On the Specificity of Antibodies

Biochemical and biophysical evidence indicates the existence of polyfunctional antibody combining regions.

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The most striking thing about an immune serum is the specific nature of its interaction with antigen. For instance, an immune serum can distinguish between two proteins having as little as a single amino acid difference between them. It can also discriminate between two chemical compounds differing in a single functional group between D and L amino acids and between many other closely related compounds (*1, 2*). Landsteiner and his collaborators and also other investigators (*1, 3*) have formulated, over a period of 30 years, a conceptual framework that has been generally accepted by immunologists. This framework can be stated simply as a series of three propositions:

1) Antibodies are specific for the antigen used to elicit the immune response, and such specificity has its base in the amino acid sequence and three-dimensional structure of the antibody molecule.

2) Antibodies may or may not bind antigens that are structurally closely related to the eliciting antigen. When they do bind, it is with interaction energies that are usually lower for the related antigen than for the eliciting antigen.

3) The humoral immune response is usually heterogeneous. Many classes and groups of immunoglobulins are produced in response even to a single antigenic determinant. The affinity of individual immunoglobulins for simple antigenic determinants covers a wide range, an indication that many different types of antigen combining sites complementary to a single eliciting haptenic determinant are present in the serum.

From the evidence on which these propositions are based, a conclusion has been generally inferred. If the immunoglobulin population constituting the immune serum is highly specific for an antigen, so must be the individual immunoglobulins constituting this population. This inference underlies arguments concerned with the number of genes needed to provide the observed wide range of antigenic specificities (*4, 5*). This inference is still a keystone in much genetic and structural thinking about immunoglobulins. Both research articles and textbooks state that the immunoglobulin site which combines with antigen is in the same location in all immunoglobulins and involves only a few, limited contact areas (*6*). In this article we will present evidence which suggests that even though immune serums appear to be highly specific with respect to antigen binding, individual immunoglobulins may not only bind a number of structurally diverse determinants, but may bind such diverse determinants to different sites within the combining region.

There are several indirect approaches that have suggested the existence of antibodies with multiple binding functions. DuPasquier and his colleagues have shown that the tadpole has only about 1×10^6 lymphocytes, yet is able to mount a specific humoral immune response directed against the dinitrophenyl determinant (anti-DNP) (*7*). Since the response to single haptens is heterogeneous, involving many different clones of cells that produce anti-DNP immunoglobulin and since there must be at least 10^5 to 10^6 cells involved in order that the antibody reach detectable levels, this is a substantial proportion of the animal's antibody producing cells. There is no doubt that the tadpole can respond to many antigens, thus there seem to be too few cells at any one time to account for the range of immune responses observed (*8*).

Williamson and his colleagues investigated the antihapten immunue potential of single mice immunized with carriers to which the hapten (*o*-nitro-*p*-iodophenyl (NIP) (*9*) has been conjugated. From previous work Williamson had developed a clone dilution transfer technique by which he was able to transfer to a large proportion of irradiated recipient mice single clones of cells producing antibody to NIP (anti-NIP). Individual NIP-binding immunoglobulins produced by these clones can be identified by the pattern they exhibit after resolution by isoelectric focusing (IEF). Williamson reasoned that if one counted the total number of clones transferred, and then identified from the IEF pattern, how many identical or "repeating" clones there were in that number, it would be possible to infer from statistical considerations how many different anti-NIP clones a single mouse could produce. The repeat frequency was in fact 5 clones out of 234 clones transferred. From this it was calculated that there is a high probability that between 8,000 to 15,000 individual cell clones in a mouse are capable of forming anti-NIP immunoglobulins. Quattrochi *et al.* (*10*) examined the two-dimensional tryptic peptide pattern obtained by the digestion of a hundred myeloma protein light chains. They looked for identical light chains from different myeloma proteins, but were unable to find any. Using similar statistical arguments, they concluded that a minimum of 1000 different light chains must exist to account for their results. All these studies may be summarized by stating that, if there is only one antihapten specificity per antibody molecule (or per cell), there do not appear to be enough lymphoid cells to account for the number of antigenic specificities. Reciprocally, there appears to be a very large number of clones involved in the production of antibodies against a single haptenic determinant. Such results gave rise to the suspicion that antibody combining regions with shared specificity for structurally diverse haptens could exist.

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We have called these "polyfunctional" antibody combining regions.

A second approach has been to look at the immune response to complex antigens such as polysaccharides and polypeptides. These studies show that interaction between antigens and the antibody combining region must extend over a substantial distance in space (*11*). This work is based on the principle that, if one can identify first one determinant P on a complex antigen which contributes to the interaction energy with the immunoglobulin and then identify a second such determinant Q which also interacts, and if the distance between P and Q on the antigen is known, it is possible to draw conclusions about the distance between the complementary contact amino acid residues in the immunoglobulin combining region. There have been many such studies (reviewed *11, 12*; see also *13*), and all of them indicate that binding can take place over distances of the order of 20 to 30 Å. This result by itself is not evidence for a polyfunctional combining region, but it does suggest that for the separate determinants P and Q (provided that they are not part of a larger antigen molecule) the antibody combining region is polyfunctional. Interpretation of some of these studies has been difficult. Many were carried out on heterogeneous antibody populations where it was difficult to distinguish between multiple binding to single immunoglobulins and binding to multiple immunoglobulins.

A third approach is inferential and has been explored by Glazer (*14*). He pointed out that the active site of many enzymes is capable of binding dyes and other organic molecules quite unrelated in structure to the substrate of the enzyme. He suggested that there was perhaps some structural or functional feature in these regions which made it possible for ligands to bind to these rather than to other regions of the protein molecule. Glazer further suggested that immunoglobulin binding regions might show an essentially similar pattern. Taken together, the studies cited so far suggest that each immunoglobulin may have more than a single specificity. A further consequence of the presence of multiple binding sites is the expectation that some immune serums will be found which are capable of binding more than 2 moles of a small hapten per mole antibody. It is interesting that there has been a recent report that

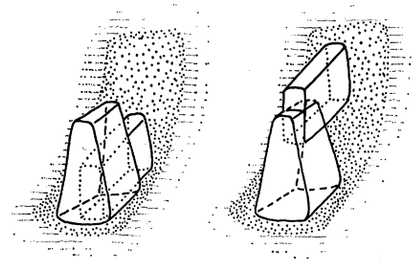


Fig. 1. Diagram showing that two proteins may occupy overlapping attachment sites (left) or have completely distinct contact amino acid residues and still overlap (right).

certain chicken serums bind between 2.7 to 4.1 moles of N^{ϵ}-2,4-dinitrophenyllysine (ϵ-DNP-lys) per mole of antibody to the immunoglobulin Fab fragment (*15*). This suggests that there may be combining regions capable of binding more than one DNP group to a single region.

Multiple Binding Functions of Homogeneous Immunoglobulins

For the last 7 years, however, homogeneous immunoglobulins derived from myeloma tumors have been available. Some of these can be shown to bind haptens (*16*). An early finding with such myeloma proteins was that a single protein bound a number of structurally diverse antigens (*16–19*). Since, however, in each instance the ligands compete with each other for attachment to the protein, it was supposed that such ligands must have common structural features such as, for instance, the nearly similar distances between oxygen atoms on one such pair of haptens, DNP and menadione, which might account for the binding of both molecules to the same site.

The first direct evidence for polyfunctional combining regions was obtained when we investigated protein 460, a mouse immunoglobulin A (IgA) myeloma protein which binds the haptens ϵ-DNP-lys and 2-methyl-1,4-naphthoquinone (menadione) with affinity constants (K_0) of 1×10^5 liters per mole and 2×10^4 liters per mole, respectively. In the course of these studies we found that there was a "hidden" sulfhydryl group whose properties affected the hapten binding of protein 460. Although this -SH group was alkylated only very slowly and incompletely by iodoacetamide and iodoacetic acid, rapid and complete reaction occurred when a sulfhydryl reagent based on the DNP group (which is known to gain access to the combining region) was used. This reagent, 5,5′-dithiobis(2,4-dinitrophenyl) forms a mixed disulfide bond with the protein and does not subsequently occupy the DNP combining site. The evidence for this is that, after formation of the mixed disulfide bond, the binding of ϵ-DNP-lys to protein 460 is unimpaired. However, protein 460 modified in this fashion no longer binds menadione with its original affinity. Combining sites for these two molecules are spatially separated on this immunoglobulin. A further search revealed two additional methods for inactivating differentially one of the two ligating functions of protein 460. When denatured with 4.3 molar guanidine hydrochloride and subsequently allowed to refold partially, the affinity for DNP was greatly reduced; but menadione binding remained intact. Treatment of protein 460 with 2 percent dimethyl sulfoxide had the opposite effect; it reduced the affinity for menadione while leaving DNP binding intact (*19*).

These results reinforced, but did not prove, the concept that the location of the DNP binding site and the menadione binding site were spatially separated. The question concerning the distance between the two sites was still unsolved. One can visualize the possibility that menadione and DNP bind to two closely overlapping sites, that perhaps one or two amino acid residues make contact only with DNP, that one or two residues may make contact with menadione, and that a number of contact amino acid residues may make contact with both ligands. Alternatively, the binding sites could be separated by some distance and could involve completely different sets of contact amino acids. Nevertheless, the volumes occupied by both haptens could still overlap, resulting in competitive hapten binding. These concepts are illustrated in Fig. 1.

One can distinguish between these two models by actually measuring the distance between the two binding sites. The best way to measure such distances is to make immunoglobulin-hapten complexes with each of the two haptens, crystallize them, and determine their three-dimensional structure by x-ray crystallography. Unfortunately, only a few immunoglobulins (or their fragments) have given crystals that are good enough for high resolution x-ray

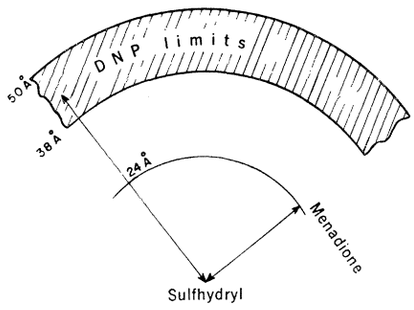


Fig. 2. The distance between the menadione and the DNP binding sites. The inner sphere segment represents the distance from the modified -SH group to the menadione binding site. The segment of the outer spherical shell represents the limits of the combining site distance of -SH to DNP.

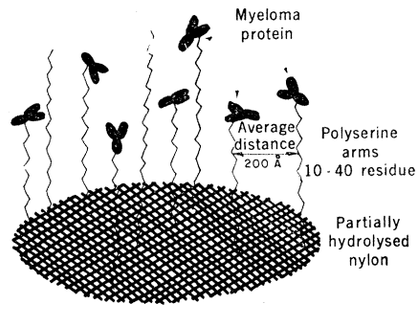


Fig. 3. Polyserine "whisker" discs used in the screening of immunoglobulins for hapten binding. The amino groups of partially hydrolyzed nylon are used as initiators for the condensation of serine *N*-carboxy anhydride to polyserine. Immunoglobulin molecules are attached to the polyserine chains with glutaraldehyde.

crystallography. None of these had been shown to bind more than one hapten. We therefore decided on a twofold strategy. We would measure by physical methods, other than crystallography, the distance between the combining sites and we would screen combining region (Fab) fragments which do give good crystals for their ability to bind structurally diverse haptens.

Distance between Subsites

One established method for estimating distances of the order found within protein molecules, is to measure the radiationless transfer of energy from a fluorescent donor molecule excited by a nanosecond pulse of light to an acceptor molecule. Such an excited probe gives off fluorescent light which is capable of being absorbed by the acceptor probe. The efficiency of energy transfer (E) is related to the donor-acceptor distance, r, by the relationship:

$$E = r^{-6}/(r^{-6} + R_0^{-6})$$

where R_0 is the distance at which E is 50 percent. It is possible to measure energy transfer by nanosecond fluorimetry and to calculate the distance r (*20*). For us, this elegant technique had one major difficulty. Menadione and DNP compete for binding to the protein; hence, it is possible to bind only one of these ligands at a time. This makes direct energy transfer experiments impossible. We solved this problem by attaching a donor probe to a third reference point and then measuring successively the distance from donor to DNP and donor to menadione (Fig. 2). The unique -SH group associated with the combining region of protein 460 after substitution with a suitable donor probe, served as a third reference point. We used two different dyes as energy donors *N*-iodoacetyl-*N'*-(5-sulfonaphthyl) ethylenediamine and *N*-(5-dimethylamino-1-naphthalenesulfohydrazinyl)-methylmaleimide. For acceptor probes we used both DNP and menadione as well as fluorescein conjugated ε-DNP-lys. Measurement of distances between donor and acceptor probes is subject to the limitation that they have no directional vectors and are thus expressed as spheres centering on the donor probe. One sphere is obtained for each -SH donor-hapten acceptor distance. Therefore, it is possible to measure the minimum distance that separates any two points located on the surface of these two spheres (Fig. 2). This minimum distance between the DNP and menadione binding sites proved to be approximately 14 Å, indicating clearly that the overlapping site model is incorrect in this instance; that there is substantial spatial separation between the sites and that the two ligands, DNP and menadione, presumably have different sets of contact amino acids (*21*).

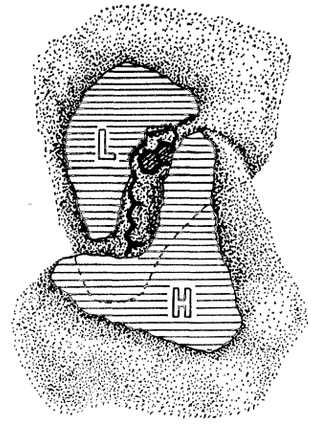


Fig. 4. Scheme of the combining region of protein New, looking into the long axis of the Fab fragment. The areas labeled L and H are occupied by the light chain and heavy chain, respectively. Between lies a depression approximately 5 to 6 Å deep in which the γ-hydroxy vitamin K_1 molecule is located.

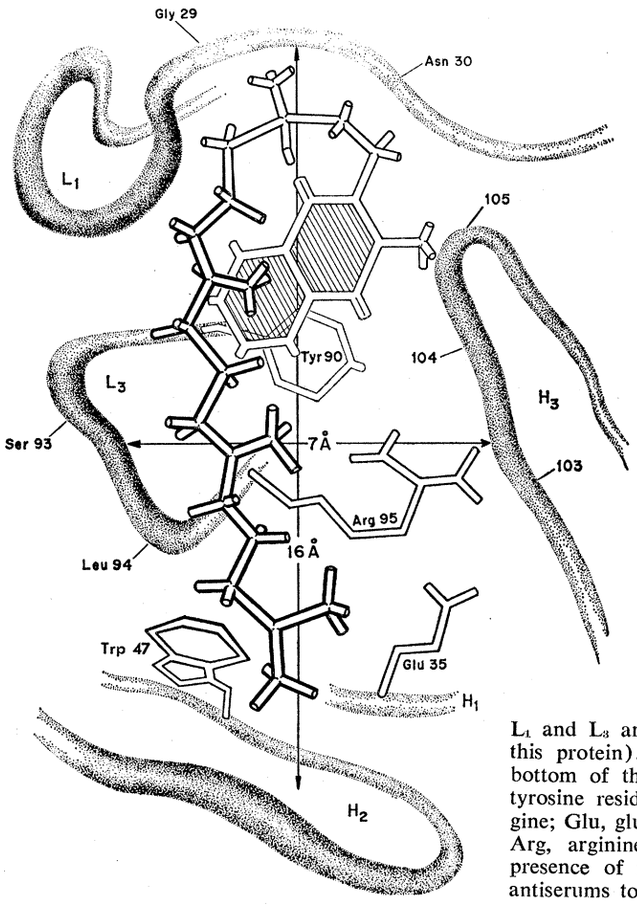
Three-Dimensional Structure of Immunoglobulin Combining Regions

While this work was in progress collaboration was begun with Poljak's group at Johns Hopkins Medical School (*22*). This group had completed the 2.8-Å structure of the Fab fragment of protein New. Protein New was not known to bind any antigen, and since we wished to look at the structure of the combining region at atomic resolution we needed to find a hapten. Therefore, we tested protein New for ligand binding activity, using a rapid screening technique (*23*). In this method, polyserine chains are attached to small, activated, nylon discs and protein New is coupled with glutaraldehyde to the polyserine chains fixed to the nylon (Fig. 3). Protein New attached to these "polyserine whisker discs" was incubated with one or another of several radioactive compounds, and then washed with buffer for 1 to 2 seconds. The retained radioactivity was then counted. Protein New bound uridine, and the affinity constant K_0 as measured by equilibrium dialysis was approximately 1×10^3 liter/mole. In the second step of the assay more than 300 nonradioactive compounds were tested for their ability to displace uridine. This assay has so far detected two structually diverse ligands which bind, with K_0 in excess of 1×10^5 liter/mole.

A crystalline complex has now been obtained of New Fab fragment and the first of these ligands, a γ-hydroxy derivative of vitamin K_1. Difference Fourier diagrams at a resolution of 3.5 Å have been constructed and atomic models of the complex have been built (*24*). These complexes show for the first time the detailed structure of at least one part of the antibody combining region. The vitamin K_1 derivative is located in a shallow depression between the light (L) chain and the

heavy (H) chain of the immunoglobulin (Fig. 4). One limb of the L-shaped cleft is surrounded by the "hypervariable" loops from the variable regions of the L and H chains. In the upper portion of this limb of the cleft, deep and at an oblique angle, lie the methylnaphthoquinone rings of vitamin K_1. The phytyl side chain forms a loop forward and upward, which then turns downward, making contact with both the H and L chains (Fig. 5). Approximately 12 amino acid residues appear to be in contact with the ligand. From comparative binding studies, using only the aromatic ring portion of the molecule, we know that the rings provide approximately half of the total binding energy ($K_0 = 1.7 \times 10^5$ liter/mole), while the phytyl side chain provides the remainder. The longest dimension over which binding takes place is approximately 15 Å. Thus far, binding of even one hapten has shown that a considerable portion of the binding cleft (one limb of which measures approximately 15 by 7 by 6 Å) is involved, and that quite separate regions bind the ring portion and the phytyl side chain. A second hapten, carminic acid ($K_0 = 1.4 \times 10^5$ liter/mole), has been found, and difference Fourier maps of this complex are being obtained. Further studies have improved the model of immunoglobulin New and the structure of its Fab′ fragment is now known to resolution of 2.0 Å (*25*).

Recently, Segal *et al.* determined the three-dimensional structure at 3.1-Å resolution of the phosphorylcholine-binding Fab fragment of McPC 603, a mouse IgA myeloma protein (*25a*). This molecule has a deeper cleft, or cavity between the light and heavy chain, approximately 12 Å deep and 20 Å long. Phosphorylcholine is bound asymmetrically to a small part of this cavity onto the heavy chain wall of the cleft. No binding function for the remainder of the cavity has yet been found. Edmundson and his colleagues have determined the structure in three dimensions of a crystalline lambda light chain dimer (*25b*). The cleft between the two lambda chains is believed to be analogous to the one found in Fab fragments. In the dimer, the cleft is even deeper than in the McPC 603 protein and has a cavity beneath the cleft, the floor of which is about 16 to 17 Å from the entrance of the cleft. Dinitrophenyl compounds, ε-dansyllysine, colchicine, 1,10-phenanthroline, methadone, morphine, meperidine-5-acetyluracil, caffeine, theophylline, menadione triacetin, and some related compounds all bind to different sites within the combining region cleft and cavity. Although it is possible that the chains in this model may be farther apart than in an actual Fab fragment, the three-dimensional data in toto suggests the intriguing (if speculative) hypothesis that the size of the combining region and the number of amino acid residues available for ligand interaction may be modulated in different immunoglobulins by the depth to which the cleft between the heavy and light chains is opened.



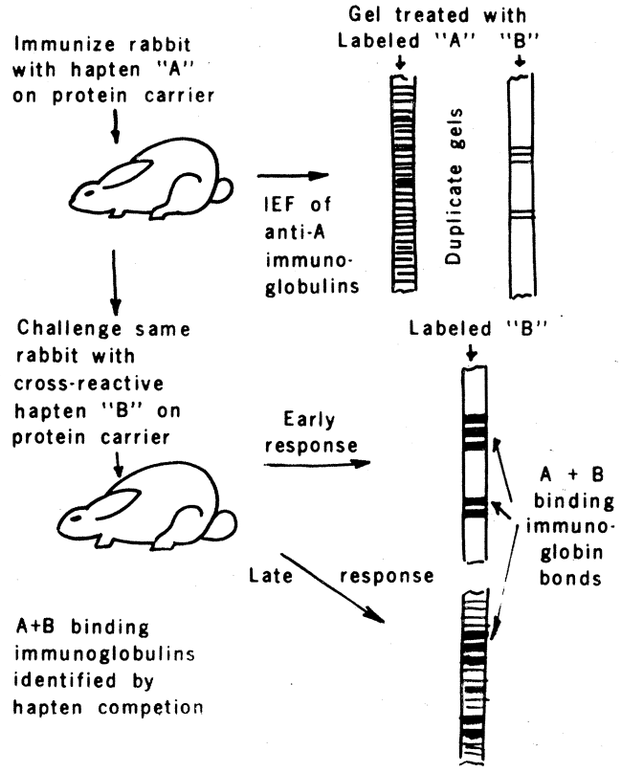


Fig. 5 (left). Drawing of γ-hydroxy vitamin K_1 bound to the combining region of the human myeloma immunoglobulin New. L_1 and L_3 are the first and third light chain variable regions (L_2 is deleted in this protein). H_1, H_2, and H_3 are hypervariable regions of the H chain. The bottom of the shallow groove between the H and L chain consists in part of tyrosine residue 90 and arginine 95 of the L chain. Gly, glycine; Asn, asparagine; Glu, glutamic acid; Trp, tryptophan; Leu, leucine; Ser, serine; Tyr, tyrosine; Arg, arginine. Fig. 6 (right). Experimental protocol for determining the presence of immunoglobulins with polyfunctional combining regions in rabbit antiserums to haptens.

Polyfunctional Combining Regions

One weakness remained in the argument that antibody combining regions are polyfunctional. It could be held that, although hapten binding to multiple subsites occurs, the binding of hapten to only one of these subsites has functional significance. We know that one of the triggering responses for "B" cell replication and antibody production, is the binding of antigen to the cell surface antibody receptors of the B cell. Such cell surface antibodies are known to have the same ligand binding specificity as the antibodies secreted by the cell (*26*). The following question can then be asked: When two dissimilar haptens bind to a single cell surface receptor immunoglobulin, are both these haptens capable of inducing production of the immunoglobulin which binds both haptens?

We tested this question in the following way (see Fig. 6). A rabbit was injected with hapten A on a protein carrier. From the immune serum we isolated the antibodies to hapten A (anti-A) and subjected them to IEF on polyacrylamide gels. We identified the anti-A bands by treating the gel with a radioiodinated derivative of hapten A, and then washed and radioautographed the gel. A duplicate IEF gel was treated with another structurally unrelated radioiodinated hapten B. A few bands that bound both A and B were found. Mutual inhibition tests were then carried out with nonradioactive A and B to see if competition, characteristic of binding to a single protein in the IEF band, could be demonstrated. Hapten pairs, A and B, if chosen at random will seldom bind to single IEF bands. The reasons for this are discussed below. We used hapten pairs that are known to give "anomalous cross-reactions" in serums. It is known that some anti-DNP serums have the ability to bind menadione, and in this they resemble protein 460, the mouse myeloma protein that binds DNP and menadione. Other examples of such anomalous cross-reactions in immune serums have been described (*27*). In our experiments, antigen B was one of five such haptens or antigens (menadione, uridine, ribonuclease, inosine, vitamin K_1) while A was ε-DNP-lys. A rabbit immunized with DNP-BGG may show perhaps 50 to 100 anti-DNP bands on IEF. Between 2 to 10 of these bands will also bind hapten B; the exact number depends on the nature of the second antigen B. These bands represent the immunoglobulin products of between one and six clones of cells that are capable of being stimulated by both antigens A and B (*28*). When the affinity constant of those bands that bind both A and B is examined, as might be expected, the affinity of the immunoglobulins in the bands for hapten A is nearly always higher than that for hapten B since these double-binding immunoglobulins were selected by hapten A. When the rabbit is subsequently challenged by hapten B, we noticed that the first immune response is almost entirely that of cells producing an antibody that binds both A and B. The IEF bands corresponding to such products may increase 80-fold in density, and no IEF bands binding A alone showed similar increases. Later on, after hapten B challenge, more anti-B bands appear, and these do not cross-react with hapten A. They also have a higher K_0 for hapten B than for the binding bands A and B. Eventually a complex anti-B response appears, consisting principally of anti-B bands with no anti-A activity (but presumably cross-reacting with other determinants). The A plus B binding bands which were present early are still present late in the immune response, but form a progressively smaller proportion of the total antibodies formed against hapten B (*29*).

This experiment tells us that in ordinary elicited antibody populations, immunoglobulin molecules exist which are capable of binding at least two, but probably several, structurally dissimilar haptens. When they do so, each of the two haptens can induce production of the immunoglobulin which binds them both, if the interaction energy is sufficiently high to trigger the response. Control experiments have ruled out the possibility that the carrier is responsible for the induction, and we must conclude that antibodies with "polyfunctional" binding regions do in fact exist. How frequently might we expect to find such polyfunctional antibodies in order to make them physiologically important? There are about 6 million organic ring compounds that have been cataloged (*30*). Not all of these are either antigenic or immunologically distinguishable. A conservative estimate would put the number of potential haptens at perhaps 2 million. Let us make a guess and say that one antibody molecule can bind 100 different haptens with reasonable interaction energy (1×10^4 liter/mole or higher). The chance that a single immunoglobulin will bind two randomly chosen haptens is then $(2 \times 10^6)/10^2$ or one chance in 20,000. Since we observe in one serum perhaps 100 antihapten antibodies resolved by IEF, the odds are still 200:1—even if all antibodies bind as many as 10^2 compounds each—of finding serums in which a single band binds a second randomly selected hapten. More recent screening work (*23*) suggests to us that in practice these odds are in the right range.

Although we had shown that in a myeloma immunoglobulin binding DNP and menadione and there were separate binding sites for these haptens, we had no evidence that the "natural" double-binding immunoglobulin species resembled the double-binding myeloma protein. We therefore prepared anti-idiotypic antibodies to two separate double-binding myeloma proteins, namely the mouse myeloma protein 460 (binding DNP and menadione) and the human IgG myeloma protein New (binding vitamin K_1 and carminic acid). Antibodies to idiotypic determinants are known to react with determinants at or very near the binding region of an immunoglobulin (*31*). We were able to show that these antibodies precipitated specifically those induced immunoglobulins in rabbits which bound with similar affinities the same two haptens that were bound to the myeloma, against which the serum had been prepared (*32*). This suggests that the combining region in the myeloma proteins may closely resemble that of their "naturally occurring" counterparts, the multiple-binding antibodies, because they share not only idiotypic determinants at the binding region, but also have similar binding constants for two dissimilar haptens.

In summary, the direct evidence we have been able to obtain in support of the existence of "polyfunctional" combining regions in antibodies is: (i) that in a double-binding myeloma protein (protein 460), the two sites which bind menadione and DNP may be separately inactivated by chemical means, (ii) that in protein 460 the menadione and DNP binding sites are separated, (iii) that antiserums to haptens contain individual immunoglobulins which bind at least two structurally diverse haptens and that the production of such immunoglobulin species is induced by immunization with both haptens, and (iv) that reaction with antiserums to idiotypic determinants

and measurements of hapten affinities (*33*) suggests that the combining regions of double-binding myeloma proteins and their "naturally occurring" antibody counterparts resemble each other (*34*).

Genetic Implications

Taken together with the indirect data that suggested the existence of multiple-binding or polyfunctional antibody combining regions, there is now considerable evidence that these are biologically important. We therefore have to see how well their existence fits the known facts about the high specificity of immune serums. The inference stated at the beginning of this paper should now be reexamined. We stated that if a serum is specific, so must be the individual immunoglobulins which constitute that serum. In 1959 Talmage (*35*), in a theoretical paper, postulated that this inference need not necessarily be true. He proposed that an individual immunoglobulin need have only a partial fit for an antigen. Thus, a number of antibodies, each having a partial fit, would produce an antiserum having a high degree of specificity. We now know that in myeloma proteins, individual hapten combining sites have in fact a high degree of specificity and will, for instance, distinguish DNP from mononitrophenols and trinitrophenols (*8*). At the same time protein 460 will bind a number of unrelated haptens at other sites within the combining region. The consequences of this, however, are exactly as Talmage first suggested. Let us suppose again that a single immunoglobulin may bind 100 determinants. If an animal is immunized with determinant A, all those cells producing A binding immunoglobulins of a sufficient affinity will respond to the antigenic stimulus by cell division and antibody production. Thus, all antibody species produced bind A. Each antibody in addition will also bind 99 other determinants, but these *need not be the same* for each antibody, and such ligating activity will be present only at a lower level (that is, 1 percent) in the antibody population, and will be diluted out. This concept is illustrated in Fig. 7. Thus, highly specific serums are compatible with component immunoglobulins, each having multiple specifications. Inman (*36*) has subjected this model to combinatorial analysis. He asked the question: How many different pairs of H plus L chain variable (V) regions would be needed to make antibodies to a large number of (2×10^6 or more) determinants? He concluded that 1×10^4 to 1×10^6 $V_H \times V_L$ region pairs would be complementary to an almost infinite number of antigenic determinants. Given free L × H permutation (this is probably not completely true), as few as 10^2 to 10^3 V genes for the H variable region and the same number for the L chain V region would suffice to produce such a number of pairs. This type of calculation brings into sharp focus the old controversy as to whether multiple inherited V region genes are responsible for the full extent of antibody diversity, or whether relatively fewer genes are inherited and that a somatic noninherited gene diversification process is responsible for expanding a small number of genes to a larger number during development of immune responsiveness. Gally and Edelman (*4*), protagonists of the somatic variation hypothesis, suggested that 1 to 10 genes for each V region subgroup are inherited. Since there are many subgroups (*37*), this number seems to approach the lower limit of Inman's calculation. Thus, polyfunctional combining regions may provide for all the specificities observed, and it may not be necessary to invoke novel mechanisms to account for the extent of antibody diversity (*38*).

Biological Implications

There would be clear epidemiological advantages to an animal if a single antibody had specificity to more than one antigenic determinant. Thus, a single antigenic determinant may ensure survival of antibodies complementary to a large number of unrelated antigenic determinants even if these determinants are no longed represented in the environment. Thus, a "memory" for a specific antibody to a pathogen may be related by the presence of a quite different infective agent, having no common determinants. This type of "linked" immune response was first recorded many years ago by Weil and Felix, who observed during World War I that many of the soldiers under their care who had previously been exposed to typhoid fever (*Salmonella typhi*) showed a highly specific increase in antibodies to *S. typhi* when they contracted an infection with *Rickettsia prowazekii*, an entirely unrelated organism having no common antigenic determinants (*39*). There is a close relation between this phenomenon and "original antigenic sin" (*40*), the power of a "related" antigen to induce production of antibodies to an antigen given a long time ago. This phenomenon may occur both when there are truly "common determinants" and when there are "linked specificities." An in-

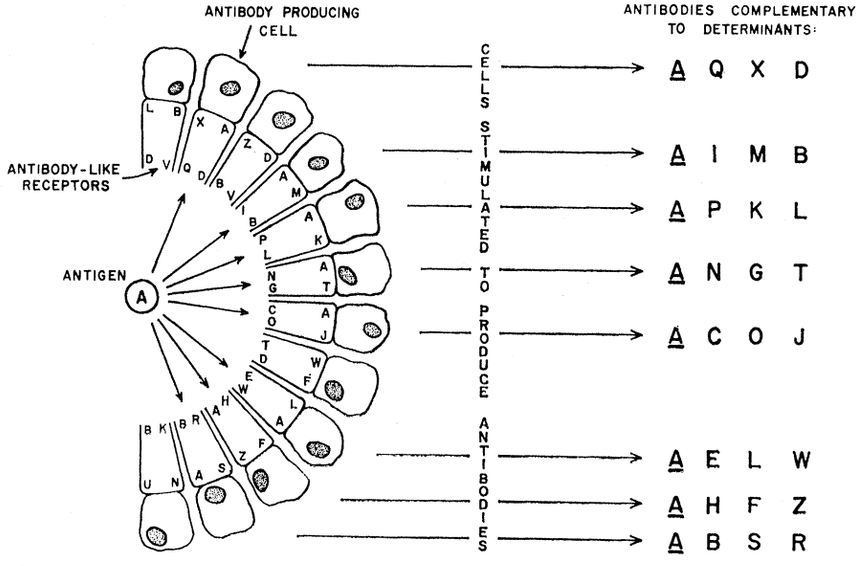


Fig. 7. Immune serum specificity as a population phenomenon. Individual B cell receptors are shown as having properties similar to immunoglobulin combining regions. For illustrative purposes, these are drawn as being each complementary to four different antigens; we suppose that this number is in fact much larger. Stimulation by antigen A causes the cells with A specificity to divide and produce antibodies directed against A. The immune serum produced will therefore react in high titer with antigen A. Each immunoglobulin also has other specificities, but because these need not be the same in every molecule, the other specificities, B to Z, will be diluted out and will react only in low titer.

teresting example in which homogeneous rabbit antibodies against streptococcal variant vaccines were used to produce effects rather like the hapten A plus B phenomenon discussed above, has been reported (*41*). Perhaps antigenic original sin, like human original sin, is a device to ensure the survival of the species.

Antibody Maturation

These experiments also suggest that "polyfunctional" combining regions may underlie the phenomenon of antibody maturation. Early in the immune response antibodies to a hapten have a relatively lower average affinity for the hapten than those later in the immune response (*42*). There is evidence that the increase in antibodies of higher average affinity is an active process of recruitment of new antibodies (*43*) and is not due to selective removal of either high or low affinity antibodies at various stages of the response. Siskind and Benacerraf (*44*) have suggested that this response is due to the fact that early in the immune response when antigen levels are high, effective stimulation of cells producing both low and high affinity antibodies will occur. Later in the immune response, when the antigen levels are low, only those cells producing high affinity antibodies will be stimulated, and there will be increased production of high affinity antibodies (*45–48*). Segre and Segre (*48*) tried to obtain direct confirmation in tissue culture for the role of antigen concentration as a driving force in this process, but were not able to do so. It must be cautioned, however, that added antigen per se may not reflect the actual concentration of antigen at the site of antibody production. Since combination of antigen with cell surface receptor is a reversible reaction, there can be no doubt that the concentration of antigen close to the receptor must affect the immune response, and the effect observed by Eisen and Siskind on the affinity of antibodies induced by various doses of immunizing antigen (*42*). Whether or not antigen concentration is important for maturation, the following facts must also be considered. When an animal is sensitized to antigen A, a small proportion of the antibodies produced will also bind an unrelated antigen B. Since the antibodies produced were "selected" by antigen A, those immunoglobulins which also bind B will do so on the average with low affinity. When primed with antigen A and then challenged with antigen B, the early anti-B antibodies produced after challenge will be predominantly of the A plus B binding type which have low average affinity for B. Only later will anti-B antibodies be produced which were "selected" by antigen B and have a higher average binding for this antigen. Hence, the antibodies to antigen B will exhibit an increase in the average binding constant with time—the phenomenon called antibody maturation. The primary challenge with antigen A would have its natural counterpart in the fact that experimental animals are not immunologically virgin and have been subjected to immunological priming by natural pathogens and perhaps also by "self" antigens. This type of mechanism could work synergistically with an antigen concentration-driven mechanism needed to produce maturation of antibody affinities.

In summary, we have discussed the experimental findings which support the idea that antibody combining regions contain subsites at which structurally diverse antigenic determinants bind. This concept provides a simple, unifying explanation for a number of seemingly unrelated immunological phenomena.

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Prehistoric Agriculture in Tropical Highlands

Settlement patterns in western Panamá reflect variations in subsistence adaptations to the tropics.

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Studies of the origin and dispersal of New World food plants have involved comparisons between two ancient and successful agricultural systems. Vegeculture, or the cultivation of starchy tubers and rhizomes, also known as root crops (manioc, yams, sweet potatoes, and so forth), may have been established in the northern South American tropics by the third millennium B.C. (*1*). Seed culture, or the selection and propagation of seed-bearing plants (maize, beans, or cucurbits), had its beginnings in Mesoamerica and possibly in parts of Andean South America in at least the sixth millennium B.C. (*2*). Recent archeological evidence suggests several centers of origin, in different periods and widely separated areas, for the most important cultivars (*3*).

The problem of agricultural dispersals is as complex as that of plant origins. Nonetheless, more serious thought has been given to the plant domestication processes, and to the evolution of subsistence systems (*4*), than to the factors influencing the adoption, modification, and success of new agricultural products and techniques. In fact the two processes, domestication and dispersal, are often equated. Yet it is important to remember that very different human selection pressures may be involved in these processes. We are reminded of this by Harris's argument (*5*) that ecosystem manipulation may have been involved in vegecultural origins, while ecosystem breakdown may have encouraged the replacement of vegeculture by seed culture by 500 B.C., in parts of northern South American tropics (*6*).

In this article we explore the introduction of seed culture into a small and somewhat marginal tropical area, where environmental conditions were in some ways favorable, in other ways rigorous. Our concern with settlement subsistence adaptations in highland Panamá may appear parochial, but it is deliberate. An understanding of complex interactions between environmental and subsistence factors in the past can only be achieved, slowly and patiently, by many case studies conducted in diverse but small and manageable areas.

This article deals with archeological developments in the upper drainage of the Río Chiriquí Viejo, in Volcán Barú, at elevations between 1200 and 2300 meters. In spite of their fertility, Central American tropical montane valleys above 1000 m were probably too cold, humid, and forested to have served as early centers of manioc or maize domestication. Apparently these valleys were also outside the aboriginal range of cold-adapted root crops such as potatoes. These factors, plus the scarcity of fish in these incident rivers and the distance from marine faunal resources, were responsible for the persistence of a hunting-gathering population until a well-developed seed complex, based on protein-rich varieties of maize and beans, facilitated human occupation by agriculturalists. The paucity of archeological sites dated before 1000 B.C. in a number of wet and cold tropical highland habitats of lower Central America may be due to the marginality of these environments for initial agricultural developments. Conversely, the explosive success of seed culture here may be related to its late introduction into lightly populated or altogether empty areas, where it met little resistance from other forms of established cultivation.

In the rich but circumscribed volcanic valleys of the Continental Divide on the Pacific side of western Panamá (Fig. 1), the spread of maize-growing peoples, probably from the adjacent lowlands and middle-altitude elevations, initiated processes leading to rank differences, social fission, warfare, and competition within and among villages. Despite the readiness of some archeologists to attribute most Central American sociopolitical developments to influences from Mesoamerica (*7*), we believe that such processes are best understood as local, although by no means uncommon or irreversible, responses to a competitive situation where ecological restrictions were being eliminated by new subsistence opportunities.

Natural Zones of the Barú Region

Volcán Barú, in the Province of Chiriquí, is the highest of several peaks in the Panamanian portion of the Talamancan Range, which extends northwest into Costa Rica and beyond.

Major events during the Pleistocene

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