

New Technologies for the Generation of Human Monoclonal Antibody

Jian Ni



About the Author: Dr. Jian Ni is Co-Chairman, Committee of Key Laboratory of Antibody Technique, Ministry of Health; Director, Committee of Monoclonal Antibody, China Medicinal Biotech Association; Visiting Professor of Shanghai Jiao Tong University, Second Military Medical University and Nanjing Medical University, Chairman and Chief Scientist of Human Antibodomics (SIP) Inc. He is Chairman, Committee of Medicine and Pharmaceuticals, Vice President, Biotechnology Association of Jiangsu; Chairman, Committee of Medicinal Biotech, Vice President, Shanghai Society of Biotechnology; President, Suzhou Biopharmaceutical Association; Chairman, Committee of Interferon and Other Cytokines, Executive Director, Chinese Society of Microbiology. Dr. Jian Ni obtained his M.D. from Second Military Medical University and Ph.D. from University of Cambridge. Dr. Ni was a Post-doctoral Fellow at the National Cancer Institute and University of California, Irvine. He is an American Society of Clinical Pathologists board certified Specialist in Immunology. Dr. Ni was a Senior Scientist of Human Genome Sciences, Inc., He has published more than 88 scientific articles and 171 issued US patents. Dr. Ni was the President of Chinese Biopharmaceutical Association, USA from 2001-2003 and a Board Director since 1999..

Introduction

Therapeutic treatment of human diseases using human monoclonal antibodies (mAbs) has proven to be effective, with few side effects. The development of mAb drugs has become a major priority within the biotech and pharmaceutical industries. MAb drug development activity has experienced explosive growth in the past 30 years since the process for creating mAbs was introduced^[1-4]. Twenty - five therapeutic mAbs and 4 Fc-fusion proteins have been approved by the Food and Drug Administration (FDA), nine of which have exceeded annual sales of \$1 billion. Numerous new technologies to perfect the design and production of mAbs for therapeutic, diagnostic, and other purposes have been developed within industry and by academics. Over the past two decades, technologies have emerged for generating mAbs derived from human immunoglobulin gene sequences by phage display and transgenic mice, human B cells directly by Human-Human Hybridoma, Hybrid hybridoma, B cell immortalization and cloning or Single-cell RT-PCR. These human mAbs provide an alternative to re-engineered, or de-immunized, rodent mAbs as a source of low immunogenicity therapeutic antibodies. There are now three marketed fully human therapeutic mAbs, adalimumab, panitumumab and Golimumab, and several dozen more in various stages of human clinical testing. In this review, I describe briefly the new technologies for the generation of human mAbs. I also discuss the trend of antibody therapies in the treatment of various human diseases and new technologies that drive the fast growth of the industry.

Antibody therapeutics: from mouse to human

Despite their apparent potential, early attempts to make use of mouse antibodies as therapeutic agents were largely unsuccessful, mainly due to the immunogenic reaction and insufficient cytotoxic activity of murine based mAbs. Newer technologies for the production of chimeric, humanized and human mAbs produced in vitro (phage display)^[5,6] and in mouse (transgenic mice)^[7] have alleviated these problems. Since the mid-1990s, antibodies have emerged as an important new class of drugs for therapeutic use across diverse clinical settings, including oncology, chronic inflammatory diseases, transplantation, infectious diseases and cardiovascular medicine. Humanized or human mAbs with low immunogenicity, enhanced antigen binding and reduced cellular toxicity provide better clinical efficacy. Higher technical and clinical success rate, along with the overcoming of technical hurdles in large-scale manufacturing, low cost of market entry and IND filing, have attracted large amounts of funds and resources towards human and humanized mAb R&D. So far, 200 companies with hundreds of new projects and targets have joined the

antibody bandwagon. This has brought billions of dollars worth of investment towards mAb R&D, as well as acquisitions and licensing deals, which together have led to this current mAb gold rush.

The 29 FDA approved antibody therapeutics include 19 unmodified IgG molecules, 2 radioimmunoconjugates, 1 antibody–drug conjugate, 3 Fabs and 4 Fc-fusion proteins; these therapeutics comprise 4 different types: 3 murine, 5 chimeric, 11 humanized and 3 human. At least 190 additional antibodies are in various stages of clinical development. One of the strengths of antibody therapeutics is that they belong to a well-established drug class that has a high success rate from the first use in humans to regulatory approval; the success rate amounting to 29% for chimeric antibodies, and 25% for humanized antibodies. This compares favorably with the 11% success rate for small-molecule drugs. The global sale of mAbs in 2008 was \$35 billion. Remicade had annual sales of \$5.3 billion in 2008. Rituxan, with 2008 sales of \$5.1 billion, was the best selling mAb and biological product. Overall in 2008, 9 mAb brands and 1 Fc-fusion protein had sales exceeding \$1 billion.

Early technologies for generating antibody therapeutics

The first developed and most widely used technology for the generation of mAbs is the use of mouse hybridomas, generated from the stable fusion of immortalized myeloma cells with B cells from immunized mice^[8]. This invention opened the door for the development of antibody-based drugs. However, because rodent mAbs comprise foreign protein sequences, most of the early drugs that entered clinical development elicited immune responses from human patients. Advances in molecular biology, involving the manipulation of gene sequences *in vitro*, and the expression of these manipulated sequences in bacterial, fungal, and mammalian cell culture systems, provided methods for re-engineering rodent antibodies to partially replace the rodent antibody sequences with functionally equivalent human amino acid sequences, thus reducing the overall immunogenicity without destroying the recognition properties of the original antibody.

Murine Hybridoma

The history of antibody therapeutics is an evolution of antibody engineering allowing the production of antibodies with increasingly fewer immunogenicity reactions in humans. In 1975, Georges Köhler and César Milstein of the UK Medical Research Council's Laboratory of

Molecular Biology in Cambridge invented hybridoma technology, later honored with a Nobel prize, which for the first time allowed researchers to fuse antibody-producing cells from immunized mice with antibody-secreting mouse cells derived from a type of cancer called myeloma, and generated hybrid cell lines that could be cloned and cultured indefinitely^[8]. In 1986, Johnson & Johnson developed Orthoclone OKT3 (muromonab) for the treatment of renal allograft rejection that was resistant to steroid and antilymphocyte globulin^[9]. However, murine antibodies are from mice and the application of murine antibodies was extremely limited in humans because it caused an immunogenicity reaction, known as the HAMA (Human Anti-Mouse Antibodies) response, including anaphylaxis and hypersensitivity reactions. This immunogenicity reaction is triggered by the human immune system recognizing the molecules as foreign because murine antibodies are different from natural human antibodies. In addition, murine mAbs consequently have a short half-life relative to human antibodies.

Chimeric antibodies

Until the early 1990s, most product candidates in development were mouse mAbs that elicited production of human anti-mouse antibodies in humans, resulting in rapid clearance, limited efficacy, and safety risks, such as immunogenicity and allergic reactions. The limitations of mouse mAbs were especially problematic in the treatment of chronic and recurring human diseases that require repeated antibody administration. These issues became the driving force for the development of numerous approaches to generate partially or fully human mAbs. In the past, making completely human monoclonals was impracticable, because there was no human myeloma cell line suitable for making hybridomas. So scientists turned to genetic engineering of both mouse antibody-producing cells and hybridomas, mixing and matching DNA from mouse and human antibody genes in an attempt to make antibodies that would not be rejected by the human immune system. The first chimeric antibodies, where 30–35% of the molecule was derived from mouse antibody sequences and around 65–70% was derived from human antibody sequences, were reported in 1984^[5–6]. Chimeric mAbs are generated by using recombinant DNA technology, where the murine Fab (fragment antigen binding) region is fused to the human Fc (fragment crystallizable) region of an antibody. Chimeric antibodies turned out to be more successful therapeutic agents than murine mAbs in overcoming the immunogenicity problems. As chimeric-antibody technology transferred to the biotech industry, a healthy pipeline of monoclonals lined up for FDA approval. In 1994, Eli Lilly of Indianapolis gained

permission to market the first chimeric antibody, ReoPro, which lessens the risk of blood clots in patients with cardiovascular disease by targeting a receptor protein on the surface of platelets. The 2 most successful mAb therapies, Biogen Idec/Genentech/Roche's Rituxan (rituximab) and Johnson & Johnson/Schering-Plough's Remicade (infliximab), are both chimeric. Their success has been attributed to the lack of immunogenicity reactions, as well as successful target selection. However, as a substantial portion of chimeric mAbs are murine, there is still a considerable risk of the human immune system recognizing the molecules as foreign and eliciting an immunogenicity reaction, known as HACA (human anti-chimeric antibody) responses.

Humanized Antibodies

The next technological development after chimeric antibodies to minimize immunogenicity reactions was humanization technology. It was initially termed reshaping and is now more commonly referred to as "humanization". This can be used to generate mAbs where 90–95% of the sequences are of human origin and 5–10% are of murine origin^[7]. Humans generate about 10% diversity in the antibodies' protein sequences during the antibody maturation process by gene splicing and mutations. Therefore the remaining 5-10% non-human portions in the humanized antibodies becomes more tolerable in humans. The technique uses genetic engineering to transfer the murine antigen binding sequences, known as the complementarity determining region (CDR), to a human antibody scaffold. The CDR region is part of the Fab region, which carries the antigen binding specificity. The CDR sequences transferred are usually optimally selected to those that confer the binding specificity and strength, keeping murine sequences to a minimum in the resultant mAbs. In 1997, Roche won approval for the first humanized mAb, Zenapax — which binds to and inhibits a receptor on activated white blood cells and suppresses tissue rejection.

A number of techniques are now used for humanization or deimmunization. As a result of the fewer murine sequences, these molecules carry a lower risk of triggering an immunogenicity reaction than chimeric and murine antibodies. Most of the antibody drugs on the market and in clinical development are humanized antibodies. There are a number of antibody technology platforms that can be licensed to develop humanized antibodies. The key players include Protein Design Labs' SMART Humanization technology, Aeres Biomedical's humanization technology, and Xoma's Human Engineering Technology.

Recently, a new humanization technology, developed by Epitomics termed "HuRAB Technology", was designed to overcome the limitation of traditional humanization technology^[10-11]. The "HuRAB Technology" uses information from the genetic evolution of the rabbit immune system: B cell maturation, to optimize and at the same time convert a rabbit mAb to human antibody molecule. The cost and time to engineer an antibody is significantly reduced. Furthermore the resulting antibodies remain and sometimes have improved binding capability compared to their parental antibody molecules.

New technologies for the generation of human monoclonal antibody

Developing chimerized or humanized antibodies is challenging. First, it can be time-consuming because murine antibodies specific to the antigen must be developed before chimerization or humanization can be carried out. Second as a result of this, engineering chimerized or humanized mAbs can be costly. Third, and most important, chimerization and humanization can both reduce binding affinity because certain murine sequences involved in binding strength may not be transferred to the final antibody molecule. As a result of this, sequence selection needs to be carefully done to ensure that binding affinity and biological activities are not reduced substantially.

There are long-term ethical issues involved with the use of humanized (CDR grafted) and chimeric antibodies, due to the limited number of mouse and primate CDRs and their foreign nature. With humanized antibodies now being used in infant populations, therapeutic and diagnostic procedures in later life will have reduced efficacy because of the increasing *in vivo* usage of mouse, chimeric and humanized antibodies. Similarly humanized, chimeric and non-human antibodies are being used effectively for initial treatment in some malignancies, because of their lymphocyte-depleting effects. However, at relapse 3-5 years later, memory cells will be present -thus lowering efficacy due to HAMA type responses. To further minimize the effort to chimerize or humanize murine antibodies, technologies were developed to generate human antibodies directly. Human antibodies are derived either from vectors carrying human antibody genes, or from human cells. Human antibodies therefore theoretically have minimal immunogenicity. Human mAbs are still perceived to have favorable safety profiles and are less rapidly eliminated from the human body, potentially reducing the frequency and amount of dosing required. Currently, several types of technologies are used to create human antibodies, such as phage display of libraries of human antibodies, transgenic

mice, Human-Human Hybridoma, Hybrid hybridoma, B cell immortalization and cloning or Single-cell RT-PCR. Most of the growing number of antibodies entering clinical trials are completely human and are derived from phage-display technology or transgenic mice that express human immunoglobulin genes.

Phage display and yeast display

Phage display involves the engineering of bacteriophages to display human mAbs on their surface^[5-6]. Genes from extensive human antibody gene libraries are inserted into a library of phage. Each phage carries the genes for a different antibody and displays a different antibody on its surface. Antibodies are selected for binding to a target antigen and the corresponding genes can be recovered and used in the development and potential manufacture of an antibody therapeutic product. The benefits of phage display are the huge size of the libraries. The larger the library is, the more likely an antibody can be isolated that binds effectively to the selected target. Certain libraries contain the genes for over 100 billion distinct antibodies. The recombination of the genes that code for the variable parts of an antibody allows further expansion of these libraries. The repertoire expressed by these phage display libraries reflects the natural B cell repertoire of the individual animal from which the cDNA was derived. Thus, depending on whether or not the donor, either animal or human subject, was previously exposed to the target antigen, these libraries can be used to harvest the primary or secondary humoral immune response. Synthetic and semisynthetic phage display libraries, where at least some of the CDR diversity is generated through the introduction of random synthetic sequences, are also used.

A number of companies are engaged in the development of human mAbs through phage display, including Cambridge Antibody Technology (CAT), now part of AstraZenica, Dyax and MorphoSys. Six fully human therapeutic antibodies developed by CAT are at various stages of clinical trials and Humira (adalimumab) was the first phage display antibody to reach the market, launched by Abbott in January 2003. It is now approved for multiple indications, including rheumatoid arthritis, Crohn's disease, and plaque psoriasis.

An advantage of phage display is the speed of identifying the initial antibody, avoiding the need for immunization in animals, which usually takes several months. However, the phage antibodies isolated in the first round of display are typically of very low affinity, and optimization (affinity maturation) of the antibody is necessary.

Affinity maturation is the process by which B-cells produce antibodies with increased affinity for antigen during the course of an immune response. With repeated exposures to the same antigen, a host will produce antibodies of successively greater affinities. A secondary response can elicit antibodies with several log-fold greater affinities than in a primary response. Such affinity maturation processes can be applied in test tubes by selecting a random, mutated antibody population. Typically in vitro affinity maturation takes a few months, too.

Yeast display is a technique initially used in the field of protein engineering. A protein of interest is displayed as a fusion to the Aga2p protein on the surface of yeast. The Aga2p protein is naturally used by yeast to mediate cell-cell contacts during yeast cell mating. As such, display of a protein via Aga2p projects the protein away from the cell surface, minimizing potential interactions with other molecules on the yeast cell wall. The use of flow cytometry in conjunction with a yeast display library is a highly effective method to isolate high affinity protein ligands (antigens) against nearly any receptor (antibodies) through directed evolution.

Advantages of yeast display over other in vitro evolution methods include eukaryotic expression and processing, quality control mechanisms of the eukaryotic secretory pathway, minimal avidity effects, and quantitative library screening through fluorescent-activated cell sorting (FACS). Disadvantages include smaller mutagenic library sizes compared to alternative methods and differential glycosylation in yeast compared to mammalian cells. It should be noted that these disadvantages have not limited the success of yeast display for a number of applications, including engineering the highest monovalent ligand-binding affinity reported to date for an engineered protein.

Transgenic mice

Transgenic mice were developed to overcome these hurdles by genetically engineering them with a 'humanized' humoral immune system^[7]. Human antibody genes, while leaving the other components of the mouse immune system intact, replace their innate antibody genes. As a result the transgenic mice produce human antibodies in response to immunization with an antigen. Once the mouse has been immunized, mAbs can either be generated through traditional hybridoma generation or by using technologies that involve the harvesting, plating and screening of B cells, followed by isolation of mAb genes and cloning into production cell lines. Using transgenic mice to generate human antibodies is relatively

simple and relies on widely used techniques. Specifically, B cells that express human antibodies are isolated from immunized mice and then cloned as with hybridomas, similar to the generation of mouse mAbs. The immune response in transgenic mice is sometimes less robust than in strains that are used to generate mouse mAbs, so an increased number of immunizations or antibody screens might be required. The binding affinity of human antibodies from transgenic mice is often high, reflecting *in vivo* affinity maturation, which is integral to the secondary immune response. This high affinity routinely obviates the requirement for subsequent *in vitro* affinity maturation or for antibody-potency enhancement using other technologies.

Abgenix's XenoMouse and XenoMax are human antibody technologies using transgenic mice to create therapeutic mAbs. Abgenix's XenoMouse is a transgenic mouse in which the mouse's antibody gene expression is suppressed and replaced with human antibody genes. XenoMouse strains have been used to generate numerous high-affinity, fully human antibodies for targets in multiple disease indications, many of which are progressing in clinical development. However, the remainder of the mouse's immune system is left intact. The mice engineered through this process have approximately 80% of the human heavy-chain antibody genes as well as a significant amount of the light-chain genes. Abgenix has developed multiple strains of XenoMouse animals, each of which produces a different class of antibody to perform different therapeutic functions.

Medarex's UltiMAB platform uses transgenic mice, where mouse genes are suppressed and replaced with the expression of human antibody genes, to generate human antibodies. The technology platform includes three key antibody technologies: Medarex's HuMAB-Mouse technology; Kirin Brewery's TC Mouse technology; and the KM-Mouse technology, a crossbred mouse that has a combination of the characteristics of the HuMAB-Mouse and Kirin's TC Mouse technologies.

In the transgenic mice of the HuMAB-Mouse technology, mouse genes encoding antibodies have been inactivated and replaced with human antibody genes. These human genes encode unrearranged human antibody genes for both the heavy and light chains. Kirin's TC Mouse technology, on the other hand, has been developed so that the transgenic mice contain the complete set of variable and constant genes, which are found at the human immunoglobulin loci. In addition, the TC Mouse technology is capable of generating all antibody isotypes, as the mouse's chromosomes have been in-

activated and replaced with human chromosomes which encode these. By combining the key features of these mice to create the KM-Mouse technology, Medarex and Kirin have generated a platform capable of making a broad range of antibodies because it has 100% of the human chromosomes for antibody genes. The ability to generate varied isotypes, in particular, allows a greater flexibility in designing therapeutic mAbs, as a particular isotype determines which effector functions of the immune response are induced.

At present, at least 33 human antibodies produced in transgenic mice are in clinical development, including 5 that are in Phase III clinical trials. In 2006, panitumumab, which inhibits EGFR signaling, became the first marketed mAb derived from a transgenic mouse platform. The path from initiation of XenoMouse technology development to regulatory approval took about 15 years, including 6 years for mouse strains derivation and mAb development and 6.5 years of clinical development. The drug has a positive risk-benefit profile in advanced chemotherapy refractory colorectal cancer and has the potential to add to the treatment of this disease in earlier lines of therapy as well as to play a role in the treatment of other malignancies. The development of panitumumab validates the XenoMouse platform as a proven technology for generation and selection of a therapeutic antibody with the desired characteristics, including affinity, specificity and isotype.

The most striking apparent difference between the phage display and transgenic mouse platforms relates to the processes employed for discovery of the drugs, the transgenic mouse derived antibodies appear to have typically been moved from lead selection directly to clinical development without undergoing lead optimization steps. Lead optimization as employed for phage display derived antibodies may not be required for transgenic mouse derived antibodies that have already undergone affinity maturation *in vivo*. This opportunity to bypass lead optimization entirely has potential advantages for the overall drug discovery process, as it might decrease timelines, and at the same time, increase the epitope diversity of the high affinity lead candidate pool.

Human-Human Hybridoma

Native human antibodies offer many compelling advantages over antibodies derived from non-human systems. As an example, native antibodies that specifically bind autologous tumor antigens frequently arise in cancer patients. These antibodies result from the interaction between cancer cells and the host immune system and

could be adapted for use as therapeutics or diagnostic reagents. In addition, the study of cloned antibodies may reveal the complexity of an effective humoral immune response while producing antibodies for the prevention and treatment of infectious diseases by passive immunization. The systematic examination of the antibodies that accompany auto-immune diseases may provide clues to the pathogenesis of these diseases as well as guide the creation of novel immuno-modulatory drugs. Unfortunately, because of the difficulty in rapidly and reliably immortalizing human B-cells and the scarcity of human B cells expressing antibodies of the desired antigen specificity and affinity, the potential utility of native human monoclonal anti-bodies has not been realized.

Despite the significant advances in mAbs technology over the last two decades, technology to develop naturally occurring and fully human therapeutic mAbs from patients without known antigens is still in its infancy. The mouse myelomas were not suitable for fusion with human B cells secreting natural therapeutic mAbs because the heterospecific hybrids quickly lost the relevant human chromosomes. The mouse-human heteromyelomas that have been used for fusion with human lymphocytes are often unstable and secrete low levels of antibodies. Human myeloma cell lines have been very difficult to derive. Attempts in numerous laboratories to use those cells for the production of human mAbs have failed despite early reports. The Epstein-Barr virus (EBV) has been used as an alternative to immortalize antibody-producing human B lymphocytes. However, such cell lines tend to be unstable with respect to growth, they are low producers of antibodies, and the EBV does not preferentially immortalize lymphoblasts engaged in antibody responses.

After 20 years of effort, Karpas et al in the University of Cambridge reported a human myeloma cell line (Karpas-707H) that can give rise to stable human hybridomas capable of producing human mAbs^[12,13]. The cell line is useful for the generation of stable human hybridomas. It can easily be fused with ouabain-sensitive Epstein-Barr virus-transformed cells as well as with fresh tonsil and blood lymphocytes, giving rise to stable hybrids that continuously secrete very large quantities of human immunoglobulins. The derived hybrids do not lose immunoglobulin secretion over many months of continuous growth.

The discovery builds on previous mouse hybridoma technique carried out by Cesar Milstein and Georges Kohler at the Medical Research Council's Laboratory of Molecular Biology in Cambridge in 1975, a discov-

ery for which they were awarded a Nobel Prize. Since 1975 researchers across the world have been trying to replicate the process with human cells. Repeated failures led to a widespread belief that it could not be done and more recently resources have been ploughed into complex and costly alternatives to the Milstein method, such as chimeric or humanized antibodies. The latest in the evolution of mAbs has been the development of human mAbs, such as phage display, transgenic mice, irradiated or SCID mouse technology, or cell-free display system. These technologies produce human mAbs, but these mAbs are not selected and produced by the human immune system, they either mature in vitro or are selected and produced by the mouse immune system. Whilst all above mentioned technologies have enjoyed some success, they are unlikely ever to create the ideal antibodies that are usually produced by the human immune system naturally.

The availability of this cell line should enable the in vitro immortalization of human antibody-producing B cells that are formed in vivo. The mAbs produced may have advantages in immunotherapy. The cell line has the potential to immortalize most of the antibodies the human body produces to fight infections, cancer, AIDS and other pathological conditions. Further molecular characterization study suggests that the hybridoma-derived antibodies are representative of antibodies from populations of human lymphocytes and at different stages in the maturation of the response, and that the use of Karpas 707H myeloma for human hybridoma fusions may therefore provide a valuable tool for analysis of the human antibody responses. It is an easier and cheaper technology to develop human therapeutic antibodies produced by B cells from tumor patients, and B cells from patients with infectious diseases, and to produce human mAbs no other methods can generate. With this method, no previously known antigen is needed, which saves tremendous effort in antigen identification and characterization. This method could become the method of choice for immortalizing human antibodies in a wide spectrum of pathological conditions.

Immunodeficient mouse and human hybridoma.

Recently, Kametani et al tried to prepare designed antigen-specific antibodies of completely human origin using immunodeficient mouse and human hybridoma^[14]. Non-obese diabetic/severe combined immunodeficient/IL-2 receptor gamma null mouse (NOG) mouse was used to reconstitute the human immune system with umbilical cord blood hematopoietic stem cells (CB-NOG mouse) and to prepare human-derived Her-2-epitope-

specific antibodies. Hybridoma lines were prepared by fusing the human myeloma cell line Karpas707H. Hybridoma lines were successfully prepared with spleen B cells obtained from the immunized CB-NOG mouse. One of these cell lines produced human IgM against the epitope peptide that can recognize surface Her-2 molecule.

Hybrid hybridoma

Human–mouse heteromyeloma cell line H73C11 with electrofusion

Human mAbs may be generated by electrofusion of human B lymphocytes with a human or mouse heteromyeloma line. In addition to a fusion protocol optimized for the fusion partners, the activation of B lymphocytes is crucial for fusion and hybrid efficiency. Schmidt E et al tested a large panel of different mitogens and/or cytokines and applied a variety of depletion/isolation protocols to normal human PBMC followed by electrofusion with the human–mouse heteromyeloma cell line H73C11^[15]. The highest yield of secreting hybridomas was obtained in response to PHA stimulation of undepleted PBMC. Furthermore, CD19+ B lymphocytes were identified as the major source of antibody-secreting hybrids. For optimal fusion efficiency, CD19+ B cells were shown to require direct physical contact with other cell populations, most probably T lymphocytes, during the stimulation process. This data highlights the importance of an adequate stimulation prior to electrofusion and may be helpful to further facilitate the development of human mAbs.

Mouse/human heterohybridoma K6H6/B5 cells and morphogenics

Generation of hybridomas secreting human mAbs has been previously reported. However, this approach has not been fully exploited for immunotherapy development. It was previously reported as the use of transient regulation of cellular DNA mismatch repair processes to enhance traits (e.g., affinity and titers) of mAb-producing cell lines, including hybridomas. This process, named morphogenics, could be used to improve suboptimal hybridoma cells generated by means of *ex vivo* immunization and immortalization of antigen-specific human B cells for therapeutic Ab development. Li J et al presented a platform process that combines hybridoma and morphogenics technologies for the generation of fully human mAbs specific for disease-associated human antigens^[16]. Human B cells are immunized *ex vivo* in the presence of human antigens and then immortalized by means of cell fusion. Alternatively, selected donors are identified

whose sera have high immunoreactivity to antigens of interest. Hybrid cells derived from these individuals' B cells are screened for secretion of antigen-specific mAbs. They generated mAbs specific to a number of human antigens, including human mesothelin and granulocyte-macrophage colony-stimulating factor (GM-CSF). One mAb showed strong neutralizing activity against human GM-CSF and is now considered for preclinical development for autoimmune disease indications. Moreover, these hybridoma cells have proven suitable for genetic optimization using the morphogenics process and have shown potential for large-scale manufacturing.

SP2/0-derived cell lines ectopically expressing mIL-6 and hTERT

The native human antibody repertoire holds unexplored potential for the development of novel mAb therapeutics. Current techniques that fuse immortal cells and primary B-lymphocytes are sub-optimal for the routine production of hybridomas that secrete human mAbs. Scott K et al have found that a murine cell line that ectopically expresses murine interleukin-6 (mIL-6) and human telomerase (hTERT) efficiently forms stable human antibody-secreting heterohybridomas through cell fusion with primary human B-lymphocytes. The hybrid cells maintain secretion of human antibodies derived from the primary B-lymphocytes through multiple rounds of cloning. Using splenic B-lymphocytes from a patient immunized with a *Streptococcus pneumoniae* capsular polysaccharide vaccine, they have succeeded in creating hybridomas that secrete human mAbs specific for *S. pneumoniae* antigens. Using peripheral blood lymphocytes, they cloned a human antibody that binds a viral antigen. These experiments establish that SP2/0-derived cell lines ectopically expressing mIL-6 and hTERT will enable the rapid cloning of native human mAbs^[17].

Cell sorting by Flow cytometry and Single-cell RT-PCR

Dohmen SE et al described a new method to analyze anti-D-specific immunorepertoires at the single B cell level and concomitantly provides a means to create recombinant immunoglobulins, with retention of the original heavy- and light-chain pairings found in anti-D-specific B cells^[18]. Single B cells from hyperimmunized anti-D donors were expanded in the EL4.B5 system and mRNA was amplified by single cell RT-PCR from the progeny of single B cells producing anti-D antibodies. The naturally occurring heavy- and light-chain gene combinations of the anti-D immunoglobulins were analyzed by automated cycle sequencing. This new method permitted the direct analysis of anti-D-specific B cells at the single

cell level. The immunorepertoire analysis showed that the IGHV genes of our anti-D-specific single B cells were restricted to genes similar to those described in hybridomas and in anti-D-specific phages. Moreover, it was possible to produce recombinant IgM and IgG with anti-D specificity from the mRNA of antigen-selected single B cells. It is suggested that this approach could be applied for the selection of human mAbs from immunized donors and for the analysis of Ig-gene repertoires at the single-B cell level.

Pre-existing neutralizing antibody provides the first line of defense against pathogens in general. Wrammert J et al reported that after booster vaccination there was a rapid and robust influenza-specific IgG+ antibody-secreting plasma cell (ASC) response that peaked at approximately day 7 and accounted for up to 6% of peripheral blood B cells [19]. These ASCs could be distinguished from influenza-specific IgG+ memory B cells that peaked 14-21 days after vaccination and averaged 1% of all B cells. Importantly, as much as 80% of ASCs purified at the peak of the response were influenza specific. They used the immunoglobulin variable regions isolated from sorted single ASCs to produce over 50 human mAbs that bound to the three influenza vaccine strains with high affinity. This strategy can generate multiple high-affinity mAbs from humans within a month after vaccination and help to resolve a major, long-standing obstacle in the field of medicine: the rapid production of fully human mAbs. Antibody or serum therapy has been demonstrated to treat a plethora of diseases effectively, but it is not widely used because sometimes fatal anaphylactic responses and serum sickness can happen. These obstacles can only be overcome by using fully human mAbs. With a modern resurgence of interest in mAb therapy, we anticipate that antibodies produced from post-vaccination ASCs will generate substantial advances for the treatment of infectious diseases.

B cell immortalization and cloning human mAbs from memory B cells

Active vaccination together with passive vaccination, also called serotherapy, acting through the administration of preformed specific antibodies has been one of the great contributions of immunology to medical treatments. MAbs can be used to offer immediate protection against a variety of toxins and pathogens, including emerging ones, such as severe acute respiratory syndrome (SARS) or H5N1 influenza, for which polyclonal human Igs from hyperimmune sera are not available in sufficient amounts. MAbs represent an ideal alternative to hyperimmune sera. They can be produced by immortalizing

memory B cells with Epstein–Barr virus (EBV) or by fusing a B cell with an appropriate partner cell to produce hybridomas. These methods have a very low efficiency, and therefore alternative strategies have been developed. These alternatives include the humanization of murine mAbs through protein engineering, the selection of antibodies from phage display, libraries of human antibody fragments, and the immunization of transgenic mice carrying human Ig loci, followed by the production of mAbs using hybridoma technology. Although these methods have led to the development of several therapeutic mAbs against cytokines or surface molecules, their impact on infectious disease therapy has been less pronounced. Indeed, the number of therapeutic antibodies against infectious agents is still limited, and only one is currently in use to prevent respiratory syncytial virus (RSV) infection in newborns, but there is an obvious advantage to using human B cells to produce mAbs. Firstly, humans can mount powerful immune responses, which include antibodies with long complementarity-determining region 3 (CDR3) regions. Secondly, antibodies are fully human and have been selected in a human body, minimizing the risk of cross reactivity with self-antigens. Thirdly, the human immune response is directed against the virulent pathogen and can target all the components necessary for infection and virulence, which are usually invisible to the immune system of a different.

An improved method of EBV transformation of human B cells was recently described based on the addition of a TLR agonist during EBV transformation and cloning [20, 21]. Using this method, neutralizing and non-neutralizing mAbs against a variety of targets including viruses such as SARS, coronavirus, toxins, and parasites were isolated. As an example, 35 independent mAbs were isolated from one individual who recovered from a SARS infection, neutralized virus infection in vitro with high potency. This method may be used not only to isolate therapeutic antibodies for passive vaccination but also to analyze the antibody repertoire in immune or vaccinated.

The intrasplenic Hu-PBL-SCID model

Human mAbs have therapeutic potential against infectious diseases and cancer, but their production has been hampered by ethical constraints preventing the isolation of Ag-specific activated B cells by in vivo immunization. Alternatively, severe combined immune deficient (SCID) mice, transplanted i.p. with human (Hu)-PBLs, allow the in vivo stimulation of human Ab responses without the usual constraints. Unfortunately, human B cells only represent a minor fraction of the surviving graft, they are scattered all over the animal to isolate for subsequent im-

mortalization procedures. To prevent this dispersion and to provide the human B cells with a niche for expansion and maturation, SCID mice were engrafted with Hu-PBL directly into the spleen[22]. Simultaneously endogenous murine NK cell activity was depleted by treatment with an antimouse IL-2 receptor beta-chain Ab. During engraftment, human B lymphocytes became activated, divided intensely, and differentiated into plasmacytoid cells. In vivo exposure to a recall Ag after cell transfer induced expansion of Ag-specific B cell clones. One week after inoculation, human B cells were abundant in the spleen and could easily be recovered for fusion with a heteromyeloma line. This resulted in the formation of stable hybridoma cell lines that secreted Ag-specific HumAbs. Thus transplantation of human lymphoid cells in the spleens of immune deficient mice represents a model for the study of human T cell-dependent B cell activation and proves to be an excellent tool for the successful production of Human mAbs.

The intrasplenic Hu-PBL-SCID model provides a direct and easy route to the rich memory compartment of human B cells that can be exploited for the production of a wide variety. Human mAbs could not only be developed from Hu-PBL derived from vaccine induced immune donors (e.g., HBsAg) but also from Hu-PBL derived from a donor carrying the infectious agent (e.g., HCV). In the latter case, in vivo immunization with recall Ag was not even necessary to generate expanded repertoires of Abs to specific epitopes of the infectious agent. This method is currently still limited in generating Abs to Ags to which the human B cell donor has already responded in vivo. Human autoantibodies, potentially useful for immunosuppressive and immunomodulatory function, could be obtained using appropriate autoimmune donors in the model. Similarly, Abs against specific MHC determinants for treating graft rejection could be derived from Hu-PBL from selected multiparous women.

FuCell technology to produces totally human hybridomas

A biotechnology research company FuCell in Australia has developed a method to produce human cell lines that secrete totally human antibodies and other proteins with normal human glycosylation. The process uses radio frequency electric fields and special electrodes to fuse selected human lymphocyte cells to immortalize cancer cells. This creates new hybrid cell lines (hybridomas) that retain the characteristics necessary for cellular division and the mechanisms required for antibody production. The method employed by FuCell avoids the labor-intensive task of isolating the relevant hybridomas from a heterogeneous mixture of hybrid cells, a process that is

required with traditional techniques. The FuCell technology generates products with fully human amino acid sequence and ensures the correct human glycosylation of the proteins. The major advantage of this technology over competing technologies is that the resulting antibodies are naturally human, including proper glycosylation. This avoids some of the limitations encountered by alternative products, such as humanized mouse antibodies.

Summary

Although murine mAbs were the first class of mAbs to enter clinical trials, these molecules have failed to become a major class of therapeutic antibodies because of the immunogenicity problems they cause. Mab therapy has been facilitated by a number of technological advances over the past 30 years. Several different technologies have been developed since the first steps of antibody engineering 15 years ago to isolate human monoclonal antibodies. Human antibody therapeutics demonstrated broad utility in the treatment of malignant, infectious, and inflammatory diseases. Because it has not been possible to readily immortalize and clone human B-lymphocytes, most of the human mAb therapies in use and under development have been derived in non-human species. These include murine mAbs “humanized” through the creation of chimeric human/murine immunoglobulin genes, single chain antibodies encoded by human genes in filamentous phages and yeast, and antibodies made in transgenic mice that bear human immunoglobulin gene loci. These approaches produce mAbs that are fully or almost fully human in sequence. However, they do not allow the direct isolation and cloning of the native human antibody repertoire, the antibodies created by an intact human immune system that are the unique products of its selective and regulatory processes. The advent of chimeric antibodies lessened but did not eliminate the rodent content of mAbs; thus, immunogenicity remained a concern. Further elimination of rodent sequences enabled the production of humanized mAbs, followed by current technology using phage display and, finally, transgenic mice technology and several other new technologies such as Human-Human Hybridoma, Hybrid hybridoma, B cell immortalization and clone or Single-cell RT-PCR, which allows for the generation of fully human therapeutic mAbs. The reduced immunogenicity of this new generation of mAbs is expected to enhance efficacy and safety, and the ease of use. In addition to providing replacements for existing mAb drugs, new technologies have greatly facilitated the optimization and modification of mAbs, opening numerous therapeutic avenues. While technology of antibodies has been a key factor in the success of

the market to date, the therapeutic suitability of antibodies for the treatment of immune-related diseases, cancer and infectious disease is also expected to continue to drive new innovations.

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Katherine Lee, 6 Venture, Suite 250, Irvine, CA92618 ; Tel : 562-686-9786 ; Fax : 949-788-9204 ; klee@pharmaron.com