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ORIGINAL ARTICLE

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Antibodies as defensive enzymes

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Abstract Antibodies (Abs) and enzymes are structural and functional relatives. Abs with promiscuous peptidase activity are ubiquitous in healthy humans, evidently derived from germline variable domain immunoglobulin genes encoding the serine protease-like nucleophilic function. Exogenous and endogenous electrophilic antigens can bind the nucleophilic sites covalently, and recent evidence suggests that immunization with such antigens can induce proteolytic antibodies. Previously, Ab catalytic activities have been linked to pathogenic autoimmune reactions, but recent studies indicate that proteolytic Abs may also serve beneficial functions. An example is the rapid and selective cleavage of the HIV-1 coat protein gp120 by IgMs found in uninfected humans. The selectivity of this reaction appears to derive from recognition of gp120 as a superantigen. A second example is the cleavage of amyloid β-peptide by IgM and IgG from aged humans, a phenomenon that may represent a specific proteolytic response to a neurotoxic endogenous peptide implicated in the pathogenesis of Alzheimer's disease.

Keywords Abzymes · Catalytic antibodies · Serine proteases · Autoimmune disease · HIV infection

The antibody-enzyme continuum

Antibodies (Abs) and enzymes are made up of the same building blocks, the 20 common amino acids, arranged as unique linear sequences that generate discrete three-dimensional

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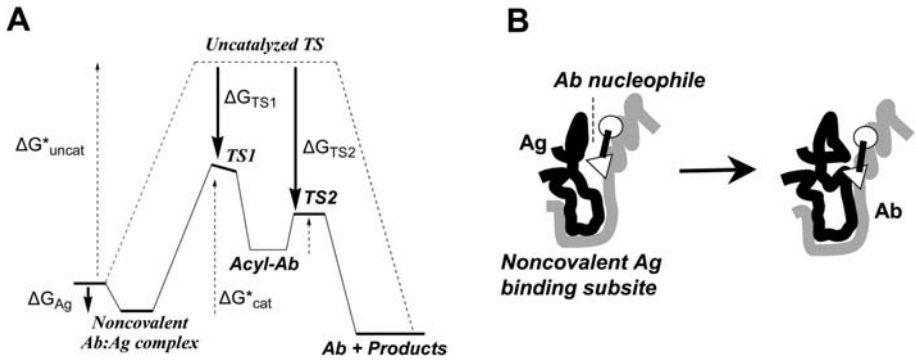

Fig. 1 **A** Energetics of peptide bond hydrolysis by Abs. Abs stabilize the antigen ground state noncovalently (ΔG_{Ag}). An unstable resonant transition state (*TS1*) is formed by nucleophilic attack resulting in generation of the covalent acyl-Ab intermediate. In the second reaction, a second tetrahedral transition state (*TS2*) is formed en route to hydrolysis of the intermediate and product release. $\Delta G^{\dagger}_{uncat}$ and $\Delta G^{\dagger}_{cat}$ correspond to activation energies for the uncatalyzed and catalyzed reactions, respectively. K_m is a function of the extent of ground state stabilization (ΔG_S). k_{cat}/K_m is a function of extent of transition state stabilization relative to the catalyst-substrate ground state complex. **B** Two-step antigen recognition by proteolytic Abs. Intramolecular interactions impart nucleophilicity to certain residues (*triangle*: nucleophile, *circle*: activating group). Initial noncovalent binding does not involve the Ab nucleophile. In the second step of the reaction, the nucleophile forms covalent complex with the antigens at a site distant from the regions responsible for noncovalent antigen binding (*Ab* antibody, *Ag* antigen)

structures capable of supporting diverse types of interactions with ligands. Abs bind and stabilize the ground state of antigens reversibly, with the affinity of binding determining the extent to which the reactants exist in free form or as immune complexes. To catalyze the chemical transformation of the antigen, an Ab must stabilize the high-energy transition state more than the ground state, resulting in reduced activation energy and acceleration in the reaction rate (Fig. 1). The ground and transition states are structurally similar except for the immediate site of the chemical reaction. In flexible substrates like polypeptides, structural changes remote from the reaction site are feasible. During peptide bond hydrolysis, for example, the planar carbonyl group converts to a tetrahedral configuration with a negatively charged oxygen atom as the transition state develops. As the double-bond character of the C-N bond diminishes, free rotation around the bond becomes more permissible, and peptide backbone and side chain movements remote from the cleavage site occur in the transition state [54], with the resultant generation of transition state-specific neoepitopes available for Ab recognition.

The genes for Abs and enzymes have undoubtedly been subjected to distinct evolutionary pressures over millions of years. The biological purpose of these molecules in higher organisms is distinguishable in a broad sense, i.e., a defense mechanism against microbial infection for the former and chemical transformation of substrates for the latter. This view, however, is overly rigid, and recent theoretical and empirical developments suggest that a less restrictive interpretation of the relationship between Abs and enzymes may be useful.

The notion that higher organisms respond to changing environmental circumstances by adaptive development of new catalysts in real-time is by no means new. In 1912, it was suggested that animals and humans synthesize proteases as a defensive response to challenge with foreign proteins [1], an idea that was held to be scientifically unsupportable in a recent critique [13]. Uncontroversial evidence for the enzymatic activity of Abs was first published in the 1970s in reports noting the sequence homologies between certain proteas-

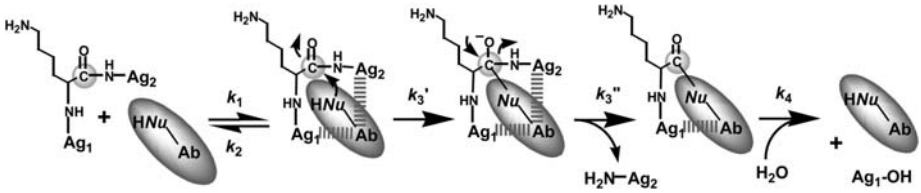

Fig. 2 Reaction mechanism of Ab catalyzed polypeptide hydrolysis. *Nu*: nucleophile; *Ag1-OH*: N-terminal antigen fragment; *NH2-Ag2*: C-terminal antigen fragment; $K_d = k_2/k_1$; k_{cat} (turnover number) is determined by the rate-limiting step, i.e., the smallest of k_3, k_3'' and k_4. The active site nucleophile attacks the carbonyl carbon of the scissile bond(s) in the antigen, forming the tetrahedral transition-state complex. The C-terminal antigen fragment is released and the acyl-Ab complex is formed. Hydrolysis of the acyl-Ab complex results in release of the N-terminal antigen fragment and regeneration of the catalysts

es and Bence Jones proteins {monoclonal Ab (mAb) light chains produced by multiple myeloma patients; [16]}, and the transaminase [65] and esterase [32] activities of polyclonal Abs raised by immunization with antigen ground states.

Empirical evidence for proteolysis by Abs was first obtained using autoantibody preparations [59]. Subsequently, additional reports have suggested an association between other immunological disorders and Ab catalysis. Intact Abs and Ab light chains with protease, nuclease and amylolytic activity have been identified in autoimmune disease [4, 67, 68], lymphoproliferative states [43, 56] and alloimmune reactions [36]. Examples of antigens that can be utilized as substrates by these Abs include peptides like vasoactive intestinal peptide [4] and Arg-vasopressin [42], proteins such as thyroglobulin [38] and Factor VIII [36], DNA [68], RNA [48] and polysaccharides [67].

Unlike the rare occurrence of antigen-specific Ab proteases, it appears that promiscuous peptidases are intrinsic components of the immune repertoire. Readily detectable hydrolysis of model tripeptide and tetrapeptide substrates by Abs from healthy humans and unimmunized mice has been reported, particularly for IgMs, the first Ab isotype produced in the course of B cell differentiation [29, 60]. The chemical reactivity of the Abs from healthy individuals probably extends beyond peptide bond hydrolyzing activity, evident from observations that human milk contains IgAs with protein kinase activity [49], and all of randomly picked mAbs catalyze hydrogen peroxide synthesis [85]. These observations indicate that catalytic activities can arise in Abs by fully natural processes.

Catalytic mechanism

Formation of covalent intermediates is an established catalytic pathway for enzymes as diverse as serine proteases, glycosidases, lipases and synthases [17, 28, 80]. Underlying the reaction is the presence of unusually strong nucleophilic groups capable of attacking the weakly electrophilic sites in the substrate, e.g., the carbon atom of the peptide bond (Fig. 2). The hydroxyl side chain of serine residues, for example, ordinarily displays poor reactivity, but hydrogen bonding with His/Asp residues within the catalytic triad of serine proteases confers increased nucleophilic reactivity to this group. As precise positioning of certain residues is necessary for the intramolecular activation of the nucleophiles, it has commonly been assumed that activated nucleophiles are formed only rarely in proteins, and when present, the sites must be indicative of enzymatic activity.

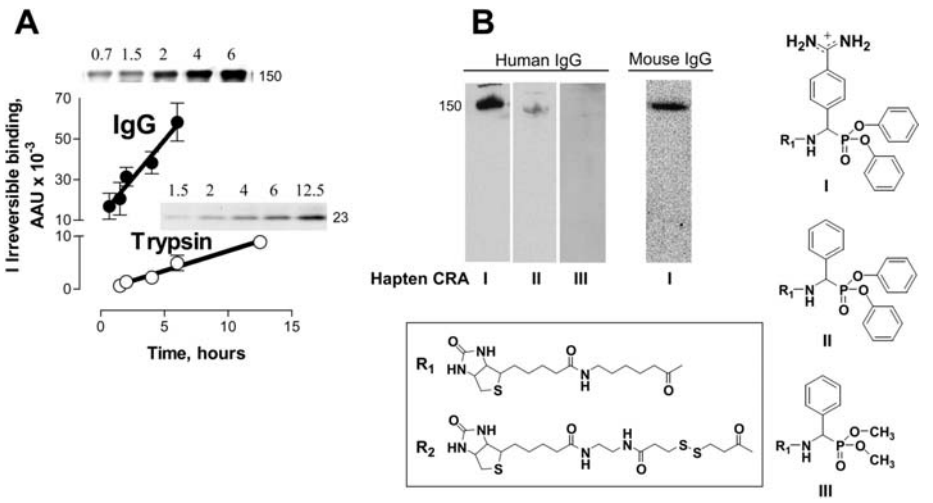

Fig. 3 Hapten CRA binding by IgG and trypsin. **A** Time course of IgG-hapten CRA I and trypsin-I binding. Y-axis values are intensities of the 150-kDa (IgG) or 23-kDa (trypsin) adduct bands expressed in arbitrary area units (*AAU*). CRA 1, 100 μM. *Inset*, streptavidin-peroxidase-stained blots of SDS-polyacrylamide gels showing biotin-containing adducts (*top*, IgG; *bottom*, trypsin). **B** Representative streptavidin-peroxidase-stained blots of SDS-polyacrylamide gels showing adducts of I with human and murine (BALB/c) serum IgG (1 μM). A weak reaction of IgG with II was observed by exposing the gel for a prolonged period (4 h) and no reaction with III was evident. Hapten CRA, 10 μM, 60 min. Data from [61] (*CRA* covalently reactive analog)

Recently, we employed small, electrophilic phosphonate diesters (haptens) as probes for the presence of nucleophilic sites in Abs. Like peptide substrates, these compounds react covalently with activated nucleophiles [52], but unlike the acyl-enzyme adduct, the covalent phosphonyl-enzyme adduct is generally not hydrolyzed, permitting detection of the phosphonylated complexes by techniques such as denaturing electrophoresis. The hapten phosphonate diester displayed irreversible binding to every IgM and IgG studied [60, 61]. The reaction occurred at rates comparable to trypsin (Fig. 3) and IgMs displayed nucleophilic reactivity exceeding the IgGs. Similarly, each of 15 randomly picked single chain Fv constructs (scFv; VL and VH domains linked by a short peptide) from a human library formed the covalent adducts, suggesting that the nucleophilic reactivity is a V domain property [61]. Contrary to the initial assumption, it may be concluded that nucleophilic sites are widely distributed in Abs.

The importance of the nucleophilic reactivity in catalysis is evident from observations that the phosphonate diesters inhibited the proteolytic activity of a variety of catalytic Abs [4, 58, 60, 61]. Site-directed mutagenesis of the L chain subunit of a VIP-cleaving Ab has provided information about the structure of the nucleophilic site. Replacement of L chain residues Ser27a, His 93 or Asp1 produced a loss of proteolytic activity [18, 20]. Molecular modeling suggested that these residues are positioned appropriately to permit hydrogen bonding between the Ser oxygen, the His imidazole and the Asp carboxyl oxygen. This Ab appears to utilize a catalytic mechanism akin to that of serine proteases. A similar mechanism has also been deduced for other proteolytic Abs (e.g., [44]). It is important to note, however, that Abs can develop structurally divergent nucleophilic sites. This is feasible because of the considerable sequence diversity encountered in V genes belonging to

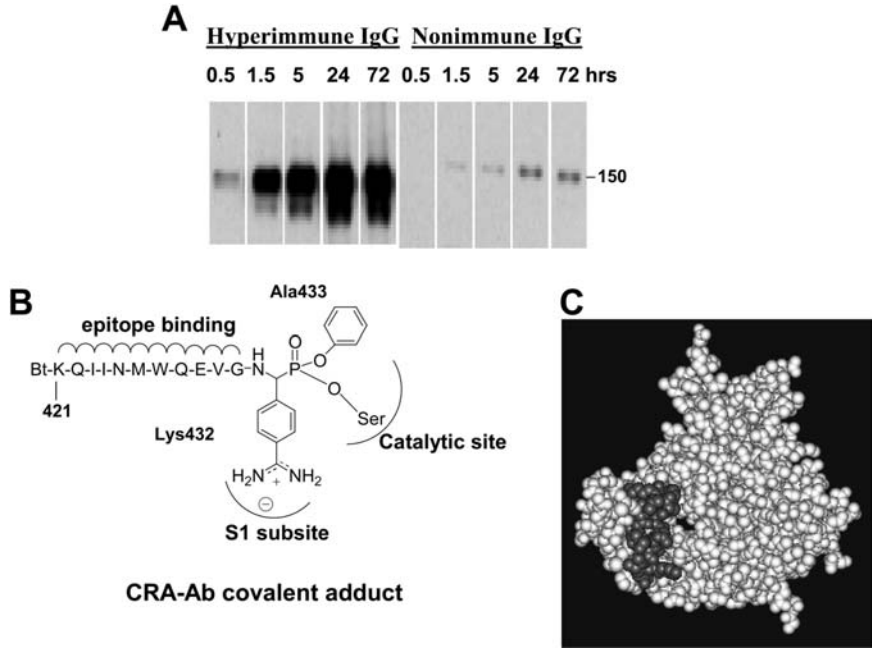

Fig. 4 Specific irreversible binding of gp120(421–433)-CRA by Abs to gp120(421–436). A Time course of formation of adducts of CRA (10 μM) incubated with IgG to gp120(421–436) (1 μM). Shown are streptavidin-peroxidase-stained 152-kDa adducts in nonreducing SDS-electrophoresis gels formed with anti-gp120(421–436) IgG and equivalent concentrations of nonimmune IgG. Data from [61]. **B** Model of noncovalent and nucleophilic binding between the peptidyl CRA and the Ab combining site. **C** Residues 421–433 (*dark gray*) of gp120 from the published crystal structure of the protein [35]

different germline families and introduction of further diversity by adaptive maturational processes. Residues other than Ser are known to serve as the nucleophiles if properly activated by spatially neighboring groups. For example, Tyr and Thr hydroxyl groups and Lys amine groups serve as activated nucleophiles in certain enzymes [26, 83].

Specific, high-affinity recognition of individual antigens is a distinguishing feature of mature Abs. The haptenic phosphonate diesters do not bind Abs with the high affinity typical of polypeptide antigens. Polypeptide covalently reactive analogs (CRAs) containing the phosphonate group located within peptide epitopes have been designed to study the extent to which Ab nucleophilic reactivity is coordinated with non-covalent antigen recognition (Fig. 4). Abs raised to a synthetic peptide of the HIV coat protein gp120, the tumor-associated antigen epidermal growth factor receptor (EGFR) and VIP are described to bind covalently to the CRA derivatives of the gp120 peptide, soluble EGFR and VIP, respectively [50, 61, 72]. The Abs reacted with the polypeptide CRAs at rates exceeding the reaction with the hapten phosphonate by several orders of magnitude. These findings suggest that the nucleophilic site is located within the larger antigen binding site at which noncovalent interactions responsible for traditional high-affinity Ab-antigen reactions occur.

Despite their robust nucleophilic reactivity, no detectable cleavage of the specific antigens recognized by several anti-gp120 and anti-EGFR Abs was evident [61]. Similarly,

notwithstanding their excellent reactivity with haptenic phosphonate diesters, the proteolytic reactivity of the Abs detected using model peptide substrates was several orders of magnitude smaller than conventional non-Ab proteases. This may be understood from consideration of the catalytic mechanism. The nucleophilic reactivity is a necessary but not sufficient condition for proteolysis, because completion of the catalytic cycle entails additional events after formation of the acyl-Ab intermediate, i.e., hydrolysis of the intermediate and product release (Fig. 2). Thus, the nucleophilic reactivity is broadly distributed in Abs, but only a small population of Abs expresses antigen-specific proteolytic activity.

Innate and adaptive components of Ab chemical reactivity

Formal proof that proteolysis is an innate immunity function came from observations that the catalytic residues of the VIPase light chain cited in the preceding section are encoded by a germline V_L gene (Ser27a, His93, Asp1) [20]. The adaptively matured light chain displaying the activity was observed to contain four replacement mutations compared to the germline protein at positions remote from the catalytic site. These residues were reverted by mutagenesis to the germline configuration protein without evident loss of proteolytic activity.

B cells undergo activation by binding of the antigen to the B cell receptor (BCR; surface Ig associated with Igα/Igβ). Igμ chain-containing BCRs expressed on splenic B cells were observed to account for most of the nucleophilic reactivity of the cells, revealed from study of the irreversible binding of haptenic phosphonate diesters using flow cytometry, microscopy (Fig. 5) and denaturing electrophoresis methods (Fig. 6) [60]. Just as noncovalent antigen binding induces B cell differentiation, nucleophilic BCR stimulation holds the potential of inducing cell maturation. The phosphonate diesters are serine protease inhibitors modeled on the structure of diisopropylfluorophosphate (DFP). DFP is reported to inhibit mitogen-induced B cell division [27, 33]. The DFP-sensitive activity was cell-associated and preferred Arg-containing substrates [34], but its molecular identity was not determined. B cell signal transduction induced by Abs to μ chains was also inhibited by serine protease inhibitors [45], and the anti-μ Ab treatment resulted in appearance of a serine protease activity on the cell surface [7]. These considerations suggest the hypothesis that nucleophilic stimulation of BCRs by endogenous or exogenous covalently reactive ligands may drive the B cells into maturational pathways (Fig. 7).

Thus, there is no bar to adaptive improvement of the nucleophilic sites in germline-encoded Abs. On the other hand, the generation of antigen-specific proteolytic Abs may be limited by processes that govern B cell maturation over the course of the immune response. When the BCR is occupied by the antigen, B cells are driven into the clonal selection pathway. Rapid catalysis by the BCR is predicted to result in release of the antigen fragments, depriving the B cells of the stimulatory signal necessary for their proliferation. Under normal circumstances, therefore, the catalytic activity can be improved by adaptive maturational processes only to the extent that the rate of product release remains slower than the rate of BCR transmembrane signaling necessary to induce cell division.

Per unit valency, IgM Abs have been observed to express proteolytic activities two to three orders of magnitude superior to IgG Abs [60]. The catalysis assays were conducted in solution phase and at excess concentrations of the substrate. These conditions do not

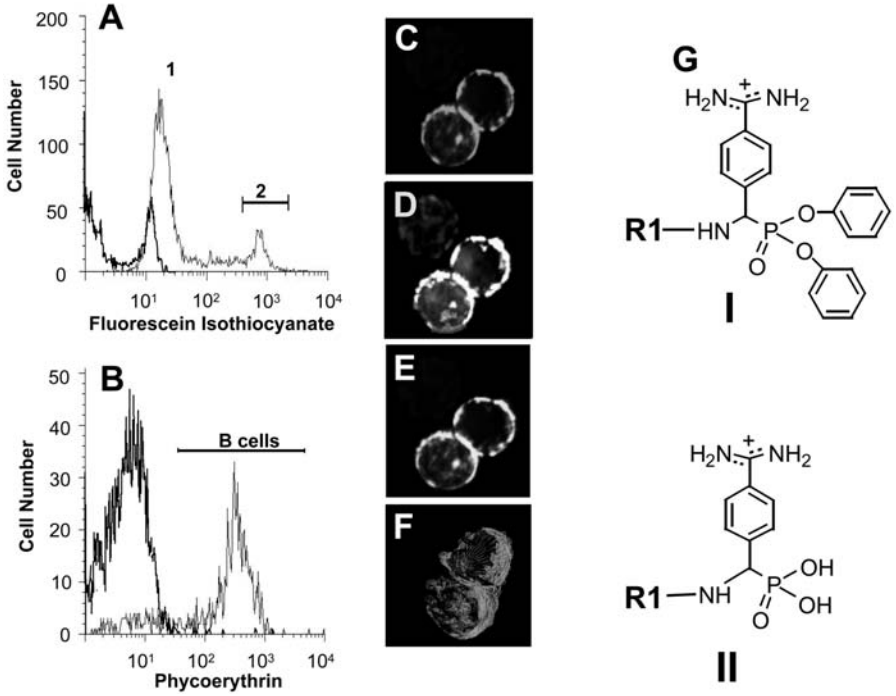

Fig. 5 Covalent reactivity of B cell surface Ig. Hapten CRA I reactivity with spleen cells. **A** Flow cytometry of murine splenocytes (naïve BALB/c mouse) stained with biotinylated hapten CRA I (*gray line*) and compound II (*black line*; both compounds 100 μM, 4 h; streptavidin-FITC (50 μg/ml). Twenty five thousand cells counted. **B** Anti-CD19 Ab staining (*gray line*; phycoerythrin conjugate) of hapten CRA I-labeled cells; streptavidin-FITC 1 μg/ml). *Black line* shows staining with the phycoerythrin conjugate of the isotype-matched control antibody. **C–F** Deconvoluted (five iterations) fluorescence acquisitions showing two B cells labeled with CRA I (streptavidin-FITC, 1 μg/ml, **C**) and phycoerythrin-conjugated anti-CD19 Ab (**D**). **E** shows a merged rendition of the FITC and phycoerythrin probes. **F** is a 3D wire frame model of the FITC emission patterns compiled from 30 individual sections and then subjected to split screen extraction. Blue counterstain, 4′,6-diamidino-2-phenylindole. **G** Hapten CRA I and compound II. From [60]

support binding of a single peptide molecule by more than one Ab valency. Therefore, multivalent binding by non-interacting sites (avidity effects) does not account for the superior activity of decavalent IgM compared to the divalent IgG. The following explanations for the superior IgM activity are tenable. First, loss of catalytic activity may be attendant to V domain somatic diversification after isotype switching from IgM to IgG. Second, distinctive IgM constant domain characteristics may be important in maintaining the integrity of the catalytic site, in which case isotype switching itself may result in reduced catalytic activity. These explanations are not mutually exclusive. Both explanations are consistent with the argument that catalysis is a disfavored phenomenon in the advanced stages of B cell development (as efficient BCR catalysis will result in reduced BCR occupancy). Monovalent Fab prepared from the IgM displayed reduced proteolytic activity, suggesting that maintenance of the constant domain architecture of IgM may be important for proteolysis [55, 60]. Altered antigen binding activities of the same V domains expressed as full-length Abs belonging to different IgG isotypes have been described (e.g.,

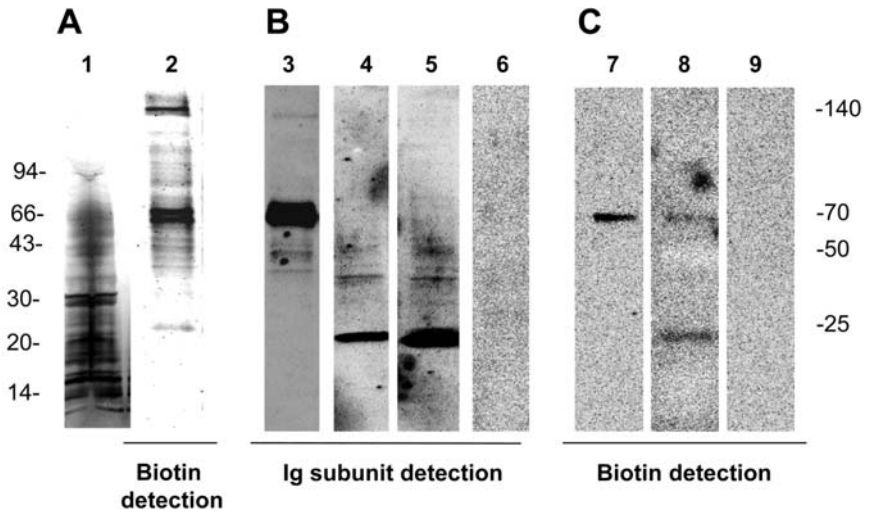

Fig. 6 Immunochemical identification of hapten CRA I-labeled Ig subunits in B cell extracts. **A** SDS-gel electrophoresis lanes showing extract of B cells labeled with hapten CRA I (100 μM, 4 h) following staining with silver (*lane 1*) and peroxidase-conjugated streptavidin (*lane 2*). Migration of marker proteins shown on left. **B** SDS-gel immunoblots of hapten CRA I-labeled B cell extract stained with Abs to μ (*lane 3*), λ (*lane 4*), κ (*lane 5*) and γ (*lane 6*) chains. **C** Streptavidin-peroxidase-stained SDS-gels showing hapten CRA I-labeled proteins recovered by affinity chromatography of splenocyte extract on immobilized anti-μ (*lane 7*), anti-κ/λ (*lane 8*) and anti-γ Abs (*lane 9*). From [60]

[46]). Similarly, allosteric combining site activation due to filling of the individual IgG valencies has been proposed previously [79]. The temporal sequence of events as the individual IgM combining sites bind antigen has not been elucidated. At excess antigen, only five of the ten IgM combining sites are thought to be filled (e.g., 10), suggesting that favorable allosteric effects on antigen binding, if present, must be restricted to conditions of limiting antigen concentrations.

The design of immunogens that induce catalytic Ab synthesis on demand is an important research area, holding the potential of yielding catalysts that could be employed to inactivate undesirable antigenic targets. Such immunogens should not themselves be hydrolyzed by catalytic BCRs so that B cell clonal abortion can be avoided. Initial hapten immunogens proposed as the route for generating transacylase Abs with protease and esterase activities mimicked the reaction transition state by means of an oxyanion attached to a tetrahedral phosphorus atom [77]. This design predicts the de novo formation of an oxyanion stabilizing structure over the course of adaptive V domain diversification that mediates catalysis exclusively by noncovalent stabilization of the reaction transition state. In our own approach, we showed the feasibility of improving the natural nucleophilic reactivity of Abs by means of immunization with the CRAs. Mice immunized with a gp120-CRA preparation produced Abs that recognized gp120 specifically and proceeded to hydrolyze this protein [57]. Previously, immunization with a hapten phosphonate diester was reported to induce Abs with aldolase activity [81]. Importantly, the increased nucleophilicity of the Abs obtained by this approach can only improve the initial step in the catalytic cycle, i.e., the formation of the covalent intermediate. Incorporation of additional

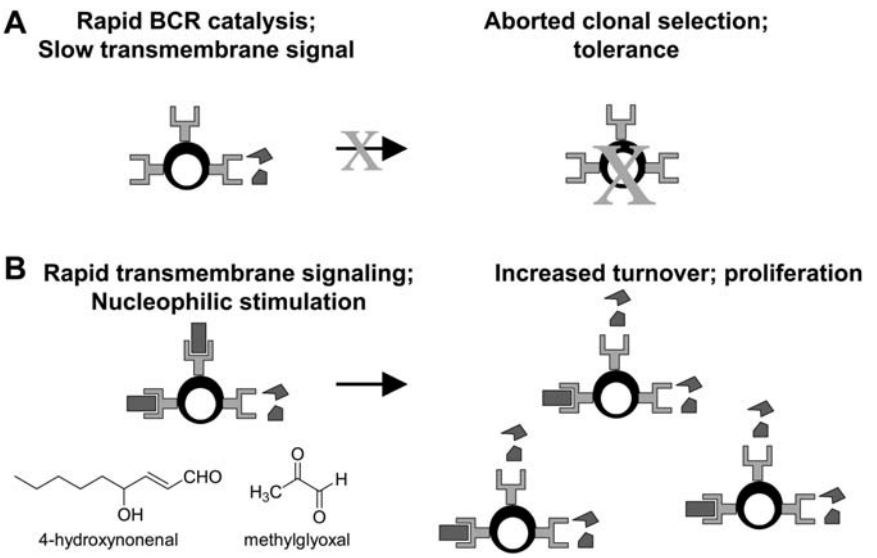

Fig. 7 Adaptive catalyst selection. Most Ab responses tend to disfavor improved catalytic turnover, because antigen digestion and release from the B cell receptor (*BCR*) will induce cessation of cell proliferation. However, there is no hurdle to increased BCR catalytic rates up to the rate of transmembrane BCR signaling. Under certain conditions, further improvements in the rate are hypothesized, e.g., if BCR transmembrane signaling rate is increased, as in CD19 overexpression, or upon stimulation of the B cells by an endogenous electrophilic antigen

structural features in the immunogens will likely be necessary to induce Abs capable of efficiently completing the cycle.

A possible consequence of Ab nucleophilic reactivity is the stable, covalent binding to the antigen devoid of artificially introduced electrophiles. Although without turnover capability, such irreversibly binding Abs offer the potential advantage of permanent antigen inactivation. Dissociation of antigen from the reversibly-associated Ab-antigen complexes, in contrast, releases antigen molecules with unimpaired bioactivity. There is no physiological bar to development of BCRs that bind ordinary antigens irreversibly, as the consequence of the irreversible binding is prolonged BCR occupancy. Two examples of covalent antigen-Ab complexes are published [37, 64], and we have also observed stable, SDS-resistant binding of HIV gp120 by certain Abs. A lesser manifestation of Ab nucleophilicity is the formation of a complex with resonant electrons and partial covalent character (a familiar example of a weak bond with partial covalent character is the hydrogen bond).

Autoantibody catalysis

Antigen-specific catalytic autoantibodies have been identified in several autoimmune diseases (reviewed in [76]), suggesting that the restrictions on synthesis of antigen-specific catalysis may be more readily surmounted in autoimmune disease than in the healthy immune system. For instance, VIP-specific catalytic autoantibodies have been observed only in subjects with disease [4], even though healthy humans also produce VIP-binding Abs

[3]. The V domains of proteolytic autoantibodies are adaptively matured, judged from their high affinity for VIP and their extensively mutated complementarity determining regions (which is typical of antigen-specific Abs) [78].

The mechanisms permitting the synthesis of antigen-specific proteolytic autoantibodies in autoimmune disease remain speculative at present. One hypothesis concerns the rate of B cell transmembrane signaling necessary to induce cellular proliferation. In principle, the BCR catalytic activity could improve adaptively if transmembrane BCR signaling is accelerated, as this will allow stimulation of cell division prior to product release from catalytic BCRs. Several reports have linked autoimmunity with dysfunctional B cell signaling due to altered levels of CD19, CD22 and Lyn, proteins contained within the BCR complex. CD19 diminishes the threshold for antigenic stimulation of B cells [24] and CD22 increases the threshold [51]. Lyn, a Src protein tyrosine kinase, is implicated in transduction of antigen-stimulated BCR signaling [23]. Dysfunction of these proteins is associated with increased autoantibody production.

Alternatively, covalent BCR binding by endogenous compounds may induce proliferation of B cells expressing proteolytic BCRs. This is supported by observations that immunization with model polypeptide CRA stimulates the synthesis of proteolytic Abs [57]. Potential endogenous CRAs are naturally occurring serine protease inhibitors and reactive carbonyl compounds, which are known to bind covalently to nucleophiles [6, 12]. For example, a positively charged derivative of pyruvate is reported to react covalently with the Ser nucleophile of trypsin and thrombin ([82]; the positive charge is located at the P1 subsite and does not participate in the covalent reaction). Additional candidate CRAs are electrophiles produced by lipid peroxidation and protein glycation reactions (Maillard's reaction), processes that occur at enhanced levels in autoimmune disease [2, 40]. Examples are 4-hydroxy-2-nonenal and malondialdehyde generated by lipid peroxidation and glyoxal, methylglyoxal and pentosidine generated in sugar metabolism reactions.

Defense functions of proteolytic Abs

Evidence for the role of autoantibody catalysts in disease pathogenesis has been reviewed previously [76]. Here, we describe the emerging scenarios under which proteolytic Abs can be viewed as fulfilling beneficial roles.

Promiscuous proteolytic Abs

These are IgMs and IgGs found in healthy individuals that cleave short model peptides with the sole sequence restriction that a basic residue (Lys/Arg) must be present on the N-terminal side of cleavage site (Fig. 8) [29, 60]. IgA and IgE Abs have not been examined for this activity. As the reported proteolysis screening experiments were limited to a few Abs and a few commercially available substrates, the upper limit for catalytic rates is not known. The promiscuous catalysts are conceptually analogous to the low-affinity Abs with polyreactive antigen-binding patterns. The latter type of Abs constitute the majority of circulating Abs in humans, and, because of their large concentration, can bind significant amounts of the antigen despite their low affinity [9]. Grabar [22] suggested that low-affinity Abs can help clear antigens by uptake of immune complexes via opsonization and

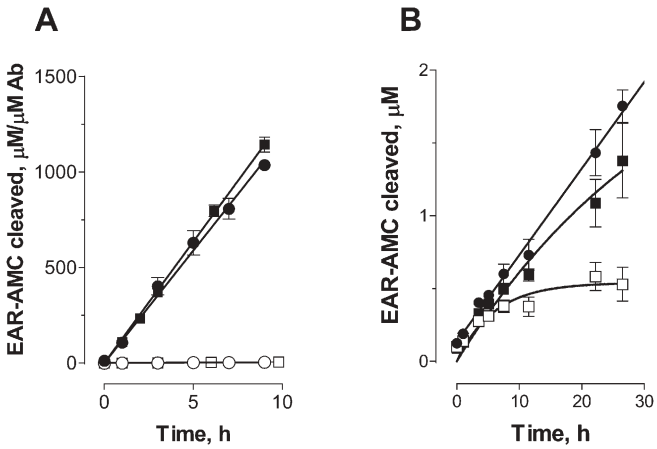

Fig. 8 Proteolytic activities of IgM and IgG Abs. **A** Cleavage of Glu-Ala-Arg-AMC (400 μM) by polyclonal murine IgM (*filled circles*), human IgM (*filled squares*), polyclonal murine IgG (*empty circles*) and polyclonal human IgG (*empty squares*). IgM, 5 nM; IgG, 160 nM. **B** Inhibition of polyclonal murine IgM (5 nM) catalyzed Glu-Ala-Arg-AMC (400 μM) cleavage by hapten CRA I (*filled squares*, 30 μM; *empty squares*, 100 μM). *Filled circles*, progress curve without inhibitor. Values are means of triplicates ± SD. From [60]

Fc receptor-mediated pathways. The proteolytic activity of the Abs provides an independent route to antigen clearance, without the need for the involvement of cellular elements.

The quantitative importance of antigen hydrolysis by the Abs depends on the kinetic properties of the Abs, Ab concentration, antigen concentration, and the time of contact between the reactants. The K_m of the proteolytic Ab is usually a good approximation of the equilibrium dissociation constant (K_d, $1/K_a$), and the k_{cat} (turnover number) defines the number of antigen molecules hydrolyzed/Ab molecule/per unit time at excess antigen concentrations ($>>K_m$). Model peptide substrates were cleaved by the promiscuous IgM preparations from human sera with apparent k_{cat} as large as 2.8/min and K_m 120 μM [60]. Serum IgM concentrations are 1.5–2.0 mg/ml (~2 μM), about three to four orders of magnitude greater than conventional proteases (for example, thrombin found at ng–μg/ml in serum as a complex with antithrombin III; [11]). If catalysis proceeds at the rate observed in vitro, 2 μM human IgM with turnover 2.8/min will cleave ~24,000 μM antigen present at excess concentration over 3 days (corresponding to the approximate half-life of IgM in blood; rate computed as [Ab] × k_{cat} × 3 days).

In view of the impressive turnover, consideration of the promiscuous proteolytic Abs in clearance of autoantigens that accumulate to large concentrations in vivo is warranted. Examples are albumin and IgG in blood, and polypeptides that accumulate at locations close to their synthetic site, such as thyroglobulin in the lumen of thyroid follicles. The promiscuous proteolytic Abs can also be conceived as a protective mechanism against microbial infection in certain situations, i.e., at sites of sepsis where bacterial antigens accumulate to concentrations approaching Ab K_m values.

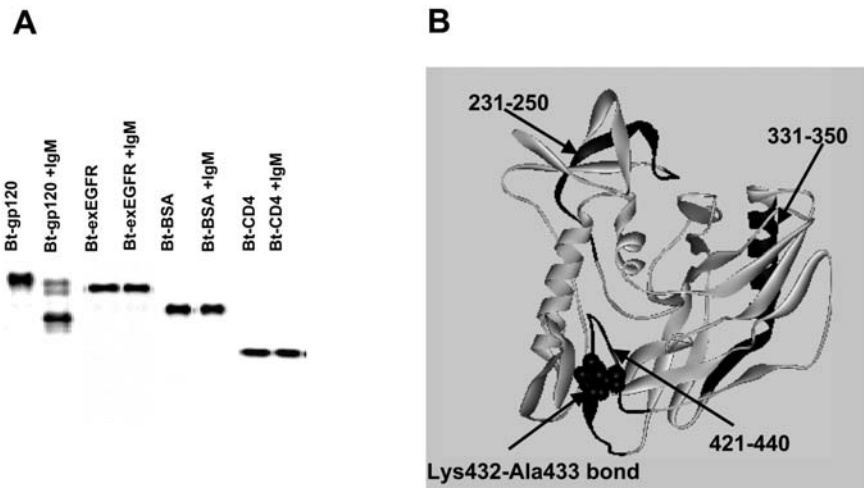

Fig. 9 Selectivity of IgM-catalyzed gp120 cleavage **A**, and the Lys432-Ala433 bond cleaved within the gp120 superantigenic site **B**. **A** Streptavidin-peroxidase-stained reducing SDS-polyacrylamide gels showing Bt-gp120, Bt-sEGFR, Bt-BSA and Bt-sCD4 incubated for 22 h in diluent or polyclonal human IgM (50 nM). Bt-protein, 0.1 μM. **B** Model of gp120 superantigenic site (adapted from [21, 30, 35]) and the cleavage site recognized by IgM (shown in *black*; Lys432-Ala433 bond; peptide determinants 231–250, 331–350, 421–440)

Specific gp120-cleaving IgMs

Viral antigens such as the HIV-1 coat protein are usually found at low concentrations in infected individuals. As the antigen concentration is limiting, catalyst competence is measured as the k_{cat}/K_m parameter. Under these conditions, low-affinity proteolytic Abs cannot be anticipated to degrade large proportions of the gp120, and specific, higher affinity Abs are necessary for effective protection.

Screening of large polypeptides for cleavage by IgMs has identified a selective proteolytic activity directed against gp120 in uninfected humans (Fig. 9A) [55]. The selectivity for gp120 can be traced to findings that gp120 is a B cell superantigen, defined as an antigen that is specifically recognized by Abs found in the preimmune repertoire (as opposed to the adaptively diversified Abs synthesized following infection or experimental immunization with the protein). Superantigen binding is usually mediated by contacts at conserved regions encoded by germline V genes. The superantigenic character of gp120 derives from the recognition of discontinuous gp120 peptide segments, including the segment composed of residues 421–433 [21, 30]. Two lines of evidence support the attribution of the proteolytic specificity to recognition of the superantigenic character of gp120. First, the CRA derivative of the synthetic gp120 peptide corresponding to residues 421–433 displayed covalent binding to the proteolytic IgMs at levels exceeding the hapten CRA devoid of the gp120 peptide sequence. Second, one of the peptide bonds cleaved by the IgMs was located within the superantigenic determinant (Lys432-Ala433) (Fig. 9B).

The evident selectivity of the catalytic IgMs for gp120 can not arise from the local chemical interactions at dipeptide units, as the same dipeptide units are present in other poorly cleaved proteins. As noted previously, adaptively matured Abs obtained by experimental immunization have been described to express antigen-selective proteolytic activity

attributable to noncovalent recognition of individual epitopes [57, 71]. A role for noncovalent gp120 recognition in the IgM-catalyzed gp120 reaction is supported by the comparatively small K_m for the reaction, about two orders of magnitude lower than the K_m for the promiscuous proteolysis by the IgMs. The noncovalent recognition of the gp120 superantigenic determinant, therefore, appears to facilitate nucleophilic attack on susceptible electrophilic groups by the Abs. This model of IgM-catalyzed gp120 hydrolysis is analogous to the mechanism of antigen-specific IgG Abs [71], except that the noncovalent interactions are dominated by contacts at conserved V domain regions instead of the adaptively mutated regions.

Apoptosis of neurons and T lymphocytes induced by monomeric gp120 shed from HIV has been implicated, respectively, in the dementia [31] and decline of $CD4^+$ T cells occurring in AIDS patients [70]. The IgM catalyzed cleavage of monomeric gp120 shed from HIV may protect against these effects of gp120. A caveat is the possibility of inhibition by naturally occurring serine protease inhibitors. In the absence of inhibitors, blood-borne IgM found in humans at 2 mg/ml in blood may be computed to hydrolyze 50% and 90% of gp120 present at concentrations $<<K_d$ in 4.6 min and 15.5 min, respectively (assuming K_d 31 μM, k_{cat} 2.1/min, corresponding to the values reported for a polyclonal human IgM preparation in [55]; computed from the equation $P_t=Ag_0[1-e^{(-k[Ab0]t)}]$, where P_t is product concentration at time t, k is k_{cat}/K_m, and $[Ab_0]$ and $[Ag_0]$ are Ab and antigen concentrations at time 0, respectively. In comparison, a reversibly binding IgM with the same K_d and zero turnover will bind only 6.6% of the available gp120 at equilibrium {computed from the equation $[Ab—Ag]^2-[Ab—Ag]\,([Ab_0]+[Ag_0]+K_d)+[Ab_0]\,[Ag_0]=0$}.

Similarly, if cleavage of trimeric gp120 found as a complex with gp41 on the HIV surface proceeds at the rate observed for the monomeric protein, only short time periods are needed to hydrolyze the majority of viral gp120 (gp120 concentrations in infection remain <<observed K_d; e.g., 10^6 HIV copies/ml with 100 gp120 molecules/virion correspond to ~2×10^{-13} M gp120; [66]). The recognition of gp120 residues 421–433 supports a protective role for IgM Abs, as this region of gp120 also contributes certain amino acids important in host cell CD4 binding [53]. Fragments generated by cleavage at the IgM-sensitive Lys432-Ala433 bond are reportedly devoid of CD4 binding activity [62].

IgG Abs that bind the gp120 superantigenic site noncovalently are described as resistance factors to HIV infection [75]. Our initial studies suggest that polyclonal human IgM can neutralize the infection of peripheral blood mononuclear cells by primary HIV-1 isolates under low serum conditions (Hanson, Karle and Paul, to be published elsewhere). Previously, Berberian et al. have discussed their unpublished studies suggesting HIV neutralization by Abs from uninfected subjects in the absence of serum (Note 11 in [5]).

β-Amyloid (Aβ) peptide cleaving Abs

Accumulation of Aβ1–40, 1–42 and 1–43 aggregates in the brain has been proposed as a causal factor in Alzheimer's disease. Several groups have observed the presence of IgG autoantibodies in humans capable of binding Aβ peptides [19, 25, 84]. Not all autoantibody responses are deleterious. The IgG autoantibodies to Aβ peptide found in healthy individuals are suggested to be beneficial, based on observations of their decreased levels in patients with Alzheimer's disease, and their ability to inhibit formation of Aβ peptide aggregates in vitro [84]. Trials of pooled intravenously administered immunoglobulins as a

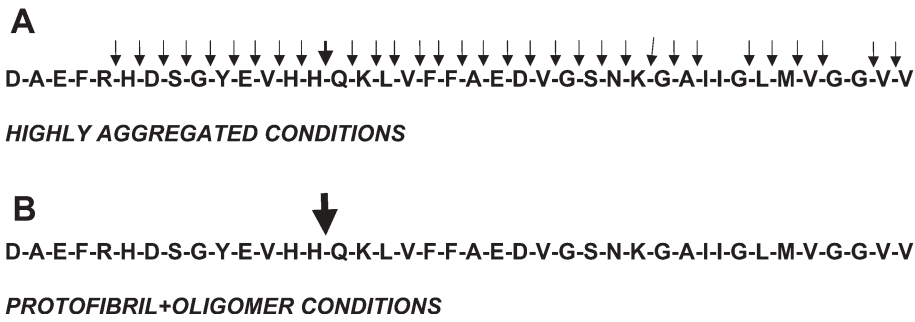

Fig. 10 Peptide bonds in Aβ1–40 cleaved by IgL hk14 under conditions of extensive peptide aggregation (1 mM; **A**) and protofibril/oligomer formation (0.1 mM; **B**). 2.5 μM IgL, 72-h incubation. Cleavage site determined by mass spectroscopy. Data in **A** from [63]

therapy for Alzheimer's disease are ongoing [15], and Abs generated by immunization with Aβ peptides and their analogs are reported to protect against cognitive decline in mouse models of Alzheimer's disease (e.g., [69]).

Sierks and coworkers [63] recently reported that two Ab light chain previously found to cleave VIP also cleave Aβ1–40, presumably reflecting the low-level promiscuous peptide recognition property of these catalysts. Interestingly, one of the light chains, clone hk14, displayed distinct cleavage profiles depending on the aggregation state of the substrate peptide (Fig. 10). Only a single peptide bond, the His14-Gln15 bond, was cleaved by the light chain at 0.1 mM Aβ1–40 [73], a concentration at which the peptide preparation contained monomers, oligomers and short fibrils, determined by atomic force microscopy. In comparison, multiple cleavage sites suggesting an exopeptidase-like activity were evident at 1 mM Aβ1–40, at which concentration large, macroscopic aggregates of the peptide were visible. Studies on synthetic peptide fragments suggest that Aβ residues 25–35 constitute the major neurotoxic determinant (e.g., [8]). As the cleavage patterns differ depending on the aggregation state of the peptide, careful study of the bond selectivity is important in identifying catalysts that could ameliorate the toxicity of the peptide. Cleavage of Aβ1–40 by the light chain under conditions favoring the exopeptidase-like activity attenuated the toxic effect on cultured neuroblastoma cells but cleavage of the peptide at the Lys16-Leu17 bond by another light chain did not [39].

Recently, we studied Aβ1–40 cleavage by polyclonal IgMs and IgGs from young (<35 years) and old humans (>70 years) without evidence of neurodegenerative or autoimmune disease using HPLC and mass spectroscopy methods [74]. IgM and IgG preparations from aged humans were observed to cleave Aβ1–40, with the IgM displaying 183-fold greater activity than the IgG (Fig. 11). The IgMs from young humans cleaved Aβ1–40 at lower levels, and the activity was not detected at all in IgGs from the young humans. The levels of Aβ1–40 cleavage by identically purified Ab preparations were different, suggesting that the activity is a polymorphic function associated with the variable domains. Cleavage of the peptide occurred primarily at the Lys28-Gly29 bond, with smaller levels of cleavage evident at Lys16-Leu. A monoclonal IgM with Aβ1–40 cleaving activity was identified by screening IgM preparations from patients with Waldenström's macroglobulinemia. Incubation of micromolar Aβ1–40 concentrations with nanomolar concentrations of the monoclonal IgM blocked the formation of peptide fibrils, evident by atomic force microscopy. These observations suggest: (1) Abs capable of cleaving Aβ1–40 are

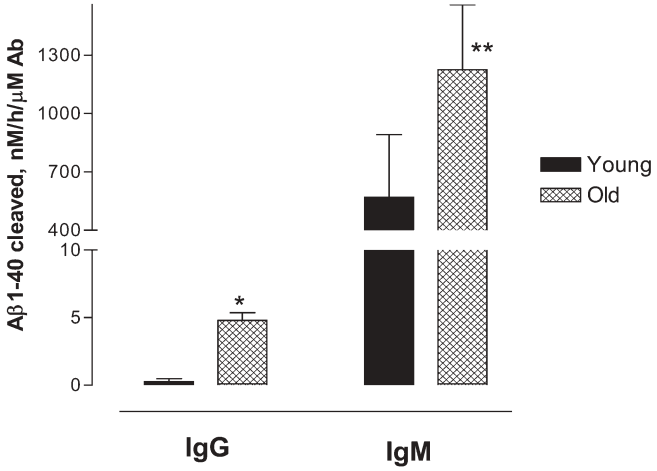

Fig. 11 Cleavage of Aβ1–40 by human IgM and IgG. Aβ1–40 (100 μM) incubated for 3 days at 37°C with IgG (1.6 μM) or IgM (34 nM) pooled from six subjects each of age <35 years (*young*) or >72 years (*old*). Reactions analyzed by reverse phase-HPLC (gradient of 10–80% acetonitrile in TFA, 45 min; detection: A_{220}). Rates computed from the area of the Aβ1–28 fragment peak interpolated from a standard curve constructed using increasing amounts of synthetic Aβ1–28. *P<0.0044; **P<0.035. Two-tailed unpaired t-test

constituents of the physiological immune repertoire; and (2) the proteolytic function of the Abs may improve adaptively as a function of age.

Aβ peptides in the central nervous system and peripheral blood are thought to exist in a state of equilibrium, with discrete transporter systems mediating the inflow and outflow of the peptides from the brain. Aβ peptide concentrations in peripheral blood are in the low nanomolar range. According to the peripheral sink hypothesis, clearance of blood-borne Aβ peptides results in increased outflow of the peptides from the brain. Indeed, peripheral injection of an anti-Aβ peptide Ab in transgenic mice prone to develop symptoms of Alzheimer's disease (mice overexpressing the amyloid precursor protein gene) resulted in diminished peptide levels in the brain [14]. Passage of the Abs into the brain is also possible. Anti-Aβ Abs are found associated with amyloid plaques in the brains of Alzheimer's patients [47]. Whether this is because of damage to the blood-brain barrier as a result of the disease or a reflection of a physiological transport process is not settled.

In view of these considerations, it may be hypothesized that catalytic anti-Aβ Abs found in aged humans may fulfill a protective function. A counterargument is the possibility of Ab-induced inflammatory reactions. In the brain, microglial uptake of Aβ-containing immune complexes via Fc receptor binding is proposed to help clear the peptide and also induce inflammatory mediator release from the cells [41]. Catalytic Abs can clear the peptide without the aid of inflammatory cells, and efficient catalytic Abs will not form stable complexes with the peptide. Thus, the catalytic function may mitigate the opportunity for inflammatory Ab effects.

Conclusions

From the data summarized in this review, it appears that Abs can frequently express nucleophilic reactivities rivaling that of conventional proteases. B cell clonal selection pressures, however, usually limit the antigen-specific proteolysis to low levels. Autoantibodies seem to bypass the regulatory hurdles for catalyst synthesis, possibly due to dysfunctional B cell signal transduction or stimulation by unusually electrophilic immunogens. Autoantibodies to Aβ may be an example in which the proteolytic function helps protect against the toxic effects of the peptide. IgM Abs catalyze the cleavage of HIV-1 gp120 selectively, which could serve as an innate resistance factor to the virus. No adaptive maturation of the Abs is required in this instance, because gp120 recognition as a superantigen confers selectivity to the reaction. Understanding the mechanisms underlying Ab proteolysis will likely facilitate the development of novel immunogens for production of antigen-specific proteases on demand, as suggested by the initial success in developing proteolytic IgG Abs in response to electrophilic polypeptide analogs.

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