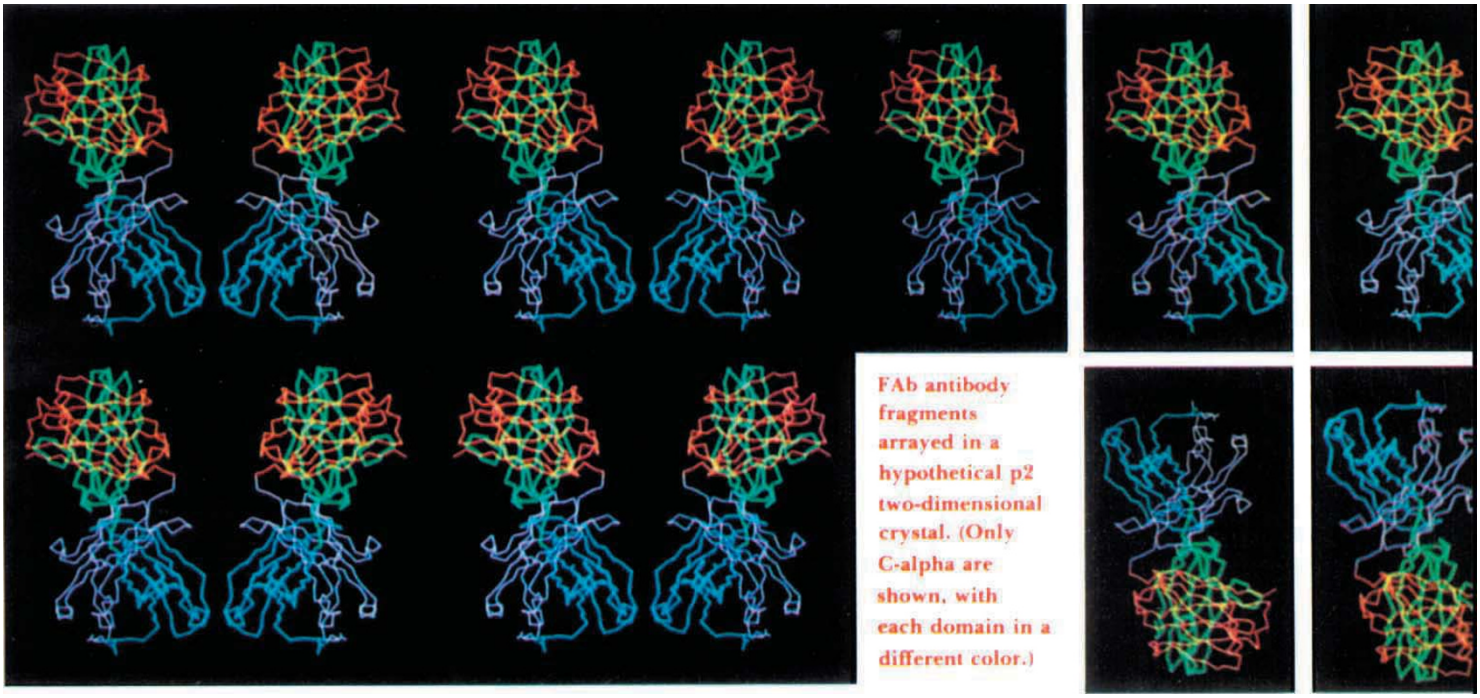
MONOCLONAL ANTIBODIES INTO THE '90s:



FAb antibody fragments arrayed in a hypothetical p2 two-dimensional crystal. (Only C-alpha are shown, with each domain in a different color.)

THE ALL-PURPOSE TOOL

To meet the challenges of a dazzling array of antigens, the B cells of the immune system have developed mechanisms for producing antibodies from more than 10^{12} different genetic combinations. Biotechnologists have exploited antibodies' selectivity and specificity—to detect and purify various ligands; to diagnose, treat, and even vaccinate against a wide range of diseases; to bind receptors and catalyze reactions.

Diagnosis

The diagnostic use of antibodies dates back four decades to the introduction of precipitin-based methods[1]. The introduction of radioimmunoassay (RIA) by Yalow and Berson[2] revealed the extreme sensitivity possible with high-affinity antibodies. Since then, fast, sensitive immunoassays—RIAs, enzyme immunoassays (EIAs or ELISAs) and fluorescence immunoassays (FIA)—have been applied to a wide variety of molecules[3].

The literature teems with diagnostics improved—or made possible—by monoclonal antibodies[4]. Monoclonals against unique microbial epitopes have made it possible to discriminate between closely related organisms—and even among subtypes[5]. They can distinguish between closely related molecules: between morphine and heroin[6]; between testosterone and related steroids[7]; and even between enantiomers of the same molecule[8]. Furthermore, pharmacokinetic studies may use MAbs to monitor levels of drugs and their metabolites[9], or to study the difference in the clearance of two enantiomers[10]. Such selectivity is impossible with conventional poly-

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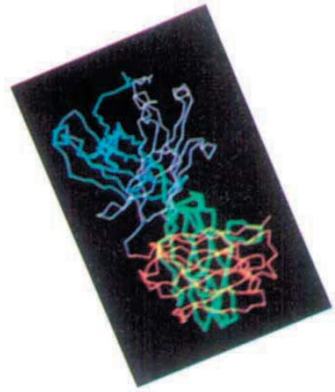

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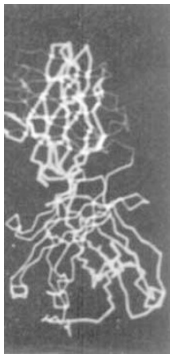


Table 1. Clinical Effects of Human Monoclonals Against Solid Tumors

Tumor	No. of Patients	Clinical Effects	Comments
Malignant melanoma[1]	8	2 Complete remission 2 Partial remission 2 Objective response	Anti-GD2 (IgM) antibody No side effects
Glioma[2]	1	Not available	Antibodies administered in a subcutaneously implanted culture chamber
Breast carcinoma[3]	6	Location	^{111}In-labeled IgM antibodies for tumor localization

[1]Irie, R.F., et al. 1986. PNAS 83:8694-8.
[2]Watson, J.V., et al. 1983. Lancet (Jan. 15):99-100.
[3]Burnett, K.G., et al. 1987. pp 253-265 in: Human Hybridomas: Diagnostic and Therapeutic Applications, Marcel Dekker, New York.

clonal antisera.

Monoclonals do have some disadvantages, however. Cross-reactivity is one[11], a direct consequence of the reagent's monoclonality. For the same reasons, a monoclonal antibody may sometimes be *too* selective. Small changes in antigen structure—due to genetic polymorphism, heterogeneous glycosylation, or slight denaturation—may so change the epitope that the antibody cannot bind[12].

The growing understanding of monoclonal antibodies' distinctive features has obviously made it possible to design new types of high-performance assays. The two-site immunoassay, for example, uses a pair of antibodies, each recognizing a different epitope: one antibody binds the analyte to a matrix; the other, linked to a marker, reveals the analyte's presence[13]. This assay performs well in comparison with standard RIA and EIA[14] and has a definite advantage when one must use cross-reacting antibodies[15]. Cross-reacting substances are unlikely to carry both antigenic determinants specifying the monoclonal pair.

So far, murine monoclonals have accounted for most applications. In general, the antibody's origin has no impact on the assay. Some human antigens (such as the Rh antigens), however, have stymied efforts to produce murine monoclonal antibodies with the desired specificities. In this case, human monoclonal antibodies have solved the problem[16]. Human MAbs are now also available against other bloodgroup antigens—including A, A1, Rh(G), Rh(c), Rh(E) and Kell[17]. In the future, human monoclonals will likely replace polyclonal antisera for blood typing[18].

Affinity Chromatography

Immobilized antibodies and antigens have long been used as affinity reagents[19]. The method does suffer from low binding capacity and specificity limited by the quality of the antibodies. Harsh elutions are often necessary, causing irreversible changes in the antigen or antibody. As a result, antibody immunoadsorbents never achieved the widespread use originally expected.

The introduction of monoclonal antibodies, however, began a new era—allowing columns with any desired specificity and high binding capacity, requiring only mild elution conditions. The nearly infinite supply of reagents made large scale industrial applications feasible.

Today, affinity columns have found their greatest use in purifying high-priced biologicals isolated from tissue and recombinant cell culture[20]. As a rule 50–100 g of monoclonal antibody is needed to purify 1 g of a low molecular weight peptide or 2-5 g of protein.

The affinity purification designer must choose antibodies carefully. When covalently bonded to solid supports, polyclonal antibodies often lose antigen-binding capacity. Monoclonal antibodies lack the diversity of structures found in polyclonal antisera. Thus, the antibody with the best binding properties in solution may not necessarily retain its affinity when coupled. In a recent study, 11 monoclonal antibodies of comparable affinity for asparagine synthetase were tested after covalent binding to Sepharose. Their antigen-binding efficiency varied widely, from 0.02 to 1 mg bound antigen/mg bound antibody[21]. It has been suggested that coupling an antibody via its F_c-portion to Protein A-Sepharose would affect binding capacity less than direct covalent linkage[22]. In any case, the choice of a monoclonal for affinity separation should be based on the antibody's performance when linked to the solid support, not in solution.

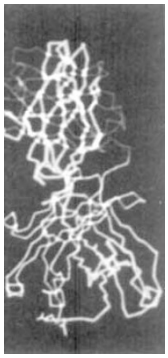
The chosen MAb should allow mild elutions. Very low-affinity antibodies (e.g. $K_a < 10^4\ M^{-1}$) have proven successful in polyclonal[23] and (more recently) monoclonal[24] applications. The target substance cannot be recovered from very dilute solutions, however. Some monoclonal antibodies switch from high to low affinity in response to relatively small changes in elution conditions[25]. Such "switch" antibodies have been made for several proteins: One prostatic acid phosphatase purification showed almost complete (95 percent) removal of enzyme from solution and a total yield in elution of 60–65 percent.

Therapy

Antibody-mediated immunotherapy was first used over sixty years ago: hematopoietic tumors were treated with hyperimmune sera from rabbits. It was not, however, until the introduction of monoclonals[26] that antibody-based therapy of different diseases could be systematically investigated. As analytical tools, monoclonals have helped researchers identify a number of tumor-associated antigens, virus and lymphokine neutralizing epitopes, endotoxins, and other important structures. The repertoire of cell-surface antigens has also been investigated on a number of human tumors, using murine monoclonal antibodies. No real tumor-specific antigens have yet been identified—except for the idiotypic T- and B-cell receptors in lymphoproliferative diseases[27].

This is not entirely surprising. The mouse immune system identifies foreign cells mainly by transplantation and blood group antigens. It might therefore overlook small antigenic changes specific for a human tumor cell. For example, mouse monoclonals exhibit none of the human antibody's fine-tuned specificity against the polymorphic structures of human histocompatibility antigens[28]. Thus, the mouse immune system might ignore the small structural changes specific to human tumor-cell surfaces—conformations readily recognized by immunocompetent human cells.

Human MAbs might thus be advantageous for treating human neoplasms. And, *in vivo*, human monoclonals might not elicit as strong an anti-immunoglobulin response as mouse immunoglobulin. Murine monoclonals have made it possible to detect a large variety of tumor-associated antigens expressed little—or not at all—by normal cells. Many experimental and clinical therapeutic systems have applied such antibodies—directed against melanomas, carcinomas, or sarcomas, for example—in different modalities and administration schedules, with varying degrees



of success[29]. Thus, unconjugated, complement-fixing, or antibody-dependent cellular cytotoxicity-mediating antibodies have been tested. Their main advantage is their relatively low systemic toxicity. Antibodies conjugated to toxins or radioactive isotopes have also been used, but unspecific uptake in normal tissue remains a problem.

Patients sensitized to mouse proteins have suffered a number of side effects—such as fever, rashes, vomiting, urticaria, bronchospasm, tachycardia, and dyspnoea[30]. These side effects are normally transient and disappear as soon as the infusion of mouse antibodies stops[31].

Much more serious is the reduction of therapeutic effect as the patient mounts an anti-mouse response. The patient's immune response first targets the therapeutic monoclonal's constant region, but it focuses on an idiotypic response after only a few injections[32]. These reactions can produce secondary allergic reactions, due to immune complex deposition in various tissues—the kidneys, liver, and lung. Therapeutic antibody dosage, the target antigen's tissue distribution, and the antibody's reactivity all affect the anti-mouse immunoglobulin response. Human monoclonals should solve most of these problems, though it remains to be seen just how these will be used.

At present, human monoclonals have been used only as native unconjugated molecules[33] or as radiolabelled imaging agents[34]. Current developments in immunoconjugates—especially those using small, highly toxic compounds—should be of utmost importance when applied to human antibodies[35].

Today, few human monoclonals can be used to treat tumors, infectious diseases, autoimmune conditions, and drug overdoses. Clinical studies are very scarce (Table 1), mostly because of the technical barriers to routine production. This is changing. Recent progress in *in vitro* immunizations[36] and immortalizing human B cells by EBV infection[37] have made it possible to obtain antigen-specific human hybridomas at significantly higher frequencies.

Antibodies as Vaccines

In 1981, two groups (Nisonoff and Laoyi[38] and Roitt, et al.[39]) first proposed using antibodies as vaccines—a logical consequence of Jerne's idiotype-anti-idiotype network theory[40]. Antibodies obviously bind to antigen epitopes. They can also serve as antigens themselves—to be recognized by still other antibodies which bind to their variable regions (idiotypes). Some of these anti-idiotypic antibodies carry the internal image of the original antigen: They can therefore act as antigenic stand-ins, eliciting an antibody response.

Clearly, not all idiotypes can serve as vaccines: some will produce a protective immunity; some will not. There are at least two reasons for this: First, only a few anti-idiotypes mimic the antigen and bind to the primary antibody's (Ab1) antigen-binding area (paratope). The other antibodies target parts of the Ab1 variable region that do not participate in antigen recognition and binding. (Note, however, that anti-idiotypes binding outside the paratope can sometimes elicit production of antibodies against the nominal antigen[41]. They probably will not generate a relevant T-cell immunity, however.) Second, some anti-idiotypes seem to induce suppressive rather than protective immunity in the vaccinated animal, even though they elicit both B and T cell responses against the antigen[42].

Anti-idiotype vaccines could replace microbes and microbial toxins, both hazardous to the patient. Anti-idiotypes also offer peptide alternatives to some microbes' primarily glycan epitopes[43]—particularly important for vaccinating infants against bacterial polysaccharides[44]. And modern hybridoma processes can produce these in almost limitless supply—a particularly significant consideration where carbohydrates make up the antigen's most important structures, putting them, at least for now, beyond the reach of recombinant DNA technology.

Anti-idiotypic antibodies have reportedly produced vaccines against microbial antigens such as parasites, bacteria and viruses (Table 2). (See recent reviews by Bona[45] and Kennedy, et al.[46].)

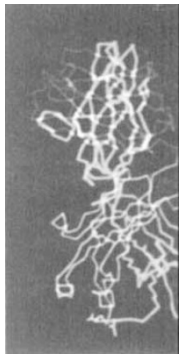
Anti-idiotypic cancer therapy is obviously another area of great interest. Although Hollinshead[47] has demonstrated the benefits of vaccinating lung tumor patients with autologous tumor extracts, immunization with tumor-associated antigens has generally been rather disappointing.

There are many reasons for this. Tumor-associated antigens are often unidentified, hard to purify, and "self." Anti-idiotypic antibodies have been tried in several experimental systems for vaccination against tumors (Table 2). Such idiotype-based antigens could potentially outperform conventional tumor-associated antigens—especially since they are easy to mass-produce and are free of the tumor viruses that may be present in conventional tumor-extracted antigens. Furthermore, by putting the epitope in a new molecular environment—such as an anti-idiotypic antibody coupled to KLH or tetanus toxoid—one may mobilize T-cell clones that would not otherwise participate in the anti-tumor-antigen response.

Several laboratories have demonstrated immunity to tumor-associated antigens after vaccination with anti-idiotypic antibodies—melanoma, virally induced sarcomas, bladder tumor, B- and T-cell lymphomas, and colon carcinoma (see Table 2). The 17-1A murine monoclonal has had therapeutic effects in patients suffering from colorectal cancer. This may not be a direct effect. Rather, the 17-1A may induce anti-idiotypic antibodies, immunizing the patient[48]. H. Mellstedt (personal communication) recently obtained a set of human monoclonal anti-idiotypic antibodies following EBV transformation of B cells from patients treated with 17-1A[49]. (These should be ready for the clinic soon.)

H. Kohler and his group have developed and characterized several anti-idiotypic antibodies which induce T- and B-cell immune responses. Although these antibodies bind to the same paratope, some induce protective immunity, and some do not. The anti-idiotypic antibody that failed to induce protective immunity elicited suppression rather than protection[50]. The reason is unclear. It may depend on the anti-idiotypic antibody's ability to activate T-effector cells via direct idiotype binding[51]. Protective antibodies induced by vaccination with irradiated tumor cells reacted with the anti-idiotype—giving protective immunity. On the other hand, serum antibodies (from individuals with growing tumors) bound to the anti-idiotype—inducing suppression[52]. This suggests an organic relationship between tumor development and the type of antibody response evoked. The results also caution us: Anti-idiotypic vaccination can have complex effects on the immune system.

We can now produce enormous numbers of different antibody specificities as internal images, with a corresponding variety of biological functions. Thus, research has produced anti-idiotypic antibodies containing internal images of such molecules as insulin, angiotensin II, and adenosine, and to adrenergic, nicotinic and opiate compounds (see Table 3). Such antibodies can bind to their respective receptors, and some of them have demonstrated an agonistic effect.



Antibodies as Enzymes

Recent inquiries have focused on still other exciting possibilities: Antibodies' new-found ability to form structures that complement enzyme substrates. Antibody-antigen interactions obviously resemble enzyme-substrate interactions in their affinity and binding specificity. There is, however, one important difference between the two groups of molecules. Antibodies interact with their ligands in stable, low-energy configurations; catalytic enzymes bind to unstable, high-energy transition forms of their substrates. The enzyme's binding energy then helps break a chemical bond in the substrate molecule[53]. By analogy, an enzymatic antibody's structure should complement the substrate's transition state—which usually exists for an inconveniently short time. So investigators have produced stable, low-energy analogs of transition states for a variety of compounds. Using these, they have produced several "abzymes" (Table 4).

Antibodies with catabolic activity against complex substances—such as proteins and nucleic acids—could be of great importance[54]. Such antibodies could possibly cleave protease-resistant amide bonds and might display greater substrate specificity than common proteolytic enzymes. A dependence on surrounding structures could be built in, allowing antibody—rather than proteases—to cleave only specific proteins[55].

Producing Optimal Antibodies

Different applications require different antibody qualities: high-affinity for diagnosis, switch or low-affinity for affinity chromatography, various affinities and isotypes for therapeutic antibodies of human origin. Human monoclonals may be preferable for affinity-purifying human therapeutics. To match these requirements, we must be able to design and produce MAbs with pre-defined properties. The immunotechnologist's obvious goal will be to prepare antibodies of the desired xenotype, isotype, specificity, and affinity.

We can now manipulate these parameters using the techniques of molecular biology[56]—the same techniques have been used to construct chimeric antibodies, i.e. antibodies with mouse variable domains and human constant domains[57]. These "humanized" antibodies may eliminate some problems associated with the anti-mouse-immunoglobulin immune response. Also, the effector functions can be tailored as required. Thus IgG1 and IgG3 human isotypes have been shown to be the most effective in complement and cell-mediated lysis[58]. They would therefore be selected for destroying tumor cells.

Sequence comparisons[59] of variable heavy (V_H) and variable light (V_L) chain domains have shown that each domain has three hypervariable regions (CDR1-3), flanked by four conserved framework regions (FR1-4). Antibodies with different specificities show greatest variability in the CD regions, and these regions evidently form the principal determinant of the antigen combining site. Thus, both V_H and V_L domains are implicated in antigen binding, although their relative contributions are unknown. Chimeric antibodies have been taken a step further: Each of the CDR1-3 regions of the heavy and light variable regions has been transplanted into a human framework. Reichmann, *et al.*[60] recently reported the elegant modification of a human IgG1 antibody by transplanting just the antigen-binding sites of a rat anti-human lymphocyte antibody (CAMPATH-1). Grafting of CDRs into human FRs

Table 2. Infective and Other Diseases Treated with Anti-Idiotype Vaccines

Antigen source	Anti-idiotype source	Species vaccinated
Streptococcus pneumoniae[1]	mouse monoclonal	mouse
E. coli K13[2]	mouse monoclonal	mouse
Trypanosoma cruzi[3]	rabbit serum	mouse, rabbit, guinea pig
Hepatitis B surface antigen[4]	rabbit serum	chimpanzee
Polio virus type II[5]	mouse monoclonal	mouse
Rabies virus[6]	rabbit serum	mouse
Reovirus[7]	mouse monoclonal	mouse
Cytomegalovirus[8]	mouse monoclonal	mouse
Human immunodeficiency virus[9]	rabbit serum	mouse
Murine sarcoma[10]	mouse monoclonal	mouse
Murine bladder tumor[11]	mouse monoclonal	mouse
Murine lymphoma[12]	mouse monoclonal	mouse
Murine B-cell lymphoma[13]	mouse idiotypic IgM	mouse
Human B-cell lymphoma[14]	mouse monoclonal	human
Human melanoma[15]	rabbit serum	mouse
Human T-cell lymphoma[16]	mouse monoclonal	mouse
Human colon carcinoma[17]	goat serum	human
Human colon carcinoma[18]	human monoclonal	—

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Table 3. Anti-Idiotypic Antibodies with Receptor-Binding Ability

Antigen	Source of Anti-Idiotype	Activity	Receptor
Insulin[1]	Rabbit serum	Agonist	Insulin
Angiotensin II[2]	Rabbit serum	—	Angiotensin II
Adenosine[3]	Mouse monoclonal	Agonist	Adenosine I
Alprenolol[4]	Rabbit serum	Agonist	Beta adrenalin
Nicotine[5]	Mouse monoclonal	—	Rat brain nicotine
Morphine[4]	Guinea pig serum	Agonist	Opiate

[1]Sege, K. and Peterson, P.A. 1978. PNAS 75:2443-7.
[2]Couraud, P.-O. 1987. J. Immunol. 138:1164-8.
[3]Ku, H.-H., et al. 1987. J. Immunol. 139:2376-84.
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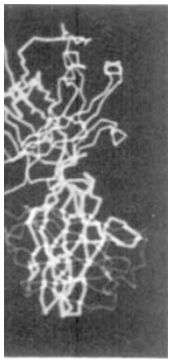

Table 4. Antibodies with Enzymatic Activity

Enzymatic Activity of Antibody	Substrate	Acceleration of Reaction
Chorismate mutase[1]	Chorismate	10^2
Esterase[2]	Hydroxyester	1.7×10^2
Esterase[3]	Carboxyl ester	9.6×10^2 2.1×10^2
Esterase[4]	Carbonate ester	7.7×10^2
Esterase[5]	Coumarin ester	1.5×10^3
Esterase[6]	Carbonate ester	8.1×10^2
Esterase[7]	Carbonate ester	6.3×10^6 1.2×10^5

[1]Hilvert, D., et al. 1988. PNAS 85:4953-5.
[2]Napper, A.D., et al. 1987. Science 237:1041-3.
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[6]Jacobs, J., et al. 1987. J. Am. Chem. Soc. 109:2174-6.
[7]Tramontano, A., et al. 1988. J. Am. Chem. Soc. 110:2282-6.

did not turn out to be simply a matter of replacing the six human complement-determining regions of the human antibody with those from the rodent immunoglobulin. The resulting human antibody reacted poorly with the CAMPATH-1. This suggested an error in packing the reshaped domain in the human FRs, reducing antigen reactivity by a factor of about 40. Only after site-directed mutagenesis did researchers obtain a V_H exhibiting restored antigen binding[61]. This illustrates that hypervariable regions are not isolated within the antigen binding site. Rather, they make a number of contacts with residues of the framework. This might prevent "humanizing" from becoming the general approach, unless antibody engineers can foresee interactions between the hypervariable and framework regions[62].

Monoclonals derived from human B cells recognize epitopes not detected by xenogeneically derived antibodies. This poses another, more serious, problem for mouse/human chimeric antibodies—implying that only today's few mouse MAbs with acceptable specificities are worth turning into chimeric antibodies for eventual clinical application. Human/human chimeric antibodies could overcome all of these difficulties—if the human V_H and V_L domains produced by *in vitro* immunization were cloned into a vector containing the desired human constant gene segments.

Human V_H and V_L regions could be enzymatically amplified via polymerase chain reaction with a set of degenerate primers[63]. These variable regions can then be modified by site-directed mutagenesis to obtain higher affinities or specificities. They can then be cloned directly into a sequencing vector or joined together with (for example) an IgG1 constant region domain—resulting in a human/human chimera.

Shaping Monoclonals by Cell Biology

Using cell biological techniques, the researcher may influence antibody affinity and isotype at two levels: during *in vitro* immunization and in existing hybridomas. Hybridoma cells spontaneously switch isotype at a frequency of 10^{-5} to 10^{-6} /cell/generation[64]. These switched cells can be identified and isolated using a number of techniques, including fluorescence-activated cell-sorting and sequential sublining[65]. Some workers have also used large-scale sequential sublining to select for hybridomas producing higher-affinity antibodies[66]. Other investigators have exploited the antigen-specific membrane Ig expressed on most hybridomas[67]. These cells were repeatedly allowed to bind to surfaces coated with bound antigen. The cells that bound most firmly and resisted extensive washings were found, quite naturally, to produce high-affinity antibodies. By one estimate, the procedure was sensitive to one mutation in 10^8 cells, leading to a 10-fold increase in affinity[68]. Several mutations in the antibody variable region might be necessary to produce still higher affinities: The probability is extremely low. One must have recourse to other methods: reshaping the existing antibody, or concentrating on the immunization step—guiding B-cell development towards production of high-affinity antibody *before* immortalization.

The latter approach has become a distinct possibility with the advent of *in vitro* immunization techniques. Although still in its infancy, this technique has shown great potential. It will allow production of both murine and human antibodies to a variety of antigens, including self antigens[69]. *In vitro* immunization procedures often yield IgM antibodies exhibiting affinity comparable to a primary *in vivo* response[70]. In the murine system, at least, the isotype can be switched from IgM to IgG1 or IgG2a by adding IL-4 or IFN-γ, respectively[71]. Cocultivating B-cells with cloned T-cells of the TH1 or TH2 subtype, producing either IL-4 or IFN-γ, can also influence isotype[72]. In addition, the affinity of antibodies produced after *in vitro* immunization depends on the amount of antigen added to the cultures; low levels of antigens favor development of cells producing higher-affinity antibodies[73]. A combination of *in vivo* and *in vitro* immunizations produces antigen-specific hybridomas, mostly of the IgG isotype[74]. Development of a secondary response depended on the presence of antigen during the *in vitro* stimulation. Generating IgG required at least a two week interval between the primary *in vivo* immunization and the secondary *in vitro* stimulation[75]. Furthermore, the IgM distribution pattern shifted towards higher affinities, and the IgG affinities appeared higher than obtained after immunization *in vivo* only[76].

In vitro immunization typically takes 5 to 8 days. This period may conceivably be too short to allow proper Ig switching and affinity maturation. Culturing normal B-cells over longer periods has met with great difficulties. Treating B-cell-containing mononuclear blood cell preparations with the lysosomotropic agent L-leucyl-L-leucine methyl ester (which quantitatively eliminates lysosome-rich large granular lymphocytes, cytolytic $CD8^+$ T-cells and monocytes) prolongs the survival time of cultured B-cells to at least 40 days (Danielsson, et al., personal communication). In the future, it might thus be possible to cultivate normal B-cells over still longer periods, either by removing inhibitory cells or by the controlled addition of cells delivering the required signals. To produce high-affinity antibodies, it would be desirable to selectively activate those B cells that recognize specific antigens. One could control proliferation and differentiation by limiting amounts of the antigen.

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