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ALTERNATIVE TECHNOLOGIES TO GENERATE MONOCLONAL ANTIBODIES

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Several new technologies to generate and modify established hybridomas that produce monoclonal antibodies have recently been presented and further development should make them more suitable for diagnostic and therapeutic techniques. Different proteolytic procedures have been used for the fragmentation of intact antibodies to Fab$'_2$ and Fab fragments and recombinant DNA techniques have made it possible to obtain chimeric, humanized, Fv fragments and single-chain Fvs. A review of the new approaches is presented and the future implications are discussed.

The introduction of hybridoma technology in 1975 by Köhler & Milstein (1) started a revolution in immunochemistry, making it possible to generate monoclonal antibodies with predefined specificities and uniform affinities. Such murine monoclonal antibodies (MAbs) could be conjugated to drugs, isotopes or toxins and used as targeting components for diagnosis or therapy in cancer, and in cardiovascular, infectious and other diseases. The potential to further increase the usefulness of such immunochemical reagents has led to intense efforts to further develop recombinant DNA technologies, both from practical and theoretical approaches.

Two major basic concepts in the improvement process emerge. Because murine immunoglobulins and their derivatives are immunogenic in humans, repeated injections in patients elicit a human anti-mouse antibody (HAMA) response, which hampers the efficiency of the biological affects of the antibodies (2). This is the major reason why prolonged treatment with murine antibodies cannot be safely done, or even recommended during long periods. The other concept is more theoretically founded. Basic knowledge of how the diversity of the antibody repertoire is generated makes it possible to isolate the immunoglobulin genes and artificially manipulate the recombinational events that normally take place inside the lymphocytes to create the vast patterns of specificities that characterize the immune system (3). It is thus possible to completely circumvent the classical hybridoma technology, characterized by immunization of animals and cell-cell fusions, and instead generate genetic libraries of heavy and light chains from which a presumably wider range of MAb specificities can be produced. From a theoretical point of view, this procedure can be used to produce an even larger repertoire of specificities and affinities (4).

The different types of antibodies in use are depicted in Fig. 1. On the left, an intact MAb (of rodent origin) is shown. This antibody retains all native functional capacities and participates in antibody-dependent cell-mediated cytotoxicity reactions (ADCC), complement binding, Fc receptor binding and placental transfer (5). It can only be produced by eukaryotic cells, because of the complex assembly of disulphide bonds. Furthermore, eukaryotic cells are required to generate the complex glycosylation patterns needed by several specific glycosyltransferases and glycosidases. This antibody does not appear in the urine, due to its large size. Its biological half-life time is 8–21 days. Intact antibodies usually display comparatively high affinities. The dark areas, representing the complementarity-determining regions (CDRs), represent the peptide stretches that give each MAb its antigenic specificity. Using recombinant DNA technologies (6), all the constant regions of a mouse MAb can be exchanged with constant regions

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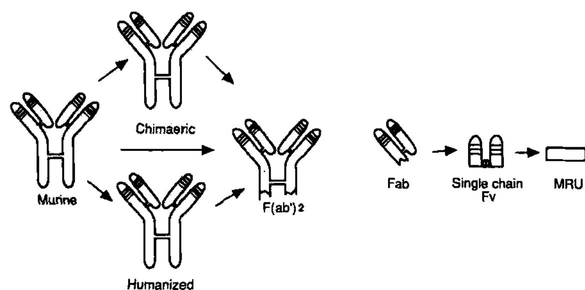

Fig. 1. Different types of monoclonal antibody derivatives generated by proteolytic cleavages or recombinatnt DNA technologies. The three black lines represent the CDR regions in the variable domain of the antibody. (Modified reproduction from Nature 1989; 342: 99. Permission granted by publisher.)

derived from human antibodies, to produce chimeric antibodies. These antibodies display lower immunogenicity in humans with all effector functions intact.

Further improvements can be obtained by replacing only the CDR regions from the mouse MAb into a complete human framework of peptide stretches, i.e., also exchanging the regions in between and flanking the CDR stretches (7). Such antibodies elicit a minimum of antibody responses, and are usually referred to as humanized antibodies. It is reasonable to believe that the most antibodies used in the future will be derived either from humans (human monoclonal antibodies) or from humanized murine antibodies.

By proteolytic degradation with pepsin of intact IgG MAbs Fab$'_2$ fragments can be obtained. This IgG derivative, generated by hydrolysis on the C-terminal side of the hinge region, retains the divalent binding properties of intact IgG, but has lost almost all functional effector properties of the intact antibody. The loss may in some cases be an advantage. Fab$'_2$ fragments are thus rapidly excreted in the urine, especially those fragments not targeting tumour cells; thus these fragments do not contribute to background radiation. Also the Fab fragments, generated by proteolytic cleavage with papain are rapidly cleared from circulation. However, due to their monovalency, they present lower affinities to their antigens in comparsion to divalent derivatives.

Smaller derivatives of the antibodies can be produced either as Fv fragments (fragments containing only the variable heavy and light chain regions) or single chain Fvs (fragments containing the heavy and light chain peptide stretches covalently connected by a short peptide (8)). These fragments can be obtained by recombinant DNA technology, and can be expressed in prokaryotic systems. All the fragments mentioned above usually show lower immunogenicity than the intact initial murine antibodies. At least in the initial phase after in vivo injection, the fragments penetrate deeper and faster into tissues that express the target antigens. However, it should be emphasized that the small Fv or ScFv fragments completely lack constant regions, which necessitates specific affinity procedures for their purification and practical handling. No isotype-specific antisera can be used for their detection. Up to now, Fv fragments have been used mainly for (NMR) nuclear magnetic resonance and crystallographic studies.

Recombinant DNA technologies to improve antibody properties

The following reasons can explain the rapid development in this field during the last few years:

- Both mouse and human Ig genes can be more safely stored as genes instead of hybridomas frozen in liquid nitrogen.
- The constant regions can be deleted or modified to convert murine antibodies to less immunogenic, chimeric or humanized antibodies (by CDR grafting).
- The different effector functions in the constant domains can be modified or exchanged.
- The specific peptide stretches in the CDR regions can be modified by in vitro mutagenesis, which affects both affinity and specificity of the antibody, and causes further maturation of the antibody and its properties.
- New expression systems i.e., prokaryotic systems, can be developed for Fv and ScFv fragments. This may have biotechnological implications for the future large-scale production of engineered antibodies.
- By making genomic libraries of genes (derived from rodents or human) that encode immunoglobulin heavy and light chains, it is possible to artificially generate a vast collection of specificities with a repertoire which is probably larger than that seen in vivo, due to the lack of T-helper cell participation, and the potential generation of antibodies with comparatively lower affinities than those observed in vivo.

Three major technological prerequisites or improvements have accelerated developments: 1) the genetics behind the diversity of the antibody repertoire is known (9), 2) polymerase chain reaction (PCR) technology, which makes it possible to amplify selected DNA pieces by framing the selected region by single chain PCR-primers (10), and 3) the expression of small Fv or ScFv fragments in bacteria (11).

The overall strategy to clone and express immunoglobulin genes from previously produced hybridomas is shown in Fig. 2. Messenger RNA with poly A-tails, which encodes heavy and light chains, can be isolated by oligo (dT) cellulose chromatography. The corresponding cDNA can be amplified by PCR to generate cDNA for individual heavy and light chains. The average size of cDNA for the heavy chain is approx. 2–3 kb. On the genomic level, however, the size of the heavy chain is approximately 12 kb, which is beyond the limit of current PCR technology. One of the most widely used modifications of the heavy chain cDNA is to exchange the constant regions of the heavy and light chains of rodent IgG MAb with the

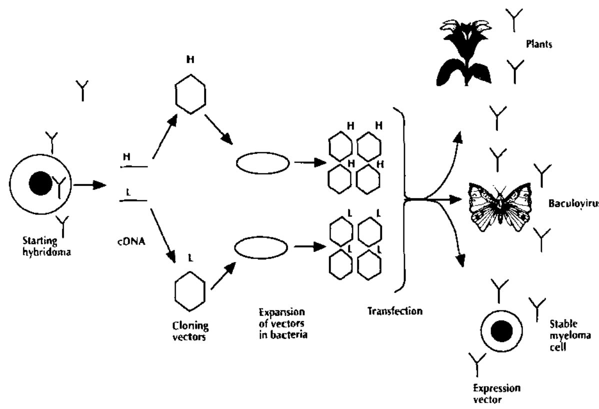

Fig. 2. The overall strategy for cloning and expression of antibody genes. (Modified reproduction from 'Monoclonal antibody and immunosensor technology' by P.C. van der Vliet, editor, published by Elsevier 1991. Reproduction permission granted by publisher).

constant regions of a human myeloma protein of a chosen class and subclass.

Another alternative is to replace the framework in the CDR regions, or modify the constant domains by adding or deleting different sequences. Several antibodies have been modified in order to improve their properties.

When modified, antibody genes have to be expanded in shuttle vectors, which can be plasmid or viral in nature. Many vectors are commercially available, but must be adjusted to the expression system used. The expression of antibody genes in mammalian cells requires vectors with a specific promoter sequence, an enhancer sequence and a sequence to indicate the addition of poly (A) to the end of the transcript (12). To obtain a functional antibody with heavy and light chains properly assembled, both these chains have to be expressed in the same cell. They are usually transfected to the expression cell on separate vectors (Fig. 2). During Ig synthesis in normal plasma cells, several crucial events occur to produce the final antibody. Heavy and light chains must be properly assembled and the leader sequence required for secretion must be present and later removed. Furthermore, glycosylation must be executed very specifically for secretion from the plasma cell to take place. Neither bacteria (*E. coli*) nor yeast cells are able to express intact immunoglobulins properly, due to the absence of adequate glycosylation and proper assembly of the different chains. Fab and Fv fragments, however, can be expressed in this way. Non-IgG-secreting eukaryotic myeloma cells are able to secrete intact properly assembled and glycosylated IgG; these cells are generally referred to as transfectomas. Immunoglobulins or fragments have furthermore been empressed in baculovirus and even in plants (13). The functional properties of these antibodies are still partly unknown.

With PCR-generated libraries of the entire immunoglobulin genome from either humans or immunized mice, it is possible to express in a bacterial vector system either the variable domains or the variable plus the constant domains of the immunoglobulin genome, which can be screened after expression (14–16). This makes the conventional fusion procedure unnecessary. With such techniques the antibody repertoire can be significantly enlarged, and many low affinity chain combinations are also generated. Conventional screening of all combinations (average 10^{12} clones) requires substantial effort, but the recently reported expression of antibodies on the surface of phages carrying in their genomes the DNA for the same antibody has had a singificant impact on the selection procedure. Such systems have now been further developed, making it possible to identify specific clones among millions of different competing antibodies. The specific improvement that makes this screening possible uses 'fused' proteins, one derived from the phage and expressed on its surface, and the other comprising the immunoglobulin heavy chain, also expressed on the surface of the phage (17, 18). This heavy chain can covalently bind light chains produced by the same phage by disulphide bonding. The phage carries in its genome the DNA for this specific antibody. From a vast number of phages, it is possible to select those that bind to the antigen when coated on plastic surfaces, and thus to enrich fractions specific for the selected antigen. Once identified, the DNA for the corresponding antibodies can be reintroduced in other systems and manipulated, so that the antibody derivative does not remain as a fusion protein.

It is of course relevant to speculate about the potential and significance of the systems described above. Initially, these were technologies seeking relevant biological applications, not biological problems seeking a solution. But as in other scientific fields, the methodology enables new questions and problems to be raised.

The possibility of establishing true human hybridomas or human antibodies will probably dominate the immediate future. Developments might include hybridomas or genes (derived form individuals) which clinically respond to defined tumours or bacteria—antigens which normal individuals cannot be exposed to for ethical reasons. Antibody genes from such individuals may be of immediate significant value. Future advances might include the generation of specific vaccines against tumour cells or bacteria, to trigger antibody responses to defined antigens. Another area in which genetically engineered antibodies may be of interest is in biological targeting to visualize inflammatory processes, tumors, or specific cell surface proteins.

Further into the future, the identification of CDRs able as isolated entities to exert biological functions is exciting. New classes of self-targeting pharmaceuticals will appear, and the amino acid sequence information gained may further facilitate the preparation of small molecular recognition elements. Such recognition elements may have a significant impact in the future clinical medicine and oncology.

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