A Theory of Delayed Hypersensitivity

The main features of this phenomenon are explicable in terms of high-affinity humoral antibody.

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For the past 40 years or more it has been traditional to classify hypersensitivity reactions into two main divisions, the immediate type and the delayed type. The immediate type is regularly associated with the presence of circulating antibody, which determines the specificity of response. The delayed type, on the other hand, is generally assumed to be independent of such antibody and is attributed to acquired specific reactivity borne by cells —that is, it is assumed to be an expression of "cellular" hypersensitivity. The experimental basis for the latter view is provided largely by two observations: (i) the delayed response can be evoked in a sensitized animal without the presence of a detectable level of circulating antibody of the appropriate specificity; and (ii) the response can be transferred to a recipient animal with lymphoid cells derived from a hypersensitive donor, whereas passive transfer of serum from such a donor is ineffective (*1*, *2*). Despite its attendant conceptual difficulties when viewed from a biosynthetic point of view, the notion of cellular hypersensitivity has gained widespread acceptance. The limited significance of the experimental evidence which is used to support this idea, and indeed the logical inadequacy of this evidence, have, however, been occasionally pointed out (*3*, *4*).

In this article we shall present an analysis of the phenomenon of delayed hypersensitivity which is based on the obligatory participation of circulating antibody as the component which determines the specificity of the hypersensitive response. We have been stimulated to approach the problem in this way by the quantitative knowledge gained during the past few years about the affinities of antibodies for their homologous antigenic determinants and the broad range of such affinities. The heterogeneity of antibody (*5*) which is involved therein plays, in our view, a key role in the various forms of the general phenomenon of hypersensitivity, as well as in other aspects of the immune response. These recent advances have provided the foundation for the leading idea of our analysis—namely, that the affinity of an antibody for its homologous antigen can be sufficiently great for the antibody to form a stable union with the antigen at concentrations of the uncombined antibody which are too low to be detected by current methods.

We wish to emphasize at this point that our discussion is restricted to the primary phase of the delayed response —namely, the sequence of events which culminate in the specific interaction of the antigen. Subsequent processes, which are undoubtedly of great significance in the emergence of the definitive lesion, fall outside the province of the present analysis.

Quantitative Aspects

On the basis of the most sensitive methods currently available for the detection of antibody, we may calculate the order of magnitude of the maximum value for the concentration of circulating antibody which may be assumed to exist in an individual whose serum appears devoid of antibody. Studies of delayed hypersensitivity to diphtheria-toxoid preparations have shown that a delayed skin response can be observed in guinea pigs possessing less than 0.015 microgram of antitoxin per milliliter of serum, as measured by the rabbit skin test for antitoxin (*6*). Since nontoxic protein antigens and their homologous antibodies appear to have been present as contaminants in these experiments, it is not certain that the delayed responses observed were due to the toxoid-antitoxin system (*7*). We shall, nevertheless, assign a value of 1×10^{-10} M, which corresponds to the value just given for antitoxin, as the maximum concentration of circulating antibody which, in our hypothesis, is responsible for the delayed response. It is important to note in this connection that passive cutaneous anaphylaxis (PCA) is widely used as an assay for serum antibodies in studies concerned with demonstrating the absence of antibody in the sera of individuals giving delayed skin responses. This assay, when applied to undiluted serum, seems to require antibody at concentrations of about 10^{-7} to 10^{-8} M (*8*). Hence, many sera that are believed to be devoid of antibody on the basis of a negative PCA reaction may contain up to 3 to 30 micrograms of antibody per milliliter. Nevertheless, for purposes of argument, we shall take 10^{-10} M as the maximum concentration of circulating antibody in individuals who exhibit delayed skin responses. The figure of 10^{-10} M allows us, on the basis of the law of mass action, to specify that the *minimum* value for the intrinsic association constant (K_A) of the antigen-antibody complex is of the order of magnitude of 10^{10} liters per mole. That is, at a concentration of free antibody of 10^{-10} M, the major portion of the antigen will be complexed with antibody only if the affinity of the two reactants is great enough to provide a K_A of at least 10^{10}. Although this value for K_A is much higher than that found for several antibody-hapten and antibody-antigen systems, it poses no serious difficulty because, as has been clearly demonstrated by Berson and Yalow (*9*), in some human anti-insulin sera a portion of the antibody population will combine with insulin with an average K_A of the order of 1×10^{10}. Since this represents an average value it is quite possible that antibodies may exhibit affinities of 10 or even 100×10^{10}.

In this connection it must be emphasized that the biosynthesis of high-affinity antibodies may be accompanied by the production of low-affinity molecules (for example, molecules with K_A of 10^5). Experience with the dinitrophenyl-lysyl group (*10*, *11*) demon-

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strates that, even against a single small determinant, a 10,000-fold variation in K_A (from 10^5 to 10^9) does occur. This undoubtedly represents a minimum range of variation, since the extreme values actually measured probably represent averages of heterogeneous populations. In the absence of any information to the contrary it must be assumed that production of antibody, even to a single determinant, yields a population of molecules whose affinities cover a very wide range, with perhaps a million-fold variation in K_A.

It appears that, given the quantitative situation as we have just described it, the distinctive features of delayed hypersensitivity can be accounted for on the basis of a low concentration of circulating antibody of high affinity. In Fig. 1 we have provided a diagrammatic representation of the dynamic and competitive relations involved, in order to make clear the inferences which may be drawn from our assumptions.

Role of Continued Antibody Synthesis in Delayed Response

The consequences of the low level of circulating antibody with respect to the slow evolution of the delayed type of response are quite evident. For the accumulation of a sufficient quantity of antigen-antibody complex to yield observable tissue damage, continued biosynthesis of antibody is required, to replace antibody which is removed from circulation by combination with antigen. That is, continued biosynthesis is required to maintain the concentration of uncombined antibody at a level which is high enough to favor the formation of complexes. One would expect to find, therefore, that the rate of synthesis of antibody can be the limiting step in the evolution of the delayed response. By contrast, in the immediate type of reaction, the formation of complexes would show a temporal dependence due to the diffusion or transport processes which are involved in bringing antigen and antibody into proximity.

A rough estimate of the quantitative relationship between the amount of antigen needed and the concentration of antibody can be made as follows. Let us suppose that 5 micrograms of antigen of molecular weight 50,000 are used for a skin test (see, for example, *6* and *12*); this is equal to 10^{-10} mole of antigen. On the assumption that one-tenth the molar quantity of antibody is needed for an observable response, 10^{-11} mole of antibody would have to be provided. This means that, with the maximum allowable concentration of antibody ($10^{-10}M$), the antibody contained in 100 milliliters of serum would be necessary for a positive response. This amount is about 4 times the total serum volume of a 500-gram guinea pig. Thus, dependence of the delayed response on continued biosynthesis is to be expected. This dependence may, in fact, be even greater than we have just indicated, since effective antibody concentrations below $10^{-10}M$ may well exist.

In human subjects the passive transfer of 100 milliliters of serum imposes no serious physiologic burden on the recipient. In this case, however, dilution of the transferred serum by the recipient's extracellular volume would greatly reduce the concentration of transferred antibody, and so the transfer would be expected to be unsuccessful. For example, in a 70-kilogram man infused with 100 milliliters of serum having an antibody concentration of $10^{-10}M$, the final concentration of antibody is likely to be about $10^{-12}M$. Even if only 10^{-12} mole of antibody were needed, rather than 10^{-11} mole as assumed earlier, dilution of a tolerable volume of serum in a recipient guinea pig (for example, 10 ml at $1 \times 10^{-10}M$) would lower the antibody concentration to such an extent that the formation of antigen-antibody complexes would be greatly limited. From the foregoing argument one might anticipate that, under special circumstances (for example, if the serum antibody were of very high affinity or the quantity of gamma globulin transferred were unusually large), the delayed response might be effectively transferred with serum gamma globulin.

From the inference that the quantity of antibody localized at the site of the response in a guinea pig is of the same magnitude as the mass of circulating high-affinity antibody, it follows that the rate of production of such antibody should provide for its replacement in a period of about 24 hours. This demand implies a much more rapid turnover of the antibody than of total gamma globulin (*13*). The basis for this difference may be found in the specific removal of the antibody by the formation of complexes with antigen, followed by "immune elimination" of the complex and its possible use in accelerated antibody synthesis (Fig. 1).

The successful passive transfer of delayed hypersensitivity with the lymphoid cells of a sensitized animal (*14*) is easily understood on the grounds that the transferred cells provide for the continued production of antibody in the recipient, so that progressive accumulation of antigen-antibody complexes may ensue. In this connection it is pertinent to recall that sensitized lymphoid cells are capable of producing humoral antibodies in a recipient animal (*14*, *15*). The successful transfer of delayed hypersensitivity in man with disrupted cells (*16*) is a provocative observation whose relevance to the present discussion is at present unclear.

Since the quantitative argument just elaborated supposes that, as an extreme case, the antibody contained in 100 milliliters of serum (at $10^{-10}M$) is necessary for the delayed response, it is necessary to inquire whether the cutaneous blood flow is great enough to permit a volume of blood equivalent to 100 milliliters of serum to perfuse a skin test site during the 24-hour period over which the delayed response evolves. This requirement would seem to be satisfied by the fact that cutaneous blood flow normally varies over a range of about 0.05 to 0.5 milliliter of blood per minute per gram of skin (*17*). Actually, when the inflammatory response is once initiated, vascular dilatation in the skin test site becomes pronounced, and it is likely that an increase in blood flow then occurs at this site, as well as an increase in the extent to which serum proteins leak through capillary walls.

Role of Circulating Antigen

In the induction of delayed hypersensitivity the quantity of the sensitizing antigen and the mode of its administration are particularly significant (*2*). Microgram quantities of antigen have been found to be especially effective (*2*, *6*, *12*). By contrast, for the induction of large amounts of circulating antibody, much larger quantities of antigen are required—that is, milligrams. Evidently a condition which must be satisfied in the induction of delayed hypersensitivity is that the level of circulating antigen be relatively low. This requirement can be readily understood in terms of our major assumption. On the one hand, a minimum level of antigen is required, to stimulate the formation of antibody by a sufficient number of cells to maintain the necessary concentration of serum antibody. On the other hand, a rate of release of

antigen much beyond this level will result in the selective removal of the high-affinity antibodies which are necessary for the delayed response. Thus, a delicate balance between these opposing factors must be maintained by means of the experimental conditions. The maximum level of circulating uncombined antigen can, therefore, be specified to be about $10^{-10}M$—of the same order of magnitude, that is, as the maximum concentration of the antibody.

It must be understood in this connection that we are not assuming that only high-affinity antibody is generated under the conditions selected. There could be formed (and we have no reason to assume otherwise) antibody of lower affinity (for example, with K_A of 10^5). However, such antibody would not participate, at the low concentration specified, either in the development of the hypersensitive state or in the delayed response. More generally, it may be noted that when an antibody population that is heterogeneous with respect to affinity for a particular determinant is exposed to that determinant, there will occur a selective combination of the determinant with the antibody of highest affinity.

The interaction of circulating antigen with the high-affinity antibodies of the sensitized animal provides an explanation for the anamnestic response which such an animal exhibits. Salvin and Smith (*12*), for example, have shown that guinea pigs given a primary injection in the footpad of 0.5 microgram of hen egg albumin in saline develop only the capacity to make a delayed response. Injection of 15 micrograms of the homologous antigen in adjuvant into these animals within 10 days of the primary injection results in an anamnestic response, with production of detectable circulating antibodies and hypersensitivity of the Arthus type. Of a similar nature were the experiments of Pappenheimer *et al.*, in which guinea pigs were given a single intradermal injection of 3 micrograms of bovine serum albumin (BSA) in the form of a washed specific precipitate formed in excess rabbit antibody and suspended in an oil-water emulsion (*18*). These animals showed delayed skin reactions but no circulating antibody. After intravenous injection of 1 milligram of I^{131}-BSA, an accelerated elimination of circulating antigen was observed, starting on about the 4th day. Thus, just as in the case of more conventional immune responses, a second administration of antigen to an animal exhibiting the delayed response rapidly elevated the concentration of serum antibody—in this case from a nondetectable to a detectable level. Our interpretation of this phenomenon is that the high-affinity antibodies responsible for the delayed response form complexes with the antigen of the second injection and that these complexes, possibly because of their increased susceptibility to phagocytosis, are utilized more effectively than uncombined antigen to stimulate the production of antibodies.

On the basis of these anamnestic reactions the view has been expressed that "delayed hypersensitivity is an early, immature, and essential phase in the development of circulating antibody" (*12*). This notion can be given molecular meaning in terms of the interpretation of the anamnestic response that we have just offered. Thus, the earliest exposure of the appropriate cells to a primary injection will lead to some antibody production. This initial antibody population will include high-affinity molecules which can either make a delayed response or, by virtue of their selective combination with additional antigen, accelerate the formation of additional circulating antibody to levels that are detectable. From this point of view, then, the antibody found in the serum, even after a primary injection, would be largely the result of stimulation by antigen-antibody complexes.

The Antigenic Determinant

The critical role which we attribute to high-affinity antibody implies that the antigenic determinant associated with the delayed response must meet certain requirements with respect to chemical nature and size. The chemical nature and size must be such as to permit a net attractive interaction in aqueous solution great enough to provide a value of K_A of at least 10^{10}. At body temperature this value corresponds to a standard free energy ($\Delta F°$) of −14 kilocalories per mole of determinant. The determinant needed to provide this degree of affinity may be quite small if it is of the proper chemical nature, in particular if it is largely hydrophobic (that is, apolar) in nature. The study of the binding of 2,4-dinitrophenyllysine [(DNP)-lysine] and 2,4,-dinitrophenol by purified antibody prepared by injecting DNP-bovine gammaglobulin in adjuvant provides a useful basis for considering this matter (*11*). For DNP-lysine, $\Delta F°$ can reach −12 kilocalories per mole of hapten. For dinitrophenol, with a K_A of 2×10^6, $\Delta F°$ is about −8 kilocalories. The difference, about 4 kilocalories, can be attributed to the additional interaction with antibody of the hydrophobic $(CH_2)_4$ group of the lysine side chain. This contribution of 4 kilocalories may be compared to the decrease of 6 kilocalories in unitary free energy observed when a mole of butane is transferred from an aqueous environment to the apolar solvent, liquid butane. The affinity of the $(CH_2)_4$ group for the antibody is very probably an expression of the same kind of change of environment (*19*). We may also note that in another system, involving the interaction of a hapten with its homologous purified antibody, the removal of a phenyl group from the homologous hapten decreased the affinity by 4 kilocalories (*20*). It is clear, therefore, that a determinant equivalent to the DNP-lysyl group plus one apolar amino acid side chain could exhibit an affinity more than sufficient to meet the minimum requirement for a $\Delta F°$ of −14 kilocalories.

To state this in more general terms, we would anticipate that an antigenic determinant of a protein need be constituted of no more than three or four amino acid side chains, predominantly apolar in character, in order to provide sufficient affinity for the delayed reaction. On this basis no *great* difference in size between the antigenic determinant in immediate hypersensitivity and the determinant in the delayed type of response is to be expected, since only one amino acid side chain can make the decisive contribution which will convert an antigenic determinant incapable of eliciting the delayed response to one which can elicit it.

It is pertinent in this connection to consider recent studies which have attempted to deal experimentally with the determinant involved in delayed skin responses. By means of immunization and skin testing with a variety of related hapten-protein conjugates, Benacerraf and Gell (*21*) and Salvin and Smith (*12*) have inferred that the determinants in delayed responses represent larger segments of the immunizing antigen than those ordinarily involved in conventional serological reactions and in immediate skin responses. It has, for example, been shown that guinea pigs sensitized with picrylated ovalbumin exhibit delayed responses to this antigen, and also to native ovalbumin, but not to picrylated bovine gamma globulin

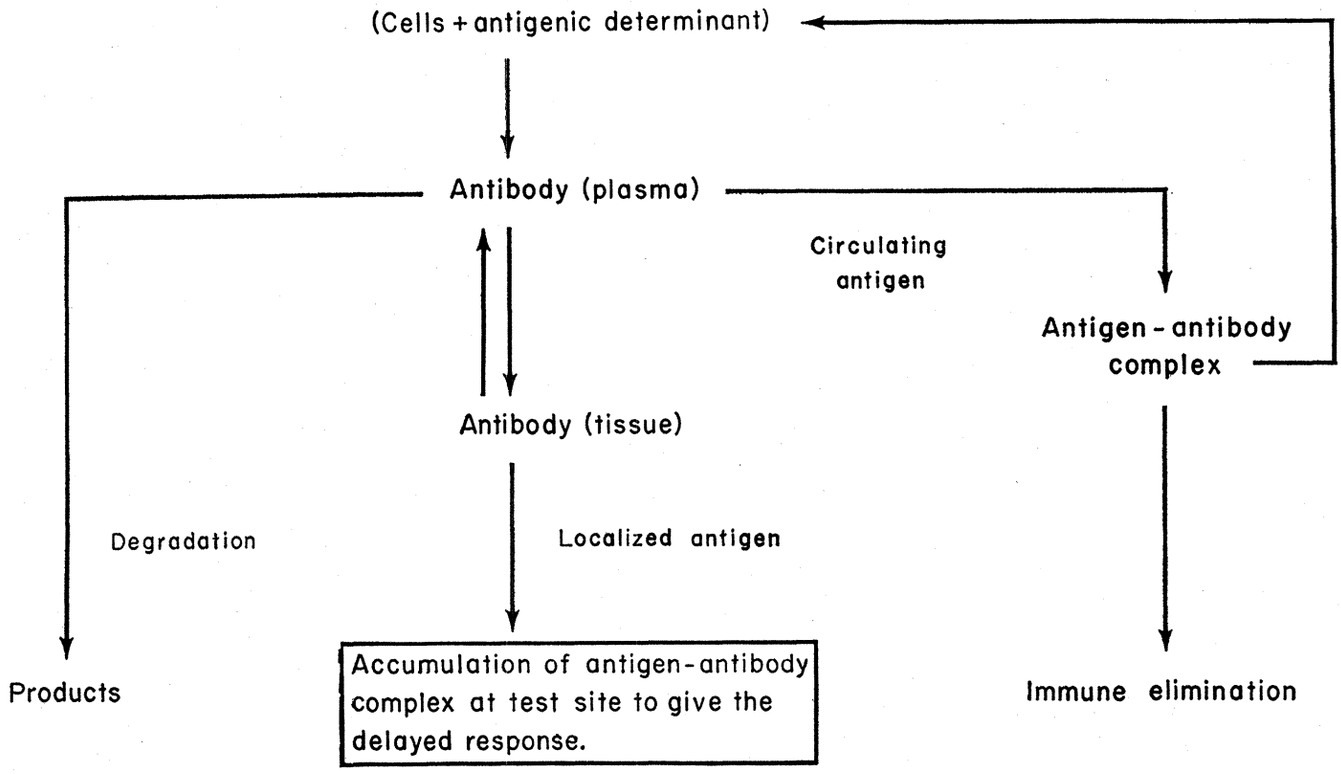

Fig. 1. Diagram depicting the dynamic and competitive aspects of delayed hypersensitivity.

(*21*). Similarly, guinea pigs sensitized with picrylated bovine gamma globulin exhibit delayed skin responses to this antigen and also to bovine gamma globulin, but not to picrylated ovalbumin (*12*). These results are discussed later in further detail in relation to contact sensitivity of the skin. It is enough to note here that the requirement for greater similarity between sensitizing and eliciting antigens in delayed responses than in conventional serological reactions and in immediate responses is readily understandable on the grounds already elaborated; that is, from the proposed dependence of the delayed response on very low concentrations of humoral antibody it necessarily follows that the relevant antibodies have high affinity for antigen.

The importance which we have, by implication, assigned to hydrophobic bonding and the quantitative estimate we have made raise a question concerning the capacity of polysaccharides to elicit delayed hypersensitivity. It appears that no instance of delayed sensitivity to polysaccharides has so far been recognized (*22*). Indeed, on the basis of the following considerations, we anticipate that polysaccharides, generally, will prove ineffective. In the case of the determinants in these antigens, the predominant attractive interaction with antibody is hydrogen bonding involving the hydroxyl groups of the sugar determinant. Because of the competition of hydrogen bonding with water, the energetic advantage achieved by the formation of a complex between determinant and antibody is expected to be much less than that arising from an apolar determinant of equal volume. If this limitation is accompanied by similar limits to the effective size of the two kinds of determinants, then the affinities achieved by polysaccharide determinants may seldom reach the values necessary for the delayed response—values that primarily apolar determinants can attain. In this connection it may be emphasized that experimental efforts designed to demonstrate delayed hypersensitivity with haptenic groups can be expected to be successful only when carried out with groups capable of inducing antibodies with high affinity for them.

Kinetic Considerations

We have already alluded to the significance of the low level of high-affinity antibodies in relation to the slow development of the delayed response. It is also pertinent to ascertain whether the rate of combination of antigen and antibody is compatible with this low level. The question is, Can the antibody, at this level, react rapidly enough with the antigen so that a substantial portion of the latter will be combined in a period of the order of hours?

For this purpose let us consider the velocity of the simple association reaction in which one antibody molecule B combines with one antigen molecule G to give the simple antigen-antibody complex BG with a rate constant k.

$$B + G \xrightarrow{k} BG$$

If we make the rather demanding assumption that the reaction proceed at such a rate that 10 percent of the test antigen will react per hour, and if we assume that the concentration of free antibody remains at some steady-state value, then k is given simply by 0.1 per hour over B, where B is this steady-state value. When B is 1×10^{-10} M, therefore, k is equal to 1×10^{9} liters per mole per hour.

The kinetic studies of Berson and Yalow (*9*) involving the combination of the univalent insulin molecule with its homologous antibody have yielded, for some sera, values of the rate constant for this system of the magnitude 1×10^{9} liters per mole per hour. Thus, the kinetic demand which our analysis makes of the high-affinity antibody is in accord with the known properties of antibody. We may note further that the multivalence of the kind of antigen

usually employed in testing for the delayed reaction would probably increase the fraction of collisions which result in the formation of complexes. Thus, larger rate constants may be expected in this case than the rate constant observed with insulin. Talmage (*23*) has also studied the rate of formation of soluble complexes of bovine serum albumin and rabbit antibody. Although his values are of the same order of magnitude as those of Berson and Yalow, they are somewhat lower and, because of the multivalence of antigen, less susceptible to unambiguous interpretation.

It is of interest to note, also, that much higher values than those we are concerned with here have been found in other protein-protein interactions. For example, in the oxidation of reduced cytochrome *c* by hydrogen peroxide catalyzed by yeast peroxidase, the velocity constant for the reaction between the preoxidase-hydrogen peroxide complex and ferrocytochrome *c* is 4×10^{11} liters per mole per hour (*24*).

Other Considerations

Delayed and immediate responses differ not only in time-course, concentration of serum antibodies, and optimal conditions for induction of the hypersensitive state but in a number of other respects. Some of these other differences —such as differences in the histopathology of the inflammatory lesion and in susceptibility to suppression by antihistaminics and corticosteroids—require no special consideration here; possibly these reflect general differences between acute and subacute inflammatory processes, regardless, to some extent, of the inciting agent or event.

It is, however, necessary to consider in some detail those special tissue and cell responses which are generally regarded as unique manifestations of delayed hypersensitivity. The compatibility of the present theory with these phenomena is dealt with below.

Cytotoxic Effects of Antigen on Isolated Cells

It has been observed that when tissue explants from animals showing delayed responses to tuberculin, or to other proteins, are maintained in vitro, a variety of mesenchymal cells exhibit morphologic alterations when the homologous antigen is added to the culture medium. Explanted cells from nonsensitive animals are not affected by the antigen at the same concentration and remain unaffected when the culture medium is enriched with serum or plasma from animals exhibiting the delayed response. Moreover, similar explants from animals with substantial serum concentrations of antibody (and immediate allergic responsiveness) fail to undergo morphologic changes on the addition of homologous antigen to the culture medium. Observations of this kind have long been regarded as providing strong support for the view that the delayed response is a manifestation of "cellular" hypersensitivity (*2*). Many investigators have failed to corroborate these observations, but the contention that such effects occur—and are specific —has been advanced with sufficient frequency and assurance (*25*) for us to assume that the observations summarized earlier in this paragraph are valid.

From the viewpoint of our hypothesis we approach these observations (i) by noting that some of the explanted cells doubtless continue to make antibody in vitro, and (ii) by recalling, from the law of mass action, that for a fixed amount of antigen the extent of antigen-antibody complex formation is related to the product of the concentration of free antibody and the affinity ($B \times K_A$). The cytopathic changes in cell cultures probably require not only that a critical mass of antigen-antibody complexes be formed but also that these complexes be localized predominantly on cell surfaces where they are likely to interfere with vital membrane functions. If we assume that the test antigens ordinarily used are adsorbed on the explanted cells, the response of the explants can be interpreted in much the same way as the delayed skin response in the intact animal as follows: antibody at exceedingly low concentration is capable of forming complexes with antigen provided that the affinity of antigen is sufficiently great and that a sufficiently high concentration of uncombined antibody is maintained by continued antibody production. This interpretation implies that explants from animals which exhibit immediate skin responses and are producing relatively large amounts of antibody may also exhibit a cytotoxic response in the absence of immune serum, provided that (i) antigen is adsorbed on the explanted cells, (ii) antibody production continues in the explant, and (iii) the antibody affinity is adequate. This situation has not been recognized experimentally; it certainly cannot, however, be excluded on the basis of existing reports, as responses of explants to antigens have been notoriously erratic.

Corneal Response

A sensitized guinea pig which exhibits an intense delayed response to an intradermal injection of antigen will often show an inflammatory response in the cornea 24 to 48 hours after injection of the same antigen into this avascular tissue of the eye. On the other hand, an animal with relatively high serum levels of antibody and immediate responsiveness to antigen injected intradermally does not ordinarily develop an inflammatory lesion when the antigen is injected into its cornea. These observations have been regarded as furnishing strong supporting evidence for the view that the delayed response is independent of the vascular system and, indeed, of the antibody contained in serum (*2*).

Although the normal cornea is essentially avascular, antibody doubtless can diffuse into it from surrounding blood vessels (*26*), and we shall here assume that in the central portion of the cornea the antibody concentration is some small fixed fraction (say, 0.1) of the plasma concentration. Our interpretation of the corneal response need not, therefore, be significantly different from that offered for the delayed skin response. Here the situation is more extreme, however, since antibody concentrations are even lower and the requirement for high K_A values (for example, 10^{11}) is correspondingly greater. According to the interpretation, the corneal response may not be obtainable in all animals who exhibit delayed skin responses—one would expect to find occasional animals who exhibit a skin response but not a corneal response. That this is indeed the case was noted several years ago by Raffel *et al.* (*27*).

According to the present view it may also be possible to elicit corneal responses in animals that exhibit immediate cutaneous responses, provided the serum antibody has sufficiently high affinity for antigen. Despite the substantial amount of work already done on corneal responses, this latter possibility cannot be evaluated from existing data. There remains a clear and unsatisfied need for further detailed correlation between corneal responses on the one hand and concentrations and affinites of serum antibodies on the other.

Contact Sensitivity

When certain protein-reactive simple chemicals (usually referred to as sensitizers) are applied on the surface of the skin or injected into the skin, an allergic state is induced after 5 or 6 days; this can be recognized by the fact that a delayed inflammatory response will subsequently occur in virtually any area of skin to which a drop of a dilute, nonirritating solution of the same sensitizer is applied. An essential step, both in the induction of this allergic state and the elicitation of the inflammatory response, is the chemical combination of the sensitizer with proteins in the skin—a process which is relatively rapid and therefore not rate-limiting for the evolution of the inflammatory response (*28*). The protein conjugates which are effective in eliciting the delayed response, and also the inflammatory lesion itself when it finally appears, are both localized at the epidermal-dermal junction (*28*). This superficial zone of skin is embryologically analogous to the cornea and, like it, very poorly vascularized (*29*). Consequently, the concentration of antibody in this area of skin is (as was suggested for the cornea) very probably only some small fixed fraction of the concentration in serum, and the formation of antigen-antibody complexes in this region would demand a high affinity between these reactants. Thus we propose that, as in the case of the delayed corneal response to injection of a protein antigen, (i) low concentrations of humoral antibody could account for the slowly evolving response to a skin-surface application of a sensitizer of low molecular weight, provided the affinity of the antibody for antigen were great enough, and (ii) the response would not be obtainable in animals with relatively high concentrations of serum antibodies if the affinity for antigen were low.

The premium which these proposals place on high affinity values in antibody furnishes an explanation for some hitherto puzzling observations. For example, contact sensitivity of the skin to 2,4-dinitrobenzenes can be induced in guinea pigs and in humans by the application of 2,4-dinitrofluorobenzene (DNFB) or any of several other dinitrobenzenes which can also combine chemically with skin proteins—for example, with lysine ϵ-amino and cysteine sulfhydryl groups (*30*). The animals with contact sensitivity of the skin thus induced have very little detectable anti-DNP antibody in their serum, and often none at all (*31*). When, however, guinea pigs are injected with substantial amounts of dinitrophenyl-protein conjugates made in vitro (with the same sensitizers, coupled in much the same way to a heterologous protein such as bovine gamma globulin), high concentrations of anti-DNP antibodies usually appear in the serum (1 to 5 mg/ml), but the animals do not exhibit contact sensitivity responses to DNFB, or to the other dinitrobenzene sensitizers (*32, 33*). Observations of this kind have largely been responsible for the view that when one individual has, at the same time, contact sensitivity of the skin and detectable serum antibodies, both apparently specific for the same determinant, the slowly evolving inflammatory lesion of the skin response is not dependent on serum antibody (*3*). As will be explained in the following paragraphs, the fallacy of this view stems from a failure to distinguish between the necessary and the sufficient components of an antigenic determinant.

In interpreting these observations we assume that the anti-DNP antibodies whose formation was induced by the injection of DNP-coupled to bovine gamma globulin (*33*) will interact only with the DNP-substituted amino acid residues (lysyl or cysteinyl) of the protein conjugates which form in a skin site tested by the application of DNFB; that is, they are not likely to be complementarily adapted to the amino acid side chains neighboring on these substituted residues.

The average standard free energy for the interaction with ϵ-DNP lysine is no less than -11 kilocalories per mole (*11*), corresponding to a K_A of about 10^8, and the corresponding values for S-DNP cysteine are undoubtedly much less negative. These values are insufficient to permit extensive formation of complexes in view of the antibody concentration expected at the epidermal-dermal junction. On the other hand, when sensitization is induced by applying DNFB on the skin, or by injecting it into the skin (so that it forms protein conjugates *in situ*), the resultant antibodies are likely to be adapted not only to DNP-lysyl or DNP-cysteinyl groups but also to one or more amino acid residues contiguous with them in the protein conjugates that develop when the skin test is performed with DNFB. If, as was emphasized earlier, one or more of these adjacent amino acids is apolar, a $\Delta F°$ of -14 or less kilocalories per mole could be achieved, thereby permitting antigen-antibody complexes to form and an inflammatory lesion to develop. Thus, the inducing antigen and the eliciting antigen would ordinarily have to be identical in order for circulating antibody to exhibit biological activity at the extremely low concentrations likely to exist in the superficial layer of skin of an animal with contact sensitivity. An antigenic determinant that is similar to, but not identical with, that which forms in vivo during the skin test would be unlikely to induce such skin sensitivity, even though it be a highly effective inducer of antibody formation.

There exists an interesting difference between the observations just described and those made when the 2,4,6-trinitrophenyl (picryl) group is used as an antigenic determinant in place of the 2,4,-dinitrophenyl group. In this case, when picrylated proteins, made by coupling picryl chloride with heterologous proteins (that is, proteins from a foreign species), are injected into guinea pigs, contact sensitivity of the skin for picryl choride is induced (*34*). That in this case it is less advantageous to have identical inducing and eliciting antigens may be ascribed to the presence of a third nitro group in the picryl determinant, which could supply a significant energetic increment over the DNP group. Thus it is likely that $\Delta F°$ for the association of anti-picryl antibodies with a picrylated amino acid residue is more negative than it is in the corresponding DNP system, and this increment could be sufficient to make the antigenic contribution of neighboring amino acyl groups unnecessary. It may be noted, incidentally, that the contribution of the nitro group to the free energy of binding could arise from hydrogen bonding to, or dipole-dipole interaction with, complementary sites on antibody, rather than from the apolar bonds emphasized earlier.

In view of the foregoing analysis, the common observation that immediate and delayed hypersensitivity can exist simultaneously, the same chemical group being required for elicitation of the two responses, may be readily interpreted. The delayed response would arise from interaction at the test site of high-affinity antibodies of low concentration with a determinant which necessarily includes one or more amino acid residues, together with the required haptenic group (for example, picryl or 2,4-dinitrophenyl). Antibodies directed only against the haptenic group, and therefore of relatively low affinity,

would not contribute to the evolution of the delayed response but, in sufficient concentration, would mediate hapten-specific immediate responses.

Transplantation Immunity

The accelerated rejection of a homograft resembles delayed responses in general in that (i) it is ordinarily not associated with detectable serum antibodies in the host that exhibits this response, (ii) it cannot be transferred with serum, and (iii) the capacity to give the accelerated rejection can be transferred with viable lymphoid cells from an "immunized" host to a normal one. Moreover, when an extract made from cells of a donor guinea pig is injected into the skin of a recipient which has previously rejected homografted skin from this donor, a slowly evolving inflammatory response is evident (*35*).

Homografts of normal or neoplastic tissue have long been known to become infiltrated with host lymphoid cells as the grafts undergo rejection, and it has been repeatedly suggested that injury and death of the grafted cells occur as a consequence of their direct contact with the host's "sensitized" lymphoid cells. It is interesting, in this connection, to recall the experiments of Weaver, Algire, and Prehn (*36*), who found that transplanted homologous cells survive indefinitely within the peritoneal cavity of a host if they are enclosed within a chamber whose Millipore-filter walls are permeable to proteins but not to host cells; they survive even when the host has been previously "sensitized"—that is, when it has been capable of rejecting an orthoptic graft from the same homologous donor. The homologous grafted cells failed to survive in such chambers only when the filters were of such porosity as to permit entry of host cells (the host having been previously sensitized with respect to the cell donor), or when lymphoid cells from another sensitized host were enclosed within the same chamber in contact with the "target" cells. These experiments are widely regarded as providing unusually clear evidence in support of the view that direct contact between host lymphoid cells and homografted cells is required for destruction of the graft. These results have, moreover, been interpreted to mean, for delayed responses in general, that the essential and vital interaction takes place between antigen and "sensitized" lymphoid cell rather than between antigen and an antibody molecule. However, the Algire-chamber results are also entirely compatible with the view that serum antibody reacts with, and is responsible for, destruction of the transplanted cells. For example, when the porosity of the chamber walls is such that host cells are excluded but protein can enter, the diffusion of serum antibody from capillaries to peritoneal fluid to chamber cavity may be so slow (*37*) that the intrachamber concentration of free antibody may never amount to more than an exceedingly small fraction of the serum antibody concentration, and hence may never achieve the concentration required for complexing with antigenic determinants of the transplanted homologous cells, even though the serum antibody affinities may be very high. On the other hand, if the chamber walls are sufficiently porous to permit host cells to enter, or if lymphoid cells from immunized hosts are actually placed in the chamber, antibody production and secretion will occur directly within the chamber and will lead, very probably, to a concentration of free antibody sufficiently high to allow complexes with "target"-cell antigens to form, with resultant destruction of the homograft.

It is important in this connection to note that there does, in fact, exist a considerable body of evidence which supports the possibility that humoral antibodies can, at least under certain circumstances, be responsible for the rejection of homograft cells (*38*), including those sequestered in Algire chambers (*39*).

When individuals with congenital agammaglobulinemia are given a variety of antigens they fail to form detectable serum antibodies but often develop delayed-type hypersensitivity (*40*). These observations, combined with a genetically determined apparent inability to synthesize gamma globulins, are regarded as additional evidence that circulating antibodies cannot act as determinants of delayed responses. Actually, however, the genetic disability in individuals with congenital agammaglobulinemia is not absolute. Most subjects with this disorder have serum concentrations of gamma globulin of about 5×10^{-6} to $5 \times 10^{-7} M$ (*41*). It is entirely possible, moreover, that such individuals do make antibodies, but at too low a level for the antibodies to be detectable. If this should be the case, the capacity of these individuals to exhibit delayed skin responses is entirely compatible with the present theory.

General Remarks

We do not claim that this hypothesis provides a comprehensive explanation of all the differences between delayed and immediate responses. We have, however, attempted to demonstrate that a specificity-determining role for circulating humoral antibody is consistent both with the known properties of antigens, of antibodies, and of their interactions and with the three essential attributes of the delayed response: (i) slow evolution, (ii) occurrence in the absence of detectable serum antibody, and (iii) transfer with viable lymphoid cells but not with serum. Some features of the delayed response, such as the histopathology of the inflammatory lesion and its susceptibility to suppression by various pharmacologically active agents, are not relevant to the hypothesis; they are not, and cannot be, explained by it. On the other hand, a number of distinctive manifestations of the delayed responses—for example, the cytotoxic effect of antigens on explanted cells, contact sensitivity of the skin, the corneal response, accelerated homograft rejection, and findings in individuals with congenital agammaglobulinemia—all seem to be readily reconcilable with the proposed hypothesis and to offer no serious obstacles to its credibility (*42*).

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Richard Joseph Block, Biochemist

Richard J. Block and his wife, together with Dr. and Mrs. Jerome A. Uram and 14 other persons, died in a plane crash on 4 February 1962, shortly after leaving Tingo María, Peru. The Americans were on a mission sponsored by the National Institutes of Health in connection with the International Program on Nutrition Studies. Characteristically, Dick Block was also interested in securing a collection of drug plants from remote areas of Peru, which, according to Theodor Binder of the Hospital Amazonico Albert Schweitzer, Pucallpa, Peru, showed promise in the alleviation of human cancer.

Throughout 32 years of continuous research in the biochemistry of amino acids and proteins, Block customarily worked on several projects simultaneously. He was aware of the staggering amount of work that still lay ahead. He was never satisfied with what he himself accomplished. His friends and colleagues often heard him say that he "retired" in 1934. What Dick accomplished in his "retirement" during these 28 years is recorded in 128 published papers, a score of patents, and four textbooks of which he was the principal author. He also contributed chapters to a number of reference volumes.

Block left numerous projects "on the fire," which are being continued by his collaborators. These include several projected books and monographs, some of which were left in the final stages of preparation. Most important of all, he left behind him friends who deeply feel the loss of such a rare human being. This was his greatest achievement. "To make and keep a friend in our lifetime is the sole purpose of our lives."

He was born in Macon, Georgia, on 4 May 1906 and received his B.S. in chemistry at Yale in 1928 and his Ph.D. in physiological chemistry at Yale in 1931. In 1930 he married Peggy Strasser of New York. After serving as a fellow at Yale, he went to Munich, Germany, to the laboratory of F. von Müller.

Before joining the New York State Psychiatric Institute and Hospital as a research associate, Block had his interests in the fields of research in biochemistry well defined, and these interests remained with him to his last day: the amino acid composition of proteins in relation to their biological properties and nutritive value; and the biological synthesis and the interconversion of amino acids, their comparative biochemistry and relationship to health and disease.

In 1932, in collaboration with G. R. Cowgill, he published five reports on the preparation and purification of a highly potent preparation of thiamine (then known as vitamin B_1). This highly active field at that time occupied the minds of numerous biochemists throughout the world, and Block was making valuable contributions to the subject. But, characteristically, his mind and heart were in his beloved amino acids, and he would do only what his mind and heart told him to do. At that time, cystine reigned supreme as the only indispensable sulfur amino acid in animal nutrition, although J. H. Mueller had isolated another sulfur amino acid, later named methionine, which challenged the supremacy of cystine.