HAPTEN-CARRIER RELATIONSHIPS OF ISOANTIGENS

A Model for Immunological Maturation Based on the Conversion of Haptens to Carriers by Antibody*

By RAYMOND A. McBRIDE,‡ M.D., and LOUIS W. SCHIERMAN, Ph.D.

(From the Department of Pathology, New York Medical College, New York 10029)

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Recognition of immunogenicity is thought to be mediated by specific receptor antibody molecules which are either freely circulating or bound to cell membranes (1–5). In conventional hapten-carrier systems an essential requirement for activation of antihapten antibody synthesis is the ability of the immune system to recognize the immunogenicity of the carrier; induction of immunological tolerance to the carrier results in reduced anticarrier and antihapten antibody synthesis following subsequent inoculation of the hapten-carrier complex (6–8). Furthermore, a secondary type immune response to a hapten is most easily achieved if the same carrier is employed in both primary and secondary stimuli; little or no response results if the carrier for the secondary stimulus differs from the carrier used for activation of the primary immune response (2). Since procedures which activate specific anticarrier antibody formation [preimmunization with carrier (2, 7) or transfer of cells containing anticarrier antibody (9)] enhance the synthesis of antihapten antibodies, it is likely that the specific carrier effect on the immunogenicity of hapten is mediated in part by anticarrier antibody. Experiments in which anticarrier antibody was injected prior to the synthetic hapten-carrier complex have not demonstrated an enhancement of antihapten antibody synthesis (2, 7, 9). These results have been interpreted as indicating that the relevant receptors are not present in the serum.

In chickens, isoantigens determined by the *B* blood group locus behave as carriers for isoantigens determined by the *A* blood group locus (10). In the isoantigen system, as with conventional hapten-carrier complexes, recognition of the immunogenicity of the carrier is necessary for an immune response to the hapten (10). The finding that the carrier effect is enhanced by circulating anticarrier antibody (11, 12) is evidence for an important role of carrier antibody in the activation of an antihapten immune response. Pincus and Nussenzweig (13) demonstrated, by means of passive antibody, suppression of the immune response to a portion of guinea pig γ_2-globulin and simultaneous enhancement of the response to antigenic determinants on another portion of the same molecule. Others have demonstrated an enhancement of the immune response to complex cellular antigens by means of passive antibody (14–17).

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The present studies were performed to explore further the role of carrier antibody in the immune response, and the results suggest that the carrier properties of isoantigenic determinants depend on their being coated with antibody.

Materials and Methods

Animals.—Partially inbred White Leghorn chickens (line G)[1] were used as erythrocyte donors. Usually, recipients were from a closely related subline (line G-B1). Blood types of donors and recipients were determined for five independently inherited systems. Previous studies indicated genetic homogeneity for other blood group systems. Breeding and management of the chickens were under our direction.

Immunization Procedures.—

Passive immunity to B antigens: Three groups of G-B1 chickens were given seven weekly intravenous inoculations of red blood cells (RBC) possessing two serologically distinct foreign blood group antigens, A_2 and B_2. The chickens were 6 wk old at the start of the experiment. Each inoculation of group 1 recipients (seven birds) consisted of 0.75 ml of a 25% washed RBC suspension in 0.15 M saline. Groups 2 and 3 received the same quantity of washed RBC. With group 2 (eight birds), the RBC were incubated for 1–2 hr at room temperature with anti-B_2 isoantiserum prior to inoculation. Equal volumes of antiserum and packed RBC were used. Two volumes of saline were added to the heavily agglutinated RBC, and the suspension was inoculated without removing unbound antibodies that might have been present. The antiserum represented a pool of seven bleedings from a single bird which had been hyperimmunized. With group 3 (eight birds), the RBC were not coated in vitro with antibody; instead, an equivalent amount of the same anti-B_2 antiserum was given intravenously to the recipient birds 1–2 hr prior to each inoculation of RBC. A fourth group (eight birds) did not receive antibody but was inoculated with the same quantity of RBC possessing only foreign A_2 isoantigens.

Preimmunization to B antigens: 11 adult G-B1 chickens were given seven weekly inoculations of RBC possessing only foreign B_3 antigens. Subsequently, seven weekly inoculations, given to these birds and nine nonpreimmunized control birds, were made with RBC possessing foreign A_2 and B_3 antigens. Inoculations, given intravenously, consisted of 1 ml of a 25% RBC suspension in saline.

In vitro coating of B antigens with antibody. B-compatible recipients: To study the specificity of passive anti-B antibody in causing an enhanced anti-A response, two groups of 8 wk old chickens received eight weekly inoculations of RBC possessing only foreign A_2 antigens. The B antigens, present on the same RBC, were coated with anti-B antibody. The genotype of the recipient birds was A^1/A^1, B^1/B^2. 16 birds were inoculated with RBC from a donor of genotype A^1/A^2, B^1/B^2. For eight recipients, the RBC were incubated prior to inoculation with hyperimmune anti-B_1 antiserum; for the remaining eight, the RBC were incubated with normal chicken serum. 10 other birds received RBC from a donor of genotype A^1/A^2, B^2/B^2; again only foreign A_2 antigens were present. Five birds received RBC that had been incubated with hyperimmune anti-B_2 antiserum, and five birds received RBC that had been incubated with normal chicken serum.

In vitro coating of A antigens with antibody: The ages and genotypes of the recipient birds, route of immunization, and quantity of RBC inoculated were the same as in the first experiment (passive immunity to B antigens). Donor RBC possessed only foreign A_2 and L_1

[1] Originally developed by A. W. Nordskog, Iowa State University.

antigens. One group (eight birds) received 11 weekly inoculations of the washed RBC. A second group (eight birds) received eight weekly inoculations of RBC that had been incubated for 1–2 hr with hyperimmune anti-A_2 antiserum, followed by three weekly inoculations of RBC that were not coated with antibody. In this experiment the RBC were washed after the incubation period to remove unbound antibodies from the inoculum.

Serological Procedures.—Recipient birds were bled (2.5 ml) at weekly intervals prior to each inoculation, and 1 wk following the last. The titers of anti-A, anti-B, and anti-L hemagglutinins were determined in separate tests with RBC possessing only the relevant foreign antigen. 1 drop of a 2% RBC suspension was added to 0.2 ml of 2-fold dilutions of sera in 0.85% saline, and, after incubation, the degree of agglutination was scored macroscopically. Titers represent the last dilution showing detectable agglutination.

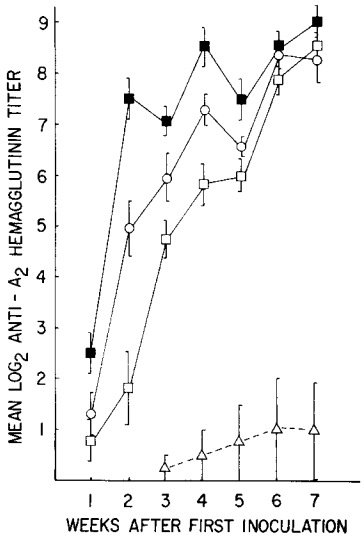

FIG. 1. The immune response to A_2 isoantigens. Recipients were inoculated with RBC possessing only foreign A_2 antigens (△– – –△) or RBC possessing both foreign A_2 and B_2 antigens which were uncoated (□——□), coated in vitro with hyperimmune anti-B_2 antibodies (■——■), or given 1–2 hr after the birds received hyperimmune anti-B_2 antibodies (○——○). Each point represents the mean titer determined 7 days after and immediately preceding weekly inoculation. Vertical bars represent standard errors of the means.

RESULTS

Effects of Passive Immunity to B Antigens.—The results obtained with passively administered anti-B_2 antibody on the production of antibody to A_2 and B_2 antigens are presented in Figs. 1 and 2. In the absence of passive antibody, it is clear (Fig. 1) that B_2 antigens have carrier properties with respect to A_2 antigens. At 7 wk the mean $\log_2$ titer of recipients inoculated only with foreign A_2 antigens was 1.0 (two out of eight recipients responded). 100% of the birds

immunized with both A_2 and B_2 antigens responded (mean titer $\log_2 = 8.5$). Thus, the A_2 antigens have a haptenic relationship to B_2.

Coating the RBC in vitro with anti-B_2 antibodies prior to each inoculation resulted in a significant enhancement of anti-A_2 antibody production (Fig. 1) and a significant suppression of B2 antibody synthesis (Fig. 2). The "helping" effect of B2 antibodies on the response to A_2 antigens was less evident when these antibodies were administered passively 1–2 hr prior to each RBC inocu-

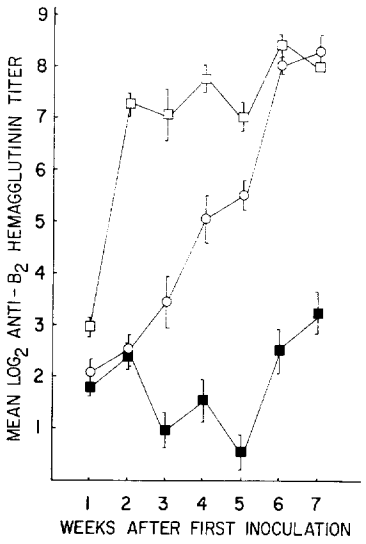

FIG. 2. The immune response to B_2 isoantigens (same recipients as in Fig. 1). Recipients were inoculated with RBC possessing both foreign A_2 and B_2 antigens which were uncoated (□——□), coated in vitro with hyperimmune anti-B_2 antibodies (■——■), or given 1–2 hr after the birds received hyperimmune anti-B_2 antibodies (○——○). Each point represents the mean titer determined 7 days after and immediately preceding weekly inoculation. Vertical bars represent standard errors of the means.

lation (Fig. 1). Furthermore, the immunosuppressive effect of anti-B_2 antibodies administered in this manner was intermediate between the normal response and the profoundly depressed response obtained with in vitro coating (Fig. 2).

Preimmunization to B Antigens.—Since passive B antibody enhances the response to A antigens, it was of interest to determine the anti-A response of birds hyperpreimmunized with B antigens alone. The results of this experiment are depicted in Fig. 3. The anti-A_2 antibody response of birds already maximally engaged in a response to B_3 antigens was as vigorous as the response of nonpreimmunized controls to the B_3 antigens. The A2 antibody titer of this latter group of recipients did not rise significantly until after the B3 antibody titer approached a maximum.

In Vitro Coating of B Antigens with Antibody. B-Compatible Recipients.—Since actively synthesized or passively administered anti-B antibodies enhance the response to haptenic A antigens, it was important to determine whether this effect is nonspecific (clumping or steric alterations in membrane-associated isoantigens) or depends fundamentally on the genetically determined ability of the recipients to discriminate between self and foreign B antigens. The results of two separate experiments in which B^1/B^2 recipients were repeatedly inocu-

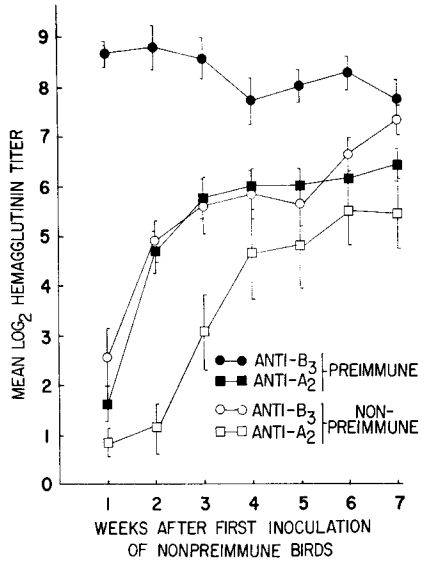

FIG. 3. The immune response to RBC possessing B_3 and A_2 isoantigens of nonpreimmunized birds (open symbols) or birds hyperpreimmunized with B_3 antigens alone (closed symbols). Each point represents the mean titer determined 7 days after and immediately preceding weekly inoculation. Vertical bars represent standard errors of the means.

lated with B-compatible, A-incompatible RBC, coated with B1 or B2 antibodies, failed to demonstrate an enhanced A antibody response; the rare animals which produced anti-A_2 in low titer were randomly distributed among the four groups receiving coated or uncoated cells.

In Vitro Coating of A Antigens with Antibody.—If anticarrier antibody is required for activation of an immune response to a hapten, it should be possible to convert some A determinants into carriers with antibody. Results presented in Fig. 4 indicate that coating RBC possessing foreign A_2 and L_1 antigens with anti-A_2 antibody activates an immune response to A_2; at 8 wk, eight out of eight recipients responded (mean $\log_2$ titer = 3.1). Only two out of eight recipients inoculated with uncoated cells of the same phenotype responded (mean $\log_2$ titer = 1.0). The fact that the mean A2 antibody titer rose 2 log units after one inoculation of uncoated cells at wk 8 suggests that the immune response of

the recipients that was "triggered" with A2 antibody-coated cells was not a maximal response.

Although the differences in mean anti-L_1 antibody titers between the two groups of recipients do not reach statistical significance, there is at least a suggestion that birds inoculated with A2 antibody-coated cells produce more L1 antibody than do birds inoculated with uncoated cells. At 11 wk, six out of eight of the group receiving coated cells produced L1 antibodies (mean $\log_2$

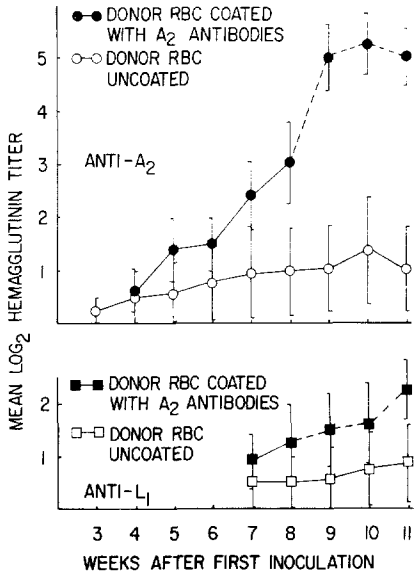

FIG. 4. The immune response to A_2 and L_1 isoantigens of birds inoculated with RBC that possessed both foreign antigens and were either uncoated (open symbols) or coated (closed symbols) with A2 antibodies. From wk 8 both groups received only uncoated RBC (- - -). Each point represents the mean titer determined 7 days after and immediately preceding weekly inoculation. Vertical bars represent standard errors of the means.

titer = 2.3). Only two out of eight recipients of uncoated cells responded to L_1 (mean $\log_2$ titer = 0.9); these were the same two birds that produced anti-A_2 antibodies.

DISCUSSION

Previous studies (10–12) and the foregoing results indicate that RBC isoantigens of chickens have hapten-carrier relationships. Isoantigens determined by the *A*, *D*, and *L* blood group loci are weak immunogens, especially in immature recipients; the majority of birds fail to produce detectable hemagglutinins even after multiple inoculations. Recipients of similar type, however, nearly always produce high titers of hemagglutinating antibodies after immunization with RBC isoantigens determined by the complex *B* blood group locus. The

hapten-carrier relationship between the *A* and *B* system antigens has been deduced from the following observations: (*a*) recipients of the type which fail to respond immunologically to A antigens alone produce anti-A and anti-B antibodies following inoculation with both foreign antigens, and (*b*) to obtain an anti-A antibody response in doubly immunized birds, the foreign A and B antigens must be present on the same RBC used for immunization; if the antigens are on separate RBC, all recipients respond immunologically to B but the response to A is not enhanced. The additional observation that the carrier property of B antigens for A antigens does not function in birds previously rendered tolerant of B emphasizes the necessity of an anti-B immune response for the initiation and completion of the events leading to anti-A antibody synthesis.

The similarities between the cooperation of genetically distinct isoantigens in the immune response and the separate antigenic determinants which comprise synthetic macromolecular hapten-carrier complexes are compelling. Benacerraf and coworkers (18) have demonstrated that certain strains of guinea pigs which fail to produce antibodies to 2,4-dinitrophenyl poly-L-lysine (DNP-PLL)[2] can be immunized with DNP-PLL complexed to bovine serum albumin (BSA), as indicated by the production of antibodies to both DNP-PLL and BSA. The prior induction of immunological tolerance to BSA in this system resulted in a reduction in the immune response to hapten and carrier after subsequent immunization with the complex (8, 18). Rajewsky and coworkers have also demonstrated, with respect to both immunity and tolerance, similar hapten-carrier relationships between B and A subunits of lactic dehydrogenase isoenzymes (6, 7).

How do immunologically distinct isoantigens cooperate in the immune stimulus? One possibility is that foreign B antigens make a unique contribution to the configuration of the A determinants [analogous to the contribution which carrier makes to the hapten (18–20)]. The specificity of the antibodies produced in response to immunization with foreign A and B antigens would be determined largely by immunogenic determinants comprising a portion of both the A and B sites. In the absence of a foreign B antigen, we would thus assume that the interaction on the RBC membrane between the B antigen and the foreign A antigen does not result in a configuration which is immunogenic in B-compatible recipients. We believe, however, that the existence of immunogenic "hybrid" determinants resulting from the interaction of foreign A and B antigens is unlikely because (*a*) the anti-A and anti-B antibodies produced by immunization with RBC which possess foreign A and B antigens can be completely absorbed after the sequential addition of RBC which possess only the specific A and only the specific B antigens (added in that order or vice versa);

[2] *Abbreviations used in paper:* BSA, bovine serum albumin; DNP-PLL, 2,4-dinitrophenyl poly-L-lysine.

such absorbed sera fail to agglutinate RBC which possess both foreign A and B antigens; and (*b*) specific tolerance to foreign B antigens abolishes the carrier property for A antigens, as mentioned previously.

Another possible mechanism for the cooperation of isoantigens in the immune stimulus is that anti-B antibody, produced in response to and following combination with B antigens, sterically alters A determinants on adjacent areas of the RBC membrane into a more immunogenic configuration. Our results obtained with RBC, possessing foreign A antigens, coated with specific anti-B antibodies prior to inoculation into B-compatible recipients do not support this possibility. Under these conditions, the immunogenicity of A antigens was not enhanced as a result of the postulated steric alterations brought about by the interaction of B antibody with B antigens.

Considerable attention is being directed to the role of antibody in the mediation and control of the immune response. Several theories of antibody formation propose that natural specific preformed antibody is the important factor in the recognition of immunogenicity (1, 4, 5, 21). Although Jerne, in his original formulation (1), focused attention on spontaneously circulating antibody, current emphasis has been placed on the results of studies which indicate the presence of globulin molecules (receptors) on the surface of lymphoid cells (22–25) which have characteristics of specific antibody (3, 26, 27). There is evidence which suggests that these cellular receptors are synthesized by the cell and possess the same specificity as the antibody produced by the cell following antigenic stimulation (27, 28). In simplest terms, antibody formation may result from the specific interaction of receptor with an antigenic determinant (3, 5, 21).

A major difficulty with theories which emphasize a role for preformed specific natural antibody (cell-bound or circulating) in the activation of an immune response is the large body of data which indicate that passively administered specific antibody usually depresses the immune response (16, 29–33). There is an apparent paradox in the supposition that the interaction of antibody with an antigenic determinant is crucial for antibody synthesis unless it is assumed that circulating antibody to a determinant, besides competing with receptors for the determinant, is essential for antibody formation to other determinants on the same particle.

The interpretation of some studies has been that immunogenic particles must contain both hapten and carrier (3, 34). Our studies with RBC isoantigens also suggest that two types of determinants are involved in activation of an immune response, one type being haptenic and the other type being carrier. Since B antigens, which are composed of multiple antigenic factors, are immunogenic, it follows that some B factors behave as carriers and others as haptens. From our observations we believe that an antigenic determinant qualifies as a carrier

only if it is foreign and is coated with antibody.[3] We assume that the determinant coated with antibody does not activate the production of antibody to itself, either because it is effectively masked by antibody (thereby blocked from interaction with cellular receptor) or because it is attached to a non–antibody-producing cell (macrophage). The macrophage may play an essential role in presenting antigenic determinants to lymphocyte receptors, and involvement of the macrophage may only come about by means of anticarrier antibody. Thus the total circulating pool of antibodies produced in response to a complex antigen such as B reflects the sum total of the activities of individual antibody-forming cells, i.e., the response of individual cells to uncoated determinants (haptens). The A antigens probably represent a narrower range of antigenic determinants, and immature recipients may lack preformed "carrier" antibody to these determinants.

A likely explanation for the enhanced response to A antigens when some B determinants are coated in vitro with B antibody is that the number of carrier determinants has been increased; hence, at an early stage of the immune response, a greater number of A determinants is presented to the lymphocyte receptors. A similar interpretation can be given to the enhanced antihapten response by birds preimmunized with carrier, where the coating of B determinants occurs in vivo. In contrast, the response to A does not develop significantly in birds responding to uncoated B determinants until a sufficient number of these determinants are converted to carriers by actively synthesized B antibody. Once a B determinant becomes coated with antibody, it is no longer an effective immunogenic stimulus for itself but becomes the essential factor for an immune response to uncoated B or A determinants. According to this interpretation it is not at all paradoxical that passively administered hyperimmune anti-B antibody (against most B factors) should suppress B antibody synthesis and simultaneously enhance antibody synthesis to other determinants (i.e., A). Furthermore, this interpretation could explain findings by other workers that small quantities of specific antibody may enhance the immune response to an antigen (14–17, 35). In this situation, a sufficient number of determinants are coated and converted to carriers for the majority of uncoated determinants, which, as a result, are rendered immunogenic.

The findings of Henry and Jerne (15) that 19S but not 7S antibodies enhanced the response of mice to sheep RBC can be accommodated into this model. Not only may the first antibodies formed following primary immunization be of the 19S class, but they may also be more restricted in their specificity

[3] Although naturally occurring B antibodies have not been demonstrated within our lines, we have found B-specific antibodies in low titer in serum from another breed. It is possible, therefore, that anti-B antibodies do occur naturally in our lines, but go undetected because of insufficiently sensitive tests.

than are hyperimmune antibodies of the 7S class. Presumably a large number of antigenic determinants on the surface of sheep RBC are foreign to the mouse, and the first antibodies formed are specific for a minority of these. Their results in which over-all enhancement was obtained using a quantity of passive 19S antibody in addition to an amount of 7S antibody, which alone was immunosuppressive, are not immediately predictable from this model. The inconsistency may be in the fact that mouse 19S and 7S antibodies differ markedly in terms of hemolytic efficiency and avidity.

It is of interest that for a given quantity of B antibody, in vitro coating of the B determinants was more effective in the suppression of B and the enhancement of A antibody synthesis than when the B antibodies were administered prior to antigen. The simplest interpretation is that in vitro coating resulted in a greater number of B determinants being masked by antibody. Thus, the prediction would be that a maximal anti-A response would occur when all foreign B determinants are coated.

If, as postulated, A antigens are nonimmunogenic because of a deficiency of natural carrier antibody (with affinity for one or more A determinants), it should be possible to enhance their immunogenicity by coating some A determinants with passive antibody. In the experiment in which birds received coated A antigens, the response was significantly greater than among recipients which received uncoated cells (Fig. 4). We believe that the gradual increase in the anti-A_2 antibody titer up to the eighth week indicates that the A2 antibodies used for coating combined with a sufficient number of the A_2 determinants to cause partial suppression. A sharp increase in the anti-A_2 response among these recipients was observed 1 wk following the inoculation with uncoated cells, an observation which at least supports this interpretation. It is of further interest to note that the L1 antibody titer was higher among the recipients of A2 antibody-coated cells. Although the differences in L1 titers between the two groups of recipients did not reach statistical significance, there was at least a suggestion that the antibody-coated A_2 determinants were serving as carriers for uncoated L_1 determinants as well.[4]

One possible explanation for the finding that the L1 antibody response was not greater than the anti-A_2 response when the A antigens were coated, as would have been predicted from the results with B and A antigens, is that a smaller proportion of the A_2 determinants were coated with antibody. If the A2 antibody response had not been enhanced to such a degree (i.e. if more A_2 dedeterminants had been coated), the anti-L_1 response might have been enhanced to a greater extent. On the other hand, the results obtained from this experiment, with A and L antigens and anti-A antibody, appear to be more closely analogous

[4] When this experiment was repeated, the A2 and L1 antibody titers of recipients of A2 antibody-coated RBC were significantly greater at 10 wk than the titers found among recipients of uncoated RBC.

to the response of birds receiving uncoated B and A antigens, where we assume that some B determinants are coated by naturally occurring anti-B antibody. On this basis, the L1 antibody titer would only begin to rise after the A2 titer was significantly elevated.

Other experiments carried out in this laboratory[5] show that tolerance to B antigens is lost by birds which receive RBC possessing the foreign B antigens as well as foreign A antigens coated with anti-A antibody. Here we assume that the coated A determinants serve as a carrier for B (which behaves as hapten in B-tolerant animals owing to lack of carrier antibody; see also ref. 36).

Our analysis of the cooperative interaction of isoantigens in the events which culminate in humoral antibody formation lead us to the following generalizations.

1. In order for a particle to be immunogenic it must have two types of determinants, a haptenic type and a carrier type. The carrier determinant is coated with antibody while the haptenic determinant is not. In the course of an immune response there is a progressive conversion by antibody of haptens to carriers. When all of the haptenic determinants have been converted to carriers, the particle is no longer immunogenic. This may be the "feedback" mechanism which controls antibody synthesis, and would explain the plateau phenomenon in the normal immune response.

2. The ability of a normal animal to respond to an antigen stems from its prior immune responses to haptens. Thus, an animal responds to antigenic determinant *a* because it possesses antibody to determinant *b* derived from a prior immune response to another antigen possessing the same determinant *b* or a similar determinant *b'*.

3. Assuming that the first immune response by an animal requires, in addition to lymphocyte receptors, humoral antibody to an antigenic determinant, one need only postulate that this "primordial" antibody is maternally derived (this seems to us to be the most likely source). Taken to the extreme, a maternally derived antibody to a *single* antigenic determinant (possibly a determinant widespread in nature) would be sufficient for the eventual development of immunological maturity of the individual. It follows from this that the capacity of an individual to respond to different antigens develops at different times. This is one reason why we regard the possibilities that a lymphocyte receptor functions as carrier antibody or that virgin lymphoid cells "leak" carrier antibody as less likely [for further discussion, see Cohn (21)]. These may have been the original sources of carrier antibody, which have been replaced, during evolution in an antigenic environment, by the more efficient maternal transmission. A strong argument for the essential role of maternal antibody in the development of immunological maturity is provided by the findings of Kerman et al. (37) and Segre and Kaeberle (38).

[5] Unpublished observations.

4. A secondary type rather than a primary type immune response to a determinant (hapten) would occur following the first exposure to the determinant if sufficient numbers of carrier determinants are present on the inoculated particles. Secondary immune responses may be due to memory cells which are the mitotic descendants of a small number of antigen-stimulated cells. A secondary type response following the *first* encounter with an immunogenic particle would cast doubt on the assumption that cells with antihapten receptors comprise a small proportion of the total population of immunocytes. Secondary immune responses of this type are easily reconciled with theories based on multipotentiality of antibody-forming cell precursors.

SUMMARY

In chickens, erythrocyte isoantigens have hapten-carrier relationships. Specific anticarrier antibody depresses the immune response to the carrier and enhances the immune response to the hapten. Antigenic determinants of "haptenic" isoantigens behave as carriers if they are coated with specific antibody. It is postulated that every humoral antibody response involves the cooperation of a carrier with a hapten and the progressive conversion by antibody of haptens to carriers. Thus a carrier is viewed as an antigenic determinant which is coated with antibody. The antibody-forming cell only synthesizes antibody to the uncoated haptenic determinants. The consequences of this interpretation for the development of immunological maturity and the secondary immune response are discussed.

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