IMMUNOLOGICAL TOLERANCE IN BONE MARROW-DERIVED LYMPHOCYTES

III. Tolerance Induction in Primed B Cells by Hapten Conjugates of Unrelated Immunogenic or "Nonimmunogenic" Carriers*

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Induction of hapten-specific tolerance can be accomplished quite successfully in several different ways and has become a subject of increasing interest and analysis in recent years (reviewed in reference 1), because this model is one in which tolerance appears to be restricted to the B-cell population and, therefore, may be more ideally suited to elucidation of some of the cellular mechanisms involved in these inactivation processes. This is particularly true since the immunoglobulin nature of B-cell receptors is now well established and increasing sophistication in the knowledge of antigen binding and movement of these receptors is being obtained (2). Since the threshold of tolerance induction in T cells is considerably lower than it is in B cells (3, 4), at least insofar as protein antigens are concerned, it is difficult to obtain a selective B-cell tolerance in vivo to conventional T-cell-dependent antigens.

In recent years, however, several investigators have reported the successful induction of true hapten-specific tolerance in vivo, which may, indeed, reflect a state of restricted B-cell tolerance. The most effective methods employed have the common feature of treating animals with haptenic determinants on molecules or substances that fail to stimulate T cells, or do so very poorly. Thus, such tolerance has been induced by administration of (*a*) hapten on nonimmunogenic moieties such as the synthetic copolymers of D-glutamic acid and D-lysine (D-GL),[1] or L-GL in PLL nonresponder guinea pigs (5, 6) or inbred mice (7, 9), (*b*) haptenic derivatives of autologous proteins (10–14), autologous or syngeneic red cells (15), or (*c*) polysaccharides such as type III pneumococcal polysaccharide (16, 17). We suggested earlier that a possible explanation for the ease of tolerance induction with the hapten conjugates of nonimmunogenic copolymers such as D-GL and also for other non- or weakly immunogenic substances may be the lack of the simultaneous occurrence of immunization and tolerance induction when these substances are administered in appropriate conditions (1, 5). If, as we suggested, the weak immunogenicity of these tolerogens reflects the relative

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[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; D-GL, D-glutamic acid and D-lysine; DNP-KLH, DNP keyhole limpet hemocyanin; OVA, ovalbumin; PLL, poly-L-lysine.

lack of stimulation of specific helper T cells, then the prediction may be made that ease of tolerance induction by any molecule in B cells will show an inverse correlation with the degree of helper T-cell activity involved. This concept has been recently extended by Mitchell in an ingenius hypothesis concerning regulatory T-cell function in antibody responses (17).

The present studies were designed to probe the role(s) of T cells in preventing or altering tolerance induction in hapten-specific B cells. This was accomplished by using hapten conjugates of normally immunogenic heterologous carriers[2] to selectively inhibit 2,4-dinitrophenyl (DNP)-primed B cells in adoptive transfer experiments in vivo. The data provide strong indications that one critical role of helper T-cell participation in humoral responses to antigens is to circumvent the development of a tolerogenic signal that, in the absence of such T-cell function, might otherwise ensue after binding of the antigenic determinants by specific precursor B lymphocytes.

Materials and Methods

The proteins, hapten-carrier conjugates, animals, immunization schedules, adoptive transfer system, antibody determinations, and statistical analyses were identical to those described in the preceding paper (9). The following additional DNP-protein conjugates were employed: DNP_8-OVA and DNP_{32}-BGG.

Depletion of T Lymphocytes. The preparation of anti-θ serum, determination of anti-θ serum cytotoxicity, and treatment of spleen cells with anti-θ serum plus complement has been detailed in previous studies from this laboratory (18).

RESULTS

Induction of Tolerance in Adoptively Transferred DNP-Specific B Cells by Nonimmunogenic or Unrelated Immunogenic DNP-Carrier Conjugates.—We have previously shown that DNP-D-GL is a highly effective tolerogenic molecule for primed or unprimed DNP-specific B cells adoptively transferred into irradiated recipients (5, 7, 9). The following experiment was designed to ask whether or not metabolizable immunogenic or nonimmunogenic DNP carriers could also serve as relatively effective tolerogens in similar conditions. For this purpose, we chose DNP-L-GL as a metabolizable but nonimmunogenic molecule (9) and DNP-OVA, and DNP-BGG as normally immunogenic DNP-protein conjugates.

The protocol and data of this experiment are summarized in Fig. 1. Spleen cells from A/J donor mice primed 2 mo earlier with DNP keyhole limpet hemocyanin (DNP-KLH) in complete Freund's adjuvant (CFA) were transferred intravenously to irradiated (550 R) syngeneic recipients (50×10^6 cells/mouse). Immediately after transfer, groups of recipient mice were treated by intraperitoneal injection of 200 μg of either DNP-D-GL, DNP-L-GL, DNP-BGG, or DNP-OVA. Controls received saline. 3 days later, all recipients

[2] The term heterologous carrier refers to any carrier molecule unrelated to that used for original priming of DNP-specific B cells.

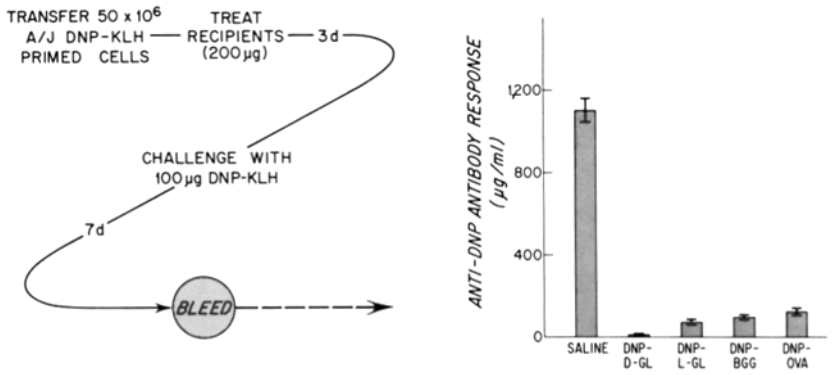

FIG. 1. Induction of tolerance in adoptively transferred DNP-specific B cells by exposure of such cells to nonimmunogenic or unrelated immunogenic DNP-carrier conjugates. The protocol is schematically depicted on the left of figure. Recipient groups were treated with saline or one of the DNP conjugates as indicated below the data bars. Each group consists of six mice. Statistical comparison of antibody responses of recipient groups treated with the various DNP carrier conjugates before secondary challenge to those of saline control recipients yielded the P values of $0.001 > P$ in all cases.

were challenged intraperitoneally with 100 μg of DNP-KLH and then bled 7 days thereafter. As shown in Fig. 1, all recipients treated with one of the DNP carriers employed were considerably depressed in their capacity to develop secondary responses to DNP-KLH as compared to saline-treated controls. Although clearly most marked in the case of DNP-D-GL treatment, diminution of at least 90% or more was obtained as well as with the other molecules employed.

Partial Reversal of DNP-Specific Tolerance Induced in Vivo With Immunogenic DNP Proteins by Serial Adoptive Transfer.—In view of the relative facility with which DNP-specific B cells could be rendered unresponsive by exposure to heterologous carrier conjugates in the preceding experiment, we felt it necessary to explore the relative reversibility of this unresponsive state as compared to that induced by the tolerogen DNP-D-GL.

The protocol of this experiment is depicted schematically in Fig. 2. Spleen cells from A/J donor mice primed 6 mo earlier with DNP-KLH in CFA were treated in vitro with anti-θ serum plus complement to remove T lymphocytes and then transferred intravenously to 550 R irradiated syngeneic recipients (20×10^6 cells/mouse). Groups of 10 recipients were then treated by intraperitoneal injection of either saline, DNP-KLH, DNP-D-GL, DNP-L-GL, DNP-BGG, or DNP-OVA. After an interval of 7 days the mice were bled, sacrificed and their spleen cells removed, pooled for respective groups, and thoroughly washed with Eagle's minimal essential medium. 15×10^6 of the respective pooled cells were then transferred intravenously together with 50×10^6 spleen cells from syngeneic KLH-primed (100 μg in CFA, 1 mo earlier) donor mice to new individual irradiated syngeneic recipient mice. All of the new recipient groups were challenged with DNP-KLH and then bled 7 days thereafter.

With the exception of the DNP-KLH-treated group, none of the groups of the first recipients produced measurable levels of anti-DNP antibodies before

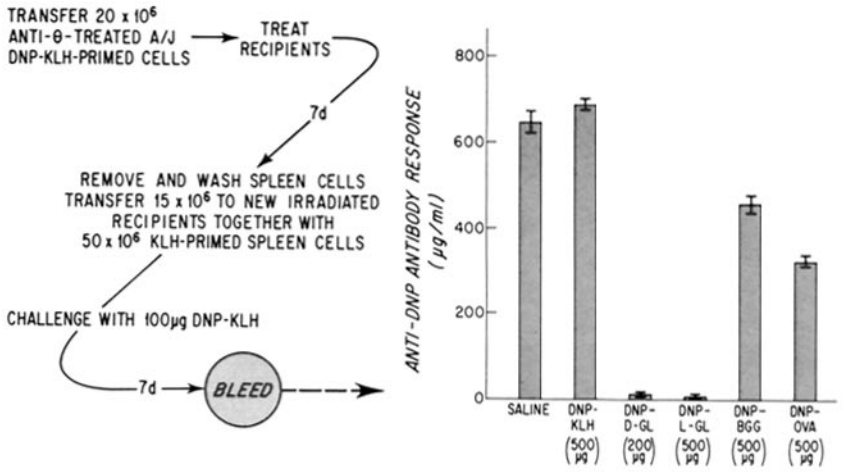

FIG. 2. Partial reversal of tolerance induced in vivo with immunogenic DNP proteins by serial adoptive transfer. The protocol is schematically depicted on the left of figure. Primary recipient groups were tested with saline or one of the DNP conjugates as indicated below the data bars. Each group consists of five mice. Relevant statistical comparisons of responses of the various recipient groups yielded the following P values: (*a*) saline vs. DNP-KLH, $0.90 > P > 0.80$; (*b*) saline or DNP-KLH vs. DNP-D-GL or DNP-L-GL, $0.001 > P$ in every case; (*c*) saline or DNP-KLH vs. DNP-BGG, $0.40 > P > 0.30$; (*d*) saline or DNP-KLH vs. DNP-OVA, $0.10 > P > 0.05$; (*e*) DNP-BGG or DNP-OVA vs. DNP-D-GL or DNP-L-GL, $0.001 > P$ in every case.

sacrifice. The group treated with DNP-KLH developed small, but detectable, anti-DNP responses (average 21.3 μg/ml) probably reflecting a combination of residual T cells in the anti-θ-treated cell population and the strong immunogenicity of KLH as an inducer of helper T cells. As shown in Fig. 2, the second recipients of the various treated cell inocula manifested variable responses depending on the nature of the treatment employed. Recipients of cells exposed to either saline or DNP-KLH in the first recipients developed very good responses to DNP-KLH demonstrating the functional integrity of the DNP-specific B cells as well as that of the fresh KLH-specific T cells from the carrier-primed donors. On the other hand, recipients of cells exposed to either DNP-D-GL or DNP-L-GL failed to develop secondary anti-DNP responses. In contrast to the latter groups, however, cells exposed to DNP-BGG or DNP-OVA were partially recovered in their functional responsiveness manifesting secondary responses that were 66% and 47%, respectively, of the control groups.

DISCUSSION

The interaction of B lymphocytes with molecules bearing specific determinants either (*a*) provides one of the programmed stimuli for proliferation and differentiation into antibody-secreting cells, or, alternatively (*b*) causes these

cells to become temporarily or permanently unresponsive. In addition to the crucial role of antigen concentration in affecting these processes, the studies described herein have emphasized the importance of helper T-cell activity in the determination whether immunity or unresponsiveness will ensue. In the absence of an appropriate and sufficient level of T-cell activation, the interaction of B-cell receptors with antigens which, in the presence of T-cell participation, would be sensed as an immunogenic stimulus renders the cell unresponsive. These findings imply that T-cell helper activity would be more effective on B cells when initiated before rather than after B-cell interaction with the epitope, and also that a B cell which has interacted with a thymus-dependent antigen is susceptible to T-cell helper effect for a relatively short period of time. Both of these postulates have been verified experimentally.[3]

In the absence of adequate helper T-cell activity, administration of DNP-OVA or DNP-BGG to recipients of adoptively transferred DNP-KLH-primed cells diminished the subsequent secondary response to DNP-KLH challenge carried out 3 days later by more than 90% (Fig. 1). This was true also in the case of administering DNP-D-GL or DNP-L-GL, but the conditions favorable to tolerance induction by these molecules may be somewhat different from those involved in unresponsiveness induced by DNP-conjugates of normally immunogenic substances such as OVA or BGG. Indeed, this is quite clearly suggested by the substantial recoveries of responses observed when B cells exposed to DNP-OVA or DNP-BGG in first adoptive recipients were subsequently transferred to new recipients together with additional carrier-primed cells. In contrast, cells exposed initially to either DNP-D-GL or DNP-L-GL failed to display any appreciable recovery in responsiveness under the same circumstances (Fig. 2).

In our initial studies on hapten-specific tolerance in guinea pigs, we demonstrated that administration of the nonimmunogenic compound, DNP-D-GL induced profound DNP-specific tolerance in both strain 2 and strain 13 inbred guinea pigs (5). Moreover, in the case of strain 13 guinea pigs, which are genetic nonresponders to L-GL (19), a comparable degree of DNP-specific tolerance was induced after administration of DNP-L-GL; strain 2 responder guinea pigs, on the other hand, developed anti-DNP antibody responses to the latter compound (5). Our interpretation of these observations was that the ease of tolerance induction with such DNP conjugates of nonimmunogenic or weakly immunogenic substances reflects the lack of simultaneous occurrence of immunization and tolerance induction when these substances are administered. If, therefore, the weak immunogenicity of a substance is primarily determined by (*a*) the absence or scarcity of effective helper T cells specific for that substance, and (*b*) the relative inability of the material to activate B cells without the functional cooperative influence of T cells, then tolerance induction by hapten conju-

[3] Katz, D. H., P. E. Newburger, T. Hamaoka, and B. Benacerraf. 1974. Hapten-specific IgE antibody responses in mice. IV. Evidence for distinctive sensitivities of IgE and IgG B lymphocytes to the regulatory influences of T cells. *J. Immunol.* In press.

gates of such materials may be interpreted to result from direct interaction, in the appropriate dose range, with specific precursors of antibody-forming cells without intervening helper cells (5). Indeed, these predictions appear to have been amply borne out by a number of models of hapten-specific tolerance in which the common feature of the tolerance-inducing molecules has been their relative inability to activate specific helper T cells (5–17).

In the studies described in the preceding (7, 9) and present papers dealing with B-cell tolerance in mice, we have reaffirmed the potent tolerance-inducing properties of DNP-conjugates of the nonimmunogenic copolymers of D- and L-GL. The additional demonstration in the present studies of the capacity to interfere with secondary anti-DNP responses with DNP-OVA and DNP-BGG under certain circumstances deserves extended comment. These conjugates are normally immunogenic in mice in a variety of experimental systems. The carriers, OVA and BGG, are capable of stimulating specific T-cell helper function in proper conditions of immunization so we know that functional T cells specific for their respective determinants do indeed, exist. How then can we explain the significant inhibitory effects obtained after exposure of cells to these compounds under certain conditions? First, it should be recalled that we have been dealing in these experiments with primed B cells, presumably largely of moderate-to-high affinity receptor type since they were taken from donors primed quite some time earlier. Second, the conditions in which inhibition by these substances was observed involved short-term exposure of cells to relatively high doses of the conjugates in aqueous form. The importance of this lies in the fact that these conditions are quite inefficient for inducing good helper T-cell function in a population of unprimed (for that antigen) T lymphocytes within a critical time period and may, indeed, for this reason cause some degree of tolerance induction in unprimed animals. Thus, where significant inhibition is best obtained, there exists a combination of conditions that on the one hand favor relatively avid antigen binding by specific B lymphocytes (i.e., primed and of high average affinity) and, on the other hand, tend to be unfavorable for induction of carrier-specific helper T-cell function.

In the context of the above reasoning, important questions are raised by the contrasting observations reported herein in which DNP-OVA and DNP-BGG were essentially comparable to DNP-D-GL and DNP-L-GL in the level of tolerance induced in one experiment (Fig. 1) but clearly different in another (Fig. 2). The probable explanation for these differences lies in the fact that in the first experiment secondary challenge was made in the same adoptive recipient that had been treated with the various tolerogenic compounds and, moreover, within a much shorter time interval after exposure. In the second experiment, on the other hand, B cells were exposed to the various substances and then removed from the milieu of the first recipient to a new recipient before being subjected to secondary challenge. In addition, in the latter case a longer interval (7 days) was employed and, perhaps most important, a new

and probably larger population of carrier-primed helper T cells was provided in the second recipients. It is difficult to ascertain what factors were most important in the partial recovery of responses observed with cells exposed to DNP-OVA or DNP-BGG; the longer time interval may have favored partial spontaneous recovery of a subpopulation of the B cells, and/or the potentiating effect of transferring cells not yet irreversibly inactivated to a new irradiated recipient environment could account for these results. In the preceding paper (9), it was clearly shown that the DNP-D-GL molecule induces an irreversible state of tolerance in B cells which can be both operationally and morphologically[4] distinguished from the unresponsiveness induced by DNP-L-GL or DNP-OVA.

The differences observed between the animals treated with DNP-L-GL and those which received DNP conjugates of heterologous immunogenic carriers, such as DNP-OVA or DNP-BGG that showed considerable recovery of responsiveness to DNP-KLH may be ascribed to the fact that L-GL does not induce T-cell immunity in the mouse, whereas OVA and BGG are T-cell-dependent antigens for which specific helper T cells are naturally found in nonimmune animals. This number of T cells specific for these carriers may have been sufficient to exert some protection against tolerance induction by DNP-BGG or DNP-OVA.

In other experimental systems under study in our laboratory in which the immune responses are under control of histocompatibility-linked *Ir* genes, the extraordinary sensitivity of B cells to the induction of tolerance in the absence of helper T cells has been demonstrated. Thus, Kapp, Pierce, and Benacerraf have observed that *H-2*^{s} and *H-2*^{q} mice which are genetically nonresponsive to the terpolymer of glutamic acid, alanine, and tyrosine (GAT), but which nevertheless can mount anti-GAT responses to a complex of GAT and methylated bovine serum albumin (MBSA) are rendered unresponsive to GAT-MBSA by very small doses of previously administered GAT.[5] Similarly, Dorf, Katz, and Benacerraf have demonstrated that the DNP conjugate of GAT is very tolerogenic for DNP B cells of nonresponder mice, even when administered in adjuvants.[6]

The mechanism by which helper T cells protect specific B cells from tolerance induction or, to put it differently, the process by which tolerance is induced in the absence of protective helper T-cell function is not understood. Experiments are in progress with the various systems mentioned above to determine if in this type of B-cell tolerance, unresponsiveness results from the direct interaction of the tolerogen with specific B-cell receptors in the absence

[4] K. A. Ault, E. R. Unanue, D. H. Katz, and B. Benacerraf. Manuscript in preparation.

[5] J. A. Kapp, C. W. Pierce, and B. Benacerraf. 1974. Genetic control of immune responses in vitro. III. Tolerogenic properties of the terpolymer GAT for spleen cells from nonresponder (*H-2*^{s} and *H-2*^{q}) mice. Manuscript submitted for publication.

[6] M. E. Dorf, D. H. Katz, and B. Benacerraf. Manuscript in preparation.

of a preceding or concomitant T-cell activation, or whether the participation of postulated specific suppressor T cells are involved in this phenomenon (20).

SUMMARY

The present studies were designed to probe the role(s) of T cells in preventing or altering tolerance induction in hapten-specific B cells. This was accomplished by using hapten conjugates of normally immunogenic heterologous carriers to selectively inhibit 2,4-dinitrophenyl (DNP)-primed B cells in adoptive transfer experiments in vivo. The data provide strong indications that one critical role of T-cell participation in humoral responses to antigens is to circumvent the development of a tolerogenic signal that, in the absence of such T-cell function, might otherwise ensue after binding of the antigenic determinants by specific precursor B lymphocytes.

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