Abstract

The ability to produce antibodies that are directed against specific antigens has played a crucial role in advancing scientific discoveries. Recombinant technologies have extended the application of antibodies beyond the research laboratory and into the clinic for the treatment of cancer and other diseases. Creative approaches using these technologies have been used to reduce the antibody to its minimal functional size, and/or make them bifunctional (immunotoxins), bispecific, or less immunoreactive (humanized). Additionally, mice that are engineered to generate antibodies of human genomic origin have been used to produce therapeutic antibodies and are being further developed. As the research and clinical demands for antibodies continue to increase, the development of improved resources (cell lines and animals) to improve production efficiency, generate larger repertoires, and deliver greater yields of antibodies is being explored, and advances in this area are discussed further in this review.

Key Words: antibody; apoptosis; hybridoma; immunotherapy; single chain Fv; tissue culture

Introduction

Soon after it was realized that antibodies with desired specificity could be mass-produced, the concept that they could be used as "magic bullets" to target disease-associated proteins was born. The development of technology to clone and sequence immunoglobulin genes provided the tools necessary to construct antibody-based molecules and fusion proteins for the treatment and diagnosis of cancer, rheumatoid arthritis, and infectious diseases. In 2003, the US Food and Drug Administration approved 14 antibody-based pharmaceuticals, of which 70 were in late-stage clinical trials (Phase II+) and > 1000 were in preclinical development (reviewed in Stockwin and Holmes 2003).

Additional growth in the area of hybridoma and monoclonal antibody production technology is projected as genomic and proteomic high-throughput programs identify new proteins that will require immunoanalyses and/or purification for further characterization. Although standard procedures for generating antibodies of desired specificity have been used for approximately 30 yr, the development of more efficient techniques and resources would be a boon for biomedical research.

Antibody Engineering

Early studies demonstrated that when monoclonal antibodies (MAbs) directed against tumor cell antigens were injected into mouse models, they inhibited the growth of tumors expressing the targeted antigens (Drebin et al. 1988). Although somewhat effective in human clinical trials, the neutralizing effects of the human antimouse antibodies that were elicited in response to the administration of these mouse-derived proteins reduced their effectiveness as diagnostic and therapeutic agents and raised concerns over the risk of treatment-associated anaphylaxis (Ritter et al. 2001). "Humanization" of the mouse-derived MAbs has been the most widely used strategy to reduce their immunogenicity for therapeutic purposes in people. To humanize a mouse MAb, its modeled structure is compared with that of human immunoglobulin (Ig) protein structures (allotypes) to identify the closest match. Recombinant approaches are then used to graft the complementarity (or specificity)-determining regions (CDRs) from the mouse-derived hybridoma Ig cDNA to the corresponding regions of the matched human Ig cDNA. The CDRs (described below), which give the antibody its affinity, are relatively small, hence the newly formed recombinant protein produced from the expression of this construct has the specificity of the mouse Ig but is less likely to be recognized as being foreign by the human immune system.

Because of their large size, it is often difficult to manipulate and express antibody genes. To overcome this limitation, various recombinant antibody-like forms and peptides have been produced using a reductionist’s approach (reviewed in Peterson (1996) (Figure 1). The smallest functional unit of an antibody to be produced has been the CDR peptides (Figure 1C). Depending on which CDR is

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Abbreviations used in this article: CDR, complementarity-determining region; CHO, Chinese hamster ovary; ETA, exotoxin A; Ig, immunoglobulin; MAb, monoclonal antibody; scFv, single chain fragment variable; Ig, immunoglobulin; TSA, transitional state analogue; VH, variable heavy; VL, variable light.

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produced, it can vary in length from eight to 20 amino acids. The CDRs may be thought of as fingertips, which make contact with an object, and the framework regions of an antibody chain as being analogous to the hand and fingers, which hold things in place. The affinity of a CDR is tested by its ability to compete with the parental antibody at its binding site. Berezov and colleagues (2001) demonstrated that a peptide designed from the sequence of the third CDR of an anti-Her2/neu antibody heavy chain sequence was able to bind to the receptor and disable its tyrosine kinase activity. Another biologically active peptide derived from an antineurokinin receptor antibody by Wijkhuisen and coworkers (2003) was capable of antagonizing substance P-induced cAMP production. These results among others exemplify the potential for antibodies to provide a scaffold from which small molecule therapeutics and diagnostic compounds can be made (Murali and Greene 1998).

Because an antibody uses multiple CDRs to bind to an epitope and peptides lack three-dimensional structure, antibody-based peptides have significantly less affinity than their multivalent parental antibodies. This characteristic may consequently limit their practical application. Recombinant approaches have led to the development of single chain fragment variables (scFvs), which are monovalent and about one third the size of an antibody (Figure 1D). The Fv section of an antibody is limited to that portion of the heavy and light chains that each contain the three CDRs and framework regions. It is smaller than a Fab (Figure 1B) in that the scFv does not include the first constant region with disulfide bounds that link the heavy and light chains together. The recombinant single chain is formed by the tandem arrangement of the heavy chain and light chain sequences joined by a flexible linker typically composed of glycines and serines (gly-gly-ser)_5. When expressed in bacteria or eukaryotic cells, the scFv folds into a conformation that is similar to the respective region of its parental antibody, and it retains comparable affinity to that of a Fab (Kortt et al. 1994).

Once produced, scFvs are amendable to various genetic modifications such as humanization and the production of novel fusion proteins to enhance their potential as therapeutic agents. The latter may be necessary to compensate for their lack of an Fc to stimulate effector function and consequential cell killing. Clinical trials using Pexelizumab, a humanized scFv that binds to the C5 component of complement, has been shown to significantly reduce myocardial infarctions associated with coronary artery bypass graft surgeries in people (Verrier et al. 2004). scFvs that adhere to various cancer-associated antigens have also been modified to deliver toxins and chemotherapeutics to solid tumors (Figure 1F). By genetically fusing a truncated form of Pseudomonas aeruginosa exotoxin A (ETA) to a humanized scFv that has affinity for the proto-oncogenic epithelial cell adhesion molecule, Di Paolo and colleagues (2003) developed an immunotoxin that inhibited the growth of lung, colon, and squamous cell carcinomas in xenografted mice. An additional cysteine residue was placed at the C-terminus of a antiendoglin scFv by Volkel and coworkers (2004) so that it could be chemically coupled to doxorubicin-loaded liposomes for specific delivery to proliferating endothelial cells, such as found in tumors. These examples illustrate the dynamic potential of recombinant antibody-fusion molecules in clinical medicine.

Several IgG/M antibodies have been reported to induce cellular growth/differentiation and apoptosis. These interactions often depend on the antibody being multivalent in order to promote bound receptor aggregation and activation. Reduction of the linker length of the scFvs to between three and 12 residues prevents the monomeric configuration of the scFv molecule and favors intermolecular variable heavy (VH)–variable light (VL) pairing with the formation of a noncovalent scFv dimer “diabody” (Holliger et al. 1993) (Figure 1E). Further reducing the linker length to fewer than three residues can, in some cases, result in the formation of trimers (Kortt et al. 1997) or even tetramers (Le Gall et al. 1999). Using this approach, Kikuchi and colleagues produced a monovalent scFv and diabody from an anti-CD47 antibody, which induced apoptosis in leukemic cells (Kikuchi et al. 2004). Interestingly, the monovalent scFv (constructed with a longer spacer) had no biological effect, whereas the anti-CD47 diabody acted similarly to its parental antibody and induced apoptosis in leukemic cells that expressed the receptor. By using a synthetic interlocking helix motif, Peterson and Greene (1998) also produced scFvs that formed bivalent dimers. The bacterially expressed bivalent anti-Her2/neu scFv was significantly more effective at causing cell-surface down-modulation of the

Figure 1  Schematic of antibody-derived molecules. (A) Antibodies (immunoglobulin G) are naturally produced by B cells and are composed of two heavy and two light chains joined by disulfide bonds. The variable regions (variable heavy [VH] and light [VL]) are stippled. (B) Fabs were the first small antibody forms made by proteolytic digestion with papaain. (C) The VH and VL regions each contain three complementarity-determining regions whose sequences can be used to synthesize CDR peptides. (D) In a single chain fragment variable (scFv) molecule, the VH and VL regions are held together by a glycine-serine linker (dark line). (E) Diabodies form when the linker spanning the VH and VL is shortened. (F) scFv-toxin fusion protein.
oncogenic receptor than the monovalent form of the molecule. Bivalency can also be provided by the fusion of an antireceptor scFv with the receptor’s ligand. Bremer and colleagues (2004) used this strategy to cross-link EGP2 (Ep-CAM) receptors, which triggered apoptosis in cancer cells expressing this receptor.

ScFvs of different specificity can also be linked together to produce bispecific antibodies that bind two different receptors on single or different cells. The latter strategy has been commonly used to enhance the activation of T cells in proximity to targeted tumor cells. This approach was used by Korn and coworkers (2004) to produce a bispecific antibody-like form with an antiendoglin scFv (found on proliferating endothelial cell in tumors, mentioned above) and an anti-CD3 scFv, which is a cytotoxic T cell-activating receptor. In tissue culture assays, the diabody facilitated killing of endothelial cells, whereas cells that did not express endoglin were unaffected. Using scFv and ligand sequences, Schmidt and Wels (1996) also constructed a bispecific fusion protein to target both the ErbB-2 and epidermal growth factor receptors (respectively) that are simultaneously expressed in various aggressive adenocarcinomas. This construct also included an ETA sequence, and the bacterially expressed fusion protein inhibited the growth of A431 tumor xenografts in nude mice. From these observations, it would appear that numerous combinations of scFvs and toxins could be made; however, the tendency for many of these synthesized fusion proteins to fold improperly and form aggregates places limitations on these creative designs.

Since the early 1990s, phage display of combinatorial heavy and light chain genes obtained from people and animals have been explored as another means of generating antibody-like molecules (Marks et al. 1991; Pini and Bracci 2000). In this approach, large repertoires of antibody variable region cDNAs are collected from the B cells and combinations of VHs and VLs are expressed in the form of scFvs on the surface of filamentous bacteriophage (Figure 2). This method allows the phages that express scFvs with the appropriate specificity to be captured from antigen-coated plates. The affinity of an scFv may be improved by mutating the CDRs of the construct and then repeating the panning selection procedure. The advantage of this approach is that cancer-specific antibody fragments can be and have been directly isolated from antibody libraries of tumor-infiltrating lymphocytes (Hansen et al. 2001) and lymph nodes (Graus et al. 1998) of cancer patients. The first phage display-derived scFv approved for clinical trial was reported by Chester and colleagues (2000) who used radiolabeled ant carcinoembryonic antigen scFvs to locate colorectal tumors for surgical removal.

The pharmacokinetic values of scFvs differ from those of antibodies and are typically cleared from the system more rapidly. This characteristic may be disadvantageous in diagnostic imaging applications in that the background noise is reduced by the more rapid clearance of radiolabeled scFvs compared with MAbs. In addition, because of their smaller size, scFvs better penetrate solid tumors.

### Mouse Engineering—Human Antibodies

A disadvantage of recombinant phage display is that the production of high-affinity antibody forms is difficult to obtain and the procedures are not familiar to most laboratories, hence the mouse continues to be the most commonly used progenitor of monoclonal antibodies and their derivatives. To avoid the multistep process of producing humanized antibodies to provide immunotherapeutic compounds, the generation of human MAbs directly from genetically engineered mice is continually being developed. This task was impossible until technology to introduce transgenes on yeast artificial chromosomes was developed, enabling the transfer of extremely large human immunoglobulin loci into the mouse germline (Choi et al. 1993). The XenoMouse® (Abgenix, Inc., Fremont, CA) and HuMAb Mouse® (GenPharm-Medarex, San Jose, CA) were the first engineered mice to carry a majority of both the human VH and VL (kappa) repertoire, and in 2002, five fully human MAbs generated from the XenoMouse were used in clinical trials (Kellermann and Green 2002). Ongoing developments are aimed at increasing the antibody repertoire even further by introducing the human Ig lambda chain locus into these mice (Nicholson et al. 1999). Microcell-mediated chromosome transfer has also been successfully utilized to transfer chromosome fragments containing human Ig genes into mice (Tomizuka et al. 1997) and cattle (Kuroiwa et al. 2002), and these genetically engineered animals may have
potential as additional sources of human MAbs and polyclonal antibodies, respectively.

**Mouse Engineering—Production Technologies**

The application and advancement of most of the technologies discussed above depend on the generation of an immune response to a specific antigen and the ability to harness the B cell component of that response for continued production and further testing. In 1975, Kohler and Milstein first reported that B cells harvested from an immunized mouse could be immortalized by fusing them with established myeloma cell lines derived from the BALB/c mouse (Kohler and Milstein 1975). The BALB/c mouse and its derived cell lines are still the current primary resource used for the generation of Mab-producing hybridoma cells. However, evidence presented in the remaining sections of this review suggests that the use of some spontaneous mutant and genetically modified mouse strains and cell lines may improve the efficiency of hybridoma/MAb production technology.

One such strain is the MRL/MpJ-lpr/lpr mouse, which has a spontaneously formed defect in the apoptosis regulatory gene *Fas*. Expression of the defective *Fas* leads to polyclonal B cell lymphoproliferation and hypergammaglobulinemia in these mice. After experiencing difficulty producing antibodies that catalyzed esterolytic activity in BALB/c mice, Takahashi and coworkers (2000) immunized MRL/MpJ-lpr/lpr mice with a transitional state analogue (TSA1). They found that this strain produced eight times as many catalytic antibody-secreting clones as similarly immunized BALB/c mice. They speculated the TSA may be recognized as a self-antigen, and hence reactive B cells were selectively eliminated in the BALB/c mice, whereas the apoptosis-resistant MRL/MpJ-lpr/lpr cells escaped this negative selection. The advantage of using this mouse strain for the generation of antibodies to other antigens, particularly those that are not very immunogenic, awaits further investigation.

The effects of antiapoptotic gene expression on B cell longevity was further demonstrated by the prolonged IgG and IgM serum titers to sheep red blood cells in inoculated Bcl-2 transgenic mice (Strasser et al. 1991). In addition, the numbers of splenocytes obtained from BALB/c mice with exogenous Bcl-2 transgenic mice were subsequently increased by two to five fold compared with wild-type BALB/c mice (Knott et al. 1996). When the splenocytes from these B galactosidase-immunized transgenic mice were used to produce hybridomas, 48% of the wells plated with the fused Bcl-2 expressing spleen cells produced B galactosidase-specific MAbs compared with only 14% of the wild-type splenocyte fusions. These results suggest that apoptosis inhibitory genes (endogenous and transgenic) may improve the efficiency of hybridoma production by increasing the numbers and repertoire of B cells obtained from each immunized mouse.

Pasqualini and Arap (2004) recently demonstrated that it may soon be possible to produce monoclonal antibodies without fusing them with myeloma cells. They demonstrated that splenocytes obtained from bacteriophage-immunized transgenic mice, H-2kb-tsA58 “Immortal-Mouse” (Charles River Breeding Laboratories, Wilmington, MA), could survive clonal selection and produce bacteriophage-specific MAbs in vitro. The key to this finding was that this mouse contains a recombinant construct that places the large T-antigen under the control of a temperature-sensitive mutant of the SV40 promoter, and thus when the splenocytes are grown at 33°C, the gene is expressed and the cells are immortalized. Additional studies are needed to determine whether this strategy will also be successful and practical in producing antibodies to additional antigens.

**Myeloma and Hybridoma Cell Engineering**

The expression of antiapoptotic genes affects the mouse’s immune response to antigens, but does expression of these genes provide any advantage to the cells’ survivability and production characteristics in vitro? Similar to the experiments using apoptosis-resistant splenocytes described above, Ray and Diamond (1994) and Kilpatrick and coworkers (1997) demonstrated that a larger repertoire of hybridoma cell lines were obtained when exogenous Bcl-2 expressing myelomas were used in fusions instead of their parental controls. These results can most likely be attributed to the protective effect of Bcl-2 expression on populations of hybridomas that would otherwise be destined for apoptosis and lost during the fusion process. Although postfusion Bcl-2 expression was not analyzed in these cells, the expression of exogenous Bcl-2 in other hybridoma cell lines was shown to suppress cell death rates under conditions of low cell density (Simpson et al. 1999), nutrient deprivation (Chung et al. 1998; Simpson et al. 1998), and increased intracellular acidity (Ishaque and Al-Rubeai 1998). In another set of studies, the increased survivability of Bcl-2 transfected hybridomas translated into increased monoclonal antibody yields (Itoh et al. 1995; Simpson et al. 1997). However, an advantage in MAb production was not noted in Bcl-2 transfected cells in two other independent studies (Bierau et al. 1998; Simpson et al. 1999).

When the genetic components of apoptosis-resistant (P3X63Ag8.653) and -susceptible (SP2/0 and D5) myeloma cell lines were compared, Bcl-XL, a protein related to Bcl-2, was found to be elevated (Gauthier et al. 1996). Similarly, we found that viability, maximal cell density, and MAb yields were markedly improved in 5-day batch cultures when Bcl-xl expression was restored to a hybridoma cell line that had been deficient in its expression (Peterson and Servinsky, submitted). These observations raise the prospects that strategies to identify and restore the expression of deficient genes that are important regulators of cell viability, such as Bcl-xl, may be an effective means to optimize MAb production.
Recently, it was demonstrated that deletion of a 60- amino acid unstructured loop from Bcl-2 and Bcl-xl enhanced the ability of the protein to prevent apoptosis (Chang et al. 1997; Figueroa et al. 2001). Chinese hamster ovary (CHO) cells that expressed the deletion mutant form of Bcl-2 (Bcl-2 delta) were also more resistant to Sindbis virus-mediated apoptosis than the parental cell line (Figueroa et al. 2001). As a result of this difference, higher yields of a heterologous protein encoded on the Sindbis virus were obtained from the Bcl-2 delta-expressing cell line. Additionally, Bcl-2 delta-expressing CHO cells adapted to serum deprivation better than the full-length Bcl-2-expressing CHO cells (Chang et al. 1997). Expression of a deletion mutant form of Bcl-xl (Bcl-xl delta) was also better at preventing apoptosis caused by IL-3 withdrawal in an immature B cell line than the full-length protein (Chang et al. 1997). Bcl-2 delta and Bcl-xl delta expression also inhibited apoptosis in two hybridoma cell lines that were studied in my laboratory; however, increases in batch MAb yields varied among the clones analyzed (N.C.P. and Servinsky, submitted). These results are similar to those obtained with the full-length Bcl family genes discussed above and are most likely attributed to the modifying effects of the backgrounds of each of the cells.

An alternative approach to increasing cell longevity in batch cultures is to decrease the cell’s production of lactic acid, which when accumulated can lead to apoptosis. By using homologous recombination to partially disrupted lactic acid dehydrogenase A expression, Chen and colleagues were able to select a hybridoma cell clone that produced 50% less lactic acid than its parental cell (Chen et al. 2001). In batch culture, cells achieved a higher density and viability, and the amount of antibody harvested from 5-day cultures was three times greater than that obtained from the parental cell cultures. As additional information about cell metabolism and protein production is gained, additional targets will be identified for modification to maximize in vitro MAb yields.

**Concluding Remarks**

Despite numerous reports of the potential for alternative mouse strains and modified cell lines to enhance MAb production technologies, the commonly accepted resources have not changed. This situation may be due in part to the inconsistent gains in increased MAb yields obtained from cultures of genetically modified cell lines. Unfortunately, reported comparisons have not involved sufficient trials with different cell lines, animals, and/or antigens to make generalized conclusions, and experiments have been performed independently with no standardization.

To evaluate effectively whether the use of genetically modified mice or cell lines can significantly improve MAb production technology, it is necessary to invest considerable effort and resources to test these resources on a larger scale under consistent, defined conditions. Academic hybridoma production centers are ideally suited to perform this task because they receive numerous antigens for custom antibody production. Additional fusions using some of the genetically modified mice or cell lines discussed above could be included with each antigen submitted, and the success rates (positive clones) and MAb production yields obtained from the alternative and conventional approaches could be compared. Given that some academic hybridoma centers perform more than 30 fusions per year, sufficient trials could be included at these centers to evaluate the practical application of these alternatives effectively. Once completed, results should be publicized to avoid future duplication of efforts and to promote the use of these newly developed resources. This endeavor would be very worthwhile because even modest gains in the development of resources to increase fusion efficiencies and/or increases in MAb yields would have a significant impact, given the large-scale use of this technology.

In keeping with a philosophy of developing alternatives to animals (Russell and Burch 1959), innovations to increase the efficiency of hybridoma production would also reduce the number of animals needed per immunization (reduction). In addition, advances in in vitro MAb production technologies would further deflate the popularity of in vivo approaches (replacement).

**References**


