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Antibody libraries in drug and target discovery

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Antibody libraries have come of age in the generation and evolution of monoclonal antibodies for therapeutic applications. Here, with an emphasis on cancer therapy, several examples are presented that illustrate the ability to design, engineer and select antibody libraries for different rationales in drug and target discovery.

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▼ The use of monoclonal antibodies (mAbs) as therapeutic agents is gaining importance in the treatment of various conditions such as cancer, cardiovascular diseases and viral infections. In concert with their clinical acceptance, mAbs have become commercially viable drugs[1]. For example, two mAbs, which were recently approved by the FDA for the treatment of non-Hodgkin's lymphoma (Rituxan®) and breast cancer (Herceptin®), already produce annual sales in the US$100 million to US$500 million range (http://www.genentech.com). Consequently, a significant proportion of biotechnology drugs that are in clinical trials or await FDA approval are mAbs and their major target indication is cancer[2]. Similar to drugs in cancer therapy, naked mAbs, such as Rituxan® and Herceptin®, exert their cytotoxic effects by interfering with ligand–receptor interactions required for tumor survival or by initiating immune responses, including cell-mediated or complement-mediated cytotoxicity. In adition, MAbs that target tumors have been conjugated to radioisotopes, chemotherapeutic agents, bacterial toxins, cytokines and enzymes in order to potentiate their cytotoxic effects[3].

Antibody libraries vs. hybridoma technology

MAbs have been generated either by the hybridoma technology[4] or, more recently, from antibody libraries[5]. The development of antibody libraries has been greatly influenced by the development of display technologies and vice versa. The physical connection of antibody phenotype (protein) and genotype (cDNA) effectively allows selection rather than screening of antibody libraries[6]. The integration of antibody libraries and phage display technology[7] approximately a decade ago[8–12] was a key event in this respect. More recently, display technologies[13,14] other than phage display have been applied to antibody libraries, including ribosome[15], yeast[16] and bacterial[17] display.

Typically, antibody libraries are selected for mAbs that bind with high affinity and specificity to a given target antigen. However, mAbs exhibit selectable phenotypes other than affinity and specificity. For example, mAbs that mediate endocytosis of phage by mammalian cells can be selected from antibody libraries, which has potential application in gene therapy[18]. Enzymatic activity is another selectable phenotype of mAbs.

Whereas the hybridoma technology is practically confined to rodents, antibody libraries allow the generation of mAbs from virtually any species whose immunoglobulin genes are known. It is conceivable that the ability to generate mAbs from a variety of species will be important for the identification of highly conserved human antigens or highly conserved epitopes of human antigens. The epitope repertoire of a given human antigen recognized by nonhuman antibodies is different for each species. As a result, epitopes that are not immunogenic in one species might be immunogenic in a different species. Highly conserved epitopes often display functional binding sites. The generation of mAbs against functional binding sites, that is, the generation of mAbs that agonize or antagonize functional interactions, is relevant for therapeutic applications[19]. Mabs from species other than mouse are of

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particular interest for the development of therapeutic antibodies that are evaluated in mouse models of human disease where antibodies are required to recognize both the human antigen in the xenografted tissue and its mouse homolog in the host tissue. By contrast, therapeutic antibodies that are derived from immune mice, either indirectly through humanization or directly through transgenic mice expressing human antibodies[20], are negatively selected against epitopes displayed by the mouse homolog.

In addition to immune repertoires, antibody libraries can be used to exploit nonimmune repertoires, that is, large naïve[21–23] and synthetic[24] repertoires, or combinations of both[25], for the generation of human mAbs[26,27]. A striking advantage of antibody libraries from large synthetic and naïve repertoires is their antigen independence (i.e. one library can be used for the selection of antibodies against any antigen). By contrast, antibody libraries from immune repertoires are individually generated from animals immunized with a particular antigen or from humans with a particular immune response, for example, against human immunodeficiency virus-1 (HIV-1; Ref. 11). Owing to this restriction of the immune repertoire, human antibodies have been primarily generated from synthetic and naïve repertoires. Some features of antibody libraries from immune, naïve and synthetic repertoires are summarized in Fig. 1, which emphasizes the interface between the three sources of antibody libraries.

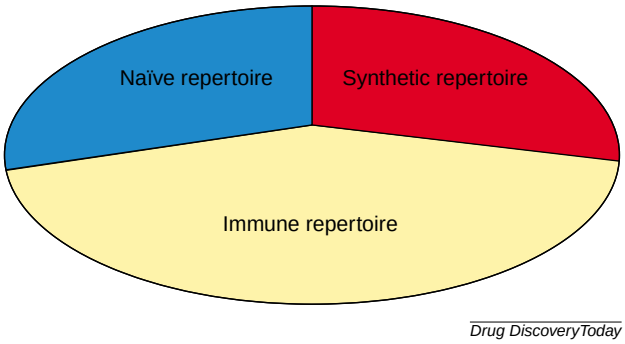

Figure 1. Sources of antibody libraries. Antibody libraries from naïve repertoires represent the primary antibody repertoire before antigen contact; antibody libraries from immune repertoires represent the secondary antibody repertoire following immunization. Accordingly, the genetic source of antibody libraries from naïve and immune repertoires is IgM mRNA from B cells and IgG mRNA from plasma cells, respectively. Antibody libraries from synthetic repertoires, by contrast, are based on synthetic oligonucleotides designed to diversify defined sections in the antibody sequence.

Structural considerations

A key module in design and engineering of antibody libraries is the immunoglobulin (Ig) domain. The Ig domain consists of ~100 amino acids, which form a sandwich of two opposing antiparallel β-sheets that surround a hydrophobic core and are linked by a disulfide bridge (Fig. 2). Probably becaue of its conformational stability and resistance to proteases, the Ig module is found in a variety of extracellular proteins (predominantly cell adhesion molecules), referred to as the Ig superfamily. It is thought that the evolutionary success of the Ig module was driven by the Metazoan evolution with its demand for stable protein modules mediating recognition and adhesion at the cell surface[28]. Although most cell adhesion molecules of the Ig superfamily are single chain molecules, they form homophilic and heterophilic intermolecular interactions that resemble the chain associations found in the antibody molecule (Fig. 2). It has therefore been suggested that the antibody molecule evolved from cell adhesion molecules[28]. With its new phenotype, the hypervariable antigen binding site, whose diversity in humans is based on the random combination of ~200 variable (V), diversity (D) and joining (J) gene segments and somatic mutation, evolved rapidly to become a central protein module of the immune system. The antigen binding site results from the convergence of six hypervariable peptide loops or complementarity determining regions (CDRs), three provided by each light and heavy chain variable domain. The six CDRs are clustered at one end of the antibody molecule (Fig. 2). It is primarily the variation in amino acid sequence in the CDRs that produces mAbs of differing antigen specificities. CDR1 and CDR2 of light and heavy chains are encoded within the V gene segments. The most hypervariable CDRs, CDR3 of light and heavy chain, are generated by the recombination of V and J gene segments or V, D and J gene segments, respectively.

Intriguingly, the same features that led to the evolutionary success of the Ig domain, namely its stability and versatility, also underlie its success in directed protein evolution driven by display technologies. In fact, the ability to functionally express and display Ig domains as Fab or single chain Fv fragments by phage, bacteria and yeast is an essential element of antibody libraries.

The Fab molecule (Fig. 2) is a 50 kDa fragment of the 150 kDa IgG molecule with a heavy chain shortened by constant domains CH2 and CH3. Two heterophilic (VL–VH and CL–CH1) domain interactions underlie the two-chain structure of the Fab molecule, which is further stabilized by a disulfide bridge between CL and CH1. The 25 kDa single chain Fv molecule comprises variable domains VL and VH connected by a polypeptide linker. Both formats have been used in antibody libraries[6].

Other molecules that consist of Ig domains, for example, the single chain T-cell-receptor molecule[29,30] and the

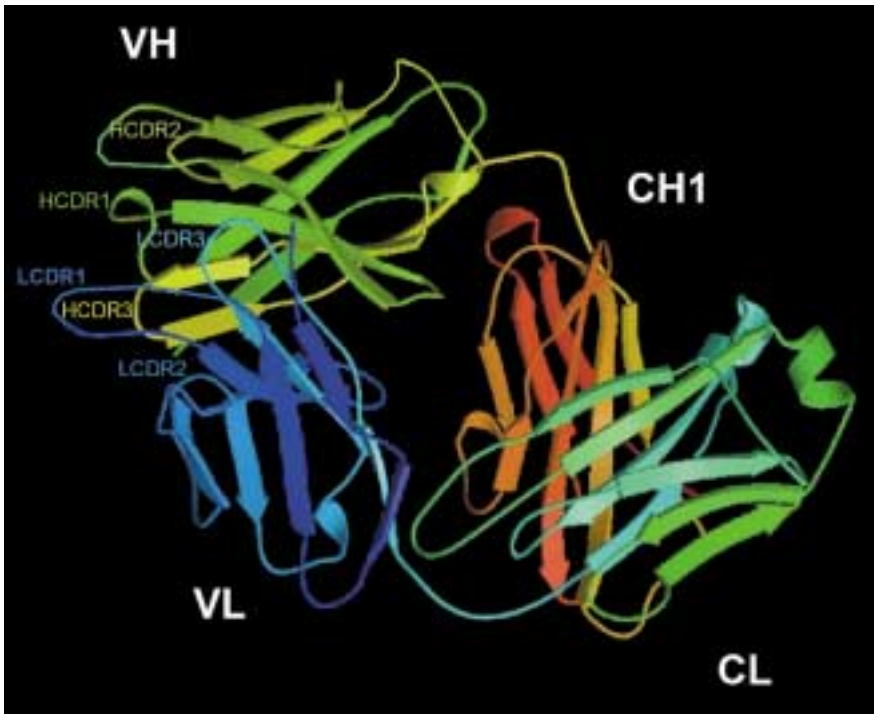

Figure 2. Three-dimensional structure of the Fab fragment of an antibody molecule. Arrows indicate β-strands and point in the amino-to-carboxyl direction. Lines indicate β-strand connecting loops. Each of the four Ig domains is formed by a sandwich of two antiparallel β-sheets linked by a disulfide bridge (not shown). The variable Ig domains of light and heavy chain, VL and VH, as well as the constant domains, CL and CH1, form dimers. Each of the two variable Ig domains, VL and VH, contain three CDRs, which are located on loops connecting the β-strands. Shown is the crystal structure of the Fab fragment of mouse mAb 33F12 as determined by A. Heine and I.A. Wilson[76].
Abbreviation: CDR, complementarity determining region.

Ig superfamily molecule cytotoxic T-lymphocyte-associated antigen 4 (Ref. 31) have been evolved *in vitro* using display technologies. In addition, the fibronectin-type III domain, which is structurally related to the Ig module, has been successfully displayed on phage[32]. An alternative strategy in protein engineering is the *in vitro* evolution of structurally distinct protein modules that have the potential to replace the Ig domain in certain applications[33,34].

In vitro evolution of antibodies

In addition to the *de novo* generation of mAbs, antibody libraries are an important tool for the *in vitro* evolution (i.e. humanization and affinity maturation) of existing mAbs. The use of antibody libraries for *in vitro* evolution has been guided by knowledge of the structural parameters that define the antibody molecule.

Humanization

A driving force in the development of human antibody libraries, as well as in the generation of transgenic mice expressing human antibodies, has been the fact that nonhuman antibodies are highly immunogenic in humans, which severely limits their clinical applications and makes antibody humanization mandatory if repeated administration is required for therapy. However, antibody immunogenicity in humans is thought to be influenced by several factors, including the nature of the antigen[35]. Accordingly, human or humanized antibodies might still elicit an immune response. The availability of sera from patients treated with antibodies will enable the nature of antibody immunogenicity to be addressed experimentally[36].

Humanized vs. human antibodies

Although the human antibody libraries from immune, naïve and synthetic repertoires initially made the humanization of antibodies from immune animals look outdated, there are now reasons to believe that humanized antibodies can challenge human antibodies in several therapeutically relevant aspects. First, 25 years of hybridoma technology yielded a number of therapeutically promising rodent mAbs, whose humanization compares well to the *de novo* generation and characterization of human antibodies for accessing clinical applications in the coming years. Second, in contrast to human antibodies derived from naïve or synthetic human antibody libraries, antibodies from immune animals were subjected to *in vivo* selection and, as a result, are more likely to recognize a given antigen selectively (i.e. without cross-reactivity to another antigen). Third, although until recently antibody humanization was based on rational design strategies, selective approaches based on display technologies have greatly facilitated the process.

Combining rational design and in vitro evolution

A frequently used rational design strategy for antibody humanization is CDR grafting[37,38]. In this approach, the six CDR loops comprising the antigen binding site of the nonhuman antibody are grafted into corresponding human framework regions. CDR grafting takes advantage of the conserved structure of the variable Ig domain, with the

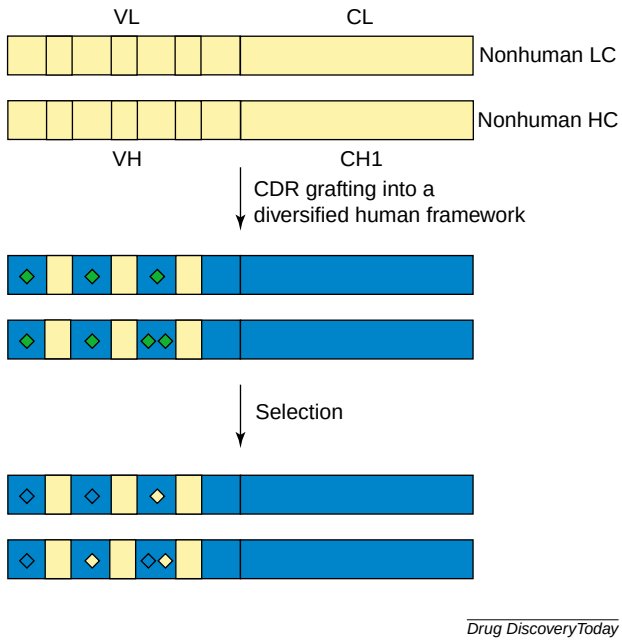

Figure 3. Selection strategy for antibody humanization that combines CDR grafting with framework fine-tuning. Schematically depicted are the building blocks of the Fab fragment of an antibody molecule [i.e. the light chain (LC) comprising variable Ig domain (VL) and constant Ig domain (CL), and the heavy chain (HC) fragment composed of variable Ig domain (VH) and first constant Ig domain (CH1)]. The three CDRs from each variable Ig domain are highlighted. Nonhuman sequences are shown in yellow, human sequences in blue. The six CDRs of a nonhuman Fab are grafted from their original framework into a human framework that contains a set of diversified residues (green diamonds). These residues are diversified to allow the selection of either the human (blue diamonds) or the original nonhuman residue (yellow diamonds).
Abbreviations: CDR, complementarity determining region.

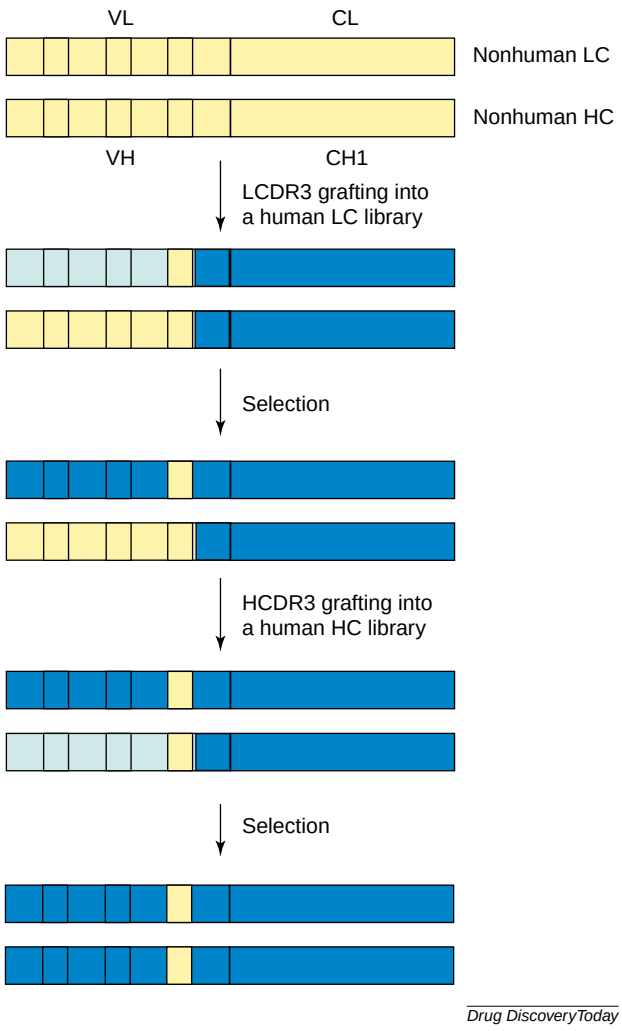

Figure 4. Antibody humanization by designed combinatorial V gene libraries. This strategy involves two selection steps for the sequential humanization of light chain and heavy chain fragments. Throughout these selections the only preserved sequences in the variable domains of light chain and heavy chain are the CDR3 sequences. Nonhuman sequences are shown in yellow, human sequences in blue. In the first step a chimeric nonhuman/human heavy chain fragment is used as a template for the selection of a human light chain V gene library (light blue) that contains the grafted CDR3 loop of the original nonhuman light chain. Likewise, a human heavy chain fragment that contains the grafted CDR3 loop of the original nonhuman heavy chain fragment is selected from a human heavy chain V gene library (light blue) in the second step.
Abbreviations: CDR, complementarity determining region.

framework regions forming a rigid, yet adjustable[39] β-sheet scaffold that displays the CDR loops (Fig. 2). In addition to residues in the CDR loops, framework residues contribute to antigen binding, either indirectly, by supporting the conformation of the CDR loops, or directly, by contacting the antigen[40,41]. Therefore, to maintain antigen binding, usually it is necessary to replace residues of the human framework in addition to CDR grafting. This fine-tuning step, which until recently required computer modeling and iterative optimization by site-directed mutagenesis, has now been subjected to *in vitro* evolution[42,43]. As outlined in Fig. 3, this strategy involves the diversification of a small number of framework residues to allow the selection of either the human or the original nonhuman residue. The diversified framework residues are chosen out of a set of key framework residues that are known to be involved in antigen binding. Key framework residues[40] have been identified structurally by crystallization and molecular modeling, as well as empirically by antibody humanization. This strategy was applied in the first humanization of a rabbit mAb[44]. The rabbit mAb had been selected by phage display from antibody libraries generated from rabbits that were immunized with human A33 antigen. Human A33 antigen is a tumor antigen that is expressed on the surface of colon cancer cells. The resulting humanized

mAbs were found to retain both high specificity and affinity for human A33 antigen[44].

Whereas the latter approach involves a very limited sequence diversification, another *in vitro* evolution strategy for antibody humanization diversifies entire V genes. This strategy (Fig. 4) was inspired by the concept of guided selection[45] and preserves only the original nonhuman CDR3 sequences of light and heavy chain while subjecting the remaining sequence to selection from naïve human V gene libraries. Using designed combinatorial V gene libraries, mouse mAb LM609 was humanized[46]. LM609 is directed to human integrin $\alpha_v\beta_3$ and has potential applicability in cancer therapy as an anti-angiogenic agent. This approach was shown to: (1) provide a rapid route for antibody humanization constraining the content of original nonhuman sequences in the final antibodies to the most hypervariable of the CDRs, (2) generate several humanized versions with different sequences simultaneously, (3) result in affinities as high as or higher than the affinity of the original antibody, and (4) retain the antigen and epitope specificity of the original antibody[46]. Designed combinatorial V gene libraries were used in several subsequent humanizations of mouse[47,48] and rabbit[49] antibodies. This strategy exemplifies an *in vitro* evolution of antibodies that is based on a combination of immune and naïve repertoires. The resulting antibodies can be evolved further by generating synthetic repertoires based on the randomization of defined sections in the antibody sequence.

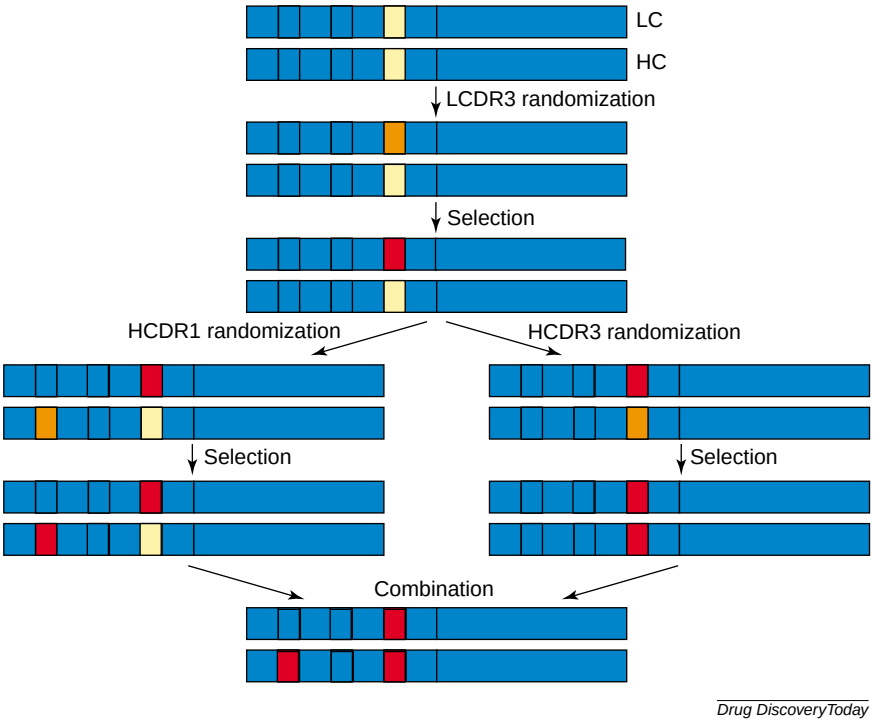

Figure 5. Affinity maturation of antibodies by CDR walking. CDR walking involves the sequential or parallel optimization of CDRs by focused mutagenesis and subsequent selection by phage display. Shown is a combination of sequential and parallel CDR optimization used for the affinity maturation of an antibody that was humanized using designed combinatorial V gene libraries as outlined in Fig. 4. Nonhuman sequences are shown in yellow, human sequences in blue, randomized sequences in orange, and optimized sequences in red. Nonhuman CDR3 of the light chain was optimized first followed by a parallel optimization of CDR1 and CDR3 of the heavy chain. Optimized CDR1 and CDR3 of the heavy chain were combined in the last step. The focused mutagenesis strategy involves the randomization of typically four to six naturally hypervariable codons in each CDR encoding sequence, a number that is limited by achievable library sizes[54]. Abbreviations: CDR, complementarity determining region.

Affinity maturation

The tumor-targeting properties of a mAb are determined by its size, avidity (i.e. number of antigen binding sites) and affinity[50,51]. Affinity maturation is a highly relevant step in engineering mAbs for therapeutic applications. By increasing the affinity to its target antigen, the therapeutic dose of a mAb is reduced whereas its therapeutic duration is increased[52]. In cancer therapy, however, too high affinities of mAbs are suspected to be counterproductive for tumor penetration[51].

The affinity maturation of mAbs using antibody libraries recapitulates the process of its natural counterpart, which is based on sequence diversification followed by selection[53]. Sequence diversification *in vitro* is accomplished by dispersed or focused mutagenesis strategies[54]. Recently, a dispersed mutagenesis strategy based on DNA shuffling[55] was combined with yeast display for the selection of antibodies with femtomolar monovalent affinity[56]. Regarding therapeutic applications, however, a focused mutagenesis strategy known as CDR walking[57] stands out as the most general approach for affinity maturation of antibodies. As outlined in Fig. 5 for the affinity maturation of humanized mAb LM609 (C. Rader *et al.*, unpublished), CDR walking involves the sequential or parallel optimization of CDRs by sequence randomization and subsequent selection by phage display. The CDRs are an obvious choice for focused mutagenesis because they comprise the antigen binding

site and they are naturally diverse, suggesting that mutations in these regions are less likely to be immunogenic. Using CDR walking, monovalent affinities of human mAbs were improved from nanomolar to picomolar range[58,59].

The described strategies for antibody humanization and affinity maturation are based on the selection of antibody libraries by display technologies. An alternative strategy is the more traditional, yet effective, screening of bacterially expressed antibody libraries using nitrocellulose filters probed with antigen[60]. This methodology has recently been applied to the parallel screening of an arrayed antibody library against a variety of antigens[61].

Catalytic antibodies

As recently pointed out by Schultz and collegues[62], 'the combination of tools and principles of chemistry, together with the tools of modern molecular biology, allow us to create complex synthetic and natural molecules, and processes with novel biological, chemical and physical properties'. Catalytic antibodies (i.e. antibodies that catalyze chemical reactions[63]) illustrate this potential at the interface of chemistry and biology. For example, a generic drug-masking strategy has been developed that can be applied to the selective activation of chemotherapeutic agents by sequential retro-aldol-retro-Michael reactions catalyzed by a mAb. Using various cancer cell lines *in vitro*, the functional unmasking of doxorubicin and camptothecin prodrugs by therapeutically relevant concentrations of the catalytic antibody was demonstrated[64]. The rationale in this concept is to combine catalytic and tumor targeting antibodies in bifunctional antibody constructs that allow the selective application of chemotherapy to the tumor[65]. Although the catalytic antibody used in these studies was generated by the hybridoma technology, it has been evolved *in vitro* by means of antibody libraries[47]. For an efficient prodrug activation *in vivo*, it will be important to further improve the enzymatic activity of catalytic antibodies by *in vitro* evolution strategies based on antibody libraries. As mentioned earlier, enzymatic activity is a selectable phenotype of mAbs. Thus, appropriately designed strategies allow for the selection of diverse catalytic activities from antibody libraries[66,67].

Target discovery

Beyond the generation and evolution of therapeutic antibodies, antibody libraries from immune, naïve and synthetic repertoires are set to become key tools in target discovery, in particular for the identification of human tumor antigens. Since the advent of hybridoma technology 25 years ago[4], a majority of human tumor antigens have been identified via mouse mAbs. Mice have been immunized with human tumor antigens by injecting human cancer cell lines or tissues. Mouse mAbs have been subsequently generated using hybridoma technology. Ultimately, mouse mAbs have guided the identification of their cognate antigen by cDNA cloning. An alternative approach for the identification of human tumor antigens is the serological analysis of recombinant cDNA expression libraries, termed SEREX, which uses autologous human antibodies from the sera of cancer patients for the screening of cDNA libraries constructed from the tumors of cancer patients[68]. More recently, tumor and tumor vasculature antigens have been identified on the transcriptional level by compiling sequence tag databases based on their differential expression in normal and malignant tissue[69].

Although the utilization of antibody libraries for target discovery has not yet been explored to its full potential, studies based on synthetic[70], naïve[71] and immune (Fig. 6) repertoires have proven the concept. In contrast to the hybridoma technology, antibody libraries can be positively and negatively selected against human cell lines, allowing

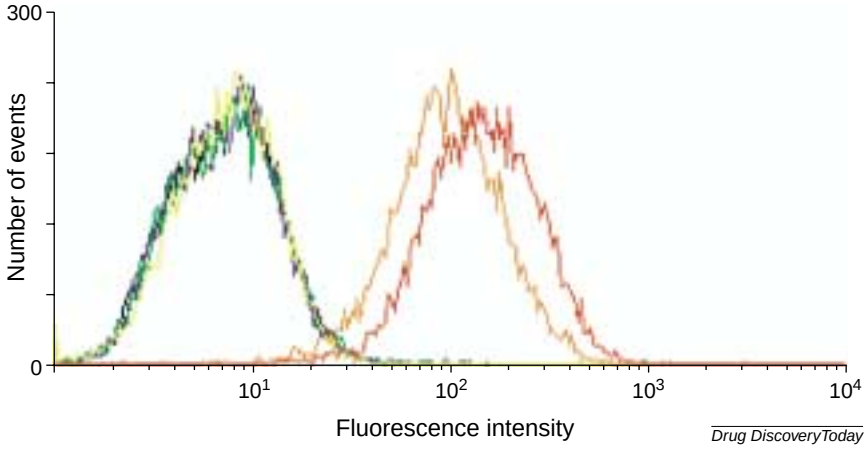

Figure 6. Antibody libraries in target discovery. Flow cytometry histogram showing the binding of phage from a rabbit antibody library to a human prostate cancer cell line after zero (blue), one (green), two (yellow), three (orange) and four (red) rounds of selection on whole cells. For indirect immunofluorescence staining, cells were incubated with phage except for the control (black). Anti-phage secondary antibodies and fluoroscien thiocyanate (FITC)-conjugated tertiary antibodies were used for detection. The y axis gives the number of events in linear scale, the x axis the fluorescence intensity in logarithmic scale. Dozens of mAbs of different sequence and selectivity were identified (M. Popkov and C. Rader, unpublished).

specificity to become part of the selection. Moreover, target discovery based on antibody libraries readily provides a targeting drug for an identified molecular target. Both the protein and the cDNA of the targeting drug (i.e. the mAb) are selected at the same time and thus can be readily channeled into *in vitro* evolution strategies such as antibody humanization and affinity maturation.

Tracking the success of peptide libraries[72,73], another promising strategy, in particular for the discovery of targets that are expressed by the tumor vasculature, is the selection of antibody libraries *in vivo*[74].

By interfering with ligand–receptor interactions or by inducing receptor clustering[75], mAbs have an impact on downstream signaling pathways that can be monitored by gene expression profiling using DNA array technology. Thus, in addition to the identification of cell surface antigens, mAbs have a potential in the definition of intracellular proteins as relevant drug targets. Paying tribute to the *zeitgeist* (current way of thinking), I envision an integration of antibody libraries, display technology and DNA array technology in an automated process designed for drug and target discovery.

With an emphasis on cancer therapy, the examples in this review were meant to illustrate the broad scope of antibody libraries from drug evolution to target discovery. Ahead of all promises, the approval of the first mAb for cancer therapy that was derived or evolved from an antibody library can be expected soon.

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References

1 Thayer, A.N. (1998) Great expectations. *Chem. Eng. News*, 10, 19–31
2 Walsh, G. (2000) Biopharmaceutical benchmarks. *Nat. Biotechnol.* 18, 831–833
3 Jurcic, J.G. *et al.* (1997) Monoclonal antibody therapy of cancer. *Cancer Chemother. Biol. Response Modif.* 17, 195–216
4 Köhler, G. and Milstein, C. (1975) Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–497
5 Huse, W.D. *et al.* (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 246, 1275–1281
6 Rader, C. and Barbas, C.F., III (1997) Phage display of combinatorial antibody libraries. *Curr. Opin. Biotechnol.* 8, 503–508
7 Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the surface of the virion. *Science* 288, 1315–1317
8 Kang, A.S. *et al.* (1991) Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc. Natl. Acad. Sci. U. S. A.* 88, 4363–4366
9 Clackson, T. *et al.* (1991) Making antibody fragments using phage display libraries. *Nature* 352, 624–628
10 Barbas, C.F., III *et al.* (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. U. S. A.* 88, 7978–7982
11 Burton, D.R. *et al.* (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc. Natl. Acad. Sci. U. S. A.* 88, 10134–10137
12 Marks, J.D. *et al.* (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222, 581–597
13 Shusta, E.V. *et al.* (1999) Biosynthetic polypeptide libraries. *Curr. Opin. Biotechnol.* 10, 117–122
14 FitzGerald, K. (2000) *In vitro* display technologies – new tools for drug discovery. *Drug Discov. Today* 5, 253–258
15 Hanes, J. *et al.* (1998) Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14130–14135
16 Boder, E.T. and Wittrup, K.D. (1997) Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* 15, 553–557
17 Daugherty, P.S. *et al.* (1998) Antibody affinity maturation using bacterial surface display. *Protein Eng.* 11, 825–832
18 Poul, M.A. and Marks, J.D. (1999) Targeted gene delivery to mammalian cells by filamentous bacteriophage. *J. Mol. Biol.* 288, 203–211
19 Cragg, M.S. *et al.* (1999) Signaling antibodies in cancer therapy. *Curr. Opin. Immunol.* 11, 541–547
20 Mendez, M.J. *et al.* (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat. Genet.* 15, 146–156
21 Vaughan, T.J. *et al.* (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotechnol.* 14, 309–314
22 De Haard, H.J. *et al.* (1999) A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J. Biol. Chem.* 274, 18218–18230
23 Sblattero, D. and Bradbury, A. (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. *Nat. Biotechnol.* 18, 75–80
24 Knappik, A. *et al.* (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* 296, 57–86
25 Söderlind, E. *et al.* (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat. Biotechnol.* 18, 852–856
26 Barbas, C.F., III (1995) Synthetic human antibodies. *Nat. Med.* 1, 837–839
27 Hoogenboom, H.R. (1997) Designing and optimizing library selection strategies for generating high-affinity antibodies. *Trends Biotechnol.* 15, 62–70
28 Hunkapiller, T. *et al.* (1989) Implications of the diversity of the immunoglobulin gene superfamily. *Cold Spring Harbor Symp. Quant. Biol.* 54, 15–29
29 Holler, P.D. *et al.* (2000) *In vitro* evolution of a T cell receptor with high affinity for peptide/MHC. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5387–5392
30 Shusta, E.V. *et al.* (2000) Directed evolution of a stable scaffold for T-cell receptor engineering. *Nat. Biotechnol.* 18, 754–759
31 Nutall, S.D. *et al.* (1999) Design and expression of soluble CTLA-4 variable domain as a scaffold for the display of functional polypeptides. *Proteins* 36, 217–227
32 Koide, A. *et al.* (1998) The fibronectin type III domain as a scaffold for novel binding proteins. *J. Mol. Biol.* 284, 1141–1151
33 Beste, G. *et al.* (1999) Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1898–1903
34 Altamirano, M.M. *et al.* (2000) Directed evolution of new catalytic activity using the α/β-barrel scaffold. *Nature* 403, 617–622
35 Clark, M. (2000). Antibody humanization: a case of the 'Emperor's new clothes'? *Immunol. Today* 21, 397–402
36 Iwahashi, M. *et al.* (1999) CDR substitutions of a humanized monoclonal antibody (CC49): contributions of individual CDRs to antigen binding and immunogenicity. *Mol. Immunol.* 36, 1079–1091

37 Jones, P.T. *et al.* (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321, 522–525

38 Riechmann, L. *et al.* (1988) Reshaping human antibodies for therapy. *Nature* 332, 323–327

39 Holmes, M.A. *et al.* (1998) Conformational correction mechanisms aiding antigen recognition by a humanized antibody. *J. Exp. Med.* 187, 479–485

40 Foote, J. and Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224, 487–499

41 Wedemayer, G.J. *et al.* (1997) Structural insights into the evolution of an antibody combining site. *Science* 276, 1665–1669

42 Rosok, M.J. *et al.* (1996) A combinatorial library strategy for the rapid humanization of anticarcinoma BR96 Fab. *J. Biol. Chem.* 271, 22611–22618

43 Baca, M. *et al.* (1997) Antibody humanization using monovalent phage display. *J. Biol. Chem.* 272, 10678–10684

44 Rader, C. *et al.* (2000) The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies. *J. Biol. Chem.* 275, 13668–13676

45 Jespers, L.S. *et al.* (1994) Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. *Biotechnology* 12, 899–903

46 Rader, C. *et al.* (1998) A phage display approach for rapid antibody humanization: designed combinatorial V gene libraries. *Proc. Natl. Acad. Sci. U. S. A.* 95, 8910–8915

47 Tanaka, F. *et al.* (2000) Reconstructing aldolase antibodies to alter their substrate specificity and turnover. *J. Am. Chem. Soc.* 122, 4835–4836

48 Beiboer, S.H.W. *et al.* (2000) Guided selection of a pan carcinoma specific antibody reveals similar binding characteristics yet structural divergence between the original murine antibody and its human equivalent. *J. Mol. Biol.* 296, 833–849

49 Steinberger, P. *et al.* (2000) Generation and characterization of a recombinant human CCR5–specific antibody: a phage display approach for rabbit antibody humanization. *J. Biol. Chem.* 275, 36073–36078

50 Hudson, P.J. (1999) Recombinant antibody constructs in cancer therapy. *Curr. Opin. Immunol.* 11, 548–557

51 Adams, G.P. and Schier, R. (1999) Generating improved single-chain Fv molecules for tumor targeting. *J. Immunol. Methods* 231, 249–260

52 Barbas, C.F., III and Burton, D.R. (1996) Selection and evolution of high-affinity human anti-viral antibodies. *Trends Biotechnol.* 14, 230–234

53 Rajewsky, K. (1996) Clonal selection and learning in the antibody system. *Nature* 381, 751–758

54 Rader, C. and Barbas, C.F., III. (2000) In *Phage display, a laboratory manual* (Barbas, C.F., III *et al.*, eds), pp. 13.1–13.15, Cold Spring Harbor Laboratory Press

55 Stemmer, W.P. (1994) Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* 370, 389–391

56 Boder, E.T. *et al.* (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10701–10705

57 Barbas, C.F., III *et al.* (1994) *In vitro* evolution of a neutralizing human antibody to HIV-1 to enhance affinity and broaden strain cross-reactivity. *Proc. Natl. Acad. Sci. U. S. A.* 91, 3809–3813

58 Yang, W-P. *et al.* (1995) CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* 254, 392–403

59 Schier, R. *et al.* (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J. Mol. Biol.* 263, 551–567

60 Wu, H. *et al.* (1999) Humanization of a murine monoclonal antibody by simultaneous optimization of framework and CDR residues. *J. Mol. Biol.* 294, 151–162

61 de Wildt, R.M.T. *et al.* (2000) Antibody arrays for high-throughput screening of antibody–antigen interactions. *Nat. Biotechnol.* 18, 989–994

62 Martin, A.B. and Schultz, P.G. (1999) Opportunities at the interface of chemistry and biology. *Trends Biochem. Sci.* 24, M24–M28

63 Lerner, R.A. *et al.* (1998) Making enzymes. *Harvey Lect.* 92, 1–40

64 Shabat, D. *et al.* (1999) Multiple event activation of a generic prodrug trigger by antibody catalysis. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6925–6930

65 Rader, C. and List, B. (2000) Catalytic antibodies as magic bullets. *Chem. Eur. J.* 6, 2091–2095

66 Gao, C. *et al.* (1998) Direct selection for catalysis from combinatorial antibody libraries using a boronic acid probe: primary amide bond hydrolysis. *J. Am. Chem. Soc.* 120, 2211–2217

67 Fujii, I. *et al.* (1998) Evolving catalytic antibodies in a phage-displayed combinatorial library. *Nat. Biotechnol.* 16, 463–467

68 Sahin, U. *et al.* (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc. Natl. Acad. Sci. U. S. A.* 92, 11810–11813

69 St Croix, B. *et al.* (2000) Genes expressed in human tumor endothelium. *Science* 289, 1197–1202

70 de Kruif, J. *et al.* (1995) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3938–3942

71 Ridgway, J.B. *et al.* (1999) Identification of a human anti-CD55 single-chain Fv by subtractive panning of a phage library using tumor and nontumor cell lines. *Cancer Res.* 59, 2718–2723

72 Pasqualini, R. and Ruoslahti, E. (1996) Organ targeting *in vivo* using phage display peptide libraries. *Nature* 380, 364–366

73 Rajotte, D. and Ruoslahti, E. (1999) Membrane dipeptidase is the receptor for a lung-targeting peptide identified by *in vivo* phage display. *J. Biol. Chem.* 274, 11593–11598

74 Johns, M. *et al.* (2000) *In vivo* selection of scFv from phage display libraries. *J. Immunol. Methods* 239, 137–151

75 Ghetie, M.A. *et al.* (1997) Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* 94, 7509–7514

76 Barbas, C.F., III *et al.* (1997) Immune versus natural selection: antibody aldolases with enzymatic rates but broader scope. *Science* 278, 2085–2092