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CURRENT PROBLEMS IN RESEARCH

Immunological Specificity

Unique combinations of selected natural globulins provide an alternative to the classical concept.

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The elucidation at a molecular level of the nature of specific biological interactions constitutes one of the most important and challenging problems of biology today. Specificity was defined by Landsteiner (*1*, p. 6) as the "disproportional action of a number of similar agents on a variety of related substrata." Specificity is a property of a wide range of biological agents such as antibodies, antibiotics, toxins, viruses, enzymes, genetic material, and plant agglutinins. Of these, only antibodies have the property that their production by the host can be stimulated by the injection of an almost unlimited range of substances. Furthermore, an antiserum reacts specifically with the antigen which stimulated its production, thereby providing a powerful tool for the detection and identification of biological materials.

It is probable that by the use of appropriate antisera any of the millions of species of plants, animals, and microorganisms can be distinguished from one another. Landsteiner, to whose name and work the subject of immunological specificity is most closely attached, extended the demonstration of the range of this specificity to a wide variety of simple synthetic compounds. It would thus appear that the number of substances which may be distinguished and identified is nearly infinite if it includes all substances which have been and are yet to be synthesized. This is, indeed, distinctive behavior.

In view of the uniqueness of antibody specificity, it is not surprising that immunologists have always considered that the relationship of an antibody to its antigen is something special. The term *anti-egg albumin* which designates an antibody to egg albumin carries with it the concept of special formation along with the concept of specificity. Whereas other types of specific interactions could be attributed to chance structural complementariness between independently fabricated molecules, in the case of antibodies this idea was generally rejected because of the infinitely large number of different molecules thought to be required. Because of these considerations, Landsteiner stated, "there remains hardly any other conclusion than . . . to assume that under the influence of antigens the formation of certain globulins (and perhaps normal antibodies) is modified in such a manner that the resulting globulins are closely adapted to the immunizing substance" (*1*, p. 148). This concept of a special relationship between antigen and antibody has been a cornerstone of immunological thinking for half a century. Widely accepted before Landsteiner's important work, it has not been seriously challenged since.

The classical line of reasoning which leads to the conclusion quoted above may be divided into the following steps.

1) In many vertebrates, the response to the injection of any one of a very large number of substances is the formation of antibodies capable of identifying the substance injected and thus distinguishing it from all other substances.

2) The antibodies so formed must be different and unique for each of the many substances to which a distinctive response can be made.

3) An animal could not possess of itself the information necessary to synthesize all of the different types of antibodies it is capable of forming in response to the varied environmental stimuli.

4) Antibodies must represent a unique modification in the synthesis of natural protein for which information is supplied by the antigen injected.

Weaknesses

While it is understandable that this line of reasoning seemed inescapable only 20 years ago, several weaknesses have developed in the argument as the result of the progress of knowledge in immunology and related fields of biology.

An increasing awareness of the diversity of antibodies in a single antiserum has emphasized the distinction between an antiserum and the antibodies it contains (for reviews, see *2–4*). Although it is necessary that a different antiserum be formed for each antigen that may be distinguished, it is possible that the large number of different antisera contain a much smaller number of different antibodies in different combinations. For example, a system consisting of only five different antibodies, A, B, C, D, and E, could distinguish nine antigens which reacted with three of the five in different combinations: ABC, ABD, ABE, ACD, ACE, BCD, BCE, BDE, and CDE. The distinguishing ability of antisera is similar to that present in this simplified system, for the injected antigen is distinguished largely by the fact that it reacts with the maximum number of anti-

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bodies. The concept that a different antibody is made for each antigen is inherent in the conclusion of step 4 in the classical line of reasoning given above. Its insertion in step 2 is an assumption which makes the final conclusion almost inevitable.

An almost unlimited number of different antibodies is not only unnecessary to explain immunological specificity, but may be an impossible assumption. The finding that the combining site on the antibody molecule is relatively small (*5*) has raised the question of whether it is possible to construct a unique antibody molecule for each of the almost infinite number of antigens. This question is particularly relevant if it is considered that not one but a whole family of different antibody molecules with different combining constants is made in response to a single antigenic determinant, and that all of these molecules appear nearly identical to the other gamma globulins of that animal in physicochemical properties, in amino acid composition and sequence, and in antigenic properties.

Another problem which has raised doubts concerning the uniqueness of antibodies is the difficulty in defining the term. Antibodies are so diverse in their physical properties and in their reactions with antigens and blend so gradually into obviously nonantibody substances that any definition is necessarily arbitrary (*6*).

While the preceding considerations cast doubt on the uniqueness of the relationship between an antibody and its antigen, other developments have brought into question the concept that antibodies are necessarily modifications of a normal protein. The analogy of induced enzyme synthesis in bacteria has demonstrated that a substance may appear where nothing was previously detectable without necessarily implying the production of a new substance (*7*). The induction by environmental stimuli of an increased production of naturally occurring animal proteins such as properdin (*8*) or ferritin (*9*) has indicated the existence of induced protein synthesis in animals. The relatively large number of genetically determined enzymes which can be synthesized by a bacterium has increased the estimates of the number of different natural proteins which can be synthesized by a vertebrate. It has been estimated that there is enough deoxyribonucleic acid (DNA) in a single human cell to encode 1000 large textbooks (*10*).

Perhaps the most compelling reasons for questioning the concept that antibodies are globulins modified by antigens are the recent observations relative to immunological tolerance—that is, the finding that except under unusual circumstances an adult animal does not synthesize antibodies to his own substances which are antigenic to other individuals. The mechanism of this obviously advantageous characteristic is not known, nor it is known to what extent, if any, this is due to restrictions imposed by heredity. That it is at least partially acquired during development might be deduced from the fact that with the exception of highly inbred animals, an individual can make antibodies against the antigens of both his parents. The most striking experimental evidence that immunological tolerance can be acquired is its establishment by the injection of foreign antigens during an immunologically unresponsive period—for example, during the fetal or immediate newborn periods (*11*), or after whole body x-radiation (*12*).

If after the initial injection into a newborn animal the concentration of antigen is maintained by repeated injections or by use of a viable, replicating cellular antigen, tolerance may last indefinitely. If the concentration of antigen falls below a critical level for a short period, subsequent injection of the same antigen may lead to antibody production (*13*). Unlike the accelerated secondary antibody response to an antigen, tolerance to an antigen in the absence of that antigen is short-lived. Probably related to immune tolerance are recent observations which indicate that antibody-producing cells are highly specialized. In a study of 456 single lymph node cells from a rabbit immunized to two different salmonellae, Nossal and Lederberg (*14*) found 33 cells that synthesized immobilizing antibody to one organism, 29 cells that synthesized antibody to the other, and no cells that synthesized antibody to both. This confirms a similar conclusion that Coons (*15*) drew from studies of doubly immunized animals with fluorescein-labeled antibody markers.

It is impossible to conclude from both of these experiments that cells do not make more than one type of protein, but only that the number of types any cell can make is greatly restricted compared with the capacity of the organism as a whole. In this sense they are highly specialized. There is a strong implication in other work that the immediate precursors of antibody-producing cells are also highly specialized. When cells from immunized animals are transferred to normal, tolerant, or x-radiated recipients, a prompt anamnestic response is obtained from an injection of the original antigen (*16*), but not from an injection of another antigen. The difference between the tolerant and sensitized animals would seem to lie in the possession by the latter of a large number of differentiated precursors of antibody-producing cells. Therefore, in some way the process of immune tolerance must be related to the process of cell differentiation and replication.

Role of Antigen in Cell Differentiation

The key issue relevant to this discussion is the role of antigen in the differentiation of antibody-forming cells. Although antigen-induced differentiation might seem an obvious analogy to substrate-induced permease synthesis in bacteria (*17*), differences arise because specific capabilities in the latter phenomenon are strictly inherited and are not associated with anything analogous to acquired immune tolerance. In order to explain acquired immune tolerance in animals, it is necessary to postulate a recognition system capable of distinguishing between the antigen and the tolerated substance (*18*). If one chooses the hypothesis that the antigen induces differentiation, it is necessary to postulate that a system for recognizing tolerated antigens resides in the undifferentiated cell. Furthermore, the recognition system must be complete in every cell for every autogenous substance and must have at least three additional properties: (i) a specificity equal to that of antibodies, (ii) an inability to be overloaded or competitively inhibited by an excess of "self," and (iii) a flexibility sufficient to add and subtract elements for the recognition of foreign substances depending on the latter's presence or absence in the cell's environment.

The alternative to antigen-induced differentiation is a progressive differentiation which is either spontaneous or induced by inherited mechanisms similar to those which cause other forms of differentiation in multicellular organisms. This inherently requires that antibodies be considered naturally occurring proteins. The advantage of this hypothesis is that it permits the control of dif-

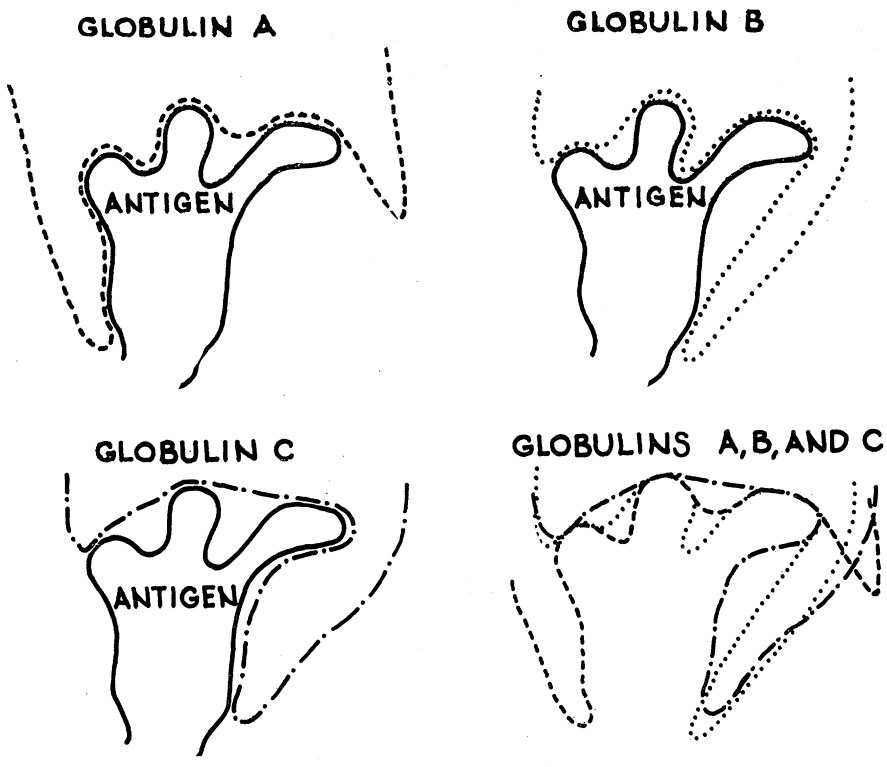

Fig. 1. Two-dimensional diagram illustrating the concept that the information and net specificity possessed by a combination of three different globulin molecules is greater than that possessed by one.

ferentiation, which must be present in any case, to substitute for the complex recognition system required by the first hypothesis. For if the combination of antigen with the first antibody formed in the early stages of differentiation has the effect of inhibiting this differentiation, the specificity and other characteristics of immune tolerance can be explained without postulating a second recognition system. As suggested by Burnet (*19*) and by Lederberg (*20*), the inhibition of partially differentiated cells might be considered a specialized example of hypersensitivity which results in selective destruction of these cells. The same result would be obtained if the "hypersensitivity" was a stimulus to another channel of differentiation. An analogy to the latter phenomenon is the inhibition of antigen development in paramecia grown in the presence of specific antisera (*21*). The action of antigen in suppressing differentiation would explain the requirement of its continued presence to maintain acquired immune tolerance. The persistence of antigen would be required if differentiation of antibody-producing cells was initiated approximately at birth and was continued throughout the life of the animal.

The concept of autogenously controlled differentiation of antibody-producing cells provides a highly satisfactory explanation for the large increase in mitoses observed in lymphatic tissue following an antigenic stimulus. If cell differentiation occurs without antigen, each individual cell type will account initially for a small fraction of the total cell population. Only by a replication of selected differentiated cell types can the capacity to produce the corresponding antibody be increased. Replication without prior differentiation is not adaptive. Replication of a cell differentiated by antigen is an unnecessary adaptive effort. The only alternative to replication of predifferentiated cells would seem to be the concept that replication is a necessary part of antigen induced differentiation.

One of the most important problems in experimental immunology today is the design of experiments to distinguish between the alternatives described above —that is, to determine the role of antigen in cell differentiation. An extension of the work of Nossal and Lederberg to two or more antigenic determinants on the same antigen or to two different antibody molecules to the same determinant would yield important information. An alternative approach is the study of immune tolerance to cross-reacting antigens and of the selective inhibition of only a fraction of the many different antibodies made to the same antigen. From the latter experiments it might be possible to determine whether acquired tolerance is the placing of a particular determinant within a self-recognition system or the selective suppression of pre-existing molecular types of natural globulin.

The major difference between the two hypotheses is that in the first instance antibody production is considered a unique biological phenomenon for which unique mechanisms may be postulated, and in the second it is considered a highly specialized example of certain general cellular processes. The ability of antibodies to distinguish an almost unlimited number of different antigens has always seemed ample justification for the first view. However, the intrinsic unlikelihood of a truly unique biological process and the difficulties presented in even defining an antibody are justification for an attempt to develop the alternative. Because an understanding of the alternative hypothesis requires an alternative concept of immunological specificity, the remaining portion of this article is an attempt to explain immunological specificity on the basis of a limited number of different naturally occurring globulin molecules.

Unique Combinations of Master Molecules

As a basis for immunological specificity the alternative to a unique antibody for each distinguishable antigen is a combination of naturally occurring globulin molecules which is unique for each antigen. The latter concept is based on the following premises.

1) A relative stable complex between a globulin molecule and some other substance can occur whenever the configurations of the two molecules permit the development of short-range intermolecular forces in excess of some critical amount. These forces have been discussed in detail by Pauling (*22*) and by Pressman (*23*).

2) The formation of a stable complex does not require a perfect fit between two complementary configurations (*23*). The heterogeneity of the antibodies combining with a single haptene and the reactions of the same antibody with differ-

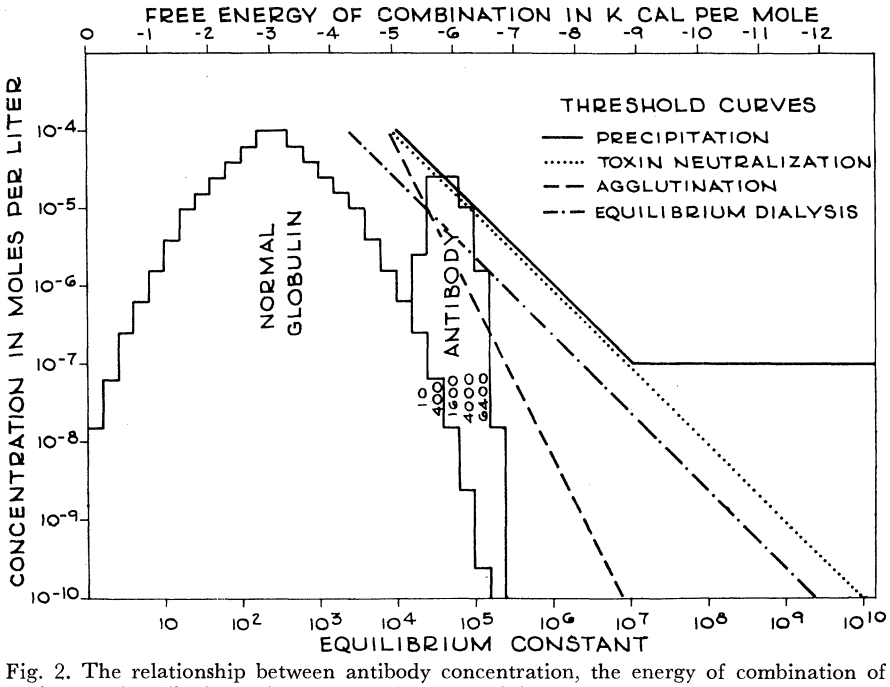

Fig. 2. The relationship between antibody concentration, the energy of combination of antigen and antibody, and the threshold required for detection. The threshold calculations are based on the Goldberg theory and the following assumptions: Agglutination involves a suspension of 10^8 particles per milliliter, each particle containing 6×10^5 sites; precipitation and toxin neutralization involve antigen molecules with a valence of 6 and antibody molecules with a valence of 2, and an optimal ratio of antigen to antibody; equilibrium dialysis requires the binding of at least 20 percent of the haptene in the antiserum compartment.

ent haptenes (*1*) provide experimental evidence for this premise.

3) A single globulin molecule may combine with a large number of different substances in the same sense that a master key may open a large number of different locks. There should be many different antigenic configurations structurally suited to combine with the same globulin if a lack of perfection in the fit in one area may be compensated by an increased binding energy elsewhere.

4) In a mixture of a large number of different globulin molecules, the dominant reactivity will be that common to the largest number of the molecules present. This is because the reactions between antigens and antisera are strongly dependent on concentration and have as well sharp thresholds below which no reaction can be detected.

5) The specificity of an antiserum containing a mixture of different globulin molecules is likely to be very much greater than that of an antiserum in which all the molecules are exactly alike. (See Fig. 1.) This follows from the probability that the number of reactivities common to all of the different molecules will be greatly restricted compared with the number of reactivities possessed by a single molecule.

6) The number of different dominant reactivities and hence the number of antigens which may be distinguished by a system of different globulin molecules is the number of different combinations in which these molecules can be mixed.

On the basis of the premises listed above, the requirements for producing an almost unlimited variety of immune sera are a moderately large number of different natural globulins with overlapping reactivities and a mechanism for selectively increasing the production of those globulins with high affinity for the antigen injected. The total number of different globulin molecules required is determined by three factors: (i) the number a of combinations required to account for the almost unlimited distinguishing ability of immune sera; (ii) the fraction b of all possible antigenic configurations with which the average globulin molecule can combine, which should be approximately equal to the fraction of different globulin molecules with which the average antigen can combine; and (iii) the number c of different globulin molecules in the average monospecific antiserum. Unfortunately, very little is known about these factors except that a is very large. It is implicit in this formulation that b is much larger than $1/a$ and probably lies between 0.001 and 0.01; it is postulated that c lies between 10 and 100. A large number of different types of globulin in an antiserum has the effect of reducing the concentration of each individual type so that the concentration reacting by chance with an unrelated antigen is below the threshold of detection. However, a structurally related antigen might react with a sufficiently high percentage of the different types to exceed the threshold and be detected in a cross-reaction.

If each globulin is so constructed that its reactions at some arbitrarily low level of affinity are restricted to approximately 1 percent of all possible antigenic configurations, and there are 5000 molecular types, each antigen will react with approximately 50 different globulin molecules. In this case there would be $5000^{50}/50!$ or approximately 3×10^{120} different combinations and an equal number of different antigens which could be distinguished. Since this is larger than the number of electrons the universe is thought to contain, it is a satisfactorily large number. If the fraction of antigens which can combine with a single type of globulin molecule is less than 0.01, somewhat more than 5000 different natural globulins are required.

In Fig. 2, the process of immunization is pictured as accelerating the production of a selected fraction of normal globulins until the concentration of these globulins exceeds the threshold required for detection. Threshold curves for precipitation and agglutination can be calculated from the Goldberg theory (*24*) if certain assumptions are made concerning the valence of the antigen and the ratio of antigen to antibody used in the test procedure (*4*). The mean equilibrium constant for antibody is that obtained experimentally by Nisonoff and Pressman for *p*-iodobenzoate (*25*). The exact shape of the distribution curve for normal globulins is unknown and undoubtedly varies according to the antigen used and the genetic and environmental history of the individual. With many antigens the distribution curve for normal globulin crosses the threshold required for agglutination but only rarely the threshold required for precipitation. The selective increase in rate of production during the process of immunization represented in Fig. 2 is analogous to the induction of enzyme synthesis in bacteria. The factor by which the rate of

production is increased by the inductive action of the antigen might be expected to be proportional to the energy of binding. The small numbers in the middle of Fig. 2 represent the factor of increase required to convert the normal globulin curve into the antibody curve.

The possession by protein molecules of affinities for a large number of substances is well recognized. Serum albumin has been shown to bind a large number of natural and synthetic substances (*26*). The number of substances which bind effectively to individual globulin molecules appears to be much more restricted, due perhaps to a greater rigidity of the molecule, as suggested by Karush (*27*). This results in a greater specificity of that binding which does occur. A similar degree of specificity was found by Boyd (*28*) in a study of a large number of plant proteins. Some possessed a highly specific agglutinating ability for red cells containing antigens A, A_1, H, and N. Boyd called these plant proteins lectins from the Latin word *legere,* "to select," and suggested that the name might be used to include "those normal antibodies of animal serum thought not to result from antigenic stimulus." In the case of serum globulins, the restricted number of substances an individual globulin will bind is compensated for by the much greater heterogeneity of the globulins. Thus, it is not surprising that normal serum contains in its globulin fraction agglutinins for the red blood cells of other species and for many bacteria. When the specificity of these agglutinins was demonstrated by his absorption experiments, as depicted in Table 1, Malkoff (*29*) reached the conclusion that a normal serum contains as many specific agglutinins as there are sorts of cells that are agglutinated by the serum. Because of the large number of different substances which could be specifically agglutinated, and because each of them amounted to several micrograms per milliliter of serum, Landsteiner rejected this hypothesis. He was able to purify the agglutinins by absorption and elution from red cells and to show that the purified agglutinins acted most strongly on the red cells used for absorption, but also agglutinated other sorts of blood. He concluded that "if one assumes that normal serum contains a sufficient number of agglutinins, each reacting with a certain proportion of all bloods, a given sort of blood will absorb from a serum all those agglutinins for which it has affinity and there will remain after absorption some that react with freshly added blood of other species. . . . One may conjecture that there exists a much greater variety of globulin molecules in a serum than would appear from physicochemical examination, some of which by virtue of accidental affinity to certain substrates are picked out as antibodies" (*1*, pp. 129, 133).

Table 1. Malkoff's results (*29*).

Blood	Unabsorbed serum	Goat serum absorbed with				
		Pigeon blood	Rabbit blood	Human blood	Pigeon and rabbit blood	Pigeon and human blood
Pigeon blood	+	0	+	+	0	0
Rabbit blood	+	+	0	+	0	+
Human blood	+	+	+	0	+	0

The preceding conclusion of Landsteiner attributed the specificity of natural antibodies to unique combinations of natural globulins. An extension of this concept to include immune antibodies might have seemed likely, because of the similarity of Landsteiner's own results with synthetic haptenes to those of Malkoff with natural agglutinins (see Tables 1 and 2). However, Landsteiner rejected this concept as an explanation of the specificity of immune antibodies, apparently because of a firm conviction that immune antibodies were different from natural antibodies. More recently, because of the failure to find differences between immune and natural antibodies, all natural antibodies have been considered to be immune antibodies produced in response to the antigens of food or intestinal bacteria. The other alternative, that all immune antibodies are unmodified natural globulins, is the thesis of this article.

Landsteiner was one of the first to demonstrate the heterogeneity of antibodies produced in response to a single antigenic determinant. A classical experiment is reproduced in Table 2 (*30*). This experiment demonstrated that antibodies to a single determinant were heterogeneous with respect to their ability to cross-react with related chemical groupings. Heterogeneity in another dimension—that is, with respect to combining constants—has been amply demonstrated by the more quantitative techniques of precipitation inhibition and equilibrium dialysis. More recently, Nisonoff and Pressman (*31*) have been able to confirm with equilibrium dialysis the experiments of Landsteiner and van der Scheer. Using a radioactively labeled *p*-iodobenzoate, Nisonoff and Pressman showed that an antiserum to this determinant was heterogeneous with respect to the ratio of combining constants to two related substances.

In general, a determination of the combining constants between purified antibodies and various haptenes has shown that the haptene used in the

Table 2. Results obtained by Landsteiner and van der Scheer (*30*). Since the test antigens contained the same proteins, unrelated to the horse serum used for immunization, the protein component could not be responsible for the differential reactions.

Immune sera for *m*-aminobenzene sulfonic acid after absorption with	Azoproteins made from chicken serum and			
	o-Aminobenzene sulfonic acid	*m*-Aminobenzene sulfonic acid	*m*-Aminobenzene arsenic acid	*m*-Aminobenzoic acid
o-Aminobenzene sulfonic acid*	0	++±	±	+
o-Aminobenzene sulfonic acid†	0	+++±	±	+
m-Aminobenzene arsenic acid*	+±	+++	0	+
m-Aminobenzene arsenic acid†	++	++++	0	+±
m-Aminobenzoic acid*	+±	+++	±	0
m-Aminobenzoic acid†	++	++++	±	±
Unabsorbed immune serum*	++	+++±	+	+±
Unabsorbed immune serum†	+++	++++	++	++±

* After standing 1 hour at room temperature.
† After standing overnight in the icebox.

azoprotein antigen for immunization and purification had a higher affinity for the purified antibodies than for any other haptene (*32*). Any modification of the original haptene usually resulted in a lower affinity for the antibodies in proportion to the degree of modification. Occasionally, however, a modified haptene was found with a higher affinity for the antibodies than the haptene used for immunization and purification (*33*). Explanations based on the concept of antibodies as uniquely modified globulins have been possible in most instances but not always (*23*). However, these results are necessary and predictable from the concept of antibodies as natural globulins. It would be expected that those globulins which have been selected by immunization and purification would have the highest average affinity for the haptene used for selection. Since this affinity is due to chance, however, it would also be expected that some of the selected globulins would have a greater affinity for some other haptene. A less likely event, but one which should occur occasionally, is that the majority of selected globulins would have a higher affinity for a haptene other than that used for selection.

If antibodies to synthetic haptenes are natural globulins selected because of a chance affinity for the antigen, it would be expected that unselected globulin (that is, globulin from an untreated animal) would have definite although lower average affinity for these substances. However, the binding of synthetic haptenes to gamma globulin may be impossible to demonstrate unambiguously because the degree of binding falls off rapidly below a critical threshold concentration. As is illustrated in Fig. 2, it might be impossible to detect the reaction between haptene and gamma globulin even though the average energy of combination were one-half that between haptene and purified antibody.

Since the total concentration of gamma globulin usually approximates 10^{-4} moles per liter, the concentration of each individual type of globulin in "normal" serum would be more than 10^{-8} moles per liter if we assumed that there were 5000 different types equally represented. However, the distribution in normal serum is undoubtedly uneven between the various types because of the uneven effect of the many internal and environmental stimuli. For example, the most avid form of diphtheria antitoxin can be detected at a concentration of 10^{-10} moles per liter (*34*). In order to explain its absence in normal serum, it is necessary to postulate that the so-called normal globulins include only those induced by fortuitous exposure to common environmental antigens in food and bacteria. In this respect a toxic substance is a selected antigen. It would not be toxic if it were antigenically similar to the common environmental antigens.

It is tempting to speculate on the relationship between specificity, heterogeneity, inducibility and acquired tolerance. At one end of the spectrum albumin has a concentration which is fixed by some internal homeostatic mechanism. It is therefore not inducible by environmental substances. It has low specificity and low heterogeneity and it is molded to self-tolerance by heredity. At the other end of the spectrum are the gamma globulins. Because of a high specificity, a large number of different types of molecules are required. Because of this heterogeneity, the economy of the animal prevents every one from being present in a high concentration. It is, therefore, advantageous that they be highly inducible. An acquired process such as immune tolerance may be the most efficient mechanism of eliminating harmful types of inducible proteins. Do the globulins in the alpha and beta fractions possess intermediate degrees of specificity, heterogeneity, and inducibility? Properdin may be a case in point. It would be of interest to determine whether the production of such a protein could be inhibited by the mechanisms of immune tolerance.

Summary

The concept of immunological specificity based on a unique combination of natural globulins is an attractive alternative to the classical concept of unique globulin molecules for each possible antigen. Many hitherto separate facets of the antibody response such as antibody diversity, cross-reactions, natural antibodies, increased mitoses in lymphatic tissue, the anamnestic response, and immune tolerance may be related through the general thesis that antibody production is not a unique biological phenomenon but a highly specialized example of certain general cellular processes.

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