Genetically Engineered Antibodies
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The technology needed to genetically engineer antibodies is evolving rapidly and the potential utility of these novel reagents is being explored with vigor. The process includes cloning of the antibody genes, their in vitro manipulation and mutagenesis, expression in a suitable host/vector system, and, for commercial production, scale-up, purification, and product evaluation. At each step, significant advances have been achieved recently. For example: at first, antibody genes were cloned from genomic libraries by using adjacent DNA probes; techniques for rapid sequencing by primer extension of total mRNA allowed more specific screening with synthesized oligomers; finally, antibody genes can now be created de novo by chemical synthesis. Moreover, such synthesis allows total control over the antibody sequence so that molecules of any configuration can be produced. New reagents created in this way include murine antibodies whose constant regions and variable-region frameworks have been replaced with human sequence to enhance immunocompatibility with patients, to switch immunoglobulin class, or both.

Additional Keyphrases: monoclonal antibodies · gene synthesis · expression vector · immunotherapy · chimeric antibodies

Monoclonal antibodies have been available for more than 15 years (1). Despite their great utility in the laboratory and in diagnostic testing, and notwithstanding their high promise and the enormous research effort expended on them, they have yet to make a significant impact as clinical tools. Monoclonals are not used routinely to treat or image tumors, to provide passive immunization against infectious diseases, or for immunosuppression after organ transplant—all uses for which they have been intended.

Among the problems encountered in the effort to translate the diversity and specificity of monoclonals into clinically useful reagents are the following:

- The human immune response to foreign antibodies.
- Difficulty in making physical contact between the antibody and the target antigen.
- Change in antigen structure during tumor development or through mutation, as well as antigen shedding.
- Low affinity, inappropriate isotype, or nonoptimal systemic half-life of immunotherapeutic antibodies.
- Difficulty in producing sufficient quantities of antibody for therapy.

In 1983, Oi et al. reported (2) that lymphoid cells can express cloned, transacted immunoglobulin genes. This demonstration opened the way to a new technology that may provide solutions to some of the problems listed above, i.e., the cloning, engineering, and expression of genes that code for antibodies. Because these methods have been shown capable of creating new kinds of antibody molecules (e.g., 3–5), and some of these are clinically relevant (e.g., 6–8), the possibility is now enhanced that the undoubted promise of immunotherapy can someday be realized.

Genetic Engineering of Antibodies

The process by which genetically engineered antibody variants can be created has been reviewed extensively elsewhere (e.g., 9, 10). The major goals of such engineering include:

- Creating immunocompatible reagents by joining rodent variable regions to human constant regions.
- Altering systemic half-life by truncation or amino acid addition.
- Isotype switching.
- Increasing affinity or avidity.
- Using gene fusion to generate bispecific antibodies.
- Using gene fusion to generate molecules that bind antigen but also possess additional activities.
- Altering effector functions such as $F_c$ receptor binding and complement activation.
- Reducing anti-idiotypic response to rodent antibodies by replacing rodent variable-region framework sequence with human sequence.

For each antibody of interest, engineering begins by cloning the variable regions, which dictates antigen specificity. DNA is isolated from hybridoma cells and cloned, usually in $\lambda$ phage vectors. The phage library is screened by hybridization with a radioactively labeled probe. Because the genomic rearrangement that results in production of functional immunoglobulin genes leaves common sequences between the variable (V) and constant (C) regions, any functional heavy-chain gene can be isolated with a single ("universal") probe. The same is true for the light chain. These clones can be verified with synthesized oligomers as described below.

Once the desired V regions have been isolated, they must be cloned into a vector appropriate for expression. If immunocompatibility is an objective, it is desirable that the expression vector contain a human C region; given that most available hybridomas are murine, the V region is usually from mouse. Moreover, panels of expression vectors now exist, each containing a different human C region. Thus, the isotype of the antibody can be chosen at will.

Expression vectors have been designed for use in bacteria, yeast, or mammalian cells (2, 11, 12). The most common hosts are murine myeloma cells, which, through mutation, have lost the ability to make endogenous antibody. Vectors for myeloma cells usually contain heavy(H)-and light(L)-chain genes individually, and these are co-introduced into the host (6, 7). In addition to the murine V and human C regions, the vectors contain a bacterial origin of replication and drug-selectable markers to identify which cells have taken up and expressed the vectors. The immunoglobulin genes are usually controlled by their own promoters and enhancers. These constructions may contain

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Received April 13, 1989; accepted June 9, 1989.
other features as described below.

When antibody genes have been engineered as desired and placed in expression vectors, they can be transfected into myeloma cell hosts. Transfection is by electroporation (13) or protoplast fusion (14). Although both H and L chain vectors are individually selectable, it is interesting that co-transfection is so frequent that selection with only one drug is necessary. Transfection frequency is about one in 1000. Drug-resistant colonies are grown, tested for secretion of antibody by enzyme-linked immunosorbent assay, and high-producing lines are identified.

**Purification of Murine V/Human C Chimeric Antibodies**

Once a cell line secreting the engineered antibody has been identified, it must be scaled-up for production. This can be done in tissue culture or by growing the cells in mice and collecting antibody from ascites fluid. Various purification schemes have been reported (e.g., 15), some of which utilize the binding activity of the antibody. My colleagues and I have used methods that rely on properties of the human C regions that distinguish chimeric from contaminating antibodies. In the case of tissue culture media, the major contaminant is bovine antibody found in serum. As shown in Figure 1 (C. Shearman and D. Lawrie, Integrated Genetics, unpublished), elution from Protein A-Sepharose can clearly separate a murine/human chimeric antibody from contaminating bovine immunoglobulins. In ascites fluid, murine antibodies constitute a major contaminant. These are removed effectively (Figure 2) by binding to Protein A-Sepharose, elution, and then passage over Sepharose complexed with anti-murine IgG (16). Both these methods yield chimeric antibody of sufficient purity for subsequent functional analysis. Moreover, these methods can be applied to any murine V/human C chimeric antibody without regard to its antigen specificity.

**Characterization of Chimeric Antibodies**

As described above, a virtually limitless array of new antibody variants can be produced by genetic engineering. The utility of each reagent for immunotherapy must ultimately be determined in clinical trials. Because such trials are laborious and costly, it is critical that novel variants be screened by various in vitro assays or in animal studies. The nature of such analysis is dependent upon the particular antibody in question. To illustrate, I refer to a murine V/human C chimeric of the murine antibody B6.2 (6), which has specificity for human breast, colon, and lung carcinomas (17). Comparisons of chimeric and murine B6.2 with

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**Fig. 2. Purification of chimeric B6.2 immunoglobulin by (A) nonreducing polyacrylamide gel electrophoresis; (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (C) isoelectric focusing**

A and B: lane 1, molecular mass standards; lane 2, unpurified ascites fluid from mice injected with cB6.2-producing transfected cells; lane 3, ascites fluid after binding and elution of IgGs from Protein A-Sepharose; lane 4, cB6.2 after purification from ascites fluid by binding to Protein A; followed by chromatography on Sepharose-bound sheep anti-murine IgG; lane 5, B6.2 purified by "Fast Protein Liquid Chromatography" (LKB, Bromma, Sweden). C: lane 1, unpurified ascites from mice injected with B6.2-producing transfected cells; lane 2, the same material after binding and elution from Protein A; lane 3, the same material after passage over anti-murine IgG-Sepharose; lane 4, purified B6.2; upper arrow, purified B6.2; lower arrow, position of purified cB6.2. From reference 16; used with permission

**Fig. 3. Indirect immunofluorescent labeling of human tumor cells**

Top of each pair of photographs (upper-case lettering): cells bound with cB6.2, then stained with rhodamine-conjugated goat anti-human x. Bottom photographs (lower-case lettering): cells of the same line bound with B6.2, then stained with fluorescein isothiocyanate-conjugated goat anti-murine IgG. The cell lines are A549 (A.a), 9812 (B.b), LS174T (C.c), SW900 (E.e), and human fibroblasts (F.f). In D,d, A549 cells were bound with total human (A) or murine (a) IgG, then stained as above. From reference 16; used with permission

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**Fig. 1. Purification of a murine V/human C chimeric antibody by chromatography on a column of Protein A-Sepharose**

About 400 ml of tissue culture supernate containing about 5 mg of chimeric antibody per milliter was passed over a 2.0 ml column. Bound antibody was eluted with a linear pH gradient of 0.1 mol/L citrate buffer from pH 6.5 to 3.0. Fractions (1.0 ml) were assayed for bovine and chimeric antibody by ELISA. The symbols designate (o): total protein, (+): bovine, and (c): chimeric antibody (Chip Shearman and Dawson Lawrie, unpublished)
general, their properties are similar to the parent murine monoclonal. Their utility in immunotherapy is being tested in various clinical trials, the results of which should be available over the next few years. It is hoped that, because of their presumed greater immunocompetability in patients, they will outperform the murine antibodies from which they are derived. Meanwhile, new kinds of antibody variants are being produced and tested (see below).

Increasing the Expression Level of Engineered Antibodies

A major issue in the widespread clinical application of genetically engineered antibodies will be the ability to produce them in large amounts. This is particularly important because many immunotherapeutic regimens involve repeated administration of relatively large doses of antibodies. Unfortunately, engineered antibodies expressed by transfection into eukaryotic hosts, e.g., myeloma cells, often are not produced efficiently. Most “transfectomas” produce 2–10 pg/10⁶ cells per day; this is only 1–10% of the production level of the best murine hybridomas. The probable reason is that the transfected genes integrate into the host chromosomes at random and, therefore, at loci that are not necessarily optimal for expression. Although antibody genes have been expressed in yeast and bacteria, there are problems in chain association, secretion, glycation, and biological activity.

We have attempted to increase the expression of transfected antibody genes by linking them to genes that can be amplified by a slow increase in the concentrations of various drugs (18). As the number of copies of the amplifiable gene increases in response to the drug, the linked antibody gene(s) is also amplified in copy number. This amplification results in an increase in gene expression and thus increased secretion of antibody.

A commonly utilized gene–drug combination is the gene coding for dihydrofolate reductase, which is amplified by the drug methotrexate. We have adapted this combination for use in myeloma cells; a representative vector is shown in Figure 6. Using this vector, we have achieved a large increase in the expression of transfected immunoglobulin genes (18). Moreover, similar amplifiable vectors are now being used to express nonimmunoglobulin genes in myeloma cells (19, and M. Hendricks, Integrated Genetics, unpublished).
The Use of DNA Synthesis in Antibody Engineering

The chemical synthesis of DNA of desired nucleotide sequence is an important new technology in molecular biology. The process has now been automated, and fragments about 100 nucleotides long can be made without great difficulty. We have been applying this technique to antibody engineering in a variety of ways.

The process of cloning desired V regions is laborious because genomic libraries must be constructed and the tiny minority (perhaps one in 10^6) of target clones must be purified from the background. Moreover, a serious problem is that aberrantly rearranged, and hence nonfunctional, V regions are often obtained from the screen (20). These problems can be circumvented by synthesizing the desired V regions de novo rather than cloning them.

Obviously, the DNA sequence of the region must be determined before synthesis. Because mRNA that specifies immunoglobulin is so prevalent in hybridoma cells, this can be accomplished directly (21). Poly A+ mRNA is isolated from the hybridoma and hybridized with a short "universal" DNA primer synthesized with the knowledge of the sequence of the common C region. The duplex is then extended by using reverse transcriptase, and the product is sequenced. This sequence can be used to make specific probes to screen libraries.

The more innovative approach is to synthesize the entire V region. Although the sequence is too long (400–500 nucleotides) to synthesize in one piece, several alternative methods are available to assemble the synthesized fragments. One technique is to synthesize pairs of opposite strands with restriction sites at their ends and short overlaps. These ends are annealed, then the structure is filled in with DNA polymerase. The resulting double-stranded fragments are then ligated through the restriction sites at their ends to form the finished structure. Depending on the length, intermediate cloning may be required to generate sufficient material for the next step. Care must be taken that the engineered restriction sites do not interfere with either the desired amino acid sequence or with physiologic codon usage. The number of known restriction sites is so large, however, that this is rarely a problem.

The advantage of polymerase is that it reduces the amount of DNA synthesis required. The disadvantage is that the error rate is relatively high. Because even a one-base error can render the construct nonfunctional, each piece must be sequenced after synthesis and after each cloning step.

A second method involves synthesis of both complementary strands in their entirety. A 10- to 15-nucleotide "overhang" allows ligation of serial double-stranded fragments. Surprisingly, one simply mixes a relatively large number of single-stranded, overlapping fragments. This complex reaction results simultaneously in opposite-strand hybridization and overlapping pair ligation to form the completed structure in a single step. The synthesized V region is then cloned, sequenced (for verification), and inserted into an appropriate expression vector.

As described above, one advantage of total V region synthesis is ease and speed. A second advantage is avoidance of cloning aberrant V regions. More importantly, however, this method gives absolute control over the structure of the resulting gene. Thus, any variant can be created easily. Further, mixing of nucleotides at nucleotide addition steps during the gene synthesis generates multiple amino acids at particular positions. This new form of in vitro mutagenesis is extremely powerful.

Replacement of Complementarity-Determining Regions

Any antibody variant can be made by using the technique of total gene synthesis. Technical limitations include possible problems with message or protein stability, chain association, glycation, or secretion. The major limitations, however, are the creative imagination, the knowledge of antibody structure/function needed to design improved antibodies, and the resources necessary to test them.

A major goal of antibody engineering is to make immunocompatible therapeutic agents. I have described chimeric antibodies containing human C regions. These reagents, however, are still capable of eliciting host immune response against portions of the rodent V regions (22). As an example of the use of total gene synthesis, I now describe a new antibody variant designed to reduce further, or eliminate, host response to therapeutic antibodies.

It has been known for some time that most antigen specificity resides in defined portions of the V regions called "hypervariable" or "complementarity-determining" regions (CDRs) (23). Spatially, the CDRs are looped away from the V region framework, which consists of a β pleated sheet (24). These CDR loops form the antigen-combining sites. Comparison of V region sequence among immunoglobulins from a wide variety of sources shows that sequence variation is far greater in the CDRs than elsewhere in the V region (25). Consequently, it should be possible to transplant the CDRs from a rodent antibody into a human framework (3, 10) and to maintain the antigen specificity of the rodent parent antibody while eliminating host response to the V region framework. Further, because the CDRs are so variable, even within a species, they are unlikely to elicit an immune response. Thus, host immune response should be reduced greatly, or eliminated, by CDR replacement.

The three H-chain and three L-chain CDRs are interspersed with framework sequence. Thus, total gene synthesis represents the most practical way to make CDR-replaced variants. In our hands, the process is carried out as shown in Figure 7. Poly A+ mRNA is isolated from the target rodent hybridoma and sequenced as described previously. A computer search is made to identify a closely related pair of human H and L chains. Alternatively, one can utilize the most related H and L chains and assume that they will later be capable of association. The next step...
Steps in CDR Replacement

1. Isolate poly A+ mRNA from the target hybridoma and sequence the H and L chain V regions by primary extraction.

2. Computer search to identify relaxed human H and L chain V exons.

3. Design "civilized" H and L chain V exons which incorporate mature CDRs into a human framework. Include unique restriction sites at the CDR/FR borders and maintain physiologic codon usage.

4. Synthesize "civilized" V exons and confirm by sequencing.

5. Ligate "civilized" V exons into expression vectors, then transfect and select.

6. Test the engineered product.

Fig. 7. Procedure to generate a CDR-replaced antibody; arrows indicate the positions of engineered, unique restriction sites

is to design the CDR-replaced variant by incorporating the rodent CDRs into the human frameworks. Unfortunately, the exact borders of the CDRs are not clear and probably vary among different antibodies. Thus, it is not always obvious exactly which amino acids to change to human and which to leave as rodent. The goal, of course, is maximal antigen binding with minimal host immune response. Much of our research is directed towards learning rules that will help dictate these choices. When suitable CDR-replaced sequences for H and L chains have been designed, they are synthesized, cloned, sequenced, expressed, and tested. A further feature of the scheme shown in Figure 7 is that unique restriction sites have been engineered at the CDR/framework borders. In the future, new antibody specificities will be generated by insertion of synthesized CDRs at these sites rather than by synthesis and assembly of the entire V regions. This will result in a significant saving of effort.

One CDR-replaced antibody has been described by Riechmann et al. (10), and others are being evaluated in our laboratory and elsewhere. Many other engineered antibody variants are also being produced and studied. At the least, this work will result in a greater understanding of immunoglobulin structure and function. New molecules will be produced that can be applied successfully for immunotherapy.

References