ELSEVIER

Antibody engineering for therapeutics

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With the acceptance of antibodies as therapeutics, a diversity of engineered antibody forms have been created to improve their efficacy, including enhancing the effector functions of full-length antibodies, delivering toxins to kill cells or cytokines in order to stimulate the immune system, and bispecific antibodies to target multiple receptors. After years of *in vitro* investigation, many of these are now moving into clinical trials and are showing promise. A potential new type of effector function for antibodies, that is, the generation of reactive oxygen species that may effect inflammation or bacterial killing, has been elucidated. In addition, the field has expanded beyond a concentration on immunoglobulin G to include immunoglobulin A antibodies as potential therapeutics.

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Introduction

Fueled by the success of therapeutic antibodies over the past decade, research into antibody engineering has expanded significantly. Early engineering work concentrated primarily on making the therapeutic tolerable to the human immune system (e.g. humanization), the generation of antibodies (e.g. via the use of transgenic mice, and phage and ribosome display technologies), and novel antibody formats (e.g. diabodies and scFv [single-chain variable region fragment]) (Figure 1; Box 1). Although studies in these areas continue, new areas have blossomed — so much so that they cannot all be encompassed by a short review. This review covers selected subjects from 2001 onwards; for subjects not covered here, a selection of recent reviews are available covering therapeutic antibodies in general [1–4].

Bispecific antibodies

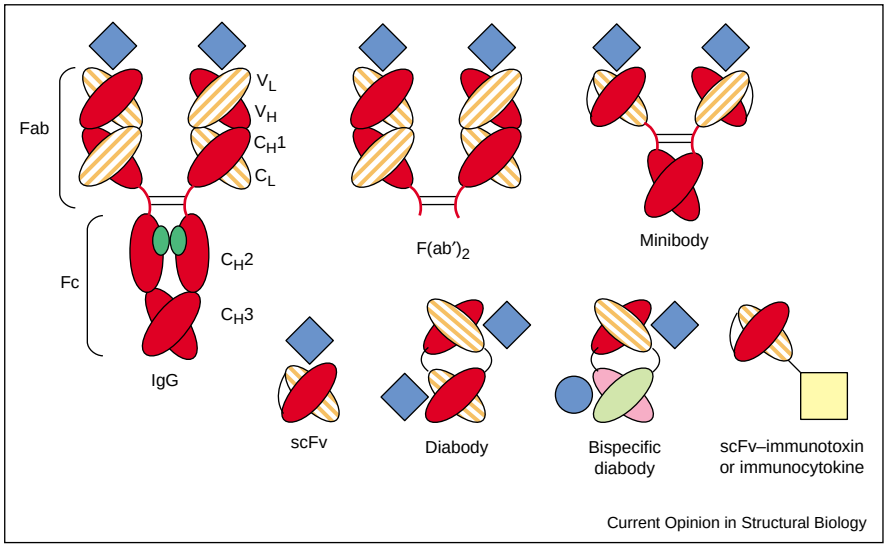
Bispecific antibodies bind to two different epitopes, most often on two different antigens, and can be constructed as full-length IgG or smaller fragments, such as $F(ab')_2$ [an $F(ab')_2$ consists of two disulfide-bonded Fab; each Fab, or antigen-binding fragment, consists of the variable light (V_L), constant light (C_L), variable heavy (V_H) and constant heavy domain 1 (C_H1)] and diabodies [5] (Figure 1). Although the field has significant potential, a major challenge has been the production of material of sufficient quantity and purity to meet clinical needs. At the forefront are bispecific antibodies in which one arm targets FcγR (Fc gamma receptor); these are designed to recruit immune system effector cells to kill tumor cells. A phase I/II trial using an anti-CD30 X anti-FcγRIII antibody reported one complete and three partial remissions, and four cases of stable disease among sixteen patients [6]. Pretreatment with interleukin (IL)-2 cytokine resulted in augmented antitumor activity, putatively through increased levels and activation of effector cells such as natural killer cells. Another trial evaluated an anti-CD30 X anti-FcγRI bispecific antibody for Hodgkin's lymphoma [7]; one complete and three partial remissions, and four stable diseases were reported among the ten patients. An anti-HER2 X anti-FcγRI antibody in combination with interferon-gamma (IFN-γ) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (again to increase FcγRI expression and activate effector cells) was evaluated in breast and prostate cancer patients to determine pharmacokinetics and safety [8,9]; although toxicity was acceptable, no responses were reported. An *in vitro* analysis of peripheral blood polymorphonuclear cells and monocytes isolated from these patients showed that their effector cells were loaded with the bispecific antibody and could phagocytose HER2-positive SK-BR-3 tumor cells [10].

As with the clinical trials, the bulk of preclinical studies have involved bispecific antibodies in which one arm targets FcγR [11,12]; a more recent, exciting addition to the fray has been bispecific antibodies with one arm directed against the high-affinity IgA receptor (FcαRI; CD89). *In vitro* and *in vivo* studies have shown not only that targeting FcαRI can effect tumor cell killing equivalent to anti-FcγR [13] but also that, on certain effector cells (e.g. granulocytes), anti-FcαRI may be more efficacious than anti-FcγR [14•]. Similarly, IgG and IgA (instead of anti-FcγR and anti-FcαR) may be equally efficacious, but trigger different cell types and systems [15•]. Simultaneous engagement of FcγRI and FcαRI has been reported to enhance tumor cell killing even more [16•].

Antibody–cytokine fusion proteins

Cytokines can effect stimulation of several immune cell types, including monocytes, macrophages, natural killer cells, dendritic cells, and T and B cells. Treatment of patients with cytokines can modulate immune responses, but their use also evokes serious toxicity. If cytokines

Figure 1



Antibody constructs commonly used for therapeutic applications. Fab and $F(ab')_2$ fragments can be generated by papain or pepsin digestion, respectively, or by genetic engineering; all other forms are generated by genetic engineering. scFv are composed of V_L–linker peptide–V_H (or vice versa); monovalent scFv and diabodies can be obtained by variation in length of the linker peptide. Minibodies comprise two scFv–hinge–C_H3 chains covalently connected by disulfide bonds. Heavy chain domains are red and light chain domains are striped. Target molecules are shown as blue boxes or blue circles. Carbohydrate covalently attached to each IgG heavy chain is shown as a green oval.

could be concentrated at the intended target (e.g. tumors), the systemic toxicity of cytokine treatment might be diminished or abolished. Engineered proteins in which a cytokine is fused to an antibody have been developed to address this problem [17•].

A variety of cytokines have been linked to full-length antibodies or scFv fragments through the N or C termini of the antibody. Antibody–IL-2 fusion proteins have shown some efficacy against a variety of tumors in mouse models, not only decreasing tumor size [18,19•,20] but also reducing metastasis [21]. The antitumor effect of these constructs may arise from the activation of infiltrating effector cells, as evident from the efficacy of IL-2 fusion proteins with scFv, as well as with full-length antibodies [18,19•]. However, the presence of an IgG Fc (the portion of an immunoglobulin composed of constant heavy domains 2 and 3) may further augment efficacy by providing two binding sites for an effector cell: one through the cytokine receptor and one through an FcγR. This not only may activate the cell via the cytokine but may also simultaneously provide FcγR engagement to effect antibody-dependent cellular cytotoxicity (ADCC); indeed, a fusion protein composed of IL-2–mouse IgG2b Fc (effectively, IL-2 was substituted for the antibody F[ab]s) showed enhanced tumor protection and decreased metastasis compared to IL-2 or IgG2b alone [21]. To further increase the effectiveness of these types of fusion proteins, a recent study reported the construction of novel antibody fusions in which two different cytokines (IL-2 and IL-12, or IL-4 and GM-CSF) were either fused in tandem to the C terminus of an antibody heavy chain, or one cytokine was fused to the C terminus while the other was fused at the N terminus of the heavy or light chain [22••]. *In vitro* and *in vivo* models showed enhanced efficacy, but, as the authors point out, access of these large constructs to *in vivo* solid tumors needs evaluation.

Antibody–IL-12 fusions have also shown antitumor efficacy. One study showed increased efficacy of antitumor activity of IL-12 fused to the N terminus of the variable heavy domain of a fibronectin-specific scFv (compared to

Box 1 Glossary of terms.

ADCC	antibody-dependent cellular cytotoxicity
CDC	complement-dependent cytotoxicity
Fab	portion of an immunoglobulin comprising the variable light (V_L), constant light (C_L), variable heavy (V_H) and constant heavy domain 1 (C_H1)
Fc	portion of an immunoglobulin composed of constant heavy domains 2 and 3 (C_H2 and C_H3)
FcαRI	high-affinity IgA receptor (also known as CD89)
FcγR	Fc gamma receptor
FcRn	neonatal Fc receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Ig	immunoglobulin
IL	interleukin
PEG	polyethylene glycol
scFv	single-chain variable region fragment

a nonspecific construct) and an increase in tumor-infiltrating effector cells [23]; thus, extracellular matrix proteins may be an alternative to membrane-bound targets on tumor cells. Whereas several studies have correlated effector cell activity with tumor cell killing, results from another antibody–IL-12 fusion protein underscore that T cells may also potentiate tumor rejection [24•]. Finally, a recent report showed that anti-HER2–cytokine fusion proteins enhanced *in vivo* response to vaccination with HER2 extracellular domain protein [25•], revealing a new potential use for these engineered constructs.

Immunotoxins

Immunotoxins are antibodies that have a conjugated or genetically linked toxin, and are designed to deliver the toxin to target cell-surface molecules, followed by internalization of the immunotoxin–target complex into the cell and subsequent cell killing by a mechanism dependent on the toxin. One member of this class of antibody, Mylotarg®, a calichaemicin-conjugated anti-CD33, has already been approved for the treatment of acute myelogenous leukemia.

In clinical trials of constructs composed of protein toxins, some therapeutic success has been reported, although the immunogenicity of non-human-derived toxins has been problematic [26]. In addition, several protein toxins (e.g. ricin A chain [27] and *Pseudomonas* exotoxin [28]) have been shown to elicit vascular leak syndrome, characterized by an increase in vascular permeability resulting in interstitial edema and organ failure; vascular leak syndrome may also occur with antibody–cytokine fusions [29]. A structural motif in protein toxins and IL-2 that may initiate vascular leak syndrome has been identified and the protein toxins have been re-engineered to alter the motif, thus preventing vascular leak syndrome [30••]. One unique form of immunotoxin utilizes cytotoxic RNase fused to an antibody [31], a cross between more traditional immunotoxins and antibody-directed enzyme prodrug constructs [32]. The use of human RNase may obviate the immunogenicity and toxicity seen with plant/bacterial-derived protein toxins.

As with antibody–cytokine fusion proteins, a protein toxin can be fused at either the N or C terminus of an antibody or antibody fragment. During the design, consideration of the natural protein toxin may be important. An example of this is evident in a study using a diphtheria toxin fragment fused at either the N or C terminus of an anti-CD3 scFv [33••]. The toxin–scFv form showed reduced binding to CD3 (probably through steric hindrance) compared to the scFv–toxin construct; however, the toxin–scFv was 100-fold more potent in cell killing. This may be due to the fact that, in the toxin–scFv construct, the scFv replaces the toxin cell-binding domain and effects optimal internalization, processing and activation of the toxin domain subsequent to cell binding.

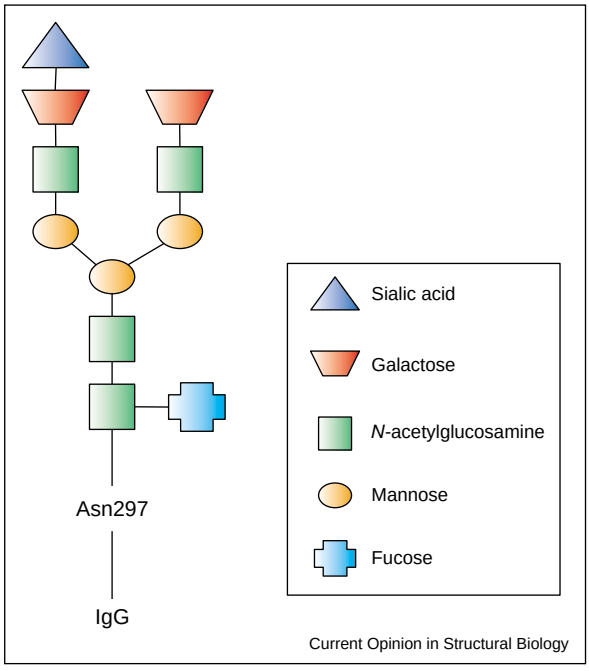
Related to immunotoxins, inasmuch as they use antibodies to deliver toxins, are immunoliposomes — self-assembled lipid bilayers that have toxins inside; polyethylene glycol (PEG) may also be incorporated into the lipid layer. Antibody fragments (e.g. Fab or scFv) are linked to the lipid or PEG. The antibody directs the immunoliposome to the target, followed by fusion of the liposome with the cell and delivery of the toxin inside the cell. Use of antibody fragments that have been selected for superior internalization signaling [34•,35] may enhance the function of the immunoliposome [36]; alternatively, judicious choice of a target that internalizes well can also accomplish this [37•]. Recent studies on immunoliposomes coated with anti-HER2 scFv have shown that delivery of doxorubicin via immunoliposomes improved the antitumor efficacy and reduced the toxicity compared to the systemic administration of doxorubicin, liposomal doxorubicin (i.e. without the anti-HER2 scFv or Fab fragment coating the liposome) or anti-HER2 antibody alone [36,38•]. Another study used immunoliposomes coated with scFv targeting the ED-B domain of B-fibronectin [39]. *In vivo* mouse studies showed that, whereas the immunoliposomes provided only a modest increase in the rate of tumor accumulation (compared to non-scFv-coated liposomes), the reduction in tumor growth was 62% (day 5) and 90% (day 8) over that of untreated animals or animals treated with scFv-coated liposomes without toxin; however, in contrast to the anti-HER2 study [38•], there was no statistical difference in tumor reduction between scFv-coated immunoliposomes and non-scFv-coated. One difference between the two studies is that the anti-HER2 liposomes target a cell-bound protein, whereas the anti-ED-B targets an extracellular-matrix-associated protein expressed around nascent blood vessels in the vicinity of tumors, but not actually on the tumor cells (see also [23]).

Engineering the effector functions of antibodies

Monoclonal antibodies elicit four effector functions: ADCC, phagocytosis, complement-dependent cytotoxicity (CDC) and half-life/clearance rate. ADCC and phagocytosis are mediated through the interaction of cell-bound antibodies with FcγR; CDC through the interaction of cell-bound antibodies with a series of proteins that constitute the complement system; and half-life by the binding of antibody to FcRn (neonatal Fc receptor). If a therapeutic antibody utilizes ADCC, induces FcγR-bearing cells to activate a cytotoxic T-lymphocyte response or merely uses the FcγR-bearing cell as a cross-linking agent, improving the binding of the antibody to FcγR could improve its efficacy. Recently, new methods for enhancing the antibody–FcγR interaction have been reported.

In IgG, the Asn297-linked carbohydrate comprises a core oligosaccharide, including fucose (Figure 2), that may contain various additional monosaccharides attached to

Figure 2



Schematic of carbohydrate forms attached to Asn297 of human IgG heavy chains. Therapeutic monoclonal antibody preparations exhibit heterogeneity in their carbohydrate. The common prevalent forms are: G0, a core carbohydrate consisting of (*N*-acetylglucosamine)(fucose)-(*N*-acetylglucosamine)-mannose-(mannose-*N*-acetylglucosamine)$_2$; G1, in which only one arm of the core has galactose attached; G2, in which both arms of the core have galactose attached. Additional forms include attachment of sialic acid to galactose and presence of a third mannose-*N*-acetylglucosamine arm.

the core. Two recent independent studies have reported that lack of the fucose moiety on the antibody carbohydrate significantly enhances the binding of antibody to FcγRIII and consequent ADCC [40•,41•]. In addition to changing the Fc carbohydrate, the protein sequence can be altered. A complete mapping of human IgG1 amino acids that affect binding to human FcγR [42••] resulted in several IgG1 variants with improved binding to specific FcγR; the variants that showed superior binding to FcγRIII also enhanced *in vitro* ADCC. In conjunction with the crystal structures of several human FcγR [43] and reports of the differential response of patients with the two human polymorphs of FcγRIIIA [44•,45], engineering human IgG to enhance the Fc–FcγR interaction, especially for the lower-affinity human FcγRIIIA(F158) polymorphic form, may lead to more efficacious therapeutic antibodies.

Another effector function of IgG involves its half-life. In some instances, it might be advantageous to either decrease or prolong the half-life of an antibody. FcRn is structurally related to MHC class I molecules, comprising an α-chain that noncovalently associates with β_2-microglobulin [46•]. FcRn regulates homeostasis of IgG [47•] and controls transcytosis across tissues [48,49]. It has been shown that the alteration of specific amino acids in murine IgG that improve binding to murine FcRn also results in increased half-life in mice [50]. The IgG–FcRn interaction is pH dependent, with IgG binding at pH 6.0 but not at pH 7.4 (the pH of blood) [50]. The epitope on human IgG1 for human FcRn has been mapped and altering some IgG1 residues enhanced binding to FcRn [42••]; notably, these alterations improve binding only at pH 6.0 and not at pH 7.4. Another recent study has underscored the requirement for improved binding only at the lower pH — IgG variants that bound better to FcRn at both pH 6.0 and pH 7.4 exhibited a decreased half-life [51•]. The demonstration that FcRn is expressed in brain microvasculature and that it may actively transport IgG from brain to blood [52] opens the potential for the use of FcRn-non-binding antibodies in treating brain tumors; if the difficulty of introducing a therapeutic antibody into the brain can be overcome, lack of transport from brain to blood might increase the residence time of the antibody at the tumor.

A series of interesting reports starting in 2000 [53] has detailed a potential new effector function of antibodies, that is, the ability of antibodies to catalyze the conversion of singlet molecular oxygen and water to form hydrogen peroxide [54,55] and ozone [56••,57,58•]. Production of these toxic molecules does not seem to perturb the antibody, but can cause bacterial killing and inflammation [56••]. One potential source of the singlet molecular oxygen may be from antibody-induced activation of the NADPH oxidase pathway, as proposed from a study of complement-independent platelet lysis by anti-GPIIIa49-66 antibodies [59•]. If this holds true, it warrants greater attention to the target of therapeutic antibodies. For some targets, activation of the NAPDH oxidase pathway, subsequent generation of reactive oxygen species and ensuing inflammation might be advantageous; for other targets, this might be deleterious. Along with the biochemical studies, several crystal structures have elucidated the portions of the antibody that may play a role in catalysis [54,58•]. Whether it is possible to engineer antibodies to remove (or possibly enhance) this effector function remains unclear.

Conclusions

The potential of engineering increasingly efficacious therapeutic antibodies has never been brighter. As in all forms of human endeavor, problems will arise, but, as in the past history of antibody engineering, creativity in generating new types of antibodies will overcome these problems. For example, the development of smaller antibody formats that can more effectively penetrate solid tumors is currently being addressed by the evaluation of scFv, diabodies and minibodies [60••,61,62], and the ability to generate lower-cost antibody fragments in

bacterial systems and endow them with a reasonable half-life has been fruitful [63,64]. With numerous clinical trials of 'naked', radiolabeled bispecific and conjugated antibodies underway, hopefully the next few years will see additions to the therapeutic antibody armament already available to patients.

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