IMMUNOLOGICAL TOLERANCE IN BONE MARROW-DERIVED LYMPHOCYTES

I. Evidence for an Intracellular Mechanism of Inactivation of Hapten-Specific Precursors of Antibody-Forming Cells*

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The establishment of thymus-derived (T)[1] and bone marrow-derived (B) cell cooperative interactions in humoral immune responses has led to a reappraisal of the conceptual and experimental approach to the study of tolerance. From an early point of relative confusion about the target cell for tolerance induction, the very elegant experiments of Chiller et al. (1, reviewed in reference 2) have clearly elucidated the critical kinetic and dose threshold differences for tolerance induction in T and B lymphocytes insofar as thymus-dependent antigens are concerned.

Nonetheless, it is still not known how both types of immunocompetent cells are rendered specifically unresponsive. A great deal of this confusion stems from apparently contradictory observations reported from studies of the frequency of specific antigen-binding cells in normal, immune, and tolerant animals. This is a crucial issue since on it rests the correct interpretation concerning the fate of tolerant cells. Some investigators have observed clearly diminished numbers of such antibody-forming cell precursors in tolerant animals (3, 4), whereas others have demonstrated specific antigen-binding cells present in relatively normal numbers in such animals (5–8). Reasonable explanations for these differences concern the different target cells involved in the various systems studied, the degree of specific tolerance existing at the time cells are examined, and the affinity for the antigen of the target cell population.

For this and related reasons, a model where tolerance is more or less restricted to

the B cell population may be more ideally suited to elucidation of some of these problems. 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 (9). Since the threshold of tolerance induction in T cells is considerably lower than it is in B cells (1, 2), at least insofar as protein antigens are concerned, it is difficult to obtain a selective B cell tolerance in vivo once the T cells have already been tolerized. Recently, however, several investigators have reported the successful induction of true hapten-specific tolerance in vivo which may, indeed, reflect such a state of restricted B cell tolerance (4, 10–12). The model of 2,4-dinitrophenyl (DNP)-specific tolerance which we have previously described in inbred guinea pigs (4), involved treatment of guinea pigs with a "nonimmunogenic" DNP conjugate of the copolymer of D-glutamic acid and D-lysine (D-GL). After treatment with DNP-D-GL such guinea pigs manifested profound DNP-specific tolerance as reflected by their inability to respond to a challenge with the immunogenic conjugate, DNP-ovalbumin (OVA). The tolerant state in this model appears to be expressed predominantly in the population of DNP-specific antibody-forming cell precursors, and in this sense has been interpreted by us to reflect a central mechanism (4, reviewed in reference 13). More recent studies have demonstrated a preferential depression of the high affinity antibody response in this model (14).

The present studies were undertaken to establish conditions for induction of DNP-specific tolerance with DNP-D-GL in inbred mice. A unique feature of this tolerance model in guinea pigs was the relative ease with which tolerance could be induced in an animal previously primed to DNP. It was of particular interest, therefore, to obtain DNP-specific tolerance in an adoptive transfer system in mice. Such a model would offer considerable advantage in further experimentation designed to approach questions concerning the existence and mechanism of intracellular events responsible for the tolerant state. In the experiments herein, we present data on various parameters of tolerance induction in such an adoptive transfer system and also describe conditions for tolerance induction with DNP-D-GL in vitro. Utilizing this system, we have found that the tolerant state is not broken by serial adoptive cell transfer and, moreover, that such observations do not reflect carry-over of tolerogen. The evidence presented provides a forceful argument for the concept of central tolerance in B cells as reflecting sub- or intracellular inhibitory events.

Materials and Methods

Proteins and Chemical Reagents.—The copolymers of D-glutamic acid and D-lysine (D-GL) and L-glutamic acid and L-lysine (L-GL) were obtained from Pilot Chemicals, Inc., Watertown, Mass. Both isomers had an average molecular weight of 115,000 and a ratio of G:L of 60:40. Keyhole limpet hemocyanin (KLH) was purchased from Pacific Bio-Marine Supply Co., Venice, Calif. Hen ovalbumin (OVA) 5 times recrystallized and bovine gamma globulin (BGG) were obtained from Pentex Biochemical, Kankakee, Ill. All other chemical reagents used were, in general, identical with those described in previous related studies (15).

Hapten-Carrier Conjugates.—The following DNP conjugates were prepared as previously

described (15, 16): DNP$_9$-KLH, DNP$_{32}$-BGG, DNP$_8$-OVA, and DNP$_{20}$-L-GL. The preparation of DNP$_{37}$-D-GL has been described in detail elsewhere (4). Subscripts refer to the average number of moles of DNP per mole of carrier.

Animals.—Mice of the inbred lines BALB/c and A/J were obtained from Jackson Memorial Laboratory, Bar Harbor, Maine. All mice were used at 8–12 wk of age.

Adoptive Transfer System.—A/J mice, 8–12 wk of age, received primary immunization with 100 μg of DNP-KLH emulsified in complete Freund's adjuvant (CFA) intraperitoneally. At various times (1–3 months) thereafter, these DNP-KLH-primed mice were killed and their spleens removed. Single-cell suspensions in minimal essential medium (MEM) (Eagle) were prepared, washed, and transferred intravenously or intraperitoneally to syngeneic, irradiated (450–500 R) A/J recipients. In general, the tolerogen was administered intraperitoneally in saline immediately after cell transfer. Secondary antigen challenge with DNP-KLH in saline was performed intraperitoneally 3 days later. All mice were bled 7 days after secondary challenge from the retroorbital plexus and serum anti-DNP antibody levels were determined as described below. Modifications of this general adoptive transfer scheme are described in appropriate sections in Results.

Measurement of Anti-DNP Antibodies.—Serum anti-DNP antibody levels were determined by a modified Farr technique (17, 18) using ^{3}H-labeled DNP-ε-amino-N-caproic acid (15). Using standard curves constructed for individual mouse strains in a manner identical with that described previously for inbred guinea pigs (15), percentage of binding was converted into amount of anti-DNP antibody in micrograms per milliliter of serum.

Statistical Analysis.—Serum antibody levels were logarithmically transformed and means and standard errors calculated. Group comparisons were made employing Student's *t* test. In those mice in which no specific antigen binding could be detected in the serum, a value of 0.01 μg/ml was arbitrarily assigned to allow logarithmic transformation of the data.

RESULTS

Specific Suppression of Anti-DNP Antibody Production in BALB/c Mice As a Result of Administration of DNP-D-GL.—

When DNP-D-GL treatment precedes primary immunization: Normal BALB/c mice received a series of injections of 200 μg of DNP-D-GL intraperitoneally in saline daily for 3 successive days. Control mice received saline injections during this period. 1 wk later, all mice were primarily immunized with DNP-KLH (500 μg intraperitoneally in saline daily for 3 successive days). This was followed 14 days thereafter (day 0) by secondary immunization with 500 μg of DNP-KLH intraperitoneally in saline. All animals were bled on days 0 and 7 and determinations of serum anti-DNP antibody levels were made.

As shown in Fig. 1, control mice which had received pretreatment with saline alone developed primary anti-DNP antibody responses to DNP-KLH, as evidenced by antibody levels on day 0, and manifested very brisk anamnestic anti-DNP responses by day 7 after secondary challenge. In contrast, the synthesis of anti-DNP antibodies was markedly suppressed in mice which had received pretreatment with DNP-D-GL. This was true both for the primary response, as evidenced by the absence of detectable anti-DNP antibody on day 0, and for anamnestic responses to the secondary challenge with DNP-KLH.

When DNP-D-GL is administered as an intervening treatment between primary and secondary immunization: BALB/c mice were given a primary immunization course consisting of 500 μg daily of DNP-KLH intraperitoneally in saline. 1 wk later, one group of these mice received an
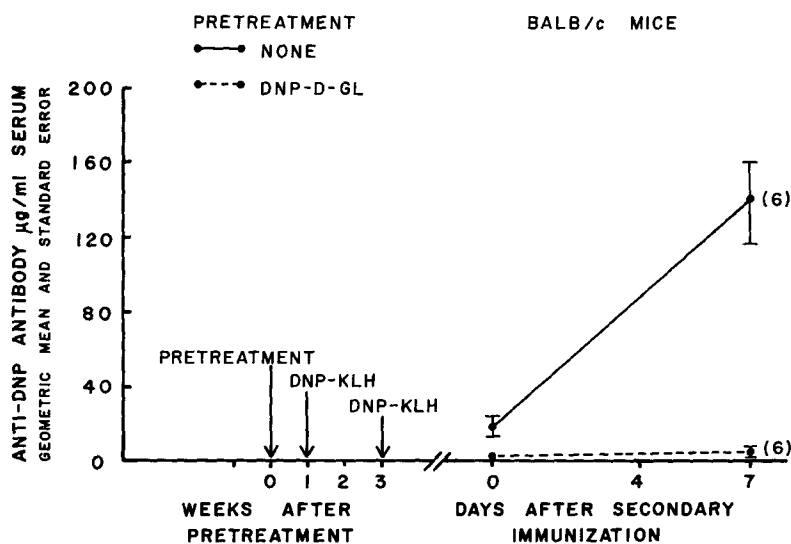
FIG. 1. Specific suppression of anti-DNP antibody production in BALB/c mice as a result of administration of DNP-D-GL before primary immunization. Normal BALB/c mice received a series of injections of 200 μg of aqueous DNP-D-GL intraperitoneally (i.p.) daily for 3 successive days. Control mice received saline injections during this period. 1 wk later, all mice were primarily immunized with DNP-KLH (500 μg i.p. in saline daily for 3 successive days). This was followed 14 days thereafter (day 0) by secondary immunization with 500 μg of aqueous DNP-KLH i.p. Serum anti-DNP antibody concentrations just before secondary challenge and on day 7 are illustrated. Numbers in parentheses refer to the number of animals in the given groups. Statistical comparison of the responses of untreated and DNP-D-GL-treated animals yielded a P value of 0.001 > P.

intervening series of injections of 200 μg of DNP-D-GL intraperitoneally in saline daily for 3 successive days. A control group received no intervening treatment. 2 wk later (day 0) all mice received a secondary immunization with 500 μg of DNP-KLH intraperitoneally in saline. The results are presented graphically in Fig. 2.

Mice which received no intervening treatment displayed normal levels of anti-DNP antibodies 3 wk after primary immunization (day 0) and developed sharp anamnestic anti-DNP responses by day 7 after secondary challenge. On the other hand, mice which had received intervening injections of DNP-D-GL had no detectable anti-DNP antibody on day 0 and were incapable of mounting secondary anti-DNP responses.

The above experiments demonstrate in inbred mice precisely what we reported earlier in inbred guinea pigs (4), namely, that administration of an appropriate dose of a DNP conjugate of a nonimmunogenic carrier molecule (D-GL) results in profound DNP-specific tolerance. This is true irrespective of whether DNP-D-GL is administered to a normal animal before primary immunization, or to a previously primed mouse in which anti-DNP antibody
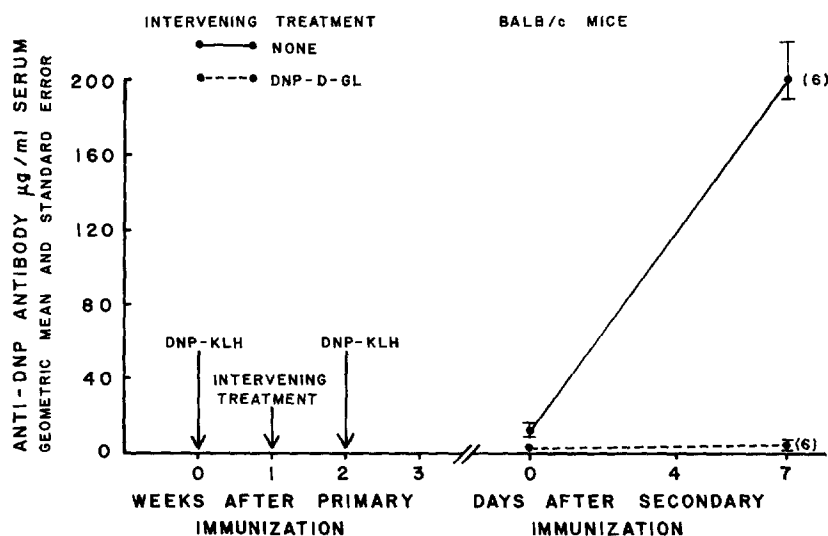
Fig. 2. Specific suppression of anti-DNP antibody production in BALB/c mice as a result of administration of DNP-D-GL as an intervening treatment between primary and secondary immunization. 1 wk after primary immunization with DNP-KLH (500 μg i.p. daily for 3 successive days), one group of BALB/c mice received an intervening series of injections of DNP-D-GL (200 μg i.p. daily for 3 successive days). A control group of primed mice received saline. 2 wk later (day 0) all mice were secondarily challenged with 500 μg of DNP-KLH i.p. Serum anti-DNP antibody concentrations just before secondary challenge and on day 7 are illustrated. Numbers in parentheses refer to the number of animals in the given groups.

Statistical comparison of the responses of untreated and DNP-D-GL-treated animals yielded a P value of $0.001 > P$.

production has already been induced. The latter point is illustrated even more forcefully in the adoptive transfer experiments described below.

Induction of DNP-Specific Tolerance with DNP-D-GL in an Adoptive Cell Transfer System in A Strain Mice.—A somewhat unique feature of the DNP-specific tolerance induced by DNP-D-GL is the relative ease with which the tolerant state can be established in a previously immunized animal (4, and preceding experiments). This provides a potential advantage over other models of tolerance since delineation of intracellular events in this phenomenon requires sufficient quantities of specific cells for any such study to be meaningful. In this and subsequent sections, we describe experiments in which we have established conditions for induction of DNP-specific tolerance in an adoptive transfer system utilizing DNP-KLH-primed mouse spleen cells.

In the prototype experiment (Table I), 50×10^6 spleen cells from A/J donor mice, which had been primed with 100 μg of DNP-KLH in CFA 46 days earlier, were injected intravenously into individual syngeneic, irradiated (500 R) recipients in two groups (A and B). Recipients in two other groups (C and D) were injected with 50×10^6 spleen cells from identically primed donors which had also been treated with 1.0 mg of aqueous DNP-D-GL intraperitoneally 7 days

TABLE I

Induction of DNP-Specific Tolerance in Adoptively Transferred DNP-KLH-Primed A Strain Spleen Cells by the Administration of DNP-D-GL

Group	Protocol*		No. of recipients	Anti-DNP antibody‡ Day 7 after secondary challenge
	Treatment of donors of DNP-KLH-primed cells	Treatment of recipients of DNP-KLH-primed cells		
				μg/ml
A	None	None	10	424.8 (1.21)
B	"	500 μg DNP-D-GL	10	0.02 (1.50)
C	1.0 mg DNP-D-GL i.p. 7 days before sacrifice	None	10	28.2 (1.34)
D	" "	500 μg DNP-D-GL	10	0.01 (1.0)

* Irradiated (500 R) A/J mice were injected intravenously with spleen cells (50 × 10⁶/ recipient) from syngeneic donor mice which had been primed with 100 μg of DNP-KLH in CFA 46 days earlier (groups A and B). Recipient mice in groups C and D were injected with spleen cells from donors which had been identically primed with DNP-KLH but also treated with 1.0 mg of aqueous DNP-D-GL intraperitoneally 7 days before sacrifice. Immediately after transfer, recipients were either treated with 500 μg of aqueous DNP-D-GL intraperitoneally (groups B and D) or not treated (groups A and C). 3 days later, all mice were secondarily challenged with 100 μg of DNP-KLH intraperitoneally in saline.

‡ The data are expressed as geometric means of serum anti-DNP antibody levels 7 days after secondary challenge. Numbers in parentheses represent standard errors. A comparison of geometric mean antibody levels gave the following results. Comparison of group A with group B and group C with group D yielded *P* values of 0.001 > *P* in both cases. Comparison of group A with group C yielded a *P* value of 0.001 > *P*.

before sacrifice. Immediately after cell transfer, recipients were either treated with 500 μg of aqueous DNP-D-GL intraperitoneally (groups B and D) or not treated (groups A and C). 3 days later, all animals received secondary challenge with 100 μg of DNP-KLH intraperitoneally in saline and were bled 7 days thereafter.

Two points are noteworthy about the data presented in Table I. First, the results of groups A and B illustrate the virtual abrogation of the adoptive secondary anti-DNP response to DNP-KLH by the administration of DNP-D-GL to recipient mice. Second, the striking suppression of anti-DNP responses in recipients of cells from DNP-KLH-primed donors which had been treated with one dose of DNP-D-GL manifests the profound nature of the tolerogenic effect of this nonimmunogenic substance, since these donors were immunized with DNP-KLH in complete adjuvant. Moreover, it is of considerable importance that the tolerant state was still expressed after adoptive transfer (group C) since this is not the case in some models of tolerance in which this has been studied (19–22). The latter point will be approached in greater detail in a subsequent section below.

Experiments were also carried out to characterize other parameters of the adoptive transfer tolerance model. In one experiment (not shown), the effective period of tolerance induction was studied by administering 200 μg of DNP-D-GL

to recipients immediately after adoptive transfer of DNP-KLH-primed cells, and then challenging groups of recipients with DNP-KLH at various times (1, 6, 24, 48, or 72 hr) thereafter. Clearly, maximal tolerization occurred in all treated recipients irrespective of the time interval between administration of DNP-D-GL and challenge with DNP-KLH. A final preliminary experiment was performed to determine the dose relationship of DNP-D-GL to tolerance induction.

Spleen cells from A/J mice primed with DNP-KLH 30 days earlier were injected intraperitoneally (50 × 10^6 cells per recipient) into irradiated (450 R), syngeneic recipients. Immediately after cell transfer, groups of recipients were either treated with varying doses of aqueous DNP-D-GL intraperitoneally, or were not treated. 3 days later, all mice were secondarily challenged intraperitoneally with 100 μg of DNP-KLH and then bled 7 days thereafter.

As depicted graphically in Fig. 3, the lowest dose (1 μg) of DNP-D-GL suppressed the secondary anti-DNP response by only 50% as compared with controls. While considerably more suppression (90%) was obtained with 10 μg of DNP-D-GL, doses of 50 μg or greater were required for complete abolition of the anti-DNP response. This dose-response relationship is consistent with our recent observations in guinea pigs in which we have shown preferential depletion of high affinity antibody-forming cells after treatment with DNP-D-GL (14). Finally, it should also be noted that in these latter three experiments the DNP-KLH-primed cells were injected intraperitoneally indicating that either route of adoptive cell transfer may be used.

Induction of DNP-Specific Tolerance by Incubation of DNP-KLH-Primed Cells In Vitro with DNP-D-GL before Adoptive Transfer.—Having established conditions for induction of tolerance in DNP-primed cells in an adoptive transfer system by treating recipients in vivo with DNP-D-GL, it was of interest to determine whether or not the same result could be obtained by incubating such cells in vitro with DNP-D-GL before transfer.

Two types of experiments were performed along these lines. In the first experiment, spleen cells from A/J mice primed 2 months earlier with 100 μg of DNP-KLH in CFA were cultured in slightly modified Mishell-Dutton conditions (23). At a cell density of 30 × 10^6 cells/ml, these primed cells were incubated with either DNP-D-GL (3 μg/10^6 cells) or saline. At intervals of 24, 48, and 72 hr, cells were harvested from the dishes, washed three times with MEM, and counted. Equal numbers of viable (trypan blue exclusion), saline-control cells and cells incubated with DNP-D-GL were transferred intraperitoneally to respective groups of irradiated (450 R), syngeneic A/J recipient mice. Secondary challenge with 100 μg of DNP-KLH intraperitoneally was performed immediately after cell transfer and the mice were bled 7 days later.

As shown in Fig. 4, incubation with DNP-D-GL resulted in suppression of the secondary adoptive transfer responses to DNP-KLH which varied in degree with the length of in vitro incubation. Thus, cells incubated for 24 hr with the tolerogen developed adoptive secondary responses which were suppressed by
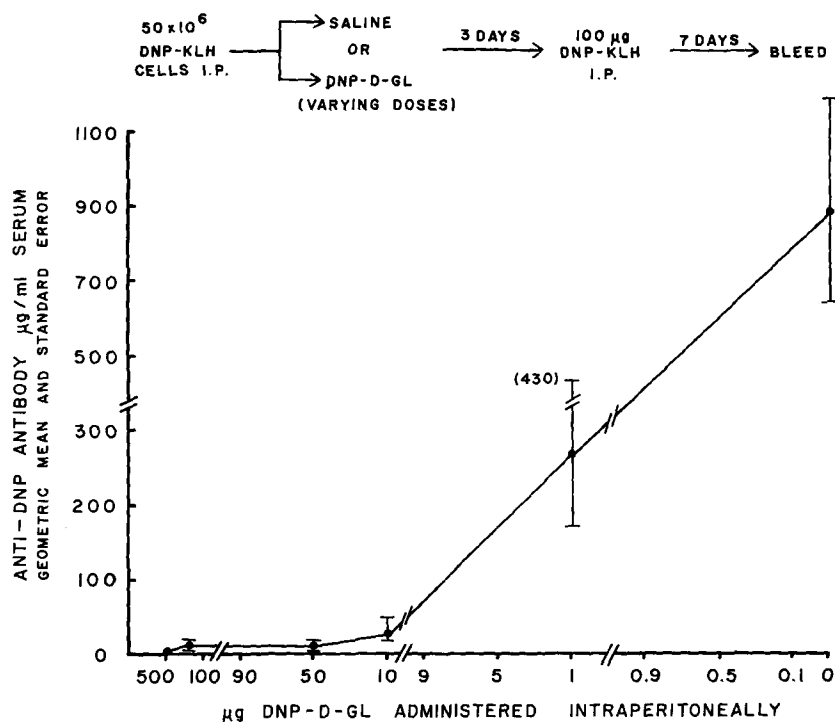
Fig. 3. Dose-response relationship of DNP-D-GL to induction of DNP-specific tolerance in the adoptive cell transfer system in A strain mice. Spleen cells from A/J donor mice, primed 30 days earlier with 100 μg of DNP-KLH in CFA, were injected intraperitoneally (50 × 10⁶ cells/recipient) into irradiated (450 R), syngeneic recipients. Immediately after cell transfer, groups of recipients (6 mice/group) were treated with varying doses (1, 10, 50, 200, or 500 μg) of aqueous DNP-D-GL intraperitoneally, or were not treated. 3 days later, all mice were secondarily challenged with 100 μg of DNP-KLH in saline i.p. Serum anti-DNP antibody levels on day 7 after secondary challenge are illustrated.

75% as compared with those obtained with control cells incubated with saline. Prolongation of the culture period to 48 or 72 hr resulted in levels of suppression of 97 and 91%, respectively, in cells incubated with DNP-D-GL as compared with controls.

A second type of experiment was carried out in which DNP-KLH-primed A/J donor spleen cells (30 days after priming) were incubated in stationary tubes with or without DNP-D-GL (3 μg/10⁶ cells) for short periods of time in a standard 5% CO₂-air atmosphere. After 1 or 4 hr, cells incubated in vitro with saline or DNP-D-GL were washed three times and then injected intraperitoneally (66 × 10⁶ cells/recipient) into irradiated, syngeneic recipients. Additional control mice received DNP-KLH-primed cells from the same pool which had not been incubated at all in vitro. Certain groups of recipients were challenged with 100 μg of DNP-KLH intraperitoneally immediately after cell transfer whereas other groups did not receive secondary
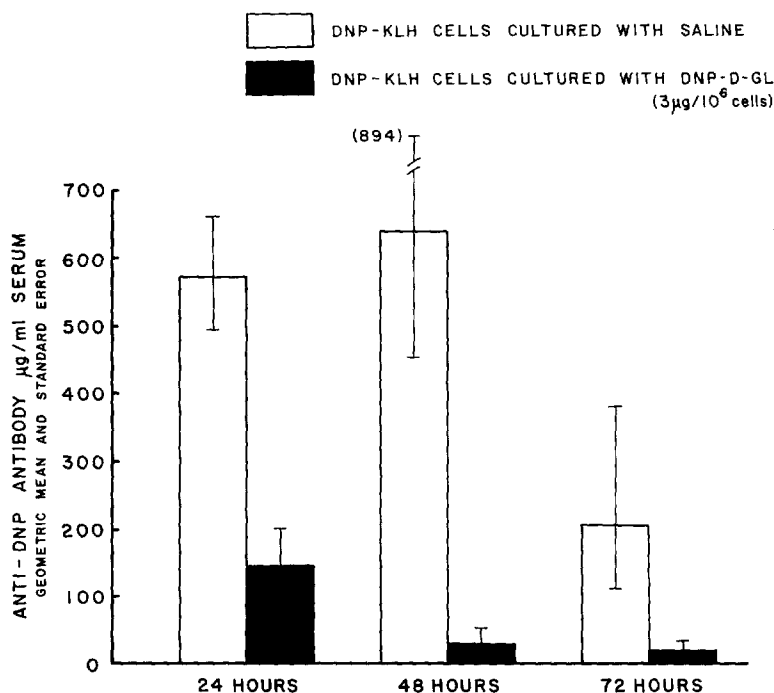
DNP-KLH CELLS CULTURED WITH SALINE

DNP-KLH CELLS CULTURED WITH DNP-D-GL
(3μg/10⁶cells)

Fig. 4. Induction of DNP-specific tolerance in vitro by incubation of DNP-KLH-primed cells with DNP-D-GL in Mishell-Dutton cultures before adoptive transfer. Spleen cells from A/J mice, primed 2 months earlier with 100 μg of DNP-KLH in CFA, were cultured in slightly modified Mishell-Dutton conditions (cell density 30 × 10⁶/ml) with either saline or DNP-D-GL (3 μg/10⁶ cells). At intervals of 24, 48, and 72 hr, cells were harvested from the dishes and washed three times with MEM. Equal numbers of viable saline control cells and cells incubated with DNP-D-GL were transferred intraperitoneally to respective groups (5 mice/group) of irradiated (450 R), syngeneic recipient mice. The numbers of viable cells of each type transferred to individual recipients were 42 × 10⁶ at 24 hr, 38.5 × 10⁶ at 48 hr, and 20 × 10⁶ at 72 hr. All mice were secondarily challenged with 100 μg of DNP-KLH in saline i.p. immediately after cell transfer. Serum anti-DNP antibody levels on day 7 after secondary challenge are illustrated. Statistical comparisons of the responses of recipients of saline control cells and cells exposed to DNP-D-GL yielded P values of $0.005 > P > 0.001$ in all cases.

challenge until 3 days after cell transfer. All mice were bled 7 days after secondary challenge. The results of this experiment are illustrated graphically in Fig. 5.

Incubation with DNP-D-GL for either 1 or 4 hr resulted in significant reductions of the adoptive secondary anti-DNP responses in all cases as compared with controls. However, there was a not inconsiderable difference in the degree of tolerance observed in groups receiving cells incubated for only 1 hr which was related to the time of secondary challenge. Thus, when DNP-KLH challenge was performed immediately after cell transfer, cells preincubated with DNP-D-GL were suppressed by 73% as compared with control cells incubated with sa-
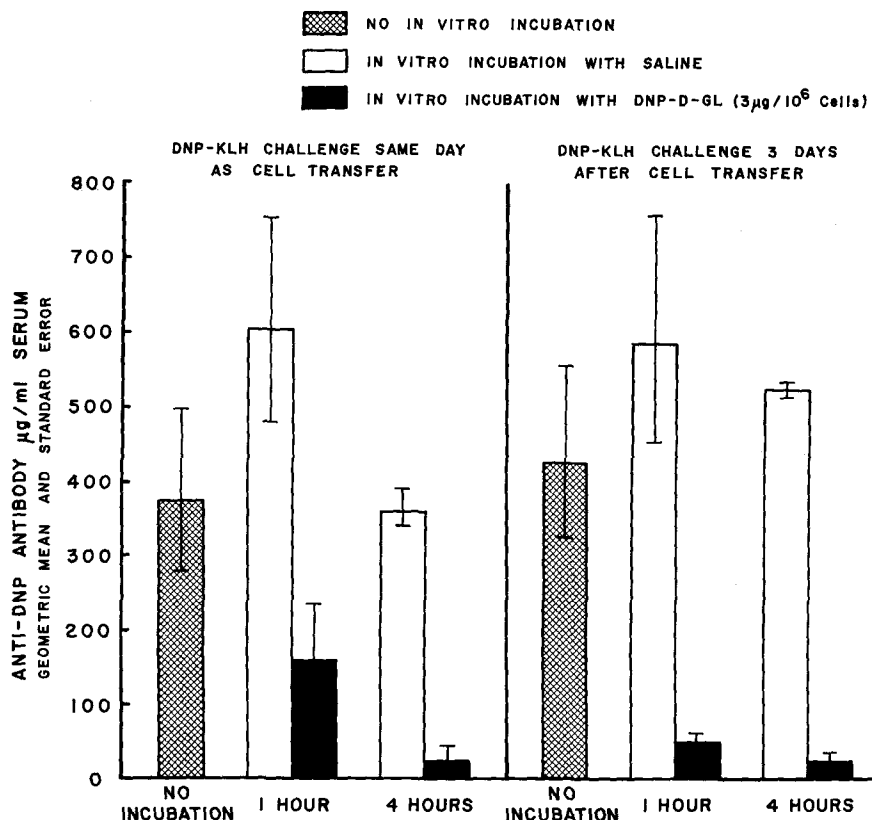
FIG. 5. Induction of DNP-specific tolerance in vitro by incubation of DNP-KLH-primed cells with DNP-D-GL in stationary cultures before adoptive transfer. Spleen cells from A/J mice, primed 30 days earlier with 100 μg of DNP-KLH in CFA, were incubated in stationary tubes with or without DNP-D-GL (3 μg/10^6 cells) for 1 or 4 hr in a 5% CO_2-air environment. At the end of the incubation, cells were washed three times and then injected intraperitoneally (66 × 10^6 cells/recipient) into irradiated (450 R), syngeneic recipients. Additional control mice received DNP-KLH-primed cells which had not been incubated at all in vitro. Certain groups of recipients were challenged with 100 μg of DNP-KLH in saline intraperitoneally immediately after cell transfer (left panel) whereas other groups did not receive secondary challenge until 3 days after cell transfer (right panel). Mean serum anti-DNP antibody levels of groups of 5 mice on day 7 after secondary challenge are illustrated. Statistical comparisons of the responses of recipients of saline control cells and cells exposed to DNP-D-GL yielded the following results. (a) Left panel: 1 hr, 0.05 > P > 0.025; 4 hr, 0.01 > P > 0.005. (b) Right panel: 1 hr, 0.005 > P > 0.001; 4 hr, 0.005 > P > 0.001.

line; when secondary challenge was delayed until 3 days after cell transfer, the level of suppression was 91%. This difference, as well as the fact that, in general, the degree of DNP-specific tolerance obtained by in vitro incubation with DNP-D-GL was less than that obtained by in vivo administration of tolerogen,

may reflect the operation of as yet unknown in vivo mechanisms which may serve to facilitate the intracellular tolerizing events.

Maintenance of DNP-Specific Tolerance after Serial Adoptive Cell Transfers.— Recently, independent investigators have reported that removal and transfer of lymphocytes from tolerant animals to nontolerant, syngeneic, irradiated recipients results in rapid loss of the tolerant state (20–22). These and related observations concerning "self-recognition" phenomena in vitro have raised serious questions about the nature and mechanisms of central immunologic tolerance. When dealing with tolerance models involving both T and B lymphocytes, the matter of interpretative construction of results must of necessity be extremely complex. However, when the tolerant state being studied is shown to be an isolated B cell-specific tolerance, the issue becomes rather clear-cut: in such instances, tolerance either reflects a central (i.e., intracellular) inhibitory state or a surface (i.e., receptor-blocking) event. If the former is true, one would expect tolerance not to be easily reversible and not to depend (once fully induced) on the constant presence of tolerogen; the converse reasoning applies to the latter alternative. In the first experiments described above (Table I), we observed that DNP-KLH-primed cells obtained from donors which had been treated with DNP-D-GL 7 days before adoptive transfer appeared to maintain a significant degree of tolerance in the untreated irradiated recipients. This result suggests a true central tolerizing event in this model. We performed the following series of experiments to approach this question more completely.

50 × 10⁶ spleen cells from A/J mice primed with DNP-KLH 46 days earlier were injected intravenously into two groups of syngeneic, irradiated (500 R) recipients. Two additional groups of recipients were injected with cells from identically primed donors which had been treated with 1.0 mg of DNP-D-GL intraperitoneally 7 days before transfer. Immediately after cell transfer, recipients were treated with either DNP-D-GL or saline and then challenged 3 days later with DNP-KLH. 7 days after secondary challenge, mice in each group were bled and killed. Suspensions of their spleen cells were prepared and adoptively transferred (50 × 10⁶ cells/recipient) to groups of new irradiated, syngeneic recipients who were then divided into subgroups which were either subjected to DNP-D-GL treatment (immediately after cell transfer) or not. 3 days after cell transfer, these new recipients were challenged with DNP-KLH and then bled 7 days later.

The protocol and results of this experiment are summarized in Fig. 6. As shown in the earlier experiments, the secondary anti-DNP response in the first adoptive transfer was abolished by DNP-D-GL treatment of either the recipient immediately after cell transfer (groups II and IV) or of the DNP-KLH cell donors 7 days before transfer (group III). When these first transfer recipients were then used as donors for the second adoptive cell transfer, the results very clearly show that such manipulation does not result in a loss of the tolerant state. Thus, group 1 recipients of cells which had never been exposed to the tolerogen developed very good anti-DNP antibody responses. In contrast, recipients of cells which had been exposed to DNP-D-GL in the first transfer,
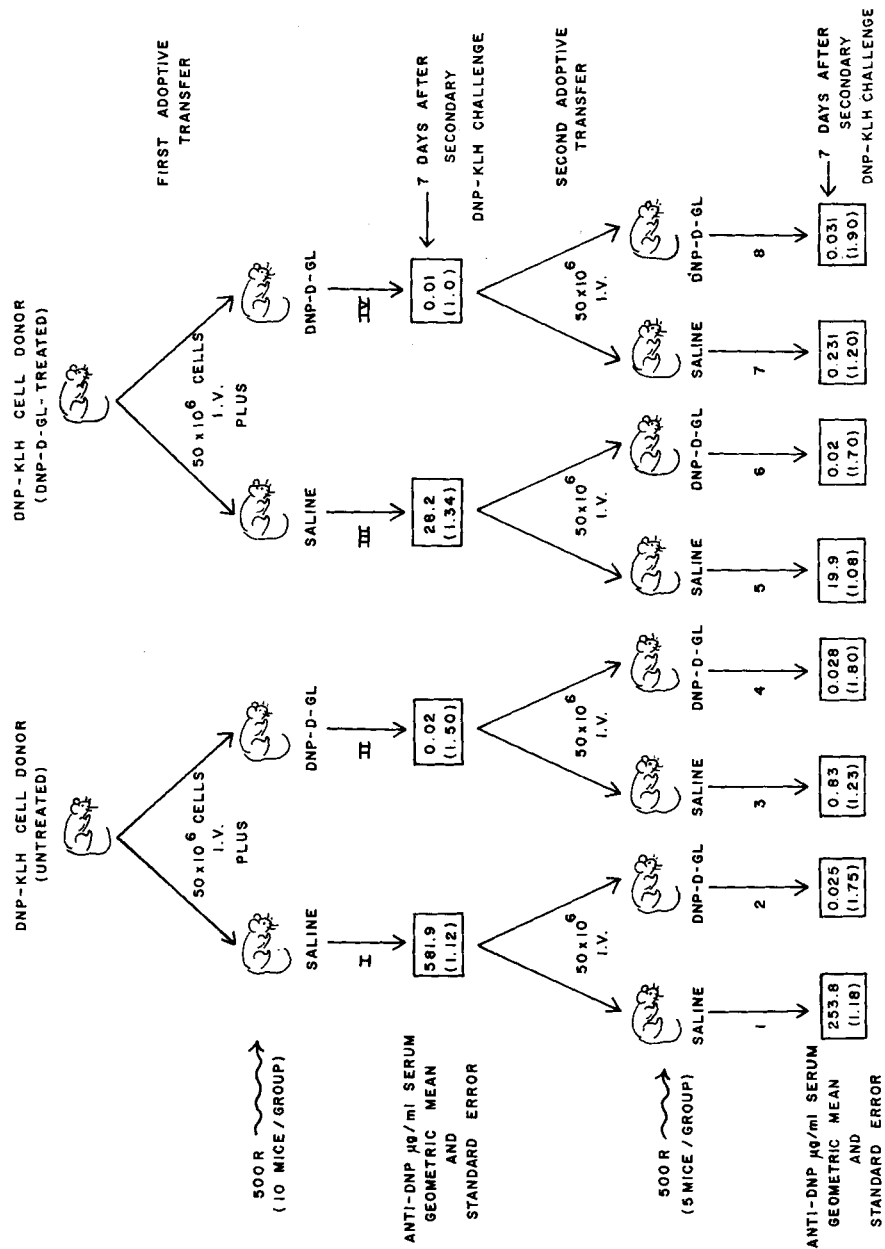
FIG. 6. Maintenance of DNP-specific tolerance after serial adoptive cell transfers. 50×10^6 spleen cells from A/J mice primed with DNP-KLH 46 days earlier were injected intravenously into two groups (I and II) of syngeneic, irradiated (500 R) recipients (10 mice/group). Two additional groups (III and IV) of recipients were injected with cells from identically primed donors which had been treated with 1.0 mg of DNP-D-GL intraperitoneally 7 days before transfer. Immediately after cell transfer, recipients were treated with either DNP-D-GL groups (II and IV) or saline (groups I and III) and then challenged 3 days later with 100 μg of DNP-KLH intraperitoneally in saline. 7 days after secondary challenge, mice in each group were bled and killed. Suspensions of their spleen cells were prepared and adoptively transferred (50×10^6 cells/recipient) to groups of new irradiated, syngeneic recipients who were then divided into subgroups (5 mice each) which were either subjected to DNP-D-GL treatment immediately after cell transfer (groups 2, 4, 6, and 8) or not (groups 1, 3, 5, and 7). 3 days after cell transfer, these new recipients were challenged with 100 μg of DNP-KLH intraperitoneally in saline. Serum anti-DNP antibody levels of groups I–IV and groups 1–8 on day 7 after their respective secondary challenges are illustrated. Statistical comparisons of group I with groups II and III yielded P values of $0.001 > P$ in both cases. Comparison of group 1 with group 5 yielded a P value of $0.005 > P > 0.001$. Comparison of group 1 with groups 2, 3, 4, 6, 7, and 8 yielded P values of $0.001 > P$ in all cases.

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but not subsequently (group 3), manifested profound DNP-specific tolerance. Even more striking, however, is the fact that a highly significant degree of tolerance was evident in group 5 recipients whose cells had not been exposed to DNP-D-GL since the original donors were so treated 24 days earlier. It follows, therefore, that recipients in group 7 should be tolerant, as indeed they were. As expected, essentially no secondary response was obtained in recipient mice treated with DNP-D-GL after the second transfer (groups 2, 4, 6, and 8).

Elimination of the Possiblity of Carry-Over of Tolerogen As the Explanation for Maintenance of the Tolerant State in Serial Adoptive Cell Transfers.—These results immediately raised questions in our minds as to the possibility that we were not only serially transferring cells but small tolerogenic doses of DNP-D-GL as well. We approached this problem by repeating and modifying the preceding experiment in part.

Thus, groups 1 and 2 of the first adoptive transfer were set up as shown in Fig. 6. On day 7 after DNP-KLH challenge, the animals were bled (yielding results comparable to those shown in Fig. 6) and their spleen cells adoptively transferred intravenously to new recipients (50×10^6 cells/recipient). Certain groups of recipients of cells rendered tolerant by DNP-D-GL in the first adoptive transfer were also injected, on the same day, with varying numbers of spleen cells from DNP-KLH-primed donor mice. Comparable groups of mice which received only the respective numbers of these "fresh" DNP-primed cells were established as controls. 3 days later all mice were secondarily challenged with 100 μg of DNP-KLH and then bled 7 days thereafter.

The data from the second transfer of this experiment are depicted graphically in Fig. 7. The left panel of this figure reiterates the observation made in the preceding experiment, namely that the DNP-specific tolerant state is maintained in cells transferred to a second recipient (solid bar). However, when these tolerant cells are transferred simultaneously with freshly obtained DNP-KLH-primed cells they do not exert a suppressive effect on the adoptive secondary anti-DNP response (open bars of right panel, Fig. 7). This was true even when relatively low numbers (12.5×10^6) of fresh DNP-primed cells were employed. It is not immediately clear why the combination of tolerant cells and fresh DNP cells gave somewhat better responses than fresh cells alone, although the differences are not statistically significant. This experiment, therefore, argues strongly against the possibility that DNP-D-GL has been serially transferred in quantities sufficient to maintain the tolerant state in these cells, and points emphatically to the existence of a central, intracellular mechanism of specific paralysis.

Evidence That Tolerance Induced by DNP-D-GL is Not Merely Reflective of Blocking of Surface Receptors.—In the previous experiments, we were able to induce DNP-specific tolerance in a classical adoptive cell transfer system either by (a) preincubating DNP-KLH-primed cells in vitro with DNP-D-GL, or (b) administering DNP-D-GL either to the DNP-KLH-primed cell donor mice 7 days before cell transfer or to the recipients of such cells immediately after

TEST FOR TOLEROGEN CARRY-OVER — SECOND TRANSFER

☐ NONTOLERANT DONORS   ☐ TOLERANT DNP CELLS + FRESH DNP CELLS
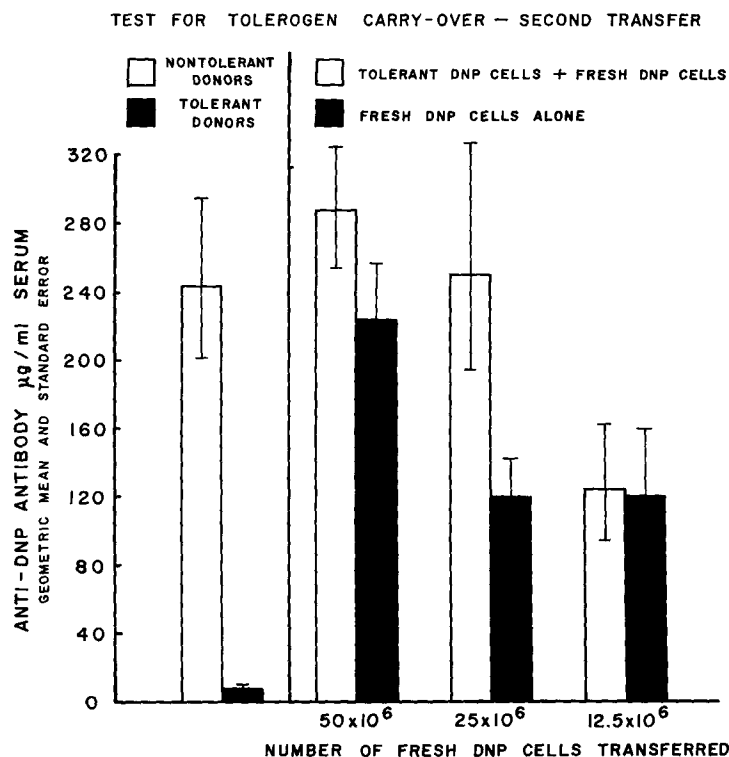
■ TOLERANT DONORS   ■ FRESH DNP CELLS ALONE

FIG. 7. Elimination of the possibility of carry-over of tolerogen in serial adoptive cell transfers. The experimental groups I and II of Fig. 6 were established using spleen cells from A/J donor mice primed 51 days earlier with 100 μg of DNP-KLH in CFA. The results obtained in these two adoptive transfer groups were comparable with those shown in Fig. 6. Spleen cells from these first transfer recipients were then adoptively transferred intravenously to new recipients (50 $\times$ 10^6 cells/recipient). Certain groups of recipients of cells rendered tolerant by DNP-D-GL in the first adoptive transfer were also injected, on the same day, with varying numbers of spleen cells from DNP-KLH-primed donor mice. Comparable groups of mice which received only the respective numbers of these fresh DNP-primed cells were established as controls. 3 days later all mice were secondarily challenged with 100 μg of DNP-KLH and then bled 7 days thereafter. Mean serum anti-DNP antibody levels of groups of 5 mice on day 7 after secondary challenge are shown.

transfer but before secondary challenge with DNP-KLH. One of the critical questions raised by such results is whether the suppression of antibody formation reflects blocking of receptor molecules present on the surface of DNP-specific B lymphocytes. Although the preceding experiments demonstrating maintenance of the tolerant state through serial adoptive transfers argues against a predominantly surface mechanism, they fail to provide direct evidence on this point. The following experiments were designed and carried out to explore this question more fully.

Relationship of time of administration of DNP-D-GL to adoptive cell transfer recipients to the induction of DNP-specific tolerance and failure to induce tolerance in vivo with immunogenic DNP-carrier conjugates: We reasoned that one appropriate way to examine this was to make a comparative study in which the tolerogenic substance, DNP-D-GL, or immunogenic DNP-carrier conjugates, DNP-OVA or DNP-BGG, were administered to the adoptive transfer recipients either before or after cell transfer and secondary challenge.

Spleen cells from A/J donor mice, which had been primed with DNP-KLH 30 days earlier, were injected intraperitoneally (36 × 10⁶ cells/recipient) into syngeneic, irradiated (450 R) recipients. Secondary challenge with 100 μg of DNP-KLH intraperitoneally was performed immediately after cell transfer. Four groups of recipient mice had been treated 3 days before irradiation, cell transfer, and secondary challenge with intraperitoneal injections of 500 μg of either DNP-OVA, DNP-BGG, or DNP-D-GL in saline, or saline alone, while another four groups of recipient mice received identical treatments 2 days after irradiation, cell transfer, and challenge. All mice were then bled 7 days after secondary challenge.

The results are summarized in Table II. Recipients treated with saline alone,

TABLE II

Relationship of Time of Administration of DNP-D-GL to the Induction of DNP-Specific Tolerance in Adoptively Transferred DNP-KLH-Primed Spleen Cells, and Failure to Induce Tolerance with Immunogenic DNP Conjugates

Group	Protocol*		Anti-DNP antibody‡ Day 7 after secondary challenge
	Time of treatment of recipients	Treatment	
			μg/ml
A	3 days before cell transfer and	None	370.7 (1.24)
B	secondary challenge	500 μg DNP-OVA	319.8 (1.38)
C		500 μg DNP-BGG	218.3 (1.42)
D		500 μg DNP-D-GL	52.3 (1.14)
E	2 days after cell transfer and	None	297.0 (1.14)
F	secondary challenge	500 μg DNP-OVA	312.7 (1.25)
G		500 μg DNP-BGG	278.9 (1.31)
H		500 μg DNP-D-GL	11.1 (1.10)

* 36 × 10⁶ spleen cells from A/J donor mice which had been primed 30 days earlier with 100 μg of DNP-KLH in CFA were injected intravenously into individual irradiated (450 R), syngeneic recipients. Secondary challenge with 100 μg of aqueous DNP-KLH intraperitoneally was performed immediately after cell transfer. Recipient mice were treated with 500 μg of aqueous DNP-OVA, DNP-BGG, or DNP-D-GL intraperitoneally either 3 days before cell transfer and secondary challenge (groups B, C, and D) or 2 days after cell transfer and secondary challenge.

‡ The data are expressed as geometric means of serum anti-DNP antibody levels of groups of 5 mice 7 days after secondary challenge. Numbers in parentheses represent standard errors. A comparison of geometric mean antibody levels gave the following results. Comparison of group A with groups B and C yielded P values of 0.80 > P > 0.70 and 0.30 > P > 0.20, respectively. Comparison of groups A, B, and C with group D yielded P values of 0.001 > P in all cases. Comparison of groups E, F, and G with group H yielded P values of 0.001 > P in all cases.

DNP-OVA, or DNP-BGG displayed comparable secondary anti-DNP antibody responses irrespective of whether such treatment was administered 3 days before (groups A, B, and C) or 2 days after (groups E, F, and G) cell transfer and challenge. On the other hand, and in marked contrast, recipients treated with DNP-D-GL at either time relative to transfer and challenge (groups D and H) manifested profoundly suppressed secondary anti-DNP antibody responses. These results provide two reasonable arguments against a receptor-blocking concept as the explanation for the DNP-specific tolerance being studied. First, if receptor blocking alone were responsible, it is difficult to understand why DNP-OVA or DNP-BGG, which are most probably bound by receptors on DNP-specific B cells (but, in this circumstance, fail to trigger such cells), failed to competitively inhibit the response to DNP-KLH. Second, and even more important, is the fact that DNP-D-GL exerted a tolerogenic effect even when it was administered 2 days after secondary challenge with DNP-KLH (group H) at a time when, presumably, a competitive receptor inhibition could no longer operate effectively.

Failure to reverse tolerance induced in vitro with DNP-D-GL by trypsinization of cells before adoptive transfer to irradiated recipients: The second approach to the question of receptor blocking took advantage of the capacity, shown earlier, to induce a significant level of tolerance by incubating primed cells in vitro with DNP-D-GL.

Spleen cells were obtained from A/J donor mice which had been primed with 100 μg of DNP-KLH in CFA 30 days earlier and boosted with same 15 days before sacrifice. These primed cells were incubated in Mishell-Dutton conditions at a density of 30×10^6 cells/ml with 3 μg/10^6 cells of either DNP-D-GL, DNP-OVA, DNP-KLH, or saline alone. After 48 hr, the respective cell groups were harvested from the dishes and washed three times with MEM. Each pool was divided into two samples. One sample was left untreated while the second sample was treated with trypsin as follows: 30×10^6 cells were incubated for 20 min at 37°C in 1.0 ml of a freshly prepared solution containing 150 μg/ml trypsin and 10 μg/ml DNAase (Worthington Biochemical Corp., Freehold, N.J.) in MEM. After trypsinization, the cells were washed three times. Groups of irradiated (500 R), syngeneic recipient mice were injected intravenously with either untreated or trypsinized cells from the respective culture groups. All mice were secondarily challenged with 100 μg of DNP-KLH intraperitoneally in saline immediately after cell transfer and then bled 7 days later.

The results are summarized in Table III. It should be noted from the outset that a considerable disparity exists between the numbers of untreated and trypsin-treated cells transferred to each recipient. This resulted from a considerably higher cell loss than was expected from trypsinization. Recipients of saline-incubated cells (groups A and B) displayed very good adoptive secondary anti-DNP responses whether or not the transferred cells were trypsinized. Recipients of cells exposed to DNP-D-GL in vitro (groups C and D) were markedly suppressed in their secondary responses (95% or more as compared with controls). Most importantly, treatment with trypsin did not abolish the unresponsive state. In striking contrast are the results obtained in recipients of cells incubated with DNP-OVA. If such cells were transferred without trypsini-

TABLE III

Failure to Reverse Tolerance Induced in Vitro with DNP-D-GL by Trypsinization of Cells before Adoptive Transfer to Irradiated Recipients

Cells incubated in vitro with:	Protocol*					
	No trypsin			Trypsin-treated		
	Group	No. of cells transferred	Anti-DNP‡	Group	No. of cells transferred	Anti-DNP‡
			µg/ml			µg/ml
Saline	A	20×10^6	2471.3	B	10×10^6	1621.0
DNP-D-GL	C	20×10^6	107.4	D	13×10^6	55.8
DNP-OVA	E	20×10^6	172.4	F	10×10^6	1212.1
DNP-KLH	G	20×10^6	1384.7	H	7×10^6	447.3

* Spleen cells from A/J mice, primed and boosted 30 and 15 days, respectively, with 100 µg of DNP-KLH in CFA, were incubated for 48 hr in vitro, with saline, DNP-D-GL, DNP-OVA, or DNP-KLH (the latter three at a dose of 3 µg/10^6 cells). At the end of the culture period, cells were thoroughly washed and then divided into two samples. One sample of each type was then incubated (20 min, 37°C) with trypsin (5 µg/10^6 cells) and then washed. The second sample of cells was left untreated. Groups of irradiated (500 R), syngeneic recipients were injected intravenously with untreated or trypsinized cells of the type and in numbers as indicated above. Immediately after cell transfer all mice were challenged with 100 µg of DNP-KLH intraperitoneally in saline.

‡ The data are expressed as geometric means of serum anti-DNP antibody levels of groups of 5 mice 7 days after secondary challenge. A comparison of geometric mean antibody levels gave the following results. Comparison of group A or group B with groups C, D, and E yielded P values of 0.001 > P in all cases. Comparison of group F with group E also yielded a P value of 0.001 > P. Comparison of group A with group G yielded a P value of 0.30 > P > 0.20.

zation (group E), a very significant level of suppression (93%) occurred. Treatment of such cells with trypsin, on the other hand, restored the adoptive secondary anti-DNP response to essentially normal levels (group F). Incubation of cells with the homologous antigen (DNP-KLH) did not significantly alter the adoptive secondary response (group G). It is not immediately clear to us why the response of group H recipients of trypsinized cells was somewhat lower.

Since trypsin presumably removes surface receptors (and any associated antigen), these results indicate that (a) the suppression observed in group E reflected blocking of surface receptor molecules by DNP-OVA; and (b) the failure of trypsinization to reverse unresponsiveness of DNP-D-GL-exposed cells must, by converse reasoning, reflect more complex inhibitory events not predominantly related to reversible surface membrane factors.

DISCUSSION

In the studies reported here, we have shown that administration of the DNP derivative of the copolymer of D-glutamic acid and D-lysine to inbred mice induces a state of DNP-specific tolerance in such animals irrespective of their immune status at the time of treatment. These results confirm and extend to

mice our previous observations on DNP-specific tolerance in inbred guinea pigs (4). In the latter studies, we presented evidence for a central mechanism of tolerance which is expressed predominantly in the population of DNP-specific antibody-forming cell precursors. This interpretation is derived from the finding that the tolerance is hapten-specific and that the frequency of both DNP-specific, antigen-binding lymphoid cells and anti-DNP, antibody-forming cells is significantly lower in guinea pigs tolerized with DNP-D-GL than in normal or immune animals (4, 14). Moreover, subsequent studies in the guinea pig model have demonstrated that a very marked depression, both in plaque-forming cells secreting high affinity anti-DNP antibody and in high affinity serum anti-DNP antibody, exists in this model, indicating a preferential tolerization of precursor cells bearing high affinity receptors (14).

A rather unique feature of the DNP-D-GL tolerance model is the relative ease with which tolerance can be induced in an animal previously immunized with an immunogenic DNP-carrier conjugate (4). In the present studies, we have taken advantage of this feature to establish conditions for tolerance induction in an adoptive transfer system in mice. (These studies have been performed under the conscious assumption that basic mechanisms of cell inactivation are fundamentally the same in primed and unprimed immunocompetent cell populations.) Thus, spleen cells from DNP-KLH-primed donor mice normally developed very good adoptive secondary anti-DNP antibody responses to DNP-KLH upon transfer to syngeneic, irradiated recipients. However, exposure of such DNP-primed cells to the DNP-D-GL tolerogen completely, or almost completely, abolished the adoptive secondary response. This was true irrespective of whether the DNP-primed cells were exposed to DNP-D-GL in the donor animal before adoptive transfer or in recipient mice after transfer. In the latter situation, it was possible to show a very clear dose-response relationship for tolerance induction with DNP-D-GL which is consistent with the above-mentioned observations on preferential depletion of high affinity antibody-forming cells in guinea pigs (14).

Incubation of DNP-KLH-primed cells with DNP-D-GL in vitro under varying culture conditions also resulted in depression of the adoptive secondary response of such cells although the kinetics and degree of tolerance induction in this way were slightly different from that obtained by in vivo tolerization. Thus, treatment of adoptive transfer recipients in vivo resulted in virtually complete tolerance induction within 1 hr after administration of an appropriate dose of DNP-D-GL. In contrast, when DNP-KLH-primed cells were incubated with DNP-D-GL in vitro for 1 hr, washed, and then transferred to irradiated recipients, the adoptive secondary response to DNP-KLH challenge performed immediately after cell transfer was depressed by 73% but not completely abolished. Allowing such cells to reside in the recipient for 3 days before secondary challenge resulted in a higher level of tolerance (91% suppression). The kinetics of tolerance induction in vitro also varied somewhat with the culture

conditions employed. Incubation in stationary tubes for 4 hr resulted in greater than 90% suppression as compared with 75% suppression obtained after 24 hr incubation in Mishell-Dutton conditions. Nonetheless, essentially complete tolerance occurred after incubation for 48 hr or longer in the latter conditions. These kinetic differences, as well as the fact that, in general, the degree of DNP-specific tolerance obtained by in vitro incubation with DNP-D-GL was less than that obtained by in vivo administration of tolerogen, may reflect the operation of as yet unknown in vivo mechanisms which may serve to facilitate the intracellular tolerizing events.

In view of the very detailed studies by Chiller et al. (1) on kinetics of tolerance induction to deaggregated human gamma globulin (HGG), which demonstrate a rather long latent period for initiation (8 days) and completion (21 days) of tolerance in bone marrow cells (although recent studies indicate that peripheral B lymphocytes in spleen become tolerant within 3 days; J. M. Chiller, personal communication), it is essential to explain the extremely rapid kinetics of tolerization observed with DNP-D-GL. This is perhaps best explained by the critical difference, which may likely exist, in the cell types involved in the two systems. Thus, tolerance induction to HGG or other thymus-dependent antigens very clearly involves the establishment of tolerance in both T and B lymphocytes, the former being rendered tolerant much more rapidly and with lower concentrations of tolerogen (1, 2). It is conceivable that in such situations tolerance induction in B lymphocytes follows a rather inefficient course until a state of absolute tolerance in all T lymphocytes of corresponding specificity has been established. In the case of DNP-D-GL, on the other hand, tolerance induction may involve exclusively the specific B lymphocyte population. As the copolymer of D-GL is either nonimmunogenic or only marginally immunogenic in guinea pigs (4) and mice (unpublished observations), it seems likely that T lymphocytes specific for this substance do not exist or are nonfunctional. Based on this assumption, we have previously hypothesized that the tolerant state resulting from DNP-D-GL treatment reflects direct interaction of DNP-specific B lymphocytes with the substance, in appropriate concentrations, in the absence of a concomitant T cell influence (4, 13). This hypothesis is strengthened by our observation that a nonspecific T cell activation caused by a graft-versus-host reaction (allogeneic effect) (24) results in the development of immunity rather than tolerance to DNP-D-GL in both guinea pigs (4) and mice (25). In this context, it appears that tolerance induction in B lymphocytes does proceed along a more rapid kinetic course than in a thymus-dependent system such as that of Chiller and Weigle (1, 2). Indeed, this reasoning is supported by previous studies on tolerance induction in vitro to thymus-independent antigens such as polymerized flagellin (POL) (26) or Escherichia coli lipopolysaccharide (LPS) (27) where the kinetics of induction have been rapid, as in our experiments here, resembling those observed with thymus cells in vivo. It is probably also relevant to note that attempts to induce specific tolerance in vitro with thymus-dependent antigens have been generally unsuccessful (28).

A critical question in any model of tolerance concerns the mechanism by which suppression of antibody formation occurs. Specifically, what happens to a tolerant cell? Does it exist in a functionally unresponsive or unrecognizable state, or is it eliminated from the system? The available evidence from studies on antigen-binding cells may appear conflicting in that some investigators have found normal numbers of such cells (5–8) while others have found diminished numbers (3, 4) in tolerant animals. These apparent contradictions may be readily resolved, it seems to us, by the following considerations.

(a) In the case of tolerance in thymus-dependent antigen systems, the presence and frequency of antigen-binding cells (representing B cell precursors of antibody-forming cells) will depend on the nature of the target cell involved and the degree of tolerance existing at the time cells are examined. Hence, where tolerance exists predominantly among T cells, one would expect to find relatively normal numbers of antigen-binding cells. However, where B cell tolerance is achieved, even for a thymus-dependent antigen, such as HGG, Chiller has recently observed that specific antigen-binding cells are significantly diminished in tolerant animals (J. M. Chiller, personal communication).

(b) In both thymus-dependent and thymus-independent systems, the detection of antigen-binding cells in a tolerant animal in which some degree of B cell tolerance exists may depend upon the receptor affinity of the cells being studied. In view of the fact that tolerance results in preferential diminution of high affinity antibody-forming cells and antibodies (14, 29–31), antigen-binding cells detected in such circumstances may be predominantly of low affinity receptor type, whereas high affinity cells may be significantly diminished.

(c) Finally, one must bear in mind the nature of the tolerant state induced. This point appears to be particularly applicable to the tolerance induced to the polysaccharide of Type III pneumococcus (S_{III}) and to $E.$ $coli$ lipopolysaccharide (LPS), both of which are thymus-independent antigens (32–36). As recently reviewed by Howard (20, S_{III}) and by Möller and Sjöberg (22, LPS), some outstanding common features shared by these tolerance models include (a) the presence of increased numbers of specific antigen-binding cells in tolerant animals which, nevertheless, possess markedly diminished numbers of antibody-forming cells; (b) the rapid loss of tolerance upon transfer of cells from tolerant donors to irradiated, syngeneic recipients; and (c) the relative incapacity of such substances to be catabolized. Howard (20) has concluded that the tolerant state to S_{III} is therefore reflective of a complexity of events involving three different mechanisms that include continuous peripheral neutralization of secreted antibody, and predominantly reversible and some irreversible inactivation of B lymphocytes. These complex events do not appear to play the predominant role in the tolerance achieved with DNP-D-GL which is most likely reflective of intracellular inactivation.

In the context of the above considerations, we have utilized several approaches in the present studies to probe the questions of mechanism of tolerance induction and fate of tolerant cells in the DNP-D-GL model. One

immediate question concerned the possibility that suppression of antibody formation reflects blocking of surface receptor molecules on DNP-specific B lymphocytes. This possibility has been ruled out by the following observations: (a) The failure to induce tolerance in the adoptive transfer system by treatment of recipients of DNP-KLH-primed cells with immunogenic conjugates of DNP-OVA or DNP-BGG under circumstances where profound tolerance was induced by DNP-D-GL. If receptor blocking alone is responsible for this DNP-specific tolerance, one might expect that DNP-OVA or DNP-BGG, which are most probably bound by receptors on DNP-specific B cells (but, in this circumstance, fail to trigger such cells), would competitively inhibit the response to DNP-KLH.

(b) More importantly, the capacity to induce tolerance with DNP-D-GL even when it was administered 2 days after adoptive cell transfer and secondary challenge. In this case, exposure of the cells to DNP-D-GL occurred at a time when, presumably, a competitive receptor inhibition could no longer operate effectively.

The most conclusive evidence that DNP-D-GL tolerance involves more sophisticated events than receptor blockade derives from the failure of enzymatic treatment by trypsin of cells tolerized by DNP-D-GL in vitro to reverse, or even diminish, the level of unresponsiveness manifested by such cells. If these cells still had been capable of responding to DNP-KLH but could not do so because all of their surface receptors were competitively blocked by DNP-D-GL, then this situation should have been corrected by trypsinization. Indeed, the results obtained in the very same experiment by incubation with DNP-OVA offer perhaps the best example of the sharply contrasting mechanisms that may contribute to unresponsiveness. Thus, since suppression of the adoptive secondary response after DNP-OVA incubation was readily, and completely, reversed by trypsinization, it is clear that unresponsiveness in this case resulted from effective (and reversible) receptor blockade. This illustrates, moreover, that such a mechanism can significantly inhibit antibody production, though the prediction seems valid that unresponsiveness of this type is probably very transient in nature. Failure of trypsinization to reverse the unresponsiveness induced by DNP-D-GL, on the other hand, supports the conclusion that such cells have been inactivated via intracellular mechanisms. A tolerant state of this type would be predictably long lasting.

The other observations reported here that bear on the issues cited above are those dealing with the serial transfer of tolerance. Thus, cells rendered tolerant by DNP-D-GL manifested the unresponsive state through as many as two serial adoptive transfers to irradiated, syngeneic recipients. This was true, furthermore, over a period of time of at least 24 days from the initial, and only, exposure of such cells to the tolerogen. The possibility that maintenance of tolerance through such serial transfers was due to transfer of tolerogenic doses of DNP-D-GL was definitively ruled out by the demonstration that simulta-

neous adoptive transfer of large numbers of tolerant cells with nontolerant DNP-KLH-primed cells did not diminish the secondary response of the latter to DNP-KLH.

If, as we propose, the tolerance induced by DNP-D-GL is a restricted B cell tolerance, then these results on serial transfer of the tolerant state are informative with respect to understanding the mechanism of tolerance in B lymphocytes. The results obtained with thymus-independent antigens depends very much upon the system used. Thus, as mentioned above, tolerance induced in vivo to certain thymus-independent antigens, S_{III} and LPS, is characteristically (and rapidly) lost after adoptive transfer (20-22). Tolerance to other thymus-independent antigens is both long lasting and transferable as in the case of the fructose polymer, levan (J. Miranda, cited by Howard [20]). This thymus-independent antigen induces a state of B cell tolerance which is not lost upon adoptive transfer. Similarly, in the system of tolerance induction in vitro to thymus-independent antigens such as POL and DNP-POL and with antigen-antibody complexes (prepared in a critical ratio), the tolerant state has also been successfully transferred to irradiated recipients (recently reviewed by Diener and Feldmann [37]). Moreover, these investigators have also shown that removal of cell-bound antigen by trypsinization did not reverse the unresponsive state induced by high doses of POL in vitro, provided the period of tolerance induction was of sufficient length (37). Our findings with DNP-D-GL in the present study are quite consistent with these results and provide a strong argument that tolerance among specific B lymphocytes can be, and in its absolute sense should be, a reflection of irreversible inhibition of cell reactivity to antigen. Moreover, it is most likely that irreversible inactivation is not a unique feature of certain thymus-independent antigens, but pertains to tolerance (in both B and T cells) in thymus-dependent systems as well. We should emphasize, however, that the concept of irreversibility as used here pertains to the individual cells which have been exposed to the tolerogen and not necessarily to the future progeny of the stem cell clone bearing specificity for the tolerogen.

The precise nature of events at the cellular and subcellular levels that result in specific unresponsiveness are as yet unknown. Nonetheless, as recently reviewed by us (13), certain observations permit the general conclusion to be made that the possible interpretation of a given antigenic signal by a specific cell, i.e. as a tolerogenic or as an immunogenic signal, will most likely be determined by the existence of several variables such as (a) the density and valence of determinant binding at B cell surface receptors, and (b) the presence, absence, and/or extent of T cell regulatory function at the time the signal is received. The existence of tolerance induction systems such as the in vitro model developed by Diener and Feldmann and colleagues (37) and the DNP-D-GL model described here may provide certain advantages in studies designed

to probe the nature of subcellular events that follow transmission of the tolerogenic signal.

SUMMARY

Administration of the 2,4-dinitrophenyl (DNP) derivative of the copolymer of D-glutamic acid and D-lysine (D-GL) to inbred mice induces a state of DNP-specific tolerance in such animals irrespective of their immune status at the time of treatment. Taking advantage of the relative ease with which DNP-D-GL can induce tolerance in an animal previously primed with an immunogenic DNP-carrier conjugate, we have established conditions for tolerance induction in an adoptive cell transfer system. Thus, the adoptive secondary anti-DNP antibody response of DNP-keyhole limpet hemocyanin (KLH)-primed spleen cells was completely, or almost completely, abolished by exposure of such cells to DNP-D-GL either in vivo or in vitro. Tolerance induction in vivo occurred irrespective of whether the DNP-primed cells were exposed to DNP-D-GL in the donor animal before adoptive transfer or in recipient mice after transfer. In the latter situation, it was possible to show that tolerance induction in this model occurs very rapidly (1 hr) and with relatively low doses of tolerogen (50 µg). Incubation of DNP-KLH-primed cells with DNP-D-GL in vitro under varying culture conditions also resulted in depression of the adoptive secondary response of such cells, although the kinetics and degree of tolerance induction in this way were slightly different from that obtained by in vivo tolerization.

Utilizing the adoptive transfer tolerance system, it was possible to approach certain questions concerning the mechanism of tolerance induction and fate of tolerant bone marrow-derived (B) lymphocytes in the DNP-D-GL model. The possibility that suppression of anti-DNP antibody from the DNP-D-GL reflects blocking of surface receptor molecules on B lymphocytes has been ruled out by several experimental observations. The most conclusive evidence on this point derives from the failure of enzymatic treatment with trypsin to reverse the tolerant state induced by in vitro exposure of primed cells to DNP-D-GL, whereas trypsinization completely restored the immunocompetence of DNP-KLH-primed cells rendered unresponsive by exposure to DNP-ovalbumin in vitro. The present studies also demonstrate that the tolerant state induced by DNP-D-GL represents a predominantly irreversible inactivation of specific B lymphocytes. This conclusion is derived from experiments in which it was found that tolerance was maintained through as many as two serial adoptive transfers performed over a period of time of at least 24 days from the single exposure of such cells to the tolerogen. Moreover, the possibility that maintenance of tolerance through such serial transfers was due to inadvertent transfer of tolerogenic doses of DNP-D-GL was definitively ruled out. It appears, therefore, that DNP-specific tolerance induced by DNP-D-GL is an example of irreversible inhibition of cell reactivity to antigen reflecting yet-to-be-determined events at the intra- and subcellular levels.

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