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Human antibody libraries: A race to engineer and explore a larger diversity

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1. ABSTRACT

Several recombinant antibody libraries associated with different screening technologies have been generated since the first steps of antibody engineering 15 years ago, in order to isolate human monoclonal antibodies. In this race to isolate antibody with virtually any specificity, innovative strategies have been developed to clone natural antibody repertoires or to increase library diversity beyond the scope of the immune system. After the *in vitro* transfer of the natural diversity, the second generation of partly or completely man-designed libraries was based on the available structural data of the antibody binding. Efficient selection strategies have proven critical in exploiting the potential of a library's diversity. The development and improvement of screening methods such as phage display, yeast display, ribosome display and robotic platforms have provided innovative tools to efficiently screen and sort out the desired binding specificities of billions of antibodies. Efforts to improve diversity exploration have been mainly focused on screening conditions of display techniques and the new emerging techniques. Here we review some of these prominent approaches in the field of human recombinant antibody libraries.

2. INTRODUCTION

The advent of the hybridoma technology by Kohler and Milstein in 1975 marked the beginning of antibody technology (1). Since this historical discovery to produce monoclonal antibodies using murine immortalized B cells, research in the field of therapeutic antibodies has made considerable progress. However, despite FDA approval in 1986 of the first therapeutic antibody (anti-CD3) developed by Johnson & Johnson, the "magic bullets" did not live up to expectations. Indeed, the production of monoclonal antibodies in the mouse posed several problems that needed resolving. With the development of genetic engineering techniques 70% of the mouse monoclonal antibody could be exchanged for a human portion. To further minimize the immunogenicity of these chimeric antibodies, the complementary determining regions (CDRs) from the mouse antibodies were conserved and reintroduced into the human variable domains. However, these humanized antibodies with 95 % of human sequences needed intensive engineering work to conserve the specificity against their targets. Developments in PCR technology have provided a solution allowing human antibody repertoires to be cloned, thereby creating large

libraries of fully human recombinant antibodies. These recombinant antibody libraries, combined with high throughput screening techniques, offer an alternative to traditional immunization of animals and hybridoma technology to specifically isolate high affinity monoclonal antibodies against diverse targets. The main advantage of these techniques is that they permit the direct isolation of human antibodies.

In vivo, the diversity of the human antibody repertoire is created by (i) combinatorial possibilities of joining among 51 V, 23 D and 6 J DNA segments to form functional variable heavy chain (VH) genes and similarly 70 V (40 Vkappa and 30 Vlambda) and 5 J segments to form variable light chain (VL) genes; (ii) nucleotide deletion and addition in the CDR3, at the splice junctions of V, D and J segments of the VH and at the junction of V and J segments of the VL; (iii) the random association of VH and VL; and finally (iv) somatic hypermutation that introduces mutations specifically in the V-genes in Ag-stimulated B cells. In order to mimic the immune system *in vitro*, many research groups have set up engineering technologies to increase the diversity of the antibody libraries. The major goal of the different researchers working in this field has been to develop larger human antibody libraries and create a wider diversity to facilitate the isolation of antibodies of every conceivable specificity. However, the quality of the libraries is influenced by several important factors including antibody formats, display levels, sequence diversity, expression level, tendency to multimerize, compatibility with *in vitro* screening, affinity maturation and the ease of conversion to other antibody formats, display or selection systems. Here we discuss the race to engineer a greater diversity and explore the huge complexity of human antibody libraries with high throughput screening methods.

3. DIFFERENT KINDS OF HUMAN ANTIBODY LIBRARIES

In 1989, the molecular engineering methods enabled the bypassing of hybridoma technology by the cloning of antibody genes from lymphocytes of immunized animals and the construction of the first recombinant antibody libraries (2, 3, 4). Two years later, the first human antibody libraries were described (5, 6). Since then, the VH and VL gene pools of most antibody libraries have been amplified and cloned from B-cells of diverse lymphoid sources such as peripheral blood, bone marrow, lymph nodes, spleen or tonsil (Table 1, non exhaustive list). As described in Table 1, the VH and VL are either used individually as a single domain or combined randomly to form an antigen-binding fragment (Fab), a single-chain fragment variable (scFv) or a disulfide-stabilized Fv antibody fragment (dsFv). The scFv and Fab are the most common formats used in recombinant antibody libraries. Which is the preferred choice between these two formats is an ongoing controversy. Fab fragments are more stable than scFv due to an additional domain-domain interface resulting from the CH1-CL association. Antibody affinity seems higher when the same V-genes are expressed as Fab compared to scFv (7). However, the scFv format has been used more frequently due to a better tolerance and expression in bacteria and greater expression in display compared to the heterodimeric Fab.

3.1. Immune and nonimmune antibody libraries

The source of antibody genes constitutes the major determinant of diversity. The variable region gene repertoire can be isolated from unimmunized (naïve library) or immunized donors (immune library) with PCR primer pairs characteristic of all the VH, Vk and Vl gene families. The antibody libraries constructed from immunized human donors are biased towards certain specificities present in naturally immunized or infected donors. These immune libraries are usually of modest size ($1x10^8$ clones), however they take advantage of the *in vivo* affinity maturation process and high affinity antibodies are often selected from these libraries (6-12). The drawbacks of this type of library are the obvious limitations of bias towards certain antigens and also the difficulty in obtaining antigen-related human B lymphocyte sources.

In contrast, the naïve libraries are derived from natural unimmunized rearranged V genes (5). The naïve libraries must be larger (higher than $1x10^9$) to compensate for the fact that they have never encountered the particular antigen against which antibody fragments are to be selected. Experience has shown that in general, the larger the library, the higher the chance of isolating specific antibodies with specific binding characteristics. The first developed libraries (10^7-10^8 clones) allowed the isolation of antibodies with target affinities in the micromolar range, similar to those obtained in a primary immune response (5). Over the following years, larger naïve libraries of 10^9-10^{10} developed by Vaughan *et al* (1996), Sheets *et al* (1998), de Haard *et al* (1999) and Little *et al* (1999) (13-16) yielded antibodies with affinities higher than 10^{-8} M. The generation of antibodies by conventional hybridoma technology is subject to the constraints of the immune system. Hence, the development of these recombinant antibody libraries enabled these constraints of immunization to be overcome. The scFv library developed by Cambridge Antibody Technology (CAT) demonstrated the possibility of isolating antibodies against compounds normally cytotoxic or immunosuppressive (13). Furthermore, the library developed by Sheet *et al* (1998) allowed the isolation of several antibodies against the same target with affinities ranging from 4 nM to 220 pM (14). As resumed in Table 1, to make the scFv or the Fab libraries as diverse as possible, some researchers isolated the *in vivo* rearranged V genes from large numbers of healthy donors: 50 (16), 58 (17), 11 (18). Others like Cambridge Antibody Technology combined 43 healthy donors and different B-cell supplies (15 peripheral blood lymphocyte samples (PBL), 4 tonsils and 24 bone marrows) to make a recombinant antibody library with large diversity (13). Each investigator aimed to use sets of primers corresponding to all reported VH, Vk and Vl gene families (5, 19) in order to amplify all known V-genes. The VH and VL genes were usually cloned separately into two distinct VH and VL libraries and assembled by overlap extension PCR or by a two step cloning. In this way de Haard *et al* (1999) developed a very large Fab library, of over 10^{10} clones, using a highly efficient two-step cloning method. In

Table 1. Diversity and kind of human recombinant antibody libraries

Library name	Institute or company	Library size	Screening system	Source	Origin	Diversity	Library type	Format	Ref
Mark 1991	MRC	$1.9x10^8$	PD	PBL	2 healthy donors	Random combinatorial VH/VL	Naive	scFv	5
Burton 1991	Scripps Res. Inst.	10^7	PD	BM	HIV-1-positive donors	Random combinatorial VH/VL	Immune	Fab	6
Hoogenboom & Winter, 1992	MRC	$2.2x10^7$	PD	GL		Random combinatorial VH segments +synthetic CDRH3 /VL	Semi-synthetic	scFv	24
Barbas 1992	Scripps Res. Inst.	$5x10^7$	PD		Human tetanus toxoid binding Fab	Random combinatorial 1 VH segment + synthetic CDRH3 / 1 VL	Semi-synthetic	Fab	23
Akamatsu, 1993	PDL	$1.7x10^7$	PD	GL		Random combinatorial VH segments + synthetic CDRH3 /Vk segments + synthetic CDRL3	Semi-synthetic	scFv	26
Nissim 1994	MRC	$1x10^8$	PD	GL		Random combinatorial: 50 VH segments + synthetic CDRH3 / 1 Vl3	Semi-synthetic	scFv	25
Griffiths 1994	MRC	$6.5x10^{10}$	PD	GL		Random combinatorial VH segments + synthetic CDRH3 /VL segments+ synthetic CDRL3	Semi-synthetic	Fab	22
de Kruif 1995	Univ. Utrecht	$3.6x\ 10^8$	PD	GL		Random combinatorial VH segments + synthetic CDRH3 / 7 VL	Semi-synthetic	scFv	28
Vaughan 1996	CAT	$1.4x10^{10}$	PD	15 PBL 4 tonsils 24 BM	43 healthy donors	Random combinatorial VH/VL	Naive	scFv	13
Pini –Viti 1998	Siena; Zurich; Genova	$3x10^8$	PD	GL		Random combinatorial of 1VH segment (DP47)+ synthetic CDRH3 / 1 VL segment (DPK22) synthetic CDRL3	Semi-synthetic	scFv	27
Sheets 1998	Univ. California	$6.7x10^9$	PD	3 spleens 2 PBL	5 healthy donors	Random combinatorial VH/VL	Naïve	scFv	14
de Haard 1999	Univ. Maastricht	$3.7x10^{10}$	PD	PBL spleen	4 healthy donors + 1 patient (gastric carcinoma)	Random combinatorial VH/VL	Naive	Fab	15
Little 1999	Univ. Heidelberg	$4x10^9$	PD	PBL	50 healthy donors	Random combinatorial VH/VL	Naive	scFv	16
n-C0DeR 2000	BioInvent	$2x10^9$	PD		healthy donors (CDRs)	Random combinatorial of 1VH segment (DP47)+ CDRH / 1 VL segment (DPL3) +CDRL	Semi-synthetic	scFv	30
HuCAL-scFv HuCAL-Fab	Morphosys	$2x10^9$ $2.1x10^{10}$	PD			Random combinatorial of 7 VH segments + degenerated CDRH3 / 7 VL segments + synthetic CDRL3	Synthetic	scFv Fab	35, 37
Sblattero & Bradbury, 2000	SISSA, Trieste	$3x10^{11}$	PD	PBL	40 donors	Random combinatorial VH/VL with cre-lox system	Naive	scFv	20
Feldhaus 2003	PNNL MIT	$1x10^9$	PD	Spleen Lymph node	58 healthy donors	Random combinatorial VH/VL	Naive	scFv	17
Weaver-Feldhaus 2004	PNNL	$3x10^9$	PD	PBL	Immunized with pentavalent botulinum toxoid	Random combinatorial VH/VL by yeast mating	Immune	Fab	7
Eeckhout 2004	Univ Gent Domantis	$1.3x10^{10}$	PD	PBL	11 healthy donors	Random combinatorial VH/VL	Naive	scFv	18
Fellouse 2005	Genentech	10^{10}	PD		Humanized Fab	Random solvent expose residus of CRDL3, CDRH1, CDRH2, CDRH3 with a binary code (Y/S)	Synthetic	Fab	31
FAB-310, FAB-410	DYAX	$4.5x10^{10}$	PD	PBL	35 patients (auto-immunes)	Random combinatorial VH/VL + synthetic CDRH1, CDRH2, FR1 and FR2)	Semi-synthetic	Fab	34
MutalBank	MilleGen	$1x10^9$	PD; ICS	PBL	healthy donors + 100 patients	Random mutagenesis VH and VL + Random combinatorial VH/VL	Semi-synthetic	scFv	21

ND : Non determined ; MRC : Medical Research Council, UK ; CAT : Cambridge Antibody Technology; PDL : Protein Design Labs ; MIT: Massachusetts Institute of Technology; PNNL: Pacific Northwest National Laboratories; SISSA: International School for Advanced Studies; PD: Phage display; YD: Yeast display; ICS: IntraCellular screening; PBL : peripheral blood lymphocyte, GL: germline gene, BM: bone marrow. Ref: Reference

general, the large libraries (10^9-10^{10}) were formed by carrying out a considerable number of ligations and hundreds of bacterial transformations, "brutal force cloning" (15). The major bottleneck of library construction is the transformation efficiency in bacteria and in eukaryotic cells. A few years later, new applications of *in vivo* recombination in bacterial host and in yeast mating allowed the generation of very large naïve antibody

libraries (20, 10). The method described by Sblattero and Bradbury (2000) used the reversibility of Cre-catalysed recombination (20). A relatively small primary library was firstly created in a phagemid vector in which the VH and VL genes were separated by two nonhomologous lox sites. The VH and VL were then recombined by infecting the phagemid into Cre-expressing bacteria at high multiplicity of infection. The library generated still remains the largest reported scFv library ($3x10^{11}$ clones) yielding a selection of antibodies having affinities between 90-15 nM against a vast number of different protein antigens. Weaver-Feldhaus *et al* (2004) used the yeast mating of haploid strains to combine the VH and VL genes in a large immune library ($3x10^9$) of diploid forms displaying Fab fragments. The affinities of the selected Fabs for botulinum toxin ranged from 0.8 to 2.1 nM (7).

Immunogenicity is another important factor to take into account when the antibody library is intended to serve in therapeutic antibody development. The risk of immunogenicity may be reduced by using antibodies that are as human as possible. Random introduction of variability in the entire variable domains has been used in the evolution of existing selected antibodies (see paragraph 4 of this manuscript). However, the mutagenesis processes used were unnatural and induced modifications able to change the nature of the antibody and increase the risk of immunogenicity. Increasing the diversity of a large antibody repertoire had, until recently, not been used through random mutagenesis on the entire variable domain (frameworks and CDRs). Our group therefore described a library generated from *in vivo* rearranged V-genes from non-immunized donors and also from a naturally oriented population of antibodies against several pathologies (100 patients) (21). In addition, to combine the diversity of a naïve and immune library, we further increased the complexity through a random mutation technology (MutaGen™, patent WO 02/38756 A1) mimicking the *in vivo* process responsible for the antibody diversity (21). We used the natural human polymerases known as mutases (Pol beta, or else Pol iota, Pol eta or Pol kappa) *in vitro,* due to their low fidelity, on the entire variable domain (frameworks and CDRs) of large repertoires of V-genes.

3.2. Semi-synthetic libraries

The combinatorial assembly of the VH and VL genes of the immune or naïve libraries does not reflect the natural antibody repertoire due to non-conservation of the natural VH-VL pairing but does constitute the creation of a non-natural diversity. An artificial repertoire could be shaped in order to lead to a different spectrum of binding sites. If holes exist in the natural repertoire, they may be absent in an artificial repertoire and vice versa (22). One of the chosen strategies is to clone the repertoire of the *in vivo* rearranged VH and VL genes from B cells. Another strategy of designing semi-synthetic repertoires uses the V germline segments of the heavy and light chains, followed by the introduction of partial or complete degeneracy into the CDR3 loop with synthetic oligonucleotides.

The CDR3 loop of the VH genes located at the center of the antigen binding site generally contributes greatly to antigen binding. Among the CDRs in natural antibodies, CDRH3 is the most variable in size (up to 24 residues) with the most diversified sequence due to nucleotide deletions and additions during the rearrangement of the V-D-J segments. These characteristics allowed an alternative to create diversity by using collections of cloned germline VH segments fused *in vitro* to synthetic CDR3 regions and then combined with a repertoire of VL genes. Thus, semi-synthetic libraries were constructed by randomization with degenerate primers of the CDRH3 (Table 1). Several studies showed that medium size libraries ($1\text{-}5x10^7$ members) with synthetic CRDH3 have provided a successful selection of novel antibody specificities (23, 24). Larger semi-synthetic libraries ($>10^8$ members) were then built by Nissin *et al* (1994) using 50 human germline VH segments fused to CDRH3 random sequences varying in lengths of 4-12 residues and combined with one Vl3 gene (25). Other libraries combined the semi-synthetic VH with semi-synthetic Vk and Vl germline segments fused to random synthetic CDRL3 sequences (22, 26, 27). By combining the VH repertoire of the Nissim library (25) with the Vk ($9x10^4$ clones) or Vl ($7.4x10^5$ clones) repertoire, Griffiths *et al*. (1994) increased the library diversity (22). The Vk and the Vl repertoires were obtained from 26 Vk and 21 Vl cloned segments fused to partly randomized CDR3 loops of 8-10 and 8-13 residues, respectively. Furthermore, an original process termed "combinatorial infection" using Cre catalyzed recombination at the loxP site permitted the generation of a very large Fab library ($6.5x10^{10}$ individual clones) (22).

CDRH3 sequences of *in vivo* rearranged V-genes are largely derived from the D-gene segments. Rather than completely constituting a random sequence these genes encode amino acids with a propensity for loop formation. In contrast, the synthetic CDR3 may be less likely to fold properly or produce usefully shaped binding pockets. The semi-synthetic library of de Kruif *et al* (1995) fused 49 different human germline VH genes to CDR3 regions of 6-15 residues (28). However, unlike the Nissim and Griffiths libraries, the CDRH3 regions were partly degenerated with short stretches of fully randomized residues flanked by regions of limited viability selected on their frequent occurrence in natural CDRH3 (28). The principles of protein design were also used by Pini *et al* (1998) to produce an antibody library of $3x10^8$ clones on the basis of single VH (DP47 germline) and VL (DPK22 germline) segments (27). The authors chose the DP47 and DPK22 germlines for their domination of the functional repertoire, their strong representation in binders isolated from synthetic phage libraries (22) and reportedly good expression levels. In this library, sequence diversity was concentrated within regions of the antigen binding site. Degenerate primers were used to introduce random mutations at positions 95-98 in CDRH3 and positions 91, 93, 94 and 96 of CDRL3 in accordance with their role as common antigen contacts. The authors also opted for short CDR3s to limit the potential diversity of the library and reduce clone variability (27). Current knowledge of the variation in the immunoglobulin repertoire as well as commonly used antigen-contacting residues defined in all available reports of antigen-antibody complexes formed the

basis in the design of synthetic oligonucleotides to generate CDR3 diversity. A high accuracy of this type of rational design is necessary to avoid the possibility of encoding antigenic epitopes.

The CDRH3 strongly contributes to the overall binding capacity of an individual antibody. However this approach shadows the remaining five CDR-loops which also contribute to the specificity and affinity of the antibody. Another approach first developed by Jirholt *et al* (1998) shuffled all the *in vivo* formed CDRs into a specific framework region using a CDR-Implantation Technology (29). They achieved a very high diversity by means of simultaneous and random combination of six biologically derived CDRs. BioInvent Therapeutics in Sweden then used this CDR-Implantation Technology to generate the n-CoDeR library of $2x10^9$ members (30). They combined the CDRs from a cDNA library prepared from peripheral blood B cells within selected master frameworks of the VH-DP47 and VL-DPL3 germline genes by overlap extension PCR. Although producing a genetic diversity beyond what is naturally created in the immune system whilst using this process, the antibodies isolated from the library exhibited dissociation constants in the subnanomolar range (30). In contrast with the precedent semi-synthetic libraries based on *in vitro* design of synthetic oligonucleotides, this process used *in vivo* formed CDR. These proofread CDR segments, optimized by the immune system with regards to functionality, would unlikely encode antigenic T-cell epitopes. All the antibodies selected from the n-CoDeR library were described as having a correct folding. However, these combinatorial diversities resulting from the combination of different *in vivo* formed gene-segments had not been proofread by the immune system.

3.3. Synthetic libraries

A finely tailored diversity was achieved based on the structural analysis of the antigen binding sites of antibodies (31, 32). Using a fixed humanized Fab framework, a group at Genentech generated a library by randomizing the solvent accessible CDR positions with a degenerate codon encoding only four amino acid residues: tyrosine, serine, alanine and aspartate (31). The results highlighted that tyrosine side chains dominate the antigen-binding sites and although the library diversity was limited, high affinity antibodies were isolated. Remarkably, a second study by the same group showed that a binary code (tyrosine, serine) was sufficient to obtain specific antibodies against several antigens comparable to those obtained from naïve libraries. The tyrosine combined with a small serine appeared particularly well suited to mediate productive interactions (33).

The rational design of diversity also enabled libraries to focalize on a certain size of antigen. Persson *et al* (2006) recently designed and created a synthetic library biased for hapten screening based on a fixed scFv (FITC8) previously selected against a hapten (32). Haptens preferentially bind to cavities and are contacted mainly by residues centrally located in the paratope, while proteins with relatively flat binding surfaces have additional contact residues in more peripheral regions. The authors therefore created a library by introducing diversity into 11 centrally located residues in the cavity of FITC8 avoiding peripherally located residues.

The single frameworks approach has the advantage of simplicity for library design and construction. Dyax developed a large Fab library having a unique combination of Vl, Vk and CDRH3 sequences captured from human donors (autoimmune patients) and synthetic diversity in key antigen contact sites of the CDRH1 and CDRH2 of a single framework (VH3-23) (34).

Some groups have chosen the option of increasing the number of frameworks to enhance the diversity of the library as for example the fully synthetic library assembled by total gene synthesis constructed in 2000 by Morphosys and the Biochemistry Institute (Zurich Univ.). Each of the human VH and VL subfamilies, frequently used during an immune response, was represented by one consensus framework, resulting in seven master genes for VH and seven master genes for VL giving 49 combinations. They increased the diversity by replacing the VH and VL CDR3 regions of the master genes by CDR3 library cassettes generated from mixed trinucleotides and biased towards natural human antibody CDR3 sequences. Sequencing 257 members of the unselected libraries indicated a 61% frequency of correct and thus potentially functional sequences (35). The binding affinities (K_D) of the antibodies selected from this library (HuCAL-scFv) were found to be from about 1nM to the lower micromolar range (36). This HuCAL-scFv library was then later converted to the Fab format (37).

These different examples do not clearly indicate which of the naïve or the synthetic libraries is most efficient at isolating antibodies with high affinity. Each of the libraries typically produces human antibodies with K_D ranging from 10^{-7} to 10^{-9} M. However, the recovery of higher affinity antibodies is important for efficient binding to the antigenic target for therapeutic and sensitive diagnostic use. For many well studied cases, increased affinities have translated into improved biological efficacy (38). In order to obtain useful therapeutic antibodies, an optimization step of the binding characteristics is often necessary following the screening of an antibody library and the isolation of the lead antibody.

4. MATURATION OF THE ANTIBODIES

Various *in vitro* strategies have been demonstrated to optimize an antibody selected from library screening and to create binding specificities and affinities outside the reach of the immune system. These include site specific mutagenesis based on structural information, combinatorial mutagenesis of CDR, chain shuffling and random mutagenesis.

In the immune system, antibodies are affinity matured in a stepwise fashion by incorporating mutations and selecting variants under increasing selective pressures. Somatic hypermutation (SHM) of immunoglobulin genes is critical in the generation of high affinity antibodies *in vivo* and occurs only after immunization. Exposure to an antigen selects lymphocytes that produce antigen reactive

antibodies from the natural repertoire of naive B cells and triggers the incorporation of somatic mutations in the V genes. This natural process allows the selection of mutations that improve the affinity of the antibody for the antigen. During the SHM process, base substitutions are introduced into a region of 1 kb surrounding the antibody-coding sequence. This process is initiated by the activation induced cytidine deaminase (AID) protein only expressed in B lymphocytes. AID deaminates cytosine to uracil in DNA and has a strong preference for the hot spot motif WRC (W is A or T and R is A or G). Ample evidence exists that the subsequent steps of SHM involve low fidelity polymerases (mutases) such as polymerase eta, zeta and iota (39-41). Hypotheses state that the intricate positioning of C and G nucleotides allows a precise concomitant hotspot/coldspot targeting of AID activity and also errors of the natural mutases to maximize SHM in the CDRs and minimize mutations in the frameworks. The SHM of mammalian antibodies generates a high frequency (1-5% of base pairs) and dispersed mutation across the V gene region at all the nucleotides: A 33%, T 17%, G, 25% and C 25 % (42).

In an excellent review H. Hoogenboom (2005) listed fully the level of *in vitro* optimization of the antibodies (38). The mutations reported have often been restricted to the antigen binding surface (CDR) (43-45), and very impressive affinity improvements of more than 1000 fold have been obtained with a subnanomolar final affinity (27, 44). However, although the framework regions of the variable domains VH and VL are not directly in contact with the antigen, their residues can have indirect effects on binding by affecting CDR conformation. Reports have demonstrated that affinity optimization could be obtained through the association of mutations in the CDR and those in the framework variable fragments (46-49). Of these reports, Boder et al (2000) showed that the combination of yeast display and error prone PCR on a whole scFv permitted the isolation of an antibody with a femtomolar antigen binding affinity. The best antibody clone, isolated after four cycles of diversity and selection, showed 8 mutations in the framework and 6 mutations in the CDR. The impressive affinity gains indicated that the *in vitro* procedure does not suffer from the kinetic and affinity limits inherent in the immune system.

In the humanization process of antibodies, simple grafting of the CDR sequences often yields humanized antibodies that bind the antigen much more weakly than the parent murine antibody. Fine tuning of the antigen binding loops can be achieved using a library approach to replace key residues in the framework regions and select the best leads (50). Genentech developed a therapeutic antibody (Avastin) through the humanization of muMAb VEGF A.4.6.1 by modification of not only the residues involved in the six CDR but also several framework residues (patent EP1325932A2). The binding of the initial humanized antibody with the murine CDR grafted onto a human framework was undetectable. The modification of 9 residues in the frameworks through a combinatorial approach restored the affinity (50). This study by Baca *et al*. (1997) demonstrated the important role of the framework in proper structuring of the CDR loops for antigen binding. Cumbers *et al* (2002) also obtained affinity maturation *in vitro* using hypermutating B-cell lines (51). Sequence analyses have shown that changes in the CDRs are important in the creation of the original low affinity and that framework changes have more of a role in later maturation. Mutations in the frameworks seem to modulate CDR loop flexibility or affect the orientation of the VH and VL domains.

5. SCREENING PROCESS OF DIVERSITY

Although building very large diversity and high quality human antibody libraries remains an important challenge, developing efficient selection strategies is also critical to exploit the potential of the diversity created. Different selection platforms and high throughput screening strategies have been developed throughout the last decade to permit the recovery of specific antibodies to a particular antigen (Figure 1).

5.1. Phage display

Phage display technology is the most common and easy-to-use platform for antibody library screening and here we will focus mainly on the monovalent phage display system, the well-established system for antibody selection. To display a single copy of an antibody fragment on the surface of a phage particle, the antibody encoding DNA is fused to the coat protein gene pIII. The resulting fusion gene is carried on a phagemid vector and display is achieved by infecting the phagemid-carrying bacteria with a helper phage. Coupling can also be achieved using a disulfide bridge between the antibody fragment and the pIII protein (Cys Display technology patented by Morphosys, N° US 6753136). The phage display selection process or biopanning includes both *in vitro* (selection and washing) and *in vivo* (phage propagation) steps. The overall protocol and different ways to present an antigen are presented in Figure 2 (52).

Despite being a well-established technique the selection by phage display must be practiced with great caution. Indeed, binding ability alone is not sufficient to guarantee success. Selection is a complex process with many factors aside affinity characteristics playing an important role. The panning process constitutes a balance between positive selective forces (affinity, specificity) and negative selective forces (toxicity for bacterial host, poor expression and aggregation-prone antibody fragment). Moreover, the form of the antigen, the correct orientation on the solid support, the method used (solid phase or in solution), the stringency of elution and the number of washing steps all have a major impact on the selection success (53, 54). With this in mind, minor stringency changes during the selection process can lead to a selection of totally different phage antibody populations.

To help in the selection of clones over a short time frame, phage display selection is generally associated with specificity screening of tens or even thousands of individual clones in an ELISA-phage assay. This screening can be carried out on a small number of clones after only

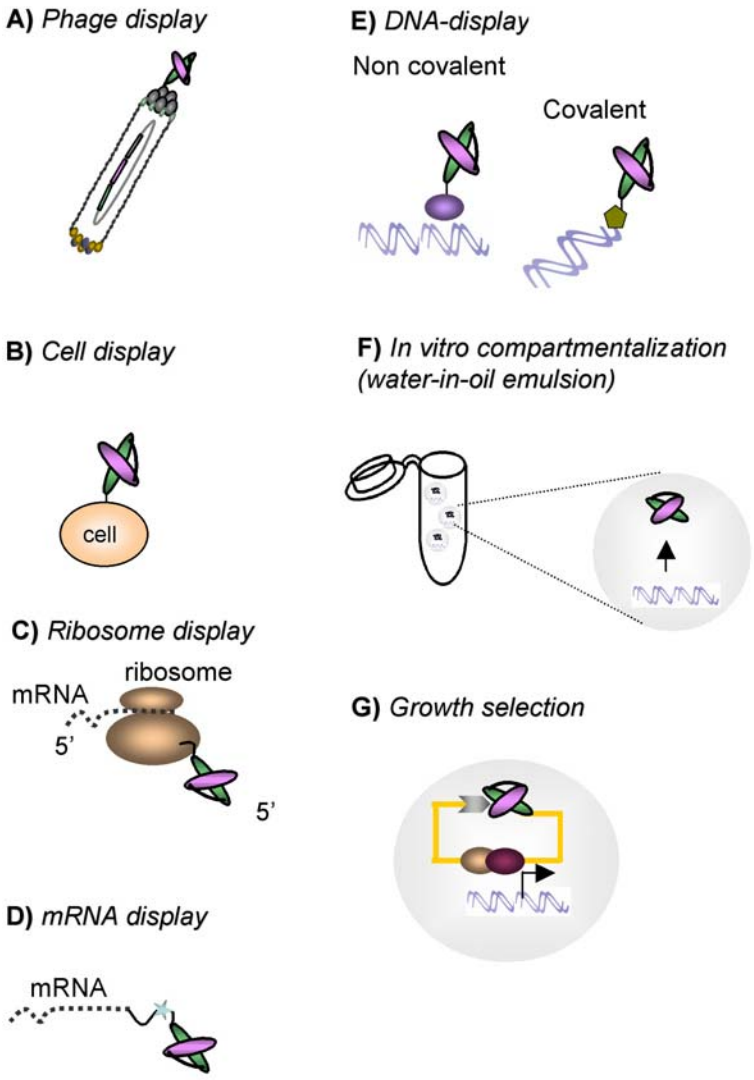

Figure 1. Schematic representation of antibody library selection platforms. Linking the antibody recombinant fragment (phenotype) to its encoding DNA (genotype) allows the selection of specific binders from fully human antibody libraries. This phenotype-genotype link enables the selection of antigen binders followed by the identification of the co-selected gene. A) The antibody fragment is displayed at the surface of the phage particle *via* fusion with a phage coat protein, usually pIII. Selection of the antibody fragment specific to a particular antigen is achieved by multiple cycles of enrichment named biopanning (see text and Figure 2 for details of the phage selection process). B) Antibodies can be displayed on the surface of cells such as bacteria *E. coli* or yeast *S. cerevisiae* and as shown more recently on mammalian cells. Cell display is not well adapted to large library screening but much more appropriate to the maturation affinity process. C) In ribosome display, the genotype-phenotype coupling takes place *in vitro* and is ensured by a non-covalent ternary complex between the nascent protein, ribosome subunits and mRNA. D) The mRNA display uses a puromycine covalent linker directly between the protein and its encoding mRNA. The advantage of this technology compared with ribosome display is that the complex mRNA-antibody fragment is more stable and can be subjected to more stringent washing. E) In non-covalent DNA-display, the DNA-protein linkage is promoted by the recognition of the bacterial RepA protein as well as its own origin of replication sequence integrated into the template DNA. In covalent DNA display a bacteriophage P2 protein genetically fused to an antibody fragment (scFv) binds to its own DNA sequence. This system has not yet been applied to human antibody screening. F) *In vitro* compartmentalization is based on the recent microdroplets technology. Briefly, an aqueous solution of antibody genes library, an *in vitro* transcription-translation system and oil surfactant are mixed to create a water-in-oil emulsion. The emulsion contains microdroplets in which no more than one gene and the corresponding expressed protein are present. G) *In vivo* based growth selection systems regroup the intracellular antibody capture technology (IACT) and the protein fragment complementation assay (PCA). *In vivo* selection procedures are based on a phenotype change (growth and/or colorimetric change) induced by an antibody-antigen interaction.

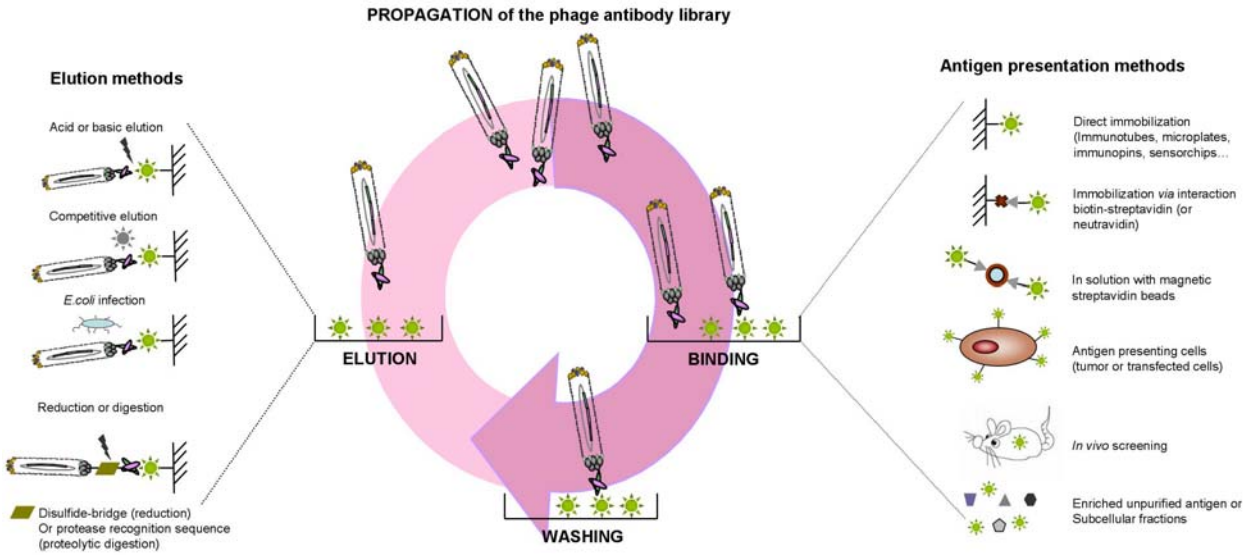

Figure 2. Phage display selection process: principles and methods. The screening of an antibody phage library is an iterative selection process named biopanning and includes many steps : i) the target bound to a solid support or free in solution is incubated with a previously batch-amplified population of unselected phage antibody library, ii) after a period of incubation, the solid support or captured target on beads are washed to remove unbound and unspecific phage antibodies, iii) remaining antibody phages containing enriched specific binders to the target can be released by elution methods (usually acid or basic elution following by a neutralization step) or directly by infecting *E. coli* in exponential growth phase, iv) the last step consists of the amplification of the DNA encoding selected antibodies by infecting *E. coli* cells and subsequent culture to produce the selected populations of phage particles. Due to residual non-specific phages not eliminated by washing, more than one round of panning is needed to permit enrichment of best affinity clones. Different elution methods are described on the left and strategies to present a particular antigen on the right.

one or two rounds of panning (low panning efforts <100 clones) or on many more clones after multiple rounds of panning (medium or high panning efforts >1000 clones) (36, 37).

The full selection process by phage display can nowadays be used in automated screening platforms combining 96-well microplate format and liquid handling robots. The target antigen can be adsorbed onto a polystyrene solid phase surface, immobilized to immunopins in 96-well format or attached to magnetic beads using a magnetic particle processor containing 96 magnetic pins (55). The automated procedure offers the opportunity to select from the human antibody library against many antigens in parallel at medium or high throughput screening levels. This integrated robotic platform associated with data analysis and storage software permit the high throughput selection of the human antibody phage display library and correlate the binding assay data and DNA sequences of each selected hit. Liu *et al*. (2002) developed another strategy, well adapted to the high throughput screening of human recombinant antibodies, to select human phage antibody libraries directly from an antigen blotted onto a membrane rendered resistant to non-specific binding by methanol treatment (56).

Despite the fact that the oldest and still dominant display platform is the monovalent phage display and that most of human antibody libraries have been constructed using this system, the potential of the multivalent system has until now been under-exploited. Indeed, using a multivalent phage display library, O'Connell *et al.* (2002) demonstrated improvement in both display efficiency and antibody selection. Nevertheless, the affinities of the selected antibodies in this case were lower compared with the antibody obtained with the same selection process applied in parallel using antibody monovalent phage display (57).

5.2. Other confirmed display platforms: Yeast and Ribosome displays

Yeast display was first used in affinity maturation experiments of single antibody fragments using off-rate selection (47, 58). Moreover, Boder *et al* (2000) obtained the highest affinity improved scFv variant by a combination of random mutagenesis and yeast display platform (47). One advantage of this selection platform is its compatibility with fluorescence-activated cell sorting (FACS) that permits an accurate characterization of the binding properties without subcloning, expression and purification of the selected clones. Briefly, an epitope tagged antibody fragment is first fused to one of the surface proteins (Aga2) of the a-agglutinin yeast *Saccharomyses cerevisiae* adhesion receptor. The scFv expression can then be monitored by flow cytometry with fluorescent labeled antibodies recognizing the N-terminal HA tag and the C-terminal c-myc tag (59). For the selection, a fluorescent or biotinylated antigen is often used to measure the strength of the antibody-antigen interaction. Until a few years ago, the yeast display was principally used to screen libraries of

modest size due to the limiting transformation efficiency of yeast. However, Feldhaus *et al*. (2003) created a non-immune human scFv library of 10^9 clones and isolated scFv with nanomolar affinity, using magnetic bead and cell sorting screening (17).

As an alternative to phage or cell display, entirely *in vitro* display strategies have been developed to enhance time and efficiency of the selection process. These systems (ribosome display, mRNA display and DNA display; Figure 1) are performed *in vitro* without the needs of transformation and amplification in the bacterial host. Among them, ribosome display is the most advanced system with picomolar affinity antibodies obtained from the HuCAL library using this technology (48). The antibody genes library is transcribed and translated *in vitro* and since the mRNA does not contain a 3'end stop codon, the ribosomes are not released and a complex ternary antibody-ribosome-mRNA is formed. Here, the amplification step is made by PCR on the DNA sequence co-selected with the binding antibodies. Optimizations are generally required, however, to stabilize the ternary complex making this technology more complicated to use than phage display. Despite this drawback, ribosome display offers the possibility to screen very large libraries (in theory over 10^{13} clones) due to the absence of the bacterial transformation step. The possibility of introducing sequence diversification by an error-prone process of reverse transcriptase at each round of selection renders this technology highly equipped to achieve the molecular evolution principle of successive diversity creation and selection. Thus, ribosome display is mainly utilized in the maturation of antibodies with affinities in the picomolar range reached with this selection platform (60, 61).

5.3. Emerging and promising screening strategies

More recently Ho *et al*. (2006) successfully displayed Fv fragments at the surface of mammalian cells (62). The authors argued that problems with protein folding, posttranslational modification and codon usage, inherent to the non-natural antibody environment of conventional display on phage, bacteria or yeast, limit the number of improved antibodies that can be obtained. To overcome these limitations, the human embryonic kidney 293T cells used in this study maintained the antibody fragments Fv in their natural, mammalian cell environment. By mixing the anti-CD22 scFv and its 2-fold improved affinity variant HA22 at a ratio in disfavor of the high affinity clone, they found a 240-fold enrichment for the best affinity scFv in a single-cell sorting selection round. Furthermore, by using this mammalian cell display system associated with mutagenesis at hotspot positions, they succeeded in obtaining a novel improved affinity clone of the scFv anti-CD22. However, the proof-of-concept of this new technology needs to be completed by a success in human antibody library screening.

A promising approach permits the screening of full length IgG antibodies from combinatorial libraries on bacterial display (63). The laboratory of Georgiou and colleagues has adapted their previously described anchored periplasmic expression (APEX) technology (64) with the production of IgG in bacteria. The clever idea was to capture the secreted IgG by a Fc-binding protein that was anchored in the inner membrane. Several antibodies with nanomolar affinities were selected from a mice immune library (10^7 clones) using flow cytometry. The direct selection of full length immunoglobulins allows to avoid the modification of binding properties often observed when antibody fragments are produced as full IgGs. It will be very interesting in the future to transfer human antibody libraries in this new technology and compare with the established display methods.

Different to the systems described above, another strategy of *in vitro* selection recently appeared on the scene of protein engineering. The *in vitro* compartmentalization technique provides an alternative way of directly linking genotype and phenotype by mimicking the compartmentalization that naturally occurs in living cells. Here, the protein and its encoding DNA are present within a water-in-oil emulsion ensuring genotype-phenotype coupling. In each aqueous droplet, a single gene is transcribed and translated to give multiple copies of the protein. Combined with microfluidic techniques and detection systems such as FACS, genes encoding proteins with desired binding characteristics could in theory be selected from large pools of genes (65). This promising technology has not yet been tested with a human antibody library however a publication reporting the first screening of a fully human antibody library is not far off.

5.4. Antibody Array

A different strategy to isolate binders to any antigen is to use screening methods in which the phenotype-genotype linking is not a requirement. In line with this, different versions of antibody array formats and methods have been developed. Sharing the common feature of immobilized antibody libraries on a solid support, such antibody arrays and microarrays can be applied to identify different patterns of expressed proteins on various sample types such as bodily fluids (serum, plasma, interstitial fluid), cell culture supernatants (secreted proteins), or resected tumor tissue (66). Nevertheless, not all antibody fragments remain functional in the microarray format and the sensitivity is dependent on the type of coating and its application.

By using antibody array in which the antibody clones are blotted to a filter the library can be screened against several antigens in parallel with a filter enzyme-linked immunosorbent assay. The Tomlinson group at the MRC proposed a high throughput method of screening antibody libraries by using robotic picking and high density gridding of bacteria producing antibody fragments followed by filter based enzyme-linked immunosorbent assay. They created high density antibody arrays based on a scFv library and obtained specific scFv fragments against a single antigen (recombinant ubiquitin), mixtures of three unpurified bacterial lysates containing three recombinant proteins and whole Hela cell extract (67). Another solution is to spot scFv from a library onto a chip surface by affinity tag means. The Borrebaeck's group demonstrated this concept using the n-CoDeR library as a source of

recombinant scFv with all individual scFvs built around one single and stable framework. They genetically fused these scFv probes to C-terminal affinity tags (his or myc-tags) and immobilized them to Ni2+ coated slides or pre-arrayed monoclonal anti-tag antibodies obtaining highly functional specific and sensitive microarrays with picomolar and femtomolar detection range (68). More recently the same group used an antibody array composed of 127 different known scFvs from the n-CoDeR library and performed comparative analysis of malignant and normal stomach tissue to identify a protein expression signature associated with *Helicobacter pylori* infected patients (69). Similarly, a cellulose binding domain (CBD) was also used as an affinity tag to coat scFv-CBD fusion fragments on cellulose-coated glass slides (70). This antibody microarray based technology associated with on-chip efficient antibody libraries should become an unavoidable proteomic technology to perform global proteome analysis in future years.

6. CONCLUSION AND PERSPECTIVE

Although the principle of using monoclonal antibodies as magic bullets in clinical use was envisaged many years ago, the true potential of monoclonal antibodies have only now started being exploited. Indeed following decades of disappointment, antibodies are finally emerging as viable therapeutic drugs. The ability to manipulate the genes encoding human variable regions has been successfully employed in the construction of fully human recombinant antibody libraries leading to the first fully human antibody approved by the FDA (Humira) in 2003. Since the nineties, many types of human recombinant libraries have been created with different schools of thought: *i)* to mimic the immune system with a two step process, the isolation of one or more binder leads (according to the primary immune response) followed by an optimization step (as with affinity maturation); or *ii)* to surpass the immune system by direct isolation of high affinity binders, with one drawback of needing high quality and very large libraries. Another dilemma concerns the framework scaffolds and the preferable option, either i) the use of natural diverse framework repertories (naive and/or immune libraries) or ii) the use of a single stable and well expressed framework associated with CDR design (semi-synthetic or fully synthetic libraries). These questions remain unanswered as different strategies have proven to be successful with advantages/disadvantages of natural repertoires *versus* synthetic libraries proving to be opposite and reciprocal.

The recent development of non-immunoglobulin scaffold libraries has also permitted the development of robust binders with high-affinity interactions (for review, see Binz *et al.* 2005) (71). Monomeric forms of these scaffolds, free of cysteines, seem to resolve some of the antibody fragment problems (stability, aggregation, production). However, this promising alternative approach is at an early stage of research and these scaffolds must be confronted with issues such as human immunogenicity and lack of effector function.

The different screening processes have shown impressive development and allow the isolation of binders from antibody libraries with higher complexities. However of all the different examples of library screening, not one clearly shows a better strategy. Moreover, although theoretically easy to use, in practice a fine tuning of the selective pressures used in the screening process is critical to ensure that “you get what you are looking for”. Mastering the screening conditions appears to be the key to isolating antibodies with desired properties.

In the near future, the development of robust high throughput discovery platforms should have major repercussions on human antibodies designed for optimal function with regards to disease and immunogenicity criteria. It will be very interesting to observe the outcome of the antibodies isolated from human libraries that are at present in preclinical and clinical trials.

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Abbreviation: VH: variable heavy chain; VL: variable light chain; Vk: variable kappa chain; Vl: variable lambda chain; CDR: complementary determining region; scFv, single chain fragment variable; Fab: antigen-binding fragment; SHM: somatic hypermutation; ;PBL: peripheral blood lymphocyte, GL: germline gene, BM: bone marrow; MRC : Medical Research Council, UK ; CAT : Cambridge Antibody Technology; FDA: Food and drug administration

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