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Engineering Antibodies for Cancer Therapy

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Abstract

The advent of modern antibody engineering has led to numerous successes in the application of these proteins for cancer therapy in the 13 years since the first Food and Drug Administration approval, which has stimulated active interest in developing more and better drugs based on these molecules. A wide range of tools for discovering and engineering antibodies has been brought to bear on this challenge in the past two decades. Here, we summarize mechanisms of monoclonal antibody therapeutic activity, challenges to effective antibody-based treatment, existing technologies for antibody engineering, and current concepts for engineering new antibody formats and antibody alternatives as next generation biopharmaceuticals for cancer treatment.

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INTRODUCTION

The concept of using natural antibodies to combat cancer predates a detailed understanding of the molecular mechanisms of antibody generation and function (1, 2). The dramatic therapeutic potential of this approach for cancer and many other diseases, captured by use of the term "magic bullets" to describe antibodies, has stimulated continual interest from the research community for many decades despite several clinical disappointments. Early attempts to induce antitumor immunity involved treatment with polyclonal antibodies derived from immune sera from a variety of sources; such therapies proved ineffective owing at least in part to poor affinity and tumor specificity as well as the technical inability to reproducibly generate the polyclonal antibodies (1).

The invention of hybridoma technology to create monoclonal antibodies (mAbs) (3) was the first major technological shift that reinvigorated the field in the 1980s. Fusion of antibody-producing murine B lymphocytes with easily cultured myeloma cell lines enabled screening of clones to identify mAbs with antigen-binding properties suitable for therapeutic applications and further yielded the ability to reproducibly generate clinically and industrially meaningful quantities of those antibodies. Although this first wave of murine mAbs demonstrated reasonable safety characteristics and led to the milestone FDA approval of the OKT3 mAb for inhibition of transplant rejection, these molecules largely failed in the clinic owing to their incompatibility with the human patient's immune system. The immunogenicity of mouse sequences [i.e., induction of the inactivating human antimouse antibody (HAMA) response], the molecules' short circulatory half-life, and their inability to efficiently recruit patient immune effectors necessary for tumor cell destruction precluded their efficacy for cancer therapy (1, 4–7). Nonetheless, several murine antibodies and antibody fragments with conjugated radioisotopes have been approved for in vivo cancer imaging (8).

Antibody engineering provided the second technological leap that drove further progress in therapeutic applications by enabling the development of partially or fully human mAbs, something that was previously problematic owing to the inability to effectively apply hybridoma technology to human lymphocytes. The first successful commercial mAb for cancer therapy (rituximab for treatment of lymphoma, which was approved in 1997) was produced by fusing murine variable domains to human constant domains (**Figure 1**) to create a chimeric protein (9). Chimeric antibodies retain the binding specificity of the murine parent antibody but demonstrate enhanced immunological function and reduced immunogenicity as a result of the roughly two-thirds of the molecule with a fully human amino acid sequence (10). Similarly, humanized antibodies are derived from a combination of mouse sequences (obtained from mAbs identified by hybridoma technology and screening) to direct binding specificity with human sequences to act as the scaffold and immunological effector. In this case, only the complementarity-determining region (CDR) loops and typically a small number of adjacent framework residues are cloned into a human immunoglobulin G (IgG) antibody, yielding a protein that is approximately 95% human (8, 11–13). Humanization of antibodies has led to several clinical successes in the past 15 years (1, 6). More recently, fully human IgG molecules have been developed using new, robust experimental tools enabling their isolation (see below). Ten mAbs for cancer therapy have received FDA approval to date (although one, gemtuzumab ozogamicin, was recently withdrawn from the U.S. market); of these ten, two are radioisotope-conjugated murine antibodies, two are chimeric, four are humanized, and two (the most recent—approved in 2006 and 2009) are fully human molecules (1, 14). Many other antibodies for cancer have been approved and marketed outside the United States, as well (15), creating an industry segment worth many billions of dollars per year (16). Human antibodies comprise the major portion of antibodies currently under development as cancer therapeutics (17).

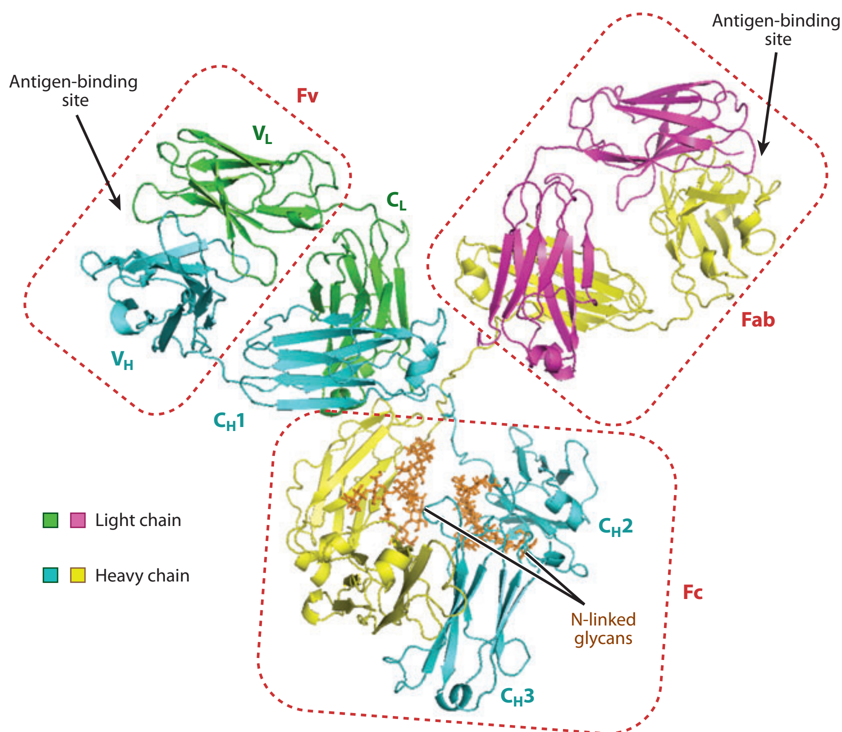

Figure 1

Anatomy of the immunoglobulin (Ig) antibody. The structure of IgG1 is shown with the variable (V) and constant (C) domains of the heavy (H) and light (L) chains labeled. Active fragments are boxed. Atomic coordinates of Protein Data Bank entry 1IGY (18) were used to generate the image. Fab, antigen-binding fragment; Fc, crystallizable fragment; Fv, variable fragment.

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THERAPEUTIC MECHANISMS

mAbs applied in anticancer therapy use numerous mechanisms, which may or may not include participation of other immune effectors. These mechanisms correlate with antibody characteristics, target location, and tumor properties. In many cases, the dominant mechanism of an antibody's therapeutic effect remains unclear (extensively reviewed in 1). Antibodies can bind to targets on malignant cells and recruit immune effector cells and complement to stimulate antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (6), which will lead to cytolysis of these tumor cells. Immune effector recruitment occurs via interaction between the crystallizable fragment (Fc) region (**Figure 1**) and Fc receptors or complement proteins. FcγRI and FcγRIII are the receptors typically desired, as they provide activating signals to a range of immune effectors including natural killer (NK) cells and mononuclear phagocytes. Fc regions also mediate recruitment of the complement-activating protein C1q, leading directly to cell lysis (19). Owing to this central role in immune effector recruitment, Fc engineering has recently become an area of great interest, and research has yielded some examples of improved effector function (20–22). CDC and ADCC furthermore can lead to upregulation of local cytokine and chemokine levels, thus recruiting additional immune effectors (1). Mechanisms of the marketed therapeutic mAbs rituximab, alemtuzumab, ofatumumab, and trastuzumab have been linked to

mediation of ADCC and CDC (23–25). Following tumor cell lysis, antitumor antibodies may also facilitate tumor antigen uptake and presentation by professional antigen-presenting cells (APCs) and thus communicate with the T cell–based adaptive immune system, although no conclusive evidence exists for involvement of the adaptive response in clinical efficacy of current antibodies (1). Nonetheless, induction of adaptive antitumor responses would be a highly desirable property of any therapeutic approach.

Typically, antibodies recognize antigens expressed on the surface of malignant cells, but they can also target soluble cytokines associated with tumor growth or metastasis. In the absence of immune effector recruitment or cell-killing payload delivery, the goal is to block or alter signal transduction in a pathway important for tumor cell proliferation and metastasis (1). For example, the first FDA-approved antisolid tumor mAb, trastuzumab, reacts with the epidermal growth factor receptor 2 (EGFR2) receptor (HER2/*neu* or ErbB2) expressed on a variety of tumor cells to disrupt signal transduction pathways promoting cell growth and differentiation (26). Thus, trastuzumab, as well as other anti-HER2 antibodies, can directly inhibit tumor cell proliferation or neoangiogenesis by interference with receptor dimerization, ligand binding, or endocytosis (24). Bevacizumab, another licensed humanized antibody, binds circulating vascular endothelial growth factor (VEGF) and interferes with VEGF receptor binding on endothelial cells, thus suppressing the angiogenesis critical to solid tumors (25). Antibodies are also able to act as agonists for several costimulatory molecules on the surface of cancer cells and thus induce cell apoptosis, although the side effects on normal tissues can be limiting (27).

An alternative strategy for the therapeutic action of mAbs is the use of immunoconjugates, in which the antibody is armed with cytotoxic chemotherapeutic agents (drugs or toxins) or radionuclides (typically β-particle emitters for therapy). The antibody provides selective delivery of these payloads to the tumor. Only two FDA-approved immunoconjugates for therapy currently exist, both involved in hematological malignancy therapy, following the market withdrawal of the only approved mAb-drug conjugate, gemtuzumab ozogamicin; the anti-CD20 mAb-radionuclide conjugates, zevalin and ^{131}I-tositumomab, are used to treat lymphoma (28, 29). Many radioimmunoconjugates are also broadly used in clinical imaging and diagnostics (30).

LIMITATIONS OF ANTITUMOR ANTIBODIES

Side effects and safety issues are common and unavoidable in antibody therapy. Treatment with rituximab, for example, has induced adverse events including late-onset neutropenia (LON), progressive multifocal leukoencephalopathy (PML), and tumor lysis syndrome (31). Additionally, drug resistance can occur in patients who initially respond to trastuzumab; several molecular mechanisms have been suggested on the basis of preclinical studies (32). Several research groups have also reported incidents of thyroid disease, cytopenias, or antiglomerular basement membrane disease after alemtuzumab treatment (33). Except for the human IgG mAbs panitumumab and ofatumumab, all other FDA-approved mAbs have at least CDR sequences derived of murine origin. Immunogenicity of foreign sequences is the most common toxicity associated with mAb therapy. The possible toxicities are either antibody-antigen binding mechanism dependent, including cardiac dysfunction, infusion reactions, infections, and transitory lymphocyte B depletion, or mechanism independent, such as hypersensitivity reactions, fever, chill, headache, and hypotension (1, 34). Some mechanism-dependent side effects of lymphoma and leukemia antibodies are related to the function of regulatory T cells (T_{reg}). For example, recognition of surface targets shared by these lymphocytes reduces T_{reg} numbers, which in turn benefits cancer elimination, but perturbation of these suppressive T cells may also increase the risk of autoimmune diseases (23, 35).

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Properties of cancer cells and their surroundings also can severely limit the effectiveness of antibody-based therapy. Cancer cells can downregulate class I major histocompatibility complex (MHC), which inhibits recognition by cytotoxic T lymphocytes (CTLs) (1). Similarly, cancer cells can mutate or downregulate surface targets for antibody recognition, creating a heterogeneous cell population. Such heterogeneity of cancer antigens is considered a major barrier to the ability to develop natural tumor immunity (1). Additionally, antibodies targeting solid tumors (which represent 85% of human cancers) must penetrate the tumor to be effective, which forms another obstacle to therapeutic efficacy (36). Compared with circulating lymphomas, malignant solid tumors require therapeutic reagents bearing higher extravasation ability, better penetration capabilities, and optimized retention properties to reach the tumor in sufficient quantities (36, 37).

Solid tumors exhibit several transport barriers for antibodies. Tumor extracellular matrix (ECM) is denser and has a tighter collagen structure than normal tissue ECM, which can slow diffusion. The blood vessels of tumor vasculature are heterogeneous, tortuous, longer, and more permeable, resulting in lower blood flow relative to normal tissue. Limitations in both types of transport methods thus cause the slow penetration of administered antibodies around tumor tissues. Moreover, systemic renal clearance and clearance by cellular uptake and degradation occur simultaneously with antibody transport through tumor tissues and decrease their penetration further. IgG and other Fc-bearing constructs can interact with the neonatal Fc receptor (FcRn), which mediates recycling of internalized molecules and return to the serum, as opposed to degradation in the lysosome, and greatly extends the antibody's half-life. Alternative therapeutic constructs (see below) lacking this feature demonstrate reduced in vivo half-life and thus reduced penetration. Renal clearance is a particular challenge for engineered molecules below ~60 kDa; clearance for these is rapid, yielding very poor biodistribution (36–38). Rapid clearance, however, is not considered detrimental for cancer imaging because long retention in the circulation can cause high background. Nonetheless, issues surrounding biodistribution—along with immunogenicity, antigen loss, and tumor heterogeneity—are recognized as a major motivation to engineer alternative cancer agents.

TECHNOLOGIES FOR ENGINEERING HUMAN ANTIBODIES

Isolation of fully human antibodies entails immunization of genetically engineered mice followed by generation of hybridomas or in vitro combinatorial library generation and application of one of several screening or selection tools (**Figure 2**). Each of these approaches has yielded clinically successful, approved biopharmaceuticals, as well as hundreds to thousands more molecules in various stages of development.

Transgenic Mice

Advances in embryonic stem cell and gene transfer methods have enabled the creation of transgenic mice with endogenous Ig genes disrupted and replaced with human gene equivalents. Transferred genes include the unrearranged V, D, J, and constant gene regions of the heavy chain and the V, J, and C regions of the light chain (39, 40). These transgenic mice mount a normal humoral immune response to immunization, including V(D)J recombination, N-region addition, class switching, somatic hypermutation, and affinity maturation to yield high-affinity (subnanomolar), fully human antibodies to a host of targets (7, 41–43). This approach has the advantage of reliably yielding molecules that combine good antigen-binding properties with low immunogenicity and solid immune effector functions in the absence of additional engineering. Recent years have seen a dramatic number of candidate molecules derived from transgenic technology enter the preclinical or clinical stages of development (reviewed in 7, 43).

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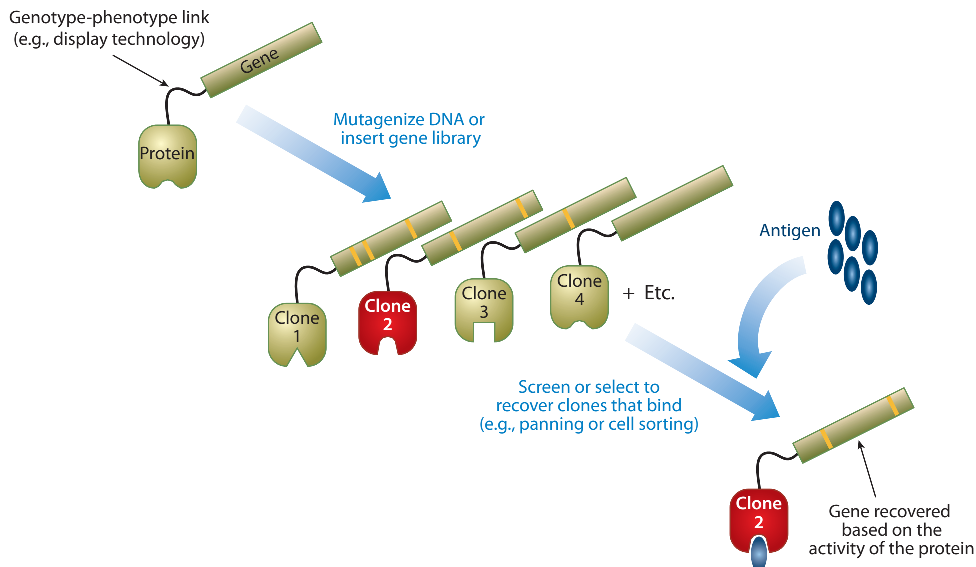

Figure 2

Schematic of the principle behind antibody library screening. The genotype-phenotype link formed by the display technology (phage, cellular, ribosome, or nucleic acid display) allows selections for active proteins to recover genetic material, which then can be amplified for further rounds of selection.

Phage Display

Since the invention of the approach more than two decades ago (44), display of proteins on filamentous bacteriophage by genetically directed fusion to coat proteins has enabled directed evolution of a vast number of proteins and peptides to engineer binding activities. Application of this approach to antibodies (see 15 for a recent review of this method) involves creating libraries of phage or phagemid vectors containing rearranged Ig V region genes as fusions to a phage coat protein gene and then introducing these libraries into bacteria for expression and packaging into phage particles. The phage particles thus bear an antibody fragment on the surface and contain the gene encoding that antibody inside the particle, enabling genetic selection by panning the phage particles on immobilized antigen (45) and thereby recovering genes encoding antibody fragments specific for the target antigen. Iterative rounds of in vitro mutation and selection can be performed to mimic in vivo affinity maturation; this can be accomplished either by introduction of random point mutations using error-prone polymerase chain reaction (PCR) or other mutagenesis techniques or by replacement of whole V genes or CDR loops with naive sequences (46–48). Highly diverse libraries of antibody variable domain sequences, surpassing those possible with in vivo immune responses (49), can be displayed on phage, and diversity can be introduced to targeted regions of the structure. In this manner, phage display (or any other display technology) enables more control over molecular parameters compared with generation of antibodies in vivo. Phage peptide and antibody libraries have been selected in vivo against tumor tissues or in vitro against cells with depletion of the library by panning on appropriate healthy tissues or cell lines to isolate novel, tumor-specific peptides or antibody fragments (50, 51). By incubating in the presence of proteases or at elevated temperatures prior to panning against structural probes, selection for structurally stable antibody fragments has also been accomplished using phage display (52).

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Phage display and other display technologies described below allow tailoring of binding specificity and affinity via investigator control of the selection conditions in contrast to in vivo affinity maturation; among the display technologies, phage display is the most mature and most widely used to date for isolation of recombinant antibodies. In comparison with some other display technologies (e.g., yeast display), phage display affords an advantage in that antibody libraries up to $\sim 10^{10}$ clones are experimentally accessible owing to the robust nature of the molecular genetics methods used in their creation. This technology has clearly been validated for therapeutic antibody discovery by its use in discovering adalimumab, a fully human anti–tumor necrosis factor-α (TNFα) antibody that quickly achieved blockbuster status following its 2002 FDA approval (53, 54), as well as its use in discovering thousands of other antibodies for a range of applications (15).

Yeast Display

A more recent technology for discovery and engineering of antibodies is yeast display. This approach entails expression of antibody fragments as fusions to a *Saccharomyces cerevisiae* cell wall protein, most commonly the mating adhesion receptor a-agglutinin (55–58). Libraries of antibody V genes or mutated antibodies can be displayed, and novel or improved antigen-binding clones can be recovered by magnetic bead–based cell separation (59–61), panning against cellular targets (62, 63), or cell labeling with fluorescent antigen followed by flow cytometric cell sorting (61, 64). A large nonimmune human V gene library containing $\sim 10^9$ clones has been constructed in the yeast display system and enabled routine isolation of high affinity (nanomolar to subnanomolar), human single-chain variable fragment (scFv) antibody fragments against several targets by flow cytometric screening (65). The use of flow cytometry affords a distinct advantage to yeast and other cellular display methods for library expression: Fitness criteria in a cell sort can be determined quantitatively on the basis of binding equilibria (66). Using cell sorting, library clones are screened rather than selected, the difference being that every clone in the library is analyzed individually in a screen, whereas in a selection clones are panned in parallel and individual members of the library are not observed experimentally until after the selection has been performed. Thus, with large libraries selections have an advantage in terms of speed, but they lack the ability to quantitatively address antibody-antigen binding parameters, because panning selects directly for resistance of the antibody-antigen bond to stringency criteria rather than concentrations. This consideration becomes increasingly important when performing affinity maturation on lead antibodies of appropriate specificity.

Yeast are eukaryotes, and thus processing and secretion of extracellular proteins parallel those found in mammalian cells. As a result, yeast have proven capable of functionally displaying many eukaryotic molecules that challenge prokaryote-based systems such as phage display, including T cell receptors (67), class I (68) and class II (69, 70) MHCs, West Nile virus envelope protein (71), a G protein-coupled receptor (72), and human proteins expressed from a cDNA library (73). Furthermore, gene libraries cloned in yeast have been found to demonstrate extremely high genetic stability, failing to show any evidence of clonal loss after more than 30 generations of growth (65). Perhaps unsurprisingly in light of these facts, a direct comparison between yeast and phage display for discovering antibodies against HIV gp120 by screening a preimmune V gene library showed that yeast display more fully sampled the scFv repertoire and returned additional binders (74): While 12 antigen-specific clones were found using phage display, 36 were identified by yeast display, including all 12 of the phage-identified clones. Thus, evidence suggests that yeast display is a powerful approach to isolate the widest possible range of lead antibodies to a given target. These molecules can then be engineered by affinity maturation (57) or convenient homologous recombination–based gene shuffling (75) to further improve binding parameters.

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Yeast display has also been used to improve the thermal stability of various proteins by mutagenesis and library screening (76–79), thus providing a route to optimize this property of lead antibodies as well. Combining antigen-binding fragment (Fab) display with the ability of yeast to mate affords a means to generate larger libraries by pairing antibody chains expressed from separate vectors; by mating yeast containing a light chain library of $\sim 3 \times 10^7$ genes with a complementary mating-type yeast containing a heavy chain library of $\sim 2 \times 10^6$ genes, a yeast Fab library of $\sim 3 \times 10^9$ diversity was created (80). Given recent improvements in the ability to efficiently transform yeast (81), generation of extremely large libraries (beyond the size that could be handled with reasonable laboratory-scale volumes of biomass) seems possible.

Other Display Technologies

A large number of other approaches to protein display (comprehensively reviewed in 82, 83) have been developed and are applicable to antibody discovery and engineering. Display and screening of scFv libraries on *Escherichia coli* have been demonstrated by fusion of the antibody fragment to the OmpA protein (84); more recently, a periplasmic display approach has been developed that allows screening of libraries of full-length, aglycosylated IgG (85). Antibodies are retained in the periplasm of outer membrane–permeabilized cells by binding to an inner membrane–anchored ZZ domain from protein A, and displaying cells are screened for antigen binding by flow cytometric cell sorting (86). This is a rapid and economical approach to directly generate functional IgG without the need for further molecular engineering.

Two eukaryotic viruses have been used for displaying heterologous proteins. Display and selection of antibody fragments on murine leukemia virus (MLV), a retrovirus, have been achieved by fusion to the envelope protein surface unit (87–89). Insect cells have been found to be an excellent expression platform for many proteins, and baculoviruses have therefore engendered interest as display vehicles as well. The baculovirus gp64 envelope protein membrane domain was used to anchor class I and class II MHC to the virion surface, which enabled screening of peptide libraries to identify antigen mimotopes against a target T cell receptor (90). Adenovirus and adeno-associated virus (AAV) have also been engineered to display novel peptides on their surfaces (including a library that was screened on AAV), typically with the goal of directing viral tropism for gene therapy applications (83, 91). Although no applications of these systems to antibody engineering have been reported, the potential exists to develop them for this purpose.

Display and screening of antibody libraries on mammalian HEK-293T cells have been achieved. To accomplish this, a library of scFv fragments were fused to the transmembrane domain of platelet-derived growth factor receptor; flow cytometric screening enabled isolation of a mutant anti-CD22 scFv that was improved fivefold in affinity (92, 93). The use of mammalian cell expression in this technique has been proposed to afford a superior environment for native folding and posttranslational modification of antibodies (93) and could enable convenient engineering of molecular properties related to, for example, interactions with Fc receptors, although the slow growth and expense of mammalian cell culture may be a limitation for some applications.

Three approaches for cell-free display have been developed and applied in the past two decades: ribosome display, mRNA display, and DNA display. Each of these uses cell-free in vitro translation or transcription/translation methods and therefore obviates the requirement to transform cells with library genetic material. This enables much larger libraries ($>10^{12}$ and in some cases approaching 10^{14} clones) to be readily addressed experimentally and further affords a cycle time for one round of selection and amplification (achieved by PCR methods) that is much faster than that for methods requiring library amplification by cell growth (82, 83, 94). The most widely used of these methods to date, ribosome display, uses the ribosome to create an

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attachment between an mRNA and the encoded polypeptide (95). A library encoded in mRNA is added to a cell extract for in vitro translation; the absence of a stop codon causes translation to pause without dissociation of the translation machinery, and low temperature can stabilize the ternary complex. Thus, panning against a target ligand enriches the bound mRNA according to the attached protein's ligand-binding affinity, and reverse transcription (RT)-PCR is used to amplify the population for further screening (94). Demonstration of picomolar-affinity scFv antibody fragment discovery by ribosome display has been achieved (96).

Like ribosome display, mRNA display is based on creation of a polypeptide/mRNA linkage during in vitro translation. In this case, a puromycin group is appended to the 3′ end of synthetic RNA; this antibiotic mimics an aminoacyl-tRNA and induces ribosome-catalyzed formation of a covalent link with the polypeptide C terminus. Multiple rounds of selection are achieved by cyclic panning and RT-PCR amplification. This method has been successfully applied to isolate antibodies (97), including in a recent demonstration of its use in the context of a powerful microfluidic approach (98).

In DNA display, antibody fragments are fused to the bacteriophage P2 protein P2A, which covalently attaches to the DNA that encoded it. This *cis* attachment activity enables genotype-phenotype linkages to form during in vitro transcription and translation (94). DNA display potentiated the selection of an antitetanus toxin scFv (99) and thus appears to represent another potentially suitable tool for cancer antibody discovery.

Each of the alternative display technologies described here presents advantages in terms of either library size or the posttranslational environment afforded the heterologous proteins relative to phage and yeast display and to one another. While the best tool for protein engineering is context dependent, it is noteworthy that none of these methods can address simultaneously the two primary respective weaknesses of phage and yeast display as applied to antibody engineering: (*a*) the lack of a eukaryotic folding and posttranslational environment (phage), which raises the question of functional library size, and (*b*) smaller (although putatively highly functional) library size (yeast). Accordingly, the potential impact of these technologies on the development of antibodies for cancer remains to be determined. In the absence of head-to-head experimental comparison, the trade-off between the issues of quantity and quality in library diversity for every display technology precludes an unambiguous conclusion regarding an optimal methodology.

ENGINEERED ANTIBODY FORMATS

Based on current knowledge of antibody mechanisms as discussed above, the optimal therapeutic protein for cancer would be a molecule that (*a*) distributes throughout the circulation without rapid renal clearance, (*b*) remains stable against proteolysis in the serum, (*c*) lacks immunogenicity, (*d*) effectively penetrates large tumor masses and small volume metastases, (*e*) does not interact with healthy cells or tissues to an appreciable degree, (*f*) remains bound to the cancerous cell or other target for a lengthy duration, and (*g*) either enters the cell via endocytosis (for proteins with conjugated drugs or radioisotopes) or efficiently recruits immune effectors or disrupts signaling functions (for unconjugated proteins). The last issue is avoided in the case of prodrug-activating antibody conjugates (100), and only issues *b*–*f* significantly impact antibodies for cancer imaging, where clearance from the circulation is required to reduce background and single high doses of the agent can be tolerated. Human and humanized antibodies have established a successful record in the clinic for treatment of certain indications precisely because they meet most of these criteria. Nonetheless, averaged over the existing antibody therapeutics, the chance of successfully treating a patient with current mAb therapy is approximately 30% (1). Current understanding of the clinical mechanisms of antibodies for cancer therapy is incomplete, and therefore precise

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design parameters for improving response rates are unclear; however, issues related to points *c–g* have been pursued to varying degrees, in terms of investigating the importance and mechanistic basis of the issue and of engineering molecules to ameliorate the presumed limitation.

As described above, modern antibody engineering approaches have largely overcome the issue of immunogenicity as well as targeting and distribution issues as they relate to antigen-binding affinity and kinetics. Progress has also been driven by better understanding of potential tumor antigen targets and their relationship to the issues of tumor selectivity and mode of therapeutic action. An important remaining challenge that has attracted considerable recent attention has been that of distribution and penetration (issues *a* and *d*), particularly with respect to the effect of antibody size. This challenge is particularly limiting for therapy and imaging of solid tumors, where mass transport barriers are more significant than for distributed malignancies. Ig-based molecular formats cannot be made smaller than ~25 kDa, whereas alternative nonantibody targeting molecules (see below) can be much smaller and thus are of interest as possible therapeutic alternatives. Although antibody fragments and small, non-antibody-based therapeutics offer a clear advantage with respect to penetration, this can be at the expense of issues *a*, *f*, and *g*, and, despite recent interest and research activity in alternative molecular formats, full-length IgG mAbs remain the dominant antibody format in clinical studies (101). A summary of some antibody-based therapeutics in development is shown in **Table 1**.

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Table 1 Selected examples of engineered antibodies in development

Antigen	Engineered antibody	Format	Development stage	Reference
Solid tumor				
EGFR	Trastuzumab variant[a]	mutated IgG1	Preclinical model	(150)
	Ertumaxomab[a]	Triomab (murine/rat IgG hybrid)	Clinical trials	(141)
	Trimeric C6MH3-B1-TNF[b]	$(scFv\text{-}TNF)_3$	Preclinical model	(142)
	-	Minibody[a]	Preclinical model	(143)
	-	TriBi-minibody[a]	Preclinical model	(143)
EpCAM	Edrecolomab[a]	Triomab	Clinical trials	(144)
	Catumaxomab[a]	Triomab	Clinical trials	(126)
CEA	Trimerbody[b]	$(scFv\text{-}collagen\ subdomain)_3$	Preclinical model	(155)
MUC1	TF10[a]	$(Fab)_2$-DNL-Fab′	Preclinical model	(156)
Circulating malignant cell				
CD20	TF4[a]	$(Fab)_2$-DNL-Fab′	Preclinical model	(157)
CD19	Blinatumomab[a]	scFv-scFv	Clinical trials	(130)
Tumor-associated stroma				
FAP	TNF-associated monomer	scFv-TNF fusion[b]	Preclinical model	(158)
	TNF-associated dimer	$(Fab\text{-}TNF)_2$ fusion[b]	Preclinical model	(158)
Mindin/RG-1	-	Diabody[b]	Preclinical model	(159)
	-	Minibody[b]	Preclinical model	(159)
Tumor vasculature				
ED-B	L19IL-2[b]	IL12-scFv fusion	Clinical trials	(160)
	L19mTNF[b]	scFv-TNF fusion	Clinical trials	(160)

[a]Bispecific.
[b]Monospecific.
Abbreviations: CEA, carcinoembryonic antigen; DNL, dock-and-lock; ED-B, fibronectin extra domain-B; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; Fab, antigen-binding fragment; FAP, fibroblast activation protein; Ig, immunoglobulin; IL, interleukin; MUC1, Mucin 1; scFv, single-chain variable fragment; TNF, tumor necrosis factor.

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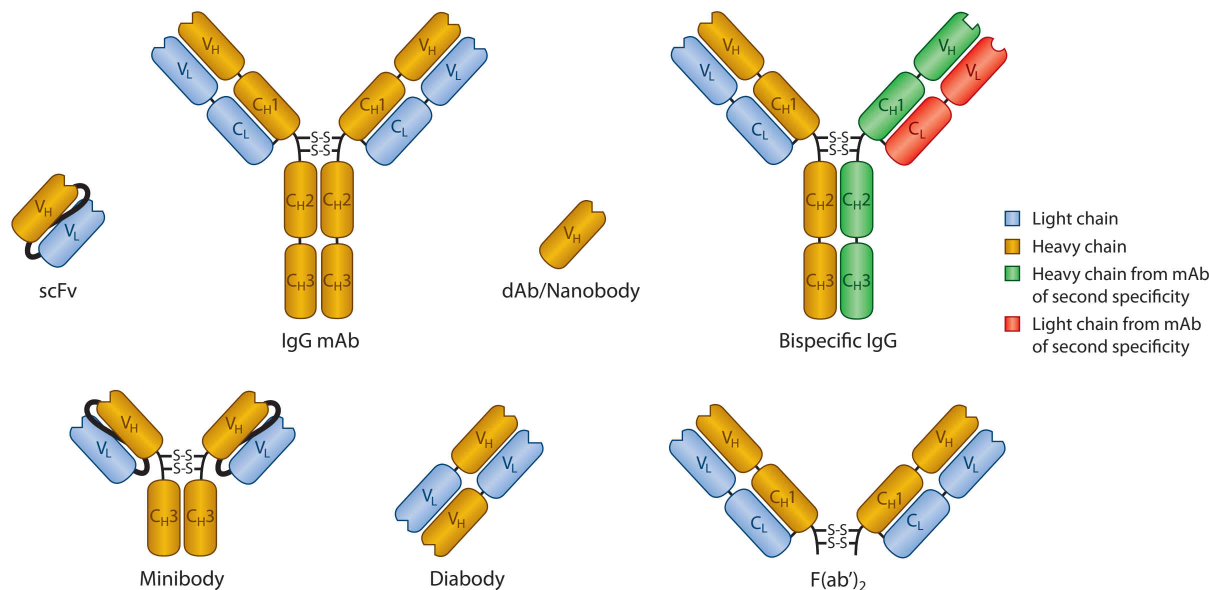

Figure 3

Selected examples of antibody formats used in cancer therapy. C, constant domain; dAb, domain antibody; F(ab), antigen-binding fragment; H, heavy chain; IgG, immunoglobulin G; L, light chain; mAb, monoclonal antibody; scFv, single-chain variable fragment; V, variable domain.

Antibody Fragments

Although all clinically successful therapeutic antibodies for cancer to date have been based on standard IgG (102), a great deal of interest in other antibody molecular formats has existed for many years. The need to efficiently penetrate into solid tumor tissue, in particular, stimulated interest in development of smaller molecules that retain antigen-binding activity and, depending on the intended mechanism, also retain immune effector functions. The modular architecture of the IgG molecule eases development of alternative formats based on this (**Figure 3**; for a comprehensive review, see 103). For instance, Fab fragments can be derived from IgG by proteolysis or produced recombinantly (103). These ∼50 kDa monovalent species retain antigen-binding activity but lack the Fc region to enable stimulation of immune function, an advantage in applications not dependent on immune stimulation for efficacy. Examples of such applications include tumor imaging and conjugated drug or radioisotope targeting, wherein the absence of interaction with complement or Fc receptors might limit some forms of toxicity (1, 17). Recombinant scFv fragments (∼25 kDa) represent minimal units of antigen binding derived from the human IgG structure; in this architecture, the V_H and V_L domains are engineered into a single polypeptide by introduction of a flexible linker (104, 105). Domain antibodies or nanobodies (∼13 kDa) are smaller still, composed of only a single, unpaired, human or mouse V_H or V_L domain (106, 107) or the naturally unpaired antigen-binding V_{HH} domain of antibodies from shark or camelid species (103, 107). Nanobodies have been shown to have potential in cancer and other therapeutic areas (108) and demonstrate intriguing biophysical properties, most notably the ability to refold after thermal denaturation and thus reacquire antigen-binding activity (107). As examples, a nanobody targeting carcinoembryonic antigen was used to deliver an enzyme for prodrug activation, resulting in potent tumor killing in a xenograft model (109), and a nanobody antagonistically targeting

EGFR inhibited tumor growth in a breast cancer xenograft model (110). In general, monovalent antibody fragments are of most interest when the antigen target is a receptor that might be activated by dimerization induced by a bivalent species such as IgG (17).

Many bivalent antibody fragments exist as well. $F(ab')_2$ molecules (∼100 kDa) are proteolytic IgG fragments that maintain the disulfide links between the two Fab regions but still lack the Fc region. These molecules have a potential advantage over monovalent fragments for some applications in that they retain the high avidity for binding to a multiple-antigen-displaying target such as a cancer cell that is afforded by two binding sites. This advantage has spurred development of several engineered bivalent architectures. Engineering the flexible linker regions in an scFv to be too short to allow intramolecular V_H:V_L domain pairing, but still allow intermolecular pairing, has been used to create bivalent diabodies (∼50 kDa) (111). Further shortening of the linker to further constrain the orientations of the domains produces trimeric structures called triabodies (112). Bivalent recombinant fragments have also been engineered by deleting various constant domains in pairs (17, 103); for example, scFv-Fc (∼100 kDa) molecules comprise an Fc region with an scFv fused to the N terminus of each C_H2 domain to create a bivalent molecule with shortened antigen-binding arms (113), and minibodies (∼75 kDa) include scFv fused only to the C_H3 domains but with an intervening hinge region for interchain disulfide bonding (103). A wealth of other approaches to dimerization or multimerization has been successfully implemented in addition to those mentioned here (103). Most of these bivalent constructions share the common feature of reduced size compared with IgG. This reflects a focus on tissue penetration and immunogenicity that is important for many applications of these reagents. Those that lack the full Fc region are likely best suited for drug, toxin, or radionuclide targeting applications for therapy or imaging, and many such molecules are currently being pursued (103). Very high binding affinity has been shown to increase the inhomogeneity of monovalent antibody fragment distribution in tumor tissue; however, a bivalent fragment with lower monovalent affinity distributed more evenly (114), which suggests that antibody-based molecular architectures of higher valency may have an advantage as drug candidates (103).

Bispecific Antibodies

Natural IgG antibodies have two specificities: The antigen-binding activity targets the molecule, and the Fc region binds to Fc receptors or complement proteins to activate immune functions, effectively bridging the target species with cells or molecules of the immune system. Natural Fc regions, however, demonstrate pleiotropic effects that depend on the IgG subclass (115). Most currently marketed antibodies are IgG1 (17), which interacts efficiently with FcγRI and FcγRIII to induce ADCC, as discussed above. Although this plurality of effects has been implicated in the clinical mechanisms of some antibodies, Fc receptor interactions can also lead to negative side effects and toxicities, and a retargeted interaction with the immune system may be warranted in some contexts, particularly those involving payload delivery as the mechanism and thus potentially benefitting from reduced immune-based side effects (17). Bispecific antibodies (bsAbs) afford a route to achieve this by combining a tumor antigen-binding activity with a second activity to augment or replace the Fc as immune effector. Furthermore, bispecificity can be used to enhance targeting by binding to multiple tumor antigens on the same cell or to cross-link different antigens to drive a signal-based effect. The antibody engineering community has enthusiastically explored these paradigms in recent years; here, we summarize selected examples of bispecific tumor targeting.

The earliest bsAbs were created by recombining natural antibodies with differing specificities using chemical cross-linking or hybridoma cell fusion, approaches that failed to reliably

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yield quality material and were amenable primarily to murine antibodies (116). In recent years, numerous other molecular formats, including all of the multivalent fragments described above, have been engineered to contain two or more antigen-binding sites with different specificities, and novel approaches to add specificity functions are continually being developed (117). For example, multispecific antibody reagents can be created by transpeptidase-catalyzed in vitro ligations of individual proteins (D.A. Levary, R. Parthasarathy, E.T. Boder & M.E. Ackerman, unpublished data) or by extension of the light chains via genetic fusion (118). Another recent example, also demonstrating the principle of improved cell selectivity, is a bispecific agent composed of two scFv fragments targeting EGFR family members, one targeting ErbB2 and the other ErbB3 (which is associated with poor prognosis and therapeutic resistance), that are joined by a flexible linker to form a single polypeptide chain. This bispecific scFv demonstrated enhanced specificity for ErbB2/ErbB3 coexpressing breast carcinoma cells in vivo and inhibited proliferation of tumor cells in vitro (119). Another engineered bsAb composed of an IgG molecule with two scFv fragments fused to the heavy chain was designed to target and cross-link two tumor necrosis factor (TNF) receptor family members, TRAIL-R2 and LTβR. This antibody demonstrated improved antitumor cell activity in vitro and in vivo, depending on the tumor cell line used (120). A bsAb directed against the CD95 death receptor and neuronal glial antigen 2 was shown to be active in killing glioblastoma cells, whereas a version targeting CD95 and EGFR was not. This divergent effect resulted in this case from a need to cross-link CD95 by binding two different cells (forming a bridged intercellular synapse); the anti-EGFR bsAb bound only to single cells, demonstrating the requirement for CD95 signaling in the activity of this agent (121).

Directly inducing cytotoxic T cell responses against tumor cells (122) represents another potentially powerful approach for cancer therapy with bsAbs. Murine antibodies with anti-ErbB2 and anti-CD3 (part of the T cell receptor complex) activities were generated by chemical cross-linking and shown to be effective at retargeting polyclonal T cells (preactivated ex vivo) toward $ErbB2^{+}$ breast cancer cells (123). Interestingly, fusion of mouse and rat hybridomas was found to yield predominantly interspecies paired IgG under certain conditions; thus, simple generation of mouse/rat hybrid bsAbs was achieved, and the resulting bsAbs were found to induce surprisingly robust stimulation of human FcγRI and FcγRIII via the murine/rat Fc region (124). One bsAb created by this technology has yielded impressive clinical results despite its nonhuman origins: Catumaxomab combines anti-EpCAM (epithelial cell adhesion molecule) and anti-CD3 activities; it is approved in Europe for treating malignant ascites and is in clinical trials in the United States (125, 126). This bsAb-format construct operates at extremely low doses, presumably limiting the typical difficulties of immunogenicity (127). T cell-inducing bsAbs have also been constructed by genetic fusion of anti-CD3 and antitumor antigen scFv. These antibodies, which are known as bispecific T cell engagers (BiTEs), have been found to mediate efficient tumor cell killing by T cells without ex vivo costimulation or T cell preactivation and at extremely low bsAb concentration (128, 129). In clinical trials of a murine BiTE against CD3 and the B cell coreceptor CD19 (blinatumomab), this low dosage enabled use of insulin pumps for continuous intravenous infusion, which allowed effective steady-state levels of the bsAb to be maintained at low total protein delivery, despite its small size allowing renal clearance (127). This culminated in a highly successful phase I clinical trial in which 100% of non-Hodgkin's lymphoma patients responded to the therapy (130). Several other targets for this bsAb technology are currently being explored (127).

Many other bsAb agents have been under investigation for cancer applications in recent years (see 131, 132 for reviews). Based on these initially promising results, further development of bsAb-based T cell targeting—directed toward different cancers, making use of additional tumor antigens, and applying inventive molecular formats—appears likely.

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ALTERNATIVE SCAFFOLDS AS ANTIBODY REPLACEMENTS

A great deal of interest has existed for several decades in developing alternative protein molecules capable of retaining the quintessential feature of the Ig-based antibody—highly specific and avid binding to a wealth of different targets from a single molecular scaffold. Alternatives have been sought to overcome some of the observed limitations of antibodies (e.g., difficult or expensive production, immunogenicity, and especially penetration limitations) and, not insignificantly, to avoid intellectual property (133). Indeed, many alternative molecular scaffolds have been investigated and developed to date, in recent years driven largely by the pursuit of much smaller (<20 kDa) targeting agents with their putatively superior transport properties. Intellectual property concerns clearly have impacted the diversity of these pursuits, probably more so than validated technological or biological limitations. Applications for these scaffolds in cancer are likely to begin with payload targeting (e.g., for imaging), although bispecific constructs based on these scaffolds are feasible and could be used to direct immune effector function. In general, the smallest scaffolds are expected to afford the best tumor penetration and lowest immunogenicity at the expense of the molecular surface area available for binding to target ligands, which roughly correlates with the maximum achievable affinity for protein-protein interactions. The optimum in this trade-off is likely to depend on details of the application, and thus all of these scaffolds might find success in certain cases. A small set of scaffolds that exemplify the concept is briefly described below (**Figure 4**).

The tenth domain of human fibronectin type III (Fn3) bears the closest structural resemblance to the Ig domain, but it is roughly 10 kDa. Its β-sheet sandwich structure provides randomizable loop regions similar to the CDR loops of antibodies (138); such engineered Fn3 domains have been dubbed Adnectins. Recently, carefully designed loop libraries, recursive mutagenesis, and yeast display were applied to rapidly engineer Fn3 domains targeted to lysozyme with low-picomolar affinity (139). An Adnectin-targeting VEGF receptor was shown to be active in blocking growth and metastasis in an in vivo pancreatic tumor model (140), which highlights the potential of this scaffold as a cancer therapeutic.

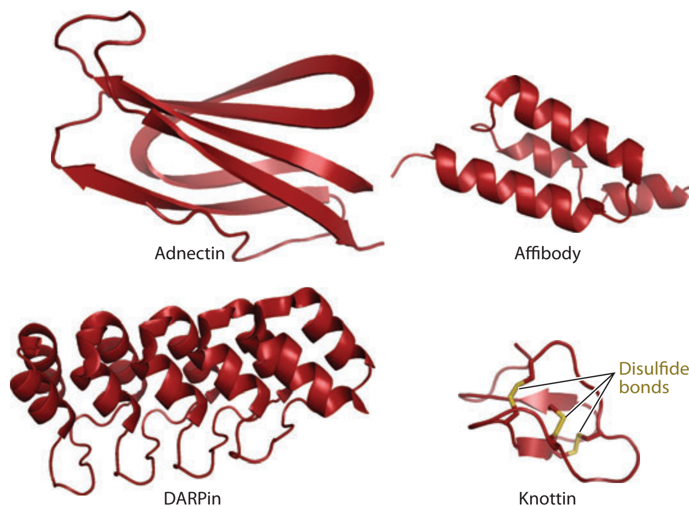

Figure 4

Selected examples of molecular scaffolds under development as alternatives to antibodies. Randomization of loops or surfaces in conjunction with display technologies is used to engineer binders to novel targets. Atomic coordinates of Protein Data Bank entries 1FNA (134), 2KZI (135), 2XEE (136), and 3E4H (137) were used to generate the images. DARPin, designed ankyrin repeat protein.

Affibodies are small (~6–7 kDa) three-helix bundles based on the Z domain of protein A (133). Rather than engineering loops, amino acids on a face of the protein composed of two helices are typically randomized to generate molecular diversity to be selected for binding, usually by phage display. Affibodies have been engineered against a variety of targets, most notably with respect to cancer relevance against ErbB2 and EGFR; affibody-targeted near-IR fluorophores and radioisotopes have been successfully applied to in vivo tumor imaging studies (141) and furthermore have shown the ability to inhibit the growth of breast carcinoma cells in vitro (142).

A domain from ubiquitous receptor proteins known as ankyrin repeat proteins has been used to develop targeting molecules as well. The repeat unit is a small (~6 kDa) domain containing two α-helices separated by a β-turn. Three or more of these units can be arranged in series to provide a binding surface with three randomizable loops on the same face of the molecule that can be engineered to identify new binding specificities (133); the resulting molecules are called DARPins (designed ankyrin repeat proteins). DARPins engineered to bind ErbB2 with high affinity efficiently targeted breast tumors in a xenograft model (143) and have demonstrated success in imaging applications (144). The modular nature of DARPins provides intriguing flexibility and presents the possibility of tailoring molecular size and binding surface area via different numbers of repeat units in order to optimize the protein for binding to different target ligands.

The knottins comprise a family of exceptionally small (~3.5 kDa) and highly stable proteins found in many species that share structural homology involving a triple-disulfide stabilized knot motif. Knottins have been engineered for binding various targets by phage (145) and yeast display (146) by screening for binders from a library containing a single randomized loop. A knottin engineered for high-affinity binding to αVβ3 integrin (146), an antigen overexpressed on numerous tumors as well as on tumor neovasculature, showed promising ability to deliver imaging agents in a glioblastoma xenograft model (147), a role for which such a small targeting agent is conceptually well suited.

A newly investigated molecular architecture that combines features of antibody fragments and alternative scaffolds is the Fcab, an isolated human IgG1 Fc fragment (~50 kDa) in which three loops at the C-terminal side of the C_H3 domains can be randomized to allow library screening for novel binding specificities. Screening by yeast display yielded an anti-ErbB2 Fcab with nanomolar binding affinity. Significantly, the engineered Fcab retained the beneficial features of the IgG1 Fc, namely extended in vivo half-life and the ability to induce ADCC against a breast cancer cell line in vitro (148). A similar recent study demonstrated that the N-terminal end of the C_H2 domain could be engineered for antigen binding (in this case to HIV gp120) (149). Combining these concepts, the Fcab scaffold obviously could represent an intriguing platform for novel bispecific reagents.

Many other alternative scaffold-based antibody replacements, such as anticalins, peptide aptamers, and avimers, have been established to varying degrees (133). Any of the display technologies described above can be readily applied to these alternative scaffolds to engineer binding or other biophysical properties and to optimize the molecules for a given application. Most of these scaffolds have the most immediate potential in cancer imaging applications. Nonetheless, despite some promising preclinical results, advantages of these molecules currently remain largely conceptual, and proof of their potential relative to more proven antibodies and antibody fragment constructs in cancer applications, in particular, awaits further study and demonstration of clinical success.

CONCLUSION

The past 20 years have seen dramatic progress in antibody engineering, and new approaches to develop these molecules have made a resounding impact on clinical use for cancer therapy. A significant expansion in the number, type, and range of diseases targeted by approved, marketed

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antibodies is likely to be realized in the near term. Although recent progress in this field has been dramatic, antibody-based cancer therapies still suffer from limitations stemming from the complicated nature of both the disease and antibody-based therapeutic mechanisms. The increasing understanding of therapeutic and efficacy-limiting mechanisms is enabling application of protein design strategies in an attempt to ameliorate these limitations. Some new general themes are emerging; for instance, whereas the focus of engineering 15–20 years ago was on improving binding affinity, attention now is commonly directed toward the trade-off between circulatory half-life and rapid penetration and the implied avidity requirements.

Impressive progress aside, the challenge in treating a heterogeneous, dynamic, and rapidly proliferating cell population such as that of a tumor remains formidable; cells lacking or downregulating antigen expression, for example, will proliferate and continue to stymie therapies unless sufficient bystander cytotoxicity can be induced reliably. Thus, the evolution-based concept that has catalyzed such dramatic progress in antibody engineering via display technologies ultimately provides one of the biggest challenges to further progress in the field. In light of this challenge, multispecific antibodies and antibody combinations capable of recruiting new immune effectors simultaneously with nonimmune mechanisms, and simultaneously targeting multiple antigens, are expected to be pursued aggressively in the near future. Alternative scaffold and antibody fragment–based constructs have been playing an increasing role in cancer research and are particularly showing early promise in imaging applications; these molecular formats may provide important new components of this multispecific/combination cancer treatment arsenal. Further advances are also likely to result from more stringent analysis of patient genetics and customization of therapies, although economic considerations could hinder large-scale implementation of personalized therapies. As noted recently, the antibody engineering field may be in need of a field-moving shift in either insight or methods to drive a continuation of the rapid progress achieved in the past two decades (16), but the inertia of current activities should sustain this field for at least another decade while we await the next breakthrough.

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DISCLOSURE STATEMENT

E.T.B. is a consultant for f-star GmbH, developer of the Fcab technology described here, and receives licensing royalties as an inventor of the yeast display technology.

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Annual Review of Chemical and Biomolecular Engineering

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