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Recombinant Antibodies as Therapeutic Agents

Pathways for Modeling New Biodrugs

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Contents

Abstract

Hybridoma fusion technology, proposed by Köhler and Milstein in 1975, started major developments in the field of monoclonal antibodies (mAbs). During the following 2 decades, their high potential as laboratory tools was rapidly exploited for biotechnology and biomedical applications. Today, mAbs represent over 30% of all biological proteins undergoing clinical trials and are the second largest class of biodrugs after vaccines. With the help of antibody engineering, mAbs have been reduced in size, rebuilt into multivalent molecules, and conjugated with drugs, toxins, or radioisotopes for the treatment of cancer, autoimmune disorders, graft rejection, and infectious diseases. Additionally, in the past few years, important advances have been made in the design, selection, and production of these new types of engineered antibodies. The present review focuses on the structural and functional characteristics of mAbs and their fragments, and also provides a walk through the most important methods used in antibody selection. In addition, the recent trends in antibody engineering for improving antibody clinical efficacy are also reviewed.

The discovery of hybridoma technology, described by Köhler and Milstein[1] in 1975, and the resulting ability to generate monoclonal antibodies (mAbs) initiated a new era in antibody research and clinical development. However, early studies on the use of murine mAbs determined that they had properties that could limit their clinical utility.[2] Firstly, the human immune system recognizes murine mAbs as foreign material and produces human anti-mouse antibodies (HAMAs) that lead to their clearance from the body, thereby limiting their therapeutic benefit. Secondly, murine mAbs were shown to have short serum half-lives and an inability to trigger human effector functions.[2-4] In an attempt to increase efficacy and reduce the immunogenicity of murine antibodies in humans, chimeric and humanized antibodies were developed using recombinant DNA technology.[5-7] This ability to manipulate antibodies into more human variants finally made antibodies suitable for clinical use.[8-10] There are currently 22 mAbs on the market that have been approved by the US FDA as therapeutic agents in a wide range of indications, including transplant

rejection, rheumatoid arthritis (RA), cancer, Crohn disease, and antiviral prophylaxis (table I). With more than 150 candidates in late-phase clinical trials, it is clear that mAbs are becoming the most rapidly growing class of human therapeutics and the second largest class of biodrugs after vaccines. This review provides an update of this fast-moving technology and covers various aspects of antibodies that are relevant to their use as therapeutic agents. We review antibodies designed as intact molecules, and then discuss the creation of smaller recombinant antibody fragments (antigen-binding fragments [Fabs], single-chain variable fragments [scFvs], and single domains) and engineered variants (diabodies, triabodies, and tetrabodies) that are now emerging as credible alternatives. In addition, we describe the most important methods used for selecting and screening different types of antibody libraries and review some of the current technologies used for enhancing antibody efficacy.

1. Structure and Antibody Function

Antibodies are part of a class of proteins called immunoglobulins. As one of the most important defenses against disease, these naturally occurring proteins are produced by the immune system in response to substances (antigens) that appear to be foreign to the human body. To identify and attack as many foreign intruders as possible, an immense number of different molecules, bearing different specificities, is required. This diversity is generated by somatic recombination and hypermutagenesis of a set of variant genes. The genetic information for this repertoire of different antibodies is stored in the B-cell pool of our lymphatic system.[11]

In vertebrates, there are five different classes of antibodies known as the immunoglobulins IgD, IgA, IgM, IgE, and IgG, which differ in their functions in the immune system.[11] IgGs are the most abundant immunoglobulins in the blood and the dominant format of therapeutic antibody (table I). This typical antibody consists of two Fabs, which are linked via a flexible region (hinge) to a constant fragment (Fc) region (figure 1). IgG antibodies are usually 'Y'-shaped molecules comprising two identical light chains (L) and two identical heavy chains (H) linked together by disulfide bonds. Heavy chains contain a variable domain (VH) and three constant domains (CH1, CH2, and CH3). By contrast, light chains contain a variable domain (VL domain) and a single con-

Table I. US FDA-approved therapeutic monoclonal antibodies

Antibody name	Trade name	Antibody type	Antigen	Therapeutic use	Company	Approval year
Muromonab	OKT3®	Murine IgG2a	CD3	Allograft rejection	Ortho Biotech	1986
Abciximab	ReoPro®	Chimeric Fab	GPIIb/IIIa	Cardiovascular disease	Centocor	1994
Rituximab	Rituxan®	Chimeric IgG1	CD20	Non-Hodgkin lymphoma	Genentech	1997
Daclizumab	Zenapax®	Humanized IgG1	CD25	Transplant rejection	Roche	1997
Basiliximab	Simulect®	Chimeric IgG1	CD25	Transplant rejection	Novartis	1998
Palivizumab	Synagis®	Humanized IgG1	RSV	RSV prophylaxis	MedImmune	1998
Infliximab	Remicade®	Chimeric IgG1	TNFα	Crohn disease	Centocor	1998
Trastuzumab	Herceptin®	Humanized IgG1	Her2	Metastatic breast cancer	Genentech	1998
Gemtuzumab	Mylotarg®	Humanized IgG4	CD33	Acute myeloid leukemia	Wyeth	2000
Alemtuzumab	Campath®	Humanized IgG1	CD52	Chronic myeloid leukemia	Genzyme	2001
Ibritumomab	Zevalin®	Murine IgG1	CD20	Non-Hodgkin lymphoma	Biogen Idec	2002
Adalimumab	Humira®	Human IgG1	TNFα	Crohn disease	Abbott	2002
Omalizumab	Xolair®	Humanized IgG1	IgE	Asthma	Genentech	2003
Tositumomab	Bexxar®	Murine IgG2a	CD20	Non-Hodgkin lymphoma	GlaxoSmithKline	2003
Efalizumab	Raptiva®	Humanized IgG1	CD11a	Psoriasis	Genentech	2003
Cetuximab	Erbitux®	Chimeric IgG1	EGFR	Colorectal cancer	Imclone Systems	2004
Bevacizumab	Avastin®	Humanized IgG1	VEGF	Colorectal cancer	Genentech	2004
Natalizumab	Tysabri®	Humanized IgG4	α4-integrin	Multiple sclerosis	Biogen Idec	2004
Ranibizumab	Lucentis®	Humanized Fab	VEGF	Wet macular degeneration	Genentech	2006
Panitumumab	Vectibix®	Human IgG2	EGFR	Colorectal cancer	Amgen	2006
Eculizumab	Soliris®	Humanized IgG1	C5	Inflammatory diseases	Alexion Pharmaceuticals	2007
Certolizumab	Cimzia®	PEGylated humanized Fab	TNFα	Crohn disease	UCB	2008

EGFR = epidermal growth factor receptor; **Fab** = antigen-binding fragment; **PEG** = polyethylene glycol; **RSV** = respiratory syncytial virus; **TNF** = tumor necrosis factor; **VEGF** = vascular endothelial growth factor.

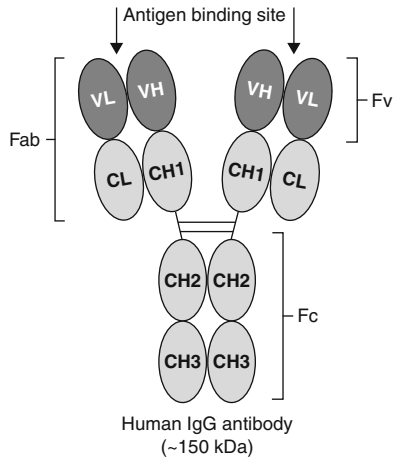

Fig. 1. Schematic representation of the structure of a conventional IgG antibody. IgG antibodies comprise a pair of identical heavy and light chains linked by disulphide bonds. Light chains contain one constant domain (CL) and one variable domain (VL), while heavy chains contain three constant domains (CH1, CH2, and CH3) and one variable domain (VH). The variable domains of both the heavy and light chains are responsible for the antigen-binding site of the molecule. The constant fragment (Fc) region recruits effector functions of the immune system. Constant light (CL) and heavy (CH) chain domains are represented in light shading. Variable light (VL) and heavy (VH) chain domains are represented in darker shading. **Fab** = antigen-binding fragment; **Fv** = variable fragment.

stant domain (CL domain). The variable domains show three regions of sequence hypervariability called complementarity determining regions (CDRs). They differ in length and sequence between different antibodies and are responsible for the specificity (recognition) and affinity (binding) of the antibodies to the antigen. The remaining V region amino acids act as a scaffold to support the loops of the CDR and are referred to as framework residues (FRs). On the other hand, the Fc region recruits cytotoxic effector functions through complement and/or interactions with γFc receptors. In addition, this non-antigen binding part also influences the serum half-life of antibodies. In this case, the Fc region binds to neonatal Fc receptors on endothelial cells and rescues antibody molecules from intracellular degradation.[10,11]

In antibody-based therapies, the goal is to eliminate or neutralize the pathogenic organism or the disease target.[8] For this purpose, mAbs achieve their therapeutic effect through various mechanisms. In some cases, antibody binding can directly and effectively block the activity of many pathogens. In other scenarios, effective treatment requires a more general immune response, and antibodies must boost effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC).[10] In ADCC responses, antibodies bind to antigens on target cells and the antibody Fc domains engage Fc receptors (FcγRs) on the surface of effector cells, such as macrophages and natural killer cells. These cells in turn trigger phagocytosis or lysis of the targeted cell. In CDC, antibodies kill the targeted cells by triggering the complement cascade at the cell surface. These effector functions contribute to the therapeutic efficacy of several antibodies in clinical settings in which the destruction of target cells is desired, such as the removal of tumor cells or virally infected cells. Strong evidence for an Fc-mediated contribution to antibody efficacy in patients is seen with the mAb rituximab.[12] This mAb is a chimeric IgG1 that specifically targets the CD20 surface antigen expressed on normal and neoplastic B lymphoid cells. Rituximab is currently indicated for both follicular and aggressive B-cell non-Hodgkin lymphomas, and its impact on clinical treatment is clearly evidenced by its high effectiveness in triggering both ADCC and CDC responses.[12-16]

2. Antibody Fragments

As previously mentioned, most marketed antibodies are composed of a full-length IgG molecule that provides for a long half-life and effector functions. However, there is a range of therapeutic applications in which other antibody formats may be more desirable.[17] For instance, in some conditions, a long serum half-life results in poor contrast in imaging applications, and inappropriate activation of Fc-receptor-expressing cells may lead to massive cytokine release and associated toxic effects. In addition, due to their high molecular weight (~150 kDa), IgG antibodies are known to diffuse poorly into solid tumors and clear slowly from the body. Therefore, to avoid Fc-associated effects in some clinical settings and address the size limitations of IgGs, smaller antibody molecules such as the Fab or the variable fragment (Fv) may be produced and become more attractive as therapeutic agents.[17-24]

Before the development of recombinant technologies, Fabs could only be generated through proteolytic cleavage of antibodies. Currently, it is possible to clone the variable (V) and the constant (C) domain genes of the light (L) and heavy (H) chains of an IgG molecule to obtain a recombinant Fab (figure 2a). Similarly, cloning the variable genes of an IgG alone permits the production of the Fv fragment of an intact antibody molecule.[25] These Fv fragments are still capable of binding to antigen. However, native Fv molecules are unstable and tend to dissociate due to the lack of covalent or disulphide bridges between the two chains. This drawback has been overcome through engineering scFv fragments by joining the VH and VL domains with a flexible peptide linker of 10–20 amino acids (figure 2a).[26,27] The most common peptide linker is highly flexible $(Gly_4Ser)_3$, but many other linker designs have been successfully employed.[28] A different approach has been to covalently link the VH and VL domains by introducing additional cysteine residues at the interface, leading to disulphide-stabilized Fv (dsFv) antibody fragments.[29,30]

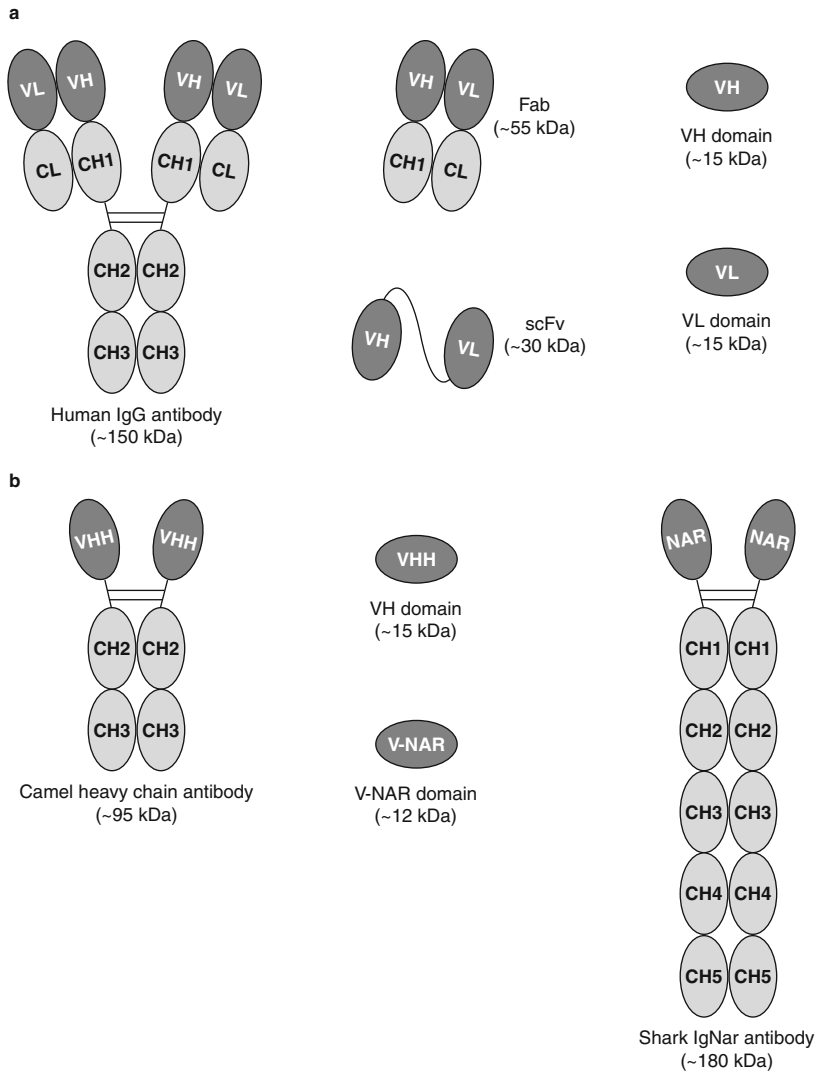

Fig. 2. Schematic representation of antibody constructs of biotechnological and clinical interest. (**a**) The engineering of antibody fragments that can be generated from an intact conventional IgG: antigen-binding fragments (Fabs), single-chain variable fragments (scFvs), and heavy and light chains only (V domains). (**b**) Camelid and shark immunoglobulins are composed only of heavy chains. They present no light chain, and the displayed V domains of both species bind their target independently. Camelid heavy chain antibodies comprise a homodimer of one variable domain (VHH) and two C-like constant domains (CH). Shark new antigen receptor antibodies (IgNARs) comprise a homodimer of one variable domain (V-NAR) and five C-like constant domains (CH). Constant light (CL) and heavy (CH) chain domains are represented in light shading. Variable light (VL) and heavy chain domains (VH, VHH, and V-NAR) are represented in darker shading.

At present, two non-IgG antibody molecules have been approved for human therapeutic use. Abciximab (Centocor), the first to be approved, is a chimeric Fab with mouse variable and human constant domains that inhibits platelet activation by blocking the platelet glycoprotein IIb/IIIa receptor.[31,32] The other antibody fragment is ranibizumab (Genentech), a humanized vascular endothelial growth factor-specific Fab that is efficacious for treating neovascular age-related macular degeneration.[33] In addition, many others are already in clinical trials. Examples include pexelizumab (Alexion Pharmaceuticals), a humanized scFv that is in phase II/III testing for reducing mortality and myocardial infarction in patients undergoing graft surgery for coronary artery bypass.[34] Trubion Pharmaceuticals is also developing TRU-015, a small modular immunopharmaceutical (SMIP™) drug candidate designed to target and deplete B cells using a balance of effector functions optimized for inflammatory disease. *In vitro* and *in vivo* studies of TRU-015 demonstrate B-cell CD20 binding, ADCC, and apoptosis activity, together with reduced CDC activity. The company is presently completing a phase IIa study with TRU-015 in RA patients with active disease.[35]

3. Single-Domain Antibodies

Fv and scFvs are usually viewed as the smallest antibody units that form complete antigen-binding sites. However, early observations in the late 1960s indicated that, occasionally, VH domains alone retain a significant part of the original binding activity.[36-38] Based on this concept, in 1989, Greg Winter's group in Cambridge, UK, isolated heavy chain variable domains with antigen affinity against lysozyme derived from an immunized murine VH library.[39] Despite such promising results, the efficient expression of VH fragments is usually confronted with folding problems, low solubility, and a high tendency for aggregation caused by the exposure of the hydrophobic VH/VL interface upon removal of the VL domain. Nevertheless, these problems have been overcome, or at least greatly reduced for some mouse and rabbit VH domains, by the identification and design of mutations that minimize the hydrophobic interface and by direct selection of highly stable single domains from phage display libraries.[40-49]

In contrast, other promising alternatives are the naturally occurring heavy-chain antibodies, devoid of light chain (figure 2b), which were recently discovered in two types of organism: the camelids (camels and llamas)[50] and cartilaginous fish (wobbegong and nurse shark).[51-53] These heavy-chain antibodies recognize the antigen through a single variable domain and can be obtained from either immunized or non-immunized animals. In camelids, the variable heavy chains are referred to as VHH (~15 kDa) and consist of four framework regions and three CDRs.[54] On the other hand, shark variable heavy chains are called IgNAR (new antigen receptor) and contain only two CDR loops because of the deletion of a large portion of CDR2, which make them the smallest natural antigen-binding site (~12 kDa).[51] In VHH and IgNAR, the third antigen-binding loop (CDR3) is often stabilized by disulfide bonds and is, on average, longer than a human or mouse VH-CDR3 loop. Therefore, these smaller antibody molecules might reach targets not easily recognized by currently available mAb therapies, such as enzyme active sites and canyons in viral and infectious disease biomarkers.[49,55] Further advantages include ease of manufacture, high stability, improved tumor tissue penetration, and rapid blood clearance.[56-59] As a result, it is clear that single-domain antibodies will quickly become one of the most promising new-generation therapeutic mAbs. Indeed, there are currently two biotechnology companies that are focused solely on single-domain antibodies as therapeutic agents. Domantis (recently acquired by GlaxoSmithKline) is one of these companies and was launched at the end of 2000 in Cambridge, UK. Its aim was to build up a series of large and highly functional libraries of fully human VH and VL domains. Today, it has more than a dozen proprietary human single-domain antibody therapeutic programs in the fields of inflammation and oncology.[49] The other biopharmaceutical company is Ablynx, which was founded in Belgium in 2002 and is engaged in the development of camelid single-domain antibodies.[17] The power of Ablynx's discovery platform has resulted so far in two VHH single domains that are in preclinical trials for the treatment of RA and inflammatory bowel disease, and other mAbs for the prevention of thrombosis associated with arterial stenosis.

4. Engineering Multivalent, Bispecific, and Bifunctional Fragments

Converting whole IgG antibodies into Fabs, scFvs, or even single-domain fragments is usually associated with a decrease in antigen-binding activity due to the loss of avidity. Nevertheless, this loss in binding activity can be compensated for by engineering multivalent antibody fragments. Indeed, several antibody conjugates have already been constructed through the use of either chemical or genetic cross-links. These molecules span a molecular weight range of 60–150 kDa and valences from two to four binding sites. For example, Fab fragments have been chemically cross-linked into di- and trivalent multimers, leading to an increase in functional affinity.[60-62] On the other hand, several strategies have been devised to genetically create multimeric scFvs. The most successful design has been the simple reduction of the scFv linker length from 15–20 amino acids to ≤5 amino acids.[17,19] While fragments with a five amino-acid linker generally result in the formation of dimeric molecules (diabodies, ~ 60 kDa) with two binding sites, further reduction often leads to the assembly of trimers (triabodies, ~90 kDa) or tetramers (tetrabodies, ~120 kDa). An alternative format is the arrangement of two scFvs connected by a flexible polypeptide linker on a single polypeptide chain.

In general, all multivalent antibody fragments are generated to be monospecific. Nevertheless, some of the previously mentioned strategies have also been applied to generate bispecific multimers. These bispecific antibodies have two different binding specificities fused together in a single molecule. Because of dual specificity, they can bind to two adjacent epitopes on a single antigen, thereby improving avidity. Alternatively, bispecific antibodies can cross-link two different antigens and be powerful therapeutic agents in some clinical settings.[23] The first approach to construct and produce bispecific antibodies was the quadroma technology that is based on the somatic fusion of two different hybridoma cell lines expressing murine mAbs with the desired specificities of the bispecific antibody.[63-65] However, this technique is complex and time consuming, and produces unwanted pairings of the heavy and light chains. Far more effective methods incorporate chemical

conjugation or genetic conjugation to couple two different Fab modules or smaller antibody fragments together.[23,66,67]

Among the first bispecific antibodies were constructs designed to redirect T cells against cancer target cells.[68,69] Target cells were killed when cytotoxic T lymphocytes were tethered to tumor cells and simultaneously triggered their activity by one arm of the bispecific antibody that interacted with the T cell receptor-CD3 complex. The use of the monomorphic CD3 complex for triggering T cells circumvented the restrictions of clonotypic T-cell specificity and enabled a polyclonal cytotoxic T-lymphocyte response against target cells bearing the antigen recognized by the second arm of the bispecific antibody. Another development is bispecific antibodies that simultaneously bind tumor cells and an activating Fcγ receptor, e.g. CD64/Fcγ receptor type I (FcγRI) on monocytes.[70,71] Their binding to Fcγ receptors can elicit effector cell activation, without competition by simultaneously binding normal IgG.

Antibody engineering has also been applied to the development of bifunctional antibodies. In contrast to bispecific antibody fragments, bifunctional molecules combine the antigen-binding site of an antibody with a biological function encoded by a linked or fused partner.[23] These include radionuclides, cytokines, toxins, enzymes, peptides, and proteins. Currently, ibritumomab, tositumumab, and gemtuzumab are three examples of FDA-approved bifunctional antibodies designed to specifically deliver cytotoxic drugs into cancer cells. Ibritumomab[72] and tositumomab[73] are both mouse anti-CD20 antibodies attached to Yttrium-90 and Iodine-131 radioisotopes, respectively. Because of their bifunctional properties they can target the surface of mature B cells and B-cell tumors, and induce cellular damage in the target and neighboring cells. In contrast, gemtuzumab is a humanized mAb that is linked to the anti-tumor agent calicheamicin, a bacterial toxin.[74] The antibody is targeted to CD33, which is expressed in approximately 90% of all cases of acute myeloid leukemia (AML), and has been approved for administration to patients who have relapsed AML.

Recently, bifunctional antibodies have also been developed to improve antibody pharmacokinetics. The small size of antibody fragments and single domains improves their ability to penetrate tumors and leads to rapid clearance from the circulation through the kidney. In some therapeutic applications, the rapid clearance is beneficial; however, in other cases, it is desirable to increase the half-life of the antibody. This can be achieved by linking the antibody fragment or single domain to polyethylene glycol (PEG). This process, named PEGylation, increases the serum half-life and simultaneously reduces the immunogenicity of proteins by chemical coupling of PEG to amino groups on the antibody.[75,76] To date, at least two PEGylated antibody fragments have progressed to clinical trials. One of these is a humanized tumor necrosis factor (TNF)-specific Fab (certolizumab pegol) that has its circulating half-life prolonged to 14 days by site-specific PEGylation in the hinge region.[77] Certolizumab pegol was recently approved by the FDA for use in adults with moderate to severe Crohn disease following favorable phase III clinical trial results. Preliminary results have also shown that certolizumab was well tolerated and efficacious in phase III clinical trials in patients with RA.

5. Selection and Screening of Monoclonal Antibodies

Mouse hybridoma technology, described by Köhler and Milstein,[1] was an important step in the development of mAbs. Mouse hybridoma is a hybrid cell produced by injecting a specific antigen into a mouse, collecting the antibody-producing cell from the mouse's spleen, and fusing it with a long-lived cancerous cell (myeloma). The resulting hybrid cell can be isolated and expanded, thus producing many identical offspring. Each of these daughter clones will secrete the immune cell product, the antibody, over a long period of time. A B-cell hybridoma secretes a single specific antibody known as a mAb.[1] Following the discovery of the Nobel prize-winning hybridoma technology, the door was open to use mouse antibodies as human therapeutics. However, as previously mentioned, murine mAbs possess several properties that limit their clinical utility. Therefore, during the past decade, several new display and screening methods have been explored and emerged to develop human/humanized high-affinity antibodies. Phage display technology and transgenic mice that express human immunoglobulin genes are currently two of the most well established platforms used to identify the growing number of human antibodies entering clinical trials. Alternative but less widely used routes are the ribosome and cell display technologies. These selection platforms will be described in the following sections (figure 3).

6. Phage Display Technology

Phage display technology had its beginnings in 1985 when Smith[78] cloned a fragment of a gene encoding the Eco RI endonuclease into the gene III insertion site of the filamentous bacteriophage f1. This strategy created a fusion protein capable of being displayed on the virion surface. Following the studies describing the display of peptides on the surface of filamentous phage, several laboratories simultaneously developed phage display systems for antibody fragments in the early 1990s.[79,80] In these systems, antibody genes are linked to the amino terminus region of the phage minor coat protein pIII. When expressed, the encoded fusion product is incorporated into the mature phage

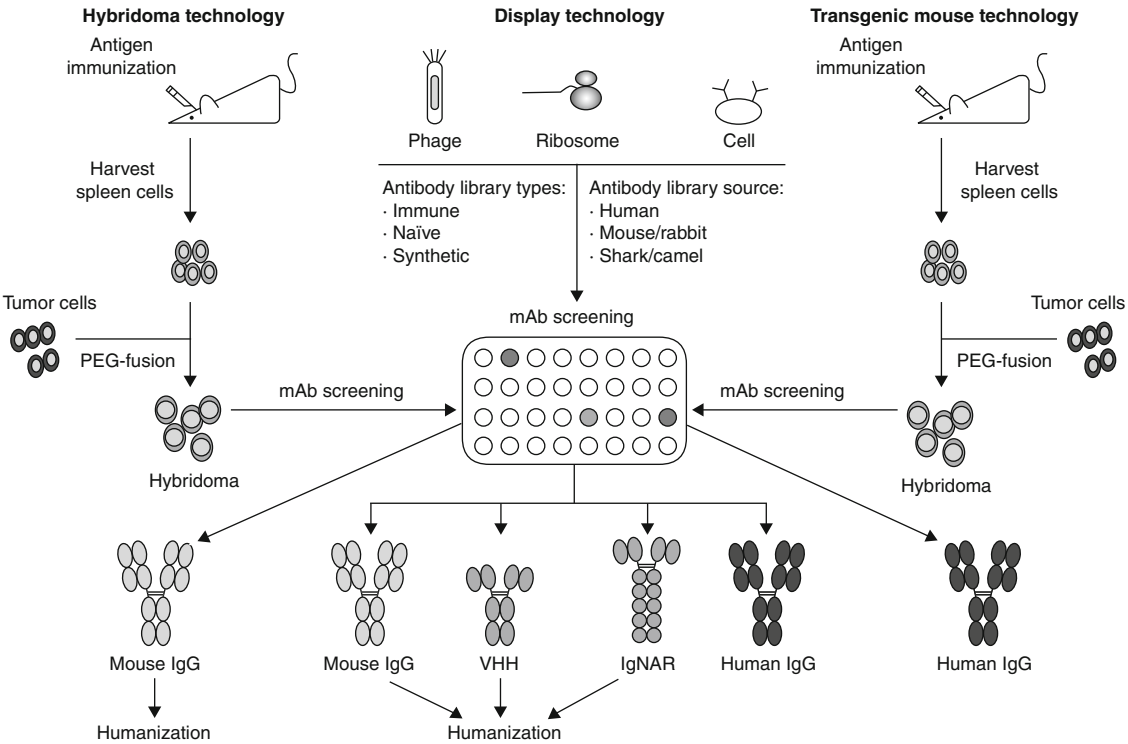

Fig. 3. Schematic representation of the different approaches for producing monoclonal antibodies. The traditional hybridoma technology for obtaining mouse antibodies is shown on the left side of the figure. The center part shows the three main procedures for obtaining antibodies through display systems (phage, ribosome, and cell display). With display technologies, antibodies can be developed from immune, naive, or synthetic libraries and derived from different sources (human, mouse, rabbit, shark, and camel). The right section of the diagram describes the procedure of obtaining human antibodies using genetically engineered mice that produce human antibodies. **IgNAR** = 'new antigen receptors'; **mAb** = monoclonal antibody; **PEG** = polyethylene glycol.

particle during normal phage biogenesis. The resulting phage particle expresses antibodies on its surface and contains the antibody encoding gene. This linkage between antibody genotype and phenotype allows the enrichment of antigen-specific phage antibodies, using immobilized or labeled antigen.[81,82] The selection process in phage display can be divided into four main steps: (i) coating of antigen; (ii) incubation of phage repertoire with antigen; (iii) washing to remove non-specific phages; and (iv) elution and reamplification of antigen-specific phages. Usually, three to six rounds of binding, elution, and amplification are sufficient to generate antibodies with high affinity and specificity. Still, this selection success hinges on a combination of display and enrichment methods, with the generation of large and highly diverse repertoires of phage antibody libraries.[83-85]

In general, three types of antibody libraries may be used: immune, naive, and synthetic.[81,82] Immune libraries are derived from the IgG mRNA of B cells from an immune source, such as immunized animals or, in some instances, human immune B cells. These libraries sometimes result in higher-affinity antibodies than those rescued from hybridomas. For example, screening of immune phage antibody libraries has been used to isolate antibodies specific for viral antigens,[86-88] bacterial toxins,[89] and cell-surface tumor antigens[90,91] with dissociation constants (K_D) as low as 10^{-10} M. However, at the same time, the need for immunization carries a number of serious limitations. These disadvantages include the time required to immunize animals, unpredictability of the immune response to the target antigen, lack of immune response to some antigens (self antigens or toxic molecules) and the fact that a new library must be generated for each desired antigen. In addition, active immunization of humans is obviously not possible for ethical reasons, and therefore immune human libraries can be generated only from B cells obtained from disease-affected patients.[81,82,85]

The second type of library is obtained from large naive repertoires of antibody fragments that can be recovered from non-immunized donors. In this case, mRNA of B cells is isolated from hematopoietic organs, and IgM variable regions are amplified using the polymerase chain reaction (PCR) with degenerate oligonucleotide primer sets and cloned directly into phage display vectors.[92-95] IgM repertoires are usually preferred to IgG because they have not been subjected to tolerance or antigen selection and thus are more diverse. The source of B cells, as well as the immunological status of the donors, cloning methodologies, and efficiencies, all contribute to the differences observed in library

diversity.[96] Most libraries make use of peripheral B lymphocytes as the source repertoire since they contain naive B cells with less bias from immunogenic stimulation. Additionally, when natural repertoire-based *in vitro* combinatorial libraries are considered, the random pairing of the light chain and heavy chain can also contribute to the diversity of these libraries. Therefore, it is clear that naive libraries have a series of advantages over libraries from immunized donors. Firstly, specific antibodies can be produced without any previous contact with the antigen, with the result that antibodies against self, non-immunogenic, or toxic antigens can be obtained. Secondly, if sufficiently large and diverse, a single library can be used for all antigens. Thirdly, antibody generation takes <2 weeks and human antibodies can be isolated.[81,82] Nevertheless, the major disadvantages are that much larger libraries must be cloned in order to isolate antibodies with affinities similar to those obtained from relatively small immune libraries, and the exact nature of the cloned V-gene repertoire is largely unknown and uncontrollable.[97] Moreover, since the antibody genes are representative of the human immunological repertoire, there is no guarantee that the clones obtained by library screening will encode antibodies amenable to expression in bacteria. In fact, poor expression and toxicity to the host bacteria are often issues with antibodies isolated from naive antibodies.[97]

These problems may be circumvented by using synthetic antibody libraries.[98] Synthetic antibody libraries are constructed entirely *in vitro* using oligonucleotides that introduce areas of complete or tailored degeneracy into the CDRs of one or more V genes. By introducing degeneracy into specific codon positions of synthetic oligonucleotides, the degree of randomization can be precisely controlled. There are examples of semisynthetic antibody libraries, which incorporate a balance of natural and synthetic repertoires and are often created to increase natural diversity while maintaining a certain level of functional diversity.

In the first synthetic antibody library, constructed by Hoogenboom and Winter,[99] a repertoire of human VH genes from 49 human germline VH-gene segments rearranged *in vitro* was developed to create a synthetic CDR3 of five to eight residues. The rearranged VH genes were cloned with a human Vλ3 light chain as scFvs for phage display. From this repertoire, many antibodies were isolated against haptens and one against a protein antigen, delivering the first proof of concept for synthetic antibodies. Subsequently, the diversity in the CDR3 regions was expanded to cover lengths ranging from four to twelve residues matching the natural length diversity of this loop.[100] This expanded library was highly successful for 18 antigens, including haptens and proteins, showing the importance of diversity in a longer CDR-H3 loop. To further increase library size and diversity, with the aim of isolating higher affinity antibodies, Griffiths et al.[101] used a novel system to add light chain diversity to the synthetically rearranged VH gene repertoire of Nissim et al.[100] In this case, 26 human germline Vκ and 21 germline Vλ segments were assembled into complete V genes using PCR with CDR3 loops partially randomized to mimic the diversity generated by V-J gene recombination *in vivo*. The heavy and light chain V-gene repertoires were combined on a phage vector in bacteria using the lox-Cre site-specific recombination system to create a large (6.5×10^{10}) synthetic repertoire of Fab fragments displayed on filamentous phage. The result was the development of antibodies against a range of different antigens with affinities comparable to those obtained from a secondary immune response in mice (up to 4 nM).[101]

Several other strategies of synthetic library construction with improved output were developed. For the most part, these libraries were generated by using germline segments of heavy and light chains where CDR loops were randomized synthetically to a length of 6–15 residues.[102] Randomization of CDR central amino acids is typically complete (3–5 residues), with limited randomization of flanking amino acids. This is usually performed to mimic the natural antibody sequences. Other laboratories have used similar approaches but strategically choose fewer germline segments for heavy and light chains that frequently occur in natural antibodies.[103] Antibodies generated in these libraries are usually subjected to affinity improvement by CDR1 and CDR2 randomization. Typically, clones with picomolar affinity are derived from a low-nanomolar parent after two or three rounds of affinity by first targeting six heavy chain residues, followed by light chain residues. The strategy of stepwise generation of affinity hopefully can be extended to other antibody clones using the same approach of library construction. These synthetic libraries appear to have evolved and improved well enough to suit therapeutic development.

The first commercial venture based on synthetic antibody technology is the Human Combinatorial Antibody Library (HuCAL®)[1] developed by MorphoSys. The library's construction used a careful selection of antibody templates by analyzing the structure, sequence variation, usage of germline genes, and hypermutation. Morphosys has arranged a group of seven VH families and seven VL germline families, where a consensus sequence of the rearranged natural antibodies was introduced in CDR1 and CDR3. In addition, selected positions in CDR-L3 and CDR-H3 were randomized in several versions of HuCAL®, where the newest one randomized all six CDR loops in specific antigen contact sites by mimicking the composition of the natural antibody

1 The use of trade names is for product identification purposes only and does not imply endorsement.

repertoire. It is recognized that the libraries worked well with test antigens, and several antibodies generated by MorphoSys are presently in various stages of clinical development, e.g. an antibody against intracellular adhesion molecule-1 (ICAM-1) for treating autoimmune diseases.[104]

Genentech has taken a different approach by using a well defined template (trastuzumab) derived from phage display libraries. Trastuzumab is a humanized antibody for which structural information and biophysical data was available. The strategy was to randomize exposed heavy chain CDR loops in a mode that mimics the natural repertoire.[105] Using a strategy of increasing CDR3 length and imitating the natural antibody repertoire, it was possible to augment affinity to the low-nanomolar range and generate clinical candidates out of this library. The affinity could be further improved to the picomolar range by stepwise randomization of light chain CDR residues mimicking the natural diversity.[106] It is of note that co-evolution of selected residues in heavy and light chain CDRs can additionally improve affinity and cross-species binding.[107] Additional studies on antibody diversity were explored by generating randomized libraries on CDR-H3 but included only four amino acids (YADS library: tyrosine [Y], alanine [A], aspartic acid [D], and serine [S]) or even two amino acids (YS library: tyrosine and serine).[108,109] This elegant study demonstrates that synthetic CDR randomization and consequent recognition requires minimal amino acid content and diversity. These libraries were capable of generating antibodies to set target antigens. From this YADS and YS library strategy, a detailed analysis of the interaction between antibodies was gathered. The result was that tyrosine dominates the direct interactions mostly through its aromatic ring structure. The serine or alanine residues seemed to help by scaffolding the tyrosine in its binding conformation because of their small size. These striking results illustrate that the chemical diversity of CDR can be incorporated into a small number of crucial amino acids, and also provide elucidation about the overabundance of tyrosine and serine residues in the natural antibody repertoire and in the antigen-binding interface.

7. Ribosome Display Technology

Ribosome display, originally described by Mattheakis and coworkers,[110] is a cell-free system for the *in vitro* selection of peptides and proteins from large libraries. It uses the principle of coupling individual nascent proteins (phenotypes) to their corresponding mRNA (genotypes) through the formation of stable protein-ribosome-mRNA complexes. This permits the simultaneous isolation of a functional nascent protein with affinity for a ligand, together with the encoding mRNA that can be used to obtain the corresponding gene. Ribosome display has recently been adapted to the screening of antibody libraries by Hanes and Plückthun,[111] and He and Taussig.[112] In this case, a large DNA library encoding antibody fragments is transcribed *in vitro*. The mRNA is purified and used for *in vitro* translation. As the mRNA lacks a stop codon, the ribosome stalls at the end of the mRNA and gives rise to a ternary complex of mRNA, ribosome, and functional protein. After *in vitro* translation, ribosomal complexes are directly used for antibody selection either on a surface immobilized ligand or in solution. To recover the library enriched for target binding, EDTA is added to destabilize the ribosomal complexes, and mRNA is isolated and purified. Then a reverse transcription reaction followed by PCR (RT/PCR) provides the cDNA template for the next round. Depending on the library complexity, type of protein scaffold, and its target, three to six rounds are required to select antibodies with low nanomolar or sub-nanomolar affinity.[111-114] To select mAbs with even higher affinities (low to mid-picomolar range), special strategies (off-rate selection) are required.[115,116]

Ribosome display has several potential advantages over phage display. Firstly, larger libraries (10^{12}–10^{14} members) can be constructed with ribosome display than with phage display. Secondly, ribosome display avoids transformation and cloning steps that can be laborious with large phage display libraries and may result in the loss of diversity. Finally, libraries can be further diversified during PCR steps in ribosome display using low-fidelity polymerases. Thus high-affinity antibodies initially not present in libraries can be generated and selected against a large array of antigens.[116]

8. Cell Display Technology

Before the advent of phage display systems, antibodies had been displayed on bacterial cells, even though isolation of specific clones was difficult due to inefficient screening methods. The recent development of high-speed flow cytometers has reactivated the efforts in cell surface display, and several high-affinity antibodies are now starting to be isolated by this method. For screening purposes, a library of cells (e.g. *Escherichia coli* or *Saccharomyces cerevisiae*)[117,118] displaying multiple copies of a different antibody variant is incubated with a fluorescently tagged antigen in a buffer solution. Cells displaying antibodies that specifically bind the ligand become fluorescently labeled and are easily isolated by fluorescence-activated cell sorting. With flow cytometry, the binding of each clone in the library to a particular ligand is easily determined quantitatively. Therefore, the brighter the fluorescent signal for antigen binding, the higher the affinity of the clone for the antigen. In addition, parameters such as ligand concentration or time for the dissociation of antibody-antigen complexes can be easily optimized. These features are particularly significant for

antibody affinity maturation and, in fact, the limited data reported so far indicate that cell surface display may be a superior screening technology for that purpose.[119-122]

9. Transgenic Mouse Technology

An alternative strategy for producing fully human antibodies is offered by transgenic mice that express human immunoglobulin genes.[123-125] In these animals, the endogenous mouse Ig genes are inactivated and replaced by large parts of unrearranged human Ig gene segments. The human DNA segments are fully functional in the mice and effectively undergo isotype switching and affinity maturation. Therefore, transgenic mice can be immunized with any desired antigen (including human proteins) and produce high-affinity human antibodies in a very similar fashion to hybridomas.[126-128] Abgenix (Xenomouse™) and Medarex (HuMAb-Mouse™) are two biopharmaceutical companies currently focused on the discovery and development of human antibody-based therapeutics derived from transgenic mouse strains. At this time, at least 33 human antibodies generated by transgenic mouse technology are in clinical development.[129] Examples of some of these antibodies are denosumab[130,131] (Amgen Inc.), a receptor activator of nuclear factor-κB (RANK) ligand-specific mAb for treatment of postmenopausal osteoporosis, and ipilimumab[132,133] (Medarex), a CTLA4-specific mAb for the treatment of melanoma.

10. Immunogenicity of Engineered Antibodies

Antibody therapeutics continue to demonstrate clinical efficacy in an ever wider array of indications. Murine mAbs have proved to be tremendously useful in diagnostics. However, when used in the treatment of patients with various ailments, their effect is not always sustained. This is often due to the development of HAMAs, which lead to clearance of the murine mAb and adverse events that are sometimes fatal. HAMAs have been the impetus for efforts over the last 20 years to reduce the murine content of therapeutic mAbs. Chimeric antibodies, constructed by linking murine variable and human constant regions,[5] were an important first step in Ab immunogenicity reduction. Although they possess greatly reduced immunogenicity relative to murine versions, chimeric antibodies still pose a significant risk of eliciting an immune response.[134] The development of CDR grafting,[7] whereby CDRs from a murine donor Ab are grafted onto human variable region acceptor FRs, was a subsequent advancement. The variety of humanization methods based on this technique [135] has produced antibodies that are more homologous to human sequences than the original chimerics mAbs, which often, but not always, result in a reduction of imunogenicity to clinically acceptable levels.[134] The main goal of CDR grafting-based humanization methods is the maximization of identity between a single donor and single acceptor sequence. The underlying assumption is that higher global sequence identity of a humanized sequence to the human acceptor results in a lower risk of immunogenicity. Nevertheless, with respect to molecular immunology, the global identity is just a fraction of the immunogenic potential.

The immunogenicity of a recombinant antibody depends on its ability to trigger either a cellular or humoral immune response. In the first case, the T cells recognize small peptides resulting from proteolytic processing of the agent displayed on the binding groove of the major histocompatibility complex (MHC) molecule of the antigen-presenting cells. In the second case, the B cells recognize the biotherapeutic agent as foreign and produce antibodies against it. In contrast to unfamiliar proteins, the native human Ab repertoire is non-immunogenic because the corresponding peptides either fail to bind to MHC-II or are not recognized by reactive T cells. Hence an administered protein will be non-immunogenic to the extent that its corresponding peptide sequences lack MHC and/or T-cell reactivity. Therefore, the identification and removal of MHC-II and T-cell reactive epitopes have become key strategies to reduce the immunogenicity of therapeutic proteins. This molecular understanding of immune reaction and tolerance highlights the limitations of humanization methods that employ CDR grafting. Standard protocols based on CDR grafting are poor engineering tools for addressing these other properties. CDR grafting typically leads to structural incompatibilities between CDRs and FRs, as well as across the VH/VL interface, resulting in substantial reductions in antigen recognition. These problems are often partially resolved by back-mutating crucial FR residues to the original murine amino acids, creating additional non-human sequences and thereby potentially restoring or generating new immunogenic potential. Because CDRs are often treated as inviolable, grafting of foreign donor CDRs onto a human acceptor framework leaves many non-human agretopes, including those within the foreign CDRs and ones generated at the FR/CDR boundaries. CDR grafting generally maximizes the donor-acceptor identity of the frameworks at the expense of linear sequence epitopes within the CDRs and at the FR/CDR boundaries.[136] This may be in part responsible for the lack of robustness in immune evasion observed for humanized antibodies. In fact, several humanized antibodies elicit a significant immune response when administered clinically,[134] and response incidences as high as 63% have been reported.[137]

11. Intracellular Antibodies

Intracellular antibodies, termed intrabodies, represent a new family of molecules that can be expressed within the context of a

cell to define or mediate function(s) of a particular gene product.[138] When fused to well characterized intracellular protein localization/trafficking signal peptides, intrabodies can be expressed in different subcellular compartments depending on the trafficking signals used. They are directed within a particular cellular compartment by the intracellular localization signals genetically fused to the N- or C-terminus of the antibody. When genetically fused to an intrabody, these short polypeptide 'signals' (e.g. PKKKRKV derived from the large T antigen of SV40) can direct the intrabody to the specific subcellular localization (e.g. nucleus) of its target antigen.[139] In principle, upon interaction with its target, an intrabody can modulate target protein function or achieve functional knockout by one of the following mechanisms: (i) modulating protein enzymatic activity by inhibiting its activity either directly, sequestering substrate, or maintaining the catalytic site in an active or inactive conformation; (ii) disrupting the signaling pathway of a target protein through interference with its protein-protein, protein-DNA, or protein-RNA interactions; (iii) diverting a target protein from its normal site of action such as sequestering nuclear localized proteins in the cytosol, targeting of cytosolic proteins to the nucleus, and retention of secreted or cell surface-expressed proteins in the endoplasmic reticulum (ER); and (iv) accelerating the degradation of target protein.

Besides specificity, other important criteria for choosing an intrabody would be the high antigen-binding affinity of the scFvs, making them attractive candidates for therapeutic purposes.[140] However, when expressed inside a cell, the behavior of scFvs is often unpredictable and does not always correlate with their *in vitro* binding affinities. Several studies have shown that the intracellular stability of an intrabody, and not affinity, is highly critical for its efficacy. The main factors contributing to scFv stability are disulfide bond formation and correct protein folding. The primary amino acid sequence of an scFv also contributes directly to its stability. In its natural environment, an antibody is directed to the secretory pathway where it is synthesized and processed to allow correct folding and disulfide formation.[138,141,142]

Once released outside a cell, the intrabody maintains its correct conformation and thus is stable in the oxidizing extracellular environment. However, in the reducing environment of the cytosol, formation of intrachain disulfide bonds does not occur. Therefore, the maintenance of correct folding is inefficient and results in protein aggregation.[139,143] This is the reason why scFvs with no disulfide bonds are used as intrabodies and not Fabs, which have a higher affinity but cannot fold efficiently in the reducing environment of a cell. Gene therapy applications of intrabodies in animal studies or eventually as therapeutic agents in human diseases needs to overcome other problems that are common for gene therapy. These issues are the development of efficient gene transfer vehicles and high level of regulated and long-term gene expression. These advances, combined with the recent improvements of viral and safer non-viral vectors, less immunogenicity, and the capability of achieving tissue-specific gene transfer through cell surface targeting, should enable intrabody technology to prosper.[143]

Intrabodies can also represent a valuable tool in functional genomics at the protein level (e.g. proteomics). Compared with other gene manipulation methods, such as gene knockout or RNA-based technologies (ribozyme, antisense RNA, or RNA interference), intrabodies have the unique advantage of targeting proteins in different cellular compartments as well as specific structural or functional motifs of a protein. The first report of the clinical application of this technology concerns an scFv specific for c-erb-B2 retained within the ER. The downregulation of the cell surface receptors resulted in the loss of the transformed phenotype. The same strategy has also been used with an anti-ras neutralizing antibody.[144,145]

12. Conclusions

Enormous strides have been made in the past decade towards the discovery, optimization, and therapeutic application of antibodies to a wide range of pathologies. Köhler and Milstein's dream of mAbs as elegantly sensitive therapeutics has finally been fulfilled. The examples presented in this review indicate that this class of drugs can be highly effective as therapeutic agents. From a stalled start, mAbs now represent approximately 30% of the novel biological entities entering clinical trials, indicating that the biotechnology community at large has finally recognized the speed and efficiency of the antibody platform.

As proof of the maturity of this field, the latest advancements in antibody design and scaffold engineering are poised to contribute to more innovative and extensive applications of biodrugs. It has been demonstrated that V-like domains provide alternative and efficient scaffolds for the presentation of paratopes capable of reaching buried epitopes and hence targeting refractory antigens. Because of the recent advances in scaffold design, repertoire construction, and selection strategies, there is currently rapid progress in the generation of specific, high-affinity mAb fragments against virtually any antigen. After intensive engineering and preclinical/clinical testing, antibody fragments are now set to join mAbs as powerful therapeutic and diagnostic agents. Increasingly, antibody fragments are also being applied to the proteomic discovery of new cancer biomarkers and exploitation at the level of robust sensitive immunosensors. Overall, it is also becoming

clear (and ironic) that the classical mAbs may yet be the commercial saviour of the high-tech proteomic and genomic revolutions.

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