprint may belong to a completely different molecule. Like all other forms of polyspecificity, this is also a basis for molecular mimicry.[30-32]

Structural data has demonstrated that antibodies bind most often to protein epitopes limited to three to seven amino acids.[33-36] Therefore, the antibody binding sites often exceed the size of the respective epitope and could engage in different interactions using its different regions. This type of polyspecificity does not depend on paratope flexibility. Another type of binding site, flexible in its free state, could attain the same rigid conformation upon binding diverse ligands.[37] A similar mechanism in the case of the NKG2D receptor was referred to as rigid adaptation.[28] Thus, plasticity could channel a flexible epitope into binding different ligands using a single conformation in the bound state.

Finally, sometime ago it was proposed that glycosylation may contribute to polyspecificity.[38] However, other groups have found no significant effect.[39] It is conceivable that the stability of the immunoglobulin molecule and, therefore, the spectrum of antigens bound by a flexible paratope could be affected by different glycosylation. These effects most probably are indirect at best.

The main mechanisms of polyspecific binding are illustrated in Figure 1.

BIOLOGICAL FUNCTIONS OF POLYSPECIFIC ANTIBODIES

Initially, the antigen binding activity of the pre-immune serum was dismissed as "background." It was later shown that this "background" activity was due to the presence of polyspecific NAbs,[40] the biological significance of which has still been questioned because of their low titers. Yet, a titer operationally is a measure of the reserve binding capacity of the undiluted serum. In real life, antigens are bound by the antibodies in the plasma without prior dilution. The concentration and affinity of these antibodies in plasma are sufficient to form immune complexes with invading pathogens or their toxins. Indeed, numerous studies in the last two decades have provided solid evidence for the role of polyspecific antibodies as an innate defense mechanism. Polyspecific NAbs have been proven to delay the dissemination of bacteria, helminths and viruses after experimental infection.[41-44]

A.L. Notkins et al. have studied in detail the biological properties of polyspecific antibodies. Unlike monospecific ones, several polyspecific monoclonals were shown to inhibit bacterial growth, enhance phagocytosis and, in the presence of complement, to lyse bacteria. Human serum IgM had been enriched in polyspecficity by immunoaffinity purification on three structurally unrelated antigens. The polyspecificity-enriched, but not the depleted human IgM, suppressed the LPS-induced matrix metalloproteinase-1 production in the absence of complement and lysed *E. coli* in the presence of complement.[45]

The two antigen binding sites of a single polyspecific IgG antibody could well bind to different antigens. Michel Nussenzweig et al. recently demonstrated the biological importance of this phenomenon naming it "heteroligation."[46] The authors observed avid binding of anti-HIV gp140 antibodies, which is highly improbable when homotypic

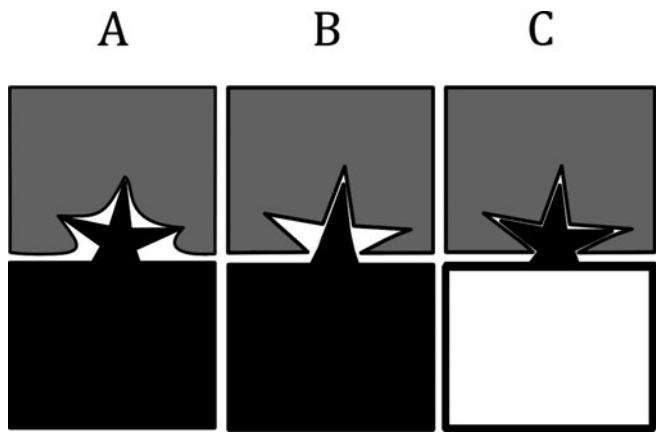

Figure 1. Schematic presentation of the main types of polyspecific binding of antibodies. The gray shapes represent the variable region. A—flexible binding, B—large paratope and C—public epitope.

because of the low copy number of gp140 on the surface of HIV virions. Most of the studied anti-gp140 antibodies proved to be polyspecific. One of the binding sites of such an antibody interacted with high affinity to its nominal antigen (gp140), while the other interacted with high affinity to a different virus surface antigen. Heteroligation may be a mechanism of virus neutralization that is more common than expected.

The beneficial activity of antibodies with natural or induced polyspecificity may go beyond preventing pathogen dissemination in the pre-immune host. Almost 20 years ago, Antonio Coutinho and Stratis Avrameas hypothesized that polyspecific NAbs that bind both foreign- and self-antigens may represent a physiological buffering system of the blood plasma that is able to prevent brisk changes of the levels of biologically active molecules—hormones, cytokines, etc.[47]

Another aspect of immune function depends also on polyspecificity. Although the germline antibody repertoire is extremely diverse, it consists of a finite number of clones that have also undergone a negative selection. Yet, it is postulated to be functionally complete. The completeness of this repertoire seems to depend at least as much on the combinatorial diversity of the B-cell receptors as on their polyspecificity. Although such a solution to the "complete repertoire" problem jeopardizes self-tolerance, this is generally overcome by subsequent control by a network of cellular interactions during an immune response.

It should be noted that there are also reports of polyspecific antibodies that have highly mutated variable regions.[48] The evolution of the antibody response in the germinal centers is driven by a competition for the antigenic signal.[49,50] Therefore, the property that is optimized is the affinity of the BCRs for the right epitope. Thermodynamically, affinity depends on mechanisms different from those that ensure specificity.[51] Reducing the entropic penalty of flexibility does not have to be the only way to increase affinity and in some cases mature antibodies may retain to some extent their polyspecificity.[52]

Despite those exceptions, germline-encoded antigen receptors are by default polyspecific.[53] The initial flexibility of the paratope seems to be a prerequisite for a successful maturation. It seems to determine the direction of the somatic evolution of

antibodies. Affinity maturation of highly flexible germline variable regions was found to produce antibodies of a significantly higher affinity as compared with those of a limited flexibility.[54] Thus, understanding antigen-specific recognition depends on understanding the mechanism of polyspecificity and its stepwise restriction by the affinity maturation process. Interestingly, the specificity of an antibody could under some circumstances also change in the opposite direction.

INDUCED POLYSPECIFICITY—POSTTRANSLATIONAL TUNING OF ANTIGEN-BINDING

Several groups, including ours, have shown that the polyspecificity of an individual antibody molecule can be inherent as well as induced. The in vitro exposure of some monoclonal or polyclonal antibody preparations to protein-modifying agents resulted in the emergence of polyspecificity. The list of these treatments includes chaotropic agents, low and high pH, high-salt concentration, elevated temperature, etc.[55-60] In their native state these antibodies behave as typical monoreactive ones. After the transient exposure to any of the protein destabilizing factors listed above they acquire numerous novel antigen-binding specificities. Interestingly, the recognition of antigens by these antibodies is characterized by physiologically relevant binding affinities. Such antibodies with novel, induced antigen-binding specificities, have been called by various authors "masked," "hidden," "cryptic," "silent," "latent," etc.[61-65] The revealing of novel antigenic specificities was observed only after exposure to mild protein-modifying conditions. Thus, exposure of polyclonal IgG to increasing concentrations of hypochlorite initially results in gradual increase in the repertoire of recognized antigens. However, at a certain point further increase in the concentration of the oxidant resulted in loss of antigenic specificities.[66] These observations can be explained by the irreversible denaturation of the immunoglobulins by higher concentrations of pro-oxidative agents (Fig. 2).

Previously, it was assumed that the generation of new antigen-binding specificities after exposure to protein-destabilizing factors was due to the dissociation of "masking" molecules. Bouvet et al. have ruled out this possibility by demonstrating that the appearance of novel specificities was due to modifications of the antibody molecules. The authors hypothesized that the transient exposure to low pH or urea resulted in conformational changes of sensitive paratopes, allowing interactions with novel, structurally unrelated epitopes.[58]

We applied biophysical techniques to investigate the molecular mechanisms responsible for the induced polyspecificity of Z2 (mouse monoclonal IgG) exposed to urea, ferrous ions or heme.[59,66-68] Using real time kinetic analyses, it was shown that the polyspecificity of Z2 induced by exposure to ferrous ions or to urea did not change the affinity to its nominal antigen.[67,68] However, the kinetic constants of the latter interaction were profoundly affected—the association and dissociation rate constants were both decreased. The slow association rate in protein-protein interactions usually implies an insufficient structural complementarity and an induced fit-type of interaction.[69,70] Indeed, using thermodynamic analysis we observed that the induction of polyspecificity by exposure to urea or ferrous ions was accompanied by unfavorable changes in the entropy of association, indicating an increased flexibility of the antigen-binding sites. To summarize, a transient exposure to protein-destabilizing agents results in structural changes in sensitive paratopes. This increases their plasticity, allowing the interaction

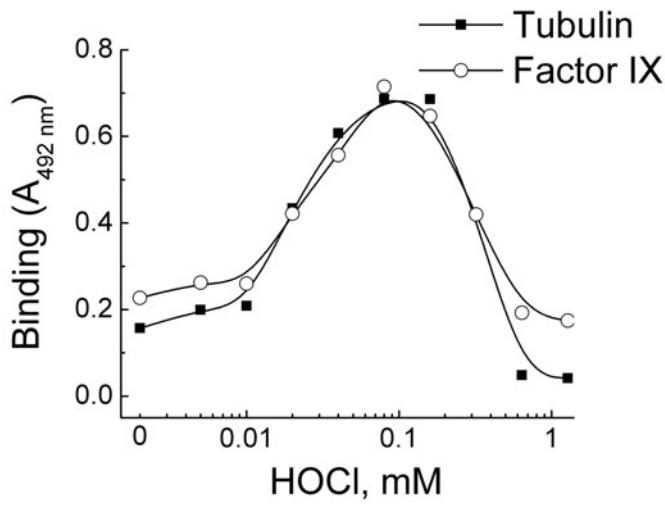

Figure 2. Effect of hypochlorite on immunreactivity of pooled human IgG (IVIG). IVIG (10 μM) was exposed to increasing concentrations of HOCL and the reactivity of 1 μM IVIG to tubulin and factor IX was tested by ELISA.

with various epitopes, most probably due to a partial denaturation of the variable regions. All factors able to induce polyspecificity are protein-denaturing agents, per se. Sensitive variable regions seem unable to return to their native conformation after such a treatment and probably comprise a larger conformational space. The unknown degree of partial denaturation of the variable regions opens the door to criticism, relating polyspecificity to stickiness due to exposed hydrophobic regions. However, the immunoglobulins modified by exposure to ferrous ions or to low pH do behave as intact antibodies. An indiscriminate stickiness due to denaturation is accompanied by aggregation. Several lines of evidence ruled this out in the case of antibodies with induced polyspecificity:

1. The molecular composition of pooled IgG exposed to ferrous ions did not differ from that of the native preparation as shown by size-exclusion analyses performed by HPLC.[71]
2. The presence of aggregated IgG after ferrous ions exposure was also ruled out by the fact that modified IgG do not cause activation of the complement as shown by the test according to Pharmacopoeia requirements for pooled therapeutic IVIG (unpublished data).
3. The antibodies with induced polyspecificity bind to a broad spectrum of structurally unrelated antigens but never to all tested antigens, i.e., they do not become indiscriminately "sticky" as would be expected by aggregated molecules. Moreover, the patterns of antigens recognized by pooled IgG differ depending on the chemical agents used to reveal the hidden specificities, thus suggesting a specific effect.[66]

The induction of polyspecificity of antibodies after exposure to heme has a different molecular mechanism (Fig. 3).[59] In contrast to protein-destabilizing factors, heme is a

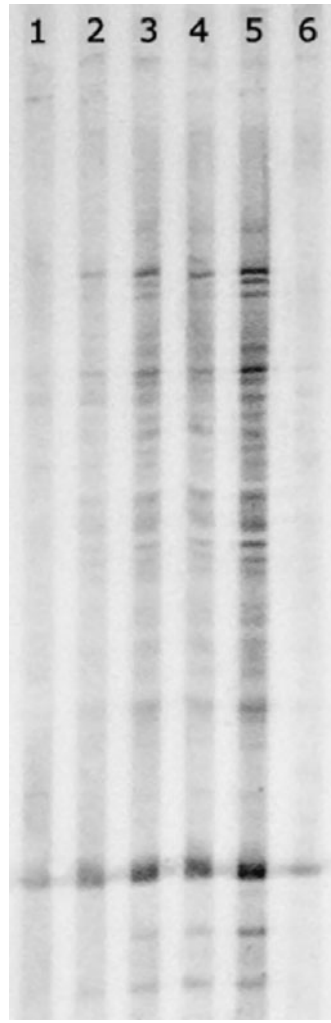

Figure 3. Induction of polyspecificity of human IgG by treatment with heme. Reactivity of IVIG to antigens from Pseudomonas aeruginosa was compared by immunoblot. Strips 1 and 6 show the binding of native IVIG. The same preparation at 10 μM was exposed to increasing concentrations of heme: strip 2–1 μM heme, strip 3–5 μM heme, strip 4–10 μM heme and strip 5–20 μM heme. The membranes were incubated with 0.5 μM IgG.

structure-forming agent, i.e., its interaction with proteins rigidifies their polypeptide chains as demonstrated by kinetic and thermodynamic analyses.[68,72] Rigidifying of the variable region is counterintuitive with regard to polyspecificity mechanisms. However, heme is a hydrophobic macrocyclic compound that is able to establish many types of non-covalent interactions (hydrophobic-, hydrogen bonds, van der Waals-, ionic- and coordinative-interactions) and to bind promiscuously to many different proteins. Thus, heme may qualify also as a prostetic group of antibody polyspecificity.[73]

IN VIVO INDUCTION OF ANTIBODY POLYSPECIFICITY—THE ROLE OF ROS

Activated phagocytes release in the inflammation sites large quantities of reactive oxygen species (ROS) and their derivatives resulting in cell and tissue damage and in oxidative modification of macromolecules.[74] In addition, inflammation leads to the liberation of free iron ions and iron-containing cofactors (heme).[74,75] The prooxidative potential of the "labile iron pool" can further exacerbate the local oxidative stress. Thus, inflammation provides locally several polyspecificity-inducing factors.[59,67] The contact of sensitive antibodies with activated neutrophils, known to secrete large quantities of ROS, results in an enhancement of the recognition of bacterial antigens.[67] Polyspecificity induced in sites of inflammation, may arm pre-existing antibodies by augmenting their capacity to act as a first line of defense.

Recent findings have demonstrated that immunoglobulins are not only passive targets of ROS but they can also catalyze their generation.[76,77] All immunoglobulins, regardless of class and antigen specificity, possess a conservative catalytic center, where oxidation of water molecules can take place. The antibody-dependent ROS production was hypothesized to synergize in antibacterial defense with that of phagocytes.[77,78] We tested the capacity of antibodies to affect recursively their own polyspecificity by the catalytic generation of ROS. Providing conditions for antibody-mediated ROS catalysis resulted in an induction of novel antigen binding specificities. These results allowed us to hypothesize that the catalysis of water oxidation by antibodies and the generation of novel antigenic specificities by the products (and/or substrates) of the catalytic process represent two synergizing parts of an innate defense mechanism.[67,79]

In numerous disease conditions such as hemolysis, ischemia-reperfusion, hemorrhages, major trauma, etc., large quantities of hemoproteins can be released into the circulation.[80,81] Outside of the cells hemoproteins easily lose their prosthetic group. There are several heme-sequestering proteins in plasma, such as hemopexin, albumin and α microglobulin that are involved in its binding and in shielding the toxicity of free heme.[80,81] However, under conditions of excessive release of hemoproteins, heme-scavenging proteins may be saturated and the concentration of free heme may reach more than 20 μM.[82] As immunoglobulins are one of the most abundant plasma proteins, they would inevitably encounter free heme in the circulation. McIntyre et al. have demonstrated that the exposure of immunoglobulins from healthy individuals to heme results in the appearance of new reactivities toward phospholipids and many other self-antigens.[83,84] The authors observed that the binding to phospholipids of heme-exposed antibodies, isolated from healthy donors, does not differ from that of autoantibodies isolated from patients with anti-phospholipid syndrome. In addition to the appearance of reactivity for self-antigens, heme exposure resulted in generation of reactivities toward particular bacterial antigens and intact bacteria.[59] This phenomenon explained the enhanced ability of heme-exposed IgG to initiate complement mediated bacterial killing.[59] Opsonization of pathogens by antibodies with induced polyspecificity may result in the deposition of complement fragments and in an increased immunogenicity, as proposed for the polyreactive NAbs. It should be mentioned that certain pathogenic bacteria induce hemolysis in order to obtain heme as vital iron-containing growth factor. Induction of novel antibacterial specificities of the heme-sensitive antibody fraction may represent a mechanism to delay the infection by hemolytic bacteria until an adaptive immune response develops.

Under certain pathological conditions, the redox agents-mediated generation of novel antigen binding specificities may also have detrimental consequences. Ischemia-reperfusion injury represents a severe inflammatory response resulting in critical tissue damage, mediated by an inappropriate activation of the complement system. It has been demonstrated that this process is initiated by NAbs.[85,86] The initial hypoxia and release of reactive oxygen species (ROS), heme and/or transition redox-active metals may result in cellular damage and in the display of cryptic epitopes on the cell surface. These new epitopes are recognized by the circulating polyspecific NAbs, the reactivity of which may have been augmented in the process. The formed immune complexes fix complement and result in tissue damage.

THERAPEUTIC POTENTIAL OF ANTIBODIES WITH INDUCED POLYSPECIFICITY

It has been suggested previously[87] and later proven[6] that some antibodies acquire polyspecificity after circulating through sites of inflammation. As mentioned above, A. Coutinho and S. Avrameas have hypothesized in 1992 that polyspecific NAbs form a physiological buffering system that controls the kinetics of circulating biologically active molecules.[47] Interestingly, inflammatory cytokines seem to be among the preferred autoantigens of NAbs.[88,89] Our recent data on the beneficial effect of the passive immunotherapy with ferrous ions- and heme-exposed IVIG preparations provide indirect evidence in support of this idea.

Normal pooled therapeutic immunoglobulins are highly polyreactive preparations. Their exposure to protein-modifying agents (see above) increases further the panel of self- and foreign antigens they bind to. These modified IVIG acquire a new clinically relevant property. They become protective when used to treat animals with experimental sepsis induced by the injection of live *E.coli*, of bacterial LPS, [58,67] of zymosan as well as by the colon puncture and ligation technique. The native IVIG preparation had no effect on survival in the same experiments and the protective activity was not due to a more efficient neutralization of LPS.[67] Sepsis is a syndrome caused by the severe and generalized activation of body defense mechanisms in response to invading pathogens. It is characterized by the uncontrolled release of pro-inflammatory cytokines ("cytokine storm") and uncontrolled activation of coagulation, complement and apoptotic pathways. The studies on the beneficial effect of modified IVIG have found lower plasma levels of pro-inflammatory molecules and normal coagulation parameters in the treated animals (manuscript in preparation). While Fe(II)- and heme-exposed IVIG are investigational drugs, immunoglobulin preparations modified by pH 4.0 exposure are already licensed and commercially available.[90]

If natural antibody reactivities are meant to buffer cytokines at times, why doesn't this compromise the signaling function in general? It is possible that, unlike IVIG with additionally induced polyspecificity, the natural buffer of a "cytokine storm" is represented by IgM NAbs. IgMs circulate almost exclusively in the plasma compartment and would protect mostly against systemic increase of cytokine levels but would not affect their local function in tissues. NAbs are mostly of the IgM isotype, produced by B1 cells and their repertoire is positively selected on self antigens during the fetal period. IgG antibodies, on their part, are mostly derived from B2-responses and belong to a repertoire selected in a different manner that includes a strong negative selection stage. It is possible that

the buffering function is an evolutionary adaptation specific for the B1-derived IgM repertoire but artificially inducible in B2-derived IgG NAbs. This hypothesis is being currently tested.

CONCLUSION

For many immunologists, the role of antibody polyspecificity in immunity remains counterintuitive. Recent studies of antibody function emphasize the importance of understanding specificity of antigen receptors as a continuous spectrum. Modern system biology thinking necessitates a careful separation of molecular interaction phenomena from system functions. Relating mechanically, the terms immunoglobulins, antibodies and specific recognition might be encouraging errors of reductionist thinking. On the other hand, as a result of understanding better natural and induced polyspecificity, the range of potential immunoglobulin-based therapeutic approaches will expand, while the existing ones are further optimized.

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CHAPTER 17

POSITIVE AND NEGATIVE SELECTION OF NATURAL AUTOREACTIVE B CELLS

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Abstract: Naturally occurring antibodies (NAbs) produced by $CD5^+$ B-1 B cells include those with specificity for thymocytes (anti-thymocyte autoantibody, ATA). Here we describe a prototypic example, encoded by an unmutated immunoglobulin μ/κ heavy chain/light chain. Studies with ATA-μ ("heavy chain only") transgenic mice demonstrated a critical requirement for self-antigen in the accumulation of B cells with this specificity and for the production of high levels of serum ATA NAb. Furthermore, analysis of B-cell development in ATA-μκ ("heavy and light chain") transgenic mice revealed two distinct responses by B cells to expression of this B-cell receptor (BCR). (1) Most B cells developing from bone marrow of adult mice were blocked at an immature stage in spleen and only escaped apoptosis by editing their BCR to eliminate the ATA specificity. (2) Some B cells differentiated to antibody-forming cells without altering their specificity, produced high levels of serum ATA, and many ATA-secreting plasma cells were observed in spleen. Finally, examination of B-cell development and ATA NAb production in ATA-μκ transgenic mice with levels of Thy-1 autoantigen varying from very low to above physiologic reveals a clear relationship between BCR crosslinking by antigen and B-cell fate. Low levels of Thy-1 autoantigen resulted in diversion of ATA B cells into the marginal zone B-cell compartment, presumably because of reduced BCR signaling. Thus, our studies demonstrate a key positive selection step in the development of NAb-producing B cells and show that most of these cells in adult mice bearing such specificities fail to reach a mature stage. Importantly, because these specificities are isolated from B-1 B cells and, when expressed as transgenes, guide development into the B-1 or marginal zone B-cell pool, we identify these B cells as a major source of natural autoantibodies in mice.

Naturally Occurring Antibodies (NAbs), edited by Hans U. Lutz.

INTRODUCTION

The discovery of a B-cell subpopulation expressing the pan-T-cell surface glycoprotein CD5, then called Ly-1, in the autoimmune-prone mouse strain NZB, led to an investigation of the antibodies produced by these cells.[1] This analysis revealed that certain natural autoantibodies were produced by $CD5^+$ B cells, based on experiments altering their numbers, either by cell sorting or by analysis of certain mutant mouse strains such as xid and MeV.[2,3] In fact, a population of $CD5^+$ (B-1) B cells was found to be strongly associated with the production of naturally occurring autoantibodies (NAbs) to a determinant exposed on mouse red blood cells by treatment with the proteolytic enzyme bromelain. Since earlier work had shown that B cells with this specificity were abundant in the peritoneal cavity,[5,6] this led to experiments with B cells washed out of the peritoneal cavity of normal non-autoimmune mice that clearly demonstrated the presence of large numbers of B cells in this site that expressed CD5,[4] and that could produce several types of NAbs.[2]

The initial experiments with this distinctive NAb-producing B-cell population focused on use of CD5 expression as a novel marker that distinguishes them from all other B cells. Later work expanded the definition of this population, based on a combination of cell surface markers, and led to renaming them as "B-1 B cells," distinguishing them from the major pool of follicular B cells, referred to as "B-2 B cells." In this chapter we describe the development and function of B cells that produce a prototypic NAb associated with the $CD5^+$ B-1 B-cell subset, termed anti-thymocyte autoantibody or ATA. This specificity is germline-encoded and typical of a class of IgM autoantibodies that are abundant in sera of certain autoimmune mouse strains, such as NZB, but that are also present in the serum of normal animals.

A NATURALLY OCCURRING AUTOANTIBODY SPECIFIC FOR THYMOCYTES

In our screen of hybridomas made from sorted $CD5^+$ B cells we isolated a panel of 15 hybridomas that secreted IgM capable of binding to thymocytes, as revealed by staining and flow cytometry analysis.[7] The binding of 8 of these could be blocked by pre-incubation of thymocytes with an anti-Thy-1 monoclonal antibody. Staining by most of these IgM was lost if thymocytes were first treated with sodium metaperiodate, an agent that oxidizes terminal sialic groups on carbohydrates. Based on the periodate sensitivity and the observation that some $Thy\text{-}1^+$ cells, particularly peripheral $CD8^+$ T cells and many $CD4^+$ T cells, were not stained by these antibodies, we hypothesized that the determinant is a specific glycosylation present on certain Thy-1 molecules whose expression is regulated with differentiation. Half of these hybridomas, including the group that produced antibody staining thymocytes most intensely, contained a recurrent rearranged immunoglobulin (Ig) heavy chain (from the V_H3609 family). The prototypic anti-thymocyte/Thy-1 autoantibody (ATA) (clone 1–6C10, from hybridoma SM6C10), paired this heavy chain with an Ig light chain from the Vκ21 family (Vκ21c).

This antibody, abbreviated as 6C10 ATA, binds to a tissue- and differentiation stage-specific N-glycosylated epitope on Thy-1 (also known as CD90), as we could immunoprecipitate a fraction of the Thy-1 peptides from a thymocyte lysate, shown by 2-D gel analysis and protein gel blotting.[8] The epitope is species-specific, as 6C10 ATA antibody does not bind to Thy-1 in rat. In mouse. the epitope is tissue specific,

because Thy-1 glycoforms expressed on neural tissue and on hematopoietic stem cells are not detected by this antibody. Curiously, we observed that the 6C10 epitope on Thy-1 ($Thy1^{6C10}$), abundantly expressed on immature thymocytes, was sharply downregulated as $CD4^+$ single-positive T cells were generated, but then was reexpressed at high levels on many peripheral $CD4^+$ T cells.[8,9] The functional significance of a NAb binding to such a developmentally regulated carbohydrate epitope remains unknown.

ATA-μ TRANSGENIC MICE: EVIDENCE FOR B-CELL POSITIVE SELECTION

The observation that the 6C10 ATA epitope was critically dependent on expression of Thy-1 suggested a novel approach to study the development of autoreactive B cells: namely by eliminating the natural autoantigen, e.g., by analyzing the development of Thy1–6C10-specific ATA B cells in mutant animals that lacked Thy-1, produced by gene targeting.[10] Since the normal frequency of B cells with the prototypic anti-thymocyte autoantibody rearrangement (V_H3609/6C10) is low, we generated heavy chain transgenic mice bearing this specific VDJ-μ and then first studied development of B cells in these mice on a Thy-1 wild-type background.[11] We found that ATA-μ transgenic mice had a high titer of serum ATA, easily demonstrable by its capacity to bind to thymocytes, as revealed by fluorescence staining. By generating monoclonal antibodies specific for ATA Ig heavy chain segments we were able to show that most B cells in these mice expressed the transgenic Ig heavy chain, paired with a diverse set of light chains. Only a small fraction of B cells paired the ATA heavy chain with a Vκ21c light chain to generate the thymocyte-binding specificity. Such cells were readily detectable in the peritoneal cavity, where they had a $CD5^+$ B-1a cell phenotype.

Importantly, the antigenic determinant recognized by the monoclonal ATA NAb is a glycoform epitope restricted to the cell surface protein CD90/Thy-1. Mutant mice that completely lack this protein have been generated by gene targeting of CD90/Thy-1, so that they do not express the ATA NAb target antigen. These mice are otherwise normal, so it was possible, for the first time, to study the development of B cells expressing a natural autoantibody, but where the natural autoantigen was missing. In contrast to results in normal wildtype $Thy\text{-}1^+$ mice, analysis of the ATA-μ transgene on a Thy-1 null (gene-deleted) background revealed a completely different picture: the serum ATA titer was essentially absent and the $CD5^+$ B-1 B-cell population in the peritoneal cavity predominantly consisted of non-ATA^+ B cells, instead expressing endogenous Ig heavy chains (Endo-IgH). Thus, when the self-antigen recognized by the ATA transgenic Ig was eliminated, entry into the B-1 B-cell pool depended on expression of different Igs that could only be produced by rearrangement of endogenous Ig genes, a process relatively rare in Ig transgenic mice. These rare cells then accumulated to occupy the B-1 B-cell pool in Thy-1 null ATA transgenic mice. Restoration of Thy-1 (self-antigen) expression in these Thy-1 null ATA transgenic mice could be accomplished by transfer of hematopoietic stem cells from Thy-1 wildtype mice, engrafting large numbers of $Thy\text{-}1^+$ T cells. When this was done, serum ATA and accumulation of ATA Tg^+ B-1 B cells were both also restored, demonstrating that both the accumulation of ATA $CD5^+$ B cells and elevated levels of serum ATA were dependent on the presence of the autoantigen, Thy-1. We described this as the first example of a positive selection of auto-reactive B cells, as diagrammed in Figure 1.

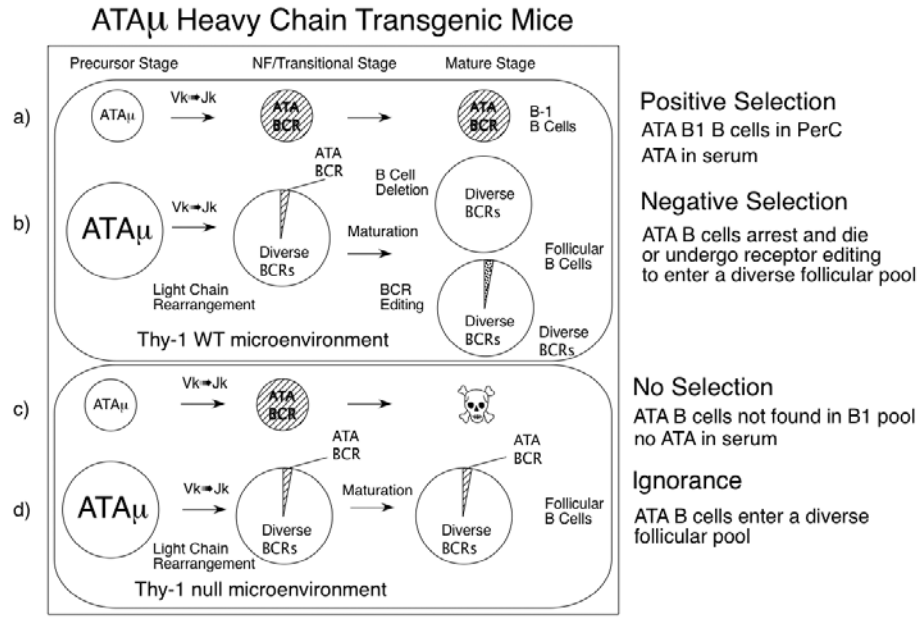

Figure 1. The ATA (anti-thymocyte autoantibody) μ heavy chain transgenic model system demonstrates a critical positive role that antigen (Thy-1 in this case) plays in the accumulation of both ATA^+ B cells and ATA immunoglobulin in serum. Precursor cells (that all contain the transgenic heavy chain, ATA μ) rearrange and begin to express light chain (indicated by Vk➠Jk) as they progress to the newly formed (NF) B-cell stage. In a normal Thy-1 wildtype microenvironment: (a) in fetal liver, B cells that pair the ATA-μ Tg heavy chain with a Vκ21c light chain (ATA BCR; pie slice with hatched fill pattern) are selected into the B-1 B-cell pool and are responsible for serum ATA IgM; (b) B cells arising from bone marrow that generate the ATA BCR are either deleted (note absence of hatched pie slice) or undergo receptor editing to a different specificity (note pie slice has a different fill pattern). Most B cells rearrange a light chain other than Vκ21c, therefore do not express a self reactive BCR (represented by the white section of the pie diagram), and so develop to typical follicular B cells. In contrast, in a Thy-1 null microenvironment: (c) B-1 B cells with an ATA BCR do not accumulate due to the absence of the Thy-1 selecting antigen; (d) bone marrow development generates follicular B cells efficiently, even for B cells that rearrange the Vκ21c light chain and express the ATA specificity. The limited numbers of cells capable of supporting ATA B-cell maturation to the B-1 B-cell fate is indicated by showing a smaller pie diagram (a and c), in contrast with the much large number (b and d) that do not support this.

In a transgenic model of a pathogenic autoantibody, B cells with an anti-erythrocyte specificity developed in the peritoneal cavity due to their sequestration from antigen, suggesting that a lack of negative selection may allow development of self-reactive B cells.[12,13] To test whether large amounts of Thy-1 antigen might negatively impact ATA B cells in the peritoneal cavity, we injected $Thy\text{-}1^+$ thymocytes into the peritoneal cavity of newborn and adult ATA Tg mice. This treatment had no appreciable effect on either the frequency of ATA B cells or the level of serum ATA, demonstrating that the ATA B cells in these mice had not simply escaped deletion by development within the peritoneum. This illustrates a clear distinction between natural and pathogenic autoantibody.

ATA-μκ TRANSGENIC MICE: EVIDENCE FOR B-CELL NEGATIVE SELECTION

To understand more about this selection process, we cloned the prototype Vκ21c light chain from the ATA hybridoma and produced transgenic mice expressing both 6C10 V_H3609 heavy chain (ATA-μ) and Vκ21c kappa light chain, referred to as ATA-μκ transgenic mice.[14] We examined B-cell development in these transgenic mice, both on wildtype (Thy-1$^+$) and Thy-1 null backgrounds. Unlike ATA-μ transgenic B cells which pair the ATA heavy chain with a diverse set of Ig light chains, in ATA-μκ transgenic animals all newly formed B cells in bone marrow express the ATA specificity (Fig. 2). That is, in "heavy chain only" ATA transgenic mice, a very small fraction (less than 1%) of newly formed B cells will pair the transgenic heavy chain with the specific light chain required to generate the ATA NAb specificity, but when both ATA Ig chains are expressed as transgenes, all rearrangements are blocked and essentially all newly formed B cells are self-reactive. The availability of ATA-μκ transgenic mice facilitated a careful assessment of the consequences of this self-reactive B-cell receptor (BCR) on bone marrow B-cell development. We found that ATA-μκ B cells in a Thy-1$^+$ environment became arrested at an immature stage in the spleen, shortly after exiting the bone marrow. By examining several independently generated ATA-μκ transgenic lines, we found two outcomes: in some lines the newly formed arrested B cells died; in other lines these cells revised their BCR, pairing the ATA transgenic heavy chain with a non-transgenic light chain produced by rearrangement of the normal (endogenous) kappa locus, thereby eliminating the ATA specificity and progressed to later stages in development (Fig. 2). Arrested B cells in spleen showed an immature cell surface phenotype by flow cytometry and had a high turnover (i.e., were short-lived), typical of immature/transitional B cells. Furthermore, also similar to immature B cells, these cells did not proliferate when exposed to antigen. Thus, in all respects these cells were similar to self-reactive B cells in "classical B-cell tolerance models" for negative selection, such as with anti-HEL/HEL, anti-MHC class I, and anti-dsDNA, where the presence of self antigen exerts a negative impact, resulting in deletion, functional silencing (anergy), or alteration of the Ig B-cell receptor (receptor editing).[15-18]

As expected, this maturation arrest did not occur on a Thy-1 null background, where instead cells progressed to a typical follicular B-cell stage (Fig. 2). These cells responded robustly to antigen exposure. Interestingly, even with the obvious negative impact of Thy-1 antigen on ATA B-cell maturation in Thy-1$^+$ ATA-μκ transgenic mice, we nevertheless detected high levels of ATA IgM in the serum; this level was significantly decreased in the absence of Thy-1, similar to results with ATA-μ transgenic mice. We sought to determine whether this ATA production derived from B-1 B cells or instead from the maturation-arrested population of B cells in spleen. First we transferred either spleen ATA B cells or peritoneal cavity cells (containing ATA B cells) from ATA-μκ transgenic mice into B-cell deficient J_H null mice and monitored the production of serum ATA. Although serum ATA was readily detectable in the recipients of peritoneal cavity cells, none was detected in recipient mice that received maturation-arrested B cells from spleen. Concerned that the staining and sorting process might have led to the functional incompetence of transferred spleen B cells, we also tried a different approach to assess production of ATA by spleen B cells. Adult ATA-μκ transgenic mice were splenectomized and then monitored for any decrease in serum ATA levels. Some decrease was found

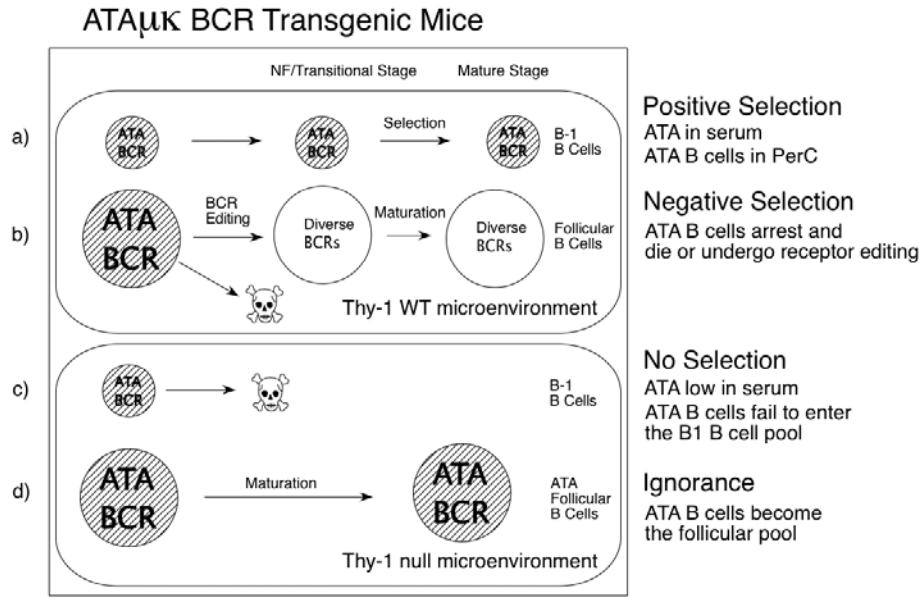

Figure 2. Analysis of bone marrow and spleen B-cell development in ATA heavy/light chain transgenic mouse lines reveals extensive negative selection (light chain editing or cell death) as a consequence of antigen encounter during adult B-cell development. In a Thy-1 wildtype microenvironment: (a) there is abundant ATA IgM in serum, derived from differentiation of some B-1 type B cells; (b) however most developing ATA B cells become arrested at a short-lived immature transitional stage in spleen and only survive by altering their specificity (receptor editing) away from ATA to a diverse BCR repertoire. In contrast, in a Thy-1 null microenvironment: (c) ATA B cells do not enter the B-1 B-cell pool, but die instead; consequently serum ATA levels are low; (d) ATA B-cell maturation is efficient in bone marrow, generating a follicular B-cell pool consisting exclusively of the transgene-encoded (non-autoreactive) BCR.

shortly after the operation; however, ATA levels remained at a persistent high level over a two month post-op monitoring period, indicating that the spleen is not the only site for production of natural autoantibody. We concluded that most bone marrow developing ATA B cells in a Thy-1 wildtype mouse reach the spleen, become inactivated, and then either die or revise their BCR specificity away from ATA. In contrast, a minor fraction of cells in peritoneal cavity (and spleen) is responsible for ATA production, with some cells showing a B-1 B-cell phenotype.

Thus, we found both positive and negative impacts of self-antigen on B-cell development and production of serum ATA NAb in these ATA-μκ transgenic mice, indicating that at least some developing B cells with this self-reactive specificity could be selected by interaction with antigen to become antibody-forming cells. The reason for ATA B cells adopting these two different cell fates, either negative selection for most bone marrow generated cells or positive selection, with development to B-1 B cells and some differentiation to antibody-forming cells, is currently under investigation. Analysis of the kinetics of development suggests one possible explanation based on homeostasis: the B cells initially generated in neonatal animals are critically dependent on strong signaling through the BCR for their survival and respond positively to self-antigen exposure,

whereas, once such a population becomes established, with attendant production of antigen-antibody complexes, the B cells developing afterwards experience BCR signaling in conjunction with non-BCR signals (immune complexes binding to cell surface receptors) and so respond negatively.

ALTERING ATA BCR CROSSLINKING: MARGINAL ZONE B-CELL DEVELOPMENT

The experiments described above illustrate two distinct ATA BCR signaling states, one with little or no signaling in the absence of Thy-1 antigen and one with strong BCR signaling in the presence of Thy-1 (on a wildtype background). We next decided to investigate the consequences of intermediate signaling states by providing different levels of Thy-1 antigen to developing ATA BCR B cells.[19] We cloned and expressed Thy-1 as a transgene in T cells and selected mouse lines with different expression levels, likely due to variation in the number of transgene copies per genome. Sub-physiologic levels of ATA self-antigen could be obtained with transgenic lines that showed abnormally low levels of Thy-1 on a Thy-1 null background. Super-physiologic levels of the ATA self-antigen were obtained using a "high copy" line (where many copies of the Thy1 transgene were stably integrated into the genome) on a Thy-1 wildtype background. Extremely low levels of Thy-1 self-antigen exposure could be accomplished by simply using Rag-1 null (gene-deleted) mice, where T-cell development is blocked at a very early stage due to the failure of T-cell receptor rearrangement, decreasing the number of Thy1-bearing cells 100-fold. It is important to note that Thy-1 is not an integral membrane protein, but rather is attached to the cell by a glycophosphatidylinositol (GPI) linkage, and so can be shed into serum where it may act to modulate B-cell development. Considering the relative paucity of T cells in bone marrow, it is likely that developing B cells encounter shed Thy-1, and so the altered levels of Thy-1 expression result in different concentrations of Thy-1 antigen in serum. The precise nature of Thy-1 antigen that immature B cells encounter in vivo, whether intact soluble protein or else Thy-1 bound to the surface of antigen presenting cells, remains to be determined.

We had already established in prior work that ATA BCR B cells developed to follicular (Fo) B cells in the absence of self-antigen (that is, they developed similarly to the majority of B cells in spleen). The striking finding for ATA B-cell development with low levels of Thy-1 self-antigen was the decreased production of Fo B cells and instead the generation of ATA BCR B cells with a marginal zone (MZ) B-cell phenotype. These results suggest that different levels of BCR crosslinking (none vs. some) can guide developing B cells into very different B-cell subpopulations that localize in tissue differently and exhibit different functional responses. Furthermore, when the ATA BCR was bred onto the Rag-1 null background, with even lower levels of serum Thy-1, we obtained the same result: most B cells in spleens showed a MZ B-cell phenotype. This variation of ATA B-cell fate depending on the level of BCR crosslinking is diagrammed in Figure 3. Importantly these MZ B phenotype cells localized in the spleen at the border of the white and red pulp, the distinctive zone where marginal zone B cells are normally found. In addition, these cells exhibited normal functions characteristic of MZ B cells, such as rapid calcium flux when exposed to Thy1 antigen, more rapid and more intense than ATA BCR B cells with a follicular B phenotype (that were generated in the complete absence of antigen). Thus, B cells

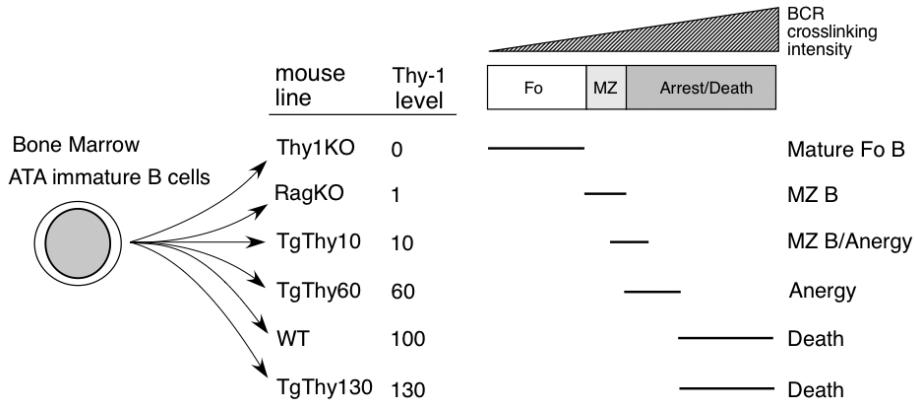

Figure 3. BCR crosslinking intensity and B-cell fate. Newly formed (immature) bone marrow ATA B cells that encounter different levels of Thy-1 self-antigen experience different cell fates: (1) those encountering 100–130% of physiologic Thy-1 level experience maturation arrest and die; (2) those encountering 60% of physiologic Thy-1 level become arrested as anergic cells and persist; (3) those encountering 1–10% of physiologic Thy-1 level enter the marginal zone B-cell pool if appropriately signaled by Notch-2[20] or else become arrested as anergic cells; and (4) those encountering no Thy-1 (0%, Thy-1 null background) mature to constitute a follicular B-cell pool.

with the same ATA BCR could enter functionally and phenotypically different B-cell populations depending on the strength of BCR cross-linking that they experienced: the follicular fate with little or no crosslinking, the MZ B-cell fate with some BCR crosslinking and appropriate non-BCR signals, or the B-1 cell fate with a higher level of BCR crosslinking. An important caveat for recruitment to the B-1 cell fate is that most bone marrow B cells did not progress to a long-lived mature B-1 cell fate when their BCR was cross-linked (signaled) at this high level, as described above. Since a significant portion of the B-1 cell pool is generated during fetal/neonatal life, we consider this issue in the next section.

DEVELOPMENTAL CHANGES IN NATURAL AUTOANTIBODY B-CELL SELECTION

Our earliest cell transfer experiments designed to fully reconstitute $CD5^+$ B-1 B cells in irradiated recipients were unsuccessful when we employed bone marrow from adult (> 2 mo old) mice as a source of B-cell progenitors. Instead we could repopulate $CD5^+$ B-1 cells (and all other B-cell populations) when we used progenitors from fetal or newborn animals.[4] This prompted us to suggest that the $CD5^+$ B-1 B-cell generation was developmentally biased, predominantly from precursors present in fetal or neonatal animals. In later experiments we purified pro-B stage cells ($B220^+CD43^+CD24^+$) from fetal liver and bone marrow by cell sorting, then transferred the cells to SCID mice that lack lymphoid cells.[23] These experiments demonstrated that most committed B cells in liver did not develop into classic follicular B cells in this transfer model, whereas those from bone marrow could. Furthermore most bone marrow pro-B stage cells did not

generate B-1a ($CD5^+$) phenotype B cells. It seems reasonable to hypothesize that intrinsic differences at the pre-B and immature B-cell stage in developing fetal/neonatal B lineage cells allow maturation of cells with moderately self-reactive BCRs. Indeed, in the neonate, in the absence of a developed immune system capable of supplying non-BCR survival signals, such self-reactivity may facilitate B maturation, thereby resulting in a repertoire that favors the production of natural autoantibodies. In the context of BCR vs. non-BCR survival signals, it is significant to note that only B-1 B cells are intact in BAFF null mice (that lack this well-characterized non-BCR B-cell survival signal), demonstrating that B-1 B-cell development and maintenance is BAFF independent.[21,22]

In contrast, intrinsic differences at the pre-B and immature B-cell stage in developing bone marrow B lineage cells appear to favor those cells with BCRs that do not show such high levels of self-reactivity. Bone marrow B cells that do generate such BCRs are likely arrested at an immature stage, hence non-functional and short-lived, or else die by apoptosis, unless they revise their BCR by receptor editing, as demonstrated in several transgenic models of B-cell tolerance. Probably some cells with moderately self-reactive BCRs can enter the marginal zone pool or possibly the $CD5^-$ B-1b B-cell subset. Thus, as shown in Figure 4, we propose an overall model for B-cell selection and production of NAbs that changes with development, and where the level of BCR crosslinking strongly influences entry into one of the three mature B-cell pools, Fo, MZ, or B-1.

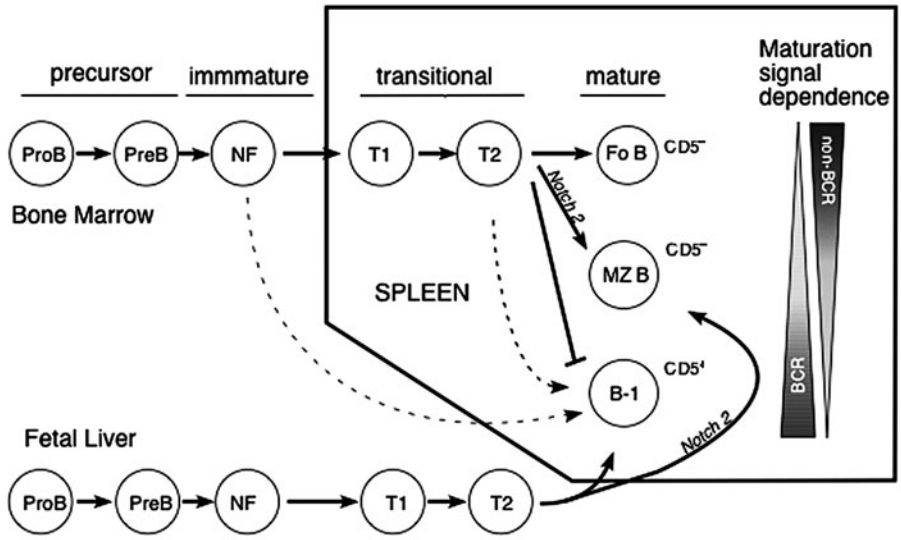

Figure 4. Overall model for development of B cells into three mature subpopulations, follicular (Fo), marginal zone (MZ) and B-1, dependent on signals from the BCR and additional cell surface receptors (such as growth factor receptors). In contrast to the largely negative selection experienced by bone marrow newly formed (NF) or transitional (T1, T2) B cells, a portion of B cells (predominating in fetal/neonatal life) respond to antigen encounter by adopting a B-1 B-cell fate and can produce natural serum autoantibody. The intermediate BCR signaling that results in entry into the MZ B-cell fate also requires Notch2 signaling, shown on the lines leading to this cell fate. The failure of most bone marrow ATA B cells to enter the B-1 pool is indicated by a blocked line. Solid lines indicate major pathways; possible minor pathways are indicated by dashed lines.

IMPORTANCE OF SPECIFICITY OF NATURAL AUTOANTIBODIES

Our analysis of B-cell development and serum autoantibody production in the ATA transgenic mouse lines has highlighted an important feature of this process: specificity. In the ATA-μ transgenic model, greater than 80% of the serum antibody consists of the transgenic heavy chain paired with a specific light chain, resulting in an antibody recognizing a distinct glycoform of the Thy-1 protein. The affinity of this antibody is sufficient for its use as a staining reagent.[9] In the same transgenic line on a Thy-1 null background most of the serum antibody is not transgene-derived and the B-1 B cells that can be detected in the peritoneal cavity show endogenous heavy chain expression, presumably selected for by diverse self-antigens unrelated to Thy-1. Thus, at least for the V_H3609/ 6C10-encoded antibody, only the Thy-1^{6C10} determinant can serve as the selecting antigen, generating an antibody where this heavy chain is paired with a specific light chain, Vκ21c. This means that it is probably not generally correct that the B cells serving as the source for most NAbs are selected by chance for "cross-reactivity," and therefore exhibit "poly-reactivity." In the example described here both the antibody and the determinant recognized are very specific and so these ATA B cells are highly dependent on the unique Thy-1 glycoform for positive selection. However, this does not rule out the possibility that certain other BCR structures (Ig heavy and light chains) have been selected into the germline because of particular cross-reactivities between self-determinants and epitopes present on pathogens, one explanation for the functional significance of some NAbs. It seems likely that the population of natural autoantibody is heterogeneous, generated by several mechanisms. Clearly a significant portion derives from B-1 B cells and some likely comes from MZ B cells, both populations selected by self-reactivity into these functionally distinct populations.

CONCLUSION

$CD5^+$ B-1 B cells are a major source of NAbs in healthy mice. In this chapter we have highlighted a key feature illustrated by our studies of ATA produced by B-1 B cells: the critical role that antigen plays in establishing the long-term B-1 B-cell pool and inducing production of NAbs. Selection by self-antigen probably holds for all $CD5^+$ B-1 B-cell-derived NAbs, but remains to be assessed for NAbs that may be generated by other mechanisms. $CD5^+$ B-1 B cells appear to require higher levels of BCR signaling than B cells in other subsets, particularly follicular B cells.[24] In the future it will be important to determine the mechanism(s) mediating positive selection of natural autoreactive B cells, to understand how their population size is maintained throughout life, and to delineate the function(s) of produced NAbs within the immune system. It is already clear that certain specificities among B-1 B cells play a critical role in host defense.[25,26] Finally, it is important to note that the breakdown of normal growth regulation in such a long-lived, self-reactive cell population carries serious implications for development of autoimmune pathologies and even B-cell leukemia,[27,28] highlighting the clinical relevance of such work.

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CHAPTER 18

NATURALLY OCCURRING ANTIBODIES/ AUTOANTIBODIES IN POLYCLONAL IMMUNOGLOBULIN CONCENTRATES

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Abstract: It was a long way from the use of hyperimmune animal sera for the treatment of toxin-producing infections to the production of polyclonal, polyspecific human immunoglobulin preparations and the use of NAbs as therapeutic tools for autoimmune and inflammatory diseases. Some highlights of the development of knowledge in blood fractionation techniques, basic science and clinical wisdom are reviewed in this chapter. Proudly we mention the outstanding contribution of Swiss scientists and clinicians in the development of IVIG as clinical tool for some otherwise untreatable diseases or taking advantage of its low adverse event profile in long-term treatment of other chronic autoimmune and inflammatory diseases. This chapter summarizes some of the characteristics and the effects in humans of NAbs which are present in IgG concentrates. We call attention to the fact that the human data remain, at least in part, incomplete, among others because even with the most efficient large-scale techniques available not more than approximately 50% of the total IgG in plasma can be fractionated into an immunoglobulin G concentrate.

INTRODUCTION

In the late 1970s a few European scientists embarked on the study of physiological autoantibodies[1,2] and one group studied their role in tissue homeostasis and focused on the interaction of NAbs with senescent RBC in the process of selective removal of 20 to 25 g of senescent human RBC per day.[3-5] For such studies NAbs were isolated from immunoglobulin concentrates containing > 97% IgG. Embarking on NAbs research was tough at a time when immunology was governed by 'T-cell only' and the T cell

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was forging the chances for getting grant proposals accepted. Meanwhile the existence of NAbs has been acknowledged even by those having had a strong T cells oriented view of immunity[6] and many clinical and laboratory investigations using IgG NAb have established a role of NAbs for therapeutic purposes.

The chapters of this book give an update on the present knowledge about NAbs. It is reassuring that human IgG NAbs exist and apparently not all self-reactive human IgG is associated with disease. In this chapter we are reviewing aspects of human NAbs in polyclonal, polyspecific immunoglobulin concentrates.

NAbs IN IMMUNOGLOBULIN CONCENTRATES: HISTORICAL ASPECT OF THE DETECTION OF THEIR THERAPEUTIC USE

It was shortly after the introduction of the first 'close to native' immunoglobulin concentrate for intravenous use, IVIG, which was termed initially IgG-SRK (SRK = Swiss Red Cross), that the immuno-modulatory effect of IVIG was reported for the first time in an international journal.[7] Although there is no direct link demonstrable between the clinical outcome and the availability of IgG-SRK, such availability for sure has paved the road for the rapid spread of getting clinical advantage from the immuno-modulatory and anti-inflammatory effect of IVIG for the benefit of patients suffering from various autoimmune and inflammatory diseases. Quite soon it became obvious that the Fc and the $F(ab')_2$ parts of the involved IgG molecules were necessary for an optimal immuno-modulatory and anti-inflammatory effect of IVIG.

Before the door could be opened for the routine clinical use of the effects of NAbs contained in IVIG, some important milestones in polishing processes of 'standard' IgG had to be achieved. Subsequent to the very first report on anti-diphtheria toxin activities in plasma,[8] from 1893 onward, the first immunoglobulin concentrates were hyperimmune preparations isolated from animal plasma (reviewed by Peter Gronski[9]) (for details on the various IgG concentrates see Addendum). Beside immunization by animal proteins, these "antitoxins" induced adverse events (AEs) in recipients. The frequency and intensity of these AEs could be reduced by ammonium sulfate precipitation and further by a "method for purification of antitoxins and the like" and the „disaggregation" or "autolysis" of the antitoxins by pepsin treatment.[10-12] The detection of antibody deficiency in humans strengthened the clinical importance of immunoglobulin concentrates as a therapeutic tool. The very first report about primary antibody deficiency dates back to 1952 and was an abstract by several authors, describing five patients.[13] In the full publication the number of patients was'reduced' to one, published by a single author.[14] Through the work of Edwin Cohn and his collaborators,[15,16] a human "standard" IgG preparation was already available in 1952 and was ready to be used for replacement therapy. Replacement in the first patient was via the subcutaneous route of application.[14] From the very beginning, the treatment of severe infections in normogammaglobulinemic patients,[17] or the replacement therapy in antibody deficiency aimed at a rapid increase of IgG in the circulation, which was most efficiently achieved by application of the immunoglobulin concentrate via the intravenous route. However, antibody deficient patients proved particularly reactive to "standard" IgG.[18] During the isolation procedure IgG molecules are inevitably altered and tend to form aggregates that remained in the "standard" IgG preparations because the production process did not include polishing steps to get rid of the altered/aggregated IgG molecules. The particular reactivity of antibody deficient

patients made it impossible to apply "standard" IgG preparations via the intravenous route. Interestingly, the intramuscular application of "standard" IgG concentrates became clinical practice for replacement therapy (IMIG), not the originally used subcutaneous route.[19] Dose limitations and the slow increase of IgG in the circulation of antibody deficient patients remained a driving force for the development of an IVIG. A first avenue taken to achieve intravenous tolerability was by heavy protease treatment (pepsin, plasmin), a process which resulted in a short in vivo half-life of the infused IgG fragments.[20-22] Another avenue taken to achieve intravenous tolerability was by irreversible or partially reversible chemical modification of the IgG molecules.[23-27] All these early IVIG products suffered from partial loss in their effector functions.[28]

The formulation of the first 'close to native' IVIG with full Fab and Fc effector functions was achieved mainly by five Swiss (Alfred Hässig, Silvio Barandun, Hans Nitschmann, Peter Kistler and Henri Isliker) after more than two decades worth of laboratory and clinical research. These five were embedded in a fertile environment of interest in protein chemistry and they had the same strong interest for fractionation methods of human plasma and in particular they were searching for an IgG concentrate with intravenous tolerability. The interest in protein chemistry was created by Alexander von Muralt, professor of physiology in Berne. Von Muralt stayed from 1928 to 1930 at the Harvard Medical School and was working in the department of Edwin Cohn.[29] After World War II, von Muralt was instrumental in helping Swiss science catch up with the level of the USA and Great Britain by motivating and supporting scientist to stay for a few years in US and UK laboratories. One of those scientists was Hans Nitschmann, professor of organic/protein chemistry who was studying cold ethanol fractionation in Cohn's laboratory. Back to Berne, together with his scholar Peter Kistler and the strong support of the Central Laboratory of the Swiss Red Cross Blood Transfusion Service, they developed the 'Kistler-Nitschmann-Lergier' cold ethanol fractionation method.[30,31] The widely used Cohn-Oncley fractionation method developed in Boston[15,16] provided plasma proteins at high purity at quite low recovery. In contrast the 'Kistler-Nitschmann-Lergier' method of cold ethanol fractionation provided better recoveries for "standard" IgG and albumin at somewhat lower purity and at considerably lower production cost. Alfred Hässig was the first head of the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. He had the mind of an MD deeply dedicated to patients and he was aware of the ethical dimensions of collecting blood. However, he combined dedication with a healthy sense for business. The precious nature of human blood/plasma as starting material for fractionation of plasma-derived medicinal products (stable blood products) was for him a driving force for the strong support of Kistler and Nitschmann[32] and very soon also the other Swiss listed above. The development of the Kistler-Nitschmann-Lergier cold-ethanol fractionation method by the Central Laboratory of the Swiss Red Cross in Berne allowed the sister Red Cross organizations to introduce this technique at no costs for development. The consequence was that plasma fractionation in post-war Europe was mainly performed by the Kistler-Nitschmann-Lergier technique in a non-for-profit environment of Red Cross organizations. In the USA, under the need for albumin on the battlefields, the transfer of the cold ethanol fractionation technique from Cohn's laboratory to Armour Pharma was completed already in 1943 and first reports on the use of "standard" IgG date back to 1944.[33] After the war plasma fractionation remained in an industrial for-profit environment. However, one exception existed: the American Red Cross let toll-fractionate US plasma in Berne. Independent of the fractionation method used, the final product was a 16% "standard" IgG concentrate for intramuscular application (IMIG).

In the early post WW II days of plasma fractionation albumin and blood coagulation factors were the main plasma products while "standard" IgG concentrate made only a small percent of what could have been obtained by the newly introduced 'Kistler-Nitschmann-Lergier' fractionation method. Alfred Hässig was concerned of discarding a large part of the human IgG because of lack of use and thus became the driving force in the second wave of plasma protein research, the development of a 'close to native' IVIG with full Fab and Fc effector functions. In Berne, Silvio Barandun had identified one of the two first antibody-deficient individuals in Switzerland and became interested in replacement therapy.[34] Barandun's patient, Albert L,[35] eventually became an employee of the Central Laboratory in Berne (sharpening needles for blood collection; needles at that time were reused). He got IgG replacement for free but had to accept the infusion of some experimental intravenous preparations what let him become quite an experienced person regarding AEs of intravenous replacement therapy. These AEs let rise the interest of Barandun in studying the mediators of AEs present in "standard" IgG concentrates which made them unsuitable for intravenous application. Barandun finally identified these mediators of early onset severe AEs as IgG aggregates which promoted spontaneous complement activation in the recipient's circulation.[18] It should be mentioned that Barandun was aware that there is also a late onset type of adverse events to immunoglobulin concentrates, which he termed a "phlogistic reaction." Decades later it was observed that at least in part such late onset adverse events were mediated by IgG dimer-induced release of pro-inflammatory cytokines like TNFα in the absence of relevant spontaneous complement activation. At IgG dimer levels of < 12% in IVIG such AEs could be avoided.[36,37]

The fifth person collaborating in the development of a 'close to native' IVIG was Henri Isliker who in the 1950s also visited the laboratory of Edwin Cohn. His strong skills in complement research rendered Isliker the perfect partner for Barandun. Together they showed that the aggregate content of "standard" IgG concentrate mediated spontaneous complement activation in the circulation of the recipients.[18] The effect was termed 'anti-complement activty' (ACA) and later the absence of such activity became a release criterion for IVIG.[38] The method how to get a 'close to native' immunoglobulin G concentrate suitable for intravenous application is an example of serendipity in research. Barandun and Isliker were doing in vitro experiments with pepsin treatment of "standard" IgG concentrate. One evening the technician of Isliker, Rudolf S., was in a hurry to get to a jazz concert. He lowered the pH of the IgG solution but forgot to add the pepsin. The incubation at low pH in the absence of pepsin resulted in an IgG solution devoid of ACA.[18] To make the low pH treatment a reproducible and stable step suitable for large scale fractionation, minute amounts of pepsin were reintroduced into the low pH process.[39] Adhering to the ideas of the Red Cross Organizations, the low pH/traces of pepsin treatment of IgG was never patented but was made available to sister organizations. IVIG which underwent correct low pH/traces of pepsin treatment never transmitted disease and the introduction of this production step was for Alfred Hässig the "lucky strike." The low pH treatment has remained to this day a valuable example of how to achieve pathogen safety of IVIG.

Having available an intravenously applicable immunoglobulin concentrate which did not undergo extensive enzymatic or chemical treatment during the production process and was a product containing IgGs with full biologic activity, including NAbs, allowed rapid infusion of relative high doses of NAbs. The observation by Imbach and colleagues[7] that IVIG was able to rise the thrombocyte counts in patients suffering from

immune thrombocytopenic purpura (ITP) was the first report on the effect of NAbs in an international journal. The publication opened a new and very fruitful field of research and clinical use of IVIG. Only in retrospect did it become apparent that the observation of the beneficial effect of IgG in ITP was made in Berne twice; the very first report by Gugler[40] was some 16 years before (as reviewed by Barandun et al.[41]). The Imbach publication showed the following principle of NAbs in a clinical setting: patients suffering from a pathological IgG-mediated autoimmune disease, i.e., ITP,[42,43] could benefit from normal IgG present in an IVIG which was obtained from a large pool of healthy donors. It was concluded that the transfused IgG (i.e., NAbs) from healthy individuals is able to inhibit the effects of pathogenic autoantibodies and that for the sake of benefit of the recipient the transfused IgG must be able to recognize the recipient's "immunological structures" in an allo-reactive manner. In turn the recipient's immune system recognizes the infused IgG molecules.

The publication by Imbach et al. set the dose of a treatment cycle at 2 g/kg b.w. In those initial years, having a 3% solution at hand, the total dose was given over 5 days, a quite convenient setting, i.e., Monday through Friday, guarantying freedom over the weekend for patient, nurses and treating physician. The background for the dose of 2 g/kg b.w. remains obscure. However, a major route of thinking was that the intravenous supply of a large amount of intact IgG (i.e.NAbs) should modify the immunologic condition of patients in such a way that pathological autoantibodies were displaced from the surface of platelets to a level which was clinically no more relevant.[41]

In 1984 the first specificity of NAbs as part of the immuno-modulatory effect of IVIG was reported.[44] In the development of the proof of the idiotype/anti-idiotype network concept (Id/anti-Id), as being a part of the mechanism of action of IVIG, Alfred Hässig was instrumental as well. In 1962 he was the laureate of the highest scientific award of Switzerland, the Benoist Prize (http://www.marcel-benoist.ch/index.php?option=com_content&task=view&id=91&Itemid=55). The Prize was awarded for his work on antigenic structures of paraproteins, among others.[45] He immunized rabbits with myeloma proteins and he showed that the rabbit antibodies in part recognized common structures however in part structures unique to individual myeloma proteins. At that time the terms of isotypes or idiotypes were not known yet. It is not clear when Hässig came across with Id/anti-Id network possibly being a mechanism of action of IVIG. However, in early spring 1980 he already talked about Id/anti-Id to PJS. On the initiative of Alfred Hässig later in the year 1980 Urs Nydegger joined the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. He in turn intensified his collaboration with Michel Kazatchkine. Nydegger and Kazatchkine became close friends in the late 70s while they were working in the laboratory of KF Austen, Boston. Hässig and Nydegger introduced Kazatchkine into the world of IVIG. Kazatchkine already before 1980 became interested in coagulation complications. The opportunity to compare plasma from spontaneously recovered and IVIG-induced anti-FVIII coagulation complications brought the breakthrough for establishing the mechanism of action of Id/anti-Id network.[44] Since the Sultan publication research on the mechanism of action and the clinical use of IVIG have followed the tow line of steadily growing knowledge in immunology. Today, the range of autoimmune diseases more or less successfully treated with high doses of IVIG is considerable.[46] There exists hesitation to accept that such a wide range of diseases can profit from the action of a "single" drug, like an immunoglobulin concentrate. In fact an immunoglobulin product is composed of an extremely high number of "different drugs," the immunoglobulin molecules with their extreme wide range of antibody specificities. Clinical efficacy

of immunoglobulin concentrates in various clinical conditions can be more easily understood when it is considered that such concentrates offer to all recipients the wide range of activities. The particular clinical efficacy is due to the fact that an individual patient profits from the action of that part of the IgG molecules in the immunoglobulin concentrate which he/she is missing and that the 'action' of the concentrate depends on the actual immunologic status of the patient.

THE ORIGIN OF NAbs AND THEIR FRACTIONATION INTO IMMUNOGLOBULIN CONCENTRATES

NAbs Producing Cells in Humans

Immunoglobulin concentrates derive from pooled plasma of healthy donors. The immunoglobulins in a plasma pool of healthy donors are believed to contain immune antibodies of the adaptive immune system and NAbs. Immune antibodies and NAbs are fundamentally different. NAbs, when defined as being physiologic and being able to bind to altered/senescent structures of the human body conserved during the evolution (molecular mimicry) are physiological self-reactive antibodies with tissue homeostatic activities while NAbs able to recognize the same conserved structures on pathogens are antibodies of the first-line-defense.[47] The challenging question is whether immune antibodies and NAbs are produced by fundamentally different B-cell subpopulations. The ultimate origin of B cells in humans is the fetal liver[48] and the omentum.[49] In human fetal bone marrow surface-bound IgM positive ($sIgM^+$) B lymphocytes are present by 17 weeks of development.[50,51]

During embryonic life of mice distinct waves of B cells are produced and B-cell subpopulations emerge which differ in their capacity to respond to antigens. At birth about 50% of the lymphocyte population is of the B1 phenotype[52]: $sIgM^{high}$, $sIgD^{low}$, $CD11b^{low}$. Particularly the $CD5^+$ B1 cells of the mouse, the B1a lymphocytes, are lacking mechanisms of V-gene rearrangement, isotype switching, somatic hypermutation and synthesize IgM in an immunization-independent manner.[53,54] The B1a cells of mice are the candidates for NAb producing cells. It is not entirely clear which B-cell subpopulation in humans might be equivalent to B1/B1a-lymphocytes.[55] $sIgM^+$, $CD5^+$ B cells appear by 14–24 weeks of embryonic life. Such cells account for about 40% of the B cells.[56] Furthermore, $sIgM^+$, $CD5^+$ B cells of the human fetal spleen secrete self-reactive IgM.[57] After birth a significant part of the $sIgM^+$, $CD5^+$ B lymphocytes are homed in the peritoneal cavity.[58]

In summary, the knowledge regarding the progenitors of NAb producing cells in humans, the innate-like B cells, remains quite limited. There is evidence that innate-like B cells in humans exist in istotype switched forms.

The Human Plasma as Starting Material for Immunoglobulin Concentrates Containing NAbs

Human plasma is available in quite large, still increasing but not unlimited quantities. The human origin adds some ethical aspects and declares plasma as a high-value starting material for fractionation. Furthermore, safety standards for blood collection and processing which have been established by authorities and the blood fractionation industry, makes plasma an expensive starting material. To ensure the best use of the

precious starting material, plasma has to be pooled to enable cost-effective processes. Pooling is considered essential in ensuring antibody diversity and immuno-modulatory potential of immunoglobulin concentrates. Complying with the directives of authorities, the pool size has to be within the limits of 1000 to 60,000 donors. Last but not least, ethical considerations and maintenance of economical fitness of manufacturers requires that from one liter of plasma as many different proteins should be isolated as ever possible.

Collection and processing of plasma have to follow strictly current Good Manufacturing Practice (cGMP) and the recommendations by authorities. Plasma can be collected either from whole blood after separation from blood cells or with help of machines which are returning the blood cells to the donor. Plasma obtained by the former method is termed 'recovered' plasma and accounts for about 25% of plasma collected worldwide. Plasma collected through apheresis is termed 'apheresis' or 'source' plasma and accounts for about 75% of all plasma collected worldwide. Differences between the two plasma types might exist, at least in protein contents of the plasma donated. More recent comparisons of the plasma protein content indicate that the type of collection is less decisive than the total volume donated per year and donor. Donation of recovered plasma is restricted to maximal 1.5 L per year per donor. In Europe the maximal volume of apheresis plasma allowed to be donated is 10–35 L per year and donor, depending of the member state, while in the USA up to 90 L per year and donor are allowed to be collected. Indeed, the immunoglobulin concentration drops following donation of high volumes by a single individual and the frequent donations might generate an acute-phase-like condition, as is seen by an increase of a known acute-phase protein, C1-esterase inhibitor.[59] It is not known whether the differences in protein content also influence the quality of immunoglobulin concentrates and particularly of NAbs. Based on studies on the strength of anti-measles virus antibodies in a single brand of IVIG there are hints that such differences might indeed transfer into a final product.[60] If one assumes NAbs to behave similarly as immune antibodies, qualitative differences might be expected for NAbs in immunoglobulin concentrates derived from plasma pools collected from high- and low-volume donors.

A particular starting material is plasma for polyclonal hyperimmune globulin preparations (hyper-Ig). The plasma origin of hyper Ig is either high-titer plasma selected from common blood donations or it derives from selected reconvalescent donors or from donors immunized and boosted. There exists evidence that the relative NAb content of high-titer plasma or plasma from individuals immunized and boosted differ compared with plasma of normal donors. It can be expected that following immunization or boosting the mechanisms of counter regulation let rise e.g., anti-idiotypic antibody titers and might have consequences on product characteristics.[61] To the best of our knowledge a clinical role of NAbs in hyper-Ig has not been investigated.

Possible Influences of Fractionation Techniques and Polishing Steps on the NAb Content of IVIG

Little is known whether the fractionation technique and the polishing steps applied to obtain immunoglobulin concentrates might influence the NAb content relative to that of the total immunoglobulin in the final preparation. Today there are two principal techniques of isolation of IgG in use, i.e., the cold ethanol fractionation and its variants[15,16,30,31] and the more recent chromatographic methods.[62-65] The latter allows to achieve an increase in recovery at high purity (Fig. 1).

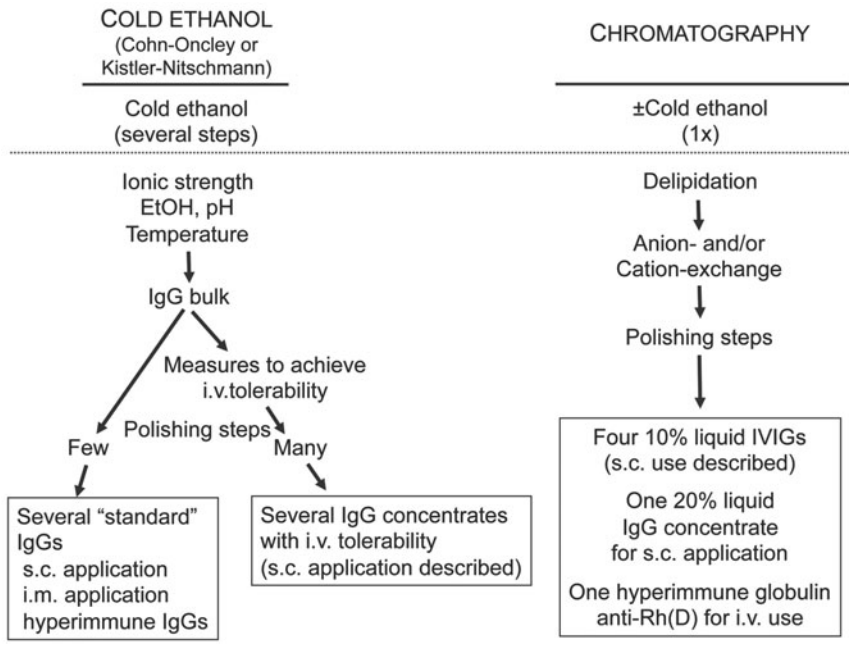

Figure 1. Outline of the various fractionation methods and the resulting immunoglobulin concentrates. In general fractionation starts with separation of cryoprecipitates from the starting plasma pool followed by the separation of albumin and immunoglobulins which is achieved by ethanol precipitation. The resulting gamma globulin paste can further be purified by several steps of cold ethanol precipitation following Cohn-Oncley[15,16] or Kistler-Nitschmann-Lergier.[30,31] The resulting "standard" IgG concentrate is a relative crude fraction. After application of a few polishing steps old-fashioned intramuscular, subcutaneous and hyperimmune preparations are obtained. These preparations are not suitable for intravenous use. The IgG bulk can undergo processes to reach intravenous tolerability (see Addendum for more details). IVIGs of our days undergo mild polishing procedures which ensure full functional integrity of the IgG molecules in the preparation. IVIG might be provided in lyophilized or liquid form and preparations have been/are applied at strengths of 3–12%. IVIGs also have been applied via the subcutaneous route. IgG paste undergoing the chromatographic avenue of purification first has to be delipidated. Ion-exchange chromatographic steps are followed by polishing steps in order to get the final product. The IVIG products are 10% ready-to-use solutions, while the unique chromatographic product tentatively developed for subcutaneous application is a 20% ready-to-use solution. There exists one chromatographically purified hyperimmune globulin anti-Rh(D). The purification process of the anti-Rh(D) takes advantage of the relative narrow range of isoelectric points of the anti-Rh(D) IgG molecules.

Despite the many efforts in optimizing protein recovery from the precious starting material, the pooled human plasma, the loss of IgG from fractionation step to fractionation step sums up to a total of ≥ 50%.[66] It is neither known whether during the fractionation and the polishing steps the relative loss of NAbs and that of immune antibodies is balanced, nor whether the loss varies form technique to technique. However, we like to report from two hints indicating that selected chromatographic production steps might result in enrichment or depletion of NAb populations relative to total IgG in IVIG. An antibody population can be enriched in case the population is restricted to a relative narrow range of isoelectric points. Applying the correct conditions, IgGs with isoelectric points outside the range are discarded while the population of interest is enriched. This principle is applied in a modern chromatographically purified anti-Rh(D) hyperimmune globulin.[62] Depletion

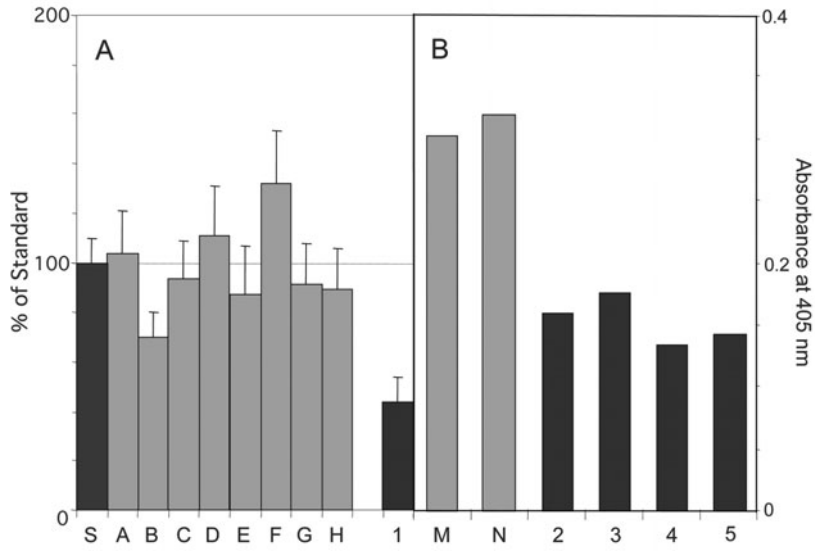

Figure 2. Examples of the effect of a polishing step on NAb content of the final product during production of IVIG. Two brands of IVIGs were analyzed for their content in anti-C3 NAbs.[68,69] The preparations were used without any further processing. An ELISA assay was performed as described elsewhere, using plates to which C3b was covalently bound[67] at an IgG concentration of 200 μg/mL. The purification of the IgG preparations (A-N, S) differed from those labeled 1–5 only in a chromatographic polishing step applied to No. 1–5, which was introduced in order to reduce the IgA content.[70] The introduction of the polishing step resulted in a diminution of the anti-C3 NAb content relative to that of total IgG. Panel A depicts standardized results, among which one lot was arbitrarily selected as standard (S). In a first run parent lots A-H were compared with a single lot of the polished product (1). Being aware of the pitfalls of misinterpretations of results due to lot-to-lot variations of anti-pathogen and immuno-modulatory antibodies[71] in a single brand of IVIG, the analysis of anti-C3 NAbs was extended to 4 polished preparations (2–5) (panel B). The reduced level of anti-C3 NAbs in the polished lots could be confirmed. Please note: in panel B no standardization was performed; the scaling of the ordinate is different from that in panel A.

of an antibody population by a particular polishing step during IgG fractionation might occur as well. We have compared various IVIG brands for their content in IgG anti-C3 NAbs that modulate complement C3 amplification as described elsewhere.[67-69] Among the various preparations analyzed, there were two which differed only by introduction of chromatographic polishing steps in order to reduce the content in IgA.[70] Several lots of the parent IVIG showed the inherent but limited lot-to lot fluctuation known to exist for anti-pathogen, immuno-modulatory and anti-inflammatory antibodies.[71] In a single lot of the preparation undergoing the additional chromatographic polishing step, the titer in anti-C3 NAbs was considerably lower (Fig. 2A). To make sure the difference was not just due to extreme lot-to-lot variation, the analysis was repeated with two lots of the parent product and four lots of polished preparations. The difference in titers of anti-C3 NAbs in the two products could be confirmed (Fig. 2B). Finally, it was possible to elute from the polishing chromatographic column beds IgG molecules highly enriched in anti-C3 NAbs (Fig. 3).

We conclude that donation practices, fractionation and polishing methods may have an effect on the NAb content relative to total IgG in an immunoglobulin concentrate.

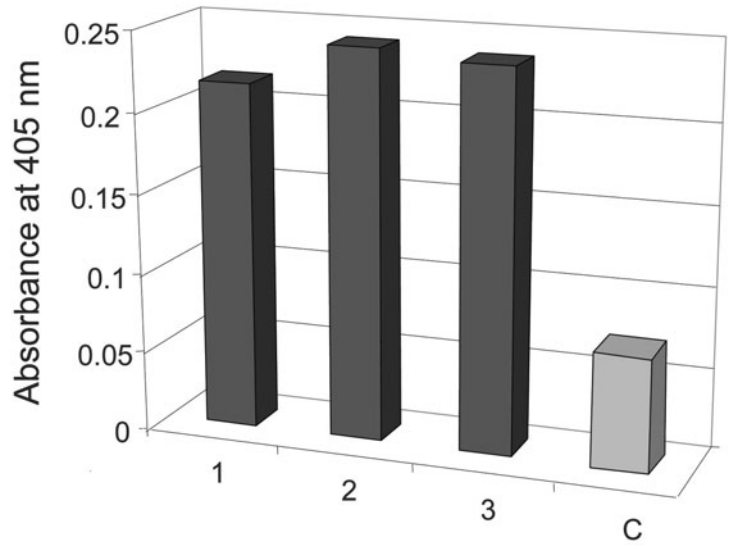

Figure 3. Enrichment of anti-C3 NAbs by ion-exchange chromatography introduced as polishing step to eliminate IgA from IVIG. Chromatography column beds from experiments shown in Figure 2 were acid-eluted and assayed for the relative content in anti-C3 NAbs. Results from three individual desorption experiments are shown (1–3). A lot of the parent IVIG served as control (C). The ELISA assay was performed with 182 μg /mL IgG of each preparation.

Some Aspects of NAbs in IVIG Produced by Large-Scale Fractionation

NAbs and Dimers in IVIG

As mentioned above, the interaction of exogenous NAbs with the recipient's immune system and tissue is in a strict sense an allo-immune type of reaction. This includes the Id/anti-Id interaction among infused IgG molecules and immunoglobulins of the recipient. In IVIG Id/anti-Id interactions can only occur between IgG molecules. These interactions are not observed in an immunoglobulin concentrate isolated from a single donor most likely because the counterparts being immunoglobulins of other Ig classes and these are removed during the fractionation process. However, as the number of donors increases from whom the plasma pool was generated, the number of molecules able to interact with each other is increasing due to alloimmune type of Id/anti-Id interactions. Thus, in IVIG from a single donor no dimers are found while with increasing numbers of donors the concentration of IgG dimers in the pooled plasma rises,[72] and such dimers can be found in IVIG.[73]

Dimer formation in liquid IVIG is a challenge for the fractionation industry. Immunoglobulin solutions, when stored, are forming an equilibrium between dimers monomers and probably oligomers.[74,75] Only a part of the dimer population is of the Id/anti-Id type. The other populations of dimers are most likely results of hydrophobic interactions of IgG molecules. Apparently IVIG with high dimer content is passing ACA release criterion because dimers are poor complement activators,[76] while dimers

apparently induce release of pro-inflammatory cytokines, particularly TNFα leading to severe adverse events in recipients.[36,77] On the one hand the challenge for the fractionating industry is to limit dimer/oligomer formation over the shelf-life of a product without removal of IgG molecules potentially able to form dimers because the IgG molecules able to form dimers might harbor a particular fraction of IgG, including NAbs, and Id/anti-Id dimers might be of advantage for the treatment of autoimmune and inflammatory diseases. Recently it emerged that the fraction of dimers in IVIG apparently have enriched some properties usually considered being of benefit for patients affected by autoimmune and inflammatory diseases[78-80] or for patients needing first-line defense because of infection.[80a] Thus, after the crude fractionation and the polishing steps appropriate stabilizers have to be added in order not to reduce the content in IgG molecules having the potential to form dimers, while keeping effective complex formation low. Two amino acids have proven efficacy in this regard: glycine and L-proline.

Terminal Sialic Acid on IgG and Immuno-Modulatory Potential

In recent years it emerged that some of the clinically well established, beneficial effects of IVIG might be due to particular carbohydrates in the Fc portion of the IgG molecules. For example, the anti-inflammatory effect of human IVIG in an animal model of ITP appears to be due to the presence of sialic acid residues within the biantennary carbohydrate bound to Asp 297 of the heavy chain (Fc portion).[81] The conclusion was based on sialic acid enriched IgG concentrate that was affinity purified from IVIG on *Sambucus nigra* lectin. The sialic acid enriched IgG concentrate revealed a 10-fold higher anti-inflammatory effect than the starting total IVIG.[82] Stadlmann et al.[83] showed, however, that IVIG binding to the *Sambucus nigra* lectin was dependent on N-glycans located in the variable region, while the N-glycans of the CH2 domain remained inaccessible to the lectin. Hence, it remains unclear whether the observed function of lectin-purified IgG concentrate is indeed dependent on sialic acid residues in the Fc-part of the IgG molecules. The lectin-purified IgG concentrate appears to induce an anti-inflammatory effect by binding to DC-SIGN instead of FcγRIIB.[84] However, others have shown that α2,6 sialylated IgG and DC SIGN are dispensable for the IVIG-mediated anti-inflammatory effect on human dendritic cells.[85] Others further demonstrated by using differently glycosylated mAbs that increasing levels in sialylation resulted in decreasing activity of antibody-dependent cellular cytotoxicity.[86] Glycosylation of IgG heavy chain will continue to provide difficulties, not only with regard to purification and synthesis, but also with regard to carbohydrate-imposed conformational properties. It is not known whether the accessibility/efficacy of the terminal sialic acid is the same or not whether located on certain IgG subclasses or particular IgG NAbs. This will certainly be a subject of research in the near future.

What Might Be the Clinically Appropriate Immuno-Modulatory Dose

The background for the immuno-modulatory dose of a treatment cycle of 2 g/kg b.w. remains obscure (see above), but it is believed to be the hallmark of clinical efficacy. It is of particular interest whether the repeated rapid doubling or even tripling of levels of circulating IgG in a single treatment course is or is not of fundamental importance for

obtaining a clinically sufficient immuno-modulatory effect. There is little doubt that the content of individual NAbs with an immunoregulatory potential in IVIG is low and this makes high dosing plausible. Studies comparing various doses for immunomodulation are scarce and the need to confirm the high dose is repeatedly expressed by clinicians. A corner point of immunomodulation by NAbs in IVIG is ITP. Godeau and collaborators found that the initial treatment with 1 g/kg b.w. of IVIG appeared to be more effective than 0.5 g/kg b.w. in adults with acute ITP and that some adults who did not respond to 1 g IgG/ kg b.w. responded to an even higher dose.[87] Benesch and collaborators in a study of pediatric ITP confirmed this observation by reporting that "for patients with very low platelet counts, doses higher than 0.6 g/kg seem to be more effective".[88] In one of the very rare clinical trials studying 'personalized' doses for ITP an initial dose of 0.8 g/kg body weight was comparably efficient to standard dosing when those patients showing no sufficient platelet count rise were re-treated on day 3.[89] Last but not least an IgG level in serum close to normal or within the normal range apparently is not sufficient to prevent ITP in patients suffering from primary antibody deficiency.[90-92] Another cornerstone for an immuno-modulatory and anti-inflammatory effect of NAbs in IVIG is Kawasaki disease (KD). In KD a minimal effective dose of 1 g IgG/kg b.w. was found while 2 g IgG/kg b.w. was more effective.[93] Others confirmed in KD the minimal effective dose of 1g IgG/kg body weight.[94] There are hints that not the total dose given at one cycle might be the only decisive parameter for clinical outcome but also the kinetic of increase of exogenous IgG in the circulation. In KD patients the infusion of a total dose of 1.6 g IgG/kg b.w. in 4 daily doses was less efficient than one single shot of 1 g IgG/kg body weight.[95] Taking into account results from other autoimmune and inflammatory diseases benefiting from NAbs in IVIG, the rapid and sufficient elevation of NAbs in the circulation seems to be the key for clinical efficacy. There is some chance to confirm or withdraw the clinical importance of peaking IgG and/or maintaining high IgG levels in the circulation for immunomodulation by dividing the intravenous dose given all three to four weeks into aliquots given subcutaneously e.g. once a week (SCIG). The results of the increasing number of reported studies treating various chronic inflammatory autoimmune conditions with SCIG will provide new insights.[96-107] Applying SCIG does not lead to peaking of IgG in the circulation, but does increase circulating IgG. First results seem to indicate that maintenance of IgG levels in the circulation close or above the normal range might be beneficial in some chronic autoimmune and inflammatory conditions.

CONCLUSION

The route leading to the detection of a clinical benefit from the NAb-containing polyclonal, polyspecific immunoglobulin concentrate was a long, hard clinical and scientific work and was helped by serendipidity. Low adverse event profiles in chronic autoimmune and inflammatory diseases, despite the high costs, render the clinical use of IVIG and its NAbs attractive.

DEDICATION

We like to dedicate this book chapter to late professor Alfred Hässig, who was a great mentor of our work. Alfred Hässig strongly and continuously supported our scientific work. A part of his always generous support was a huge amount of outdated IVIG for free.

ADDENDUM

Hints and reports on the presence of functional NAbs in polyclonal, polyspecific human immunoglobulin concentrates with an IgG content > 93%. The table does not list all related publications which might have been published. For details on fractionation, see also Figure 1.

Type of Immunoglobulin Concentrate[a]	Hints and Reports for the Presence of Functional NAbs
Early IgG Concentrates not Suitable for Intravenous Use[15,16,31]	
"Standard" IgG for intramuscular or subcutaneous application[108-113,b]	*Direct, retrospectively recognized demonstration of NAbs:* • First description of IgG-induced platelet rise in an ITP patient[40] *Indirect evidence for or against functional NAbs* • Effect reported for chronic autoimmune peripheral neuropathies[97]
Hyperimmune globulins of the first generation for intramuscular or subcutaneous application[b]	*Direct demonstration of NAbs:* • Anti-idiotypic antibodies in anti-Rh(D) hyperimmune globulin[61] *Indirect evidence for or against functional NAbs from clinical trials* • No effect seen in one patient with chronic inflammatory demyelinating polyradiculoneuropathy[114] • Effect reported in one patient with autoimmune neutropenia[115]
Modified Immunoglobulin Concentrates Suitable for Intravenous Use	
Enzymatically digested[c] • Pepsin treatment[20] • Plasmin treatment[22]	PEPSIN-TREATED: *Direct demonstration of functional NAbs:* • Inhibition of the binding of anti-gpIIb-IIIa anti-platelet antibodies[116] • Diminished inhibition of FcγRII-dependent platelet activation[117]

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Addendum (continued)

Type of Immunoglobulin Concentrate[a]	Hints and Reports for the Presence of Functional NAbs
Modified Immunoglobulin Concentrates Suitable for Intravenous Use (continued)	
	Indirect evidence for or against functional NAbs from in vitro and clinical trials: • 5S IgG showed beneficial effects in sepsis/SIRS,[118] presumably by complexing anti-hinge NAbs[119] • Abrogated effects on DC maturation and function otherwise seen with intact IgG[120] • Effect reported in ITP; in some studies the effect is partially lost[121-123] • No effect in myasthenia gravis[124] • Ineffective in Kawasaki disease[93] *Comment:* • NAb activity must be impaired in case Fc-effector function is part of the biologic activity PLASMIN-TREATED *Direct demonstration of NAbs:* • Ex vivo effect on T cells subsets[125] *Indirect evidence for or against functional NAbs from clinical trials:* • Partial to full effect in acute or chronic ITP[121,125-127] • Limited effect in one patient with AIHA[128] • Some effect in Kawasaki disease[129]
Chemically modified[c] • Limited S-sulfonation[24,130] • Reduction or reduction and alkylation[23,25,27] • β-propiolacton[26] (beside IVIG several hyperimmune globulins)	S-sulfonated IVIG *Direct demonstration of NAbs:* • Ex vivo effect on T cells subsets[125] • Inhibition of the binding of anti-gpIIb-IIIa anti-platelet antibodies[116] *Indirect evidence for or against functional NAbs from clinical trials:* • Can mediate platelet count rise in ITP patients[121,126] • Effect reported in Kawasaki disease[131] • Effect reported in Guillain-Barré syndrome[132] REDUCED/ALKYLATED IVIG *Direct demonstration of NAbs:* • Not reported *Indirect evidence for or against functional NAbs from clinical trials:* • Effect reported in chronic ITP[133] β-PROPIOLACTON-TREATED IVIG

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Addendum (continued)

Type of Immunoglobulin Concentrate[a]	Hints and Reports for the Presence of Functional NAbs
	Direct demonstration of functional NAbs: • Diminished inhibition of FcγRII-dependent platelet activation[117] *Indirect evidence for or against functional NAbs from clinical trials:* • Effect reported in myasthenia gravis[124] • Effect reported in post-transfusion purpura[134] • Effect reported in Guillain-Barré syndrome[135] • Reduced[123] or similar effect[133] in ITP when compared with other IVIG brands • Inferior to other IVIGs in Kawasaki disease[136,137]
'Unmodified' Immunoglobulin Concentrates for Intravenous Use (the IVIGs)	
• Manufacturing entirely by one of the variations of the cold ethanol fractionation[d] • Manufacturing predominantly by chromatography[63;138;139,e]	IVIGs Obtained by Cold Ethanol Fractionation • *Direct demonstration* of a wide range of NAb specificities (among others: see chapters of this book) • *Evidence based* support for presence of functional NAbs (among others: see chapters of this book) IVIGs Manufactured Predominantly by Column Chromatography *Direct demonstration of NAbs:* • Efficacy reported in a cell culture model of immune neuropathy[140] • Presence of $A\beta_{1-42}$ antibodies[141] • Anti-HLA antibodies and inhibition of C3 deposition[142] • Anti-α-synulcein antibodies[143] • Maturation of TLR 4-primed peripheral blood monocytes[120] • Antiinflammatory effect[144] • CD200-dependent suppression NK cell cytolytic activity in vitro[145] *Indirect evidence for or against functional NAbs from clinical trials:* • Case report of effect in fulminant myocarditis[146] • Randomized controlled study shows efficacy in chronic inflammatory demyelinating radiculoneuropathy[64] • Dose finding study shows no efficacy in relapsing-remitting multiple sclerosis[147]

continued on next page

Addendum (continued)

Type of Immunoglobulin Concentrate[a]	Hints and Reports for the Presence of Functional NAbs
'Modern' Immunoglobulin Concentrates for Subcutaneous application	
Subcutaneous application of modern IVIGs in clinical trials[148–151,e]	*Direct demonstration of NAbs:* • See 'unmodified' immunoglobulin concentrates for intravenous use *Indirect evidence for or against functional NAbs from clinical trials* • Not reported
Developed for subcutaneous application exclusively[152-156,f]	*Direct demonstration of NAbs:* • See 'unmodified' IVIGs *Indirect evidence for functional NAbs:* • Support for efficacy by results from treating chronic autoimmune and inflammatory conditions[96-101,103-107,157,158]

AIHA: autoimmune hemolytic anemia
ITP: Immune thrombocytopenia
NK cell: natural killer cell
PTP: post-transfusion purpura
TLR: toll-like receptor

[a]Parameters of early IgG concentrates can be obtained from Römer et al.[159,160]
[b]Provided as 16% solutions for clinical use
[c]Marketed to be used in a strength of 5% or 10%; irreversible chemical modification by β-propiolactone in order to achieve intravenous tolerability or partially reversible chemical modification by limited S-sulfonation or reduction/alkylation
[d]In clinical use in a range of strength of 3–12%; lyophilized or liquid formulation provided by several manufacturers
[e]Liquid prepartions for clinical use as 10% solutions
[f]Liquid preparations for subcutaneous application at 10%, 16% and 20% protein strength

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