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COMMENTARY

ANTIBODIES AS MODELS FOR RATIONAL DRUG DESIGN

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Passive immunization has a venerable history. While the treatment of pneumococcal pneumonia with rabbit antisera was abandoned when sulfonimides were introduced, there is still a place for tetanus and diphtheria antitoxins in the infected, but unimmunized patient. More recently, monoclonal antibodies specific for human cell surface determinants have found application in the reduction of transplantation rejection as well as in the treatment of certain lymphoid malignancies; yet these applications in pharmacology are quite limited. Now that antibodies are far better understood as chemical entities, and the manufacture of single molecular species has been simplified by cell fusion techniques, can they be used as drugs in a broader sense?

How does the pharmaceutical industry develop drugs?

There are three common routes to the development of a new drug:

1. The structure of a natural substance that is known to have favorable pharmacologic properties is modified by the medicinal chemist to yield a great many variants. These are screened until one having suitable pharmacologic properties is identified. Examples include the development of β-adrenergic blockers from the known structure of agonists such as epinephrine and the derivation of the structure of converting enzyme, captopril, from the structure of the snake venom peptide, teprotide.

2. The structure of an existing drug of interest is modified. Often modification is by trial and error, with many compounds having to be tested. The semisynthetic penicillins are an example of this approach.

3. Organic compounds are screened at random for a specific action. Early attempts to identify cancer chemotherapeutic agents are an example of this route to new drugs.

Each of these approaches relies on serendipity—a hope that trial and error will yield something of value. Thousands of compounds must be tested before a useful one is found. The consequences of this mode of searching are staggering development costs and a failure to identify drugs that have optimal characteristics for the desired application (e.g. side effects may often be the product of a lack of selectivity).

The optimal drug has very high specificity and affinity for its intended receptor. This implies a very precise fit to a receptor site, utilizing the strongest intermolecular forces possible. The most straightforward way of accomplishing this is to create the maximum number of interatomic interactions between receptor and ligand. Small molecules seldom offer a high degree of selectivity. For example, epinephrine interacts with both types of α- and β-adrenergic receptors, whereas somewhat larger organic compounds are capable of being more selective.

Of all classes of compounds, antibodies provide the greatest range of specificities and affinities. The number of interatomic interactions between an antibody combining site and a large ligand such as a receptor far exceeds those between the common small ligand or drug and its corresponding binding site. Increased selectivity and affinity result. The very large number of potential antibody specificities that exists provides an opportunity to create selectivity of a finer degree than is possible with simpler compounds.

How does all this diversity arise? The overall structures of antibodies of differing specificity are so similar that a superficial examination fails to differentiate among them. Much of the structure is highly conserved. There is a small region of the molecule, however, that is highly variable in its structure (the complementarity region) and makes up the surface that binds antigen. It is the amino acid sequence in this region (approximately sixty amino acid residues) that determines the nature of the antigen recognized. Recent work has uncovered the mechanisms responsible for variation in the amino acid sequence of the complementarity region.

The antibody molecule comprises four polypeptide chains, two identical light and heavy chains. The variable region of the light chain is the product of two genes: V, which occurs in several hundred copies, and J, of which four copies have been identified [1–4]. V and J of the light chain may occur in any combination. The variable region of the heavy chain is the product of three genes: V, in several hundred copies, D, in ten or more copies, and J, in four copies [5, 6]. As in the light chain, any permutation and/or combination of these genes may occur. These mechanisms alone account for 10^7 different antibodies. In addition, somatic point mutation has been shown to occur. Thus, the number of possible antibodies must actually exceed 10^{10}.

The antibody molecule is arranged into six rather discrete spatial domains. A single domain that has a size of 25,000 daltons and is capable of binding antigen with the same affinity as the intact antibody, which is six times as large (Fig. 1) [7], may be isolated by enzymatic cleavage [8]. A detailed analysis of

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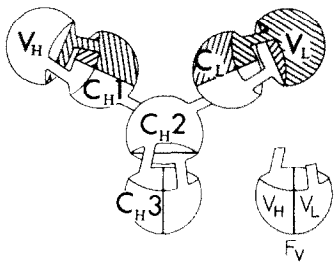

Fig. 1. Schematic representation of the antibody molecule based on crystallographic studies [7]. V refers to variable domains, while C indicates constant domains. The antibody molecule is arranged into six discrete spatial domains. A single domain may be isolated by enzymatic cleavage comprising V regions from light and heavy chains [8] that has a size of 25,000 daltons and is capable of binding antigen with the same affinity as the intact antibody which is six times as large. (Modified from Ref. 7.)

crystal structures of immunoglobulins suggests that the essential region of the combining site, containing the complementarity residues that contact the antigen as well as essential supporting amino acid residues, may comprise only 12,000 daltons.* Thus, the antibody combining site, while quite large in comparison to most drugs, is of manageable size.

The specificity of antibodies

Some insight may be gained into the repertoire of selectivity by examining a set of monoclonal antibodies specific for digoxin [9]. These antibodies were obtained first by immunizing A/J mice with a conjugate of digoxin and hemocyanin and then fusing their spleen cells with a nonimmunoglobulin-secreting myeloma line, Sp2/0, according to the general method of Köhler and Milstein [10]. After cloning and amplification by the ascites method, each antibody was determined to be a single molecular species by many criteria, including amino acid sequence analysis. The properties of six of these antibodies are summarized in Table 1 and compared to a sheep polyclonal antibody prepared by conventional immunization. It should be noted that all of the antibodies listed have very high association constants for digoxin, ranging from 5×10^8 to 5×10^9 for the monoclonal antibodies and in excess of 10^{10} for the polyclonal antibody. Each antibody is examined with respect to its ability to bind to a set of digitalis glycosides that differ structurally in varying degrees when compared to digoxin. The specificity profile of each antibody is unique. Antibody 25–54 has a similar affinity for digoxin and digitoxin and thus does not recognize the hydroxyl group on position 12 in the C ring of the steroid. It shares this property with all the monoclonal antibodies except 40-020, which binds digoxin with an affinity 100 more than digitoxin. Two of the monoclonal antibodies, 35-20 and 26-10, bind digitoxigenin with affinities nearly equal to digoxin, indicating that the digitoxose sugars play no role in their binding site. The other monoclonal antibodies are able to differentiate significantly between these two compounds. All of the antibodies bind deslanoside with the same affinity as digoxin, indicating that their combining site does not include the terminal digitoxose sugar. Only antibody 26-10 is able to bind acetyl strophanthidin with similar affinity to digoxin, while it cannot bind ouabain. Selective substitutions on the A and B rings of the steroid ring appear to be of importance here, some being tolerated and others not. In all of the other antibodies, either substitution on the A and B rings (acetyl strophanthidin or ouabain) is not compatible with binding. The polyclonal antibody is a mixture of many individual antibodies, but its average specificity characteristics seem to resemble those of monoclonal antibody 40-020.

The pattern that emerges is that of a series of binding sites that offer exquisite resolution at the submolecular level. A molecule that has a size of 850 daltons can be dissected so that only segments of its structure are recognized and small differences easily measured.

Antibodies to receptors

An important area of pharmacologic intervention is receptor blockade. How are antibodies to receptors generated? Very few receptors have been iso-

* J. Novotny, E. Haber and M. Karplus, unpublished data.

Table 1. Specificity of hybridoma antibodies for various cardiac glycosides relative to digoxin*

Hybridoma clone or antisera	Cardiac glycoside inhibitors					
	Digoxin	Digitoxin	Digitoxigenin	Deslanoside	Acetyl strophanthidin	Ouabain
Dig 25-54	1.0	1.8	54.0	4.3	>100	>100
Dig 35-20	1.0	4.6	2.7	3.7	110	333
Dig 26-10	1.0	1.3	3.7	2.6	1.5	66
Dig 40-020	1.0	110	>600	2.9	>600	>600
Dig 40-120	1.0	1.3	109	0.9	433	>500
Dig 40-040	1.0	2.7	59	ND†	>45	>450
Sheep antidig serum	1.0	152	860	ND	ND	$>10^5$

* Modified from Ref. 9.
† Not determined.

lated in pure form for use as antigens. It is possible to utilize two different approaches to the generation of anti-receptor antibodies. The first may require either no purification or only a partial purification of the receptor. In essence, purification is effected by taking advantage of the selection inherent in the cloning hybridoma cultures. If one considers that the immunized animal responds to the very many antigenic determinants present on a cell membrane by proliferation of different lymphocyte clones, it should be possible to select one of these clones corresponding to a single antigenic determinant, such as the desired receptor. Modern hybridoma technology facilitates this selection, but constraints are placed on the availability of clones by the very nature of the immune response. When the antigen of interest is represented by only a very small fraction of the antigenic determinants in the cell membrane, it is unlikely that it will evoke an immune response. This is particularly a problem when, as in many physiological receptors, there are only 10,000–50,000 copies per cell. While antibodies to differentiation and histocompatibility determinants that are rather abundant on the cell membrane may be routinely obtained by immunization with whole cells, there are few instances in which hormone or neurotransmitter receptor antibodies have been elicited in this manner.

While complete purification is often very difficult, affinity chromatography with one of the ligands of the receptor may effect rather dramatic concentration of the antigen of interest. An example may be cited in the partial purification of the β-adrenergic receptor from cardiac tissue [11]. The polyclonal antibody produced in response to immunization with material that was concentrated 7000- to 8000-fold in relation to the cell membrane was an effective β-adrenergic blocking agent. It is noteworthy, as indicated in Table 2, that the antibody was able to selectively block β_1-receptors and did not interact with β_2-receptors, a differentiation that has been difficult to achieve with drugs.

A second approach that may be more widely applicable to the vast array of physiologic receptors that as yet cannot be purified is the application of the immunologic principle of antiidiotypy. While the receptors may not have been purified for use as antigens, their ligands are usually readily available in quantity. Agonistic substances are often either peptides of modest size or organic compounds, both readily synthesized. For some receptors, a variety of antagonists has been created in the laboratory of the organic chemist. Could an antibody for the receptor be obtained by utilizing the ligand (agonist or antagonist) as a template? The vast diversity of antibody combining sites, as suggested above, provides the potential for creating a complementary fit to almost any shape. If a figurative plaster mold could be cast upon the surface of the ligand that bound to the receptor, then a second mold made from the first one should have a perfect fit to the receptor. The well-known immunologic principle of raising antibodies specific for another antibody's combining site (antiidiotypic antibodies) may be utilized as the vehicle for molding the desired shape.

Certain refinements are needed to achieve the desired end. Only some of the atoms of a ligand bond to the receptor. To achieve the desired result, the first antibody must bind the ligand generally in the same way as the receptor does, and thus the same atoms and interatomic interactions must be utilized. This goal may be achieved either by conventional immunization techniques or by the use of monoclonal antibodies. When conventional immunization is employed, many antibodies to the immunogen are formed, each binding to it in a somewhat different manner. The antibodies of this polyclonal response may be fractionated by the use of ligands of different structure. By virtue of being receptor ligands, they are all capable of binding to the receptor and must have some common structures. If appropriately selected, structures irrelevant to binding are not shared. Those components of the polyclonal antibody mixture that bind to all possible ligands must be most similar to the receptor. The most practical way of effecting fractionation with polyclonal antibodies is sequential affinity chromatography. When monoclonal antibody techniques are employed, it is simply necessary to use selection techniques that will identify those antibodies that have the property of binding to all possible ligands.

This general approach has now been applied to the insulin receptor [12]. I shall review our own work on the β-adrenergic receptor [13]. There is a wide variety of structurally different β-antagonists available; all, however, share a common structure, a propanolamine side chain.

Immunization of rabbits with an alprenolol–protein conjugate [14] elicited an antiserum which was passed over an acebutolol affinity column; the fall-through volume was discarded. Elution of the affinity column was carried out with *l*-propranolol, and the eluent antibody was characterized after the *l*-propranolol had been removed by dialysis. The specificity profile of this antibody fraction closely resembled that of the β-adrenergic receptor. All β-adrenergic agonists and antagonists tested bound with considerable affinity, in some cases similar to

Table 2. Inhibition of isoproterenol-stimulated adenylate cyclase by antibody*

	Cardiac adenylate cyclase (pmoles cAMP · mg^{-1} · min^{-1})	Inhibition (%)	Hepatic adenylate cyclase (pmoles cAMP · mg^{-1} · min^{-1})	Inhibition (%)
Control	37.5 ± 6.9		41 ± 6	
Preimmune globulin	36.5 ± 8.5	0	41 ± 6	0
Immune globulin	27.3 ± 6.5	76 ± 8	39 ± 7	0

* Modified from Ref. 11. Results are means ± S.E.; N = 3 or 5.

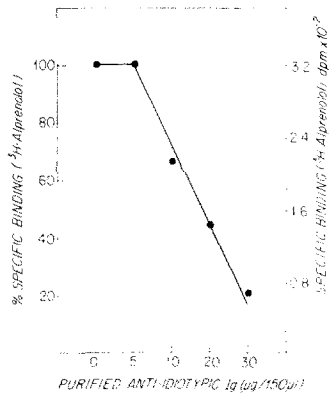

Fig. 2. Effects of DEAE-purified Ig fraction of antiidiotypic serum on (−)-[^{3}H]alprenolol (2 nM) binding to turkey erythrocyte membranes. (Modified from Ref. 13.)

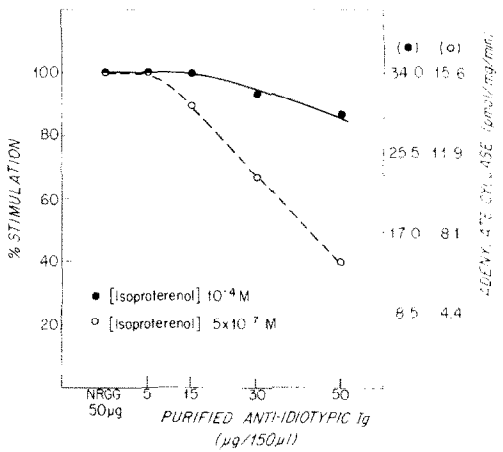

Fig. 3. Ability of antiidiotypic Ig fraction to inhibit adenylate cyclase stimulation in turkey erythrocyte membrane. Inhibition was tested at isoproterenol concentrations of 5×10^{-7} M and 10^{-4} M in the presence of 10^{-4} M guanosine triphosphate. In contrast to the effects seen at the nonsaturating isoproterenol concentration of 5×10^{-7}, only a slight amount of inhibition is seen at the highest Ig concentrations in the presence of 10^{-4} M isoproterenol. (Modified from Ref. 13.)

that of the receptor. This fraction also resolved *l* and *d* stereoisomers of isoproterenol. Thus, the antibody fraction could be considered as a qualitative, but not a strictly precise quantitative, model for the β-adrenergic receptor. Antibodies specific for the combining sites of the first antibody set were then raised by immunization of allotypically matched rabbits. The immunogen was identical to the immunoglobulins of the recipient animals except for the variable region of the molecule. Because of tolerance to self-determinants, the immunized animals made antibodies only to unique structures on the immunogen (antiidiotypes). Figure 2 shows the inhibition of binding of a labeled β-antagonist, [^{3}H]alprenolol, to a turkey erythrocyte membrane preparation by purified antiidiotypic antibody. Binding of the ligand, alprenolol, to the idiotype (the first generation antibody that had been raised in response to alprenolol) is largely inhibited by antiidiotype (second-generation) antibody. The antiidiotype also appears to be an inhibitor of adenylate cyclase activation by β-adrenergic agonists. Increasing concentrations of isoproterenol progressively inhibit adenylate cyclase production in turkey erythrocyte membranes at an isoproterenol concentration of 5×10^{-7} M. At a higher concentration of isoproterenol (10^{-4} M), less inhibition is observed, as would be consistent with the competitive nature for this interaction (Fig. 3).

Thus, the antiidiotype behaves as a true β-adrenergic antagonist, competing with both agonists and antagonists for the receptor site. The obvious potential uses of such receptor-specific antibodies are: the recognition of structural differences among subsets of β-adrenergic receptors [15]; a more rigorous examination of their respective physiologic roles utilizing reagents of greater resolution; the isolation of receptors with antibody affinity chromatography; and the ultimate application of antibody fragments as drugs.

Idiotypic antibodies elicited by immunization are generally present in very low concentration, which retards their usefulness as physiologic probes and certainly excludes their consideration in any study requiring a large amount of material. When monoclonal antibodies are employed, selective affinity chromatography with a series of ligands may be omitted. One simply employs cloning methods to select the monoclonal antibody that has the appropriate profile. Homcy and Sylvestre [16] have described the specificity profile of a monoclonal antibody raised in BALB/c mice in response to immunization with an alprenolol–protein conjugate. This antibody binds *l*-propranolol with an affinity two orders of magnitude higher than *d*-propranolol, resembling the receptor in its stereoselectivity. Acebutolol, another β-adrenergic antagonist, is bound with a lower affinity than propranolol, also resembling receptor specificity. A potential use for an antibody of this type that resembles a receptor is in the screening of potential receptor-blocking drugs.

How may antibodies be used as drugs in a practical sense?

There are three requirements that must be met before one can envision the widespread application of antibodies as drugs: (1) the molecule must be reduced to the minimal size required to bind antigen; (2) it must be nonimmunogenic; and (3) it must be constructed in such a manner that it may be absorbed by the gastrointestinal tract.

The pharmacology of the smallest antibody fragment available that has been shown to bind antigen, Fv, has not as yet been studied, though some insight can be gained by examining the biodistribution and immunogenicity of Fab, a fragment twice as large. The immunoglobulin molecules that bind two moles of antigen per mole may be cleaved into smaller fragments by the enzyme papain [17]. The resultant antigen-binding fragment, Fab, binds one mole of antigen, whereas the remainder, Fc, contains the complement-binding site. Fab has a number of desirable properties when compared with the intact molecule, IgG: equilibrium distribution in extracellular

Table 3. Kinetics of distribution and elimination of sheep IgG and Fab in the baboon*

	$T_{\frac{1}{2}}$ distribution (hr)	$T_{\frac{1}{2}}$ clearance (hr)	Volume of distribution (ml)	Clearance ($ml \cdot kg^{-1} \cdot hr^{-1}$)
IgG	4.0	61.0	53.0	0.54
Fab	0.30	15.3	370.0	21.0

* Modified from Ref. 18.

fluid is achieved more rapidly; the volume of distribution is greater; and the fragment is eliminated with a far shorter half-life (Table 3) [18]. In addition, when injected intravenously, Fab is less immunogenic than IgG [18]. The immune complexes that may be formed are smaller than those that cause nephrotoxicity (comprising a single antigen molecule with several Fab attached), and complement cannot be fixed because the relevant binding sites on the Fc have been lost.

While smaller antibody fragments reduce the risk of immunogenicity, it is unlikely that they will eliminate it entirely. Human immunoglobulins may now be produced by cell fusion techniques *in vitro* [19, 20]. An even more promising potential development is the application of the techniques of molecular genetics. Immunoglobulin genes are now routinely cloned for study [2–5]. It is not at all farfetched to envision their manipulation and expression as antibody fragments carrying the minimal structure necessary to effect antigen binding. Recombinant DNA methods permit the introduction of human immunoglobulin framework sequences so that tolerance to intrinsic proteins can prevent the mounting of an immune response.

Gastrointestinal absorption of antibodies provides a more formidable problem. While there was a time when it was believed that peptides of any size were not significantly transported across the gastrointestinal mucosa, recent work has demonstrated that, with appropriate structural changes, functional peptides of moderate size may be absorbed with considerable efficiency. Veber and colleagues [21] have constructed a derivative of the peptide somatostatin that is absorbed after oral administration and has equal biologic potency to the natural hormone administered parenterally.

Antibodies in vivo

Our own laboratory has utilized antibodies as *in vivo* diagnostic or therapeutic agents in three different applications: the study of experimental hypertension, the reversal of digitalis intoxication, and the diagnosis of myocardial infarction.

Renin-specific antibodies. Though renin has been known to play a role in circulatory control since the classic work of Goldblatt in the 1930s [22], its precise importance in a number of specific circumstances has been in doubt. Much has been learned from the application of inhibitors directed at several of the steps in the sequence leading to the production and action of the final product of renin, angiotensin II [23]. Most of the inhibitors used, however, lack specificity. The competitive inhibitors, such as saralysin, of the action of angiotensin II on receptors are partial agonists. The angiotensin-converting enzyme is identical to the enzyme that inactivates bradykinin [24], and thus its inhibitors also affect the kinin system. In addition, compounds such as captopril have been shown to stimulate prostaglandin synthesis [25]. Since both the kinin and the prostaglandin systems have an effect on vasoregulation, it is difficult to define the specific part that renin plays when interpreting experiments in which these inhibitors are used.

Antibodies specific for angiotensin II as well as for angiotensin-converting enzyme have been utilized as physiologic tools, but they have not provided a solution to these difficulties. Active immunization of rabbits with coupled angiotensin II failed to alter the development of hypertension after renal encapsulation or constriction of one renal artery and contralateral nephrectomy [26–31]. Immunization of two-kidney, Goldblatt-hypertensive rats ameliorated the hypertension in some studies [27, 32] but not in others [29]. Furthermore, prior immunization with angiotensin II did not prevent the development of hypertension induced by constricting one renal artery in the rat [33]. The inconsistency of these results may be explained by a subsequent study demonstrating circulating unbound angiotensin II in immunized rabbits [34]. Lack of clear definition of the equilibrium between circulating angiotensin, antiangiotensin antibodies, and angiotensin vascular receptors *in vivo* makes such studies difficult to interpret. In fact, immunized animals have very high concentrations of bound angiotension II and normal concentrations of the free hormone [35].

Antisera to purified rabbit and canine pulmonary converting enzymes [36–38] were less than optimally useful in that administration of antibodies to the homologous enzyme resulted in an immediate immune-dependent lethal reaction in both rabbits [39] and dogs.* When antibodies to a heterologous enzyme (rabbit) were administered to rats in high doses, a slow decline in blood pressure was observed in the two-kidney, Goldblatt-hypertensive rat [37] with very different kinetics from those observed with peptide or chemical inhibitors of converting enzyme. Thus, the value of these antisera in physiologic studies is uncertain.

A specific antibody for renin should be a highly selective antagonist. Renin antibodies have been used as physiologic reagents for many years [40, 41], yet the specificity of those reagents was and still is in doubt since we now known that the preparations

* V. J. Dzau, unpublished observation.

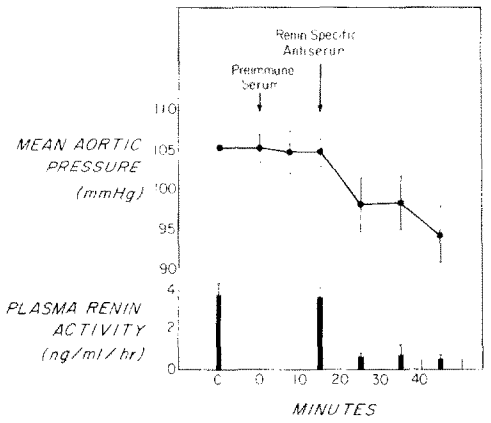

Fig. 4. Effects of preimmune serum and renin-specific antiserum on plasma renin activity in salt-depleted dogs. Preimmune serum had no effect, whereas renin-specific antiserum lowered plasma renin activity, causing blood pressure to fall. (Reprinted from Ref. 43, by permission of the American Association for the Advancement of Science.)

then used as immunogens contained less than 1% of the enzyme. Dzau *et al.* [42] purified canine renin some 600,000-fold in an eight-step process that yielded a product homogeneous by several criteria. Antibodies specific for purified canine renin raised in a goat inhibited the pressor action of the enzyme but did not modify the capacity of either angiotensin I or II to raise blood pressure [43]. This antibody preparation did not have any effect on the hemodynamics of the unanesthetized, sodium-replete dog, while a significant hypotensive effect was noted in the sodium-depleted dog when the renin-specific antiserum was injected intravenously (Fig. 4). Parallel to the fall in blood pressure, a decrease in both plasma renin activity and angiotensin II concentrations was observed, indicating that the antibody was exerting its effect by inhibiting the enzymatic action of renin on its substrate.

As discussed above, antibody fragments may have significant advantages in physiologic investigation. As can be seen from Fig. 5, the hypotensive effect of intact antibody is very persistent, in this instance for more than 24 hr, because antibody is only eliminated by metabolism, with the half-life for endogenous immunoglobulins measured in days or weeks depending on the species and the immunoglobulin isotype. If immune complexes form, elimination is more rapid by the reticulo-endothelial system. When there is concern about renal function, the presence of immune complexes is likely to cloud interpretation. In hemodynamic studies, vasoactive peptides released by activation of complement may have independent effects. Fab, as discussed above (see Table 3), has a number of advantages. The immune complexes that may be formed are smaller than those that cause nephrotoxicity (comprising a single antigen molecule with several Fab attached), and complement cannot be fixed because the relevant binding sites on the Fc have been lost. In Fig. 6 the results of intravenous injection of renin-specific Fab into sodium-depleted dogs are demonstrated. When compared with intact antibody (Fig. 4), it is apparent that the initiation of hypotension is more rapid. It is now possible to conclude, without equivocation, that renin plays a significant role in the maintenance of cardiovascular homeostasis in the sodium-depleted subject. Similar studies with renin-specific Fab have demonstrated the effect of renin in initiating acute renovascular hypertension.* Here also

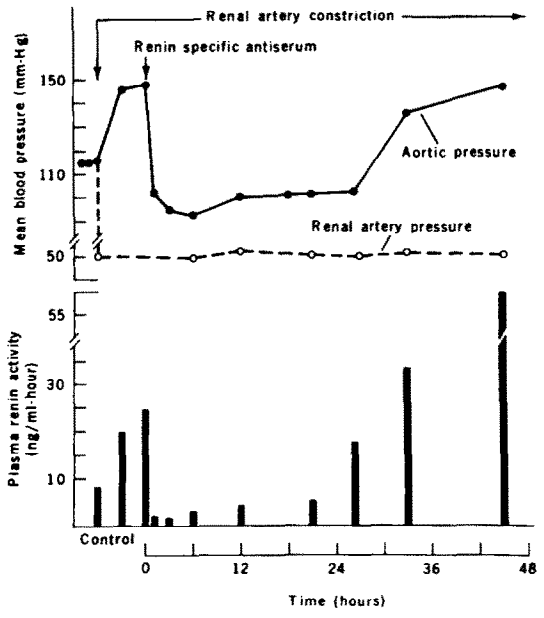

Fig. 5. A representative experiment illustrating the duration of action of the antiserum. After renovascular hypertension was produced, administration of renin-specific antiserum resulted in a sustained suppression of systemic blood pressure and plasma renin activity below control levels for 24 hr. (Reprinted from Ref. 43, by permission of the American Association for the Advancement of Science.)

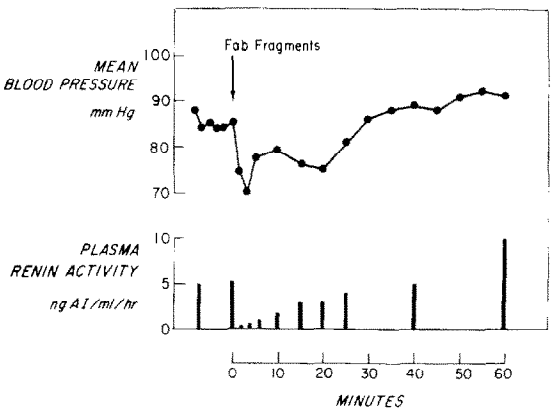

Fig. 6. Effect of renin-specific antibody Fab on plasma renin activity and blood pressure in a sodium-depleted dog. Renin-specific antibody Fab caused a transient fall in blood pressure associated with suppression of renin activity in plasma. (Reprinted from Ref. 23, by permission of the Biochemical Society.)

a more rapid initiation and a shorter duration of effect were noted.

Digoxin-specific antibody in the clinical reversal of toxicity. The digitalis glycosides are of great value in the treatment of congestive heart failure and, consequently, are frequently used in clinical medicine. Unfortunately, they are characterized by a very close toxic-therapeutic ratio. Digitalis intoxication is one of the most frequent adverse drug reactions reported. There is no specific antidote, and the cardiac arrythmias that are a feature of digitalis intoxication are commonly fatal. We reasoned that if an antibody specific to the digitalis glycosides had a higher affinity for the drug than for the physiologic receptor, it should be possible to transfer the ligand from the receptor to the antibody simply by mass action. For optimal effectiveness, diffusion distances should be minimal and the antibody should be in high concentration in extracellular fluid in proximity to the receptor. It would also be desirable to remove the antibody–drug complex rapidly from the body. Conventional antibody does not allow these goals to be satisfied.

Digoxin-specific antibody has been purified from sheep antiserum utilizing immobilized ouabain. Fab was then isolated after papain cleavage [44]. After demonstration of safety and effectiveness in animal studies, clinical investigations were initiated. At the time of this writing, twenty-six patients with life-threatening digitalis intoxication have been studied in a multicenter national trial [45]. Twenty geographically dispersed university-based centers participated, and patients were admitted to the trial if they presented with a life-threatening rhythm disturbance or hyperkalemia and were resistant to conventional therapeutic approaches. The patients ranged in age from 19 months to 35 years, and the overdoses occurred either during the course of therapy (N = 13) or as a result of accidental or suicidal overdose. Figure 7 shows the outcome. In each case a dramatic reversal of the signs or symptoms of intoxication occurred. Five patients died, one because inadequate quantities of Fab were available, and the remaining four because irreversible brain or cardiac damage had occurred by the time the drug was administered.

The history of a recently reported case is typical of the group [46]. The patient was a 34-year-old woman who took 20 mg of digitoxin, a massive overdose, with suicidal intent. She appeared to be well on admission to the hospital, except for nausea, but soon lapsed into a series of life-threatening arrhythmias that included multiple ventricular fibrillations (treated with countershock), as well as asystole (treated with ventricular pacing). At the time the antibody Fab became available to the physicians treating her, she was in shock, aneuric, and exhibited dilated pupils. Her serum potassium was elevated, a grave prognostic sign in digitalis intoxication [47]. Within an hour after the intravenous administration of antibody Fab, her atrioventricular conduction had returned, and she was soon in normal sinus rhythm. No further dysrhythmias occurred. The patient was discharged from the hospital without sequellae several days later. Figure 8 demonstrates the initial marked increase of serum digitoxin concentration in this patient as tissue-bound drug equilibrated with

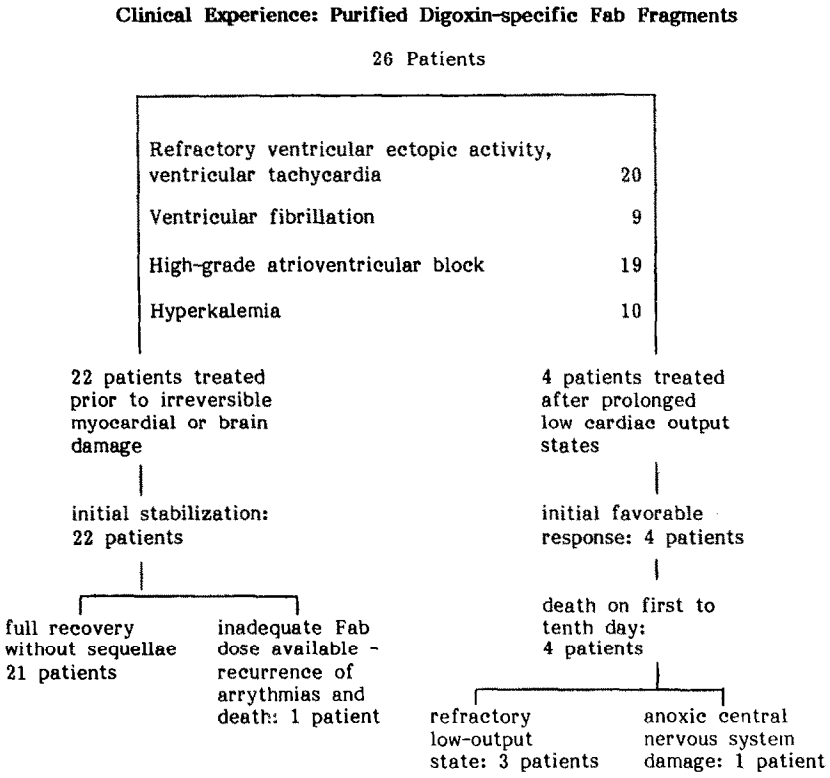

Fig. 7. Clinical results of Fab in reversing life-threatening digitalis intoxication. (Reprinted from Ref. 45, by permission of the Massachusetts Medical Society.)

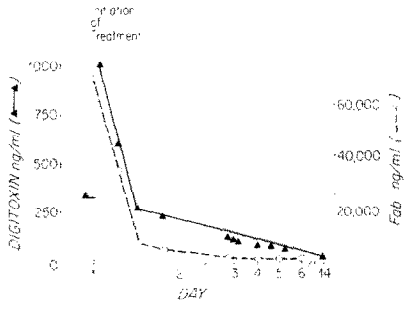

Fig. 8. Blood levels of digitoxin and Fab fragments after intravenous administration of antibody Fab to a 34-year-old woman suffering a series of life-threatening arrythmias as a result of a massive overdose (20 mg) of digitoxin. Within an hour of intravenous administration of antibody Fab, atrioventricular conduction had returned. (Reprinted from Ref. 48, by permission of Academic Press.)

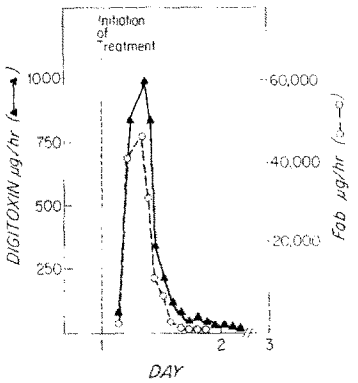

Fig. 9. Urinary excretion of digitoxin and Fab fragments following intravenous administration of antibody Fab to a 34-year-old woman suffering from digitalis intoxication (20 mg). (Reprinted from Ref. 48, by permission of Academic Press.)

the antibody (antibody-bound drug is pharmacologically inactive), followed by rapid clearance of both drug and Fab. It should be noted that the half-life of digitoxin in man is normally 3.5 days with hepatic metabolism of the drug being the major source of removal. It is apparent that excretion was markedly accelerated by the antibody Fab, the half-life seemingly reduced to about 12 hr. Figure 9 shows that both antibody and Fab appeared in the urine largely within the first day after Fab administration.

It should be apparent that this approach could be applied to many other drugs or toxins. Of particular interest is the acceleration of excretion of a substance that is normally metabolized slowly. Fab is capable of altering the route of disposal from hepatic metabolism to renal excretion [49].

Imaging of myocardial infarcts. The diagnosis of myocardial infarction often does not present a significant challenge to the clinician. Symptoms may be pathognomonic and so-called objective findings unequivocal. Quite often, however, when it is most important to know, associated injury or disease masks symptoms and distorts the results of laboratory tests, none of which are entirely specific. Beyond the question of diagnosis is quantification. There have been many attempts at investigating methods for arresting the progress of infarction and salvaging as much tissue as possible. It is very difficult to evaluate the efficacy of these interventions without an objective measure *in vivo* of the extent of cardiac tissue necrosis. Up to the present time, quantification of infarct size has only been possible in retrospect. The hallmark of cell death is loss of membrane

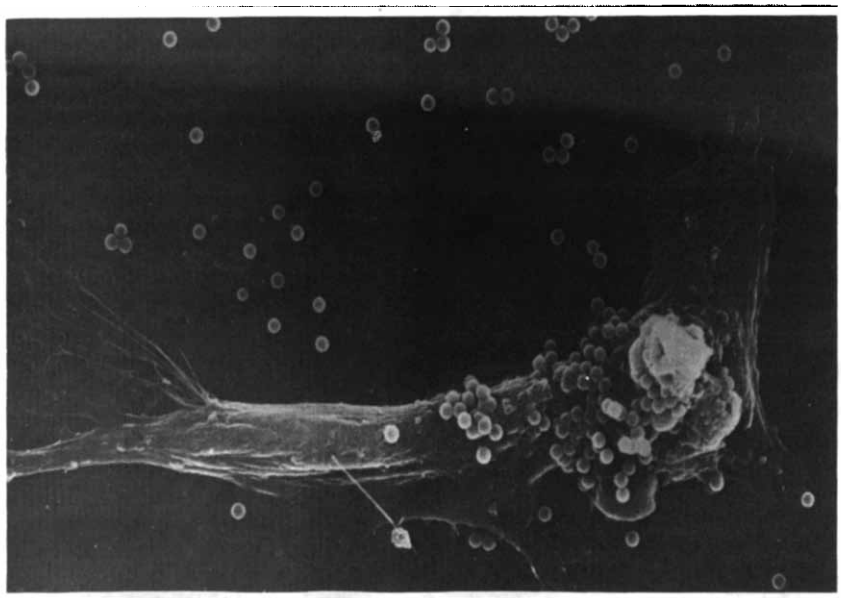

Fig. 10. Scanning electron micrograph of an ischemic neonatal mouse myocyte (magnification ×2400). The spheres adherent to the cell are covalently bonded to myosin-specific antibody. Non-ischemic cells do not bind significant numbers of spheres. (Reprinted from Ref. 51, by permission of the British Medical Association.)

integrity; when there is no longer a physical separation between inside and outside, the cell ceases to exist. This principle is commonly employed in clinical practice in diagnosing tissue infarction. Intracellular enzymes are lost to extracellular fluid, and their increased concentration in the blood may be used as a measure of tissue necrosis. An alternative approach is the inward diffusion of a marker that is normally excluded from cells. We have selected myocardial infarction as a model and radioactively labeled myosin-specific antibody as a marker. Myosin is the principal protein of the cardiac cell. Its covalent structure is unique to the heart [50], allowing the development of antibodies that differentiate between cardiac and either skeletal- or smooth-muscle myosins.

In the intact organism, cardiac myosin is protected from extracellular fluid by the plasma membrane of the cell. When cell death occurs and the membrane breaks down, myosin is exposed to extracellular fluid. It is then available to react with labeled antibodies or antibody fragments. A visual demonstration of this phenomenon is seen in Fig. 10. The figure shows a scanning electron micrograph of part of a neonatal mouse cardiac myocyte that had been rendered ischemic by prolonged exposure to nitrogen and then incubated with 1-μm diameter, polystyrene spheres covalently bound to myosin-specific antibody. Myofibrils are seen protruding from a hole in the cell membrane. Antibody-coated microspheres clearly bind to the myofibrils. Not only is myosin very insoluble in physiological fluids so that membrane breakdown does not result in antigen loss, but it persists for considerable periods after ischemic necrosis [52]. This should permit identification of infarcted myocardium days to, perhaps, several weeks after the initial event. In our initial exploration of this concept, a canine myocardial infarction model was used [53]. The left anterior descending coronary artery was ligated, and 4 hr later ^{125}I-labeled, myosin-specific antibody or antibody fragments were injected intravenously. At varying times subsequent to the injections, animals were killed, the hearts perfused with triphenyltetrazolium chloride, and the myocardium was examined [54]. Figure 11 (left) shows a section of a heart treated in this way. The light area represents a largely subendocardial infarction. The central panel of the figure is a tracing of the slice superimposed on an autoradiograph. The exposed area corresponds to the infarct as revealed by the triphenyltetrazolium stain. The right panel shows specific radioactivity in the area of the infarct relative to myocardium on the posterior wall. It is apparent that the major concentration of radioactivity was in the subendocardial region, with lesser concentration in the subepicardial region that had been subjected only to spotty necrosis as indicated in the triphenyltetrazolium stain. Microautoradiographs showed that individual necrotic myocytes could be identified and differentiated from adjacent viable cells [56]. In order to demonstrate that antibody concentration was specific and not simply the result of passive diffusion of a macromolecule into infarcted cells, specific antibody labeled with ^{131}I and nonimmune globulin labeled with ^{125}I were injected simultaneously into the coronary arteries [57]. At the center of the infarct, the antibody had concentrated 34-fold in relation to normal myocardium, while the nonimmune globulin was only 7-fold in excess. In normal tissue, as expected, concentrations are equal. When compared to a marker of relative flow (the distribution of radioactive microspheres that had been injected into the left atrium), it was clear that the concentration of labeled antibody was inversely related to relative blood flow [53, 54]. There seems to be sufficient collateral circulation in this ischemic model to provide delivery of antibody, even to regions of minimal blood flow. It would be very desirable to apply this method to the detection, localization and quantification of myocardial infarcts in the living subject. Optimal imaging with a gamma or positron camera requires the labeling of antibodies with radionuclides of appropriate half-life and affinity. We have accomplished this by covalently linking diethylene triamine pentaacetic acid (DTPA) to antibody or antibody fragment and then binding cationic radionuclides by chelation [57]. Successful images have been obtained using the following radionuclides linked to antibody: [^{111}In]DTPA-AM-Fab [57], [^{68}Ga]DTPA-Fab [58], and

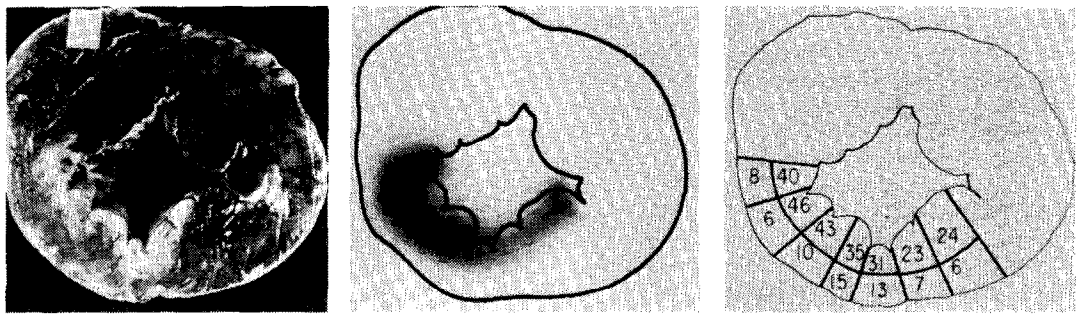

Fig. 11. Histochemically delineated myocardial infarction in a ventricular slice (left) seen as white or lighter-colored regions, and normal myocardium seen as darker regions. The corresponding macroautoradiograph is shown in the center panel with the outline of the ventricular slice; the corresponding ratios of antibody uptake are demonstrated in the indicated areas (right). Ratios of antibody uptake were determined as antibody activity in test tissue/antibody in normal posterior ventricular myocardium. (Reprinted by permission of the American Heart Association, Inc. from Ref. 55.)

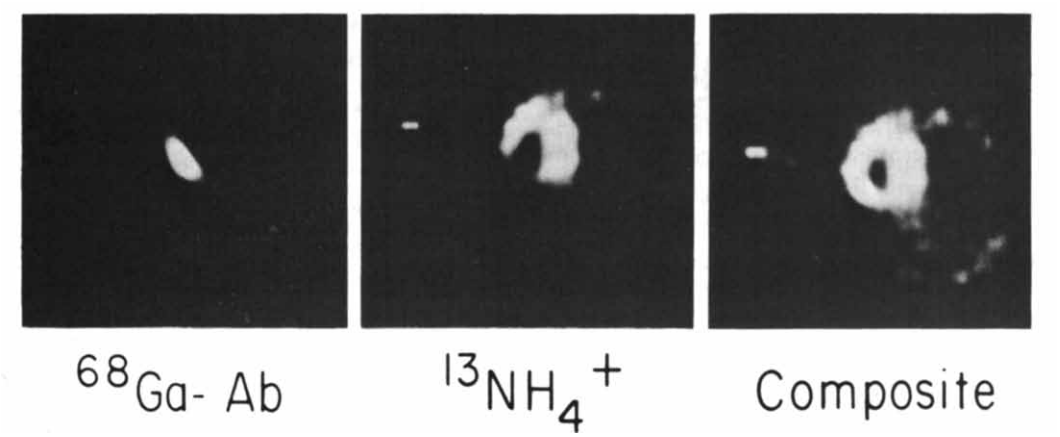

Fig. 12. Sequentially obtained positron camera ^{68}Ga and $^{13}NH_4^+$ scintigrams of dog heart. $^{13}NH_4^+$ (5 mCi) was administered intravenously to a dog that had a prior 4-hr occlusion of the distal segment of the left anterior descending artery and was imaged (center); 30 min later 1 mCi of ^{68}Ga was injected into the same artery and imaged (left panel). Right panel is a composite of both images. (Reprinted by permission of the American Heart Association, Inc. from Ref. 55.)

[^{99m}Tc]DTPA-AM-Fab [57]. An example of a positron image of an anterior infarct in a living dog using ^{68}Ga-labeled antibody is shown in Fig. 12 (left). The positron imaging technique allows tomographic reconstruction, and thus the heart is visible on cross section in the ^{13}N-ammonia image in the center panel of Fig. 12. (Ammonium ion is a potassium analog and concentrates in normal tissue.) On the left side of the image there is clearly less density, reflecting the region of infarction. Superimposition of the ^{13}N and the ^{68}Ga images (right panel) showed that the antibody was concentrated directly in the area of diminished ammonium ion uptake. The potential for applying this method to the evaluation of myocardial infarcts in a clinical setting seems very great indeed since it provides a method of great specificity and resolution.

The use of heterogeneous affinity column-purified antibody, though useful, cannot be standardized because of the variability of the immune response: quantities of antibody are necessarily limited. The application of monoclonal antibodies readily overcomes these difficulties. We have utilized monoclonal antibodies to human cardiac myosin that cross-reacted with canine cardiac myosin to image experimental infarcts in dogs and have initiated clinical studies. Figure 13 shows specific localization of monoclonal anti-cardiac myosin labeled with ^{99m}Tc in infarcted myocardium following intracoronary administration of the antibody. The use of ^{99m}Tc as the radiolabel and the selectivity of the antibody have eliminated the necessity of subtractive imaging as well as the lag period between intravenous injection and visualization by scintigraphy.

A look into the future

The future of antibodies as therapeutic agents must lie in the application of genetic engineering

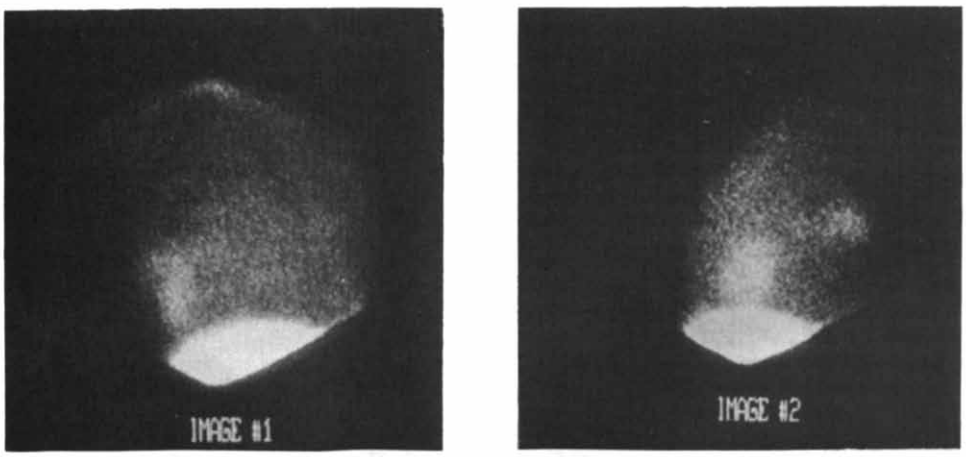

Fig. 13. Left lateral (image 1) and anteroposterior (image 2) scintigrams showing localization of ^{99m}Tc-labeled monoclonal DTPA-antimyosin (WM-2) Fab fragments 18 hr post intracoronary administration in a canine experimental myocardial infarction. The lower central activity is also due to liver activity. (Reprinted from Ref. 59, by permission of Elsevier/North-Holland Biomedical Press.)

techniques to their modification and manufacture. A minimal antigen binding site will be created by splicing the appropriate cDNA fragments. A better understanding of structure–function relationships in the antibody combining site will permit refinement of antibody specificity beyond that which random selection by the immune system provides. Here site-directed mutagenesis techniques will be applied to change the amino acid sequences of antibodies in order to increase either their affinities or specificities. Appropriate structural changes may also alter antigenicity and gastrointestinal absorptive properties. Finally, the optimally engineered molecules will be expressed by microorganisms in industrial fermenters so that their ultimate cost may not exceed that of penicillin today.

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