Mechanisms of Adverse Drug Reactions to Biologics

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Abstract Biologics encompass a broad range of therapeutics that include proteins and other products derived from living systems. Although the multiplicity of target organs often seen with new chemical entities is generally not seen with biologics, they can produce significant adverse reactions. Examples include IL-12 and an anti-CD28 antibody that resulted in patient deaths and/or long stays in intensive care units. Mechanisms of toxicities can be categorized as pharmacological or nonpharmacological, with most, excepting hypersensitivity reactions, associated with the interaction of the agent with its planned target. Unexpected toxicities generally arise as a result of previously unknown biology. Manufacturing quality is a significant issue relative to the toxicity of biologics. The development of recombinant technology represented the single biggest advance leading to

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humanized products with minimal or no contaminants in comparison to products purified from animal tissues. Nevertheless, the type of manufacturing process including choice of cell type, culture medium, and purification method can result in changes to the protein. For example, a change to the closure system for erythropoietin led to an increase in aplastic anemia as a result of changing the immunogenicity characteristics of the protein. Monoclonal antibodies represent a major class of successful biologics. Toxicities associated with these agents include those associated with the binding of the complementary determining region (CDR) with the target. First dose reactions or infusion reactions are generally thought to be mediated via the Fc region of the antibody activating cytokine release, and have been observed with several antibodies. Usually, these effects (flu-like symptoms, etc.) are transient with subsequent dosing. Although biologics can have nonpharmacologic toxicities, these are less common than with small molecule drugs.

Keywords Biologics · Mechanisms · Pharmacological · Monoclonal antibodies · Cardiotoxicity · Superagonism

1 Introduction

Biologics (biotherapies, biotechnology-derived pharmaceuticals) are a rapidly growing sector of pharmaceuticals that are approved for a variety of therapeutic areas, including the treatment of cancer, rheumatoid arthritis, multiple sclerosis, and many others. Although definitions of biologics can vary somewhat, for the purpose of this chapter they encompass products derived from living systems and include endogenous proteins and peptides, monoclonal antibodies, fusion proteins, and vaccines. Among the first biologics were proteins isolated from animal tissues, such as porcine insulin for diabetes or horse antiserum to treat diphtheria. Following the advent of technology to allow sequencing of human proteins and recombinant DNA technology to enable their production, the development of drugs such as insulin, Factor VIII, and others represented a major advance in the ability to treat serious diseases. The breakthrough of using cell fusion for the production of monoclonal antibodies from immunized mice (Kohler and Milstein 1975) was the first step that has led to the plethora of monoclonal antibodies as therapeutics, arguably the most successful class of biologics. Table 1 lists a selection of currently approved biologics in the US. Most of these biologics are manufactured through the growth of genetically modified cells in vitro that have the appropriate insertion of a sequence of DNA and a promoter sequence such that the cells secrete the desired protein into the culture supernatant. The culture of the cells and subsequent purification process to obtain the protein are extremely important and relevant in the consideration of potential adverse reactions to biologics.

Table 1 Selected marketed biologics and associated adverse events

Generic name	Target/ligand	Indication	Adverse reactions
Monoclonal antibodies			
Alemtuzumab	CD52	B-cell chronic lymphocytic leukemia	Infections, hematologic toxicity, infusion reactions, hypersensitivity
Adalimumab	TNFα	Rheumatoid arthritis, Crohn's disease	Infection, neurological events, lymphoma, hypersensitivity, infusion reactions
Abciximab	IIbIIa	Adjunct to percutaneous coronary intervention	Bleeding, thrombocytopenia
Basiliximab	CD25	Organ transplant	Hypersensitivity
Bevacizumab	VEGF	Colorectal cancer	Gastrointestinal perforations, wound healing inhibition, hemorrhage, hypertension, protein urea, infusion reactions
Cetuximab	EGFr	Colorectal cancer	Infusion reactions, skin rash, pulmonary toxicity
Daclizumab	CD25	Organ transplant	Hypersensitivity
Efalizumab	CD11a	Psoriasis	Infection, thrombocytopenia, hypersensitivity, infusion reactions
Infliximab	TNFα	Rheumatoid arthritis, Crohn's disease	Infection, neurological events, lymphoma, hypersensitivity, infusion reactions
Trastuzumab	HER2	Breast cancer	Cardiomyopathy, hypersensitivity
Rituximab	CD20	Non-Hodgkins lymphoma	Fever, infusion reactions, tumor lysis syndrome, infections, progressive multifocal leukoencephalopathy (PML)
Palivizumab	F protein of respiratory syncytial virus	Prophylaxis of serious lower respiratory tract disease	Hypersensitivity
Omalizumab	IgE	Asthma	Malignant neoplasms, anaphylaxis
Natalizumab	4 integrin	Multiple sclerosis	Hypersenisitivy, progressive multifocal leukoencephalopathy (PML)
Recombinant endogenous proteins			
Alteplase	Tissue plasminogen activator	Restoration of function to central venous access devices	Bleeding, infection
Anakinra	rhIL receptor antagonist	Rheumatoid arthritis	Infections, injection site reactions

(*continued*)

Table 1 (continued)

Generic name	Target/ligand	Indication	Adverse reactions
Betaseron	Interferon γ 1b	Multiple sclerosis	Injection site reactions, flu-like symptoms
Rebif	Interferon β 1a	Multiple sclerosis	Hepatotoxicity, flu-like symptoms, suicide and depression, anaphylaxis
Insulin aspart	Insulin	Diabetes	Hypoglycemia, injection site reaction, systemic allergy
Laronidase	a-L-iduronidase	MPS I	Infusion reaction, rash, infection
Rasburicase	Recombinant urate-oxidase enzyme	initial management of plasma uric acid levels in pediatric patients expected to result in tumor lysis	Hypersensitivity, methemaglobinemia, hemolysis
Agalisdase β		Fabry disease	Infusion reactions
Epoetin α	Erythropeitin	Anemia	Infection, hypertension, cardiovascular events, hypertension, seizures, pure red cell aplasia
Dornase α	DNase	Cystic fibrosis	Voice alteration, rash
Imiglucerase		Gaucher disease	Infusion reactions
Oprelvekin	rhIL-11	Thrombocytopenia in chemotherapy	Hypersensitivity, fluid retention, pulmonary edema, ventricular arrhythmias
Aldesleukin	rhIL-2	Metastatic renal cell carcinoma	Capillary leak syndrome, infection, sepsis, CNS, pulmonary and cardiac effects
Reteplase	Rh plasminogen activator	Myocardial Infarction	Bleeding, arrhythmias
Mecasermin	rhIGF-1	Growth failure secondary to severe primary IGF-1 deficiency	Hypoglycemia, intracranial hypertension, transaminas elevations, otitis, cardia murmur
Drotrecogin α	Rh activated protein C	Severe sepsis	Bleeding
Follitropin	Hu follicle stimulating hormone	Infertility	Ovarian enlargement, ovarian hyperstimulation syndrome, pulmonary embolism, pulmonary infarction
Tenecteplase	Tissue plasminogen activator	Acute myocardial infarction	Bleeding, arrhythmias, cholesterol embolization
Teriparatide	Parathyroid hormone	Osteoperosis	Nausea, injection site reactions
Pegfilgrastim	Pegylated-GCSF	Neutropenia	Splenic rupture, respiratory distress

(*continued*)

Table 1 (continued)

Generic name	Target/ligand	Indication	Adverse reactions
Becaplermin	rhPDGF	Diabetic ulcers	Increased mortality secondary to malignancy, erythmatous rashes
Fusion proteins			
Abatacept	Anti-CTLA4-Fc fusion	Rheumatoid arthritis	Infusion reactions, infections, malignancies, hypersensitivities
Alefacept	LFA-3 Fc fusion	Psoriasis	Malignancy, infection
Etanercept	TNF receptor Fc fusion protein	Rheumatoid arthritis	Serious infection, malignancies, hepatotoxicity, hypersensitivity, infusion reactions
Rilonacept	IL-1 receptor Fc fusion	Cryopyrin-Associated Periodic Syndromes	Injection site reactions, infections
Vaccines			
BCG	NA	NA	Lymphadenitis, osteomyletis (rare, 1 in one million), disseminated BCG infection (1-10 per 10 million)
Diptheria, pertussis, hepatitis B and tetanus toxoid	NA	NA	Injection site pain, fever
Hepatitis A	NA	NA	Injection site pain, headache, allergic reaction
Influenza	NA	NA	Injection site reaction, muscle ache, autoimmune hemolytic anemia, nervous system and respiratory disorders
Measles, mumps and rubella	NA	NA	Thrombocytopenia, hypersensitivity to egg components or neomyecin, fever, vasculitis, arthritis, pneumonitis
Polio	NA	NA	Injection site reaction, fever, anaphylaxis (rare)

Although a focus on adverse events mechanisms with biologics can leave the impression that these types of therapeutics have substantial toxicities, as a class, they are often generally well tolerated. Preclinical studies rarely identify dose-limiting toxicities, and dose range finding pilot studies are rarely necessary to progress from shorter-term toxicology testing to chronic studies. Since biologics can have highly species-specific activities, the selection of the appropriate, pharmacologically relevant species for investigating mechanisms of toxicity is crucial.

2 Mechanisms Overview

Broadly speaking, the mechanism of toxicity associated with biologics can be grouped into similar categories as those for new chemical entities (NCE); that is, those that are pharmacologically mediated and those that are not (Fig. 1). Pharmacologically mediated is defined as the interaction of the biologic with its intended target, such as the binding of anti-vascular endothelial growth factor (anti-VEGF) antibody to VEGF or the interaction of an endogenous protein such as interleukin 12 (IL-12) with its relevant receptor. Interaction with this intended target can result in an anticipated biological effect, such as the reduction in blood glucose by insulin, or in a previously unanticipated effect such as cardiotoxicity secondary to HER2 downmodulation on myocytes after the administration of anti-HER2 antibody. Nonpharmacological effects are those unrelated to the interaction with the intended target and include, for example, hypersensitivity reactions secondary to an immune response to the protein or acute phase reactions due to the Fc region of a therapeutic antibody. From a purely quantitative perspective, the most frequent adverse events in patients taking biologics are hypersensitivity reactions, thus falling into the nonpharmacological category. However, in terms of the diversity of types of adverse events, by far the majority are pharmacologically mediated. In fact, excepting the two examples cited above (hypersensitivity and acute phase reactions), almost all adverse events to biologics, whether initially understood or not, turn out to be pharmacologically mediated. Why is this?

Proteins are catabolized into amino acids that are indistinguishable from endogenous amino acids, which are then recycled into other proteins via new protein synthesis or are excreted. There are therefore no metabolites per se, no production

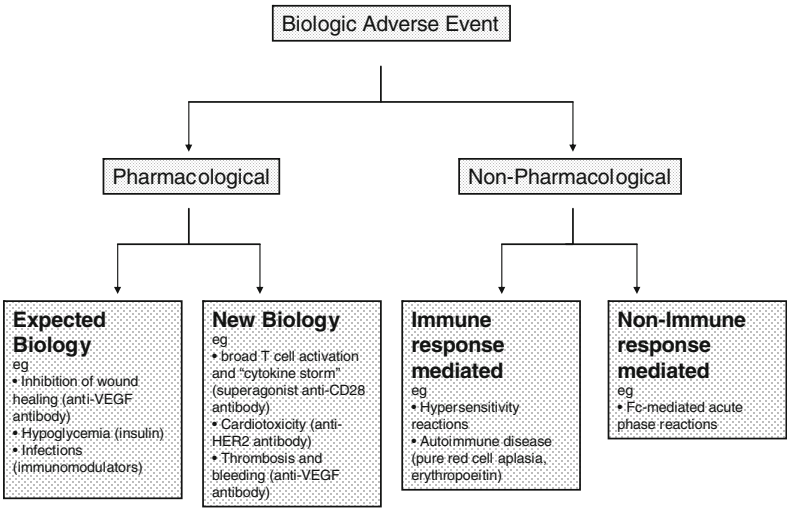

Fig. 1 Categories for mechanisms of toxicity associated with biologics

of new chemical entities, no xenobiotic that may have a previously unpredicted and non-pharmacological interaction with a cell system. Proteins are so critically dependent on primary (sequence), secondary (folding), and tertiary structure for their proper interaction in the in vivo system that catabolism results in a biologically inert molecule (with the exception as noted above of potentially serving as an immunogen). It therefore follows that the majority of the toxicological effects of an exogenously administered protein reside from its interaction with the relevant ligand or receptor in the in vivo system.

It does not, however, follow that all effects of biologics are relatively benign. Although many are well tolerated, there are examples of serious and even life-threatening toxicities occurring through pharmacological interactions, including two, IL-12 and an anti-CD28 superagonists, that are reviewed in this chapter. The examples selected for discussion in this chapter cover monoclonal antibodies, including those that are designed to inhibit the target (anti-VEGF antibody, anti-HER2 antibody), and those designed to agonize the target (anti-CD28 antibody). Although monoclonal antibodies and endogenous proteins share similar issues and mostly common mechanisms, a unique mechanism relative to the latter can involve the inadvertent removal of the native endogenous protein as a result of an autoimmune response to the exogenously administered therapeutic. Erythropoietin is an example of this and is discussed relative to the implications for product quality and the implications of a change in how the material is vialed. Finally, combination toxicity is relatively rare with biologics. They are not metabolized by the cytochrome P450 system, as are many NCEs, and therefore do not present interaction concerns with other NCEs based on cytochrome P450 induction or inhibition. However, though uncommon, it is possible for an interaction to occur on the basis of intersecting biological effects, and the anti-HER2 antibody toxicity in combination with anthracyclines is discussed as an example of this.

3 Pharmacologically Mediated Toxicities

3.1 Cardiotoxicity with a Monoclonal Antibody: Trastuzumab

Trastuzumab is a humanized monoclonal antibody against HER2 (ErbB2) and is used for the treatment of breast cancer in patients whose tumors overexpress this tyrosine kinase receptor on their cell surface. Trastuzumab treatment as a single agent resulted in overall response rates of 15–30% with substantially increased benefits in combination with other chemotherapies including anthracyclines. However, beginning in late phase clinical trials, cardiotoxicity was noted in a percentage of treated patients. Congestive heart failure occurs in 1–4% of treated patients, and 10% of patients have decreased cardiac function. The incidence of cardiac dysfunction increases in combination with exposure to anthracylines, over and above the known cardiotoxicity associated with these latter drugs as single agents.

Thus trastuzumab will serve in our discussion, both as a relatively rare example of a combination toxicity associated with a biotherapeutic, as well as an example of what appears to be a pharmacologically mediated mechanism of toxicity (a more common category with biotherapeutics).

Although a complete understanding of the desired pharmacological mechanism of action of trastuzumab is not yet established, it is thought to have activity through several mechanisms, all involving sequelae following binding to tumor surface HER2. Trastuzumab can kill tumor cells as a result of antibody-mediated cellular cytotoxicity (ADCC) through the activation of natural killer cells expressing the Fc gamma receptor. Trastuzumab may also downmodulate the expression of HER2 on the surface of the tumor cell removing or reducing the tumorigenic effects of HER2 overexpression such as the promotion of angiogenesis. Other possible mechanisms as well as the above have been reviewed recently in an article by Valabrega and colleagues (Valabrega et al. 2007). Of most relevance to the discussion of the mechanisms of cardiotoxicity is the inhibition and/or downmodulation of HER2 expression, for, as it turns out, HER2 likely has a role in the survival of normal healthy myocytes under situations of stress.

Before discussing this, it should be noted that the traditional range of safety studies to support the registration of the drug did not detect cardiotoxicity in normal healthy animals, either as a single agent or in combination with anthracyclines. Tissue cross-reactivity studies did not detect binding of the antibody to the heart using immunohistochemistry techniques.

Studies with murine knockouts have demonstrated that ErbB2, its coreceptor ErB4, and the ligand for the latter, neuregulin, are essential for the normal development of the heart. However, prior to the clinical cardiotoxicity observed with trastuzumab, there was little knowledge about the role of HER2 in the adult heart. A conditionally mutated mouse with a deficiency of ErbB2 only in the ventricle was developed to study this. These mice, although viable, developed cardiomyopathy as adults, including chamber wall thinning, decreased contractility, and chamber dilation (Crone et al. 2002). Cardiomyocytes prepared from these mice were more susceptible to cytotoxicity following incubation with anthracyclines than myocytes from normal mice. Taken together, this and other work has led to the overall hypothesis that the ErbB2/ErbB4 complex provides a survival mechanism to myocytes in the face of cellular stresses that could otherwise lead to cell death. With the reduction in ErbB2 (HER2) levels associated with trastuzumab treatment, the cells have less protection against a subsequent challenge with a stressful stimulus such as anthracycline exposure, leading to cell death.

An understanding of this two hit hypothesis led to the question of whether the clinical cardiotoxicity could be ameliorated by avoiding coadministration of trastuzumab with anthracylines by staggering the treatment regimen, thereby allowing ErbB2 levels to have recovered to a sufficient degree to protect the myocytes against anthracyline toxicity. Indeed, a small clinical trial to test this hypothesis showed promising results by maintaining the overall benefits of trastuzumab and chemotherapy treatment while greatly reducing the incidence of cardiotoxicity (Joensuu et al. 2006).

3.2 Interleukin-12

Interleukin-12 (IL-12) is a heterodimeric cytokine, which regulates cell-mediated immunity (Trinchieri 1993). This cytokine is produced by monocytes/macrophages, B cells, neutrophils, and dendritic cells in response to stimuli produced during infections. IL-12 has been studied for its therapeutic potential in the treatment of cancer and infectious diseases (Gollob et al. 2000) and included an evaluation in a phase II trial for the treatment of renal cell carcinoma. Administration of IL-12 in the phase II trial resulted in serious adverse events resulting in hospitalization and deaths (Leonard et al. 1997). The mechanism of this toxicity was investigated by analyzing the clinical data and by investigative toxicology studies in several animal models. The toxicity appears to have been associated with an exaggerated pharmacological activity of IL-12.

IL-12 has been found to have several biological activities, principally enhancing natural killer (NK)-mediated cytotoxicity and promoting T-helper cell type I immune responses. The latter occurs through the action of IL-12 on Th naïve cells to promote differentiation to Th1 cells. Although IL-12 stimulates the production of a variety of cytokines by cells in the immune system, the increased production of IFN-γ by NK and T cells is considered a principal mediator of its pharmacologic activity. IL-12 treatment has been shown to have anti-tumor and anti-metastatic activities in mice in vivo which involve direct and indirect T cell effector mechanisms including IL-12-induced secretion of IFN-γ (Weiss et al. 2007). The biology of IL-12 has been reviewed elsewhere (Trinchieri 1993; Del Vecchio et al. 2007).

IL-12 (500 ng kg^{-1}) administered to patients in a phase II trial resulted in serious adverse events that involved the liver (elevated transaminases, hyperbilirubinemia) and hematopoietic system (leucopenia, thrombocytopenia), and included severe fatigue, dyspnea, and stomatitis (Leonard et al. 1997). Twelve patients were hospitalized and two died, even though the same dose was tolerated in a phase I trial (Gollob et al. 2000). The investigation of this toxicity focused on several areas including: patient characteristics, test material differences, pharmacokinetics, regimen comparison, and animal models. The patient population in the two trials was similar, with similarity in age and race, the proportion who had previously received IL-12 treatment, and cancer status. A change in the manufacturing process, described as minor, was made between the phase I material and phase II material. However, in vitro biological activity and biochemical characterization, including amino acid composition, N-terminus sequencing, gel electrophoresis, peptide mapping, carbohydrate analysis, in vitro stimulated proliferation assays, and peripheral blood lymphocyte IFN-γ induction assay, were all comparable between the materials used and were not sufficient to account for the dramatic toxicities seen in phase II patients. Although only limited data were available due to the abbreviated nature of the trial, the pharmacokinetics of IL-12 were not reported to be significantly different between the patient groups. The regimen employed between the two trials was different: in phase I, patients were administered a single dose of

IL-12 14 days prior to subsequent consecutive doses whereas in Phase II, patients were administered consecutive doses without a pretreatment dose.

The mechanism of toxicity that was elucidated with animal models and confirmed by analysis of the clinical data was found to be associated with the expected induction of IFN-γ by IL-12 and was significantly affected by the regimen employed. Animals including mice and nonhuman primates demonstrated similar toxicities associated with IL-12 treatment as seen in patients (Car et al. 1999). Using recombinant murine IL-12, mice administered IL-12 at 500 ng/day had a high incidence of mortality that was ameliorated when the same dose was administered 7 days prior to consecutive daily dosing. This reduction in toxicity correlated with reductions in serum IFN-γ concentrations. Interestingly, different strains of mice were found to have differing sensitivities in terms of severity of toxicities observed as well as qualitative differences, including the absence of muscle toxicity in the strain of mice known to be deficient in inducible isoenzymes, phospholipase A2 (Car et al. 1999). Muscle toxicity was not reported in humans. Additional experiments in mice included coadministration with neutralizing antibodies to IFN-γ as well as administering IL-12 to a strain of mice deficient in the IFN gamma receptor. These studies confirmed that the toxicity seen in mice is associated with the overproduction of IFN-γ. The analysis of the clinical trial data further confirmed that the patients in the phase II trial had higher levels of IFN-γ even though the pharmacokinetics of IL-12 were similar when compared to patients in the phase I trial. Furthermore, the direct administration of IFN-γ in humans results in some of the same effects, although less severe, as those observed with IL-12 treatment. The mechanism by which an earlier "priming" dose of IL-12 blunts the subsequent IFN-γ response is not understood. However, because IFN-γ production by IL-12 was established as a necessary and desired component of the antitumor pharmacological activity of IL-12, this toxicity appears to be in the class of exaggerated pharmacological activity.

3.3 Multiple Adverse Events with a Monoclonal Antibody, Bevacizumab

Bevacizumab is a recombinant humanized IgG1 antibody that binds to and inhibits the biologic activity of human vascular endothelial growth factor (VEGF). VEGF is an endothelial-specific survival factor (Gerber et al. 1998b), mitogen, and angiogenic factor, which also has significant effects on vascular permeability (Ferrara et al. 2004). VEGF activity is mediated through its interaction with two receptors on endothelial cells, KDR and Flt1, which are high affinity tyrosine kinase receptors. Bevacizumab is indicated for first- or second-line treatment of patients with metastatic carcinoma of the colon or rectum. Bevacizumab was the first in the class of angiogenesis inhibitors that, by inhibiting the formation of new vessels from preexisting vasculature, proved the potential of this therapeutic approach to cancer treatment originally proposed by Judah Folkman more than 30 years ago. In

combination with chemotherapy, treatment with bevacizumab has extended survival by 4.5 months. Adverse events associated with bevacizumab treatment in patients have included gastrointestinal perforations, wound healing complications, arterial thromboembolic events, proteinurea, hemorrhage, and hypertension. At first glance, at least some of these events would seem to comprise mechanisms that are unlikely to be related to the pharmacological action of inhibiting angiogenesis; however, as the story has unfolded, many of them have been found to have an association with biological activities of VEGF that were previously unknown or due to a complex biological process that led unexpectedly to toxicity.

Angiogenesis is known to play a role in a range of physiological processes including wound healing, embryogenesis, and corpus luteum formation. It is therefore not surprising that bevacizumab administered to cynomolgus monkeys at doses up to 50 mg/kg led to physeal dysplasia in both sexes and an absence of corpora lutea in females (Ryan et al. 1999). Longtitudinal bone growth occurs at the physis and is characterized by an organized sequence of chrondocyte differentiation through zones of resting, proliferating, and hypertrophied chrondocytes. Vascular invasion of the hypertrophied chrondocytes and subsequent mineralization is a critical component in the net lengthening of the bone. Both physical disruption of the blood supply, as well as disruption through bevacizumab administration, results in a significant increase in the width of the physis also characterized by disorganization of the chrondocyte columns and absence of vascular invasion. More recently, VEGF has also been shown to be a survival factor for chrondocytes (Zelzer et al. 2004). The net result in these adolescent monkeys was the disruption in the normal growth and closure of the bone plate. Ovarian and uterine weights were significantly reduced in females by administration of bevacizumab at doses of 10 and 50 mg/kg (but not 2 mg/kg). Microscopically, decreased ovarian weights correlated with an absence of corpora lutea in the high dose group (Ryan et al. 1999). Cyclical angiogenesis is a necessary part of the life cycle of a healthy ovary. The development of corpora lutea is associated with the proliferation of vessels in the theca interna, which invade the ruptured follicle after release of the egg, to form a capillary network around the luteal cells. VEGF expression has been demonstrated to be time- and location-specific relative to the corpora lutea cycle. Other means of VEGF inhibition in rats have likewise demonstrated inhibition of corporal lutea formation. In summary, both effects on bone growth and corpora lutea formation appear to be directly related to the inhibition of new vessel formation through inhibition of VEGF by bevacizumab treatment. What then of the other adverse events associated with bevacizumab treatment?

The label for bevacizumab describes an increased incidence of arterial thromboembolic events, such as cerebral infarction, transient ischemic attacks, and others, when combined with chemotherapy compared with chemotherapy alone. Although the potential mechanisms underlying these events have not been fully elucidated, two main hypotheses involve either endothelial cells lining the vessels becoming prothrombotic or direct activation of platelets. Given the complexity of the mechanisms maintaining a patent vasculature, these may not indeed be mutually exclusive. Under normal physiological situations, endothelial cells lining the blood vessels

play an important role in preventing coagulation. These cells produce a variety of molecules that: prevent platelet aggregation (NO, ecto-ADPase, PGI2), inhibit thrombin formation (TFPI, thrombomodulin), or breakdown fibrin should a thrombus be formed (tPA). Incubation of endothelial cells with VEGF has been shown to shift them to a prothrombotic state through increased expression of tissue factor and other proteins. Under these circumstances, they are then able to activate platelets. This suggests that elevated levels of VEGF can produce a prothrombotic state, and that, therefore, reduced levels of VEGF via bevacizumab treatment should have an antithrombotic effect. However, as noted earlier, as well as promoting new vessel growth, VEGF is also a survival factor for endothelial cells (Gerber et al. 1998a). If levels of VEGF become too low, perhaps in combination with other factors, endothelial cells may become apoptotic. Apototic endothelial cells are procoagulant. This, then, describes a U-shaped curve in the relationship between VEGF concentration and prothrombotic conditions on the endothelial surface of blood vessels (Fig. 2), with both high levels and low levels producing factors consistent with a prothrombotic state. Other hypotheses providing a potential link between bevacizumab and arterial thrombosis include direct affects on platelets. Platelets are known to play an important role in arterial thrombosis. In an in vitro system, incubation of bevacizumab, heparin, and VEGF in the right proportions with platelets resulted in platelet activation. Further research is needed to determine whether this could occur in an in vivo system and to further understand the relative contributions of the endothelial and platelet interaction of VEGF and their relationship to bevacizumab-mediated arterial thrombotic events. For further reading on these and other mechanisms of toxicity associated with bevacizumab treatment,

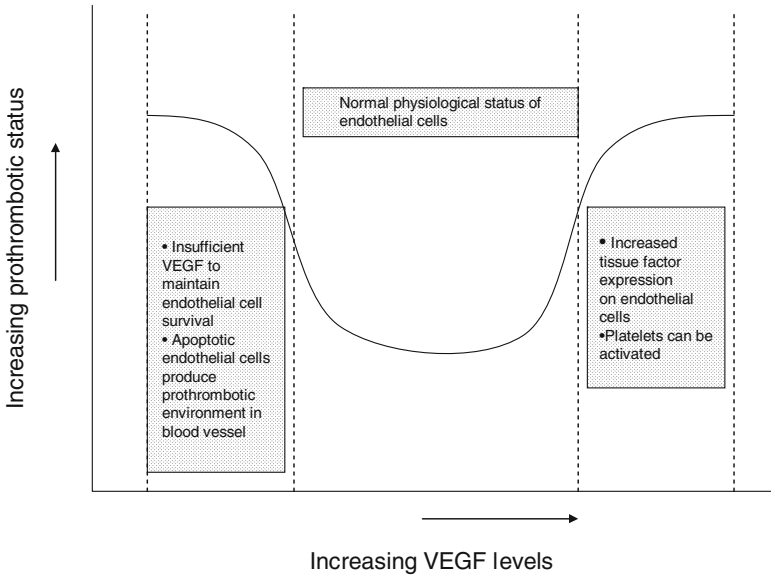

Fig. 2 Relationship between VEGF concentration and prothrombotic activity

Drs Verheul and Pinedo have published a comprehensive overview (Verheul and Pinedo 2007).

In summary, many of the adverse events associated with bevacizumab treatment appear to be related to its pharmacological action to inhibit VEGF. Some of the adverse events such as inhibition of wound healing were expected pharmacologically mediated toxicities, whereas others (such as arterial thrombosis) were unexpected as both the knowledge of the biology of VEGF and its impact on complex balanced systems developed in parallel with the clinical experience with bevacizumab.

3.4 Superagonist Anti-CD28 Monoclonal Antibody

In 2006, a phase I trial with a superagonist monoclonal antibody to CD28 (TGN1412) in six healthy volunteers produced life-threatening adverse events that required intensive care (Suntharalingam et al. 2006). Although all the volunteers survived, the event resulted in focused regulatory and scientific investigation to understand the mechanism of toxicity and to improve the manner in which phase I trials are evaluated and conducted.

Normal T cell stimulation requires two simultaneous signals: one that is antigen-dependent via the T cell receptor, and a second that is antigen-independent via costimulatory receptor stimulation, CD28, which is one of the costimulatory partners in T cell activation. Monoclonal antibody agonists to CD28, therefore, require a second antigen-dependent signal to result in T cell activation. However, about 10 years ago, a family of agonist anti-CD28 antibodies were found that did not require a costimulatory signal for T cell activation and were designated "superagonists". These antibodies could stimulate T cell activation without the need for any other factor. Although the presence of a T cell receptor was necessary, it did not need to be ligated (Schraven and Kalinke 2008). The pharmacologic rationale for TGN1412 was based on the observation that, when a superagonist anti-CD28 antibody was administered to rodents, there was a preferential expansion and activation of a regulatory T cell subset, and thus this approach might have promise in the treatment of autoimmune disease. Within 90 min of a single intravenous dose of 0.1 mg kg^{-1} TGN1412, the volunteers developed headaches closely followed by myalgia, nausea, diarrhea, vasodilation, lymphopenia, and hypotension. After 12–16 h, they became critically ill with lung injury, renal failure, and disseminated intravascular coagulation. These changes were associated with a rapid increase in proinflammatory cytokines and a significant depletion in lymphocytes and monocytes (Suntharalingam et al. 2006). Preclinical safety studies were conducted with TGN1412 prior to the trials, in cynomolgus monkeys and rhesus monkeys, and they did not identify any toxicity.

Subsequent studies have not answered all of the questions surrounding the mechanisms of toxicity; however, they have indicated that the toxicity is mediated through the pharmacological activity of the antibody to activate T cells by CD28 agonism, producing a proinflammatory state of sufficient magnitude to result in

high circulating levels of proinflammatory cytokines known as a "cytokine storm". This should not be confused with a first dose reaction or acute phase reactions to be discussed below, which result in transient and more modest effects and are associated with the induction of much lower levels of cytokines. The latter is mediated via the Fc portion of the antibody molecule whereas the TGN1412 effects were produced via the intended interaction of the antibody with its target, albeit resulting in unintended effects.

When human PBMCs were incubated with TGN1412 in the aqueous phase in vitro at a wide range of concentrations, no cytokine release or lymphocyte proliferation was produced. This was consistent in studies conducted prior to the trial or subsequently. However, the manner in which the TGN1412 antibody was presented to the PBMCs proved crucial because when the antibody was first air dried onto the plate, wet coated on top of anti-Fc antibodies, or coated on top of endothelial cells, all resulted in the release of cytokines into the medium and lymphocyte proliferation (Stebbings et al. 2007). It was thus apparent that, when the antibody was presented to human lymphocytes in a fashion more similar to the in vivo situation, it was able to stimulate a pro-inflammatory activity. Therefore, a clustering of the antibody on the surface of the lymphocytes appeared to be needed to produce activation. A primary role for the Fc portion of the antibody was ruled out by the observation that a Fab fragment of the antibody lacking the Fc portion was likewise able to stimulate cytokine release and lymphocyte proliferation (Stebbings, personal communication). The dose-response relationship observed in vitro was bell-shaped, with a peak effect at concentrations that were in the range of extrapolated estimates with the starting dose used in the trial. Incubation of cynomolgus monkey whole blood with TGN1412 using the same conditions resulted in the activation of lymphocytes (demonstrated by increased expression of IL-2R and blast transformation), but it did not lead to cytokine release or lymphocyte proliferation (Stebbings et al. 2007). Therefore, TGN1412 does bind to and activate CD28 in nonhuman primates, but appears to have a qualitatively different cellular response to the CD28 activation. Although the extracellular domain of CD28 is 100% identical between nonhuman primates and humans, there are three amino acid differences in the transmembrane region that may have a role in this differing signal.

Further supporting a pharmacologically mediated role for TGN1412-mediated toxicity, studies with a superagonist CD28 antibody (JJ316) in rats demonstrated an analogous response with initial T cell activation, acute lymphopenia, and cytokine release when the superagonist is administered to rats (Muller et al. 2008). However, this is followed by a subsequent second phase of activation that is predominantly associated with regulatory T cells. During the initial phase, there was a dramatic redistribution of T lymphocytes to secondary lymphoid organs from the periphery. Proadhesive changes on the cell surface were accompanied by strong activation including upregulation of CD25, CD69 and pro-inflammatory mediators. Therefore, the pharmacological activity of CD28 superagonism does include general T cell activation; however, in humans, the effect appears to be much more pronounced than in rats, and where this proceeds quickly to an expansion of regulatory T cells in rats, this was not apparent in humans or was overwhelmed

by the potent initial activating event. In summary, while there are many aspects of the TGN1412 mechanism of toxicity still to be understood, it does appear to fall in the category of a pharmacologically mediated toxicity albeit one that involved new understanding of the immunoregulatory role of CD28.

4 Non-pharmacologically Mediated Toxicities

4.1 Acute Phase Reactions

An adverse reaction often associated with the initial administration of some biologics, and particularly monoclonal antibodies, is an immune stimulation that is termed an acute phase response. The symptoms include fever, flu-like symptoms, fatigue, and anorexia. Usually, these symptoms diminish with subsequent injections. In MS patients who experience this reaction, they have, in addition, an exacerbation of their neurological symptoms presumably as a result of increasing the autoimmune response resulting in worsening brain lesions (Moreau et al. 1996). Under normal physiological circumstances, this response is associated with conditions of inflammation, tissue damage, or infection, and functions to remove infectious organisms and activate tissue repair processes (Gribble et al. 2007). Biologics associated with an acute phase response or "first-dose-effect" include monoclonal antibodies to CD52 (alemtuzumab), to CD3 (muromonab) and CD20 (rituximab). Although a number of different proteins either increase or decrease during an acute phase response, the cytokines IL-1, IL-6, and TNFα are considered the initiators (Baumann and Gauldie 1994). The main sources of these cytokines in an acute phase response can be macrophages via toll-like receptor activation or, as discussed below, NK cells.

The exact mechanism by which some biologics produce a significant acute phase response while others do not is unclear, but has been investigated in more detail for alemtuzumab (Wing et al. 1996). Using ex-vivo whole blood and nonadherent mononuclear cell cultures, these investigators examined the mechanism and time-course of IL-6, TNFα, and IFN-γ release upon incubation with alemtuzumab. Cytokine release in vitro was found to have the same time course (TNFα and IFN-γ first, IL-6 second) as measured in patients, and was not due to the presence of endotoxin. The postulated mechanism involved the interaction of the IgG1 isotype with the low affinity Fc receptor (FcγR) on the surface of immune cells. This was supported by the 40–50% reduction in cytokine release when an anti-CD56 IgG1 was used with a mutation such that it does not bind the FcR. An antibody to the FcγR1 (CD16) inhibited the cytokine release which further supported the involvement of this receptor. Cytokine release was not found to be a consequence of complement activation as a C1q- mutant antibody stimulated equivalent levels of TNFα as the unchanged antibody. Although isotype was found to be important (IgG1 antibodies with their capacity to bind the FcR having the highest likelihood of stimulating an acute phase response), the target to which

the antibody binds was also found to be important. An IgG1 anti-CD4 antibody was found to induce only a modest cytokine release, and the authors speculate that this may be related to the antigen density since CD52 expression is about 20 times higher than CD4 on the cell surface. This is supported by studies that show IgG1 antibodies induce cytokine release in whole blood cultures in proportion to the antigen density on the lymphocytes (Wing et al. 1995).

In summary, acute phase responses are due to the release of cytokines, particularly TNFα, IFN-γ, and IL-6. This can occur through direct stimulation of immune cells by the biologic, or indirectly via binding of the Fc portion of the antibody to the low affinity Fc receptor on other immune cells. The density of the antibody's target also plays a role as well as the antibody isotype and therefore its FcR binding affinity. Although this might be avoided by changing the isotype of the antibody, in some cases the desired mechanism of efficacy requires FcR activity such as is the case with alemtuzumab. In these cases, pretreatment of patients with methylprednisone, a steroid that inhibits cytokine synthesis, has been found to be a successful approach to mitigating the acute phase response.

4.2 Immunogenicity

Biologics are sufficiently large and complex as to elicit immune responses directed to the protein. For the most part, the principal response elicited is a T cell-dependent humoral response. The development of an antibody response to a biologic in most cases has no adverse consequences (Schellekens 2002a; Shankar et al. 2006). An antibody response is not an adverse event in itself. However, an antibody response to a biologic can have consequences that fall into three main categories: hypersensitivity reactions, reduction in efficacy, and the induction of autoimmune disease (Schellekens 2002b).

A review of the labeling for marketed biologics demonstrates that the majority contain warnings with regard to potential idiosyncratic reactions or hypersensitivity reactions (Table 1). The incidence of these types of reactions, though, has dramatically reduced from the original biologic products that were based on isolated animal proteins (e.g., porcine insulin) and murine monoclonal antibodies. This was made possible by the application of recombinant DNA technology and the movement towards proteins with fully human sequences. Serum sickness is a result of the deposition of antigen–antibody complexes in the tissues, where normal clearance mechanisms for such complexes have become overwhelmed, and is associated with fever, skin lesions, gastrointestinal symptoms, lymphadenopathy, and proteinuria. Tissue damage occurs as a result of inflammatory processes stimulated via interaction with the complement system, activation of macrophages, and direct interaction with basophils and platelets. Serious cases of "serum sickness" or immune-complex disease are now rare, and only isolated cases have been reported with recombinant therapeutic proteins (D'Arcy and Mannik 2001).

Immune responses can ablate or reduce efficacy, either by binding directly to the biologically active site of the molecule (e.g., the CDR on an antibody) and inhibiting

its pharmacological activity, or by increasing the rate of clearance of the protein, thus reducing exposure levels to below those needed for efficacy. Good examples of this are proteins derived from *E. coli* or plant origin such as streptokinase trichosanthin that produce strong immune responses. Other examples include Factor VIII, GMCSF, and IFNβ. How "adverse" this is for the patient depends on the nature of the disease and the availability of alternative therapies.

Some biologics are exogenous proteins that are intended to replace an absence of the endogenous protein or to supplement a deficit in production of the endogenous protein. It would therefore be theoretically possible for antibodies generated in response to the exogenously administered protein to cross-react with the endogenous protein and ablate its biological activity. Depending on the role of the endogenous protein and whether it is the sole contributor to a biologically important system, a serious autoimmune reaction can result. Fortunately, despite the relatively large number of endogenous proteins marketed or in development, this has occurred with only two products; erythropoietin and megakaryocyte-derived growth factor (MDGF). The mechanisms associated with aplastic anemia as a result of epoetin treatment are discussed in more detail below. Pegylated MDGF was in development for the increase of platelet yields in blood donors as well as in cancer patients. In normal healthy volunteers, thrombocytopenia was observed and correlated with the development of antibodies to MDGF, resulting in the cessation of clinical development (Wire 1998). Antibodies to peg-MDGF apper to have inhibited the endogenous production of this factor, impairing the ability of megakaryocytes in these individuals to produce normal levels of platelets.

The reasons why some proteins induce no or low titer antibodies, while others in rare instances result in autoimmune-inducing antibodies, is not well understood. Certainly, sequence homology plays a significant role as evidenced by the immunogenicity of murine monoclonal antibodies as compared to chimeric (human constant region, murine variable region) and humanized antibodies (mouse complementarity region grafted onto human variable and constant regions; Clark 2000; Presta 2006). However, as hopes of eliminating immunogenicity entirely have faded with the realization that fully human sequenced proteins can still be immunogenic, other factors that can influence immunogenicity are apparent (Schellekens 2002a). These include glycosylation (reduced or no glycosylation being more immunogenic), the effector status off an antibody, host cell products, contaminants and process-related impurities (see section below), route of administration (IM and SC routes are generally more immunogenic than IV), formulation (freeze drying can increase aggregation and thus immunogenicity), dose and regimen, and of course, patient factors.

4.3 Manufacturing and Product Quality

Biologic products are derived from living systems, such as the culture of mammalian or bacterial cells that secrete the desired product into the culture medium; as such, their production constitutes a complex manufacturing process. Subsequent steps isolate and purify the product. Due to this very different process from the

synthesis of chemicals, different quality issues arise that, if not properly controlled, can cause adverse effects. These can include the presence of aggregates, host cell proteins, and viruses. The protein molecules themselves are also complex; differences such as in folding and glycosylation can have a significant effect on the activity of the drug (Schellekens 2002a). These complexities in generating a reproducible drug product have led to substantial discussions regarding the requirements needed to generate follow-on biologics or biosimilars (Covic and Kuhlmann 2007). The principal mechanism of toxicity associated with these product changes is via an induction or enhancement of immunogenicity to the drug substance which can lead to an immune-mediated toxicity (Kromminga and Schellekens 2005). These can include hypersensitivity reactions, immediate or delayed, or even induction of an antibody directed to self-antigens where an endogenous protein is the target of the immune response. An example of this is erythropoietin α used in patients with chronic renal failure. Erythropoietin is a growth factor for red blood cells and in some patients antibodies develop capable of cross-reacting with and neutralizing endogenous erythropoietin leading to pure red cell aplasia (PRCA). Cases of PRCA increased dramatically beginning in 1998 which appeared to coincide with the removal of human serum albumin from the formulation and its replacement by polysorbate 80 and glycine (Locatelli et al. 2007). The likely mechanism of toxicity was an interaction of the polysorbate 80 with the uncoated rubber stopper of the prefilled syringe, leading to the release of a leachate that acted as an adjuvant. This was supported by studies that demonstrated the rate of PRCA was far lower with the same formulation delivered using syringes with coated stoppers versus uncoated stoppers (4.61/10,000 patient years vs 0.26/10,000 patient years; Boven et al. 2005). Since the introduction of prefilled syringes with coated stoppers, with the same formulation, the rate of PRCA has returned to very low levels.

From the earliest types of biologics that were isolated from animal or human tissues or plasma, the possibility of infection as an adverse effect of treatment was a significant concern. A well-known example of this was the high incidence of HIV and hepatitis C infection in patients receiving factor VIII to treat hemophilia. Since then, biologics made from recombinant technology and those that are human plasma-derived have had good viral safety records by employing controls on source materials and the mode of purification (Farshid et al. 2005). More recently, concern has shifted to defense against potential infection by transmissible spongiform encephalopathy such as scrapie and bovine spongiform encephalopathy. Unfortunately, these agents are difficult to detect and remove (Rohwer 1996), although robust purification procedures such as used in the preparation of a product from bovine lung was found to clear a spiked contamination of a mouse-adapted scrapie (Kozak et al. 1996). To date, there have been no reports of TSE as a result of a contaminated biotechnology-derived therapeutic. Ironically, the formulation changes made for erythropoietin described above, which indirectly led to many cases of PRCA, was made as part of the effort to remove human-derived products from recombinantly derived therapeutics from concern of TSE.

5 Vaccines

Vaccines represent such a distinctive type of biologic, with a patient population (including infants), regimen, formulation, and production method very different from recombinantly produced biologics that they are here assigned their own section. The mechanisms involved in adverse reaction to vaccines has been recently reviewed in detail (Siegrist 2007) from which much of the following information is summarized. Although a distinctive type of biologic, adverse reactions to vaccines can nevertheless be categorized as associated with the intended effect of the vaccine (to induce a protective immune response to an antigen), i.e., pharmacological, and those unrelated to the intended effect, i.e., nonpharmacological.

5.1 Pharmacological Effects

Excess replication of a live vaccine can lead to disease and can be the result of an immune deficient individual, or due to the selection of a too virulent strain, or because of reversion of a live strain to wild-type. The development of aseptic meningitis as a result of administering the live mumps vaccine is one example of this. In the case of the polio vaccine where both live and inactivated vaccines are available, there is a 1:750,000 risk of the development of polio as a result of reversion of the live virus strain. The inactivated vaccine does not carry this risk, but it is not as effective as the live vaccine.

Other adverse reactions with an association with the intended effect of the vaccine are represented by local inflammation and systemic inflammatory reactions. Local inflammatory reactions are caused by the large local deposit of antigen and the subsequent infiltration of macrophages. This is thought to be mediated via Toll-like receptor signaling. Since different formulations containing the same antigen component induce different degrees of local reaction, it is clear that there is both an antigen- and adjuvant-driven element to the nature and severity of the local reaction. Systemic inflammatory reactions, characterized by fever, nausea, and myalgia are a consequence of the release of cytokines from the liver following immune cell activation. These cytokines then have a systemic effect to produce fever, myalgia, and vascular effects. Host factors are a significant driver of these reactions: age (the young and the old are likely to have weaker reactions), gender (more common in females than males), genetics, and previous vaccine dose (stronger reaction with subsequent doses due to stronger amnestic cytokine responses.

5.2 Non-pharmacological Effects

Fainting as a result of the vagal reaction, shortly before or after injection, is an adverse reaction that can, of course, occur with some of the biologics, which are likewise injectables. Allergic reactions can occur to the formulation components of

the vaccine, this has, for example, been reported with thiomersal. It is possible for the vaccine to induce antibodies that, through molecular mimicry, can react with self-antigens. An example is the induction of antibodies cross-reactive to platelet surface glycoproteins which result in thrombocytopenia. This has occurred with both the measles (1:6,000) and rubella (1:3,000) vaccines. The production of antibodies that autoreact with antigens in the myelin sheath of peripheral nerves can result in peripheral neuropathies secondary to inflammation of the myelin and sometimes further axon loss. The mechanism for how these self-antibodies result from vaccination with, for example, the influenza vaccine remains unknown.

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