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Advances in Monoclonal Antibodies

Stephen M. Edgington

Stephen M. Edgington is Senior Editor of Bio/Technology Magazine, 65 Bleecker St., New York, NY 10012, e mail p01000@psilink.com

"I have no fear that the thought which forms the basis of serum therapy will ever disappear out of medicine." Emil Behring in an address to the 1895 Naturalist Congress

Should developing countries consider monoclonal-antibodies (MAbs) as a means to fight tropical diseases? There are a number of economic and technological questions to consider in attempting to answer this question.

"Developing countries should consider transferring a technology only after it has passed the research and development stage," says John Preston (Massachusetts Institute of Technology, M.I.T., Cambridge, MA). Preston's years of experience in transforming bench-top inventions into commercial ventures gives him a unique perspective on what is required. According to Preston, the key for short-term economic success is to wait until someone else has tried it --and then improve on their mistakes. Preston says the reason for this is simple. Tweaking a system to make it more cost effective or improve its quality is much easier than starting with a blank piece of paper.

Antibody-based therapies seem to meet Preston's criteria for adoption. While antibody therapies have been in use since the 1890s, during the past 25 years MAbs have steadily

matured as diagnostic and therapeutic tools. At present, diagnostic MAbs make up 75 percent of the estimated $740 million U.S. market for antibodies. MAb tests for blood type, pregnancy, drugs, and infectious diseases are the major money winners. But these numbers pale in comparison to the $8 billion dollars in sales that therapeutic MAbs are projected to generate by the year 2000.[1] More than half of the 90 MAb therapeutics presently in preclinical or clinical testing in the U.S. should emerge from regulatory trials by the year (see Figure 1.). These MAb therapeutics include treatments for gram-negative sepsis, cancer, autoimmune diseases, H.I.V., and other virally transmitted diseases.

For developing countries, this forecast for explosive growth must be tempered by the realization that economic barriers to entry are high for the types of Mabs presently in clinical trials. At present, late-stage MAb drugs range in cost from $2000-$4000 per dose.[2] When one considers that World Health Organization studies suggest that most developing countries spend $5 per person for health care annually,[3] it is little wonder that drug developers have paid little attention to MAb therapies for tropical diseases.

While it may appear that MAb therapeutics are too expensive to every be applied to the endemic diseases in developing countries, there are reasons to believe this situation will change. For one, a MAb drug for malaria is now in pre-clinical development. Secondly, new technologies such as phage antibodies and combinatorial library screening methods promise to develop MAb diagnostics and therapeutics at an unprecedented rate--and cost. These techniques have a relatively low barrier to entry making them within the reach of almost any standard lab within a metropolitan area.[4] Moreover, the relatively inexpensive

scale-up costs suggests that developing countries may build on this research to develop commercial applications.

But perhaps most important, these new technologies will go far to identify the molecular shapes of the invading pathogens and pinpoint the best targets for intercession. This will not only enable new disease treatments, but will also be a powerful tool in the parallel development of new vaccines. As the history of antibody therapies suggests, passive immunity is only a stopgap measure for an acute situation. Freeing developing countries from the heavy burden of tropical diseases demands the cheap, ongoing immunity that only vaccines can provide.

Passive immunity

The first successful use of antibody therapy in humans is suggested to have occurred in 1891.[5] Anecdotally, a young boy in Berlin was reported to have received an injection of diphtheria antitoxin that saved his life on Christmas day. While this can not be documented, it is known that the previous year Emil Behring published a paper demonstrating that diphtheria antitoxin could produce passive immunity to a lethal dose of diphtheria toxin in animals.[6] The results were so impressive that by 1893 Faberwerke Hoechst prepared enough sheep-serum diphtheria antitoxin to mount the first large-scale trial in humans.[7] The success of these trials led, in turn, to trials in Paris using horse-serum antitoxin. Here again, the results were impressive: diphtheria-related mortality dropped 50 percent.

While the antibody serum was successful, it wasn't without its problems. "Serum sickness," as it was known, accompanied administration of the drug in the form of fever, rashes, and joint pains. At times, patients went into anaphylactic shock. Unbeknownst to Behring and the researchers of the day, the human immune system was reacting against the foreign horse antibodies. The gradual improvements Behring made in reducing these side effects can be seen today as blind luck: ammonium-sulfate precipitation of the serum reduced side effects by fractionating out the antibodies from the other serum proteins. Pepsin digestion further reduced the side effects in this gammaglobulin fraction by creating smaller, less immunogenic antibody fragments.

Despite these problems, the serum had widespread use until 1913. Then, inactivated diphtheria toxin, injected directly, was shown to produce active immunity without serum sickness. From that point on, the vaccine supplanted antitoxin therapy.

But, for certain diseases, a form of serum therapy continues today. Agammaglobulinaemic patients--those without sufficient levels of naturally occurring IgGs--are still given pooled IgGs collected from human serum as a means of producing passive immunity. But, as a common practice the approach is limited.

From this brief history, it is readily apparent that inducing passive immunity through serum antibodies has always been most successful as means to either neutralize a pathogen in an acute disease--such as diphtheria--or as a stopgap measure in a chronic disease where no other therapies exist--such as agammaglobulinaemia.

Hybridomas

In 1975, the elegant experiments of George Kohler and Cesar Milstein (Medical Research Council, Cambridge, U.K.) revolutionized the way antibodies could be obtained.[8] The team developed an *in vitro* method for generating large quantities of a purified antibody to a known antigen--the first MAb. The technique involved immunizing a mouse repeatedly with the antigen of interest, and then removing the mouse's spleen. Since the spleen is a reservoir of proliferating B cells--the cells that secrete antibodies in reactions to a specific antigen--the scientists could use it as an antibody library. By "checking out" the B cell that responded to the antigen challenge, and then fusing it to a non-secreting myeloma cell line, they created a "hybridoma." The major problem with this method was that one could never be sure that the resulting hybridoma maintained good binding affinity and specificity until this labor-intensive process had been completed. Compounding this, the amount of antibody secreted by the hybridoma varied widely between hybridomas. Despite these drawbacks, since 1977 this technique has been the bread and butter of the MAb industry, allowing the development of antibodies for basic research and diagnostics.[9]

But the mouse-based hybridoma technology has been limited in its therapeutic applications because rodent antibodies--much like sheep or horse serum--cause an immune reaction against them. This results in side effects similar to serum sickness and decreased effectiveness due to short half lives.

The creation of human monoclonal antibodies--an apparent solution to these problems--has not proven to be an easy undertaking. Human B cells proved to be refractive to attempts to immortalize them: whether fused with myeloma cells or infected with Epstein

Barr virus (EBV), the B cells eventually lose their ability to produce antibodies.[10] Those EBV-transformed B cells that do remain stable are problematic because they generally produce minute yields of low-affinity IgM antibodies. Many of the headline-grabbing failures of MAbs in clinical trials have been IgM antibodies produced by these methods. The reason for these difficulties can be traced to the hybridoma system itself. Humans, unlike lab animals, cannot be hyperimmunized with antigens of interest, nor have their spleens harvested, in the hopes of producing a breakthrough drug. Instead, researchers use cultured lymphocytes, generally from the peripheral blood, as a source of B cells in the hopes of producing a high-affinity antibodies. But, in general, these cells produce low affinity Mabs. Last, but not least, mouse Fc portions of the antibody are not recognized by human Fc receptors (see Figure 2.). This neutralizes the effector function of the antibody--the recruitment of T killer cells, macrophages, and complement to destroy the invading pathogen. With no killer function, their therapeutic value is nullified for many diseases.

Steps to humanization

To overcome this lack of effector function, researchers chemically linked plant and bacterial toxins, enzymes, radioactive nuclides, and cytotoxic drugs. This demonstrated that rodent MAbs were an effective delivery system for site-directed killing, but many of the most promising targets, such as surface antigens on cancer cells, produced low-affinity rodent MAbs. Since these low-affinity MAbs tended to cross react with other non-target antigens, a method was needed to assure that these lethal materials were delivered to the correct address.

This led to the production of bispecific MAbs--antibodies that were specific for a different epitope on each arm. Because the two arms recognized different target-specific antigens, they produced higher specificity than the respective antibodies from which they were derived.

Initially, chemical cross linking was used to link the two separate arms. But, this proved to be unsatisfactory from a production standpoint--often yielding less than 10 percent of the desired antibody from the starting mixture. An alternative method fused two hybridomas to create antibodies that were bispecfic.[11] But the time, cost, and difficulty in creating two hybridomas, and then fusing them, prevented wide-spread use of this method as well.

Applying the tools of molecular biology to reshape antibody structure was a major step in overcoming the problems of rodent-based MAbs. For the first time, researchers could genetically fuse enzymes, toxins and cytokines to MAb binding fragments--avoiding the problems of chemical linkage and decreasing the production problems associated with hybridomas.[12] Not only were these "fusion proteins" much simpler to produce than chemically cross-linked Mabs, in many cases they could be expressed in bacteria rather than hybridomas. In contrast to the hybridoma technology, that depended on a mammalian cell to produce two separate chains and having them associate inside the cell correctly in order to be functional, in bacteria the heavy and light chains of the variable (V) domains were expressed as a single chain with a short peptide tether. This tethered approach allowed the two chains to naturally associate as they were expressed. This not

only greatly reduced the cost of production, but allowed researchers to proceed much more quickly in developing and testing new "chimeric antibodies."

Since specificity was still a major issue in ensuring delivery of highly toxic substances even with these chimeric antibodies, the next generation of human MAbs sought to overcome this problem by reengineering the mouse Fc region with a human Fc region. This reengineering process restored the molecule's natural effector function (see Figure 2.). While the method worked in pre-clinical testing, clinical trials demonstrated that more than 50 percent of the patients' immune systems recognized the rodent portion of the molecule and mounted an immune response to it.[13]

The reason for this immune recognition was eventually traced to the six hypervariable loops known as the complementarity determining regions (CDRs). These CDRs form a framework that make up the antigen-binding surface on the antibody (see Figure 2.). Because mouse CDRs are different from those of humans, the immune system sees it as an invading pathogen. "Grafting" human CDRs onto the rodent framework successfully disguised the antibody from the human immune system while maintaining the same specificity. But most often, the antibody's affinity decreased--requiring further tweaking of individual amino acids to restore equivalent binding. So far, the strategy has produced clear clinical benefit in certain instances.[14]

Phage antibodies

The use of PCR (polymerase chain reaction) techniques to rapidly clone the variable (V) region of antibody genes paved the way for new ways to generate antibody heavy and

light chain combinations.[15] Since V genes have similar sequences on each end, it was possible to form PCR primers that hybridize to the ends and amplify the variable sequence in the middle. Starting with human B cell RNA or DNA it is possible to generate antibody gene libraries by this technique.

These cloned genes were expressed in Escherichia coli. Filimentous bacteriophage (phage) were used to deliver the antibody gene to the bacteria: the antibody variable regions are incorporated into the genome. Once the phage injects its single-stranded DNA genome into the bacteria it is incorporated into the bacterial genome. As the bacteria replicates, it passes the viral genome on to its daughter cells. In this way, each bacterium becomes a factory for antibody production. This method allowed expression and screening of as many as a million transfected genes at a time.[16]

Researchers soon learned to take additional advantage of the phage delivery system: The pencil-shaped viruses have on their tips several copies of a gene III protein used to attach to the E. coli's cell wall. Researchers found that fusing an antibody's variable region to this attachment protein still allowed the phage to attach to the bacteria.[17] As the bacteria replicated they formed and extruded a perfect replica of the invading phage--with the phage antibody on its tip. In a single overnight incubation one milliliter of bacterial supernatant contains 10^{11} phage particles. Running this population through an affinity column allowed researchers to easily select the best binders from this pool. Having found the best binders, the sequence can then be excised and put into the expression system of choice.

Combinatorial libraries and the immune system

Phage libraries enable researchers to simulate the humoral immune system *in vitro*. By analogy, each phage which contains a randomized variable gene ligated to its gene III region mimic a resting B cell. When one introduces an affinity column linked antigen to this *in vitro* system, the bacteriophage expressing the antibody on its surface bind to the antigen--just as a B cell does in the immune system. Through growth in bacteria, one can amplify the selected phage antibody, sequence the antibody gene, and then use it to produce large amounts of soluble antigen-specific antibody--duplicating the result of a humoral response to an invading pathogen.

Phage antibodies have the possibility of extending the molecular shape repertoire beyond what occurs in natural antibodies: the heavy and light V genes are often amplified separately and then recombined randomly when ligated into the phage. This means that antibody recognition that is normally suppressed or weak is now achievable through use of the phage antibody system. Target candidates can now include self antigens, carbohydrate moieties, and tumor surface antigens.

The technology also allows the development of polyclonal phage antibodies-- an antibody "cocktail" of different monoclonals raised against a specific antigen. It has long been understood that polyclonals are usually a far more effective means to detect and neutralize a pathogen than a single monoclonal. The serum antibodies initially used to treat diphtheria were precisely these kinds of polyclonal mixtures. But the hybridoma system did not facilitate the easy development of polyclonal mixtures. For rapidly mutating viral

infections, the phage polyclonal antibody approach should produce much more reliable means of detection and treatment.

Phage antibody technology's new range of molecular shape recognition is forcing a complete rethinking of antibody recognition and binding. It is now becoming routine to tweak antibody binding affinity --regardless of the antibodies origin. This ability to tinker with molecular shape allows drug makers to identify promising lead compounds *in vitro* and then refine the binding characteristics in animal testing and even in clinical trials. Strategies for this type of "affinity maturation" extend from simple point mutations in the variable region, to shuffling either the complementary heavy or light chain to create new libraries. Since there is a physical limit to the number of potential shapes one can easily screen in a single library, strategies are emerging that allow researchers more confidence in searching this molecular "shape space" for the best answer.

Refining phage selection strategies to produce high affinity antibodies brings all these elements into play. For example, researchers recently attached an antigen to the gene III protein in a group of infection-competent phage, and then expressed antibody fragments from a library on a group of infection-incompetent phage. In the same way that a B cell must recognized an antigen as a signal for replication, the antibody phage had to recognize the antigen phage before it could infect the bacteria and be replicated.[18] This method has proven to be an extremely powerful way to search antibody shape space.

Searching shape space

This concept of searching shape space has extended beyond phage antibody systems and now include the synthesis and testing of combinatorial libraries made from peptides, DNA, RNA, and the mixing and matching of chemical analogs to all three. Instead of employing phage technology, researchers rely on either peptide or nucleotide synthesizers and PCR amplification to create large libraries of molecular shapes that will bind to a particular target. As the cost of synthesis goes down, these approaches become increasingly appealing.

The method has the advantage of being able to start with materials that exhibit properties that are desirable in the final product. Fro example, by synthesizing libraries that contain chemical moieties known to increase *in vivo* half life, researchers can find the best binders from pools that already address later-stage delivery problems. This can provide considerable cost savings for drug development--too often drugs go all the way to human clinical trials before they are found to be ineffective.

The phage antibody technology and the combinatorial library techniques should complement each others' development. The phage antibody technology promises to enhance these combinatorial approaches by mimicking the ligand binding of cell surface receptors. At present, less than 20 soluble receptors are available for screening purposes. By developing phage antibodies that mimic the ligand binding of these uncloned receptors, rapid screening for ligand mimetics should be possible. The combinatorial approach should enhance the phage antibody methods by providing new ways to screen for the most effective binders.

In addition, the recent development of transgenic mice that mimic the human immune system should extend the range of both phage antibody and combinatorial approaches.[19] These transgenic mice can serve as human-like preclinical tests for therapeutics generated by either method. In addition, hyperimmunization of these mice should enable the development of human hybridomas to antigens never previously possible. Particularly for human diseases that there is no mouse counterpart, this should become a powerful method for generating and testing diagnostics and therapies for tropical diseases.

Conclusions

Compared to the expense of hybridoma technology, both the phage antibody system, and the combinatorial libraries systems are much more within the range of possibility for developing countries. Although these technologies are considered cutting edge, their relative simplicity, speed in developing lead compounds, and the enhanced control over the final product recommend them to labs with limited resources.

Beyond this, adopting these techniques place researchers solidly in the mainstream of emerging trends--enhancing the potential for training and funding by industrialized countries.

As there is a steady migration of diseases to developed nations that were previously unknown--or thought eradicated--the demand for this type of researcher may be on the increase. As an example, trypanosomiasis, also known as Chagas' disease, never previously seen n the U.S. is now becoming increasingly prevalent.[20] What's more, new targets for intervention in the progression of these diseases are being found. These targets

suggest themselves to screening through either phage antibody technologies or combinatorial library methods. For example, the Chagas' disease parasite, Trypanosoma cruzi, was recently found to require a functional cysteine protease in order to mature. So far, *in vivo* tests in mice have shown that enzyme inhibitors are able to significantly reduce T. Cruzi growth.[21] Further screening promises to develop new diagnostics and therapeutics.

While it may seem cruelly ironic, as these diseases spread in industrialized countries, they suddenly become attractive targets for drug developers: In the U.S., the outbreak of less than 10,000 cases of a disease qualifies it for orphan drug status--despite the fact that it may affect millions of people world wide. An FDA (Food and Drug Administration, Rockville, MD) representative recently outlined the advantages for drug developers to consider developing treatments for these diseases:[22] Orphan drug status would allow the drug to go on an accelerated track through U.S. clinical trials and could mean government participation to help defray costs. In some instances, it would also mean that the drug maker could charge for the drug during certain phases of the trials as a means to recoup expenses.

As business becomes defined by a global perspective rather than the national, the interdependence of nations has become more fully acknowledged. In the past, developing countries have looked to industrialized countries for solutions to their problems. When it comes to tropical diseases, this has not been a winning strategy. The adaptation of biotech-oriented MAb technologies may be one step developing countries can take toward finding solutions that help solve their economic and health problems.

References

1. Market Intelligence Research Corporation. Monoclonal Antibody Markets. Mountain View, CA

2. Chalfin, D., Holbein, M., Fein, A., and Carlon, G. 1993. Cost-effectiveness of monoclonal antibodies to gram-negative endotoxin in the treatment of gram-negative sepsis in ICU patients. (intensive care unit). *JAMA* **269**:249-255.

3. Rowe, P. 1993. Tropical diseases (second annual meeting of the National Institute of Allergy and Infectious Diseases International Centers for Tropical Disease Research). *Lancet* **341**:1273.

4. Medynski, D. 1994. Synthetic peptide combinatorial libraries. *Bio/Technology* **12**:709-719.

5. Singer, C., and Underwood, E.A. 1962. *A short history of medicine*, 2nd ed. Oxford University Press, Oxford.

6. Behring, E., and Kitasato, S. 1890. Uber das zustandekommen der diptherie--Immunitat und der tetanus--immunitat bei thieren. *Deutsch Med. Wochenschr* **49**:1113-1114.

7. Gronski, P., Seiler, S., and Schwick, H. 1991. Discovery of antitoxins and development of antibody preparations for clinical uses from 1890 to 1990. *Mol. Immu.* **28**:1321-1332.

8. Kohler, G., and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**:495-497.

9. Williams, A., Galfre, G., and Milstein, C. 1977. Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: Differentiation antigens of rat lymphocytes. *Cell* **12**:663-673.

10. Steinitz, M., Izak, G., Cohen, S., Ehrenfeld, M., and Flechner, I. 1980. Continuous production of monoclonal rheumatoid factor by EBV-transformed lymphocytes. *Nature* **287**:443-445.

11. Pastan, I., and Fitzgerald, D. 1991. Recombinant toxins for cancer treatment. *Science* **254**:1173-1177.

12. Gross, G., Waks, T., and Eshhar, Z. 1989. Expression of immunoglobulin-T cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl. Acad. Sci. U. S. A.* **86**:10024-10028.

13. Meridith, R., Khazaeli, M., Plot, W., Saleh, M., Liu, T., and Allen, L. 1992. Phase I trial of iodine-131-chimeric B72.3 (human IgG4) in metastatic colorectal cancer. *Journal of Nuclear Medicine* **33**:23-29.

14. Hale, G. et al. 1988. Remission induction in non-Hodgkin lymphoma with reshaped monoclonal antibody CAMPATH-1H. *Lancet*:1394-1399.

15. Orlandi, R., Gussow, D., Jones, P., and Winter, G. 1989. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. U. S. A.* **86**:3833-3837.

16. Huse, W. et al. 1989. Generation of a large combinatorial library of the immunoglobulin library in phage lambda. *Science* **246**:1275-1281.

17. MacCafferty, J., Griffiths, A., Winter, G., and Chiswell, D. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**:552-554.

18. Duenas, M., and Borrebaeck, C. 1994. Mimicking the strategy of the immune system by linking antigenic recognition and phage replication: Clonal selection and amplification of phage displayed antibodies. *Bio/Technology* **12**:in press.

19. Lonberg, N.et al. 1994. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* **368**:856-857.

20. Kirchoff, L. 1993. American trypanosomiasis (Chagas' disease--a tropical disease now in the United States. *New Eng. J. Med.* **329**:639-645.

21. McKerrow, J. The cysteine protease of Trypanosoma cruzi (Cruzain) as a target for drug design. NIAID International Centers for Tropic Disease Research, Third Annual Meeting, April 27-29

22. Haffner, M. Opportunities for clinical studies of orphan products. NIAID International Centers for Tropical Disease Research, Third Annual Meeting, April 27-29, 1994

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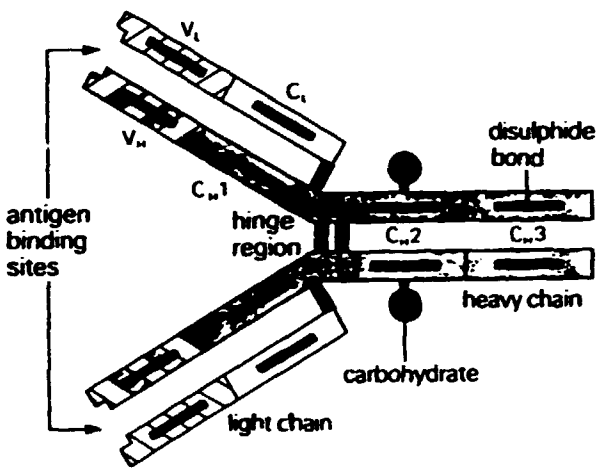
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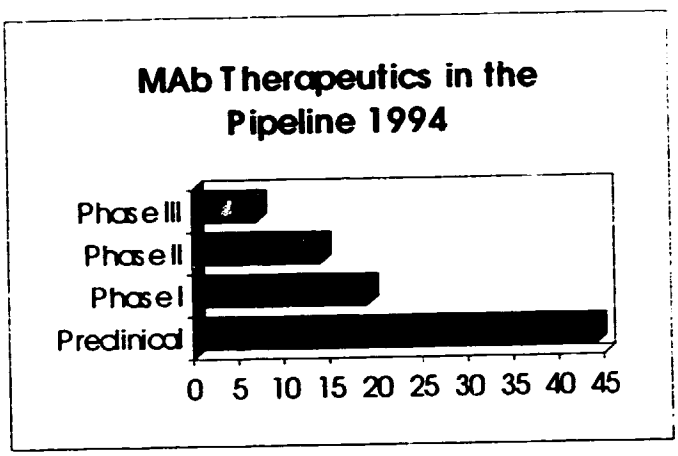
Figure 1.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