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SPECIAL ARTICLES

Antibody Engineering: Current Status and Future Development

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Since its introduction, the production of monoclonal antibodies by hybridoma technology has revolutionised almost every field of clinical medicine.[1] The high specificity of monoclonal antibodies has overcome many difficulties intrinsic to the use of polyclonal antibodies, and enabled them to be widely used in diagnosis and treatment of disease, as well as for isolation and purification of proteins and other substances.

Monoclonal antibodies are normally derived from somatic hybridization between non-secreting myeloma cells and immune spleen cells, usually of mouse or rat origin. The clinical use of these antibodies in human is limited in part due to the production of an anti-globulin response to the non-human immunoglobulins. This makes the repeated use ineffective and a severe hypersensitivity reaction may also occur. Xenogeneic antibodies are also not well-fitted to destroy cell *in vivo* because complement and cellular effectors, such as K cells and phagocytes, are not efficiently recruited.[2,3] Ideally, antibodies for *in vivo* clinical applications should be of human origin. Immortalization of human antibody-secreting cells has, unfortunately, been found to be very difficult. Recently, human monoclonal antibodies have been produced from Epstein-Barr virus transformation of human B lymphocytes, or from heterologous fusion between human B lymphocyte/plasma cells and mouse or rat myeloma cells, or the combination of both techniques.[4,5] However, some problems remain, notably the low yield of antibody-producing cells from somatic hybridization, difficulties in obtaining high affinity antibodies, low antibody production, and the instability of the resulting hybridoma cells. Human monoclonal antibodies derived from these techniques, in which IgM is predominant,[6] may not have the appropriate constant region for the desired effector functions. Isolation of the isotype switch variants of human antibody-secreting cells was found to be virtually impossible. Other problems include the bio-ethical difficulty in immunizing human against many dangerous agents, such as pathogens and tumours. Although *in vitro* immunization is theoretically possible, the successful application of this technique still looks distant.

Recombinant DNA technology is a powerful alternative technique for producing human monoclonal antibodies. Genetically engineered chimeric human/mouse monoclonal antibodies have been developed by replacing the Fc region of the murine immunoglobulin molecules with the human constant regions.[7-9] Moreover, the framework regions of variable domains of rodent immunoglobulins have also been replaced by their human counterpart.[10-12] This technique of antibody engineering has enormous potential to be used for producing tailor-made antibodies with special physical and functional properties. Another advantage of this technology is that antibody molecules can be designed to have specificity and effector functions which may not occur naturally.

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Theoretical Basis

Immunoglobulin molecules are composed of two parts, variable and constant regions. The former contributes to specific antigen binding. Study of the three dimensional structure of immunoglobulins revealed that the antigen binding site is constructed from six complementarity determining regions (CDRs), three from the heavy chain and three from the light chain variable regions, separated by the framework regions (FRs). Amino acid sequences of the CDRs are highly variable, but those of the framework regions are relatively more conserved. [13,14] The immunoglobulin constant region is required for effector functions of the antibodies, notably complement activation and Fc receptor binding. Different isotypes of immunoglobulins may have different effector functions, for example human IgG1 and IgG3 are the most effective for complement and cell-mediated lysis. [15] Immunoglobulin molecules are composed of 2 independently folding light chain domains, and 4 or 5 heavy chain domains, depending on the isotypes. Each domain has about 110 amino acid residues.

At the genetic level, Immunoglobulin variable region genes are rearranged by randomly joining the V, D, and J genes of the heavy chain gene, and by the joining the V and J genes of the light chain gene, to form the variable gene domains. Constant region gene domains are arranged in germ line as individual exon domains [16] (Fig. 1). Distinct immunoglobulin polypeptide domains are each encoded by individual exons to form the complete immunoglobulin molecule. Most of the splice junctions at the end of each exon show the same splicing pattern. This exon domain arrangement enables easy genetic manipulation of immunoglobulin genes, such as deletion and replacement of exons.

Cloning and manipulation of immunoglobulin genes

Rearranged variable region genes for cloning and expression may be derived from the rearranged germ line gene DNA or from mRNA-derived cDNA. Germ line or cDNA libraries can be screened using probes specific for the rearranged immunoglobulin DNA. Plasmids for expression of immunoglobulin genes can be classified into 2 groups for genomic or cDNA constructions. The genomic exon structure of immunoglobulin genes allows easy manipulation between each exon without having to worry about the reading frame or nucleotide change, since this can be done in the non-coding regions. The cloned rearranged variable domain exons can be put upstream to the constant region exons, which will then be transcribed, processed, and expressed as a single mRNA. In most cases, each individual exon may also be deleted or replaced without affecting the synthesis of the molecules. [17] The cDNA-type construct is more difficult to manipulate since special attention must be paid because nucleotide change introduced as a consequence of cloning or subsequent manipulation may affect the production and function of the resulting immunoglobulin proteins.

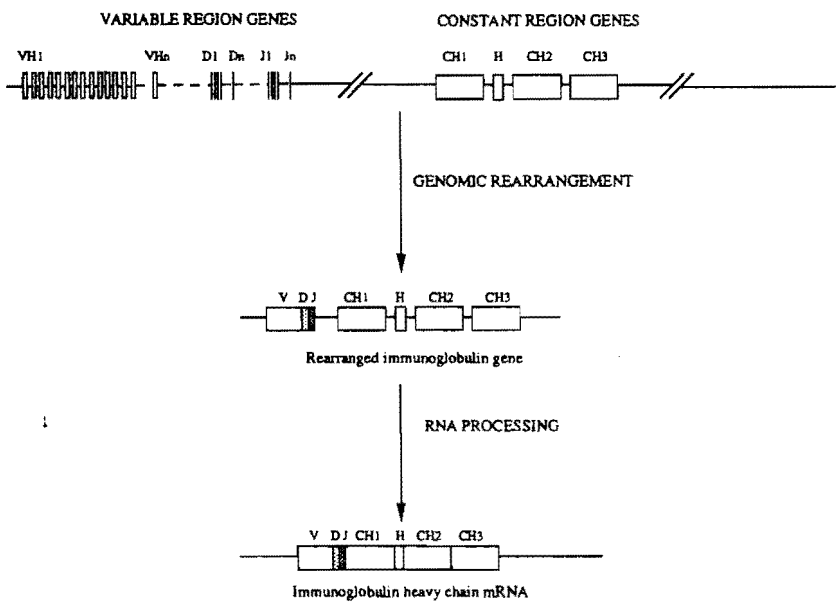

Fig. 1 Schematic diagram (not drawn to scale) showing the arrangement of germ line heavy chain immunoglobulin genes, rearranged immunoglobulin genes, and immunoglobulin mRNA.

Expression plasmids must contain biochemically selectable markers for selection of the successfully transfected host cells. Both heavy chain and light chain genes have to be introduced into the same host cells, either sequentially or at the same time. They may be cloned into the same expression plasmid, or, more commonly, into different plasmids containing independent selectable makers. [7,18] The expression of genetically engineered heavy and light immunoglobulin gene are under the influence of their normal control elements, ie. immunoglobulin promoters and enhancers, or of viral control elements, present in the expression plasmids. [2,19,20]

Expression of engineered immunoglobulin genes

The system for the expression

of genetically engineered immunoglobulin genes should be able to express both the heavy and light chain genes at high levels, glycosylate, assemble and secrete the complete functional antibody molecules. The most effective and commonly used system uses the murine myeloma or hybridoma cell lines since these cells are naturally well-equipped for producing and secreting functional antibodies. Genetically engineered genes can be introduced into host cells by electroporation or protoplast fusion, with stable transfection frequencies between 10^{-4} to 10^{-5} recipient cells. Yields of the secreted genetically engineered antibodies vary between experiments, and levels between 1-10 μg/ml are usually achieved although higher level of expression may occur. Production of large amounts of antibody required for clinical uses may be limited by the complexity and cost of large-scale cell culture. Although many attempts have been made to increase level of production of the transfected genes, such as by modifying the control elements or increasing the number of copies of the transfected genes,[21,22] it is possible that other unknown factors are necessary for the high level of expression of immunoglobulin genes. Other eukaryotic expression systems, such as COS cells, are also under investigation.[23]

Efforts have been made to produce antibody molecules in non-mammalian expression systems, such as in bacteria *Escherichia coli* or yeast, but these have until recently met with limited success.[24] Immunoglobulin polypeptides expressed in *E. coli* are usually insoluble and these polypeptides are also proteolytically degraded.[25,26] Other problems include the formation of incorrect disulphide bonds since the bacterial system does not support a proper protein folding environment. The effector functions of antibody molecules may also be affected by the lack of glycosylation. However, expression in *E. coli* may be useful for immunoglobulin fragments such as Fv and Fab fragments,[27,28] single-chain Fv,[29,30] and single-domain antibodies,[31] which may not need glycosylation and disulphide bond formation. For large scale production of antibodies, handling of bacterial cells may be easier and cheaper than manipulating mammalian cells.

Expression in yeast system also seems to be very attractive since it is capable of glycosylation and forming correct disulphide bonds. Functional antibody molecules have been successfully produced in yeast, although in a very low yield.[32] Yeast-derived chimeric antibody exhibited antibody-dependent cell-mediated cytotoxicity but not complement-dependent cytotoxicity.[33] This may be due to the incorrect glycosylation. In another

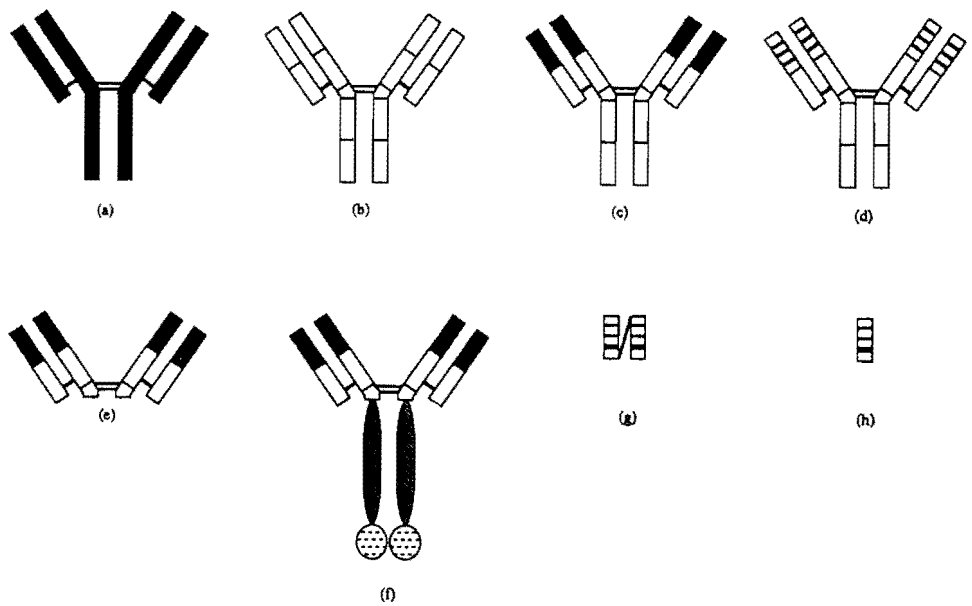

Fig. 2 Schematic diagram (drawn to scale) representing genetically engineered molecules resulting from antibody engineering. (a) murine immunoglobulin; (b) human immunoglobulin; (c) chimeric human/mouse immunoglobulin; (d) CDR-grafted humanised immunoglobulin; (e) chimeric $F(ab')_2$ fragment; (f) hybrid chimeric immunoglobulin-effector fusion protein; (g) CDR-grafted single chain Fv; (h) CDR-grafted single domain antibody fragment (dAb).

recent development, active antibody molecules have also been obtained from transgenic tobacco plants.[34]

Production of Chimeric Antibodies

Myeloma cells transfected with the rearranged rodent immunoglobulin genes were found to produce and secrete functional antibody molecules.[35-38] Two genes encoding both the heavy and light chains have to be present, and both heavy and light chain polypeptides have to be synthesized, assembled, and secreted to create a complete antibody molecule. Chimeric immunoglobulin genes have been genetically engineered by replacing the constant region exons of mouse immunoglobulin genes with the human constant region exons, then transfected into murine myeloma cells. These genes are faithfully expressed, and chimeric human/mouse monoclonal antibodies have been found to be secreted.[7-9] These chimeric antibody molecules have the variable regions encoded by murine genes while the entire constant regions are of human origin (Fig. 2). The binding affinity and specificity of the chimeric antibodies are, as predicted, comparable with those of the original rodent immunoglobulins. Since the constant regions can be easily manipulated, this technique is also very useful to produce other antibody isotypes that have previously been difficult to clone, for example IgE, by replacing constant region genes with the appropriate isotypes.[9]

Several chimeric antibodies, with specificities to tumour-associated antigens and other antigens, have been produced.[15,28,39-50] Their *in vitro* biological activity, specificity, affinity, biodistribution, and pharmacokinetics are currently being investigated. Some antibodies are now being used in the clinical trials.[51]

Winter and his colleaques have extended this approach further to make the "more human" monoclonal antibodies by replacing the complementarity determining regions of rodent immunoglobulins with those of human, a technique known as CDR grafting.[10-12] The genetically engineered genes were transfected into myeloma cells and functional antibody molecules were found to be secreted. These antibodies have only the CDRs that are derived from rodent, and theoretically should be seen by the human immune system as complete human immunoglobulin molecules (Fig. 2). Preliminary data showed that the specificity and affinity of the CDR-grafted antibody were comparable to those of the original antibodies. Whether these results can be generalised to be used with other antibodies, or whether these CDR-grafted antibodies have lower immunogenicity than the chimeric antibodies, have yet to be investigated.[52-54]

Although these chimeric or CDR-grafted monoclonal antibodies may minimize the anti-globulin response to heterologous rodent antibodies, they are, theoretically, still able to elicit the anti-idiotypic and anti-allotypic immune responses. The latter may be minimized by selecting the appropriate allotypes of antibodies which are most compatible with the patients' own immunoglobulins. The clinical significance of these anti-idiotypic and anti-allotypic response to the therapeutic chimeric or CDR-grafted monoclonal antibodies is not clearly known.

Expression of functional fragments of antibody molecules

Complete antibody molecules may have limited access to some tissues due to their size. Several attempts have been made to genetically produce small antigen-binding fragments of immunoglobulins. Biologically active Fab and Fv fragments have successfully been produced in myeloma cells and in *E. coli*.[27,28,55] The variable region genes of both heavy and light chains can be genetically linked by nucleotides encoding the spanning peptides, and expressed in *E.coli* system. Functional single chain polypeptides, so called single-chain Fv (Fig. 2), capable of specific binding to the same antigen as the original antibody, have been produced.[29,30] These molecules are composed of only the heavy and light chain variable regions and have a molecular size of about 1/6 of the complete antibody molecules. Moreover, the heavy chain variable region genes of the antibodies to keyhole-limpet hemocyanin and to lysozyme have been cloned and expressed in *E.coli*.[31] The resulting single domain polypeptide (dAb), containing only a VH domain (Fig. 2), can bind to the antigen with the affinity comparable to the complete antibody molecule. In this experiment, a repertoire of the variable region genes of immune spleen cells was amplified by polymerase chain reaction, then cloned and expressed in an *E.coli* expression system. Cell culture supernatants from each clone were then analyzed and some were found to be able to bind to the immunized antigen. Interestingly, some dAbs were found to specifically bind to another antigen not used for immunization. Since it may be possible to generate libraries of variable immunoglobulin domain expression vectors, these "universal" libraries may possibly be used for screening and selecting the antigen-specific dAb without having to amplify and clone immunoglobulin genes from every antigen-specific hybridoma cell lines or immunized animals and may supersede the hybridoma technology. In another experiment, a large combinatorial library of the immunoglobulin repertoire has also been generated in bacteriophage lambda vectors. Large numbers of monoclonal Fab fragments against a hapten can be screened from this expression library.[56] These techniques also lead to the rapid production of monoclonal antibodies without the use of live animals.

These antibody-like fragments which are much smaller than the

native immunoglobulins may be useful for *in vivo* clinical applications by virtue of their penetration. On the other hand, they may have a disadvantage due to the rapid plasma clearance.

Hybrid antibody molecule with novel effector functions

The inadequacies of monoclonal antibodies to be therapeutically or diagnostically efficient on their own have led to efforts to increase their efficiency by attaching them to various agents such as bacterial or plant toxins, fluorescent dyes, radionuclides and cytotoxic drugs. Direct coupling of antibodies to effector compounds has some major disadvantages. Chemical manipulation can both inactivate antibody binding sites and cause crucial alterations in the effector agents,[57] thus decreasing the efficiency of the immunoconjugates. Problems may also arise if the covalent bonds between the carrier antibody and the effector compound needs to be split for full biological action since such bonds may not be easily broken.[58]

The constant region of immunoglobulin may not be necessary for the expression and secretion of the molecule since the Fab or Fv-like fragments can be secreted from the transfected cell lines.[59] Large genetically engineered immunoglobulin molecules with two CH1, hinge, and CH2 domains have also been produced.[60] In addition, the CH2 and CH3 exons can be replaced by enzymatic moieties.[61] Neuberger and colleagues have successfully constructed and expressed antibody fusion proteins in which the nuclease from *Staphylococcus aureus* (SNase) or the Klenow fragment of *Escherichia coli* DNA polymerase I were joined to the CH2 immunoglobulin exons of the engineered heavy chain expression plasmids.[61,62] In another experiment, most of the heavy chain constant region of an immunoglobulin, with specificity to fibrin, was replaced by a gene coding for the β chain of tissue plasminogen activator (tPA). The resulting hybrid proteins exhibited the proteolytic activity of the original tPA, while retaining the specificity to fibrin.[63] Hybrid antibody-toxin fusion proteins, such as ricin and bacterial lymphotoxin may be another alternative of the magic bullet. Such hybrid molecules have enormous potential uses in immunoassays and immunotherapy.[61,64] Hybrid immunoglobulin molecules may also be used as carriers to obtain large amounts of proteins that are difficult to isolate or available only in very small quantities.

Genetic manipulation allows new effector functions to be added to the antibody molecules. The success of this technology depend on the stability, toxicity and secretion of the fusion proteins. In addition, they have to be folded in a manner that both antigen-binding domain of immunoglobulin and the novel effector domains remain functionally active.

Antibodies with two distinct binding ends show great promise as targeting agents and for improving immunoassays. The production and purification of these bispecific antibodies is still very difficult.[20] Bispecific antibodies have been produced by introducing two sets of immunoglobulin heavy and light chain genes into myeloma cells or by transfecting a set of heavy and light chain genes into secreting hybridoma or transfectoma cell lines. Chimeric bispecific human antibodies have been identified.[20,65] Since only the variable regions are derived from the parent hybridomas, the constant regions of the two heavy chains of chimeric immunoglobulins can be selected to allow total random association of heavy chains for the best yield of bispecific antibodies. Bispecific antibodies that cannot be produced due to the inability of the parental heavy chains to form stable molecules can also be engineered by replacing the heavy chain with a suitable class or subclass. Yields of bispecific molecules can be increased by selecting a suitable pair of constant regions. Transfectomas secreting bispecific antibodies also have a smaller number, and less complexity, of chromosomes compared with hybrid hybridomas obtained from cell fusion. Since only small chimeric genes are introduced and then integrated into host genome, the resulting transfectoma cell lines should be more stable. Genetically engineered single-peptide bispecific antibodies (such as VH1-VL1-VH2-VL2) may also be constructed using peptide linkers between each variable domain. This construct will allow 100% yield of bispecific molecules.[66]

Homologous recombination and transgenic animals

Human monoclonal antibodies can also be produced by gene targeting homologous recombination in murine hybridoma cells. Murine hybridoma cell lines secreting antibodies to tumour-associated antigen have been transfected by plasmid vectors encoding heavy chain constant region exons of the human IgG1 isotype, and homologous recombination between mouse and human heavy chain exons was observed at high frequencies. The antibodies secreted by these transfected murine cell lines retain the binding specificity but have the human IgG1 isotype.[67]

In another approach, a small repertoire of human immunoglobulin heavy chain gene exons was introduced into mouse germ line DNA by transgenic animal technology. Immunoglobulin molecules secreted by lymphocytes cloned from these transgenic mice were found to have the constant region of human origin. Creating transgenic mice which carry the whole repertoire of human antibody heavy and light chain genes may lead to the production of complete human monoclonal antibodies. Hybridomas derived from these immunised transgenic mice would

be able to produce human immunoglobulins which lack any sequences of mouse origin.[68]

Applications of engineered antibodies

Most chimeric human/mouse monoclonal antibodies have comparable specificity and affinity to those of the original mouse antibodies. Their effector functions, such as complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC), are similar to those of human antibodies.[15] Pharmacokinetics and biodistribution of these antibodies in the xenogeneic mice system are comparable to the human antibodies.[44] These "humanized" genetically engineered antibodies should be useful for many *in vivo* clinical applications by virtue of their reduced immunogenicity.

A chimeric human/mouse, IgG1/k, monoclonal antibody with specificity to human colon cancer were injected into 10 patients with metastatic colon carcinoma. Plasma half life of this chimeric antibody was six times longer than murine IgG. This chimeric antibody was found to be less immunogenic than its murine counterpart. Antiglobulin antibody directed to the variable region of the therapeutic chimeric antibody was detected in only one patient. No toxic or allergic reactions was observed during the course of the trial.[51] Preliminary result of a clinical trial using a genetically engineered CDR-grafted human/rat antibody, CAMPATH-1H, injected into 2 patients with non-Hodgkin lymphoma showed no detectable anti-globulin response to the therapeutic antibody.[53]

Genetically engineered monoclonal antibodies are very useful tools for investigating the structure-function relationships of the immunoglobulin molecules. For example, a panel of different hinge-length antibodies were constructed and systematically investigated. The result showed that the hinge region was essential for manipulating complement-binding activity.[60] Matched sets of immunoglobulin molecules with the same variable regions but having different constant region isotype will be very useful for studying the effector functions of antibody molecules.[15,46,69-72] Genetic manipulation by exon-shuffling and site-directed mutagenesis has also been used for studying the region of constant region domains required for effector functions.[73,74]

Antibody engineering can be used for rescuing unstable antibody-secreting human EBV-transformed or hybridoma cells. Immunoglobulin genes may be cloned from these human cells and then expressed in the appropriate host cells. Cloning of human immunoglobulin genes directly from peripheral blood mononuclear cells or lymphocytes from solid organs is also a wonderful possibility.

Conclusion and future development

Protein engineering technology enables the construction of specific antigen binding molecules with a variety of structures. Variable domain genes can be easily manipulated and joined to sequences encoding peptides that mediate the desired effector functions. These engineered proteins will be useful for immunodiagnosis and immunotherapy.

Several groups are now investigating suitable systems for cloning and expression of the genetically engineered genes.[14,60,75] Variable region genes can be easily amplified and cloned by polymerase chain reaction and inserted into the "cassette" sets of the appropriate expression plasmids.[14,20,31] This system will enhance and speed up the production of more chimeric antibodies that will certainly find their way for wide ranges of clinical applications.

Ultimately, the aim of antibody engineering is to produce novel synthetic antibody-like molecules with the desired specificities and effector functions. The potential applications of such molecules are enormous and will certainly revolutionize our knowledge of immunology in the near future.

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