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Review

A Review on The Development, Production Strategies, and Utilization of Monoclonal Antibodies

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ABSTRACT

Over the last 20 years, monoclonal antibodies have become the backbone of biological therapeutics for the treatment and diagnosis of several diseases. The rising incidence of cancer and other immunologic diseases promoted the increasing investments of the global pharmaceutical industry in monoclonal antibody development. The R&D has focused on the highest efficacy which is majorly correlated with the antigen-binding specificity and the lowest immunogenicity of monoclonal antibodies. This review aims to provide a brief description and explanation of each stage in the development path of mAbs.

Keywords: monoclonal antibodies, industry, cancer

INTRODUCTION

As pharmaceutical technology focuses on a new area of personalized treatments, the monoclonal antibodies represent the biggest point of interest. Healthcare professionals, as well as the pharmaceutical industry, rely on these new therapies due to their superiority over traditional treatments such as the creation of an immortal cell line producing the specific antibody for a unique antigen. They bind to a single site (epitope) of an antigen (1).

The earliest mAbs were used as laboratory agents for immunoassays. They were initially tailored in the laboratory to provide reproducible and highly specific immunoassays such as particle agglutination, enzyme-linked immunosorbent assays, radioimmunoassays, immunofluorescent-antibody assays, and immunohistology (2). However, they expeditiously developed into therapeutic and diagnostic agents. Since their discovery, they have been used widely for the diagnosis of several diseases such as leukemia, breast cancer, multiple sclerosis, arthritis, asthma, psoriasis, melanoma, and transplant rejection (3). Currently, they represent a group of therapeutics treating millions of patients with revenue that is expected to reach $125 billion by 2020 (4).

The first monoclonal antibodies were isolated from the mouse by hybridoma technique by César Milstein and Georges Köhler in 1975 which brought them in a Nobel prize in 1984 (5). Orthotone (OKT3) was the first monoclonal antibody approved in 1986 for the prevention of kidney transplant rejection. After this first approval in 1986, there was a long gap until a new monoclonal antibody was registered (6). In the late 90 s first chimeric monoclonal antibodies were approved. This was followed by humanizing and fully human mAbs afterward. The sales and growth of the monoclonal antibody market have expeditiously increased. Up to date more than 70 mAbs and 15 biosimilars were approved for clinical use. Today hundreds of monoclonal antibodies, including full-length mAbs, Fc fusion proteins, Fab fragments, drug-antibody conjugates, are either under review or in phase III studies. These new treatments have diverse clinical applications and multiple targets mostly in oncology and hematology followed by autoimmune diseases, chronic inflammatory diseases, infectious diseases, transplantation, cardiovascular diseases and orphan diseases with a few patient populations (7). With this review, each stage in the development path of mAbs will be described and explained in detail.

Development of monoclonal antibodies

Antibodies are immune system components with the ability to bind target proteins and activate other functions of the immune system. They can remain in circulation for a few weeks. All antibodies have a basic structure that consists of two identical heavy and two identical light chains. The heavy chain carry one variable (VH) and three constant (CH) regions while the light

chain carry one variable (VL) and one constant (CL) region. These two chains are attached to each other by disulfide bridges (8). An antibody can be separated into two regions; Fc and Fab. Fab region is the upper part and it is the variable region where antigen recognition and binding occurs. Fc region is the lover part and this constant region responsible for the interactions with other immune system components and activates biological reactions in the system (9).

Monoclonal antibodies (mAbs) are single epitope-specific antibodies generated from a single B-lymphocyte clone. They are tailored to recognize unique binding sites, epitopes on an antigen. This is what makes these antibodies more specific and more preferred agents than polyclonal antibodies (10).

Monoclonal antibodies generated by hybridoma technique

Köhler and Milstein were the pioneers of this technique which involves the utilization of immortalized B lymphocytes to produce monoclonal antibody. They isolated B-lymphocytes from the spleen of mice after immunizing them against a unique epitope on a single antigen. This method is based on the fusion of B-lymphocytes which have a limited lifetime in cell culture and myeloma cells with suppressed secondary purine nucleotide synthesis pathway and selection of only hybridoma cells that can survive in culture conditions with suppressed primary synthesis pathway (11).

The first process step involves incubation of myeloma cells treated with 8-azaguanine containing medium for 1 week. This results in a mutation in the gene sequence encoding hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) enzyme synthesis which crucial for the secondary purine synthesis pathway therefore the secondary purine synthesis pathway of myeloma cells is blocked (12).

The second step of the process is the chemical or electro-fusion of myeloma cells with unlimited lifetime but blocked secondary purine synthesis pathway and B-lymphocytes with both pathways active but a limited lifetime. After fusion, the cells are cultured in medium containing HAT (hypoxanthine-aminopterin-thymidine). Mutagen aminopterin used in HAT selection blocks the de novo (primary) purine synthesis pathway and manipulating them to utilize the secondary pathway. Myeloma cells with blocked secondary synthesis pathway cannot survive in this culture conditions as well as B-lymphocytes with a limited lifetime in vitro since the cultivation takes 1-week. Only the hybridomas (myeloma cells engaged with B-lymphocytes) can remain alive since they acquired immortality from the myeloma cells and selective-resistance from B-lymphocytes (myeloma cells carrying HGPRT mutation are unable to synthesize nucleotides via primary pathway since this pathway is inhibited by aminopterin in the HAT selective medium). This preliminary hybridoma culture is still polyclonal because each primary B-lymphocyte generates its specific antibody to medium. This mixture of polyclonal antibodies needs to be diluted and separated into hundreds of culture well plates. After separation, each well is screened fort he targeted antibody activity. Positive wells containing hybridomas with desired antibody activity are recloned and tested again for activity (1).

The first approved monoclonal antibody was Orthoclone (OKT3 – Muromonab-CD3) generated from mouse cell culture for application in humans against T lymphocytes to prevent organ reject (host versus graft reaction) after kidney transplantation. CD3 was the cognate antigen for this mouse IgG2a antibody which binds selectively to this antigen and inhibits its activity on T-lymphocytes. However, due to adverse events reported such as the development of human anti-mouse antibody (HAMA) response, application of this therapeutic monoclonal antibody was limited. Also, problems like the genetic instability and low yielding profile of hybridomas and the rare availability of suitable myeloma cell lines precluded the successful production of these early monoclonal antibodies (7).

These stumbling blocks fostered the pursuit of next-generation production techniques. Many different monoclonal antibody expression systems were tested and each having limitations. *E. coli* was a highly suitable expression system for antibody single-chain variable regions and Fab fragments. It was also an advantage that *E. coli* does not contain glycosylating enzymes that inhibit protein-protein interactions which in turn can prevent potential immune reactions. However, such a small microorganism was insufficient to synthesize a relatively larger molecule consisting of four individual chains (13).

Chimeric Monoclonal Antibodies

Right after OKT3 approval, a researcher Greg Winter discovered that the mouse monoclonal antibodies can be humanized by combining human constant antibody regions and animal antigen binding (variable) regions. The technique of manipulating and recombining genes to generate the next level of therapeutic monoclonal antibodies is known as chimeras (14). To prevent HAMA response in humans, the development of human chimeric monoclonal antibodies has been accelerated. Production of human chimeric mAbs, such as Abciximab (ReoPro, approved in 1994 for the inhibition of platelet aggregation) uses the known murine hybridoma technique however after that involves an additional process of replacing the constant region of the generated murine antibody with the corresponding constant region from a human antibody (15).

By replacing the murine antibody constant region with the human constant antibody region as much as possible, the HAMA response could be reduced to a point. Chimeric antibodies were two-thirds human with four murine variable regions and eight human constant regions. However, even when all the constant regions were replaced with human regions HAMA response could not be prevented. This was a limitation for long term administration of these human chimeric antibodies at repeated doses (16).

Humanized Monoclonal Antibodies

To overcome the limitation of some human chimeric antibodies still causing HAMA response when administered, a technique with a genetic engineering approach was proposed. This approach was an extension of chimera strategy to generate much more humanized monoclonal antibodies, such as Daclizumab (Zenapax, approved in 1997 for the prevention of acute organ

rejection in patients with renal transplants) which is composed of murine (10%) and human (90%) antibody sequences (17).

To optimize antigen-binding affinity of the antibodies and minimize the immunogenicity, several rational methods to humanize murine antibodies were characterized. Each was designed to achieve better results, minimizing problems faced with the previous strategy. Three main variants were described to design a method; designation of regions determining the antibody affinity/specificity, source of human gene sequences being framework region donors (maybe germline or mature gene sequences), designation of residues other than the domain determining affinity/specificity and decide the target for back mutation for restoring or improving the specificity/affinity of the humanized monoclonal antibody. Each approach described below is mainly based on the same design cycle but has different strategies with a different set of variants such as antibody structure, binding affinity, and source of human sequences. These rational methods can be listed as; CDR grafting (using mature of germline sequences), re-surfacing, superhumanization, and human string content optimization (9).

The technique called CDR grafting involves replacing only the complementary determining region residues (CDRs) and grafting these into a human framework. Since only 25% of the variable region residues are CDRs and involved in antigen interaction, CDR-grafted antibodies were more than 90% humanized. These grafted monoclonal antibodies are sometimes called mosaic mAbs. This technique was a further step of initially generated mouse mAbs (18). However, humanized monoclonal antibodies had an unintended consequence of altered antibody specificity. The binding affinity was changed since human framework residues could alter the CDR orientation. For example, when only the antibody heavy chain was CDR grafted the binding affinity of the antibody was decreased 10-fold. This same antibody suffered 100-fold loss of binding affinity when both chains were CDR-grafted. This CDR loop conformation alterations and loss of binding affinity were attributed to the absence of some murine residues referred as vernier zone residues in the final humanized antibody. These residues are located closely to CDRs in the β-sheet framework regions (19). Therefore, CDR grafting by vernier zone retaining method was proven to be more advantageous and this technique has been the most prevalent method for the humanization of antibodies. Trastuzumab (Herceptin, approved for the treatment of HER2 positive breast cancer) was one example of the mAbs produced by grafting CDRs of murine antibody to human IgG framework with retaining amino acids of vernier zones. Since in this method more murine amino acids (of CDRs and some vernier zone residues) were retained in the final antibody compared to the antibodies produced by straightforward grafting, inducing immunogenicity in humans is more likely (20).

Germline humanization technology is another humanization method in which human germline genes are the main source of human framework regions based on the resemblance of the human CDR structures to those of the murine antibody to be humanized. The structural similarity is assessed with the residue-to-residue homology score of the murine CDRs to human candidates having the same canonical structures (21). Compared to mAbs humanized with framework regions in IgG, humanized mAbs with germline framework regions are expected to cause lower immunogenicity. This is because the germline genes are less somatically hypermutated where human mature sequences carry somatic mutations generated randomly. This was the basis of the idea of humanizing murine antibodies with these germline sequences. Antibodies produced with germline sequences are less immunogenic however IgG-derived sequences are sometimes more advantageous due to their higher antigen-binding affinity (16).

Antibody resurfacing method is an alternative antibody humanization strategy to CDR grafting described by Pedersen at al. This method involves maintaining the core of the murine variable region and the CDR framework but replacing the potentially antigenic surface residues of the framework region with the human surface residues. This method was based on the idea that the surface residues are the only reason for the HAMA response to variable murine regions. mAbs humanized by this method show a little change in affinity and stability (22).

Superhumanization is another humanization strategy that aims to lower the immunogenicity caused by murine CDRs. It also proposes to use human germline sequences to make humanized antibodies. The strategy was based on structural similarities between human and mouse CDRs instead of frameworks. The most critical step of this strategy is to identify human germline V genes for CDR residues that have the same canonical structure as the CDR residue structure of the mouse antibody of interest. In the identified sequences, remaining nonhomologous CDR residues are converted to the mouse antibody sequence. The antibodies constructed by this strategy retain antigen-binding affinity and ability since they are CDR-grafted to have a design that has the minimum alteration from human sequences so that they are called "superhumanized" (16).

In 2007, Lazar et al. described a novel technique for the humanization of antibodies based on a new immunologically relevant criteria of antibody humanness called human string content (HSC). This strategy compares the mouse sequences with the human germline gene repository and the deviations are scored as HSC. Then the target sequence is humanized by HSC maximizing instead of using its global identity therefore, it allows the sampling of human diversity from different germline sequences in the framework and CDR regions and multiple diverse candidate sequences are generated (23). This method was performed for the humanization of four different antibodies with varying antigen specificities (24). Resulting variable regions of the antibody were fundamentally different from those of CDR-grafted antibodies, HSC optimized antibodies being immunologically more human and it was derived from several human germline sequences. Additionally, these antibodies have a better or comparable antigen-binding affinity without being subject to affinity maturation process (9).

Human antibodies

As mentioned above, several different approaches were developed to overcome the immunogenicity problems and antigen-binding affinity loses of antibodies. Each strategy had its advantages and disadvantages. Comparing one to another, a further strategy to develop more compatible antibodies was necessary.

Transgenic animals for mAb production

The most genuine method was to start from original human genes to generate the most coherent human antibody. In 1994, new technologies for human monoclonal antibody production from transgenic mice were first described by two different groups by the publication of two transgenic mouse lines, the HuMabMouse (25) and the Xeno-Mouse (26). They reported the development of mice with four germline modifications of heavy and light chain genes; two of them were targeted disruptions and other two with human transgenes. These two groups used different technologies to engineer and introduce human sequences; one used pronuclear microinjection other used fusion of yeast protoplast to introduce yeast artificial chromosome-based transgenes (25, 26). In the light of these reports and further studies, transgenic animals with human genes were developed to generate fully human antibodies with high antigen affinity. Genetically-engineered animals were found to be robust engines to derive diverse repertoires of full human variable regions which also possess intrinsic drug-like properties such as high specificity, high potency, solubility, manufacturability and low immunogenicity risk (27).

This technique involves the development of transgenic animals carrying human immunoglobulin (Ig) genes in the germline and the genome is rearranged to express human Ig chains from mouse B lymphocytes. These mice were engineered in a way to prevent the expression of murine Ig chains by silencing the endogenous murine Ig loci by gene targeting techniques. Then, to these animals with the ability to express human antibody genes, the conventional immunization strategy is applied and the mAb technique (activated B cells being fused with mouse myeloma cells, testing/screening, freezing) is followed (28). Panitumumab (Vectibix, approved in 2006 for the treatment of epidermal growth factor-receptor –EGFR– positive colorectal cancer) was the first fully-human therapeutic antibody derived from a transgenic mouse. In preclinical mouse models, panitumumab was proven to be more potent compared to mouse antibody m225, the parent of a chimeric EGFR antibody which is already approved and marketed, cetuximab. Since the fully human antibody, panitumumab does not carry mouse sequences and has a higher affinity, it appears to be non-immunogenic and requires a lower dosing schedule than chimeric antibody (29). Another fully human monoclonal antibody against EGFR is zalutumumab is an IgG1 antibody and was also more potent in preclinical xenograft models (30).

Ipilimumab, approved by the FDA in 2011 for the treatment of melanoma, targets T-cell inhibitory receptor cytotoxic T-lymphocyte antigen (CTLA-4) demonstrated enhanced immune responses which mediate tumor rejection in mouse tumor models and it was shown to stimulate a humoral immune response when co-administered with vaccines (31).

Denosumab, another monoclonal antibody generated in transgenic mice, is a fully human IgG2 monoclonal antibody that specifically binds to receptor activator of nuclear factor kappa-B ligand (RANKL), represses bone resorption markers in patients with various metastatic tumors with no reported severe adverse events (32).

These animals produce fully human antibodies without the need for laboratory humanization processes. Several different strategies including yeast protoplast fusion with embryonic stem cells and pronuclear microinjection were developed to achieve transgenic animals generating human antibody repertoires (33). Oocyte microinjection of gene loci on yeast or bacterial artificial chromosomes or transfection of embryonic stem cells were used in rodents. Manipulated fibroblasts with human chromosome fragments of artificial human chromosomes were used to derive transgenic ruminants (34). The largest human immunoglobulin repertoire was created by an innovative technology called micro cell-mediated chromosome transfer (MMCT) in which micro cells derived from human fibroblasts were fused with mouse embryonic stem cells which resulted in generation o pluripotent cell lines carrying a human chromosome or fragment (35). Tomizuka *et al.,* using this technique, generated embryonic stem cell lines and chimeric mice carrying human chromosome fragments of chromosome 2 and 14 including immunoglobulin (Ig) kappa light chain and heavy chain genes. Also, they have achieved to generate chimeric mice carrying intact human chromosome. They reported that transferred chromosomes were stably preserved and human immunoglobulin genes were expressed properly in chimeric tissues (36). Gene silencing was achieved either by gene targeting either in fibroblasts or embryonic stem cells, via DNA microinjection by zinc finger technology. Comparisons demonstrated that fully human antibodies from transgenic animals are not produced as efficiently as wild type immunoglobulins. This limitation was attributed to imperfect relation between human antibody constant region and mouse endogenous signaling components (34). Therefore, the production of antibodies by this technology was limited with the antibodies which can be recognized by the mouse immune system (37).

Display Technologies in mAb production

Therapeutic monoclonal antibodies have been developed successfully by the help of display technologies that involve the display of antibodies or antibody fragments on the phage, yeast or bacteria surfaces. Phage display technology, being an *in vitro* selection system is not related to the *in vivo* immune response like in transgenic animal technique. Therefore, display technologies can be a remarkable tool for antibody discovery. It is the primary and a widely used in vitro antibody production and selection technology (38).

The technique was first demonstrated in 1985 by George P. Smith and since then several research groups have focused on this technique and contributed to the improvement of applications that have advanced the therapeutic antibody field. In 2002, adalimumab (Humira, approved for moderate-to-severe rheumatoid arthritis therapy alone or in combination with methotrexate or other disease-modifying anti-rheumatic drugs) was the first human mAb approved by FDA (39) and is currently the

best selling mAb in the market. Phage display technology enables the isolation of human mAbs specific to almost any antigen by clonal antibody selection in prokaryotic systems. Phages are viruses that can infect bacterial cells. In recombinant DNA technology, the most commonly used vectors are bacteriophages that infect the recombinant DNA host *E. coli*. Such recombinant DNA vectors contain extensions of foreign DNA, that can be expressed in the host when the vector replicates. This is one of the most essential features of a recombinant DNA vector. A phagemid vector provides these essential features and allows the expression of foreign DNA which is intentionally inserted in the vector as expressed in fusion with a phage protein to be displayed on the surface of the phage (40). A variety of bacteriophages have been used in phage display, most commonly used were filamentous bacteriophages which are highly productive. Filamentous phages are non-lytic phages that carry single-strand DNA. The Ff, M13, f1, and fd are essential phages that are commonly used in phage display, M13 being the most frequently used (41). The most important property of M13 is that unlike other phages, it can be easily purified and used. Upon binding to its specific receptor, the replication of M13 is initiated. So that, bacterial cells are infected with M13 phages. In other words, the bacterial cell is the M13 phage production factory of which cell growth is slowed down due to stress caused by the M13 production. Upon binding to the bacterial cell, the single-stranded DNA of M13, with the help of bacterial cytoplasmic enzymes, is converted to double-stranded DNA (known as the replicative form – RF) (42).

The vectors generated by fusing portions of phage and plasmid genome are called phagemid vectors which are designed for cloning and fusion protein expression. Phagemids contain a gene sequence encoding the coat protein that will get integrated to the foreign DNA to be expressed, and also a stop codon which will allow host-specific fusion protein or soluble partner expression. During assembly, for fusing into a phage particle, the phagemid carries an additional morphogenetic single-stranded DNA replication signal (38). Phagemids can retain themselves as plasmids, enabling the expression of target protein in the bacteria, but they do not carry the other genes to retain phage assembly. So, the phagemid-transformed host cells are essentially infected with a helper phage for the generation of viable phage particles. The bacteriophage itself is the helper phage, that is designed for the supply of the proteins essential for phage assembly.

The diversity of antibodies from antibody repertoires is dependent on the initially estimated complexity of the fragment gene sequence library. Since the antigen-binding region is a heterodimer, the complexity of the library is enriched by the random fusion of heavy-chain and light-chain sub-libraries (40).

Antibody libraries are enormous collections (more than 1010 genes) of antibody gene sequences encoding different antibodies with unidentified features. They represent an fundamental source for antibody discovery by *in vitro* selection methodologies especially phage display, and their construction is very crucial. There are two leading types of antibody libraries: immune libraries and universal libraries.

Immune libraries are where blood cell samples are collected from immunized donors, which, are limited to vaccinated donors, patients who suffered a disease or an infection, for human antibodies. In medical research, these libraries are commonly used for the discovery of antibodies against targets of infectious pathogens such as HIV, West Nile virus and immunized cancer patients. Immune libraries can also be constructed from immunized animals. Use of transgenic animals carrying human antibody gene sequence repertoire enables the discovery of human antibodies from such libraries (38). The advantage of these libraries is that the affinity maturation is already completed in the organism and isolated antibodies present high affinity and specificity to the target antigen. On the other hand, the limitations of immune-libraries are; the necessity to build a new library for every single antigen, expensiveness, ethical issues, and laborious process and that the desired antigen has to be immunogenic *in vivo* (43).

Universal libraries constructed using naïve natural human antibody gene sequence repertoires are related to the human antibody germline to some extent and demonstrate lower incidence of immunogenicity. These libraries are also named "single-pot" libraries that store an extensive repository of antigen specificities almost counting in every possible antigen (44). Naïve antibody libraries are developed from realigned V genes of B lymphocytes from non-immunized naïve donors, such as the IgM repository. The antibodies in these libraries have already been selected *in vivo* for functional B lymphocyte receptors, including the low immunogenicity and low toxicity selection. Affinity engineering also can be done using phage display (45).

Other universal libraries, other than naïve ones, which are constructed fully with natural human IgM repository, additionally semi-synthetic or synthetic universal libraries exist. They consist of solely synthetic gene sequences or a combination of synthetic and natural gene sequences, respectively. Semi-synthetic antibody libraries are derived either from circumstantial V gene sequences from pre-B lymphocytes or by utilizing an antibody framework consisting of one or diverse CDRs together with randomized sequences. Fully synthetic antivody libraries are employing with 7 human VL and 7 VH frameworks provided with synthetic CDR cassettes (38).

Summaries of isolation steps of phage display generated antibody are described as following;

Gene fragment production: The first step for the identification and isolation of monoclonal antibodies from a phage-displayed library is the constitution of antibody-library (46). High grade mRNA from human PBMCs (peripheral blood mononuclear cells) is extracted and cDNA is reverse-transcribed. The synthesized cDNA carries the genetic codes for all antibodies specific to various antigens consisting of numerous lymphocyte clones with an approximation of 109 to 1011 clones (28). The VH and VL polymerase chain reaction products, making up the Ig-encoding repository, are bound into a phageamid, a phage display vector (46).

Gene fragments cloning in the phagemid: Genes encoding different clones of antibodies are fragmented by restriction enzymes and cloned into phagemid vectors, then displayed on the phage surface. Optimum isolation of antibodies is dependent

on the sequence diversity of gene fragments at this step. So far, several different phages such as phage λ, T4, T7, and M13 have been identified for displaying antibody fragments. Some of these vectors assist to the antibodies displayed on the surface of phage to retain their functionality. But the most applicable is the utilization of phages like M13, which does not damage the bacterial cells. The phagemid vectors require a helper phage to exit from the host cell and enter the medium.

Selection of specific phages: Due to the diversity in the gene sequence for the targeted antibody, a variety of antibody clones display on the phage surface after cloning. Specific clone which will recognize the targeted antigen is selected by a process called biopanning. Using the specific antibody – antigen-binding property, the phage carrying the targeted antibody can be isolated because all surface antibody fragments are functional. This step involves the enrichment by biopanning of the targeted antibody present on the phage surface based on their affinity to bind target antigen (28). For in vitro selection, target antigens are immobilized on a solid surface. Polystyrene surfaces, such as immuno-tubes and 96-well immuno-plates have high protein binding capacity and are used widely for immobilization of antigens. Also, magnetic beads with protein G/A, streptavidin, maleimide, or N-hydroxysuccinimide may be utilized for the immobilization of antigens for biopanning in vitro (46). The target antigen is immobilized, phages are bounded to the target immobile antigen, non-bonded phages are washed and removed, bonded phages are eluted, bacterial cells are re-infected so that eluted phages are amplified, recombinant phages are purified and antibodies on the phage surface are re-expressed. All these steps of biopanning are repeated approximately 5 times to finally isolate the specific antibody with the highest affinity to the target antigen (28). This process is a crucial step of phage display that permits the isolation of mAbs in a short period of almost two weeks, far more rapidly than conventional hybridoma method.

The biopanning technique is not only limited to previously identified recombinant proteins. Actually, the phage display method can also be used to extract antibodies against tumor cell surfcae antigens that were not identified previously. Phage display method is very useful for applications in cancer stem cell (CSC) research, and antibodies eith the ability to bind known SCS markers such as CD44 and CD133 have already been characterized from phage display libraries (46).

Screening: The main aim of the step is to isolate antibodies with the highest affinity to the target. Different methods are used for screening such as immunoassay, immunoblotting, isolation due to fluorescent features, immunocytochemistry. Immunoassay screening involves the coating of the antigen on a solid phase, the addition of phage displaying antibody and finally the detection of surface proteins by a secondary antibody. Following incubation and washing, in the appropriate wavelength, the signal values and therefore binding is measured (28).

The abovementioned display technology has provided advantages for the therapeutic mAb discovery, however, it also has disadvantages. The largest phage display library consists of approximately 10 billion human antibody sequences derived from naturally occurring gene sequences. Using phage display enables the screening of many antibodies at once and the identification and isolation of specific antibodies of interest (47). Additionally, the characterisation of mAbs with phage display method is a completely in vitro process. Therefore, immunological tolerance is not a restriction for the process, this enables the characterisation of antibodies against antigens with suboptimal immunogenicity or those that are not possible or hardly possible to obtain with animal immunization techniques. The in vitro feasibility of the method may be especially helpful when determining antibodies against pathogens with mutations which can cause an outbreak of infectious diseases (46). The disadvantage of this technology is that libraries are not constructed of full-length antibodies; gene sequences are not actual human sequences but are based on human sequences and the proteins are not expressed by human cells but are expressed by bacteria. To partially overcome this situation, yeast, a eukaryote, are used for some post-translational modifications which are not possible in procaryotes, however, the modifications may be still different from human (48).

Fully human monoclonal antibodies generated by phage display or in transgenic animals usually show a lower incidence of HAMAs than their humanized counterparts, however, immune responses persist and differ by product and indication (16).

Future perspectives

Here, we summarize the development path of monoclonal antibodies, the strategies to improve their therapeutic effects and their increasing importance in clinical use. The antibody therapeutic market has undergone rapid expansion in recent decades and monoclonal antibodies have become an attractive therapeutic option especially for the treatment of cancer, infectious diseases and autoimmune diseases due to their flexibility and specificity. Monoclonal antibodies have a precise target selectivity compared to traditional small molecules. Recently, mAbs are designed as fusion proteins, antibody-drug conjugates, antibody fragments and bispecific antibodies. These new methodologies have provided a broader therapeutic potential. New methods such as single B cell antibody technology, human antibody mice have been described to generate fully human antibodies however phage-display technology represents the most advantageous platform due to its economical and efficient in vitro methodology. More recently advanced Technologies such as next generation sequencing, single cell sequencing and robotic screening have a great potential to improve therapeutic antibody discovery. Each challenge faced during this journey was responded with sophisticated engineering strategies such as immunomodulators, bispecific mAbs, antibody-drug conjugates, and CAR-T cells. The continuous and accelerated development of mAb therapeutics will be fostered by the identification of novel biomarkers in order to enhance specificity and efficacy and antibody therapeutics market will become a major driver of the biopharmaceutical industry.

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