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Monoclonal and recombinant antibodies, 30 years after. . .

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Abstract. In 1975, the hybridoma technology provided, for the first time, an access to murine monoclonal antibodies. During the two following decades, their high potential, as laboratory tools, was rapidly exploited, but *in vivo* applications were still very limited. Nowadays, antibodies, omnipresent in both diagnostic and research domains, are largely invading the domain of therapy. A wide array of novel technologies, including phage display and transgenic mice, to isolate fully human antibodies and engineer these molecules, has been implemented. The natural propensity, of the antibody molecules, to metamorphosis makes them an ideal response to new applications and therapeutic challenges. The present review is a tentative update of the different antibody "formats" and a walk through the techniques recently applied to antibody engineering. In addition it also addresses some specific issues such as the development of expression systems suitable for large-scale production of recombinant antibodies.

Keywords: Recombinant antibodies, engineering, immunotherapy, phage display, expression system

1. Introduction

As much as eighteen antibodies have been, to date, approved by the US Food and Drug Administration (FDA) for various therapeutic applications (Table 1): three murine, five chimeric, nine humanized and one human. Antibodies and their derivatives account for more than 30% of all biopharmaceuticals under current development. As a result of growth in existing markets for mAbs therapeutic and the opening of new ones, the global market is projected to increase to US$ 16,7 billions in 2008 [1,2]. Moreover, at least 130 mAbs are currently under clinical trials, including 55 humanized Abs and 37 fully human products. Additionally, new technologies including conjugated Abs and antibody fragments are expected to rise in importance (currently 19.7% of the antibody clinical pipeline) [2]. Monoclonal antibodies are set to play a significant role in the treatment of a wide number of indications in various therapeutic domains, such as oncology [3–5], asthma, transplant rejection, inflammatory, autoimmune, cardiovascular [6,7] or infectious diseases [8]. In cancer therapy, the goal is to induce, upon antibody administration, the direct or indirect destruction of cancer cells, by specific targeting the tumour (Rituximab®) or the vasculature that nourishes the tumour (Avastatin®). Numerous anti-inflammatory antibodies already on the market (Remicade®, Humira®) or under clinical trials (IDEC C9.1) have also shown the great potential of mAbs. Although the recombinant antibody technology allow a large diversity of potential applications such as sensitive detection (piezoimmunosensors) [9], prevention of dandruff [10], or removal of environmental contaminants [11]), this review will focus on the therapeutic potency of mAbs and their derivatives, illustrated by innovations progressively and continuously introduced during the past 30 years.

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2. Antibody engineering: A quest for humanity (**chimerization, humanization, de-immunization**)

In 1975, Georges J.F. Köhler and César Milstein developed and described the hybridoma technique for the generation of monoclonal antibodies [12] (Fig. 1), consisting in immortalizing mouse antibody-producing cells through fusion with tumour cells. Mouse hybridomas were the first reliable source of monoclonal antibodies (mAb), facilitating the large-scale production of murine mAb (Mumab) generated against any antigen. These first generations of murine mAb, as therapeutic agents, suffered a number of drawbacks: short serum half-life, insufficient activation of human effector functions and development of human anti-mouse-antibody (HAMA) responses in patients, especially when repeated administrations were necessary [13]. These problems have recently been overcome, to a large extent, using genetic engineering techniques. Different strategies have been developed to avoid, mask, or redirect this human immune response: 'chimerization' by fusion of mouse variable regions to human constant regions [14], 'humanization' [15] and 'de-immunization' by removal of T-cell epitopes [16,17] (Table 2).

2.1. Chimerization

In chimeric antibodies, the murine constant regions are replaced with human equivalent regions, on the basis that the constant region contributes a significant component to the immunogenicity. In addition, this allows for a better interaction with human effector cells and the complement system. This strategy led to therapeutic successes such as basiliximab (Simulect®: IgG1 anti-CD25, Novartis) or cetuximab (Erbitux®: IgG1 anti-EGFR, ImClone). Chimeric antibodies were perceived as less foreign, and therefore less immunogenic than mouse monoclonal antibodies, nevertheless human anti-chimeric antibody responses (HACA) have been observed (for example with infliximab/Remicade® [18]).

2.2. Humanization

In such a context, antibody humanization technology was developed, and made possible by the transfer of all xenogeneic CDRs (complementary determining regions) onto the framework of a human Ab. Different approaches of CDR-grafting have been tested according to the human template used as scaffold: sequences with known crystal structure [19–21], rearranged somatic sequences, unmodified germline sequences [22] or consensus sequence [23].

The first humanized Abs were constructed based on human sequences with known crystal structure, which permits the identification of residues contributing to the antigen binding. In the "best fit" strategy, the closest human sequence, usually rearranged, is used as a framework to receive the murine CDRs. Another approach for humanizing an Ab is to choose the closest human germline sequence [22]. Indeed, human Ab genes are formed *in vivo* by rearrangements of germline gene segments. Only later in B cell ontogeny, occurs a hypermutation process, tailoring the initial sequences to improve recognition of specific Ag. The body thus may be more tolerant to germline-encoded Abs. Also, clinical problems would be reduced if germline sequences were used for constructing humanized Abs. The consensus method utilizes variable light (V_L) and variable heavy (V_H) domain frameworks derived from the most common amino acid found at each position within a given human subgroup. Whatever the method, CDR-grafting might not result in the complete retention of antigen-binding properties because some framework residues can interact directly with antigen [24], or affect the conformation of CDRs loops [21]. In this case, the antibody must be engineered to fine tune the structure of the antigen-binding loops to restore high affinity. In the other hand, humanization of a xenogeneic Ab does not necessarily abolish the immunogenicity of the molecule all together, since the humanized Ab can still induce response against its xenogeneic CDRs. Not all residues within the CDRs of an Ab are essential for binding to its Ag. In fact, the Ag-binding site of an Ab usually involves only 20–33% of the residues [25]. These residues have been designated as specificity determining residues (SDRs) [26]. Therefore, a murine Ab can be humanized through grafting only restricted to its SDRs onto the human template, minimizing the immunogenicity [27].

Another strategy of humanization, termed resurfacing, was proposed by Padlan (1991) and involves the replacement of solvent exposed murine framework residues in the variable regions with human residues [28–30]. Finally, 'Guided selection' is a process that transfers the specificity of a murine mAb to novel human mAbs by creating a hybrid library of the murine heavy chain and random human light chains, then the selecting for binding antibodies and repeating the process with the human light chain isolated and a library of human heavy chains. Adalimumab (Humira®)

Table 1
FDA-approved recombinant antibodies by august 2005

Year	Product Name	Target	Product type	Technology	Indication	Developer	DCI
1986	Orthoclone OKT®3	CD3	Murine IgG2a	Hybridoma	Transplant rejection	Ortho Biotech	muromonab-CD3
1994	Reopro®	GpIIb/gpIIIa	Chimeric Fab		Cardiovascular disease	Centocor/Eli Lilly	abciximab
1997	Rituxan®	CD20	Chimeric		Non-Hodgkin lymphoma	Biogen-IDEC	rituximab
1997	Zenapax®	CD25	Humanized IgG1		Transplant rejection	Protein Design Labs	daclizumab
1998	Remicade®	TNF-α	Chimeric IgG1		Crohn disease Rheumatoid arthritis	Centocor	infliximab
1998	Simulect®	CD25	Chimeric IgG1		Transplant rejection	Novartis	basiliximab
1998	Synagis®	RSV	Humanized IgG1		Respiratory syncytial virus	Medimmune	pavilizumab
1998	Herceptin®	Her-2	Humanized IgG1		Breast cancer	Genentech	trastuzumab
2000	Mylotarg®	CD33	Humanized IgG4 toxic conjugate		Acute myeloid leukaemia	Wyeth	gemtuzumab
2001	Campath®-1H	CD52	Humanized IgG1	Reshaping	B-cell chronic lymphocytic leukaemia	ILEX Pharmaceuticals	alemtuzumab
2002	Zevalin®	CD20	Murine		Non-Hodgkin lymphoma	Biogen-IDEC	ibritumomab tiuxetan
2002	Humira®	TNF-α	Human IgG1	Phage display guided selection	Rheumatoid arthritis	Abott	adalimumab
2003	Bexxar	CD20	Murine-iodine conjugate		B cell non-Hodgkin's lymphoma	Corixa	tositumomab
2003	Xolair	IgE	Humanized IgG1	Phage display	Asthma, allergy	Genentech	omalizumab
2003	Raptiva®	CD11a	Humanized IgG1		Psoriasis	Genentech	efalizumab
2004	Erbitux	EGFR	Chimeric IgG1		colorectal cancer	ImClone	cetuximab
2004	Avastatin	VEGF	Humanized IgG1	CDR grafting directed mutagenesis FR	colorectal, breast, lung cancer	Genentech	bevacizumab
2005	Tysabri	CD40	humanized IgG4k		Multiple sclerosis	Elan /Biogen	natalizumab

Abreviations: TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor, EGFR: epidermal growth factor receptor, RSV: respiratory syncytial virus.

is the first phage-display-derived human antibody and was generated by "guided selection" starting from a murine monoclonal antibody [31,32].

Despite the reduction of proportion of murine sequences, the level of the immunogenicity of humanized Abs range from negligible to intolerable. The humanized anti-HER2/neu Ab Herceptin(gave as low as 0.1% of HAHA in breast cancer patient [33] while the humanized A33 Ab elicit 49% of HAHA among colon cancer patients treated with this Ab [34]. This immunogenicity might result from human anti-human antibody (HAHA) responses, in particular to the idiotype [35].

It will be interesting to observe the immunogenicity of fully human antibodies in therapeutic settings. To date, it is difficult to determine whether "fully human" mAbs are less immunogenic than humanized mAb as full immunogenicity data are available for only one mAb developed from phage-displayed libraries (Humira®).

Finally, anti-allotype reactions are predicted to occur during therapy of a genetically diverse population with a single antibody reagent.

2.3. Deimmunisation

As an alternative, Biovation (UK, www.biovation.co.uk) has developed the DeImmunisation(technology consisting of identification and removal of T helper (Th) cell epitopes from antibody [16,17]. Two products of DeImmunisation(are actually under clinical trials: J591, a modified antibody binding to prostate specific membrane antigen (PSMA) and the radiolabelled antibody Tromboview.

2.4. Primatized Abs

Another method for obtaining antibodies that are closely related to human Ab involves the use of non-human primates. Primate antibodies are more similar in sequence to human antibodies than are murine antibodies and are less susceptible to be immunogenic in humans. Indeed, the gene segments of macaques are as closely related to human immunoglobulin genes than human genes to each other [36,37].

Three primatized Abs directed against human antigens [IDEC C9.1, IDEC 114 and IDEC 151] are cur-

Table 2
Immunogenicity of engineered antibodies

AAR	Murine Ab	Chimeric Ab	Humarized Ab
Marked	84%	40%	9%
Tolerable	7%	27%	36%
Negligible	9%	33%	55%

According to Hwang et al. [214], Ab's immunogenicity was classified as 'negligible' if detectable in less than 2% of patients, as 'tolerable' if detectable in 2–15% of patients and as 'marked' if present in more than 15% of patients.
Abbreviations: AAR: anti-antibody response, Ab: antibodies.

rently undergoing clinical trials (Biogen Idec Inc., Cambridge, MA) and preliminary results do not show any anti-primate antibody response [38]. In addition, Biogen Idec is developing lumiliximab, a primatized anti-CD23 macaque/human chimeric antibody that inhibits the production of the IgE antibody, for the potential treatment of allergic conditions [39].

3. Fully human antibodies

Monoclonal antibodies of human origin may have greater therapeutic value, thus several methods have been developed to generate human mAb (Humab): selection from human hybridomas, from transgenic mice or from *in vitro* libraries.

3.1. Human B cell hybridoma

The recovery of stable human B cell hybridoma producing high-affinity IgG mAbs is rarely achieved, lacking of a suitable human myeloma cell line. The best results are obtained using heteromyelomas (mouse x human hybrid myelomas) as fusion partners. However, the mouse-human heteromyelomas that have been used for fusion with human lymphocytes are often unstable. As an alternative, human antibody secreting cells can be immortalized by Epstein-Barr virus (EBV) infection. However, EBV-infected cells are somewhat difficult to clone, and usually produce only very low yields of immunoglobulin [40]. Recently, an improved method of B cell immortalization by EBV involving the addition of a polyclonal B cell activator (CpG) [41] has been described.

The final argument restricting the human B cell hybridoma approach is that human circulating antibody repertoire does not generally contain specificity to "self" proteins, which represent the majority of target for human antibody therapeutics.

3.2. Mice producing human antibodies

3.2.1. Humanized SCID and TRIMERA mice

Another approach for generating human mAbs consist of transplanting a functional human immune system into immunodeficient mouse strains, such as Severe combined immunodeficient (SCID), SCID-bg, Trimera or $\gamma c^{-/-}$/RAG2$^{-/-}$ mice.

Severe combined immunodeficient (SCID) mice, lacking mature T and B cells and virtually devoid of endogenous serum immunoglobulins, can be successfully reconstituted with human peripheral blood lymphocytes (PBLs). Such mice reconstituted with a competent human immune system would represent an invaluable tool for producing large amounts of human immunoglobulins, after immunization with antigen. However, the use of SCID mouse can be limited by shortened lifespans, spontaneous production of functional lymphocytes with ageing, and residual innate immunity leading to variable levels of engraftment. Natural killer (NK) functions in particular would be detrimental to engraftment of human lymphoid cells [42]. For example, depletion of NK activity in SCID-bg mice facilitated engraftment of human PBL from anthrax-vaccinated (AVA) donors. Stable recombinant cell lines producing human monoclonal antibodies were generated by hybridoma formation and human anti-PA neutralizing mAb of high affinity were obtained [43]. Other immuno-deficient strains of mice were developed, for instance the $\gamma c^{-/-}$/RAG2$^{-/-}$ mice. In addition to T- and B-lymphocyte deficiency, $\gamma c^{-/-}$/RAG2$^{-/-}$ mice are completely deficient in NK activity [42].

Alternatively, the Trimera mouse has been developed through a three-step process [44]. Firstly, a normal mouse host is rendered immuno-incompetent by a lethal split-dose total body irradiation. Secondly, the myeloid and erythroid lineages are reconstituted by transplantation of bone marrow cells from a genetically immune-deficient mouse donor. Thirdly, the resulting preconditioned mouse is transplanted with human cells that can be maintained in the host. Even if Trimera mouse was considered as a potential source of monoclonal antibodies, its use has been, so far, restricted to a model for investigation of human diseases.

3.2.2. Transgenic mice

The availability of transgenic strains of mice expressing human Ig genes (XenoMouse®, HuMab^{TM} Mouse, Transchromo Mouse) provides an alternative method for isolating human mAbs [45]. Rearrangement and hypermutation occurrence was an indication

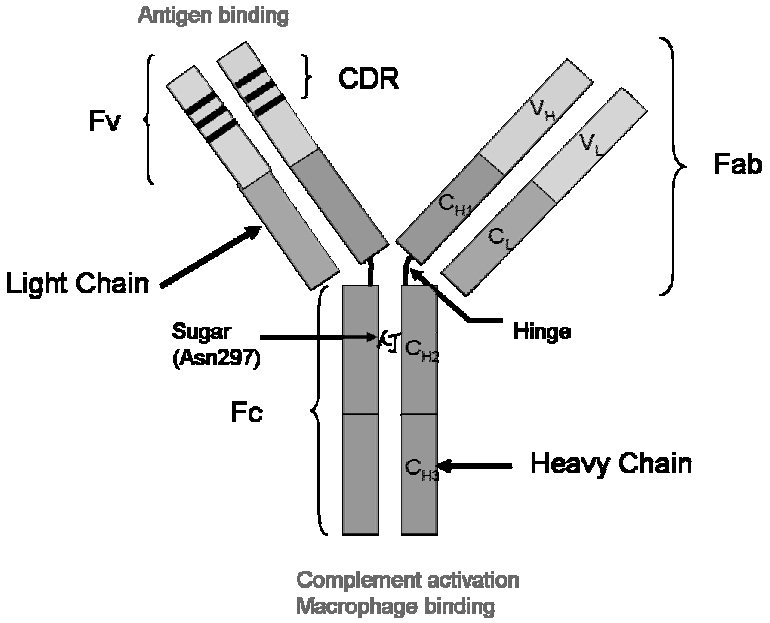

Fig. 1. Schematic representation of an immunoglobulin G (IgG). An IgG is composed of two identical light chains (each composed of two domains, labelled V_L and C_L and two identical heavy chains (each composed of four domains, labelled V_H, C_{H1}, C_{H2} and C_{H3}). Antigen-binding surface is formed by variable domains of the heavy (V_H) and light (V_L) chains. Effector functions, such as complement activation and binding to cytotoxic cells, are mediated by the Fc region.

that the endogenous cell signaling machinery of mice was compatible with human immunoglobulin sequence elements [46] and would constitute a marked advantage.

XenoMouse® strains, developed by Abgenix (Fremont, CA), were engineered by functionally inactivating the murine heavy chain and κ light chain Ig loci and incorporating megabase-sized inserts of human DNA (YAC) carrying Ig heavy chain and κ light chain loci that express the vast majority of the human Ab repertoire [47,48]. Three different strains of XenoMouse® mice have been produced, constrained to class switch from IgM to IgG1, IgG2 or IgG4. Recently, the immune repertoire of XenoMouse® strains has been increased by the introduction of the entire human Igλ locus [46]. To date, results obtained in preclinical and early clinical trials with human antibody from Xenomouse® mice confirm their lack of immunogenicity [49,50]. However, human antibodies from mice can be distinguished from human antibodies produced in human cells by their state of glycosylation, particularly with respect to their Galα1-3Gal residue [51]. This carbohydrate residue is widely distributed among non-primate mammals. Anti-Gal antibodies are produced in humans throughout life, as approximately 1% of circulating Ig [52]. Thus, antibodies bearing that residue would probably be subject to rapid immune clearance [53].

As an alternative strategy, human minichromosomes (derived from human chromosome 2 and 14) containing the complete germline clusters for heavy and κ light chains were introduced into mice, called Transchromo mice [54,55]. These mice, developed by the Kirin Brewery Company (Japan), are capable of producing every subtype of fully human Ig, including IgA and IgM. However, the instability of the transchromosome carrying the Igκ locus was particularly detrimental, as hybridoma production was less than 1% of that seen in wild type mice. An instant solution to this problem was to cross-breed the Kirin TC mouse carrying the human chromosome fragment 14 (locus IgH), with the Medarex YAC transgenic mouse carrying about 50% of the Igκ locus. The resulting mouse (KM) performed as well as normal mice with regard to immune responsiveness and efficiency of hybridoma product [55].

3.2.3. Antibody display

An approach considered as a breakthrough for producing fully human antibody is the selection from antibody gene repertoires expressed either *in vivo* on the surface of cells or filamentous bacteriophages [56,57], or *in vitro*. These strategies of selection of antibodies from large antibody repertoire depend on the linkage between genotype and phenotype, enabling recovery of the DNA encoding the selected antibody fragment.

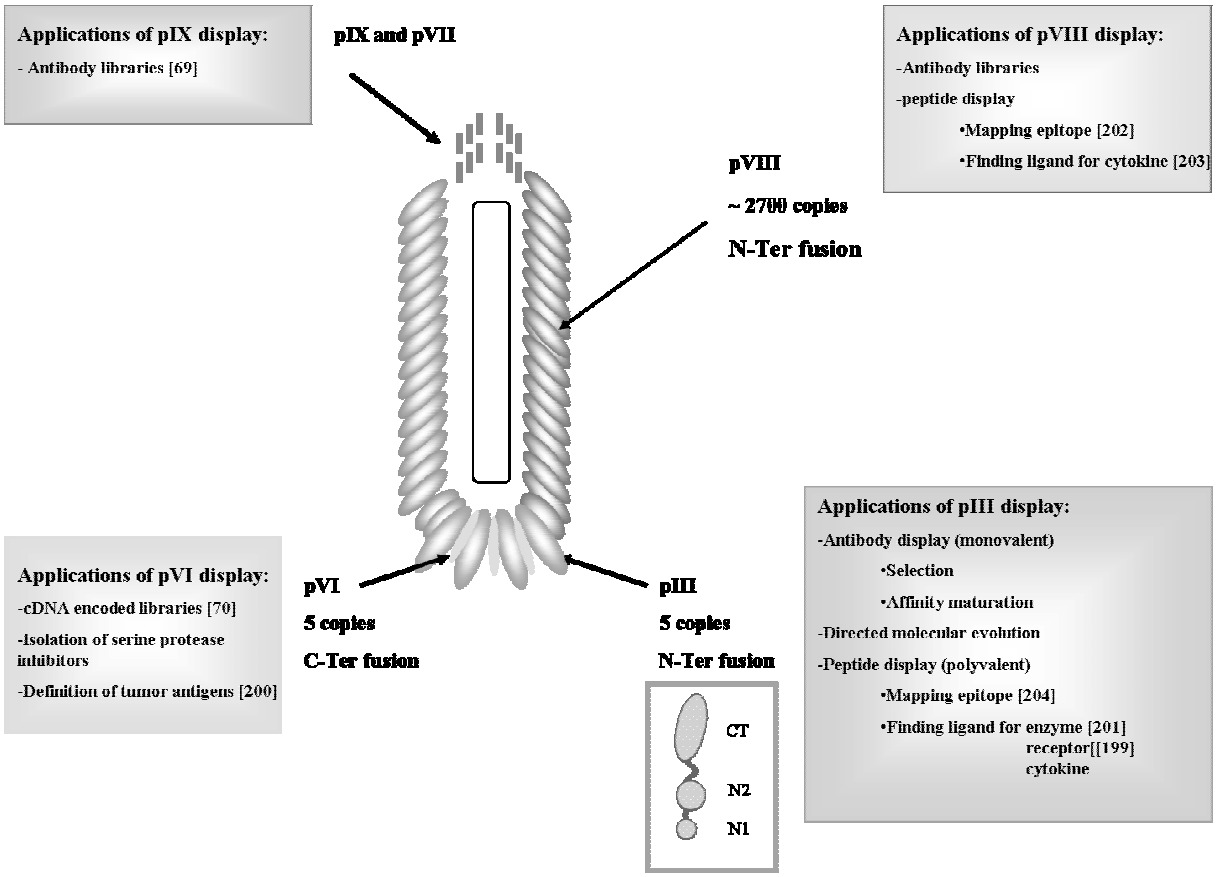

Fig. 2. Illustration of various M13 phage display applications. The large diversity of display formats of peptide or protein has allowed various applications as illustrated in this figure, including selection of high affinity antibodies, directed molecular evolution, screening of cDNA libraries, mapping epitope, and finding ligand for cytokine, enzyme or receptor.

3.2.3.1. Phage display

This strategy is currently the most widely used and well-established technique for antibody display and library screening [58]. Large repertoires of scFv or Fab antibody genes are cloned into phagemid vectors as a fusion product to one of the phage coat protein genes. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody display on the phage surface. During the screening procedure generally termed "biopanning", phages that display a relevant antibody will be retained on a surface coated with antigen while non-specific phages will be washed away. Multiple rounds of panning are made possible and necessary to select the best binders.

Naïve, Immune and synthetic repertoires

The different types of antibody libraries are distinguished by the source of their repertoires: naïve, immune or synthetic. Naïve libraries are constructed by cloning the antibody variable domain genes from non-immunized donors [59,60]. Since the description of the first antibody libraries, many efforts have been done towards the obtaining of universal large sized repertoires. The theoretical justification for large repertoires was that it should be possible to isolate, from a large repertoire (10^{10}–10^{11}), antibodies with nanomolar affinities against any antigen [61,62]. The key factor limiting the obtaining of very large repertoires is the bacterial transformation step, inherent to the library construction. Even if techniques based on so called 'combinatorial infection' [63,64] have provided an elegant means of breaking this technological barrier, the vast majority of, so far described, 'naïve' repertoires, with a complexity over 10^{10} clones, have been assembled through the massive and tedious accumulation of small-sized sub-repertoires [60].

As a definition the complexity of a given library is the overall number of different VH–VL combinations obtained at the DNA level. But the experience has shown that there is a significant difference between the encoded diversity and the displayed diversity for differ-

ent reasons such as toxicity upon bacterial expression, non-proper folding or assembly, competition between wild type and antibody-linked gene III molecules, proteolysis of the displayed moiety, and many others.

Lastly, it became obvious that the major problem was not the assembly of a large repertoire but rather its maintenance over time and the retardation of the ineluctable drift of its content. This clearly explains that the current tendency is rather focused on smaller or biased repertoires obtained from immunized subjects [60]. An immune library has two main characteristics: it will be enriched in antigen-specific antibodies and some of these antibodies will have undergone affinity maturation by the immune system.

Nevertheless, some examples of universal large sized synthetic [62,65] and semi-synthetic [66] repertoires are available. They are based on the use of a limited number of universal frameworks selected for their capacity to be over-expressed in *E. coli*. According to the technique employed, CDRs are totally synthetic or derived from a pool of naturally expressed ones.

Display formats and optimization

Display method have been developed based on fusion to the C-terminus of the λ phage tail protein pV, or both the N- and C-terminus of the capsid D protein, which is part of the phage head. Further double stranded DNA phage display systems have been described using bacteriophages T4 [67] or T7.

The filamentous bacteriophage M13 has been by far the platform of choice for antibody display (Fig. 2). Although M13 coat protein pIII and pVIII [68] display formats have been the most instrumental in the construction of antibody libraries, alternative phage display systems have been reported, like those based on the minor coat protein pIX [69] or pVI [70]. Because the success of phage display depends not only on the diversity of the library at the DNA level, but also on the efficiency with which the encoded proteins are displayed on the phage surface, extensive studies of M13 assembly and structure have enabled improvements in phage display technology [71]. Indeed, phage selections often suffer from the amplification of non-specific binding molecules. In order to alleviate this problem, three groups have developed a system coupling the binding of a displayed peptide or protein to its target with the amplification of the displaying phage: selectively infective phage (SIP) [72], selection and amplification of phage (SAP) [73], and direct interaction rescue (DIRE) [74]. The 'selectively infective phage' technology (SIP) exploit the modular structure of the phage protein pIII, which consists of three domains: N1, N2 and CT. The N-terminal N1 domain is absolutely essential for *E. coli* infection, while the CT domain is absolutely essential for phage morphogenesis. [75]. Thus, SIP consists of two components: (i) a phage particle made non-infective by replacing its N-terminal domains of pIII with an antibody fragment and (ii) an "adaptater" molecule in which the antigen is linked to N-terminal domains. Infectivity is restored when the displayed protein binds to the ligand. Consequently, phage propagation is strictly dependent on the protein-ligand interaction [76].

A vector system allowing for the display of bivalent Fabs fused to leucine zippers on phagemid virions has been reported by Lee et al. [77]. The "bivalent display" format is a way to effectively mimicking the binding avidity of natural antibodies and greatly reducing the off-rate for phage bound to immobilized antigen.

3.2.3.2. Yeast Display, bacterial display, Anchored periplasmic expression (*APEX*)

A complementary approach utilizes the display of antibody libraries on the surface of bacteria [78] or yeast cells, most commonly *Escherichia coli* and *Saccharomyces cerevisiae*. The display of Ab on the surface of bacteria is not only an alternative expression system for the screening of binders from libraries, but opens new potential applications, like the delivery of passive immunity to mucosal body surfaces [79] or whole cell catalyst [80]. Unlike phage, the relatively large size of bacteria and yeast allows screening by flow cytometry [81].

Recently, a protein library-screening technology based on anchored periplasmic expression (APEX) has been developed [82]. In this method, proteins are anchored on the periplasmic face of the inner membrane of *E. coli*. After disruption of the outer membrane by tris-EDTA-lysozyme, the inner membrane-anchored proteins bind fluorescent ligands, allowing screening by flow cytometry.

Baculovirus displaying antibody moieties such as scFv fragments have been constructed for targeting specific cell types in the gene therapy field. But as for today the baculovirus system was not considered as an attractive choice for the construction of antibody libraries [83].

3.2.3.3. In vitro display technologies

One limitation of the *in vivo* selection systems is the library size that could be generated. The efficiency of transfer of DNA into cells often limits the library size to 10^9–10^{10} members. In addition, selection in the

Table 3
Recombinant antibody fragments

Ab fragments	Composition	Features	Properties	Applications
dAb	V_H or V_L	PM: 11-15 kDa • Llama single domain Ab fragment (VHH) [114] •NAR [117-118]	Expression at high level in microbial culture Solubility, temperature stability, high stability under denaturing conditions that far exceed those of IgG, Fab or scFv [10]	• *In vitro* selection (phage display) and affinity maturation • Can be formatted into larger molecules to create drugs with prolonged serum half-life • Immunocytochemical, immunohistochemical, immunoblot analysis [205] • Intrabodies
Fv	V_HV_L	PM: 25-30 kDa	Rapid blood clearance Better tumor penetration	
scFv	V_HV_L	Stabilized by a linker $(Gly_4Ser)_3$ [211]	More stable or higher affinity for the antigen	
dsFv	V_HV_L	Stabilized by disulphide bridges [93]		
Fab	$(V_{H1}+C_{H1})$ (V_L+C_L)	PM:50 kDa	Better stability than scfv Prolonged circulating half life *in vivo*	Immunotherapy (eg Reopro®)
Fab-PEG			Prolonged circulating half-life Reduced immunogenicity Increased solubility and resistance to proteolysis [105]	Immunotherapy
Diabody Triabody Tetrabody		Reducing the linker length results in the formation of dimers, trimers or tetramers [90]	Incresased avidity high functional affinity	
$(scFv)_2$	scFv-scFv	(scFv)2 produced using disulphide bridges or by joining the two scFv fragments with a third polpypeptide linker	• Cross-linking tumor and effector cells ⇨	• Redirect cytotoxic immune effector cells for enhanced killing of tumor cells by ADCC [207]
scDiabody Tandem diabody		The formation of either a scDiabody or a tandem diabody depends on length and aminoacid composition of the linkers [206]	Drawbacks: Short half-life, Poor accessibility to tumor sites	• Enrich the tumor/normal tissue localization ratio of chemo or radiotherapeutic agents [215]
Di-Minibody		scFv fused with amphipathic helixes [100], immunoglobulin CH3 domains [101], a 'knobs into holes'device, a barnase-barstar couple [103]		• Target two tumor-associated antigens for down-regulation of multiple distinct cell proliferation/survival pathways [208]

context of the host environment (*E. coli*) could lead the loss of potential candidates due to their growth disadvantage or even toxicity for *E. coli*. The most popular *in vitro* display technologies are ribosome display [84] and mRNA display [85] based on an original idea from G. Kawasaki (US patent n° 5,643,768 and US patent n° 5,658,754). These *in vitro*-based antibody selection methods have proven to be successful in the construction and selection of libraries with a high diversity and complexities (potentially up to 10^{14} members). Inherent characteristics of the *in vitro* systems could obviously be turned into advantages:

– More easily amenable to an automated process,
– The RT-PCR step between screening rounds can be performed according to error prone conditions, thus generating an additional degree of diversity [86,87].
– An *In vitro* process is more likely to tolerate screenings under non-physiological conditions such as elevated temperature or a highly denaturant environment [87].

Other potential *in vitro* systems for antibody display are covalent antibody display (CAD) and polysome display [88]. In the covalent display technology, a protein is fused to P2A, a bacteriophage DNA-nicking protein that covalently binds its own DNA and thereby be subjected to selection regimes similar to those for phage display [89]. The polysome display is a modified ribosome display method, by exploiting the interaction between a tandemly fused MS2 coat-protein (MSp) dimer and the RNA sequence of the corresponding specific binding motif, C-variant [88].

4. Antibody design (Table 3)

4.1. Antibody fragments

For many therapeutic applications, the antibody activity (as cytokine inactivation, receptor blockade or

Table 3, continued

Ab fragments	Composition	Features	Properties	Applications
Tetra-Minibody	• MoaFv [95]: the CH1 and CL domains of the Fab fragment replaced with heterotetrameric molybdopterin synthase (MPTS). • scFv-streptavidin: fusion of scFv with streptavidin [209] • Fusion with the tetramerization domain of p53 [216]			
Immunotoxins	Plant toxins: ricin A chain [213], saponin Bacterial toxins: *Pseudomonas* exotoxin [212], cholera toxin	Potential immunogenicity of non-human-derived toxins		Tumor cells killing [104] (eg Mylotarg®)
Ab-drug	Doxorubicin Calichaemicin	Potential lost of antibody reactivity upon conjugation		Targeted delivery of drugs to a tumor site
Ab-radionuclide	Yttrium-90 (ex: Zevalin®) Iodine-131 (ex: Bexxar®)			Immunoscintigraphy Radio-immunotherapy (RIT)
Immunocytokines	A variety of cytokines (IL-2, IL-12) linked to full length Ab or scFv fragment [4]			Activation of anti-tumoral immune response
Ab-enzyme	DNase, RNase [110]	Immunogenicity?		Antibody directed enzyme prodrug therapy (ADEPT) [105-111]
Pepbodies	Fusion of a peptide, capable of binding Ab effector molecules, to scFv or Fab [125]	Easy production / full-size Abs		Recruitment of effector's functions
Troybodies	T-cell epitopes inserted into loops of the CH1 domain of APC-specific antibodies [125-140]			Delivery of T cell epitopes to antigen presenting cell (APC)

Monospecific immunoglobulin fragments are represented in white, bispecific antibodies are indicated in light grey and conjugated antibodies are shown in dark grey.

viral neutralization) does not require the recruitment of effector functions through the Fc portion. According to the therapeutic application, all of Ab's features should be taken into consideration: size, tissue penetration, distribution, half-life, effector functions, affinity, stability and immunogenicity. This can explain that smallest Ab (scFv or Fab) fragments may be preferred. Consequently, one of the tendencies of antibody engineering has been to reduce the size towards a minimum antibody fragment, retaining both binding affinity and specificity. In addition, smallest Ab fragment can be functionally expressed in *Escherichia coli* and yeast, which would dramatically reduce the cost associated with large-scale mammalian cell culture.

The Fv fragment, formed by the heterodimeric association of the two variable domains V_L and V_H of the light (L) and heavy (H) chain, respectively, can be genetically engineered into single chain Fv fragment (scFv) with a flexible polypeptide linker. The most commonly used linker is a flexible decapentapeptide (Gly4Ser)3. Changing the linker length between V-domains was capable of inducing oligomerization into diabodies, or even into higher order valency antibody fragments (triabodies, tetrabodies), potentially increasing the avidity [90]. Diabodies, the most characterized of these molecules, have shown high functional affinity, greater tumour retention and slower systemic clearance than their monovalent counterparts in pre-clinical studies [91]. To stabilize the association of the V_H and V_L domains, different linkage strategies have been proposed, for instance through disulfide bridges [92,93] and 'knock into holes' mutations [94]. An alternative format to scFv, named MoaFv, has been proposed to stabilize the Fv fragment and restore the bivalency of the antibody [95]. This MoaFv was constructed by replacing the C_{H1} and C_L domains of the Fab fragment with heterotetrameric molybdopterin synthase (MPTS).

The fragment antigen binding (Fab) is a heterodimer of V_H-C_{H1} and V_L-C_L linked together through a disulfide bond. In comparison with whole antibodies, small antibody fragments such as Fab or scFv exhibit better pharmacokinetics for tissue penetration. However, Fab and scFv are monovalent and often exhibit fast off-rates and poor retention time on the target.

The half-life of circulating antibody fragment (Fab, scFv) can be improved by site-specific coupling of polyethylene glycol (PEG) to the fragment, so called PEGylation [96]. For example, site-specific pegylation into the hinge region has prolonged the circulating half-life of an anti-TNF-α human fragment (CDP 870, Celltech) to 14 days. Covalent attachment of PEG increases the circulating *in vivo* half life of the antibody fragment, increasing the apparent size of the antibody fragment above the glomerular filtration limit. PEG may have the additional benefit of increasing solubility and resistance to proteolysis, and almost reducing immunogenicity [97]. Kitamura et al. have shown that PEG attachment to an intact murine monoclonal antibody reduced the HAMA response significantly when compared to the parent intact antibody [98].

4.2. Bispecific antibodies

Bispecific antibodies (BsAb) bind to two different epitopes and have been used to crosslink various cells and molecules. Various methods for generating bispecific antibodies have been developed: generation of hybrid-hybridoma or quadroma [99], chemical and genetic coupling of Fab fragment. Multimeric Ab fragments may be obtained by linking them to molecules which tend to self-associate, such as amphipathic helices [100], coiled-coil structures, immunoglobulin constant domains [101] and molecules which are homomultimeric. Recently, a new format of BsAb has been proposed, consisting of two scFv, one for human epidermal growth factor receptor 2 (HER2)/neu and the other for CD16, heterodimerized by a 'knobs-into-holes' device from the C_{H3} domains of the human IgG1 Fc fragment [102]. Another multimerisation system, based on the interaction between the prokaryotic ribonuclease Barnase (110 aa) and its inhibitor Barstar (89 aa) has revealed important advantages [103]. The use of such constructs is based on the exceptionally high association constant (up to 10^{14} M^{-1}) of the barnase-barstar couple.

4.3. Conjugated antibodies or "armed" antibodies

Ab fragments have been fused with a vast range of molecules to introduce different functionalities including radionuclides, toxins, or drugs for cancer cell killing, enzymes for prodrug therapy and cytokines to stimulate the anti-tumor immune response.

4.3.1. Tumor cells killing

For example, monoclonal antibodies directed to tumour-associated antigens or antigens differentially expressed on the tumor vasculature have been covalently linked to a drug such as calicheamicin (Mylotarg®, Wyeth) or a radionuclide (Zevalin®). These antibodies have been designed to deliver their toxic load directly to cancer cells, avoiding the toxicity associated with systemic administration. Imunotoxins are composed of Fv fragments of antibodies that bind to cancer cells fused to a truncated form of a very potent bacterial toxin (e.g. *Pseudomonas* exotoxin, ricin A chain). The antibody moiety specifically directs the toxin to cancer cells [104].

4.3.2. Activation of anti-tumoral immune response

Similarly, cytokine-antibody fusions (e.g. TNF-α, IFN-γ IL-12 or IL-2) have shown to initiate tumour regression in several systems [4]. These immunocytokines allow the local activation of the anti-tumoral immune response, avoiding the dilution effect associated with systemic cytokine administration. Another category of antibodies with high specificity and affinity can be developed to bind specific cytokines or their receptors, inhibiting the detrimental effect of the cytokine [3].

4.3.3. Antibody-directed enzyme prodrug therapy (ADEPT)

Enzyme may also be conjugated to antibodies in antibody-directed enzyme-prodrug therapy [105]. ADEPT involves the pre-targeting of prodrugs to tumors. An Ab-enzyme fusion protein is first administered and allows localizing to the tumor, followed by the administration of the prodrug which is activated by the enzyme at the tumor site [106]. This approach has been recently applied to various tumour therapies [107–109]. For example, a dimeric immunoenzyme comprising an anti-CD22 diabody and *Rana pipiens* liver ribonuclease was recently generated [110]. Previous clinical trials have shown evidence of tumor response; however, the activated drug had a long half-life, which resulted in dose-limiting myelosuppression. Also, the

targeting system, although giving high tumour to blood ratios of antibody conjugate (10 000: 1) required administration of a clearing antibody in addition to the antibody-enzyme conjugate [111].

4.4. Alternative immune repertoire [112]

Full-length antibodies, Fab and scFv fragments provide an antigen-binding surface comprising six CDR loops. Some target molecules are refractory to the immune repertoire, particularly those with cavities. The specific recognition of these target molecules would require a small penetrating loop for tight binding. The natural mammalian antibody repertoire does not encode penetrating loops and only rarely has the propensity to select this type of antibody [113]. In camelid species (camels, dromedaries, llamas), a significant proportion of the serum immunoglobulins is constituted by antibodies naturally devoid of light chains, called heavy-chain antibodies or HCAbs [114]. HCAbs lack the first domain C_{H1} but harbour an intact variable (V) domain (VHH) encoded by different V genes. This HCAb has been found to be physically very stable and is produced at very high levels in *Escherichia coli*. In addition, the CDR3 (up to 24 residues in llamas) is unusually long and such CDRs are especially suited for binding to the inner part of cavities [115].

On the basis of theses observations, Domantis (www.domantis.com) has developed a series of large and highly functional libraries of fully human V_H and V_L domain antibodies (dAbs). These dAbs correspond to the variable regions of either the heavy (V_H) or light (V_L) chains of human antibodies.

More recently, the immunoglobulin isotype novel antigen receptor (IgNAR) has been discovered in the serum of the nurse shark (*Ginglymostoma cirratum*) as a homodimeric heavy-chain complex, which also naturally lacks light chains. NARs are composed of five constant domains and a single variable domain with two CDR-like regions [116,117]. The variable domains of these unique molecules are candidates for the smallest antibody-based immune recognition units. A library of V(NAR) domains with extensive variability in the CDR1 and CDR3 loops displayed on the surface of bacteriophage has been produced. The content of this library was further validated through selection against the Apical Membrane Antigen-1 (AMA1) from *Plasmodium falciparum*. In addition, a recombinant V(NAR)s with nanomolar affinities for AMA1 could be produced at good yields in *E. coli*. [118].

4.4.1. Alternative scaffolds

4.4.1.1. Ankyrin repeat protein

Structural characteristics of ankyrin repeat (AR) protein incited to exploit AR domain as a scaffold for the construction of libraries of novel binding molecules [119]. Binz et al. have designed an AR module composed of fixed framework positions and randomized potential interaction positions [120]. These proteins are more amenable to be expressed in large amount; they are generally soluble, monomeric, devoid of disulfide bonds and stable under physiological conditions. Recently, the potential of AR protein has been demonstrated as intracellular kinase inhibitors [121].

4.4.1.2. Anticalin and duocalin

Lipocalins have also been recruted as scaffold for the design of artificial binding proteins termed Anticalin® (Pieris proteolab AG) [122,123]. Compared with antibodies, lipocalins, which exhibit biotechnological advantages due to their smaller size, are composed of a single polypeptide chain and they exhibit a simpler set of four hypervariable loops that can be more easily manipulated at the genetic level. Schlehuber and Skerra have also reported the construction of a functional fusion protein from two independent anticalins, a so-called duocalin [124].

4.5. Pepbodies

Pepbodies are obtained by fusion of a peptide, capable of binding one or several Ab effector molecules, to scFv or Fab fragment. These targeting fragments are able to efficiently, recruit some of the effector's functions, normally associated with the Fc fragment, such as complement activation [125]. Such pepbodies have been constructed, by introducing C1q-binding peptides into a loop of a C region domain. These pepbodies have retained the ability to bind both the antigen and C1q.

4.6. Intrabodies and transbodies

4.6.1. Intrabodies [126,127]

Intracellular Abs, also called intrabodies, are usually scFv [128] or dAbs [129], expressed within the confines of the intracellular environment. During the past decade, there has been growing interest in the use of such Ab fragment, in therapy of human diseases [130], targeting intracellular molecules such as tumour antigens and in functional genomics by blocking or modulating the activity of proteins or protein domains. Intra-

bodies can also be designed to modulate the function of its target molecule in other ways. For example, it is possible to design intrabodies to relocate its target molecule to another subcellular location.

However, the cytoplasmic expression of scFvs is generally limited by their instability and insolubility. The reducing environment of the cytoplasm prevents the formation of the intra-chain disulfide bridges [131], which are critical in folding almost all antibodies. Consequently, most Abs does not fold properly and are inactive inside cells. A number of approaches have been devised to overcome the limitation imposed by the cellular environment.

To overcome this problem, Auf der Maur et al. have proposed the "quality control" system that allows for a rapid selection of stable and soluble antibody frameworks suitable for intracellular applications [131]. This approach consists of constructing randomized hypervariable loop libraries on scFv frameworks that have been pre-selected for their intrinsic high stability and solubility in an intracellular environment.

Intracellular antibody capture (IAC) technology [132] is an approach based on selecting intrabodies starting from diverse scFv phage antibody libraries, which are initially screened against an antigen *in vitro*, and subsequently screening the selected scFv in a yeast *in vivo* antibody-antigen interaction assay.

4.6.2. Therapeutic antibody gene transfer

A tentative answer to overcome some issues in the domain of passive immunotherapy has been proposed by Bakker et al. [133]. The approach, taking advantage of the existing gene therapy protocols, is the possibility of raising therapeutic antibodies after DNA transfer *in vivo*. Genes encoding fully human antibodies targeting pathogens might be complementary to existing treatments and help controlling infectious diseases.

4.6.3. Transbodies

Recently, Heng and Cao have proposed to test the possibility of fusing protein transduction domain (PTD) to scFv antibodies [134]. PTD are short peptide sequences that enable proteins to translocate across the cell membrane and be internalized within the cytosol. This would result in a 'cell-permeable' antibody or 'Transbody'. Correct conformation, folding and disulfide bond formation can take place prior to introduction into the target cell. Thus, the process, still at hypothesis stage, has been proposed to possess several advantages over standard intrabody technology and to be potentially more efficient than the well-established gene silencing through RNA interference.

4.6.4. Antibodies and vaccination

4.6.4.1. Idiotypic network

Immunization with anti-idiotype (Id) antibodies represents a novel approach to active immunotherapy [135, 136]. It has been proposed that an anti-idiotypic Ab (anti-anti-X) may resemble the external Ag X and thus carry its 'internal image' [137]. Idiotypic-based vaccines contain neither nominal Ag nor its fragments. This includes the possibility that Id vaccines would be devoid of undesired side or toxic effects, which might sometimes be associated with some candidate antigens. In addition, one of the major problems of human cancer is 'immune tolerance', which might be tentatively broken by an appropriate anti-Id vaccine [136].

4.6.4.2. Antigenized antibodies

Antigenization of Abs, consist of the expression of foreign oligopeptides located into the hypervariable loops of an antibody molecule [138]. Immunization with antigenized antibodies is an alternative method to focus the immune response against defined epitopes of foreign antigens, and a way to deliver a peptide immunogen with an extended half-life. Musselli et al. have demonstrated that the severity of experimental allergic encephalomyelitis can be greatly attenuated by active immunization with antigenized Ab expressing synthetic peptides of the TCR, which are a key regulatory element in this disease [139].

4.6.4.3. Troybodies

Troybodies are APC-specific antibodies with T-cell epitopes inserted and "hidden" into loops of the CH1 domain (Constant region), without disrupting immunoglobulin folding. When they bind to their target APC, the T-cell epitopes are released, by antigen processing, and presented in association with MHC class II molecules to $CD4^+$ T cells. Targeting of antigens to antigen presenting cell (APC) results in an enhanced antigen presentation and T cell activation [125,140].

4.6.5. Catalytic antibodies

Among the multiple facets of the antibody molecule, one of the most intriguing is the capacity of naturally supporting a catalytic activity. A major and still controversial question is whether catalytic or more generally enzymatic activity of some natural antibodies represent function or dysfunction of these molecules [141]. According to the literature, antibody catalytic activities have been often associated to pathogenic situations such as autoimmune diseases. On the other hand, some other works have emphasized the beneficial role of cat-

alytic antibodies, considered in that context as "defensive enzymes" [142]. Serine protease or other peptidase activities have been reported for naturally occurring catalytic antibodies [143]. Remarkably, in a number of examples the light chain has been precisely characterized as the active component for proteolysis [144].

A wide array of therapeutic applications is conceivable for catalytic antibodies, including anti-viral intervention or pro-drugs activation. In the latter case the major advantage would be the lower immunogenicity of a catalyst perceived just as a natural molecule by the host's immune system. In the domain of treatment for drug addiction, interesting data have been reported concerning catalytic antibodies capable of breaking down cocaine to systemically inert molecules [145,146].

Isolating antibodies acting as powerful catalytic entities is still a great challenge; the reasons underlying that situation are essentially the following:

- Catalytic antibodies are often selected for their capacity to stabilize the transition state of a given chemical reaction, which impose to design, as immunogens, transition state analogues. The major difficulty being to chemically synthesize stable molecules mimicking a highly ephemeral structural entity, and very closely similar to the transition state in terms of 3D structure.
- The other constraint is more related to a capacity of screening against a catalytic activity, which could be very different from a standard selection based on affinity for the target.

A comprehensive overview of various strategies to overcome these issues was recently published [147, 148].

5. Expression systems

Clinical applications of antibodies often require large amount of highly purified molecules. The development of very efficient expression systems is essential to the full exploitation of the antibody potential. The expression of functional, correctly folded antibodies or antibody fragments and its scale up to commercial level is a major goal in therapeutic antibodies development. The choice of the most suitable expression system is function of the Ab format (whole antibody, monovalent or bivalent fragment), the application of the Ab (therapeutic, diagnostic, experimental tools), but factors such as scale-up, total annual production, post translational modification and regulatory issues comes also into play. While Bacteria and yeast are suitable for the production of antibody fragments, only mammalian cells or transgenic organisms can be the source of whole antibodies. The huge demand for large amounts of monoclonal antibodies is currently driving the improvement of existing expression systems or the quest for alternative production means.

5.1. Prokaryotic systems

Bacterial systems are suitable for the expression of scFv and Fab, intracellularly or within the periplasm.

5.1.1. E. coli [149]

Efficient recovery of properly folded and active Ab fragment in *E. coli* is strongly primary sequence dependent. Expression of recombinant Ab is typically achieved by fusion to N-terminal signal peptides, which target the protein to the periplasmic space of *E. coli* where chaperones (Skp, FkpA, DsbA and DsbC) assist the folding of the Ig domains and form the correct disulfide bridges to stabilize the structure. However, during accumulation of recombinant proteins in the periplasm of *E. coli*, undesired proteolytic degradations can occur. *E. coli* host strains lacking the major periplasmic proteases (DegP and Prc) have been shown to increase the yield of Fab produced in the periplasm two to three fold [150]. The yield of functional antibody fragments expressed in the bacterial periplasm depends also on the amino acid sequence, being highly variable from antibody to antibody. Alternatively, a correctly folded Fab has been produced in the cytoplasm of *E. coli* trx B gor potential of the cytoplasm is comparable to that of the mammalian endoplasmic reticulum. The expression has been improved via the co-expression of molecular chaperones such as skp [151]. An alternative to the periplasmic and cytoplasmic expression of Abs is their secretion to culture supernatants using the α-hemolysin (HlyA) system of *E. coli* [152].

Until recently it was admitted that the expression of full-length antibodies in the periplasmic domain of *E. coli* was not a reasonably achievable goal. Nevertheless, the assumption was objected by Simmons et al. (2002) who described the rapid and efficient expression of complete IgGs in the periplasm of *E. coli* [153]. The binding properties, of the recovered antibodies, were fully retained while, as expected, the recruitment of the effector functions linked to the Fc domain was lost.

The interesting question of a potential glycosylation, in *Escherichia coli*, was addressed in a work done by Wacker et al. [154]. They have established that the N-

linked glycosylation pathway found in the bacterium *Campylobacter jejuni* could be transferred into *E. coli*. Although structural differences exist between bacteria and eukaryotic cells, this raises the possibility of expressing glycosylated antibodies or antibody fragments in *E. coli*.

One can imagine that a combination of the two previously described technologies might open up the way to the production of full-length glycosylated antibodies or other complex molecules in *E. coli*. As exciting as could be such a perspective we should not underestimate both, the difficulty of transferring complete glycosylation pathways into prokaryotic hosts and the limitation of expressing large complex molecules into the periplasm of gram negative bacteria.

5.1.2. Alternative prokaryotic systems

Several gram positive bacteria have been used for expressing antibody fragment such as *Bacillus subtilis* [155], *Lactobacilus zeae* [156], *Streptomyces lividans* [157], and *Staphylococcus carnosus* [158].

As the assembly and glycosylation of full-length antibodies is not possible in bacterial systems, mammalian cell culture or transgenic organisms are more suitable for production of these molecules.

5.2. Baculovirus expression system

The baculovirus expression system has been successfully used for the expression of functionally active antibodies [159,161]. Moreover, cassette baculovirus vectors with human Fc regions have been engineered to allow transfer of specific variable regions selected by phage display and subsequent expression as a complete antibody [160]. A major disadvantage of the baculovirus expression system, however, is the catalytic properties of infectious baculovirus that narrows the window for optimal antibody production (generally 3–5 days).

5.3. Mammalian cell culture

Any human antibody IgG subclass can be generated in mammalian cell culture systems using Chinese hamster ovary (CHO) cells, NSO cells (derived from mouse myeloma) or Human embryo kidney (HEK-293) [162]. Mammalian cell culture has emerged as the method of choice for the production of most monoclonal antibodies currently commercialized, although the cost associated with scaling this system up for mass-production purpose are extremely high. Estimate of the current worldwide capacity available for antibody manufacturing using mammalian cell culture range from 413 000 L [163] to 475 000 L [164]. Morrow estimates that 25–50 new mAb products could be approved in the next five years, requiring 15 000–20 000 L each of new capacity. Also, currently available manufacturing capacities could be overhelmed in the near term (1–2 years).

Although improvements in media composition and host cell engineering have led increase of the productivity of mammalian cells [165], alternative expression systems have to be developed.

5.4. Yeast and filamentous fungi

The ability of these organisms to grow in chemically defined medium in the absence of animal-derived growth factors and to secrete large amounts of recombinant protein have made these hosts a system widely used to produce proteins that cannot be functionally expressed in *E. coli*. because of folding problems or the requirement for glycosylation.

Several antibody fragments, such as single-chain antibodies (scFvs) [166] or Fab [167] have been expressed at high yield in *Pichia pastoris*. The methylotrophic yeast *Pichia pastoris* is almost as easy to genetically manipulate as *Escherichia coli* could be, and has an eukaryotic protein-synthesis pathway [166]. In addition, antibody fragments can be produced in *Pichia* under fully validated conditions, which is essential for the production of therapeutics. Protein expression in Yeast is carried out using defined media, and recombinant proteins can be secreted into the medium, enabling purification from a starting material that does not contain a great number of contaminants.

The filamentous fungi *Aspergillus awamori* [168], *Aspergillus niger* [169] and *Trichoderma reesei* (patent n°WO 92/01797) have also been used to express antibody fragment and full-length IgG.

5.5. Transgenic plants

Antibodies extracted from plants, so-called plantibodies, have particular advantages in term of cost of production, ease of scaling up or down production to meet market demand, and freedom from animal-derived pathogens including prions and viruses [170, 171]. The first functional mAb was expressed in plant in 1989 [172]. Since then, plantibodies have been successfully produced in transgenic tobacco, soybean [173], alfalfa [174] and other plants [175]. Plants have proven to be effective systems for producing func-

tional therapeutic mAbs capable of conferring passive protection against bacterial and viral pathogens in animals [171,176].

To date, plants offer the only large-scale, commercially viable system for production of secretory IgA (sIgA) [177].

Particle bombardment allows the simultaneous introduction of multiple constructs, thereby expediting the recovery of transgenic lines expressing multimeric antibodies such as secretory immunoglobulin (sIgA) [178]. The recent availability of large amounts of secretory IgA plantibodies opens up a number of novel therapeutic opportunities for disorders of the mucosal immune system. In a human trial, the plant derived recombinant sIgA prevented oral re-colonization by *Streptococcus mutans* [175].

5.6. Transgenic animal-based production systems

The mammary gland has generally been considered the tissue of choice to express valuable recombinant protein since milk is easily collected in large volumes. Targeting the production of recombinant protein pharmaceuticals to the milk of transgenic farm animals (goat, cow) could potentially solve many of the problems associated with either microbial or animal cell expression systems: improperly folding of complex proteins and lacking of adequate post-translational modifications [179]. Several reports describe expression of antibodies in the milk of transgenic animals [180] despite limitations by the relatively long interval from birth to first lactation encountered with domestic livestock, the discontinuous nature of the lactation cycle and substantial material investments required to produce transgenic dairy animals [181].

The transgenic production of antibodies in egg white has been reported to take approximately 18 months [182]. The cost of generating non-purified material from transgenic chikens is calculated at $0.5/g. However, this cost does not account for the production of material under cGMP conditions.

Insect, plant and yeast cells are attractive as hosts for the production of recombinant antibodies as they represent potentially inexpensive and versatile expression systems. The principal limitations to the greater use of these hosts for the generation of pharmaceutical proteins are that the N-glycans generated differ from those of human glycoproteins due to the presence of some host glyco-epitopes on expressed glycoproteins which may elicit immune responses in humans, and poor pharmacological activity *in vivo* due to the rapid clearance from the circulatory system of glycoproteins with non human glycans.

5.7. In vitro system

Cell-free protein synthesis systems have been used as an alternative method of producing proteins from cloned genes. Thus, scFv [183] and Fab [184] have been reported to be synthesized in the *in vitro* systems. However, functional Abs with antigen binding activity are obtained only if disulfide formation and rearrangement is allowed to take place during the translation reaction (for example with addition of protein disulfide isomerase (PDI) or chaperones) [185,186]. Despite a certain potential for scale up, cell-free transcription/translation systems are still at a laboratory scale and within the milligram range, limiting their use as production means for research or screening purposes.

5.8. Glycosylation pattern and expression systems

All the above-mentioned expression systems are not equivalent for the post-translational modifications of antibodies, in particular glycosylation. Although the processing of the initial Glc2Man9Glc Nac2 oligosaccharide to Man8GlcNAc2 in the endoplasmic reticulum shows significant similarities between plants, insects, yeasts and mammals, very different processing events occurs in the golgi compartments [187]. Glycosylation profoundly affects the effector functions of IgG antibodies [53]. In particular, an optimal effector function is dependent upon the correct carbohydrate structure at Asn 297 of the Fc region of the two heavy chains. This oligosaccharide is buried between the CH2 domains, forming extensive contacts with amino acid residues within CH2. The terminal β1,4-galactose residues are in close contact with the protein backbone. It has been suggested that the Gal residues are critical for antibody performance, not only in effector functions but also in correct antibody folding [53]. Differences in glycan usage between human and expression hosts (mammalian cells, insect cells plants, yeasts) have caused some concerns that recombinant mAbs could be immunogenic or even allergenic. Consequently, several groups investigated the possibility of humanizing glycosylation pathways in various expression systems to produce human-like glycoproteins [187,188]. Glycosylation engineering of mammalian cell lines by stable transfection with genes encoding terminal human glycosyltransferases allows to obtain products with tailored glycosylation in high yields [189]. Umana et al. showed that the efficacy of antibodies could be improved by enhancing the potency of their natural immune effector functions [190]. Stable overexpres-

sion of n-acetylglucosaminyltransferase III, an enzyme not naturally expressed in CHO and NSO cells, in recombinant antibody-producing cells generate IgG with high levels of bisected, non fucosylated oligosaccharides in the Fc region [190]. On the other hand, the CHO cell line has been engineered to express the missing enzyme α,2–6 sialylytransferase [191], and feeding with galactose has proven to improve galactosylation of produced antibodies. Insect cells, like other eukaryotic cells, modify many of their proteins by N-glycosylation. However, the endogenous insect cell N-glycan processing machinery generally does not produce complex, terminally sialylated N-glycans such as those found in mammalian systems [188]. Similarly, glycoengineering has been used to improve the baculovirus-insect cell system [192].

In plant, N-linked glycans may be processed to include potentially antigenic and/or allergenic β(1,2)-xylose residues attached to the α-linked mannose of the glycan core and α(1,3)-fucose residues linked to the proximal Glc-NAc [193]. However, recent studies suggest that these glycans have limited immunogenicity. Even if plant glycan patterns may not represent a problem in terms of human health, they may affect conformational epitopes, or clearance of plant derived antibodies. In attempt to "humanize" glycans on antibodies produced in plant, the human β-1,4-galactosyltransferase was stably expressed in tobacco plants [194].

Most efforts to humanize yeast glycosylation pathways [195] have focused on the deletion of specific yeast genes involved in hypermannosylation (α-1,6-mannosyltransferase Och1p), and the introduction of genes catalysing the synthesis, transport and addition of human sugars (α-1,2 mannosidase, galactosyltransferases) [196]. This approach has been successfully used in *Pichia pastoris* [197,198].

6. Recombinant antibodies: to the infinity and beyond. . .

Recent statistical data, concerning recombinant products entering the market, indicates that at least one out of three molecules is an antibody or an antibody-derived molecule. It is reasonable to think that the current tendency is promised to increase in the coming years. This can be explained by a favourable conjunction between a sustained demand from the market and the relative maturity of the antibody engineering techniques. In such a context one should not underestimate the tremendously complex patent situation governing the recombinant antibody world. Nevertheless, for scientists as well as for the pharmaceutical industry, the antibody is an inexhaustible source of inspiration. Multi-faceted protein, the antibody has been able to undergo successive metamorphosis making it always fitted with the needs of the medical and industrial worlds.

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Abbreviations used in this paper:

AAR: anti-antibody response, Ab: antibody, ADCC: antibody-dependant cellular cytotoxicity, APC: antigen presenting cell APEX: anchored periplasmic expression, BsAb: bispecific antibody, CAD: covalent antibody display, CDR: complementary determining regions, cGMP: current good manufacturing practice, CHO: chinese hamster ovary, EBV: Epstein-Barr virus, Fab: antigen-binding fragment, Fc: crystallisable fragment, FDA: Food and Drug Administration, H: Heavy chain, HACA: human anti-chimeric Ab, HAHA: human anti-humanized Ab, HAMA: human anti-mouse Ab, HCAb: Heavy chain antibody, HEK: Human embryo kidney, L: Light chain, mAb: monoclonal antibody, NAR: new antigen receptor, NK: natural killer; PBL: peripheral blood lymphocyte, PEG: polyethylene glycol, PSMA: prostate specific membrane antigen, scFv: single-chain Fv fragment, SCID: severe combined immunodeficient, SIP: selectively infective phage, TNF: tumor necrosis factor, YAC: yeast artificial chromosome.

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