REVIEWS

Potent antibody therapeutics by design

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Abstract | Antibodies constitute the most rapidly growing class of human therapeutics and the second largest class of drugs after vaccines. The generation of potent antibody therapeutics, which I review here, is an iterative design process that involves the generation and optimization of antibodies to improve their clinical potential.

Adverse event
An untoward medical occurrence in a patient who has been administered a pharmaceutical product. This occurrence does not necessarily have a causal relationship with the treatment.

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Since the mid-1990s, antibodies have emerged as an important new drug class. Indeed, 18 antibodies are now approved (17 have been marketed, and 1 withdrawn) for therapeutic use in the United States across diverse clinical settings, including oncology, chronic inflammatory diseases, transplantation, infectious diseases and cardiovascular medicine (TABLE 1; see Supplementary information S1 (table)). These approved antibody therapeutics include 14 unmodified IgG molecules, 2 radioimmunoconjugates, 1 antibody–drug conjugate and 1 Fab. At least 150 additional antibodies are in clinical development[1].

One of the strengths of antibody therapeutics is that they belong to a well-established drug class that has a high success rate from the first use in humans to regulatory approval: 29% for chimeric antibodies, and 25% for humanized antibodies[1]. This compares favourably with the ~11% success rate for small-molecule drugs[2]. Moreover, much of the development and clinical experience that is gained from the generation and optimization of one antibody is applicable to other antibodies, thereby streamlining certain activities and decreasing some of the many risks that are intrinsic to drug development. In general, antibodies are well tolerated by humans, although infusion reactions (particularly for the first dose) are common but usually manageable[3]. For example, most patients treated with rituximab (Rituxan; Genentech, Inc. and Biogen Idec Inc.; and MabThera; F.Hoffman-LaRoche Ltd; a CD20-specific monoclonal antibody) experience mild to moderate first-infusion reactions that include fever and chills, and these reactions occur less frequently with subsequent doses. Infusion reactions with rituximab are commonly attenuated by premedication and by incremental increase in the rate of infusion of rituximab. A key strength of antibodies as therapeutics is that their clinical potential can readily be increased by improving their existing properties — as I review here — or by endowing them with new activities[4–6].

The limitations of antibody therapeutics, include the restriction of targets to those on the surface or exterior of host cells or invading pathogens. Advances in the expression of functional antibodies within cells — known as intrabodies — show that these antibodies are useful research tools, but the potential of intrabodies as therapeutics remains to be shown[7,8]. Another limitation is that antibody drugs are expensive[9], which limits their use to serious medical conditions. Many factors contribute to the high cost of antibody therapeutics, including the large expense of drug development in general, the high cost of manufacturing and the large total doses that are often required. In addition, the intellectual property that is often associated with the generation, optimization and production of antibodies commonly leads to a series of stacking royalty payments, which further increases the cost. Although antibody therapeutics are often safe and well tolerated, rare but serious adverse events have been reported for several antibodies[10,11], including 2 deaths out of 5,000 patients treated with natalizumab (Tysabri; Biogen Idec Inc. and Elan Corporation, plc; an $\alpha_4\beta_1$-integrin- and $\alpha_4\beta_7$-integrin-specific monoclonal antibody)[9] (TABLE 1; see Supplementary information S1 (table)).

The development of potent antibody drugs has evolved into an iterative design process (FIG. 1). The design strategies involved[4–6] are reviewed here, with an emphasis on IgG, because this is the format of antibody that is used by almost all approved antibody drugs[1]. In addition, antibody fragments are discussed, because their clinical importance is growing[1,12] and because fragments are commonly used in the engineeri ng of antibody properties[13]. This Review article explores the complex relationship between the goals for antibody therapeutics, the available tools for antibody generation and optimization, and the tunable properties of antibodies.

Table 1 | **Monoclonal antibodies approved for therapeutic use in the United States***

Product name, antibody name and company‡	Antibody format	Antigen	K_d (nM)	Proposed mechanisms of action	Approved indications
Orthoclone OKT3; muromonab-CD3 (Ortho Biotech Products, L.P.)	Mouse IgG2a	CD3	0.83	Blocking of function of T-cell-expressed CD3; reversal of graft rejection	Prophylaxis of acute kidney-transplant rejection
ReoPro; abciximab (Centocor, Inc.)	Chimeric Fab	gpIIb–gpIIIa and $\alpha_v\beta_3$-integrin	5	Receptor binding and antagonism; inhibition of platelet aggregation	Prevention of platelet-mediated clots in coronary angioplasty
Rituxan; rituximab (Genentech, Inc. and Biogen Idec Inc.); and MabThera (F.Hoffman-LaRoche Ltd)	Chimeric IgG1	CD20	8.0	Sensitization of cells to chemotherapy; induction of apoptosis, ADCC and CDC	Non-Hodgkin's lymphoma and rheumatoid arthritis
Zenapax; daclizumab (F.Hoffman-LaRoche Ltd)	Humanized IgG1	CD25	0.3	Receptor binding and antagonism	Prophylaxis of acute kidney-transplant rejection
Simulect; basiliximab (Novartis AG)	Chimeric IgG1	CD25	0.1	Receptor binding and antagonism	Prophylaxis of acute kidney-transplant rejection
Synagis; palivizumab (MedImmune Inc. and Abbott Laboratories)	Humanized IgG1	RSV gpF	0.96	Binding and neutralization of RSV; inhibition of viral fusion and replication	Prophylaxis against RSV infection in children at high risk
Remicade; infliximab (Johnson & Johnson and Schering Plough Corporation)	Chimeric IgG1	TNF	0.1	Ligand binding and receptor antagonism	Crohn's disease, rheumatoid and psoriatic arthritis, ulcerative colitis and ankylosing spondylitis
Herceptin; trastuzumab (Genentech, Inc. and F.Hoffman-LaRoche Ltd)	Humanized IgG1	ERBB2	5	Sensitization of cells to chemotherapy; inhibition of angiogenesis and proliferation; induction of ADCC	Metastatic breast cancer that overexpresses ERBB2
Mylotarg; gemtuzumab ozogamicin (Wyeth)	Humanized IgG4, calicheamicin conjugated	CD33	0.08	Induction of double-stranded DNA breaks and cell death (caused by calicheamicin)	Acute myeloid leukaemia that expresses CD33
Campath; alemtuzumab (Genzyme Corporation and Schering AG)	Humanized IgG1	CD52	10–32	Induction of ADCC and CDC	B-cell chronic lymphocytic leukaemia
Zevalin; ibritumomab tiuxetan (Biogen Idec Inc.)	Mouse IgG1, ^{90}Y-labelled	CD20	14–18	Induction of cell death by radiation; induction of apoptosis	Non-Hodgkin's lymphoma
Humira; adalimumab (Abbott Laboratories)	Human IgG1	TNF	0.1	Ligand binding and receptor antagonism; induction of CDC	Rheumatoid and psoriatic arthritis
Xolair; omalizumab (Genentech, Inc. and Novartis AG)	Humanized IgG1	IgE	0.17	Ligand binding and receptor antagonism; reduction in release of allergic-response mediators from mast cells and basophils	Persistent asthma
Bexxar; ^{131}I-tositumomab (GlaxoSmithKline)	Mouse IgG2a, ^{131}I-labelled	CD20	1.4	Induction of cell death by radiation; induction of apoptosis, ADCC and CDC	Non-Hodgkin's lymphoma
Raptiva; efalizumab (Genentech, Inc. and Serono S.A.)	Humanized IgG1	CD11a§	3	Receptor binding and antagonism; inhibition of leukocyte adhesion to other cells	Plaque psoriasis
Erbitux; cetuximab (ImClone Systems Inc. and Bristol-Myers Squibb Company)	Chimeric IgG1	EGFR	0.2	Receptor binding and antagonism; inhibition of cell proliferation; induction of apoptosis; sensitization of cells to chemotherapy and radiotherapy; inhibition of angiogenesis, invasion and metastasis; induction of ADCC	Metastatic colorectal cancer, and head and neck cancer
Avastin; bevacizumab (Genentech, Inc. and F.Hoffman-LaRoche Ltd)	Humanized IgG1	VEGF	1.1	Ligand binding and receptor antagonism; inhibition of angiogenesis and metastatic disease progression	Metastatic colorectal cancer
Tysabri; natalizumab (Biogen Idec Inc. and Elan Corporation, plc)‖	Humanized IgG4	α_4-Subunit of $\alpha_4\beta_1$-integrin and $\alpha_4\beta_7$-integrin	0.3	Receptor binding and antagonism; inhibition of leukocyte adhesion to their counter receptors	Multiple sclerosis

*Sources include drug prescribing information, company websites and REF. 1. See Supplementary information S1 (table) for references. ‡Products are listed in order of first regulatory approval, from Orthoclone OKT3 (1986) to Tysabri (2004). §Also known as α_L-integrin. ‖Natalizumab (Tysabri) was voluntarily withdrawn from the market by its manufacturers because of safety concerns, in early 2005 (REF. 10). Five of 5,000 patients treated with natalizumab developed progressive multifocal leukoencephalopathy, including two who died[9]. The US Food and Drug Administration has recently lifted the clinical-trial hold on natalizumab, and Biogen Idec Inc. and Elan Corporation, plc, plan further safety testing of natalizumab in patients with multiple sclerosis. ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; EGFR, epidermal-growth-factor receptor; gp, glycoprotein; K_d, antigen-binding affinity; RSV, respiratory syncytial virus; TNF, tumour-necrosis factor; VEGF, vascular endothelial growth factor.

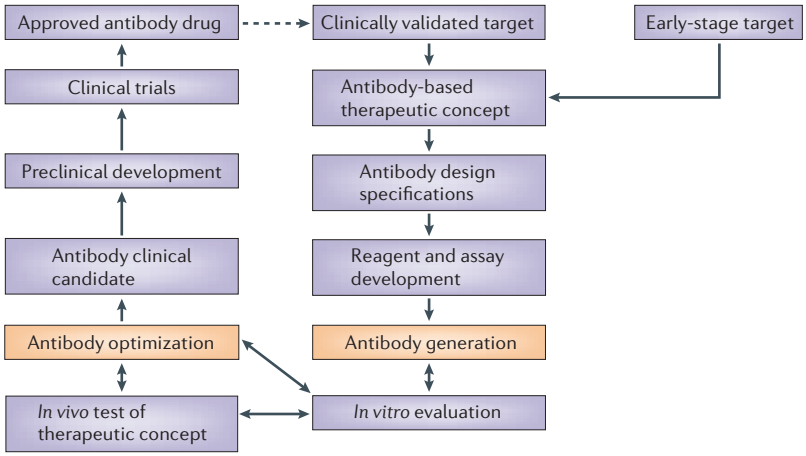


Figure 1 | **Iterative design of antibody therapeutics.** The first step in the generation of antibody therapeutics, which is one of the most difficult steps, is the selection of a target antigen. This is reviewed elsewhere for oncology targets[141]. A concept for therapeutic intervention using an antibody is then devised, ideally on the basis of knowledge about the target, including its role in the pathobiology of disease. This therapeutic concept leads to the antibody design specification: that is, a list of crucial or desirable properties that are predicted to achieve the desired clinical outcome. The design specification guides the method of antibody generation, as well as the reagents and assays that are needed to identify antibodies with the desired properties. Panels of antibodies are evaluated *in vitro* and then *in vivo* to test the therapeutic concept and to rank the antibodies for potency and safety. Antibody optimization might be necessary or desirable to meet the design goals and can be guided by existing antibody drugs, in terms of favourable attributes to emulate and limitations to overcome. Revision of the design specifications might be required in light of *in vitro* and *in vivo* data. The antibody clinical candidate is then subjected to further preclinical and, if warranted, clinical development. Clinical-trial data — particularly if they validate the target through to the drug-approval stage — might provide the impetus for the whole process to begin again, to generate an improved drug.

Goals for antibody therapeutics

Currently available antibody therapeutics both establish this class of drugs and point to design goals for future antibodies. One need is to improve the efficacy of antibody therapeutics, as is indicated by anticancer therapy, for which antibodies are, as yet, seldom (if ever) curative. For example, a pivotal Phase III clinical trial for the treatment of metastatic colorectal cancer showed that — when included in a chemotherapy regimen (consisting of irinotecan (Camptosar; Pfizer Inc.), fluorouracil and leucovorin) — bevacizumab (Avastin; Genentech, Inc. and F.Hoffman-LaRoche Ltd; a vascular endothelial growth factor (VEGF)-specific monoclonal antibody) extended the median survival of patients from 15.6 to 20.3 months[14]. Therefore, in oncology, one of the goals for increasing the efficacy of antibodies is to improve their antitumour activity and to improve patient survival, by increasing the frequency of partial responses or, more preferably, complete responses, and by extending the duration of responses[4,11]. To improve safety, it might be useful to focus on limiting first-infusion reactions or more serious target-related adverse events. For example, the acute and severe influenza-like syndrome that is associated with administration of muromonab-CD3 (Orthoclone OKT3; Ortho Biotech Products, L.P.; a CD3-specific monoclonal antibody) is Fc-mediated and is largely overcome by attenuating the interaction between the Fc region of the antibody and the receptors for the antibody (IgG receptors; FcγRs) expressed by the patient. In general, increasing the potency of the antibody or extending its half-life in plasma might allow the dose or frequency of administration to be reduced, which might have the associated benefits of improved quality of life and/or convenience for the patient, and/or reduced cost of the drug. The high cost of antibody therapeutics that are produced in mammalian cells — commonly Chinese hamster ovary cells, NS0 mouse myeloma cells or hybridoma cells — might be reduced by using alternative hosts for production. For example, *Escherichia coli* is an established host for the generation of protein therapeutics and is gaining acceptance for the production of antibody fragments. Numerous other hosts are being explored for the production of antibodies, and these include other microorganisms, insect cells, and transgenic plants and animals[15,16].

Early tools for therapeutic antibody generation

Hybridomas. The first developed and most widely used technology for the generation of monoclonal antibodies is the use of mouse hybridomas generated from the stable fusion of immortalized myeloma cells with B cells from immunized mice[17]. The ubiquitous use and broad success of mouse monoclonal antibodies in research, including drug discovery, is in stark contrast to their paltry 3% success rate in drug development[1]. This infrequent clinical success using mouse antibodies reflects the high immunogenicity of these foreign proteins in humans, as well as the weak interactions that mouse antibodies typically have with human complement and FcγRs, resulting in inefficient effector functions (FIG. 2). In addition, mouse antibodies do not bind the human salvage receptor FcRn[18], resulting in a terminal half-life that is typically less than 20 hours[4,5]. These limitations of mouse antibodies have largely been overcome by their chimerization or humanization[16,19], advances that have ushered in the current era of antibody therapeutics. Other important factors that contribute to the success of antibodies as drugs include better choices of antibody and target antigen for intervention in the pathobiology of disease[20,21] and progress in recombinant antibody production[15,16], thereby allowing higher doses to be used.

Chimerization and humanization. Chimerization involves joining the variable (V; antigen binding) domains of a mouse monoclonal antibody to the constant domains of a human antibody[22,23], and this process necessitated an appreciation of the structure and function of immunoglobulin domains. The advent of humanization required a detailed understanding of the structure of V domains and the determinants of antigen binding. The simplest of the many available humanization strategies involves transfer of the complementarity-determining regions (CDRs; that is, the antigen-binding loops) from a mouse antibody to a human IgG[24–26]. The generation of humanized antibodies with a high binding affinity for antigen generally requires additional transfer of one or more framework-region residues from the parent mouse antibody[19].

Partial response
In oncology, a response in which there is greater than or equal to a 50% reduction in total tumour size, with no new lesions or increase in size of an existing lesion. This is often calculated as the sum of the products of the perpendicular diameters of all measurable lesions.

Complete response
In oncology, a response in which no remaining tumour can be detected by visual inspection or by clinical imaging technologies. This does not necessarily indicate that the disease has been cured.

Response duration
The time from the first response until disease progression or death.

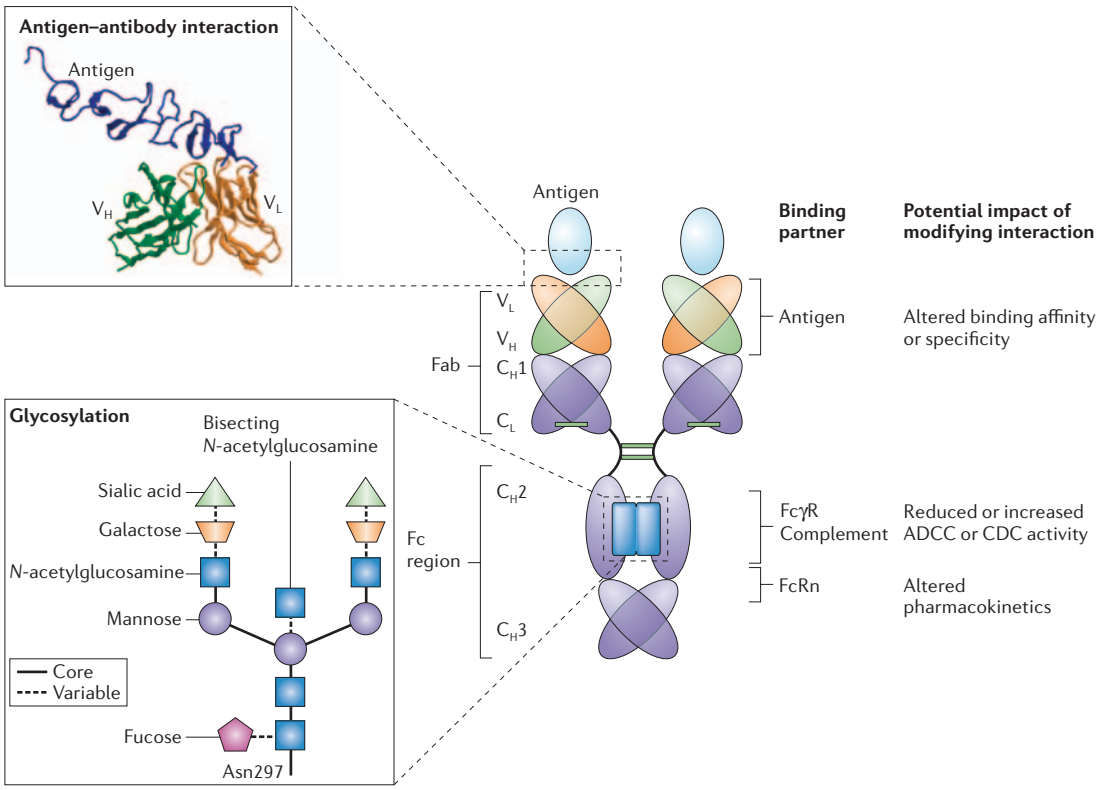


Figure 2 | **IgG structure and function.** IgG molecules are tetramers of ~150 kDa, which comprise a pair of identical heavy and light chains linked by disulphide bonds (green bars). The heavy chains contain a variable (V) domain (the V_H domain; green) and three constant (C) domains, the C_H1 domain, C_H2 domain and C_H3 domain (purple). By contrast, the light chains contain a V domain (the V_L domain; orange) and a single C domain (the C_L domain; purple). Highly selective binding of antigen (light blue) is a common hallmark of antibodies. This is mainly mediated by six loops, which are known as the complementarity-determining regions (CDRs), three of which are present in each of the V_H and V_L domains. The interaction of antibody with antigen is exemplified in this figure by the Fab of trastuzumab (Herceptin; Genentech, Inc. and F.Hoffman-LaRoche Ltd) in complex with ERBB2 (REF. 144). For clarity, only the V domains of trastuzumab and domain IV of ERBB2 are shown (see inset). The remaining portion of the V domains, which are known as framework regions, function mainly as a structural scaffold to support the CDRs. Human IgG bound to an antigen on a target cell, particularly IgG1 and IgG3, can subsequently interact through its Fc region with IgG receptors (FcγRs) expressed by effector cells or with complement component 1q (C1q), potentially supporting the destruction of target cells through antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), respectively. In addition, the Fc region of IgG can bind the salvage receptor FcRn after fluid-phase uptake by vascular endothelial cells and other cells, an interaction that contributes to the long (~21 day) half-life of human IgG. Modifying the amino-acid sequence of IgG to tailor the interaction with one or more binding partners is a promising strategy to improve the clinical potential of antibodies (TABLE 2). Effector functions (that is, ADCC and CDC) require the presence of the Fc-region glycan (dark blue) and are crucially influenced by its structure. The N-linked glycan that is attached to the conserved asparagine (Asn) residue at position 297 comprises a core structure of *N*-acetylglucosamine and mannose, plus additional carbohydrate residues, which can vary, including fucose, galactose, sialic acid and bisecting *N*-acetylglucosamine. The image in the upper left inset is reproduced, with permission, from *Nature* REF. 144© Macmillan Publishers Ltd.

Routes to completely human antibodies

Most of the growing number of antibodies entering clinical trials are completely human[1] and are derived from phage-display technology[13] or transgenic mice that express human immunoglobulin genes[27]. Phage display and transgenic mice are mature technologies that offer robust and complementary routes to the generation of potent human antibodies. Alternative, less widely used routes to the generation of human antibodies include ribosome-, mRNA- and yeast-display libraries[13], as well as human hybridomas from patients[28,29] and antibody-cDNA cloning from single lymphocytes selected on antigen[30,31]; these techniques are not discussed further here.

Human antibodies from transgenic mice. The generation of human antibodies by target-antigen immunization of mice that are transgenic for human immunoglobulin genes and have disrupted mouse immunoglobulin heavy-chain and Igκ light-chain loci was first described in 1994 (REFS 32,33). Subsequent progress has included the expression of more V gene segments by the transgenic mice, thereby expanding the potential repertoire of recovered antibodies[27]. Mouse strains that encode human antibodies with different heavy-chain isotypes have also been created to tailor effector functions. At present, at least 33 human antibodies produced in transgenic mice are in clinical development[27], including 5 that are in

Effector functions
Fc-mediated antibody properties that are involved in the destruction of target cells: that is, antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP).

Half-life
The time taken for the plasma concentration of a drug to decrease to half of its original value. Initial half-life and terminal half-life refer to the first (distribution) and second (elimination) phases of bi-exponential pharmacokinetics, respectively.

Complementarity-determining regions
(CDRs). The polypeptide loops in an antibody that are the main determinants of antigen binding. There are three CDRs in the variable domain of the immunoglobulin heavy chain and three in the variable domain of the immunoglobulin light chain.

Binding affinity
(K_d). For two interacting molecules, the ratio of their association (k_a) and dissociation (k_d) rate constants: that is, $K_d = k_d/k_a$.

Framework regions
The polypeptide segments in an antibody that together form a structural scaffold for presentation of the antigen-binding (complementarity-determining region) loops. There are four framework regions in the variable domain of the immunoglobulin heavy chain and four in the variable domain of the immunoglobulin light chain.

Phage-display technology
A technology for displaying a protein, such as an antibody fragment, on the surface of a bacteriophage that contains the gene(s) encoding the displayed protein(s), thereby physically linking the genotype and phenotype.

Phase III clinical trials: panitumumab (Abgenix, Inc. and Amgen Inc.; an epidermal-growth-factor receptor (EGFR)-specific monoclonal antibody), for treatment of colorectal cancer; ipilimumab (Medarex, Inc. and Bristol-Myers Squibb Company; a cytotoxic T-lymphocyte antigen 4 (CTLA4)-specific monoclonal antibody), for treatment of melanoma; denosumab (Amgen Inc.; a receptor activator of nuclear factor-κB (RANK)-ligand-specific monoclonal antibody), for treatment of post-menopausal osteoporosis and to avoid bone loss in hormone-ablation therapy during treatment for cancer; zanolimumab (HuMax-CD4; Genmab A/S and Serono S.A.; a CD4-specific monoclonal antibody), for treatment of cutaneous T-cell lymphoma; and golimumab (Centocor, Inc.; a tumour-necrosis factor (TNF)-specific monoclonal antibody), for treatment of rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis.

Using transgenic mice to generate human antibodies is relatively simple and relies on widely used techniques. Specifically, B cells that express human antibodies are isolated from immunized mice and then cloned as for hybridomas, similar to the generation of mouse monoclonal antibodies. The immune response in transgenic mice is sometimes less robust than in strains that are used to generate mouse monoclonal antibodies, so an increased number of immunizations or antibody screens might be required. The binding affinity of human antibodies from transgenic mice is often high, reflecting *in vivo* affinity maturation, which is integral to the secondary immune response. This high affinity routinely obviates the requirement for subsequent *in vitro* affinity maturation or for antibody-potency enhancement using other technologies. The initial output of these transgenic mice is human IgG, allowing early screening for biological function. Hybridoma cell lines that are generated from immunized transgenic mice provide an easy way to produce the corresponding human antibodies for the process of screening through to pre-clinical development and, in certain cases, early clinical development. However, recombinant cell lines, such as Chinese hamster ovary or NS0 mouse myeloma cells, often produce substantially higher antibody titres than human hybridoma cell lines and are commonly chosen to support late stage, if not all, clinical development[27]. It is often desirable to obtain species-crossreactive antibodies, allowing the biological function of the antibodies to be evaluated in animal models of disease. However, it is challenging to use transgenic mice to derive antibodies that are crossreactive with mouse antigens, because self-reactive antibody-producing cells are selected against by processes of immune tolerance induction.

Human antibodies from phage-display libraries. Phage encoding a single-chain V-domain antibody fragment (scFv) on their surface were first described in 1990, in a study that included the selective recovery of phage on the basis of antigen binding[34]. Subsequently, diverse human immunoglobulin-heavy-chain V (V_H) gene segments and light-chain V (V_L) gene segments were prepared from peripheral-blood lymphocytes of non-immunized donors, using amplification by PCR. Genes encoding scFvs were made by randomly combining V_H and V_L gene segments using PCR. The combinatorial library (fewer than 10^7 scFv genes) was then cloned for display on the surface of phage and was used to identify scFvs that bind target antigens[35]. Further progress in phage-display technology has included increases in library size (up to ~10^{11} genes) and quality, display of Fabs, and high-throughput screening methods adapted from small-molecule drug discovery[36]. Experience with phage-display libraries[36] supports the prediction[37] that increases in functional display-library size facilitate the identification of higher-affinity clones. One human antibody isolated by phage-display technology has been approved widely for therapeutic use: adalimumab (Humira; Abbott Laboratories; a TNF-specific monoclonal antibody), which is used for treatment of rheumatoid and psoriatic arthritis (TABLE 1; see Supplementary information S1 (table)). At least 14 additional antibodies that have been derived from recombinant libraries[13] are in clinical development, including 8 or more human antibodies from phage-display libraries[38].

Optimal implementation of antibody phage-display libraries requires resources and knowledge that are not as widely available as for hybridoma technology. In addition to the creation of large, high-quality libraries, expertise in phage husbandry, automation and informatics is required to facilitate screening, as well as expertise in high-throughput methods to allow the reformatting of antibody fragments and their production as IgG for further screening[13]. After their isolation from phage-display libraries, some antibody fragments have a sufficiently high binding affinity or biological potency for therapeutic applications. More commonly, antibodies need to be optimized to meet the design goals of the project, and this is readily accomplished by selection for high-affinity variants from phage-display libraries[13]. A particular strength of phage-display libraries, in contrast to hybridoma technology, is that direct selection for exquisitely specific binding properties, such as species crossreactivity[39], is sometimes possible. In addition, phage-display technology is the most well-established route for obtaining very large collections of antibodies, such as the more-than-1,000-member panel of antibodies that are specific for B-lymphocyte stimulator (BLYS; also known as BAFF)[40]. This capability might allow the identification of antibodies that have rare combinations of properties or very high potency. For example, belimumab (LymphoStat-B; Cambridge Antibody Technology, Human Genome Sciences and GlaxoSmithKline) was generated by affinity maturation of a lead BLYS-specific antibody and is in Phase II clinical trials for the treatment of rheumatoid arthritis and systemic lupus erythematosus[40,41]. Initial screening of scFv or Fab phage-display libraries is routinely carried out in the form of antibody fragments on phage or in solution, whereas IgG molecules are often required for functional assays. However, the advent of high-throughput methods for reformatting antibody fragments as IgG molecules[42–44] has largely overcome what was previously a bottleneck in deriving human antibodies from phage-display libraries.

Affinity maturation
An *in vitro* or *in vivo* process for increasing the affinity of a binding interaction, such as that between an antibody and its cognate antigen.

Species crossreactive
For antibodies, this is often used to denote binding to the corresponding antigen from two or more species: for example, humans, non-human primates and rodents.

Single-chain V-domain antibody fragment
(scFv). A small antibody fragment that comprises the variable (V) domains of the immunoglobulin heavy and light chain in either order joined by a short (~15 amino-acid residue) peptide linker.

Functional display-library size
The number of clones that is displayed with sufficient efficiency to be potentially selectable.

Epitope
The part of an antigen that interacts with an antibody.

Tunable properties of antibodies

Antibodies have numerous interdependent properties that can be tuned to improve their clinical potential. These properties include immunogenicity, antigen-binding specificity and affinity, effector functions and other biological activities, pharmacokinetics, molecular architecture, internalization after cell binding, and biophysical properties[4–6]. Several properties can be modulated by engineering the interaction between IgG and one of its binding partners: target antigen, FcγRs, complement component 1q (C1q) and the salvage receptor FcRn (FIG. 2). Two broad strategies are widely used, either individually or in combination, for the optimization of antibody therapeutics: display libraries[13,38] and structure-based design (also known as rational design)[45] (BOX 1).

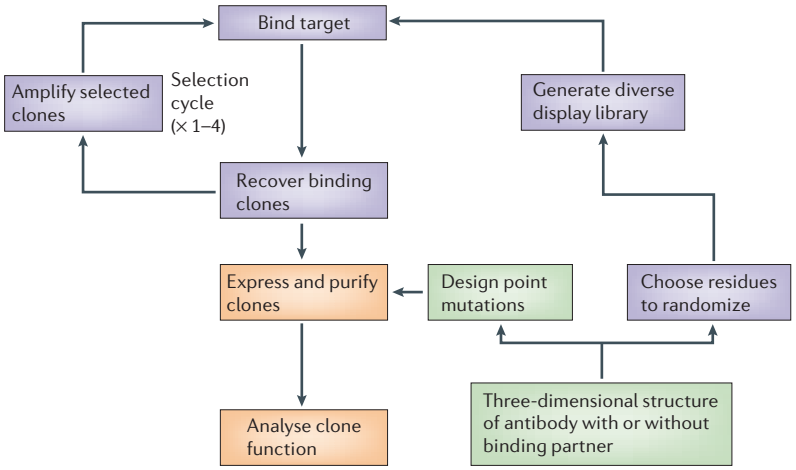
Immunogenicity. All protein drugs, including antibodies, are potentially immunogenic[46]. Indeed, patients commonly raise a mouse-antibody-specific antibody response when administered mouse antibodies, particularly after receiving multiple doses. Such responses might compromise antibody efficacy by direct neutralization, acceleration of clearance, prevention of further dosing and induction of serum sickness.

Immunogenicity data for chimeric humanized mouse antibodies strongly indicate that the replacement of mouse-antibody constant domains with human-antibody constant domains substantially reduces the immunogenicity of the antibody and that humanization might further reduce the immunogenicity[47]. The few data that are available for completely human antibodies preclude assessment of whether these antibodies are less immunogenic or have other advantages over humanized antibodies. Patients might raise a humanized-antibody-specific antibody response to humanized antibodies, but this is usually sufficiently low in titre and frequency that it does not affect treament[47]. Moreover, there are several strategies that might further reduce the risk of immunogenicity of humanized or human antibodies if this is required (TABLE 2).

Discussions of antibody immunogenicity often focus on the influence of primary amino-acid structure, with limited attention being paid to the multitude of other contributing factors, such as the presence of aggregated or misfolded antibody or contaminants, the presence of microheterogeneity in the antibody pool (for example, glycosylation or chemical modifications such as deamidation) and the presence of T-cell epitopes within the amino-acid sequence of the antibody[48]. Antibody immunogenicity is also influenced by clinical parameters, including the dose, route and frequency of administration, as well as patient-specific factors, such as disease and immune status, MHC haplotype and concomitant medication[48–50]. Quantification of any immune response to antibodies poses considerable challenges, and these challenges increase with the use of antibodies that are closer to human antibodies[50].

Antigen-binding specificity. Antibodies typically bind their target antigen with highly specific selectivity, which is a prerequisite for their use in targeted therapy. By contrast, it is less common for antibodies to bind the corresponding antigen from a different species, such as a non-human primate or a rodent, although such binding is a desirable attribute that would facilitate preclinical evaluation of efficacy and toxicity. Success at generating such species-crossreactive antibodies depends on the sequence-relatedness of the antigen in different species (including the conservation of the target epitope) and the method of antibody generation. Phage-display technology[36] and other display technologies[13,38] (BOX 1) are particularly well suited to the generation of

Box 1 | **Technologies for antibody optimization**



Antibodies are commonly optimized by selection from genetically diverse display libraries of antibody-fragment variants (purple boxes) or by structure-based design (green boxes) followed by expression, purification and functional characterization of individual variants (orange boxes). These selection and design strategies are complementary and are particularly powerful when used in combination, as exemplified by the tailoring of the interaction between the Fc region of IgG and FcRn[101].

Phage-display libraries are the most commonly used display technology for antibody generation and optimization[13,36,38]. However, other display technologies — such as yeast-, mRNA- and ribosome-display libraries — are gaining in popularity for the selection and optimization of antibodies[13,38]. Display libraries display single-chain variable-domain antibody fragments (scFvs) or Fabs, and contain the encoding DNA or RNA. They have high genetic diversity or repertoire size (commonly 10^9–10^{13}). These technologies allow the selective recovery of clones that bind a target antigen from a library, and they provide the means to amplify the selected clones for further rounds of selection or analysis. The genetic diversity in these libraries is commonly created by cloning the repertoire of the immunoglobulin heavy-chain and light-chain variable gene segments from naive or immunized individuals. Alternatively, this diversity can be achieved by using synthetic DNA to randomize the complementarity-determining regions (the antigen-binding loops) or by a combination of these two approaches. The binding step can be undertaken with the target in solution, immobilized on a surface or on cells. After extensive washing, specifically bound clones are recovered and amplified for the next round of selection. In an optional step, additional genetic diversity can be introduced during clone amplification: for example, by error-prone PCR.

Structure-based design uses three-dimensional structural information, sometimes in combination with sophisticated computational methods, to predict the site and type of useful mutations[45]. The ideal starting point for design is a high-resolution structure of an antibody in complex with the relevant binding partner: that is, antigen, complement, IgG receptor (FcγR) or FcRn. However, even a molecular model of an antibody or antigen–antibody complex can sometimes provide a useful starting point. Extensive mutational analysis has increased our understanding of, and provided a means to modulate, the interaction of the Fc region of IgG with various FcγRs[80,142], FcRn[80] and complement component 1q (C1q)[94,95,143] (TABLE 2).

Table 2 | **Designer antibodies for human therapy**

Design goal	Strategies	Potential benefits
Immunogenicity		
Minimize risk	Minimize non-human sequence (chimerization or humanization of mouse antibody, use of human monoclonal antibody, use of human germline sequences); design to remove T-cell epitopes; minimize presence of aggregated or misfolded antibody (purification, formulation, engineering of variable domains for improved stability or folding kinetics); minimize presence of contaminants (purification)	Increased efficacy; improved safety; more efficient effector functions; longer terminal half-life
Antigen-binding specificity		
Improve selectivity for antigen	Select from display libraries; screen antibody panel; use structure-based design	Prerequisite for targeted therapy
Increase species crossreactivity	Select from display libraries; screen antibody panel; use structure-based design	Facilitates preclinical development (efficacy testing, toxicology)
Antigen-binding affinity		
Increase	Select from display libraries; use structure-based design	Increased efficacy; reduced dose or frequency of administration; increased potency of ADCC
Decrease	Select from display libraries; use structure-based design	Increased localization to tumours; more homogeneous distribution in tumours
Biological activities associated with variable domains		
Isolate antibodies with potent activities	Screen antibody panel	Increased efficacy; reduced dose or frequency of administration
Improve activities of existing antibodies	Induce affinity maturation of existing antibody then screen for potency	Increased efficacy; reduced dose or frequency of administration
Effector functions		
Improve or tailor	Screen human IgG panel; engineer Fc region (point mutations, glycan modifications); increase antigen-binding affinity	Increased efficacy
Avoid or abolish	Screen human antibody panel; select IgG2 or IgG4 as isotype; use IgG with point mutations in Fc region; use IgG that lacks disulphide bonds in hinge region; use IgG with an aglycosylated Fc region; use antibody fragments	Reduced adverse events
Pharmacokinetics		
Reduce plasma half-life	Use antibody fragments; use IgG with impaired affinity for FcRn	Less whole-body exposure to antibody; improved target-to-non-target ratios
Increase plasma half-life	Use IgG with increased affinity for FcRn (at pH 6.0); modify antibody fragments (PEGylation, binding to molecules with a long half-life such as IgG and serum albumin)	Increased plasma concentrations might improve localization to target; increased efficacy; reduced dose or frequency of administration
Internalization		
Decrease efficiency	Screen antibody panel; choose target antigen accordingly	Efficient effector functions
Increase efficiency	Select antibodies that can internalize from display libraries; screen antibody panel with drug-conjugated crosslinking antibody	Improved efficacy for antibody–drug conjugate or immunoliposomes
Chemical, proteolytic and thermodynamic stability		
Increase	Design to remove 'problem sites'; select from display libraries; use structure-based design	Maintains potency; longer shelf life, improved expression yields; longer terminal half-life *in vivo*; reduced risk of immunogenicity; improved *in vivo* localization to tumour
Other biophysical properties: solubility and folding kinetics		
Increase or improve	Select from display libraries; use structure-based design	Improved expression yields; reduced risk of immunogenicity

ADCC, antibody-dependent cell-mediated cytotoxicity; PEG, polyethylene glycol.

species-crossreactive antibodies, because these technologies circumvent the induction of immune tolerance and allow screening[51] or, preferably, direct selection[39] for species crossreactivity. At least one clinical lead antibody has been engineered by rational design to bind the corresponding antigen from another species (rhesus monkeys)[52], but this strategy is too onerous and unpredictable for routine use.

Antigen-binding affinity. One of the most widely studied properties of antibodies is antigen-binding affinity (which is denoted K_d). Many library-construction strategies, in conjunction with several display technologies (BOX 1), have been used successfully for the affinity maturation of antibodies[6,13]. Two impressive early examples were the use of phage-display libraries to increase the affinity of an HIV-1 glycoprotein 120 (gp120)-specific monoclonal antibody fragment[53] and an ERBB2 (also known as HER2)-specific monoclonal antibody fragment[54] from the nanomolar to the picomolar range. Similar successes have become relatively common with the use of phage-display libraries and, more recently, with the use of yeast- and ribosome-display libraries[13]. Separate mutations that increase binding affinity are routinely combined and often further increase affinity in an additive or synergistic manner[55].

Increases in binding affinity that are achieved using display technologies are typically dominated by large reductions in dissociation rate. Nevertheless, several-fold increases in association rate have been reported for antibodies that have undergone affinity maturation using phage display[56] or yeast display[57] with targeted screening or selection strategies, respectively. Rational design using electrostatic principles has also increased the association rate of antibodies[58]. Regarding functional significance, increases in association rate had a dominant role in the affinity maturation of palivizumab (Synagis; MedImmune Inc. and Abbott Laboratories; a respiratory syncytial virus (RSV) gpF-specific monoclonal antibody)[56], allowing this antibody to neutralize RSV more potently.

Affinity maturation of antibodies is often, but not always[59], associated with increased *in vitro* biological potency[6,13]. Much less is known about the relationship between the antigen-binding affinity of an antibody and its *in vivo* properties, with tumour targeting being the most widely studied property. For example, the antigen-binding affinity of an antibody influences tumour localization, as shown for a panel of ERBB2-specific scFvs[60,61]. A threshold affinity (K_d) of 10^{-7}–10^{-8} M was required for specific tumour localization of scFvs, with uptake reaching a plateau at a K_d of 10^{-9}–10^{-11} M. The lowest-affinity scFv had the most uniform distribution throughout the tumour, whereas the highest-affinity scFv was found mainly in the perivascular region of the tumour[61]. These observations are consistent with the 'binding-site barrier' (that is, that tumour penetration by an antibody is limited by binding to its target antigen), as described by Weinstein and colleagues[62]. So, there is an upper limit of antigen-binding affinity beyond which further increases in affinity do not seem to be beneficial for tumour localization. Tumour localization of dimeric versions (known as diabodies) of these ERBB2-specific scFvs (FIG. 3) correlated inversely with the intrinsic (monomeric) affinity of the scFv, with the lowest-affinity diabody (K_d of 400 nM) localizing over twofold more efficiently than the highest-affinity diabody (K_d of 2 nM)[63]. Although the basis for this phenomenon remains to be fully elucidated, it might have broad implications for the future development of antibodies that target tumour cells. That is, simple dimerization of a low-affinity scFv can lead to considerably greater tumour localization than can be achieved by the more time-consuming process of scFv affinity maturation. Potential improvements in antitumour activity that result from increased tumour uptake remain to be explored.

The marked effect of antigen-binding affinity on the *in vitro* and *in vivo* activities of antibodies supports the idea that affinity also has a considerable impact on the clinical utility of antibodies. Indeed, extensive affinity maturation of palivizumab led to the second generation antibody motavizumab (Numax; MedImmune Inc.)[56], which proved more efficacious than the parent antibody in a mouse model of RSV infection — in terms of reduced viral load and disease severity[64] — and is in two Phase III clinical trials at present. The antigen-binding affinity of current antibody therapeutics ranges from a K_d of 0.08 nM to 32 nM (TABLE 1; see Supplementary information S1 (table)). Whether there is an optimal antigen-binding affinity for antibodies remains unexplored. So, an empirical approach is warranted for preclinical optimization of the antigen-binding affinity of antibodies, and this would ideally be guided by mathematical modelling[65].

Biological activities associated with variable domains. Antibodies can have a large range of therapeutically relevant activities, as shown by the antibodies that have been approved for use in the United States (TABLE 1; see Supplementary information S1 (table)). The potential benefits of increasing biological potency include increased efficacy, and reduced dose or frequency of administration. The choice of which antibody activity to improve might be difficult, because some antibodies have multiple activities. For example, cetuximab (Erbitux; ImClone Systems Inc. and Bristol-Myers Squibb Company) blocks binding of EGF and transforming growth factor-α to EGFR, inhibits receptor activation and cell proliferation, induces apoptosis, sensitizes tumour cells to chemotherapy and radiotherapy, indirectly inhibits angiogenesis, invasion and metastasis, and supports antibody-dependent cell-mediated cytotoxicity (ADCC)[66] (TABLE 1; see Supplementary information S1 (table)). Moreover, it might be challenging to determine the most crucial antibody activity or combination of activities through the use of animal models, let alone in patients[66].

Two broad approaches yield antibodies with potent biological activity: improving the activity of existing antibodies, and *de novo* functional screening for new antibodies. Affinity maturation of an existing antibody followed by functional screening is a commonly used

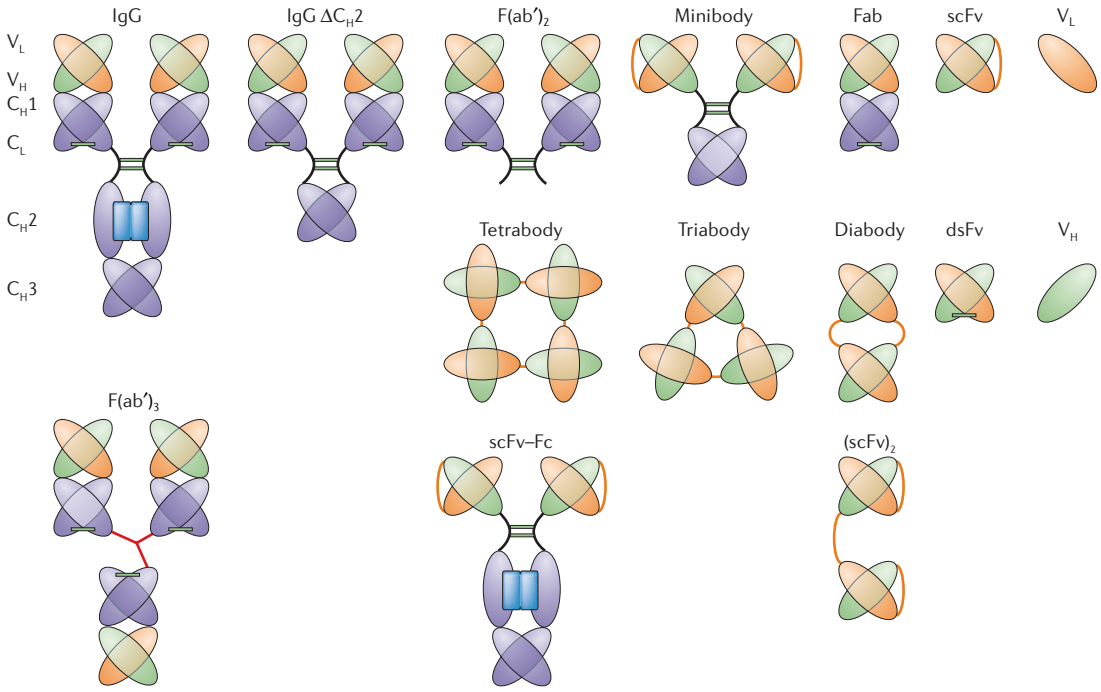


Figure 3 | **Representative antibody formats.** The modular domain architecture of immunoglobulins has been exploited to create a growing range of alternative antibody formats that spans a molecular-weight range of at least 12–150 kDa and a valency (n) range from monomeric (n = 1), dimeric (n = 2) and trimeric (n = 3) to tetrameric (n = 4) and possibly higher[12,16]. For simplicity, all antibody formats are shown as being monospecific: that is, having one or more copies of identical antigen-binding sites. However, formats with a valency of two or more have also been used to create antibodies that have two or more (up to the valency of the format) distinct antigen-binding sites, which bind different antigens or different epitopes on the same antigen. The building block that is most frequently used to create novel antibody formats is the single-chain variable (V)-domain antibody fragment (scFv), which comprises V domains from the heavy and light chain (V_H and V_L domain) joined by a peptide linker of up to ~15 amino-acid residues. Both the V_L-domain–peptide-linker–V_H-domain topology and the V_H-domain–peptide-linker–V_L-domain topology have been widely used. Constant (C) and V domains, inter-chain disulphide bonds (green bars) and glycosylation are depicted as in FIG. 2, and peptide and chemical linkers are shown as orange and red lines, respectively. C_H, C domain of immunoglobulin heavy chain; C_L, C domain of immunoglobulin light chain; dsFv, disulphide-stabilized scFv.

and frequently successful strategy for increasing the potency of antibodies (as discussed earlier). The biological activity might depend on the antibody format (for example, scFv, Fab or IgG)[51], so it is highly desirable to screen using the format that it is anticipated will be used for clinical applications. For example, a bivalent format might be required for crosslinking, and thereby activating, a receptor.

Effector functions. For antibody therapeutics, avoiding effector functions such as ADCC and complement-dependent cytotoxicity (CDC) (FIG. 2) might reduce the side-effects, or increasing effector functions might increase the efficacy. The choice of effector-function profile of an antibody therapeutic can be guided by consideration of the target antigen, therapeutic strategy and clinical setting.

Muromonab-CD3 is a mouse monoclonal IgG2a that is highly immunosuppressive and efficacious at both preventing and reversing the rejection of organ transplants[67]. Unfortunately, muromonab-CD3 is mitogenic, in that it stimulates the proliferation of T cells and the production of cytokines *in vitro*. Therapy with muromonab-CD3 leads to a massive, albeit transient, systemic release of cytokines, resulting in an acute and severe influenza-like syndrome (as mentioned earlier), which involves fever, chills, headaches, vomiting and diarrhoea. The beneficial activities of muromonab-CD3 are mediated by the V domains, whereas the undesirable mitogenic activity of muromonab-CD3 requires interaction between the Fc region of the antibody and the FcγRs of the patient[68]. An additional limitation of muromonab-CD3 is that most patients develop a mouse-antibody-specific antibody response[69].

Clinical experience with muromonab-CD3 (REF. 67) indicates that a non-mitogenic CD3-specific antibody that is minimally immunogenic or non-immunogenic might offer considerable advantages. Several strategies have yielded CD3-specific antibodies that show little or no binding to FcγRs, resulting in little or no mitogenicity. These strategies include use of the isotypes IgG2 and IgG4, and use of IgG of various isotypes with mutations that impair interaction with FcγRs or prevent the glycosylation that is required for effector functions[70–73].

At least three humanized CD3-specific antibodies with engineered Fc regions recapitulate the immunosuppressive benefits of muromonab-CD3 while ameliorating the influenza-like syndrome and antibody response, as shown in several Phase I (REFS 74–76) and Phase II (REF. 68) clinical trials.

Several antibody therapeutics — including rituximab, adalimumab, cetuximab, trastuzumab (Herceptin; Genentech, Inc. and F.Hoffman-LaRoche Ltd; an ERBB2-specific monoclonal antibody), alemtuzumab (Campath; Genzyme Corporation and Schering AG; a CD52-specific monoclonal antibody) and ^{131}I-labelled tositumomab (Bexxar; GlaxoSmithKline; a CD20-specific monoclonal antibody) — support ADCC and/or CDC *in vitro* (TABLE 1; see Supplementary information S1 (table)). These effector functions might contribute to the therapeutic efficacy of antibodies in clinical settings in which the destruction of target cells is desired, such as the removal of tumour cells or virally infected cells. The strongest evidence for an Fc-mediated contribution to antibody efficacy in patients is for rituximab: responsiveness to rituximab in patients with non-Hodgkin's lymphoma correlates with polymorphisms in FcγRIIIa[77,78] and FcγRIIa[78], which are expressed by immune effector cells. These observations implicate the interaction between the Fc region of rituximab and the FcγRs of the recipient, and probably ADCC, in contributing to antitumour activity. Polymorphic forms of FcγRIIIa that have either valine or phenylalanine at position 158 arise from mutations in the *FCGR3A* gene. Natural killer cells that are homozygous for the Val158-containing form of FcγRIIIa have a higher affinity for the Fc region of IgG1 than do natural killer cells that are homozygous for the Phe158-containing form of FcγRIIIa[79]. Moreover, effector cells that are homozygous for the Val158-containing form are commonly more potent at inducing ADCC *in vitro* than those that are homozygous for the Phe158-containing form[80]. The response rates to rituximab in patients with follicular non-Hodgkin's lymphoma are highest in those who are homozygous for the higher-affinity FcγRIIIa (the Val158-containing form)[77,78]. By contrast, no such correlation was found for responsiveness to rituximab in patients with B-cell chronic lymphocytic leukaemia[81], indicating that the antitumour activity of antibodies can vary with the clinical setting.

Effector functions have been enhanced by engineering the glycan structure or amino-acid sequence of the Fc region to improve binding to FcγRs. Glycosylation of IgG at the conserved asparagine residue at position 297, which is in the C_H2 domain (immunoglobulin heavy-chain constant domain 2), is required to support the interaction between IgG and FcγRs, which is a prerequisite for ADCC[80]. Host cell lines have been selected or engineered to express antibodies with altered glycosylation, such as increased amounts of bisecting *N*-acetylglucosamine[82] and/or reduced amounts of fucose[83] (FIG. 2). Reducing the fucose content increases ADCC activity to a greater extent than does increasing the presence of bisecting *N*-acetylglucosamine[84]. Moreover, increases in ADCC by using antibodies with a low fucose content occur independently of the FcγRIIIa polymorphism at position 158 (REF. 85). IgG molecules with specific and homogeneous human N-linked glycan structures have recently been produced in engineered strains of the yeast *Pichia pastoris* and have been shown to have improved antibody effector functions[86].

Increasing ADCC activity through engineering the amino-acid sequence of the Fc region has been greatly facilitated by a detailed mutational analysis that maps the binding site on human IgG1 for each of the activating (FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb) and inhibitory (FcγRIIb) FcγRs[80]. Fc-region variants that have many permutations in binding properties to the various FcγRs have been obtained. For example, humanized IgG1 molecules with mutations in the Fc region that increase binding affinity for FcγRIIIa can increase ADCC *in vitro*[80]. More recently, computational design methods have been used to tailor the interaction between IgG molecules and various FcγRs to modulate effector functions, achieving a more than 100-fold increase in the potency of ADCC elicited by alemtuzumab and trastuzumab variants, as well as increases in the percentage of tumour cells that are killed[87]. Efficient killing of tumour cells that express small amounts of antigen has also been shown, albeit with an unknown risk of killing non-tumour cells that express small amounts of the target antigen.

Species differences in FcγRs between mice and humans complicate *in vivo* assessment of the potential benefits of increasing ADCC activity. The use of severe combined immunodeficient (SCID) mice reconstituted with human peripheral-blood lymphocytes offers one option to address this problem[85]. Alternatively, for antibodies that target CD20, B-cell depletion of non-human primates offers a way to evaluate antibodies with increased ADCC activity[88]. Using mice that are transgenic for human FcγRs[89,90] might be a more broadly applicable approach. These preclinical challenges notwithstanding, clinical evaluation of antibodies that have increased ADCC activity seems warranted. Indeed, a Phase I/II clinical trial of a glyco-engineered (low fucose) variant of the CD52-specific monoclonal antibody alemtuzumab (GLYCART biotechnology AG (F.Hoffman-LaRoche Ltd)) is in progress for the treatment of chronic lymphocytic leukaemia. In addition, a Phase II clinical evaluation for the treatment of rheumatoid arthritis is underway for ocrelizumab (Genentech, Inc., F.Hoffman-LaRoche Ltd and Biogen Idec Inc.), a humanized CD20-specific monoclonal antibody[88] with *in vitro* ADCC and CDC activity several fold higher and lower than rituximab, respectively.

Several antibody therapeutics, including rituximab, support CDC activity *in vitro* (TABLE 1; see Supplementary information S1 (table)). First-infusion reactions caused by rituximab, such as fever and chills, correlate with the activation of complement[66], but definitive evidence that CDC contributes to the antitumour activity of rituximab in patients remains elusive[91]. Nevertheless, CDC has been shown to contribute to the *in vivo* antitumour activity of rituximab in mice, as judged by loss of antitumour activity in C1q-deficient mice[92] and diminished antitumour activity following C1q depletion with cobra venom[93]. Detailed mapping of the C1q-binding site

Severe combined immunodeficient mice (SCID mice). Mice that are homozygous for the *scid* allele of the gene that encodes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). These mice are severely deficient in functional B and T cells.

on human IgG1 led to the development of a rituximab mutant with improved C1q binding[94]. CDC activity was increased about twofold *in vitro*, albeit at the expense of slightly impaired ADCC[95]. Human CD20-specific antibodies with highly potent CDC activity against some tumour cells that express large amounts of the complement-regulatory proteins CD55 and CD59 and that are refractory to CDC induced by rituximab have been isolated from transgenic mice[96]. One of these human antibodies, HuMax-CD20 (Genmab A/S), is showing antitumour activity in Phase I/II clinical trials.

Pharmacokinetics. The terminal half-life of antibodies in plasma can be tuned over a wide range to fit clinical goals. It can range from several minutes for single-domain antibodies and scFvs to several weeks for many IgG molecules (TABLE 2). It might be desirable to increase the terminal half-life of an antibody to improve efficacy, to reduce the dose or frequency of administration, or to improve localization to the target. Alternatively, it might be advantageous to do the converse — that is, to decrease the terminal half-life of an antibody — to reduce whole-body exposure or to improve the target-to-non-target binding ratios.

Tailoring the interaction between IgG and its salvage receptor, FcRn, offers a way to increase or decrease the terminal half-life of IgG. Proteins in the circulation, including IgG, are taken up in the fluid phase through micropinocytosis by certain cells, such as those of the vascular endothelia. IgG can bind FcRn in endosomes under slightly acidic conditions (pH 6.0–6.5) and can recycle to the cell surface, where it is released under almost neutral conditions (pH 7.0–7.4)[97,98]. Mapping of the Fc-region-binding site on FcRn[80,99,100] showed that two histidine residues that are conserved across species, His310 and His435, are responsible for the pH dependence of this interaction. Using phage-display technology, a mouse Fc-region mutation that increases binding to FcRn and extends the half-life of mouse IgG was identified[101]. Fc-region mutations that increase the binding affinity of human IgG for FcRn at pH 6.0, but not at pH 7.4, have also been identified[80,102]. Moreover, in one case, a similar pH-dependent increase in binding (up to 27-fold) was also observed for rhesus FcRn, and this resulted in a twofold increase in serum half-life in rhesus monkeys compared with the parent IgG[102]. These findings indicate that it is feasible to extend the plasma half-life of antibody therapeutics by tailoring the interaction of the Fc region with FcRn. Conversely, Fc-region mutations that attenuate interaction with FcRn can reduce antibody half-life, as has been elegantly shown by the pharmacokinetic optimization of an scFv–Fc to allow tumour imaging by positron emission tomography[103]. Modulation of serum IgG concentrations has recently been achieved by tailoring the interaction between an IgG Fc region and FcRn[104]. An IgG engineered to bind FcRn with higher affinity and reduced pH dependence potently inhibits the interaction of endogenous IgG with FcRn, thereby resulting in a rapid reduction in the IgG concentration in mice. These FcRn-blocking antibodies, which are known as Abdegs (antibodies that increase IgG degradation), have potential therapeutic application for the reduction of IgG concentrations, for example, in patients with antibody-mediated diseases[104].

Using antibody fragments instead of antibodies reduces the terminal half-life of the therapeutic from many weeks to a few hours or less, which is too short for many therapeutic applications[12]. The terminal half-life of antibody fragments can be extended many fold by endowing them with the ability to bind long-lived molecules, such as IgG[105] and serum albumin[106]. Antibody fragments with a wider range of terminal half-lives (from a few hours to weeks) can be generated through PEGylation (that is, conjugation to polyethylene glycol, PEG)[107]. At least two PEGylated antibody fragments have progressed to clinical trials[12], including certolizumab pegol (Cimzia; UCB; a humanized TNF-specific Fab′ conjugated to PEG). Certolizumab pegol has a terminal half-life of ~14 days in patients, which is comparable to the parent IgG, and it is well tolerated[108,109]. Regulatory submission for certolizumab pegol is now complete, and this followed preliminary reports of favourable safety and efficacy data in two pivotal Phase III clinical trials for the treatment of Crohn's disease. The potential advantages of PEGylated fragments over IgG include a lack of any undesirable Fc-mediated effects, a reduction in the risk of immunogenicity and a moderate reduction in the cost[110].

Molecular architecture. Nearly all marketed antibody drugs are in the IgG format, and most contain a human Fc region of the IgG1 isotype, which in many cases supports one or more effector functions (TABLE 1; see Supplementary information S1 (table)). Human IgG2 and IgG4 are often inefficient at supporting effector functions and are sometimes chosen when effector functions are unnecessary or undesirable (TABLES 1,2; see Supplementary information S1 (table)). Few, if any, IgG3 molecules are in clinical development, possibly because the hinge region of human IgG3 is much longer and more prone to proteolysis than that of human IgG1, IgG2 and IgG4. The use of other naturally occurring forms of antibody — such as IgA[111–113] and IgM[114] — has been explored in several small-scale clinical trials. Nevertheless, IgM has limited potential as a therapeutic, because its enormous size (~1 MDa) and complexity (~21 different polypeptide chains) pose formidable manufacturing challenges. In addition, the huge size of IgM molecules seems likely to compromise their ability to reach targets beyond those encountered in the vasculature. However, receptor-mediated transcellular transport of IgM and IgA might improve their access to extravascular targets[115].

The modular domain architecture of antibodies (FIG. 2) has been exploited to create a growing number of alternative formats that span a broad range of molecular weights (from 12 kDa to more than 150 kDa), valencies (from 1 to more than 4) and antigen-binding specificities (from 1 to more than 3 antigens or epitopes on the same antigen)[12,16] (FIG. 3). The repertoire of antibody-based proteins has been further expanded and endowed with novel functions by fusion of the genes encoding the

PEGylation
The chemical modification of a protein by conjugation to one or more molecules of polyethylene glycol (PEG).

Valency
For antibodies, the number of binding sites for the cognate antigen.

antibodies with the genes encoding other proteins or protein fragments, including enzymes, multimerization domains and toxins[12].

Antibody fragments have many attributes that differ from those of IgG, and these offer potential advantages under some clinical settings. As noted previously, antibody fragments provide a simple way to avoid Fc-dependent effector functions, and after PEGylation, they can show a broad range of pharmacokinetic properties. For imaging applications, more efficient penetration of tumours and faster clearance from the body are potential advantages of using antibody fragments instead of IgG, because these properties can give rise to improved tumour-to-non-tumour binding ratios[116]. By contrast, because IgG has a longer terminal half-life than most antibody fragments, this often, if not invariably, translates into greater tumour uptake, which seems desirable for therapeutic applications in oncology[116]. A monovalent antibody fragment might be more advantageous than a bivalent form, such as IgG, for use as a pure receptor antagonist that can block ligand binding to a receptor without crosslinking and potentially activating the receptor[117].

Single-domain antibodies[118] with favourable properties — for example, high affinity, high solubility and robust expression — are now routinely identified from natural sources such as camels, llamas and sharks and by selection from V_H and V_L gene-segment display libraries from humans and other species[12]. The small size of single-domain antibodies might allow ready access to the appropriate regions of targets (such as structural 'canyons' in viruses and active sites of enzymes[12]), which are challenging, but sometimes possible[119], to reach with conventional antibodies (which have antigen-binding determinants on two V domains). Several antibody fragments have been produced at gram-per-litre titres by *E. coli* fermentation, a feat that has only recently been approached for IgG molecules (which are much larger and more complex) in this host[120].

One non-IgG antibody-based molecule, the Fab abciximab (ReoPro; Centocor, Inc.; a gpIIb–gpIIIa- and $\alpha_v\beta_3$-integrin-specific monoclonal antibody), has been approved for human therapy (TABLE 1; see Supplementary information S1 (table)). In addition, ranibizumab (Lucentis; Genentech, Inc. and Novartis AG; a humanized VEGF-specific Fab) was safe and well tolerated in a Phase I clinical trial[121] and efficacious in Phase II and III clinical trials for the treatment of wet age-related macular degeneration. Other antibody formats (FIG. 3) that have been explored in clinical trials for imaging or therapeutic applications include the scFv[122,123], $F(ab')_2$ (REF. 124), minibody[125], $(scFv)_2$ (REF. 126) and scFv–Fc (TRU-015; Trubion Pharmaceuticals Inc.). The increasing interest in antibody fragments as therapeutics is exemplified by 7 of the 15 antibodies in current Phase III clinical trials being antibody fragments[1]. In addition, there are at least 7 other antibody fragments in earlier stages of clinical development[12].

Internalization. Antibodies are often internalized after binding targets on the cell surface, but this occurs to greatly varying rates and extents. The parameters that affect internalization efficiency are poorly understood but probably include the choice of target antigen and epitope, the association and dissociation rates for antibody–antigen interaction, and intracellular events such as the relative rate of recycling to the cell surface versus degradation.

Efficient antibody internalization is desirable for certain applications, such as for delivery of cytotoxic drugs[116,127] and immunoliposomes[128]. Removal of the target antigen from the cell surface prevents the further binding of any ligands and is therefore a desirable outcome when using a receptor antagonist, a mechanism of action that is common to many antibody therapeutics (TABLE 1; see Supplementary information S1 (table)). By contrast, rapid antibody internalization after cell binding is undesirable if the therapeutic strategy requires effector functions (TABLE 2). Direct selection for antibodies that can be internalized is possible using phage-display libraries[129,130]. In addition, screens are available to identify antibodies that are potent in the form of drug conjugates[131]. In some cases, conjugation to drugs increases the efficiency of antibody internalization[132,133].

Biophysical properties. The V domains of antibodies differ widely in biophysical properties such as stability, solubility and folding kinetics, all of which can affect recombinant expression. By using phage-display libraries for the selection of antibody fragments with optimized binding activity, favourable properties such as robust expression, high stability and solubility are often selected together[12]. Direct selection for favourable biophysical properties can be achieved using chemical denaturants, high temperature, and reducing agents or proteases[13]. For example, single-domain antibodies that are aggregation resistant, soluble and well expressed by *E. coli* have been selected from transiently heated phage-display libraries[134]. Soluble expression of an scFv variant in yeast was increased 100-fold by screening mutant yeast-display libraries for improved yeast-surface display[135]. Rational design has also proven to be a highly valuable tool for improving the biophysical properties of antibody fragments[136], as exemplified by the grafting of the CDRs of one antibody into another antibody with a more stable framework region followed by structure-guided engineering of framework-region residues[137]. Improved thermodynamic stability can improve tumour targeting, as has been shown for an scFv[138]. By contrast, there are few data on the impact of improved V-domain biophysical properties when the V domains are incorporated into IgG molecules.

Perspectives

In recent years, tremendous progress has been made towards the goal of using antibodies as therapeutics, but numerous challenges remain. For example, the identification of plausible new target antigens for antibody therapeutics is aided by past successes and failures, but it is made more difficult by our limited knowledge of recently identified targets. So, the impressively high success rate for therapeutic antibodies[1] will be difficult to

maintain in light of the crucial dependence of success on the choice of target antigen. Nevertheless, if the success rate of antibody therapeutics does continue, then it will be difficult to meet production demands using established mammalian expression technologies[139]. Such a production shortfall might provide the impetus for further development of alternative antibody-production technologies[15,16], which in turn might help to provide some relief from another limitation of antibody therapeutics, high cost[9]. The concept of improving the antitumour activity of antibodies is well established, but doing so and remaining in the range of manageable toxicities remains a continuing challenge[4]. In the case of antibody modifications, it remains to be seen whether engineering the amino-acid sequence or glycan structure will affect immunogenicity, pharmacokinetics or biodistribution. An additional challenge is navigating the burgeoning range of antibody technologies for those that are best suited to the generation of antibodies with the desired properties.

Established trends in the field of antibody therapeutics include the growing number of human antibodies[1] and antibody fragments[1,12] that are in clinical development. The decade or more interval between the advent of key antibody technologies (that is, hybridoma technology, chimerization, humanization and the generation of human antibodies from phage-display libraries) and the first corresponding drug approval[3,27] seems likely to continue for new technologies, because this interval reflects the long time-frame for adoption and implementation. Three powerful factors are converging to support the emergence of antibody therapeutics that are more potent and effective: a clinical imperative to achieve a better outcome for patients; the availability of established and emerging technologies to improve antibody performance; and the commercial drive to compete. Increased competition is inevitable, because many approved antibody drugs (TABLE 1; see Supplementary information S1 (table)) and antibody drugs under investigation[3,140,141] target the same antigen and clinical indications.

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