0031-6997/82/3401-0077$02.00/0
PHARMACOLOGICAL REVIEWS

Vol. 34, No. 1
Printed in U.S.A.

Antibodies in Vivo*

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THE IMMUNE system generates a set of molecules that exhibit extraordinary specificity and selectivity. Of the various recognition molecules of the immune system, humoral antibodies have been most accessible for study on a molecular level as the resolution possible with these antibodies far exceeds that observed with any other set of compounds. In specific instances, enzyme substrate or ligand protein pairs (e.g., avidin and biotin) may show exclusive affinity for one another as well as exhibit a very high energy of interaction; yet the range of compounds recognized is very limited. Nucleic acids may have a greater potential resolution than antibodies but are only capable of recognizing either one another or a highly selective set of protein molecules. Antibodies, on the other hand, can resolve almost any compound that has a reasonable degree of complexity. A remarkable example is the resolution between the *d* and *l* stereoisomers of the simple aliphatic molecule, tartaric acid (22). More complex organic molecules that vary by only single substituent groups (such as the steroids, digoxin, and digitoxin that differ in a single OH group) may be readily separated (29). Even antigenic differences between large proteins that differ in only a single amino acid (such as hemoglobin A and S) have been discerned (25). Antigenic differences have also been studied among complex lipids, such as the prostaglandins, and among nucleic acids and the polysaccharides.

The immune system has been deliberately manipulated by clinicians in several different ways. Most notably, stimulation of the body to recognize a foreign microorganism by immunization has long been a cornerstone of preventive medicine. The passive infusion of pneumococcal-specific antisera had a brief vogue in the preantibiotic days, while tetanus and Rh antibodies are still of great value today. By providing a method for measuring substances in biological fluids that would not be possible by any other means, the immunoassay has produced a literal explosion in the use of antibodies as in vitro diagnostic agents. Yet this remarkable group of compounds has been largely neglected in its potential utility as a source of pharmaceutical agents with a wide variety of applications.

There may be several explanations for this neglect. Prior to very recent times, antibodies were available only as complex mixtures of proteins, isolated in very limited quantities from animal sera. Clearly, a hutch of rabbits does not provide the optimal source for commercial production of a pharmaceutical agent with the capacity for wide application, especially when each rabbit produces a different set of antibodies with each bleeding. Antibodies are also large proteins that are immunogenic when injected into a heterologous species. Each of these problems has been overcome by application of a new technology that has been available only in the last several years. Antibodies may now be produced by cell culture methods in infinite quantities with previously unimagined homogeneity and reproducibility (21). Antibody fragments may be produced that reside within the body for shorter periods of time and are less likely to provoke an immune response (30). We are on the threshold of producing human antibodies by the same cell-culture approach (3, 24).

I shall review the potential application of antibodies to human pharmacology in three different areas: first, the location of a unique antigen in vivo by scintigraphy with an antibody fragment to which a radionuclide has been attached; second, blockade of the physiological action of a hormone that is secreted in excess; and third, blockade of a physiological receptor. In another paper in this volume, Dr. Vincent Butler will discuss the neutralization of the toxic effect of drugs by specific antibodies.

The Specificity of Antibodies

Antibody specificity is determined by the amino acid sequence of the region of the molecule in contact with antigen. Three segments of the variable regions of both the light and heavy polypeptide chains make up the complementarity regions that actually form atomic contacts to the antigen molecule. Variation within these regions is very great and now appears to be generated by at least several different genetic mechanisms. 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, 26–28). V and J of the light chain may occur in any combination. The heavy chain's variable region is the product of three genes, V, in several hundred copies, D, in 10 or more copies, and J, in four copies (8, 32). 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}.

An example of the variety of antibodies that may be formed in response to a rather simple antigenic stimulus, a repeating, unbranched disaccharide comprised of cel-

* This work was supported by National Institutes of Health grant HL-19259.

	Amino Acid Sequences in Complementarity-determining Regions		K_o	
	First hv Region	Third hv Region	(LM^{-1})	Discrimination
	23 30 32a b 33	90 97a b c d e f		
3368	C Q A S E S I G N E - - L	C Q Q D W N S N N V V N N - - - F G G	2×10^4	$(S3)_8 = (S3)_6 = (S3)_4$
3374	——— Q N — D S W - - —	— S Y Y S I S S A - - - - - - ———	2×10^5	$(S3)_8 = (S3)_6 > (S3)_4$
K-25	——— Q B — Y S Y - - —	— T Y S Y — S T Y - - - - - - ———	2.8×10^5	———
BS-1	——— Q — Y S G - - —	— Z G S T Y G G G Y - - - - - - ———	1.4×10^6	———
BS-5	——— Q — Y S N - - —	— Z G S B Y T G T — - - - - - - ———	———	———
3381	——— E — S — W - - —	— S T D G N S D G F S — Y G ? ———	3×10^6	$(S3)_8 = (S3)_6 > (S3)_4$
3T74	— S — Q — V Y — N N Y —	— A G G Y S — S S H N A - - - - ———	7×10^6	———
3T72	——— Q ——— S W - - —	——— T — S — D — T - - - ———	1×10^7	$(S3)_8 > (S3)_6 > (S3)_4$
3T70	——— N — Y S S - - —		1.2×10^7	———

FIG. 1. Light chain complementarity region sequences and binding properties of antipneumococcal type III polysaccharaide antibodies. Amino acid residues are indicated in the standard single-letter code. Discrimination is a measure of the relative capacity of the tetra-, hexa-, and octasaccharide subunits ($[S3]_4$, $[S3]_6$, and $[S3]_8$) of the S3 polysaccharide to inhibit the binding of anti-S3 antibody to iodinated S3 polysaccharide. (Reprinted with permission of Cold Spring Harbor Laboratory, Cold Spring Harbor Symp. Quant. Biol. 41: 647–659, 1977.)

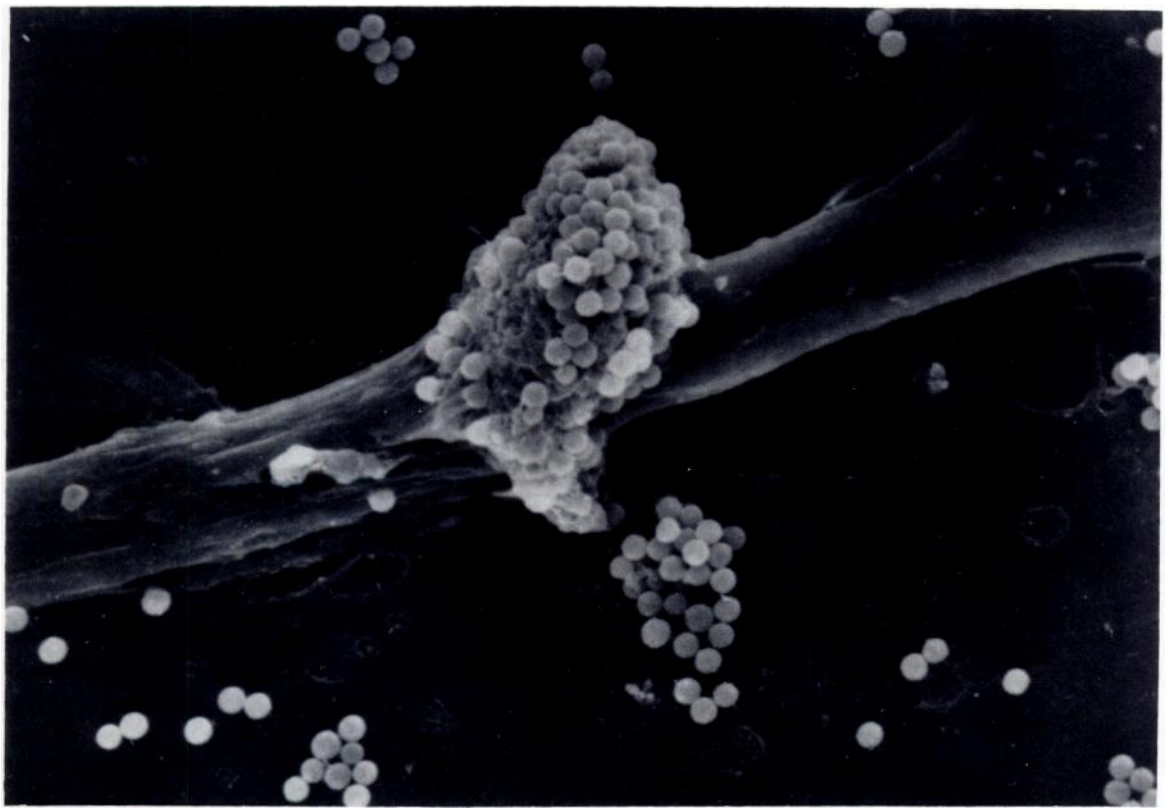

FIG. 2. Scanning electron micrograph of an ischemic neonatal mouse myocyte (magnification 3400×). The spheres adherent to the cell are covalently bonded to myosin-specific antibody. Nonischemic cells do not bind very many spheres.

lobiose units, is shown in figure 1 (12). Depicted in the one-letter amino acid code are two of the complementarity regions of the antibodies' light chains. Note that all the antibodies are different, not only in amino acid sequence, but in the length of the complementarity regions. These differences are reflected in the affinity with which the antigen is bound as well as the size of an oligosaccharide subunit that is optimally bound. This small vignette of antibody variability with respect to recognition of a single antigen allows an appreciation of the enormity of the menu from which one may choose an antibody for a specific recognition task.

Imaging of Myocardial Infarcts with Cardiac Myosin-Specific Antibodies

The hallmark of cell death is loss of the membrane 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 the diagnosing of 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. Since its covalent structure is unique to the heart (15), it allows 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 cell's plasma membrane. 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 figure 2. 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-μ diameter polystyrene spheres covalently bound to myosin-specific antibody. Cell contents are seen protruding from a hole in the cell membrane. Antibody-coated microspheres clearly bind to the herniated cytoplasm, but not to the cell membrane.

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 (9). This should allow for 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 (16). The left anterior descending coronary artery was ligated and four hours later [^{125}I]labeled, myosin-specific antibody or antibody fragments were injected i.v. At varying times subsequent to the injections, animals were killed, the hearts perfused with triphenyltetrazolium chloride, and the myocardium examined (20). Figure 3 (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 is 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 (17).

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 (20). Figure 4 shows the results of this experiment. At the center of the infarct, the antibody has concentrated 34-fold in relation to normal myocardium, while the nonimmune globulin is only 7-fold in excess. In normal tissue, as expected, their concentrations are relatively 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 (16, 20). There seems to be sufficient collateral circulation in this ischemic model to provide for delivery of antibody, even to the 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 radio-

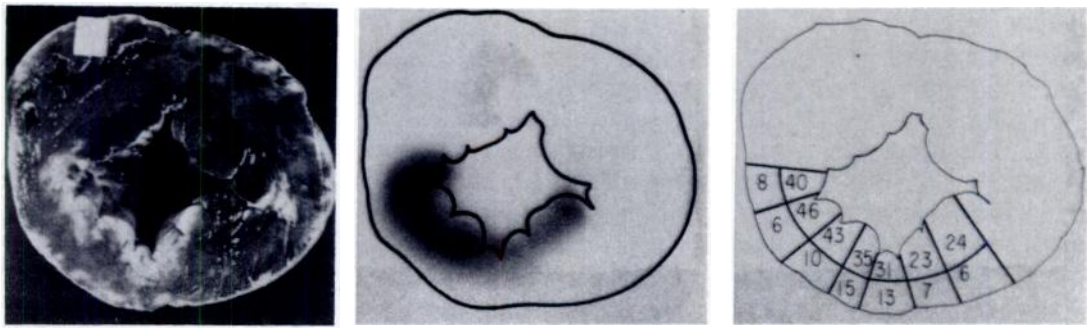

FIG. 3. Histochemically delineated myocardial infarction in a ventricular slice (left) seen as white or lighter-colored regions, and normal myocardium seen as darker regions. The corresponding macroautoradiograph is shown in the center with the outline of the ventricular slice. The right panel shows relative antibody uptake in the indicated areas. Ratios of antibody uptake were determined as specific radioactivity in relation to normal posterior ventricular myocardium. (Reprinted with permission of American Heart Association, Proc. N. Engl. Cardiovasc. Soc. **2**: 31–38, 1979.

nuclides by chelation (19). Successful images have been obtained with the following radionuclides linked to antibody: [^{111}In]DTPA-Ab Fab (19), [^{68}Ga]DTPA Fab (18), and [^{99m}Tc]DTPA-Ab Fab (19).

An example of a positron image of an anterior infarct in a living dog with ^{68}Ga-labeled antibody is shown in figure 5 (left). The positron imaging technique allows for tomographic reconstruction, and thus, the heart is visible on cross section in the ^{13}N ammonia image in the central panel of figure 5. (Ammonium ion is a potassium analog and concentrates in normal tissue.) On the left side of this 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 for a method of great specificity and resolution.

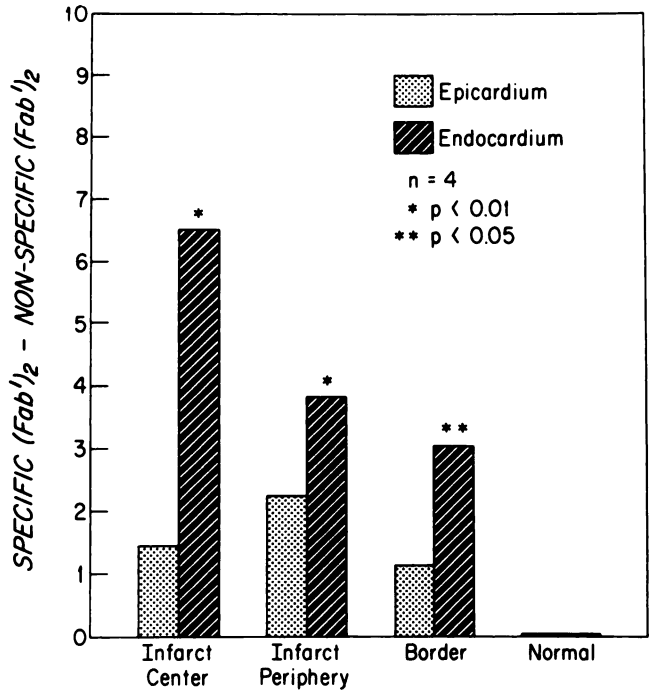

FIG. 4. Specific antimyosin $(Fab')_2$, binding in infarcted myocardium 48 to 72 hours after coronary occlusion. Specific binding is shown of antimyosin $(Fab')_2$, obtained by subtracting nonspecific relative localization of normal rabbit IgG $(Fab')_2$ from relative antimyosin $(Fab')_2$ binding, in endocardial and epicardial layers of infarct center, periphery, border zone region, and normal myocardium. (Reprinted with permission of J. Clin. Invest. **58**: 439–466, 1976.)

Blockade of the Hypertensive Action of Renin

Though renin has been known to play a role in circulatory control since the classic work of Goldblatt in the 1930s (10), 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 (11). 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 (34), and thus its inhibitors also affect the kinin system. In addition, compounds such as Captopril have been shown to stimulate prostaglandin synthesis (35). Since both the kinin and the prostaglandin systems have an effect on vasoregulation, it is difficult to define the specific part that renin plays in interpreting experiments in which these inhibitors are used.

A specific antibody for renin should be a highly selective antagonist. Renin antibodies have been used as physiological reagents for many years (4, 31), yet their specificity was then also in doubt since we now know that the preparations then used as immunogens contained less than 1% of the enzyme. Dzau et al. (7) in our laboratory purified canine renin some 600,000-fold in an eight-step process that yielded a product, homogeneous

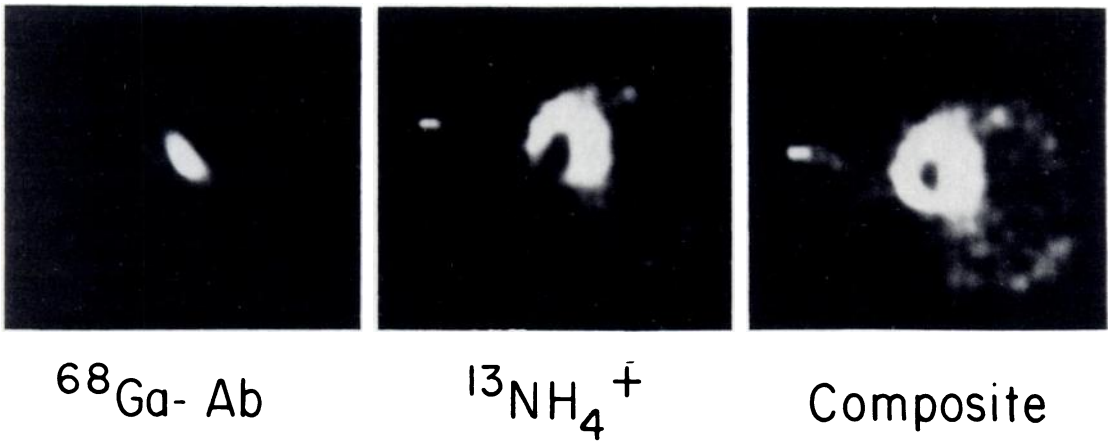

FIG. 5. ^{68}Ga and $^{13}NH_4^+$ scintigrams were obtained sequentially with a positron camera; 5 mCi of $^{13}NH_4^+$ was administered I.V. to a dog that had a prior 4-hour occlusion of the distal segment of the left anterior descending artery and was imaged (center); 30 minutes later 1 mCi of ^{68}Ga was injected into the same artery and imaged (left panel). Right panel is a composite of both images. (Reprinted with permission of American Heart Association, Proc. N. Engl. Cardiovasc. Soc. **2**: 31–38, 1979.)

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 (5). 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 immunoglobulin G fraction from an antiserum was injected i.v. (fig. 6). Parallel to the fall in blood pressure, a decrease in both plasma renin activity and angiotensin II concentrations were observed, indicating that the antibody was exerting its effect by inhibiting the enzymatic action of renin on its substrate.

Intact antibody has a number of troublesome properties when used as a drug. When the source is a heterologous species, it is an immunogen. After the first use, an immune response develops that may result in anaphylaxis, serum sickness, or at best accelerated elimination. As can be seen from figure 7, the hypotensive effect after acute renovascular constriction is very persistent, in this instance over 24 hours, 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 reticuloendothelial 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. Immunoglobulin molecules that bind 2 moles of antigen per mole may be cleaved into smaller fragments by the enzyme papain (23). The resultant Fab fragments bind 1 mole of antigen each, whereas the Fc fragment contains the complement binding site.

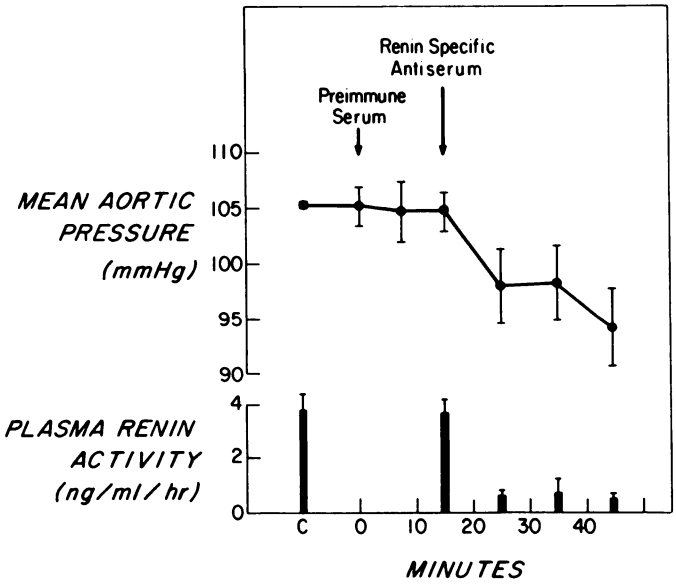

FIG. 6. In salt-depleted dogs, preimmune serum had no effect whereas renin-specific antiserum lowered plasma renin activity, causing blood pressure to fall. (Reprinted with permission of Science **207:** 1091–1093, 1980.)

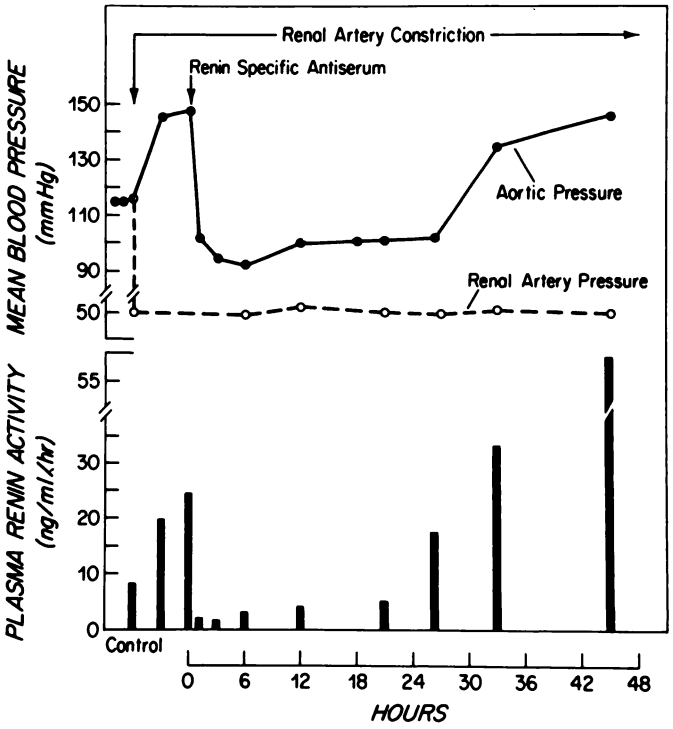

FIG. 7. A representative experiment illustrating the duration of action of the antiserum. After renovascular hypertension was produced, administration of renin-specific antiserum resulted in a sustained suppression of systemic blood pressure and plasma renin activity below control levels for 24 hours. (Reprinted with permission of Science **207:** 1091–1093, 1980.)

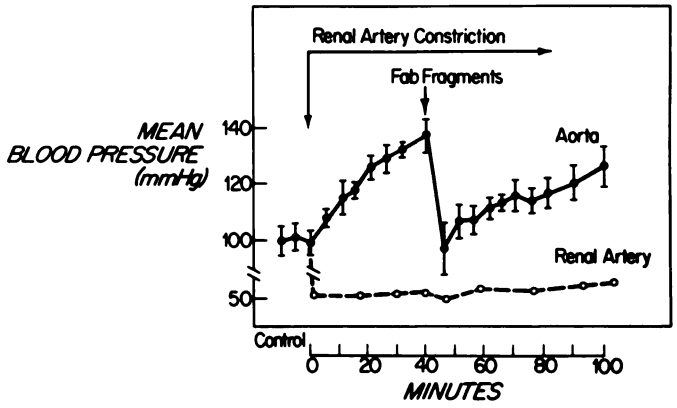

FIG. 8. The i.v. administration of renin-specific Fab results in the rapid onset of a brief hypotensive response in sodium-depleted dogs.

Fab has several desirable properties when compared with the intact molecule, IgG: equilibrium distribution in extracellular fluid is achieved more rapidly; the volume of distribution is greater; and the fragment is eliminated with a far shorter half-life (30). In addition, when injected i.v., Fab is less immunogenic than IgG (30). 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 figure 8 the results of i.v. injection of renin-specific Fab into sodium-depleted dogs are demonstrated. When

compared with intact antibody (fig. 7), it is apparent that the initiation of hypotension is more rapid, and the duration of the effect very much shorter. 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 (6).

Blockade of the β-Adrenergic Receptor

The hormone receptors of the cell's plasma cell membrane are present in very few copies (10,000 to 50,000/cell), which makes their isolation exceedingly difficult. Antibodies to partially purified receptor preparations have been elicited (33), but their utility is limited because these preparations of necessity contain antibodies to other membrane constituents. The ligands for these receptors, however, 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 have been created in the organic chemist's laboratory. 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 in the first section, 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 immunological principle of raising antibodies specific for another antibody's combining site (antiidiotypic antibodies) may be used 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. There are two ways of achieving this end, short of depending on fortuitous probability. 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 achieving 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 (14). I shall review our own work on the β-adrenergic receptor. There is a wide variety of structurally different β-antagonists available. All, however, share a common structure, a propanolamine side chain (fig. 9). Rabbits were immunized with an alprenolol-protein conjugate. The resulting antiserum was passed over

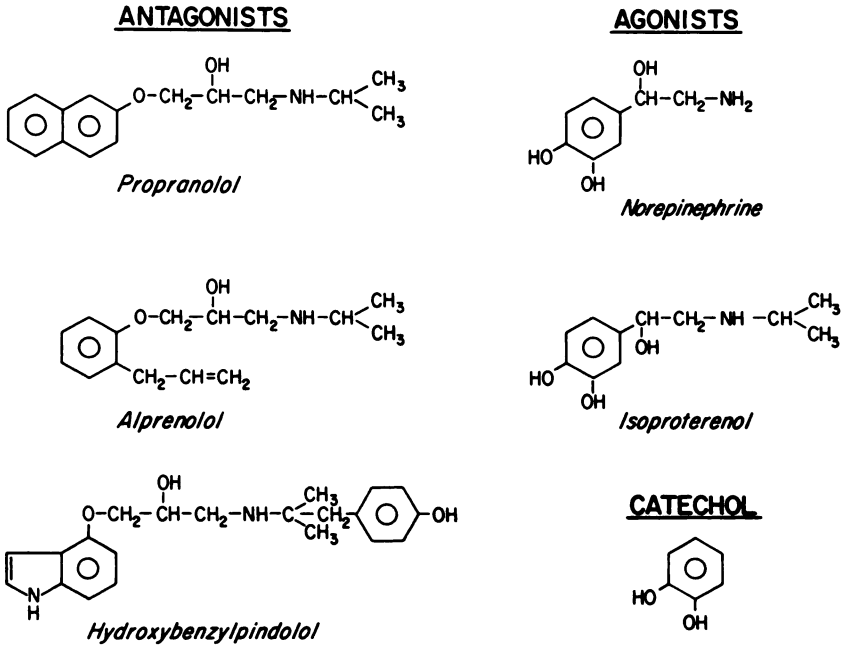

FIG. 9. Structures of β-antagonists and agonists. Note the common propanolamine side chain. Catechol, which is neither agonist nor antagonist, does not possess this structure.

an acebutolol affinity column and the fall through volume discarded. Elution of the affinity column was carried out with *l*-propranolol and the eluant antibody characterized after the *l*-propranolol had been removed by dialysis. Figure 10 demonstrates the specificity profile of this antibody fraction. Several β-adrenergic antagonists as well as agonists are bound with considerable affinity, in some cases similar to that of the receptor. This fraction also resolved *l* and *d* stereoisomers of isoproterenol (not shown). 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).

Table 1 shows the inhibition of binding of a labeled β-adrenergic antagonist, [^{3}H]alprenolol, to several kinds of binding sites by the 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). Of greater interest is the inhibition of the specific binding of alprenolol to plasma membrane preparation of diverse tissues in two different animal species. Scatchard analysis shows the binding of the antiidiotype to be competitive with β-adrenergic antagonists.

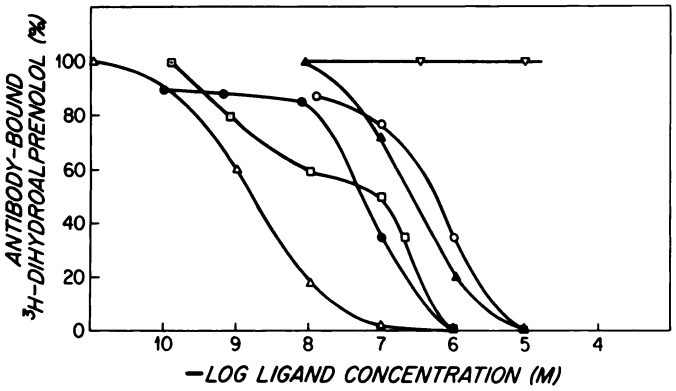

FIG. 10. Competitive inhibition of ^{3}H(−)-dihydroalprenolol binding to unfractionated antialprenolol antiserum. Rabbit immune serum (100 μl) was diluted 1:10 in buffer and incubated for 1 hour at 25°C with ^{3}H(−)-dihydroalprenolol and increasing concentrations of (±)-propranolol (△), (±)-alprenolol (□), (±)-hydroxybenzylpindolol (●), (±)-norepinephrine (○), (±)-isoproterenol (▲), or catechol (▽). Each determination was performed in triplicate and corrected for the nonspecific ^{3}H(−)-dihydroalprenolol binding to rabbit preimmune serum. The results are expressed as a percentage of the total antibody-binding capacity for the tritiated antigen. (Reprinted with permission of Circ. Res. **46:** 808–813, 1980.)

TABLE 1

*Inhibition of [^{3}H]alprenolol binding to antibodies and receptors by antiidiotypic antibody**

	Maximal Binding with Preimmune	In Presence of Antiidiotype	Inhibition (%)
	dpm	*dpm*	
Original idiotype (alprenolol)	10,853 ± 250	488 ± 250	96
Turkey erythrocyte	19,643 ± 540	6195 ± 525	68
Canine lung	6,385 ± 283	4315 ± 190	34

* Specific binding at 4–6 nM [^{3}H]alprenolol.

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 5×10^{-7} M isoproterenol concentration. At a higher concentration of isoproterenol (10^{-4} M), less inhibition is observed; this suggests a competitive nature for this interaction as well. 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 (2); a more rigorous examination of their respective physiological role with reagents of greater resolution; and the isolation of receptors with antibody affinity chromatography.

Antibodies as Drugs

The development of a new drug is often the result of empiricism, serendipity, or the deliberate modification of an existing natural product. These products of the organic chemist's laboratory are usually characterized by a lack of specificity with consequent abundance of adverse reactions. If antibodies were the basis of identification of a specific locus of action, one could choose among some 10^{11} different combining sites and thus markedly increase the potential for reacting only with the desired site. Specificity would be enhanced and side reactions diminished. The considerably larger size of the antibody combining site in comparison to conventional drugs allows for the establishment of more interatomic interactions and thus both greater affinity and specificity.

While some of the advantages of antibodies are obvious, the disadvantages, discussed in part in previous sections, must also be considered. When binding to a specific site is all that is desired, the activation of the biological effects that are an intrinsic part of an immune reaction are undesirable. The smaller Fab fragment that was discussed above as a possible solution to some of these problems still carries with it an entire domain (one-half of its mass) that is of no relevance to antigen binding. Fab from a heterologous species, while of diminished immunogenicity, is likely to cause hypersensitivity when used on a long-term basis.

The solutions to these problems are almost at hand. A much smaller fragment of antibody has been produced that retains all the binding energy and specificity of the intact molecule. Fv comprises a single domain and has a molecular size of 25,000 daltons (13). While its pharmacokinetics have not as yet been examined, I anticipate

that it will be very rapidly cleared and distributed. The advent of the hybridoma method will make possible not only the selection and large-scale production of antibodies of uniform properties, but also the reduction or elimination of the potential problem of hypersensitivity since it will be possible, as indicated above, to produce human antibodies. Will the formation of antiidiotypic antibodies defeat the long-term utility of antibody therapy? I believe that the well-recognized difficulty in raising antiidiotypes, even when the effort is undertaken with deliberation, indicates that this problem will rarely manifest itself. The next decade will see a new pharmacology based on the antibody-combining site.

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