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REVIEW

Recent advances in the generation of human monoclonal antibody

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Abstract The use of monoclonal antibodies (mAbs) has now gained a niche as an epochal breakthrough in medicine. Engineered antibodies (Abs) currently account for over 30% of biopharmaceuticals in clinical trials. Several methods to generate human mAbs have evolved, such as (1) immortalization of antigen-specific human B cell hybridoma technology, (2) generation of chimeric and humanized antibody (Ab) from mouse Ab by genetic engineering, (3) acquisition of antigen-specific human B cells by the phage display method, and (4) development of transgenic mice for producing human mAbs. Besides these technologies, we have independently developed a method to generate human mAbs by combining the method of in vitro immunization using peripheral blood mononuclear cells and the phage display method. In this paper, we review the developments in these technologies for generating human mAbs.

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Therapeutic monoclonal antibodies (mAbs) have developed into a beneficial and profitable group of products. Recent statistics show that mAbs comprise 16% of the biotechnology-based medicines applied to clinical tests. Indeed, 18 mAbs are now approved for therapeutical use in the United States under diverse clinical settings, including oncology, chronic inflammatory diseases, transplantation, infectious diseases, and cardiovascular diseases (Table 1) (Reichert et al. 2005; Carter 2006). In addition, together with the progression of genome medical science, an increasing number of target molecules in various diseases will be revealed. Thus, it is of urgent necessity to establish the technology for generating human mAbs that are specific for disease-related molecules. Here, we have reviewed the recent developments in the technology of producing human mAbs.

Technology of producing mAbs, from past to present

Mouse mAbs

The technology of producing mouse mAbs was devised in 1975 based on the use of mouse hybridomas generated by the fusion of B cells derived from

Table 1 Monoclonal antibodies approved for therapeutic use in the United State*

Generic (Trade)	Company	Antibody format	Antigen	Approved indications
Muromonab-CD3 (Orthoclone OKT3)	Johnson & Johnson	Mouse IgG2a	CD3	Prophylaxis of acute kidney-transplant rejection
Abciximab (ReoPro)	Centocor	Chimeric, IgG1 or Fab	gpIIb/IIIa, $\alpha_v\beta_3$-integrin	Prevention of platelet-mediated clots in coronary angioplasty
Rituximab (Rituxan)	Genentech	Chimeric, IgG1κ	CD20	Non-Hodgkin's Lymphoma and rheumatoid arthritis
Dalizumab (Zenapax)	Hoffmann-LaRoche	Humanized, IgG1κ	CD25	Prophylaxis of acute kidney-transplant rejection
Basiliximab (Simulect)	Novartis	Chiemric, IgG1κ	CD25	Prophylaxis of acute kidney-transplant rejection
Palivizumab (Synagis)	MedImmune	Humanized, IgG1κ	RSV gpF	Prophylaxis against RSV infection in children at high risk
Infliximab (Remicade)	Centocor	Chimeric, IgG1κ	TNF-α	Crohn's disease, rheumatoid and psoriatic arthritis, ulcerative colitis and ankylosing spondylitis
Trastuzumab (Herceptin)	Genetech	Humanized, IgG1κ	ERBB2 (HER2)	Metastatic breast cancer that overexpresses ERBB2
Gemtuzumab ozogamicin (Mylotarg)	Wyeth	Humanized, IgG4κ	CD33	Acute myeloid leukaemia that expresses CD33
Alemtuzumab (Campath)	Genzyme	Humanized, IgG1κ	CD52	B-cell chronic lymphocytic leukaemia
Ibritumomab tiuxetan (Zevalin)	Biogen Idec	Mouse, IgG1κ	CD20	Non-Hodgkin's lymphoma
Adalimumab (Humira)	Abbott	Human, IgG1κ	TNF	Rheumatoid and psoriatic arthritis
Omalizumab (Xolair)	Genentech	Humanized, IgG1κ	IgE	Persistent asthma
Tositumomab (Bexxar)	Corixa	Mouse, IgG2aλ	CD20	Non-Hodgkin's lymphoma
Efalizumab (Raptiva)	Genentech	Humanized, IgG1κ	CD11a	Plaque psoriasis
Cetuximab (Erbitux)	ImClone Systems	Chimeric, IgG1κ	EGFR	Metastatic colorectal cancer, and head and neck cancer
Bevacizumab (Avastin)	Genentech	Humanized, IgG1	VEGF	Metastatic colorectal cancer
Natalizumab (Tysabri)	Biogen Idec	Humanized, IgG4κ	$\alpha_4\beta_1$-integrin, $\alpha_4\beta_7$-integrin	Multiple sclerosis

*Sources include drug prescribing information, Reichert et al. 2005; Carter 2006 ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; EGFR, epidermal-growth-factor receptor; gp, glycoprotein; RSV, respiratory syncytial virus; TNF, tumore-necrosis factor; VEGF, vascular endothelial growth factor.

immunized mice with immortalized myeloma cells (Kohler and Milstein 1975). Researchers have created large numbers of mouse mAbs to treat or diagnose human diseases. However, the availability of mouse mAbs has been greatly limited by their high immunogenicity in humans and rapid clearance due to human anti-mouse antibody (HAMA) reactions occurring in patients. Thus, mouse mAbs exhibit greatly limited and inefficient effector functions in clinical trials. In addition, mouse mAbs do not bind to the human FcRn (Ober et al. 2001). To overcome these disadvantages of mouse mAbs, researchers have

attempted to generate chimeric and humanized mAbs derived from mouse mAbs (Kipriyanov and Le Gall 2004; Gonzales et al. 2005).

Chimeric and humanized mAbs

Chimeric mAbs are generated by replacing the constant regions of mouse Abs with those of human Abs by gene manipulation (Boulianne et al. 1984; Morrison et al. 1984). To generate chimeric mAbs by gene manipulation, we need to understand the structure-function relationship of immunoglobulin (Ig) molecule in order to retain the functional structure of mAbs. However, even when functional chimeric mAbs can be created by gene manipulation, the mouse-derived variable region of these chimeric mAbs can be immunogenic in patients. Indeed, HAMA reactions have been detected in patients receiving therapeutic chimeric mAbs. Thus, we need to further reduce the immunogenicity of chimeric mAbs by humanizing the mouse-derived variable region.

The simplest of the many available humanization strategies involves transferring the complementarity-determining regions (CDRs; antigen-binding loops) from a mouse mAb to a human IgG (Jones et al. 1986; Riechmann et al. 1988; Verhoeyen et al. 1988). The non-human content in the sequence of a CDR-grafted antibody (Ab) is, therefore, only 5–10%, although that of a chimeric Ab is approximately 30% (Gonzales et al. 2005).

Human mAbs

To further reduce the immunogenicity of chimeric and humanized mAbs, researchers have tried to establish a method to generate human-derived mAbs. To date, several methods to generate human mAbs have been established, such as the phage display method and transchromosome mice technology in which human Ig genes are expressed (Lonberg 2005).

Phage display

Phage display is one of the most widespread methods to select antigen-specific variable region genes (McCafferty et al. 1990; Hoogenboom 2002; Hoogenboom 2005). Diverse human immunoglobulin-heavy chain variable (V_H) gene segments and light-chain variable (V_L) gene segments were prepared from peripheral blood lymphocytes by PCR amplification. The gene encoding single chain variable fragment (scFv) was created by randomly combining V_H and V_L gene segments by using PCR. The combinatorial library (comprising more than 10^7 scFv genes) was then cloned into *Escherichia coli* to display scFv on the surface of the phage. After panning the phages bound to a specific antigen, antigen-specific scFv can be identified (Marks et al. 1991). To date, several improvements have been made in the phage display method in order to increase the efficiency of the acquisition of antigen-specific scFv, to augment the affinity of scFv for antigens, and to increase the specificity of scFv (Bradbury and Marks 2004). At least 14 Abs generated by the phage display method are now in clinical use (Lowe and Jermutus 2004).

Transgenic mice

Another method to generate human mAbs is to use transchromosome mice, whose Ig-heavy chain and Igκ-light chain loci are disrupted and which have transgenes encoding genes for human Ig (Green et al. 1994; Lonberg et al. 1994). Subsequent progress includes the expression of more V gene segments by the transgenic mice, thereby expanding the potential repertoire of the recovered Abs (Lonberg 2005). Transgenic mice that produce human Abs with different heavy-chain isotypes have also been created to tailor effector functions. At present, more than 33 human mAbs produced by transchromosome mice are in clinical use (Lonberg 2005). The immune response in transgenic mice is sometimes less robust than that in strains that are used to generate mouse mAbs; therefore, an increased number of immunizations or Ab screens is known to be required.

In vitro immunization

We established a method of in vitro immunization using human peripheral blood mononuclear cells (PBMC) (Ichikawa et al. 1999). In this method, PBMC were first treated with L-leucyl-L-leucine methyl ester (LLME) to remove suppressive cells and then sensitized with soluble antigen in the presence of several cytokines and muramyl dipeptide (MDP). Sensitized PBMC was transformed with

Epstein-Barr virus (EBV), and fused with mouse-human hetero myeloma host cells to create EBV-immortalized B cell hybridomas. However, we encountered difficulties in obtaining antigen-specific B cell hybridomas, such as low efficiency and loss in antigen-specificity during the long-time culture. To overcome these problems, we tried to obtain the V-region genes of antigen-specific Ab by using the phage display method. When using the DNA from PBMC immunized in vitro as template for PCR amplification, the V_H and V_L genes were easily amplified by using a smaller number of cells. However, when using the DNA from non-sensitized PBMC as template, large numbers of cells were required to amplify the V_H and V_L genes. This suggests that the generation of a sufficiently large library of scFv is a limiting step for obtaining antigen-specific scFv by the phage display method that uses DNA from non-sensitized PBMC as template. On the other hand, it was remarkably simple to amplify the V-region genes when using the DNA from PBMC immunized in vitro with a specific antigen. These results suggest that in vitro immunization enables enrichment of antigen-specific B cell population, which was evidenced by the enzyme-linked immunospot (ELISPOT) analysis of PBMC immunized in vitro. By using scFv libraries created from PBMC immunized in vitro, we obtained scFv specific for mite allergen and the TNF-α peptide through several rounds of pannings. After amplifying the V_H and V_L genes by using antigen-specific scFv as template and combining these genes with the constant region genes of human IgG, antigen-specific human IgGs were produced in mammalian cells.

To efficiently expand antigen-specific B cells in the in vitro-immunized PBMC, we optimized the culture condition for the in vitro immunization of PBMC. Firstly, we evaluated the optimal concentration of additive cytokines such as IL-2 and IL-4 in in vitro immunization to induce antigen-specific Ab production (Yamashita et al. 2002). The results demonstrated that the optimal concentration of cytokines differs among individuals; thus, preliminary experiments are required to determine the optimal concentration of IL-2 and IL-4 in in vitro immunization. Next, we searched for an adjuvant substituting for MDP, which could induce antigen-specific Ab production. Until now, we have found that CpG oligonucleotides can be used as strong adjuvants for inducing antigen-specific Ab production in in vitro immunization (paper under preparation). Finally, we investigated the immune responses that occurred in in vitro immunization. The results demonstrated that PBMC include suppressive cells and that these cells can be removed by the LLME treatment. We found that PBMC can be sensitized with antigen and produce antigen-specific Abs by the removal of these cells even without the LLME treatment (paper under preparation). Thus, we believe that PBMC can be used to produce antigen-specific Abs. Presently, we are investigating the molecular mechanisms of immune suppression caused by these suppressive cells.

Ab engineering

Affinity maturation

Engineering Abs for improving their antigen-binding affinity has been very active and is probably one of the areas of intensive studies (Chowdhury and Wu 2005). One approach is to create very large libraries of randomly mutagenized CDRs or the entire variable domains and then select for higher-affinity variants from this large collection of mutants. Randomly mutagenized libraries are created by (1) random CDR mutagenesis-mutagenesis at the antigen-binding region to increase both binding affinity and specificity; (2) chain shuffling-shuffling the V_H or V_L of a given Ab with a repertoire of the corresponding chain, thereby creating a large library of variants; and (3) error-prone PCR and DNA shuffling-generation of Ab libraries with mutations within the variable regions.

Another approach is to create small libraries by focused mutagenesis that increases the likelihood of identifying higher-affinity variants. (1) Focused mutagenesis: this approach involves randomizing every single position in each of the six CDRs into 20 different amino acids. (2) Hotspot mutagenesis: in this approach, certain hotspots are rationally selected based on known Ab structure-function relationships, and small libraries of approximately 400 clones are generated and higher-affinity variants selected (Ho et al. 2005).

Recently, activation-induced cytidine deaminase (AID) enzyme, which is responsible for the class switch recombination and somatic hypermutation of

hot spots in Ig V-domains, was discovered (Muramatsu et al. 1999). This enzyme is reported to induce somatic hypermutation in foreign genes in transfected hybridomas (Martin et al. 2002) and fibroblasts (Yoshikawa et al. 2002). Thus, AID can be used for generating engineered Abs with a higher affinity and specificity in future studies.

Improvement of the effector functions of mAbs

The therapeutic efficacy of mAbs is dependent on their ability to trigger effector activity as well as their antigen-binding ability (Chowdhury and Wu 2005). These effector functions include Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, the antigen-Ab complex binds to the Fcγ receptor (FCγR) and the neighboring effector cells such as natural killer (NK) cells and macrophages, which leads to the lysis or opsonization of target cells. In CDC, the antigen-Ab complex activates the complement system, leading to the lysis or opsonization of target cells. Therefore, it is reasonable to think that engineering Abs to improve their binding to FcγR or the complement factors by glycoengineering (Yamane-Ohnuki et al. 2004) or protein engineering may lead to improvement in their therapeutic efficacy (Chowdhury and Wu 2005).

Another approach to improve effector function is to add new effector functions to the mAbs. In particular, in mAbs used for cancer therapy, mAb fragments are fused with a range of molecules to introduce different functionalities, including cytotoxic drugs or radio-labelling for killing cancer cells, enzymes for prodrug therapy, and cytokines for stimulating antitumor immune response (Carter 2001). Further, to elicit effector functions of the therapeutic mAbs by allowing them to reach close to the effector cells or molecules of the target cells, novel mini-antibodies were designed, including diabody (dimer of scFv), triabody (trimer), tetrabody (tetramer), and sc-diabodies (Sanz et al. 2005).

Future prospects

In the future, the information drawn from genome-medical science and genome-informatics, which list the disease-related antigens useful for medical treatment, should be indispensable to develop the therapy using mAbs. Moreover, the development of a technology for the mass production of mAbs is also indispensable from the viewpoint of expenses involved. Thus, we should establish and develop a technology for the mass production of mAbs by using various cytotechnological methodologies.

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