INDUCTION OF IMMUNITY AND TOLERANCE IN VITRO BY HAPTEN PROTEIN CONJUGATES

III. Hapten Inhibition Studies of Antigen Binding to B Cells in Immunity and Tolerance*

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A critical stage in the induction of antibody responses is the interaction of antigen molecules with the surface receptors of immunocompetent cells. This interaction may result in either immunity, or its opposite, immune tolerance. The differences between the pattern of antigen binding to nonthymus-dependent lymphocytes (B cells)[1] in immunity and tolerance are not fully understood. Recently, attempts were made to study this problem in tissue culture, using dinitrophenylated polymeric flagellin (DNP POL) as antigen (1). The interaction of antigen molecules with cell membranes may be readily studied in dissociated lymphoid cell populations in vitro, where antigen concentrations are relatively uniform and catabolism negligible compared with the situation in vivo (2). Furthermore, since the response to DNP POL in vitro does not require the participation of either thymus-dependent lymphocytes (T cells) (1, 3) carrier-reactive cells, or macrophages (4, 5), only the pattern of reaction of DNP POL molecules with B cells determines the ensuing immunological events. It was found that the number of 2,4-dinitrophenyl (DNP) haptenic groups present per monomeric unit of the DNP POL molecule (i.e. the epitope density) dictated its capacity to induce either immunity or tolerance (1). Antigen molecules with few DNP groups immunized, but never induced tolerance, whereas highly conjugated DNP POL induced tolerance, but never immunity (1). Based on these observations, a mechanism of B cell signal discrimination between immunity and tolerance was proposed, based on the number

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; ATXBM, adult thymectomized, irradiated, and bone marrow-protected mice; B cells, nonthymus-dependent lymphocytes; dansyl, 5-dimethylamino-1-naphthalene sulfonyl determinant; DNP, 2,4-dinitrophenyl determinant; DNP HgG, dinitrophenylated human gamma globulin; $DNP_{12}HgG$, DNP HgG with 12 moles DNP/mole of HgG; DNP lysine, ϵ-2,4-DNP-lysine; DNP POL, dinitrophenylated polymeric flagellin; DRC, donkey red cells; FCS, fetal calf serum; HgG, human immunoglobulin G; SRC, sheep red cells; T cells, thymus-dependent lymphocytes.

of simultaneous reactions of antigenic determinants with B cell receptor molecules in a given region of the cell membrane. Many such interactions cause tolerance, whereas a smaller number causes immunity.

The present communication describes experiments in which the binding reaction of DNP POL with receptor antibody molecules was studied by the use of other DNP compounds as competitive inhibitors. Both the immunogenic manner of binding of DNP POL to B cells, and the tolerance-inducing mechanism, were studied using DNP compounds of varying valency and molecular structure. These studies have further delineated differences between the dynamic antigen receptor interactions in immunity and tolerance.

Materials and Methods

Mice.—CBA/H/Wehi mice aged 70–140 days were used for tissue culture.

Tissue Culture.—The method described by Feldmann and Diener (5) was used. Eagle's minimal essential medium was obtained from Grand Island Biological Co., Grand Island, N. Y. It was supplemented with 100 μg/ml of streptomycin, 100 units/ml of penicillin G, and 5% fetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne, Australia). Cultures were placed in a humidified incubator at 37°C in a gas flow of 10% CO_2, and either 7 or 20% O_2 in N_2 for 3, 4, or 5 days. Since similar relative results were obtained at all these times, only the results of assays at day 4 are shown. Spleen cell suspensions were prepared as described elsewhere (3).

Antigens.—DNP POL was prepared using polymeric flagellin from *Salmonella adelaide*. A Cohn fraction II human gamma globulin (HgG) preparation was obtained from Commonwealth Serum Laboratories, and further purified by batch extraction on diethylaminoethyl cellulose (6). It was dinitrophenylated by the method of Eisen (7). ϵ-2,4-DNP-L-lysine (DNP lysine) was obtained from Mann Research Laboratories, Inc., New York. "Dansyl" chloride (5-dimethylamino-1-naphthalene sulfonyl chloride) was obtained from Calbiochem, Sydney. Dansyl proteins were prepared by the method of Paul et al. (8).

Donkey red cells (DRC) were stored in Alsever's solution for 1 wk before use.

Enumeration of Antibody-Forming Cells.—Cells forming antibody (AFC) to sheep red cells (SRC), DRC, or DNP were detected using the method of Cunningham and Szenberg (9). The number of AFC to DNP was determined by the separate assay of 0.1-ml aliquots of the cultured cell suspension with both SRC and SRC coated with rabbit anti-SRC Fab′ conjugated with DNP (10).

Thymectomy.—Adult thymectomy was performed as described by Miller (11). Irradiation and bone marrow reconstitution was performed as described elsewhere (3). The resultant mice (adult thymectomized, irradiated, and bone marrow-protected mice [ATXBM]) were used as described previously (3).

Macrophage Depletion.—The active adherence column technique of Shortman et al. (12) was used, which provides a lymphocyte preparation extensively depleted of phagocytic cells.

RESULTS

Inhibition of the Anti-DNP Response to DNP POL In Vitro.—Since DNP POL immunizes B cells directly in the absence of helper cells (1, 3, 4), the mechanism of binding of this antigen molecule to B cell receptors bears much more relevance to the actual mechanism of B cell immunization than is the case with antigens whose antibody responses require the participation of helper

cells, such as T cells and macrophages. Since the antibody response to a defined haptenic determinant is being measured, it is possible that nonimmunogenic forms of the same determinant may competitively inhibit the binding of DNP POL which immunizes B cells. DNP compounds of varying valency and physical structure were used as competitive inhibitors of the anti-DNP response. As univalent (one DNP) DNP compounds, ε-2,4-DNP-L-lysine and a preparation of dinitrophenylated human gamma globulin (DNP HgG) containing an average of 0.3 mole DNP/mole of HgG were used. Poisson analysis shows that there would be only 4% of DNP HgG molecules with more than one DNP group. Multivalent DNP HgG with 12 moles DNP/mole of HgG ($DNP_{12}HgG$) was used as a DNP compound of approximately the same diameter as the receptor on B cells, a monomeric IgM molecule (13). Nonimmunogenic DNP_3POL (1) was employed as a multivalent and polymeric DNP compound.

Since these compounds do not induce anti-DNP responses in unprimed spleen cells in vitro, (unpublished data and reference 1), they can be used as competitive inhibitors. However, rather than acting as competitive inhibitors, it was possible that these agents may inhibit the response to DNP POL by inducing tolerance. It was thus necessary to investigate the capacity of these DNP compounds to induce DNP-specific B cell tolerance. A 6 hr preincubation at 37°C, as employed in studying B cell tolerance to POL (14), was used. The results shown in Fig. 1 demonstrate that neither DNP lysine, $DNP_{0.3}HgG$, nor $DNP_{12}HgG$, even at high concentrations, induce B cell tolerance under these conditions, unlike DNP_3POL which induces tolerance at concentrations of 10 μg/ml or higher. The slight diminution of responsiveness with 10^{-3} M DNP lysine, or 10^{-4} M DNP HgG were not specific since a similar partial suppression of the DRC response occurred in the same cultures.

The capacity of the four DNP compounds to inhibit the anti-DNP response of normal CBA spleen cells to 100 ng/ml DNP_1POL was investigated. The pooled data from nine experiments adjusted to percentages are shown in Fig. 2. DNP lysine and $DNP_{0.3}HgG$ were weak inhibitors, since high concentrations ($3–8 \times 10^{-6}$ M) were needed to cause a 50% inhibition of the anti-DNP response to DNP_1POL. $DNP_{12}HgG$ was a much more efficient inhibitor, since only 3×10^{-8} M was needed for 50% inhibition. An even more efficient inhibitor was DNP_3POL (2×10^{-9} M for 50% inhibition).

These experiments indicated that the structure and valency of the DNP compound was important in determining its capacity to inhibit the anti-DNP response of spleen cells to DNP_1POL, with univalent compounds being relatively inefficient compared with multivalent compounds. Although the presence of multiple determinants, permitting the formation of much more stable multiple bonds to DNP- specific receptors, was the most obvious reason for the markedly different inhibitory capacity of DNP_3POL or $DNP_{12}HgG$, there were other possible reasons. For example, it was conceivable that $DNP_{0.3}HgG$

or DNP lysine may bind to T cells or phagocytes in the spleen cell suspensions much more than $DNP_{12}HgG$ or DNP_3POL do. To exclude this possibility, competitive inhibition experiments as outlined above were performed using T-depleted spleen cell populations (ATXBM spleen) or macrophage-depleted

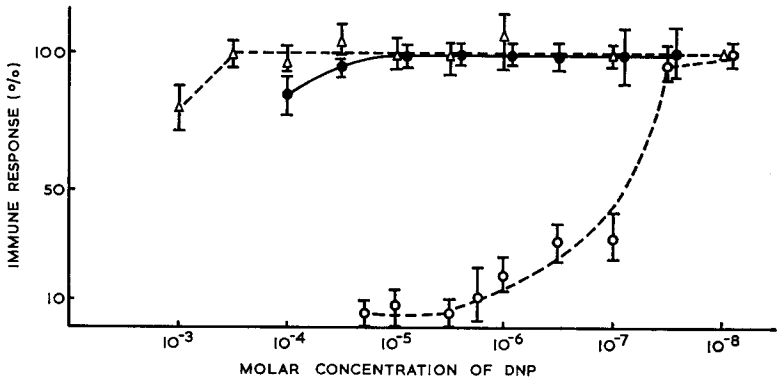

FIG. 1. Capacity of DNP compounds to rapidly induce tolerance at 37°C in vitro. △, DNP lysine; ●, $DNP_{12}HgG$; ○, DNP_3POL. Data pooled from four experiments. Each value represents arithmetic mean of six to eight cultures ± standard error of the mean. The response to DRC was monitored throughout and was normal except for the very highest concentrations of DNP lysine or DNP HgG.

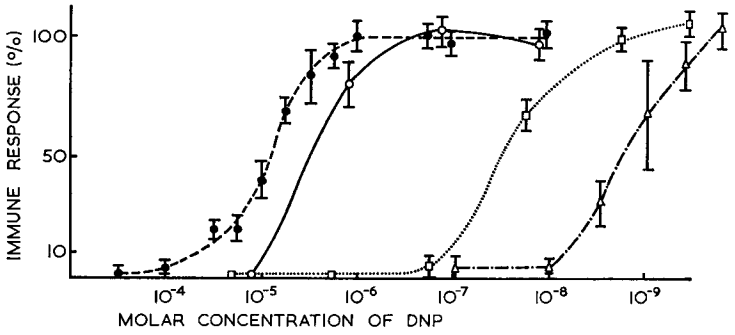

FIG. 2. Inhibition of the anti-DNP response to DNP_1POL. Inhibitors were added before the immunogen and were present for the 4 day duration of culture. The response to DRC in all the cultures was normal. Data pooled from nine experiments. Each value represents arithmetic mean of 12–24 cultures ± standard error of the mean. ●, DNP lysine; ○, DNP_3HgG; □, $DNP_{12}HgG$; △, DNP_3POL.

suspensions (adherence column filtrate) from CBA spleen. Table I demonstrates that neither T cell or macrophage depletion influenced the results, indicating that the binding of DNP compounds to cells other than B cells could not have been the reason for the variations in blocking capacity.

The culture medium used for in vitro culture contains 5% FCS. Thus it

contains 1–2 mg/ml of bovine serum albumin. Since albumin molecules are known to bind DNP conjugates (15), it was possible that some of the difference in inhibitory efficiency of different DNP conjugates may reside in varying degrees of binding to the serum albumin in the culture medium. Thus the competitive inhibition experiments were performed in a serum-free medium. These experiments were rendered possible by the capacity of DNP POL to immunize mouse spleen cells rapidly at 4°C (16). Thus various concentrations of the DNP inhibitors were added to chilled and washed normal spleen cells in serum-free medium, 30 min before the addition of the immunogen. 2 hr later the cells were washed in normal medium and cultured without the addition of any further DNP antigen, but with DRC as a control for the function of the cells. The pooled results of these experiments are shown in Fig. 3. The

TABLE I

Competitive Inhibition of the Anti-DNP Response in T- and Macrophage-Depleted Spleen Cell Populations

Cells	Concentration of DNP conjugates giving 50% inhibition			
	DNP lysine	$DNP_{0.3}HgG$	DNP_{12} HgG	DNP_3POL
	M	M	M	M
Normal spleen	8×10^{-6}	3×10^{-6}	3×10^{-8}	2×10^{-9}
T-depleted spleen	7×10^{-6}	2×10^{-6}	3×10^{-8}	2×10^{-9}
Macrophage-depleted spleen	8×10^{-6}	3×10^{-6}	2×10^{-8}	3×10^{-9}

Values are the averages of three experiments, in which the 50% end point was determined by visual estimation of the inhibition curves. T-depleted spleen cells came from ATXBM mice and macrophage-depleted spleen cells were obtained by glass bead column filtration (12).

same marked difference between the blocking capacity of monovalent and multivalent DNP conjugates was found as described in Fig. 2. However, if the competitive inhibition experiments were performed at 4°C rather than 37°C the difference between $DNP_{12}HgG$ and DNP_3POL was much less apparent.

Highly conjugated DNP proteins tend to aggregate (7). It was possible that the difference between the inhibitory capacities of $DNP_{0.3}HgG$ and $DNP_{12}HgG$ may have been due to the aggregation of the latter conjugate perhaps forming a linear molecular aggregate resembling polymeric flagellin. This possibility was tested using dansyl conjugates of HgG which also aggregate. A preparation of $DNP_{0.3}dansyl_8HgG$ was weakly inhibitory (Table II), excluding aggregation of the competitive inhibitors as a major influence on the inhibitory capacity of DNP HgG.

Inhibition of DNP-Specific B Cell Tolerance.—While it has been previously ascertained that immunity to DNP POL did not require the presence of cells other than B lymphocytes (1), the requirement for T cells in the induction of tolerance in vitro to DNP POL was not known. The induction of tolerance in normal CBA and ATXBM spleen was thus compared. Incubation of either

ATXBM or normal spleen with 100 μg/ml of DNP_3POL for $5\frac{1}{2}$ hr at 37°C induced DNP-specific tolerance (Table III). Thus only the reaction of DNP_3POL with the B cells surface influences the induction of B cell tolerance, and it was therefore possible to use DNP conjugates to investigate the mechanism of binding of antigen molecules to the B cell membrane in tolerance without the need to consider the interactions of antigen with other cells.

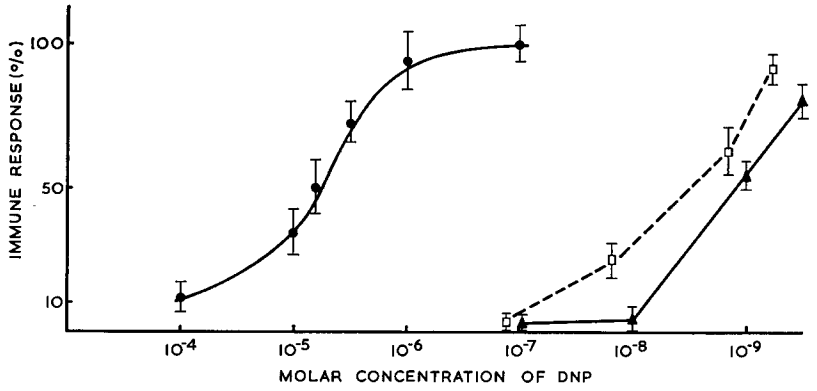

FIG. 3. Inhibition of the anti-DNP response to DNP_1POL. The inhibitors and immunogen were present for only 2–2½ hr at 4°C. Period of culture was 4 days. ●, DNP lysine; □, $DNP_{12}HgG$; ▲, DNP_3POL. Data pooled from three experiments. Each value represents arithmetic mean of 4–12 cultures ± standard error of the mean.

TABLE II

Relative Capacity of Mixed DNP Dansyl Conjugates to Inhibit Anti-DNP Responses

Conjugate	Concentration giving 50% inhibition
	M
$DNP_{0.3}HgG$	3×10^{-6}
$DNP_{0.3}dansyl_8HgG$	2×10^{-6}
$DNP_{12}HgG$	3×10^{-8}

Data pooled from three experiments was plotted, and line of best fit visually estimated to yield a 50% endpoint. $Dansyl_8HgG$ did not inhibit the anti-DNP response at nontoxic concentrations.

The results in Fig. 1 indicate that neither DNP lysine, $DNP_{0.3}HgG$, nor $DNP_{12}HgG$ induce tolerance to DNP in vitro under conditions where DNP_3POL does. DNP_1POL, regardless of concentration, does not induce tolerance (1). Thus these compounds were nontolerogenic and could be used to inhibit the induction of tolerance. Various concentrations of inhibitors were added to normal spleen cells at 4°C, 30 min before the addition of 20 μg/ml DNP_3POL. The cells were incubated for 6 hr at 37°C before being washed and challenged with 100 ng/ml DNP_1POL in vitro. Fig. 4 illustrates the pooled results ob-

tained in four experiments. These have been standardized so that the partially tolerant state, which ranged from 10–20% of the optimal response to DNP POL, was taken as zero, and the normal response as 100%. Neither DNP lysine, $DNP_{0.3}HgG$, nor $DNP_{12}HgG$ blocked the induction of tolerance; whereas DNP_1POL at DNP concentrations of more than 10^{-7} M prevented the induction of tolerance. The concentration of flagellin in DNP_1POL required to inhibit tolerance by 50% was approximately the same as in the DNP_3POL tolerogen.

TABLE III

Induction of B Cell Tolerance in T-Depleted Spleen Cells

Cells	Incubation	Antibody response (AFC/culture ± SE)	
		DNP	DRC
Normal	No antigen	840 ± 120	2860 ± 105
	100 μg/ml DNP_3POL	110 ± 50	2420 ± 260
ATXBM	No antigen	730 ± 210	105 ± 30
	100 μg/ml DNP_3POL	130 ± 45	160 ± 70

Either normal or ATXBM spleen cells were incubated for 5½ hr at 37°C as indicated above, washed twice through FCS, and cultured with 3×10^6 DRC and 100 ng of DNP_1POL for 4 days. Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Four other similar experiments confirmed these results.

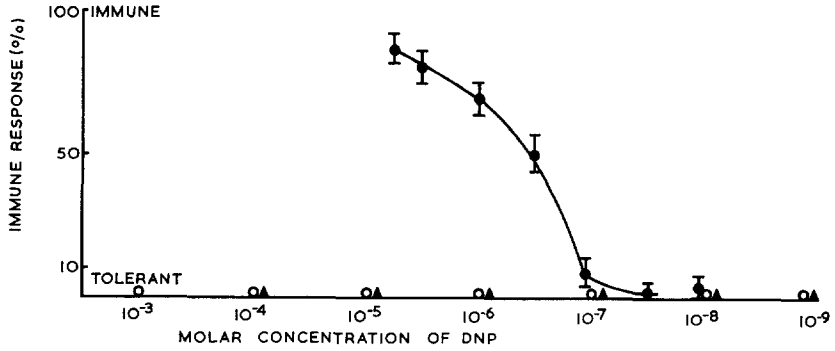

FIG. 4. Inhibition of the induction of DNP-specific B cell tolerance to DNP_3POL. ○, DNP lysine; ▲, $DNP_{12}HgG$ or DNP_8HgG; ●, DNP_1POL. Data pooled from six experiments. Each value represented the arithmetic mean of 12–24 cultures ± standard error of the mean.

DISCUSSION

Cultures of dissociated lymphoid cells provide a uniform antigenic environment for studying the kinetics of the interaction of cell membrane receptors with antigenic determinants. Using DNP POL, an antigen which immunizes

B cells directly (1, 3, 4), the initial key event in the stimulation antibody production is its reaction with B cell receptors. With this antigen complications due to involvement of T cells or macrophages are circumvented.

Since the B cell precursor of DNP-specific AFC possesses receptors which recognize the DNP determinant, only DNP compounds would react with these receptors. Thus it was possible to use DNP conjugates to investigate the nature of the binding of DNP POL to B cell receptors in immune induction. Compounds which bind to DNP receptors may inhibit anti-DNP antibody production by various means, either by inducing tolerance (1), receptor modulation (17), or by irreversible (18–19) or reversible (20–22) occupation of the receptor site, thereby preventing immunization. It was thus necessary to investigate the immunological properties of the DNP compounds to be used as inhibitors of the immune response. It was found that neither DNP lysine[2] nor DNP HgG[2] induced tolerance within 6 hr at 37°C at nontoxic doses (Fig. 1), unlike DNP_3POL. These agents have been previously reported to be nonimmunogenic and thus could be used as competitive inhibitors of the response to DNP_1POL. Fig. 2 demonstrates that DNP lysine, DNP HgG, and DNP_3POL at the appropriate concentrations inhibited anti-DNP responses to DNP_1POL. This suppression may have been due to true competitive inhibition for receptor sites, but other interpretations were possible. The first possibility is that a state of partial tolerance may be slowly induced by DNP lysine, or DNP HgG if they are present in culture for prolonged periods of time. To circumvent this and other difficulties, the competitive inhibition experiments were modified. The DNP inhibitors, and the immunogen were only present for a brief period of time (2–2½ hr), in the cold (0–4°C), conditions under which B cell tolerance is much less likely to occur (5, 14). These modifications were possible since brief incubation with DNP POL immunizes spleen cells (16). The experiments of brief duration (Fig. 3) more truly resemble competitive inhibition studies as applied to enzyme reactions than the longer term experiments (Fig. 2). Despite the differences in experimental design, the results were essentially similar.

Recently Taylor et al. (17) made the important observation that the localization of membrane immunologlobulin is dynamic, varying with experimental conditions. Highly conjugated DNP proteins could, like anti-immunoglobulin sera, cause the receptors to aggregate at one pole of the lymphocyte forming "caps," with subsequent pinocytosis of receptors. The above membrane rearrangements involved metabolic processes, as they did not occur at 4°C or in the presence of metabolic poisons. Cap formation and subsequent pinocy-

[2] It should be noted that the failure of monovalent DNP compounds to induce B cell tolerance, in conditions where DNP_3POL does, excludes the proposed mechanism of signal discrimination of Brestcher and Cohn (23). They had suggested that interaction with one combining site of a receptor antibody molecule mediated tolerance, whereas interaction with both combining sites initiated immunity.

tosis may have explained the immune inhibition caused by $DNP_{12}HgG$ or DNP_3POL at 37°C, but not at 4°C or by DNP lysine or $DNP_{0.3}HgG$ at any temperature. Thus the results obtained in Fig. 3 were not due to tolerance, or receptor modulation, and hence were due to competitive inhibition of the binding of, and subsequent response to, DNP_1POL.

The number of DNP groups present per molecule of inhibitor (or its "valency") markedly influenced their capacity to act as inhibitors. Monovalent compounds such as DNP lysine and $DNP_{0.3}HgG$ inhibited the response to DNP_1POL very poorly compared with multivalent $DNP_{12}HgG$ or DNP_3POL (Figs. 2 and 3). There were several possible reasons for this difference. The possibilities of tolerance induction or cap formation (17) by multivalent conjugates have already been discussed and excluded by the results shown in Fig. 3. Aggregation of multivalent conjugates of $DNP_{12}HgG$, perhaps yielding tolerogenic aggregates was excluded as an important factor by the use of mixed DNP dansyl conjugates, which also have a tendency to aggregate (Table II). Differences in the degree of binding of different DNP compounds to the serum albumin present in the normal culture medium was demonstrated not to be a factor of importance since virtually identical results were obtained when all the DNP conjugates only interacted with cells in serum-free medium (Fig. 3). Thus the marked difference (100–1000-fold) in inhibitory capacity of multivalent compared with monovalent conjugates was due to the formation of multiple antigen-receptor bonds, in keeping with the well-known far greater association of bivalent antibody (such as the B cell receptor) for multivalent antigens (24).

The possible modes of binding of the DNP compounds used are represented in Fig. 5. The far greater inhibitory capacity of $DNP_{12}HgG$ than $DNP_{0.3}HgG$, due to the formation of two bonds to surface receptors, could be due to the formation of bonds with both combining sites of a single B cell receptor antibody molecule. This interpretation seems much more probable than the alternative, of two bonds to adjacent receptors, since the diameter of DNP HgG can only be about the same as that of receptor antibody molecule.

DNP_3POL was a more efficient inhibitor of the response to DNP_1POL than was $DNP_{12}HgG$ (Figs. 2 and 3), indicating that its mechanism of binding differed from $DNP_{12}HgG$. Since $DNP_{12}HgG$ makes two bonds to combining sites, DNP_3POL must make more bonds, at least three. Thus dinitrophenylated polymeric flagellin makes bonds with more than one receptor antibody molecule (see below).

Thus data presented here, taken together with prior results (1, 3, 4), may be fitted into a mechanism of receptor antibody-antigen interaction which directly immunizes B cells. The first conclusion is that an antigen molecule must bind to both combining sites of a receptor antibody molecule, greatly increasing binding efficiency. This is the most likely explanation for the difference between the capacities of monovalent and multivalent DNP HgG to inhibit the

antibody response. If this occurs, then the receptor antibody molecule would be "stretched" causing conformational changes in the invariant part of the receptor, which may, as Bretscher and Cohn (23, 25) have pointed out, be a convenient method of signal transmission from the cell membrane, initiating antibody production.

The second conclusion is that B cells are not immunized by interactions at a single molecule of receptor antibody. This conclusion is based chiefly on the nonimmunogenicity of $DNP_{12}HgG$ in normal spleen cells (1), and correlates well with the described pattern of binding of these molecules to B rosette-forming cells (16), where DNP HgG, but not DNP POL, is rapidly lost from the cell surface at 37°C. The basis for the elution of DNP HgG from the B cell surface at 37°C is the rapid turnover of B cell receptors, recently discovered by Wilson, Nossal, and Lewis (26) and Cone et al. (27). This rapid turnover of

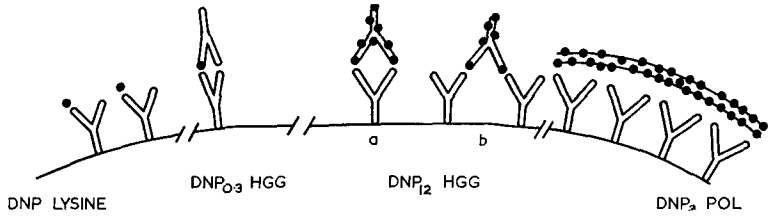

FIG. 5. Possible modes of interaction of DNP lysine, DNP HgG, and DNP_3POL with surface receptors of B cells. Note the situations with $DNP_{12}HgG$. Two modes of interaction may occur different from that which occurs with $DNP_{0\text{-}3}HgG$. The most likely of the two possible interactions are two bonds with the one receptor antibody molecule.

cell receptors induces a kinetic component into models of antigen-receptor interaction in immunity or tolerance. If it is assumed that receptors are randomly released from the membrane, then the essential multipoint binding to the B cell surface may partly reflect the requirement for an adequate duration of antigen-receptor interaction for an immunological "signal" to induce the biochemical changes of immunity.

The mechanism of antigen binding to DNP-reactive B cells in the process of tolerance induction was also investigated using DNP conjugates. To study antigen binding in tolerance, nontolerogenic DNP conjugates were used (Fig. 1). Of the conjugates used, only DNP_1POL inhibited the induction of tolerance, whereas DNP lysine, $DNP_{0\text{-}3}HgG$, and $DNP_{12}HgG$ did not. This contrasts with the results obtained in studying immune induction (Figs. 2 and 3), where all these conjugates were effective, and implies that a tolerance-inducing antigen must bind more avidly to B cell membranes than an immunogen. Since it has already been demonstrated that multiple simultaneous bonds between several antigenic determinants and cell-bound receptor antibody molecules are necessary for immunization, a greater density of such interactions must

occur in the induction of tolerance. The rapid turnover of B cell receptors at 37°C, with the consequent loss of $DNP_{12}HgG$ from the cell surface, seems the most likely reason for the incapacity of $DNP_{12}HgG$ to either induce B cell tolerance itself within 6 hr, or to prevent the induction of B cell tolerance. Cap formation and pinocytosis of receptors, which according to the studies of Taylor et al. (17) should occur with $DNP_{12}HgG$ and DNP POL, cannot be the explanation for the inhibition of tolerance, as the former was ineffective.

The data presented in this communication fits in well with the model of B cell signal discrimination proposed recently based on the very different im-

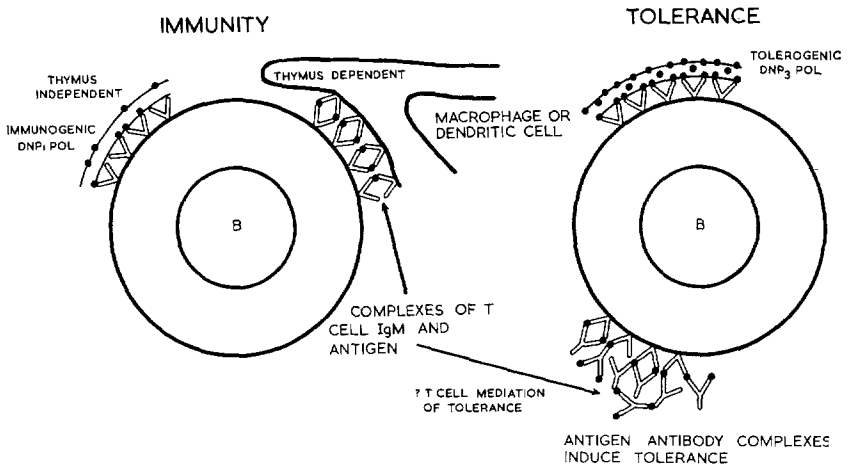

FIG. 6. Representation of the mechanism of B cell signal discrimination, based on the epitope density and competitive inhibition data obtained using DNP POL. Note that the lattice of DNP determinants on immunogenic DNP POL may resemble the lattice of T cell IgM-antigen bound on the macrophage surface, as occurs in thymus-dependent responses. Similarly, the surface of antigenic determinants on tolerogenic DNP_3POL resembles that which may be formed by complexes of antibody and antigen. It is theoretically possible that T cells may mediate tolerance by the release of complexes of T cell momeric IgM and antigen.

munological effects of high or low conjugates of DNP POL (1). This model is summarized in Fig. 6. The critical difference between the signals for immunity and tolerance is that many more interactions in a given region of the cell surface are required for tolerance. If enough receptors in a given area are not reacting with antigenic determinants, tolerance does not occur. This mechanism of signal discrimination in B cells fits in well with the mechanism of B cell immunization with thymus-dependent antigens. It has recently been found that T/B lymphocyte cooperation is mediated by a subcellular component (28, 29), which binds to the surface of macrophages (30). This cooperative factor contains antigen (30) and thus it could yield a surface or matrix of antigenic determinants resembling DNP POL, which then immunizes B cells. Furthermore

the spacing of the antigenic determinants on the surface of cooperating macrophages may be such that immunity is the only possible immunological consequence, as is the case with DNP_1POL which cannot induce tolerance (1). This model of B cell signal discrimination is also compatible with the induction of antibody-mediated tolerance in vitro (31), with mixtures of antigen and antibody in the zone of slight antigen excess. It also proposes (Fig. 6) a speculative model for the mediation of tolerance by T cells (32), by complexes of antigen and bivalent T cell receptor immunoglobulin.

SUMMARY

The capacity of dissociated spleen cell suspensions to be immunized by dinitrophenylated polymeric flagellin (DNP POL), in the absence of thymus-dependent lymphocytes or macrophages, provided a simple experimental system to investigate the mechanism of binding of antigen molecules to non-thymus-dependent lymphocyte (B cell) receptors during the induction of immunity or tolerance. Various nonimmunogenic DNP compounds were used to inhibit the anti-DNP response to DNP POL. By performing inhibition experiments of brief duration at 4°C, it was established that the inhibition of the anti-DNP response by nonimmunogenic compounds was due to competitive blockade of receptors, and not tolerance or receptor modulation. It was found that univalent DNP compounds were much less efficient inhibitors of the antibody response than multivalent DNP conjugates. The difference in inhibitory capacity between univalent and multivalent DNP human globulin (DNP HgG) suggested the importance of interaction with both combining sites of a single receptor antibody molecule. Nonimmunogenic highly conjugated DNP_3POL was a more efficient inhibitor of the anti-DNP response to immunogenic DNP_1POL than $DNP_{12}HgG$, indicating that interactions at more than one receptor molecule are involved in immunization of B cells. Recent demonstrations of the rapid metabolic turnover of receptor antibody molecules suggests that the requirement for multipoint binding (to different receptors) may simply be to maintain the antigen at the cell surface in a dynamic system.

Competitive inhibition experiments were also performed to investigate the mechanism of binding of DNP_3POL in the induction of B cell tolerance. It was found that monovalent DNP compounds or multivalent $DNP_{12}HgG$ did not prevent the induction of tolerance, unlike their capacity to inhibit immunity, suggesting that a tolerance-inducing antigen binds more avidly to the cell membrane than an immunogen. The inhibition data obtained here, together with prior results describing the differential immunogenicity of DNP conjugates of different structure, and the importance of epitope density on DNP POL conjugates, permit certain conclusions about the details of antigen-receptor interaction in immunity and tolerance. Distinctions between the mechanisms of immune and tolerance induction are discussed.

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REFERENCES

1. Feldmann, M. 1972. Induction of immunity and tolerance in vitro by hapten protein conjugates. I. The relationship between the degree of hapten conjugation and the immunogenicity of dinitrophenylated polymerized flagellin. *J. Exp. Med.* **135**:735.
2. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cells from cultures of normal mice. *J. Exp. Med.* **126**:423.
3. Feldmann, M., and A. Basten. 1971. The relationship of antigen structure and the requirement for thymus-derived cells in the immune response. *J. Exp. Med.* **134**:103.
4. Feldmann, M. 1972. Induction of immunity and tolerance *in vitro* by hapten protein conjugates. II. Carrier independence of the response to dinitrophenylated polymeric flagellin. *Eur. J. Immunol.* **2**:130.
5. Feldmann, M., and E. Diener. 1971. Antibody mediated suppression of the immune response *in vitro*. III. Low zone tolerance *in vitro*. *Immunology*. **21**:387.
6. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1970. Methods in Immunology: a Laboratory Text for Instruction and Research. The Benjamin Co., Inc., New York. 2nd edition.
7. Eisen, H. N. 1964. *Methods Med. Res.* **10**.
8. Paul, W. E., D. H. Katz, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses. II. Specific properties of carrier cells capable of enhancing anti-hapten responses. *J. Exp. Med.* **132**:283.
9. Cunningham, A. J., and A. Szenberg. 1968. Further improvements on the plaque technique for detecting single antibody forming cells. *Immunology*. **14**:599.
10. Feldmann, M. 1971. Induction of immunity and tolerance to the dinitrophenyl determinant *in vitro*. *Nat. New Biol.* **231**:21.
11. Miller, J. F. A. P. 1960. Studies on mouse leukaemia. The role of thymus in leukaemogenesis by cell-free leukaemic filtrates. *Br. J. Cancer*. **10**:431.
12. Shortman, K., N. Williams, H. Jackson, P. Russell, P. Byrt, and E. Diener. 1971. The separation of different cell classes from lymphoid organs. IV. The separation of lymphocytes from phagocytes on glass bead columns and its effect on subpopulations of lymphocytes and antibody-forming cells. *J. Cell Biol.* **48**:566.
13. Vitetta, E. S., S. Bauer, and J. W. Uhr. 1971. Cell surface immunoglobulin. II. Isolation and characterization from mouse splenic lymphocytes. *J. Exp. Med.* **134**:242.
14. Diener, E., and W. D. Armstrong. 1969. Immunological tolerance in vitro: kinetic studies at the cellular level. *J. Exp. Med.* **129**:591.
15. Revoltella, R., L. T. Adler, and A. G. Osler. 1971. Antibody weight estimates at the nanogram level. *J. Immunol.* **106**:1507.
16. Wilson, J. D., and M. Feldmann. 1972. Dynamic aspects of antigen binding to B cells in immune induction. *Nat. New Biol.* **237**:3.
17. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. de Petris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti immunoglobulin antibody. *Nat. New Biol.* **233**:225.

18. Plotz, P. 1969. Specific inhibition of an antibody response by affinity labelling. *Nature (Lond.)*. **223:**1373.
19. Segal, S., A. Globerson, M. Feldman, J. Haimovitch, and D. Givol. 1969. Specific blocking of *in vitro* antibody synthesis by affinity labelling reagents. *Nature (Lond.)*. **223:**1374.
20. Mitchison, N. A. 1967. Antigen recognition responsible for the induction *in vitro* of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32:**431.
21. Segal, S., A. Globerson, and M. Feldman. 1971. A bicellular mechanism in the immune response to chemically defined antigens. I. Antibody formation *in vitro*. *Cell. Immunol.* **2:**205.
22. Traubridge, I. S., E. S. Lennox, and R. R. Porter. 1970. Induction *in vitro* of specific plaque forming cells. *Nature (Lond.)*. **228:**1087.
23. Bretscher, P. A., and M. Cohn. 1968. Minimal model for the mechanism of antibody induction and paralysis by antigen. *Nature (Lond.)*. **220:**444.
24. Hornick, C. L., and F. Karush. 1969. The interaction of hapten coupled bacteriophage ϕ x 174 with anti hapten antibody. *Isr. J. Med. Sci.* **5:**163.
25. Bretscher, P. A., and M. Cohn. 1970. A theory of self non self discrimination. *Science (Wash. D. C.)*. **169:**1042.
26. Wilson, J. D., G. J. V. Nossal, and H. Lewis. 1972. Metabolic characteristics of lymphocyte surface immunoglobulin. *Eur. J. Immunol.* In press.
27. Cone, R. E., J. J. Marchalonis, and R. T. Rolley. 1971. Lymphocyte membrane dynamics. Metabolic release of cell surface proteins. *J. Exp. Med.* **134:**1373.
28. Feldmann, M., and A. Basten. 1972. Specific cooperation between T and B lymphocytes across a cell impermeable membrane *in vitro*. *Nat. New Biol.* **237:**13.
29. Feldmann, M., and A. Basten. 1972. Cell interactions in the immune response in vitro. III. Specific collaboration across a cell impermeable membrane. *J. Exp. Med.* **136:**49.
30. Feldmann, M. 1972. Cell interactions in the immune response in vitro. V. Specific collaboration via complexes of antigen and T cell immunoglobulin. *J. Exp. Med.* In press.
31. Diener, E., and M. Feldmann. 1971. Relationship between antigen and antibody induced suppression of immunity. *Transplant. Rev.* **8:**76.
32. Gershon, R. K., and K. Kondo. 1971. Infectious immunological tolerance. *Immunology*. **21:**903.