IMMUNOLOGICAL TOLERANCE IN BONE MARROW-DERIVED LYMPHOCYTES

II. Effects of Allogeneic Cell Interactions and Enzymatic Digestion with Trypsin on Inactivated Hapten-Specific Precursors of Antibody-Forming Cells*

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The administration of the 2,4-dinitrophenyl (DNP) conjugate of the "non-immunogenic" copolymer of D-glutamic acid and D-lysine (D-GL)[1] induces a state of profound DNP-specific immunological tolerance (1–6). In our original description of the model several years ago (1), normal or DNP-immune inbred guinea pigs treated with DNP-D-GL were found to be incapable of developing anti-DNP antibody responses following immunization with an immunogenic DNP-protein conjugate. The tolerant state was shown to be expressed exclusively in the population of DNP-specific antibody-forming cell precursors as evidenced by a significant diminution in the frequency of both DNP-specific antigen-binding bone marrow-derived (B) lymphocytes and anti-DNP antibody-forming cells in guinea pigs tolerized with DNP-D-GL as compared to normal or immune animals (1, 2). Moreover, tolerant animals displayed a very marked depression both in plaque-forming cells secreting high affinity anti-DNP antibody and in high affinity serum anti-DNP antibody, indicating a preferential tolerization of precursor B cells bearing high affinity receptors (2).

Subsequent studies in inbred mice demonstrated that administration of DNP-D-GL induced a state of DNP-specific tolerance in such animals irrespective of their immune status at the time of treatment (3). 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 established conditions for tolerance induction in an adoptive cell transfer system. Thus, the adoptive secondary anti-DNP antibody response of DNP-primed spleen cells was abolished by exposure of such cells to DNP-D-GL either in vivo or in vitro (3). Utilizing the adoptive transfer tolerance system, evidence was obtained to support the hypothesis that the tolerant state induced by DNP-D-GL represents an irreversible inactivation of specific B lymphocytes. This conclusion was based on the following experimental observations (3): (*a*) The tolerant state was maintained through as many as two serial adoptive transfers performed over a period

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

of time at least 24 days from the single exposure of primed cells to the tolerogen; (*b*) 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; and (*c*) Finally, the possibility that tolerance by DNP-D-GL reflects blocking of surface receptor molecules on B lymphocytes was argued against on the basis of the failure of enzymatic treatment with trypsin to reverse the tolerant state induced by in vitro exposure of cells to DNP-D-GL whereas, in contrast, trypsinization completely restored the immunocompetence of DNP-specific cells rendered unresponsive by exposure to DNP-ovalbumin in vitro.

The present studies were undertaken to further explore the cellular events involved in specific inactivation by DNP-D-GL. The intriguing paradox with this molecule is that it induces tolerance extremely rapidly following exposure of B cells to it under normal conditions, and yet in the presence of a strong appropriately timed T-cell influence, such as that provided by the allogeneic effect, the molecule can transmit a positive immunogenic signal (1, 4, 7–9). This provides, therefore, another method by which to test the relative permanence of B-cell inactivation by DNP-D-GL. An additional question of critical importance concerns the failure, as mentioned above, of trypsin to reverse the tolerance induced by DNP-D-GL (3). To what extent does this failure reflect the fact that D-rotatory amino acid copolymers are themselves resistant to digestion by mammalian enzymes? In the experiments herein, we present data bearing on these questions and additional parameters of tolerance induction by DNP-D-GL. The evidence obtained in these studies reinforces the concept of central tolerance in B cells induced by DNP-D-GL as reflecting sub- or intracellular inhibitory events.

Materials and Methods

Proteins and Hapten-Carrier Conjugates.—The copolymers of D-GL and L-glutamic acid and L-lysine (L-GL) were obtained from Pilot Chemicals, Inc., Watertown, Mass. Both isomers had an average mol wt of 50,000 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) 5x recrystallized and bovine gamma globulin (BGG) were obtained from Pentex Biochemicals, Kankakee, Ill. The following DNP conjugates were prepared as previously described (10, 11): DNP_9-KLH, DNP_{14}-KLH, and DNP_{20}-L-GL. The preparation of DNP_{37}-D-GL has been described in detail elsewhere (1). Subscripts refer to the average number of moles of DNP per mole of carrier for L-GL and D-GL and to the number of moles of DNP per 100,000 mol wt units for KLH.

Animals.—Mice of the inbred lines BALB/c, A/J, and (BALB/c × A/J)F_1 hybrids (CAF_1) were obtained from Jackson Laboratories, Bar Harbor, Maine. All mice were immunized or used as recipients at 8–12 wk of age.

Immunizations and Adoptive Cell Transfers.—Mice used as donors of DNP-primed spleen cells were immunized by intraperitoneal injection of 100 μg of DNP-KLH emulsified in complete Freund's adjuvant (CFA). At various times after priming (usually 3–5 mo) suspensions in Eagle's minimum essential medium (MEM) were prepared and washed. Depending on the nature of the experiment these cells were either subjected to some in vitro manipulation or transferred (see Results). Cells were transferred intravenously to syngeneic, irradiated (500–

600 R) recipients. Secondary challenge was in all cases administered intraperitoneally with the DNP-KLH in saline. Recipient mice were bled from the retroorbital plexus 7 days after secondary challenge and serum anti-DNP antibody levels were determined as described below.

In Vitro Incubation Procedures.—In experiments involving exposure of primed cells to tolerogenic molecules in vitro, the following conditions were used: for short term cultures (1–4 h), cells were incubated in closed plastic 50 ml culture tubes at a cell density of 20×10^6 cells/ml in MEM supplemented with 10% fetal calf serum. The cell suspensions were gently agitated every 15–20 min during incubation. For longer culture periods (2–4 days), cells were cultured in petri dishes under Mishell-Dutton conditions at a cell density of 30×10^6 cells/ml. In all cases, cells were thoroughly washed at termination of culture and viabilities determined by trypan blue exclusion.

Treatment of Cells with Trypsin.—Enzymatic treatment of cells with trypsin was performed as follows: 30×10^6 cells were incubated for 20 min at 37°C in 1 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 in MEM.

Measurement of Anti-DNP Antibodies.—Serum anti-DNP antibody levels were determined by a modified Farr technique (12, 13) using [^{3}H]DNP-ε-amino-*N*-caproic acid (10). Using standard curves constructed for individual mouse strains in a manner identical to that described previously for inbred guinea pigs (10), 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 0.10 μg/ml was arbitrarily assigned to allow logarithmic transformation of the data.

RESULTS

Capacity of the Allogeneic Effect to Alter or Reverse DNP-Specific Tolerance Induced by DNP-D-GL.—The allogeneic effect has been shown to be a potent T-cell stimulus capable of influencing antigen-induced activation of B lymphocytes (4, 7, 14). Indeed the original observation that this phenomenon could convert a normally strong tolerogenic molecule such as DNP-D-GL into an immunogenic signal (1) provided the strongest early evidence that the allogeneic T cells were reacting directly with DNP-specific B lymphocytes, a point which has since been directly substantiated (7, 15, 16). Moreover, Osborne and Katz have recently shown that an appropriately timed allogeneic effect can provide the necessary helper influence for generating primary antibody responses of the IgG class to DNP-D-GL (9).

The observations mentioned above provide a starting point to question certain parameters of B-cell inactivation by DNP-D-GL. The most important of these concerns the degree of reversibility of such a tolerant state inasmuch as this feature offers an indirect view into possible mechanisms involved. The following series of experiments were designed to probe the capacity of the allogeneic effect, a source of strong T-cell action, to alter the responsiveness of B cells subjected to various tolerance-inducing regimens.

When DNP-specific tolerance is induced by incubation of DNP-KLH-primed cells in vitro with DNP-D-GL before adoptive transfer: Spleen cells from CAF_1 mice primed 1–1½ mo earlier

with DNP-KLH were incubated in culture tubes with either DNP-D-GL (2 μg/10^6 cells) or saline. The cells were incubated at a cell density of 20 $\times$ 10^6 cells/ml at either 37°C or 4°C for 1 h. Afterwards, the various aliquots of cells were washed three times with MEM and then counted. Equal numbers (20 $\times$ 10^6) of viable (trypan blue exclusion) saline-control cells and cells incubated with DNP-D-GL were transferred intravenously to respective groups of X-irradiated (550 R), syngeneic CAF_1 mice. Certain groups received an additional transfer, immediately after the first, of parental BALB/c spleen cells (2 $\times$ 10^6 cells/recipient) while the remaining groups received no allogeneic cells. Immediately thereafter, animals were either challenged with 10 μg of DNP-KLH intraperitoneally or not and then bled 7 days later. The 1:10 ratio of allogeneic to primed cells employed is based on our previously reported observation on optimal parameters for the phenomenon in mice (15).

The far left panel of Fig. 1 summarizes schematically the protocol for these two experiments. The left and right data panels depict the results obtained with cells incubated at 4°C and 37°C, respectively. Essentially no substantial differences attributable to temperature of incubation were observed. In both experiments when no additional allogeneic cells were transferred, very good adoptive secondary anti-DNP antibody responses were obtained with control cells incubated with saline provided secondary challenge with DNP-KLH was carried out. Cells incubated with DNP-D-GL manifested poor responses to DNP-KLH challenge that were diminished by more than 95% irrespective of the temperature of incubation. When additional allogeneic cells were transferred, the secondary response of control saline-treated cells was enhanced in

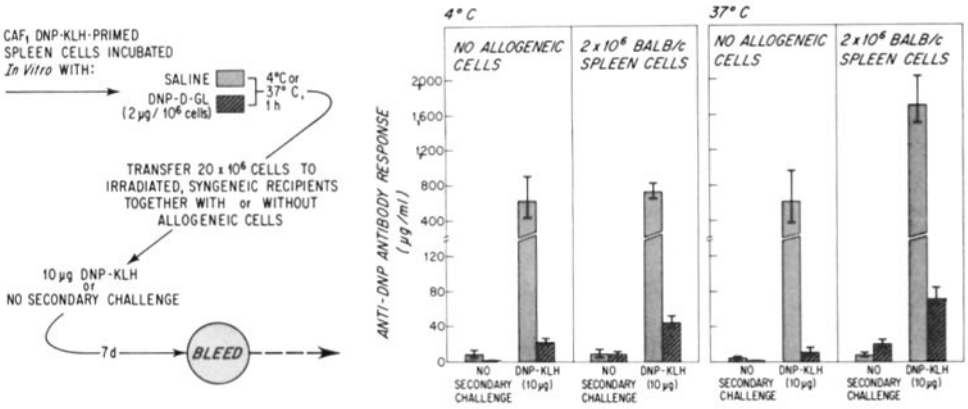

FIG. 1. Induction of DNP-specific tolerance by incubation of DNP-KLH-primed cells in vitro with DNP-D-GL before adoptive transfer. The protocol is depicted schematically on left of figure. Two experiments are shown. In one the temperature of in vitro incubation of cells with either saline or DNP-D-GL was 4°C while in the other it was 37°C as indicated. Each group consists of five mice. Relevant statistical comparisons of secondary responses to DNP-KLH challenge yielded the following P values: (*a*) cells incubated with DNP-D-GL compared with saline-treated cells, $0.001 > P$ at both 4°C and 37°C and when transferred either with or without allogeneic cells; (*b*) cells incubated with DNP-D-GL and transferred together with allogeneic cells as compared to DNP-D-GL-treated cells transferred without allogeneic cells, $0.05 > P > 0.025$ at 4°C and $0.01 > P > 0.005$ at 37°C.

one experiment (right panel, 37°C incubation) but not in the other (left panel, 4°C). The responses to DNP-KLH obtained with cells incubated with DNP-D-GL were increased as a result of allogeneic cell transfer in both experiments. Whereas these increases are clearly statistically significant when compared to responses of cells exposed in vitro to DNP-D-GL and transferred without allogeneic cells, it should be noted that the secondary responses in the presence of allogeneic cells were still diminished by more than 92% as compared to the responses of respective saline control cells in the presence of allogeneic cells. Nevertheless, the fact that a significant increase in response could be obtained as a result of allogeneic cell transfer indicates the presence of a subpopulation of DNP-specific B cells capable of being triggered by a sufficient T-cell stimulus even after exposure to the tolerogenic signal.

When DNP-specific tolerance is induced by exposure of DNP-KLH-primed cells to DNP-D-GL in vivo in adoptive recipients: This experiment utilized the system that we previously described for induction of tolerance in DNP-primed cells in an adoptive transfer system by treating recipients in vivo with appropriate doses of DNP-D-GL (3). The protocol is schematically depicted in the left panel of Fig. 2. Spleen cells from CAF_1 donor mice primed 3 mo earlier with DNP-KLH were transferred intravenously to irradiated (600 R), syngeneic recipients (50×10^6 cells/mouse) who were immediately thereafter treated with either saline or 500 μg of DNP-D-GL in saline intraperitoneally. 3 days later all mice were secondarily challenged with 10 μg of DNP-KLH i.p. 7 days after challenge these two groups were bled and then sacrificed. Serum anti-DNP antibody levels reflected excellent responses in saline control recipients (4,327 μg/ml average) and virtually no response in the DNP-D-GL-treated group (0.16 μg/ml). Their respective spleen cells were pooled, washed, and transferred intravenously to new, irradiated CAF_1 recipients (50×10^6 cells/mouse) either together with unprimed parental A/J spleen cells (5×10^6 cells/recipient) or without allogeneic cells. All mice were then immediately challenged with 10 μg of DNP-KLH intraperitoneally, and bled 7 days later.

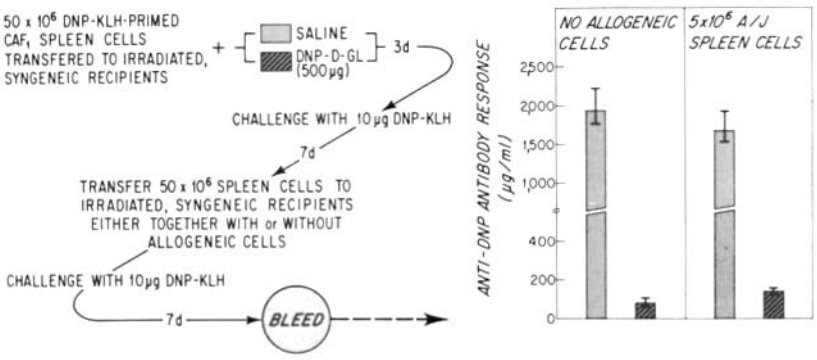

FIG. 2. Induction of DNP-specific tolerance by exposure of DNP-KLH-primed cells to DNP-D-GL in vivo in adoptive recipients. The protocol is depicted schematically on left of figure. Each group consists of six mice. Relevant statistical comparisons of antibody responses yielded the following P values: (*a*) cells exposed to DNP-D-GL compared with saline-control cells, $0.001 > P$ when transferred either with or without allogeneic cells; (*b*) cells exposed to DNP-D-GL and transferred together with allogeneic cells as compared to DNP-D-GL-treated cells transferred without allogeneic cells $- 0.10 > P > 0.05$.

As shown in the right panel of Fig. 2, very good anti-DNP responses were elicited in the second adoptive recipients of primed cells exposed only to saline in the initial recipients, and these responses were not significantly affected by allogeneic cell transfer. In contrast, cells exposed to DNP-D-GL in the first recipients developed markedly diminished responses, lower by more than 90% of controls, irrespective of whether or not allogeneic cells were included in the second transfer.

When DNP-specific tolerance is induced by in vitro exposure to DNP-D-GL of DNP-KLH-primed spleen cells from donors subjected to the allogeneic effect:—The preceding experiments failed to demonstrate any striking capacity of the allogeneic effect to reverse tolerance induction by DNP-D-GL. It has been amply documented, however, that timing of optimal allogeneic cell interaction to antigen encounter by B cells is critical for development of enhancing effects in this system (7, 8, 14, 17). It is possible that the preceding experiments did not allow sufficient time for generation of effective allogeneic cell interactions prior to the tolerogenic signal from DNP-D-GL. The following experiment was designed to circumvent this possible explanation by inducing the allogeneic effect in the donors of DNP-KLH-primed cells before their subsequent in vitro exposure to DNP-D-GL.

As depicted in Fig. 3, CAF_1 mice primed 3 mo earlier with DNP-KLH were injected intravenously with parental BALB/c spleen cells (25×10^6 cells/mouse). 2 days later, these mice and a group of comparably primed CAF_1 donors that had not received allogeneic cells were sacrificed and their spleens removed. The pooled spleen cells from each donor group were then incubated in vitro for 1 h at 37°C with either saline or DNP-D-GL ($3\ \mu g/10^6$ cells) as in the earlier experiment (Fig. 1). Afterwards, equal numbers of viable cells from the various aliquots were transferred intravenously to irradiated (500 R) CAF_1 recipients (20×10^6 cells/mouse) which were then either not challenged or secondarily challenged with 100 μg of DNP-KLH.

Recipients of primed cells from donors not given allogeneic cells that were not exposed to DNP-D-GL in vitro developed high secondary anti-DNP antibody responses to challenge with DNP-KLH (Fig. 3). Exposure of such cells to DNP-D-GL in vitro diminished the secondary response by 92% (the lower degree of tolerance as compared with the preceding experiments probably reflects the log higher challenging dose of DNP-KLH). In the case of primed cells obtained from donor mice that had received allogeneic cells 2 days before sacrifice, several points are noteworthy: first, the secondary responses to DNP-KLH of saline-treated control cells from such donors were significantly lower (around 65%) than the responses obtained with donor cells not subjected to allogeneic cells in situ. The possibility that this result is merely a reflection of inherent qualitative differences in the donor cell pools unrelated to allogeneic cell transfer may be immediately dismissed since (*a*) differences of this magnitude are not common, in our experience, when cell pools are prepared on the same day from 8–10 mice per donor pool group when all donors were primed at the same time and housed together, and (*b*) this result has been observed

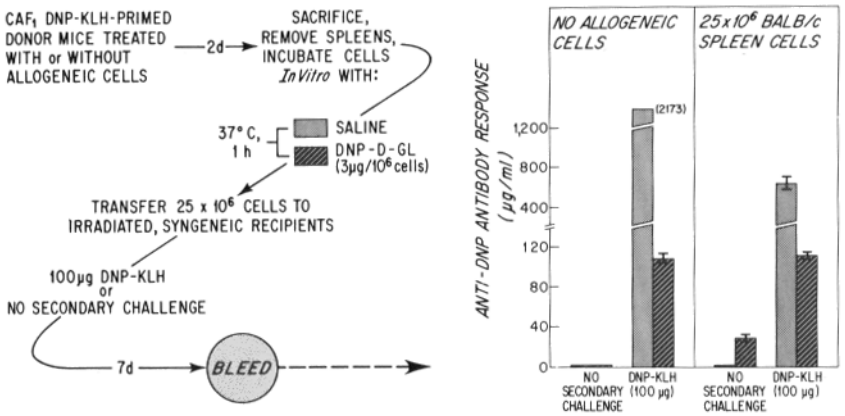

FIG. 3. Induction of DNP-specific tolerance by in vitro exposure to DNP-D-GL of DNP-KLH-primed cells from donor mice subjected to an allogeneic effect. The protocol is schematically depicted on left of figure. Each group consists of five mice. Relevant statistical comparisons of responses yielded the following P values: (*a*) Cells incubated with DNP-D-GL compared with saline-treated cells in response to secondary challenge with DNP-KLH-$0.001 > P$ when primed cells came from otherwise untreated donors; $0.005 > P > 0.001$ when primed cells came from donors treated with allogeneic cells; (*b*) cells from untreated donors compared to cells from donors treated with allogeneic cells— (*1*) in absence of secondary challenge, $0.001 > P$ when cells exposed to DNP-D-GL in vitro; $0.30 > P > 0.20$ when cells treated with saline; (*2*) in response to secondary challenge, $0.90 > P > 0.80$ when cells exposed to DNP-D-GL in vitro; $0.01 > P > 0.005$ when cells treated with saline.

by us in several experiments of this type. Second, despite the significantly suppressed responses in the aforementioned saline-control cells, the magnitude of responses manifested by the same donor cells that had been exposed to DNP-D-GL in vitro was disproportionately high in comparison to that of the saline-treated cells, and essentially equal to that displayed by DNP-D-GL-treated cells from the donor mice not given allogeneic cells. Finally, the DNP-D-GL-treated cells from donors that had received allogeneic cells produced low level, but nevertheless highly statistically significant, anti-DNP antibodies in the absence of any secondary challenge with DNP-KLH.

Taken collectively, the latter observations may indicate that the allogeneic cell transfer to primed donor mice may, indeed, have influenced the degree of tolerance induction resulting from subsequent in vitro DNP-D-GL treatment. Thus, these results may reflect an enhancing influence of the allogeneic cells on certain DNP-specific B cells binding DNP-D-GL in the face of a concomitant suppressive influence exerted by the allogeneic effect on other DNP-specific B cells and/or on KLH-specific helper T cells. Alternatively, the diminished responses of the saline-treated cells may reflect suppressive allogeneic effects on only helper T cells in which case the net effect on the responses of DNP-D-GL-treated cells might be nil. We are inclined to favor the former explanation, particularly in view of the statistically significant anti-DNP anti-

body production in the absence of any secondary challenge observed in recipients of DNP-D-GL-treated cells from donors that had received allogeneic cells.

Capacity of Enzymatic Treatment of Cells with Trypsin to Alter or Reverse DNP-Specific Tolerance Induced by DNP-D-GL.—In our previous studies with this system in mice (3), we had been able to render DNP-KLH-primed spleen cells relatively unresponsive by exposing them for appropriate lengths of time in vitro to either DNP-D-GL or a DNP conjugate of an unrelated carrier protein such as OVA. Thus, cells exposed to these reagents developed markedly diminished adoptive secondary anti-DNP antibody responses when transferred to irradiated recipients and challenged with DNP-KLH. However, a difference in the unresponsive states caused by DNP-D-GL as opposed to DNP-OVA was shown in that gentle enzymatic treatment of such cells with trypsin completely reversed the unresponsiveness in the case of cells exposed to DNP-OVA, but not in the case of cells exposed to DNP-D-GL. This observation formed a strong basis for our contention that tolerance induction by DNP-D-GL involves complex, irrevocable intra- or subcellular events following a tolerogenic signal probably generated at the surface membranes of such cells consequent to specific binding of the molecule (3).

The possible flaw in the above reasoning lies in the fact that our own concept of how trypsin might have worked in reversing DNP-OVA-induced unresponsiveness was by stripping the cell membrane of its specific receptors and any DNP-OVA attached to them. This in turn would result in resynthesis of surface receptors and the intact capability of such cells to respond. The failure of trypsinization to reverse the tolerant state induced by DNP-D-GL was explained in the context of an irreversible intracellular inactivation process not subject to manipulations at the cell surface. Another possible explanation, with altogether different implications for mechanisms of tolerance in this system is that being a nonmetabolizable molecule, DNP-D-GL is not itself subject to enzymatic degradation and may thereby prevent accessibility of other surface membrane sites to the enzyme. If this were the case, then the index of irreversibility by trypsin as a reflection of intracellular mechanisms of inactivation may be quite invalid. We have therefore performed a series of experiments to put these questions to direct analysis.

Failure to reverse tolerance induced in vivo with DNP-D-GL or DNP-L-GL by trypsinization of cells before adoptive transfer: The protocol of this experiment is schematically depicted in the left panel of Fig. 4. Briefly, spleen cells from CAF_1 mice primed 3 mo earlier with DNP-KLH were transferred intravenously to irradiated (550 R) syngeneic recipients (50×10^6 cells/mouse). Immediately thereafter, groups of recipients were injected with either saline, DNP-D-GL (500 μg), or DNP-L-GL (500 μg) intraperitoneally. 3 days later, all mice were challenged with 100 μg of DNP-KLH and then bled 7 days later. The anti-DNP antibody responses of these groups of recipients are summarized in the top right panel of Fig. 4 where it is shown that recipients treated with either

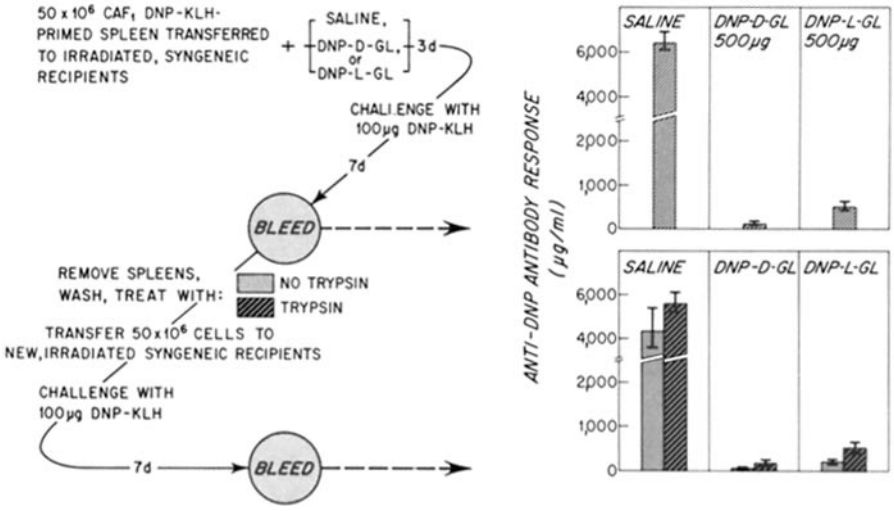

FIG. 4. Failure to reverse tolerance induced in vivo with DNP-D-GL or DNP-L-GL by trypsinization of cells before adoptive transfer. The protocol is schematically depicted on the left. Each group consists of 10 mice in the top panel, five mice in the bottom panel. Relevant statistical comparisons of responses yielded the following P values: (*a*) top panel, comparison of saline-treated controls with DNP-D-GL-treated cells, and DNP-L-GL treated cells yielded $0.001 > P$ in both cases; (*b*) bottom panel—(*1*) saline-treated control cells compared to DNP-D-GL or DNP-L-GL-treated cells, $0.001 > P$ irrespective of whether or not cells were treated with trypsin; (*2*) nontrypsinized DNP-D-GL or DNP-L-GL-treated cels compared to their respective trypsinized counterparts, $0.10 > P > 0.05$ in both cases.

DNP-D-GL or DNP-L-GL were significantly unresponsive as compared to saline controls (98% and 92% diminution by DNP-D-GL and DNP-L-GL, respectively).

The spleen cells from these respective groups of mice were obtained and then washed three times with MEM. Each pool was divided into two samples, one of which was left untreated while the second was treated with trypsin as outlined in Materials and Methods. Groups of irradiated (550 R), syngeneic recipient mice were injected intravenously (50×10^6 cells/mouse) with either untreated or trypsinized cells from the respective donor mouse groups. All mice were challenged with 100 µg of DNP-KLH intraperitoneally immediately after cell transfer and then bled 7 days later. As shown in the lower right panel of Fig. 4, cells exposed to saline in the initial recipients developed very good adoptive anti-DNP antibody responses in the final recipients; trypsinization slightly, but not significantly, improved these responses. In contrast, cells exposed to either DNP-D-GL or DNP-L-GL failed to respond in the final recipients and this inability to respond was not appreciably reversed in either case by trypsinization.

Failure to reverse tolerance induced by DNP-D-GL and reversal of tolerance induced by DNP-L-GL in vitro by trypsinization of cells before adoptive transfer:

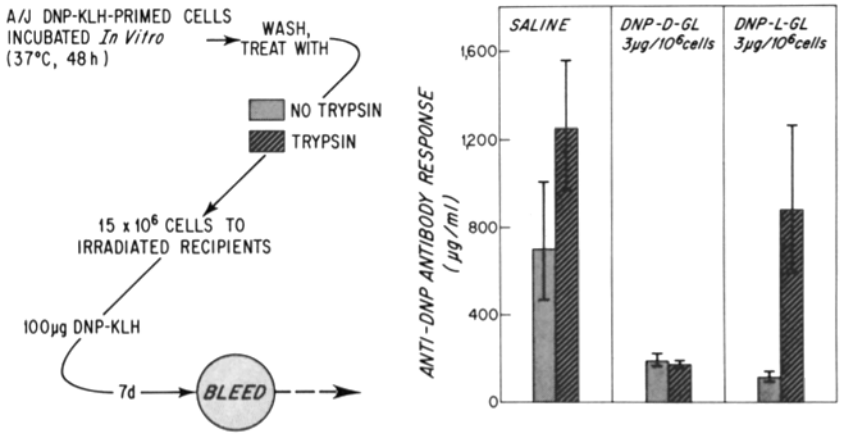

FIG. 5. Failure to reverse tolerance induced by DNP-D-GL and reversal of tolerance induced by DNP-L-GL in vitro by trypsinization of cells before adoptive transfer. The protocol is schematically depicted on the left. Each group consists of five mice. Relevant statistical comparisons of responses yielded the following P values: nontrypsinized vs. trypsinized saline control cells, $0.20 > P > 0.10$; nontrypsinized vs. trypsinized DNP-D-GL-treated cells, $0.90 > P > 0.80$; nontrypsinized vs. trypsinized DNP-L-GL-treated cells, $0.05 > P > 0.025$; nontrypsinized saline controls vs. nontrypsinized DNP-D-GL and DNP-L-GL-treated cells, $0.01 > P > 0.005$ in both cases; trypsinized saline controls vs. trypsinized DNP-D-GL-treated cells and DNP-L-GL-treated cells, $0.01 > P$ and $0.60 > P > 0.50$, respectively.

In the first experiment of this type, depicted in Fig. 5, spleen cells from A/J donor mice primed 5½ mo earlier with DNP-KLH were incubated at 37°C in Mishell-Dutton conditions at a density of 30×10^6 cells/ml with 3 μg/10^6 cells of either DNP-D-GL or DNP-L-GL, or saline alone. After 48 h, the respective cell groups were harvested from the dishes and washed three times in MEM. Each pool was divided into two samples, one of which was left untreated while the second was trypsinized. The respective cell pools were then transferred to irradiated, syngeneic recipients (15×10^6 cells/recipient) which were challenged with 100 μg of DNP-KLH and then bled 7 days later.

As shown in Fig. 5, recipients of cells incubated with saline developed good adoptive secondary responses to DNP-KLH and trypsinization of such cells resulted in a substantial increase in the magnitude of these responses. The latter finding has been consistent in many such experiments and will be discussed at greater length below. Recipients of cells incubated with DNP-D-GL were significantly diminished in their secondary responses (86% as compared with controls) and, moreover, treatment with trypsin did not abolish the unresponsive state. In striking contrast are the results obtained in recipients of cells incubated with DNP-L-GL. If such cells were transferred without trypsinization, a very significant level of suppression (88%) comparable to that induced by DNP-D-GL occurred. Treatment of such cells with trypsin, on the

other hand, restored the adoptive secondary anti-DNP response to essentially normal levels.

The capacity of trypsinization to reverse tolerance induced by DNP-L-GL exposure in vitro but not in the previous in vivo experiment (Fig. 4) may reflect a kinetic difference in efficiency of tolerance induction with this molecule in the contrasting conditions. Another experiment was performed in which the time of in vitro exposure to DNP-L-GL was lengthened from 2 to 4 days (Fig. 6). The experimental conditions were essentially identical to those of the preceding experiment (Fig. 5) except that CAF_1 donor and recipient mice were employed and cells were exposed to either saline alone or DNP-L-GL (3 $\mu g/10^6$

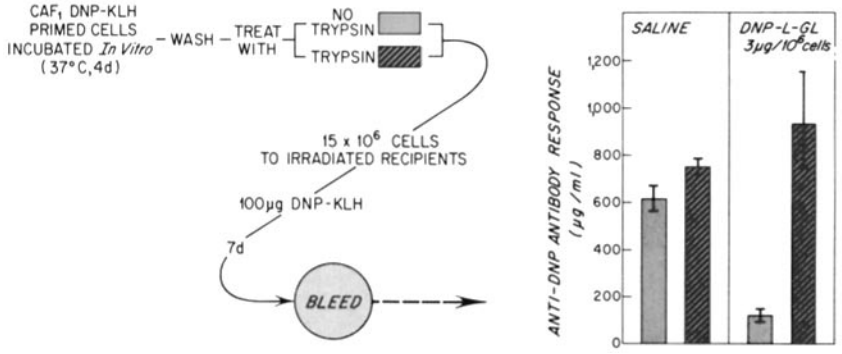

FIG. 6. Reversal of tolerance induced by DNP-L-GL in vitro by trypsinization of cells before adoptive transfer. The protocol is schematically depicted on the left. Each group consists of five–six mice. Relevant statistical comparisons of responses yielded the following P values: (*a*) nontrypsinized saline controls vs. nontrypsinized DNP-L-GL-treated cells, $0.025 > P > 0.01$; (*b*) trypsinized saline controls vs. trypsinized DNP-L-GL-treated cells, $0.50 > P > 0.40$; (*c*) nontrypsinized DNP-L-GL-treated cells vs. either trypsinized DNP-L-GL-treated cells or saline controls (trypsinized or not), $0.001 > P$ in all cases.

cells) for 4 days. The results summarized in Fig. 6 demonstrate again the unresponsive state as compared to controls induced by exposure of cells to DNP-L-GL in vitro and the complete reversal of the unresponsiveness by treatment of such cells with trypsin before adoptive transfer.

Reversal of tolerance induced by DNP-D-GL in vitro at 4°C by trypsinization of cells before adoptive transfer: The question of whether or not conditions could be found whereby trypsin could be employed to reverse (or rather prevent) the tolerance-inducing sequence of cellular events was approached by taking advantage of the fact that tolerance induction readily occurs in this system by in vitro incubation of primed cells with DNP-D-GL even at low temperatures (5, and experiments presented here). We reasoned, therefore, that perhaps at low temperature the transduction of any tolerogenic signal might be sufficiently delayed so that reversal by trypsinization would be feasible if the cells were subjected to this enzymatic treatment right away. The following experiment demonstrates that, indeed, this is true (Fig. 7).

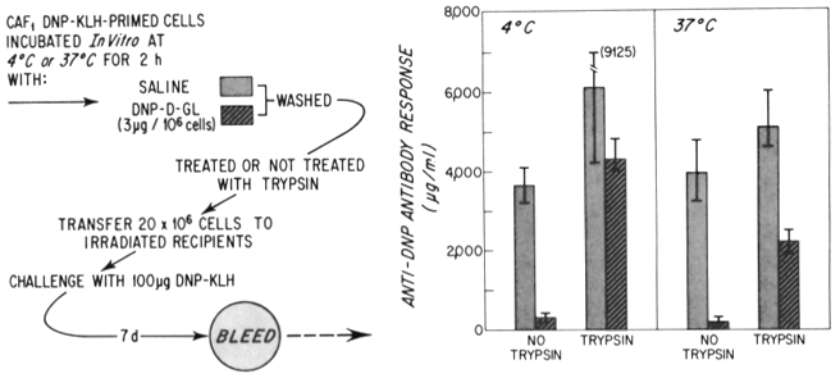

FIG. 7. Reversal of tolerance induced by DNP-D-GL in vitro at 4°C by trypsinization of cells before adoptive transfer. The protocol is depicted schematically on the left. Each group consists of five–six mice. Relevant statistical comparisons of responses yielded the following P values: (*a*) nontrypsinized saline controls vs. nontrypsinized DNP-D-GL-treated cells, $0.005 > P > 0.001$ and $0.001 > P$ at 4°C and 37°C incubation temperatures, respectively; (*b*) trypsinized saline controls vs. trypsinized DNP-D-GL-treated cells, $0.30 > P > 0.20$ and $0.005 > P > 0.001$ at 4°C and 37°C incubation temperatures, respectively.

Spleen cells from CAF_1 donor mice primed 3 mo earlier with DNP-KLH were incubated in culture tubes with either DNP-D-GL (3 $\mu g/10^6$ cells) or saline. The cells were incubated at either 4°C or 37°C for 2 h. Afterwards, the respective cell groups were washed three times in MEM and then each divided into two samples, one of which was left untreated while the second was trypsinized as before. The respective cell pools were then transferred intravenously to irradiated (550 R) syngeneic recipients (20×10^6 cells/recipient) which were challenged with 100 μg of DNP-KLH and then bled 7 days later.

As shown in Fig. 7, recipients of saline control cells incubated at either temperature developed very good adoptive secondary anti-DNP responses and, again these responses were improved by treatment with trypsin. Recipients of cells exposed to DNP-D-GL but not trypsinized before transfer displayed markedly depressed responses irrespective of the temperature of incubation (91% and 95% diminished at 4°C and 37°C, respectively). However, treatment of these cells with trypsin before transfer had a significant effect upon their subsequent capacity to respond which was determined to a great extent upon the temperature used for incubation with DNP-D-GL. Thus, cells exposed to DNP-D-GL at 4°C developed secondary responses after trypsinization that were not statistically significantly different from those of saline control cells also treated with trypsin ($0.30 > P > 0.20$). Indeed, the magnitude of the responses in this group was even greater than that obtained with saline control cells not subjected to trypsin treatment. In the case of cells incubated with DNP-D-GL at 37°C, trypsinization markedly improved the ultimate response by comparison with those of cells exposed to DNP-D-GL but not trypsinized (10-fold), but did not abolish the statistically significant difference with respect to responses of saline control cells incubated at that temperature and then treated with trypsin ($0.005 > P > 0.001$).

DISCUSSION

In the studies presented here, we have extended our previous observations on the state of DNP-specific tolerance induced by the DNP derivative of the copolymer of D-glutamic acid and D-lysine. In so doing, we have deliberately focused on the question of reversibility of inactivation at the B-cell level to develop a more cogent understanding of the cellular events that occur subsequent to binding of the tolerogenic moiety. The capacity of a strong T-cell stimulus, such as that provided by the allogeneic effect, and of gentle enzymatic treatment with trypsin to alter, prevent or reverse the tolerance induced by DNP-D-GL have been tested in a variety of ways.

As mentioned in the Introduction, DNP-D-GL represents a rather intriguing molecule in that under normal conditions it is highly tolerogenic for DNP-specific B cells. This is true over a very wide dose range both in vivo and in vitro (1–6); in contrast to other antigenic moieties considered to be relatively thymus-independent, DNP-D-GL does not appear to be capable of eliciting antibody responses even at very low doses in mice either in vivo (9) or in vitro (5).[2] However, in the milieu of a very strong T-cell stimulus, DNP-D-GL has been shown to transmit immunogenic signals to specific B lymphocytes (1, 4, 7–9). Thus, DNP-primed lymphocytes will develop secondary anti-DNP antibody responses to challenge with DNP-D-GL provided an appropriately timed transient graft-vs.-host reaction (allogeneic effect) has been induced (1, 4, 7, 8). Unprimed DNP-specific lymphocytes can also be induced to primary anti-DNP antibody production, and of the IgG class, by DNP-D-GL in the presence of an allogeneic effect (9). Indeed, it is particularly interesting that DNP-D-GL works in an allogeneic effect on unprimed B cells whereas normal immunogenic conjugates such as DNP-KLH fail to behave in the same fashion (9).

In the present experiments, we have attempted to manipulate the system of tolerance induction in a variety of ways to determine if the allogeneic effect could alter the tolerant state. It should be stressed that the main emphasis here was to attempt to alter cellular events presumably already set in motion (or perhaps completed) by reaction with the tolerogenic molecule, and protocols were designed accordingly. It is already sufficiently clear on the basis of ample direct evidence, mentioned above, that the allogeneic effect can prevent a tolerogenic signal from ensuing, indeed converting the signal to an immunogenic one. In the guinea pig system, we were unsuccessful in rescuing B lymphocytes from a state of unresponsiveness induced by DNP-D-GL by subjecting such animals subsequently to an allogeneic effect (1). The present experiments confirm this general conclusion in that cells previously exposed to DNP-D-GL, and rendered markedly unresponsive by such exposure either in vitro (Fig. 1) or in vivo (Fig. 2), failed to be appreciably reversed to a state of responsiveness by subsequent exposure to an allogeneic effect. On the other hand, when allogeneic cell interactions were induced in vivo within the DNP-

[2] D. H. Katz. Unpublished observations.

primed donor lymphoid cell population before exposure of such cells to DNP-D-GL in vitro, then some degree of B-cell stimulation could be detected (Fig. 3).

These observations point out once again the previously noted contrasting effects of the DNP-D-GL molecule on DNP-specific B lymphocytes depending on the existing conditions at the critical moment of receptor-substrate binding. In normal circumstances, binding of DNP-D-GL results in functional inactivation of DNP-specific B cells. This tolerant state is induced extremely rapidly and is not reversible once it has occurred. In marked contrast, however, is the capacity of this very same molecule to serve as a potent stimulus for differentiation of DNP-specific precursor B cells to antibody-producing cells when the initial binding event takes place in a milieu of strong T-cell activity such as that exerted by the allogeneic effect (1, 4, 7–9). In this respect, however, the critical moment of specific binding of DNP-D-GL must occur during or after the generation of this T-cell activity; if the binding event precedes the development of T-cell participation, no stimulation will occur as a result of the very rapid kinetics of the tolerogenic events within the cell population exposed to this molecule.

The series of experiments involving the effects of enzymatic treatment with trypsin were performed to clear up several uncertainties regarding the significance of our previous failure to reverse DNP-D-GL-induced B-cell tolerance by such treatment (3). In those studies, we observed that in vitro exposure of DNP-KLH-primed cells to either DNP-D-GL or to a DNP conjugate of an unrelated carrier protein such as OVA rendered such cells unresponsive upon subsequent adoptive transfer and challenge with DNP-KLH. However, trypsinization of cells before adoptive transfer reversed the tolerance in the case of cells incubated with DNP-OVA but not in the case of cells treated with DNP-D-GL (3). This observation provided a forceful argument in favor of the hypothesis that B-cell tolerance induced by DNP-D-GL represented an irreversible state of intracellular inactivation resulting from a tolerogenic signal generated at the cell surface membrane. In addition, the fact that DNP-OVA-treated cells were initially unresponsive but could be reversed by trypsinization appeared to point out at least a second distinct type of tolerance mechanism in B cells whereby transient unresponsiveness occurred due to surface receptor "blockade" presumably resulting from the binding of specific determinants by B cells in the absence of a suitable T-cell influence. The latter issue is, indeed, an important one which is considered in greater depth in the accompanying manuscript (18).

Our previous reasoning, therefore, was based essentially on the notion that trypsinization strips the cell surface membrane of its exocytic receptors and, of course, any determinants bound to those receptors. Provided no central inactivation process had occurred, such cells would be capable of resynthesizing their surface receptors and presumably of responding again to antigen. By extension of this thought, any state of unresponsiveness that could be readily

reversed by trypsinization might be considered to reflect a mechanism of surface receptor blockade. Conversely, any state of unresponsiveness not susceptible to reversal by trypsin might be considered to reflect a mechanism of intra- or subcellular inactivation. Such reasoning does not exclude the possibility that the former mechanism might not ultimately result in the latter events given sufficient time.

An objection to our previous interpretation, which we failed to take into account at that time, is the possibility that DNP-D-GL might virtually cover the surface of cells to which it becomes bound and, being itself resistant to the action of trypsin, prevent accessibility of other susceptible surface membrane components to the enzyme. This possibility has been pointed out to us by many of our friends and colleagues and prompted us to explore this question in great depth. The results of the experiments reported herein demonstrate, we believe, that the failure of trypsin to reverse tolerance under certain circumstances is, indeed, a reflection of a state of irrevocable cellular refractoriness and not explained by inaccessibility of the surface membrane components to the enzyme. This conclusion derives from the observation that under appropriate conditions of in vitro tolerance induction by DNP-D-GL, enzymatic treatment with trypsin can, in fact, prevent full tolerance from becoming established (Fig. 7). Thus, when exposure of cells to DNP-D-GL for a relatively short time in vitro is carried out at low temperature (4°C), the development of tolerance can be interceded by immediate trypsinization. Trypsin treatment also substantially improved the responsiveness of cells exposed to DNP-D-GL at 37°C but to a lesser extent than was observed with cells treated at 4°C. We interpret these data to indicate that: (*a*) the effect(s) of trypsin in reversing (or preventing) tolerance at the cellular level does not depend necessarily on the susceptibility of the tolerogenic moiety to the action of the enzyme, and (*b*) the generation of the tolerance-inducing signal involves metabolic cellular processes that can be delayed somewhat by low temperature leaving such cells relatively more susceptible to intercedent manipulations such as trypsinization.

At first glance, the failure of trypsin treatment to reverse tolerance induced by DNP-L-GL in vivo appears to be contradicted by the rather ready reversal of tolerance induced by the same molecule when in vitro conditions are employed (Figs. 5 and 6). However, the efficiency, kinetics, and duration of tolerance induction in vivo has always been of considerably greater magnitude than tolerance induced in vitro in our hands (3). Moreover, data will be presented in the following paper which documents a similar situation with tolerance induced in vivo with immunogenic molecules such as DNP-OVA under proper conditions (18). Finally, it should be pointed out that the experiments presented herein are representative of three–four experiments of each type, which all reproduce these basic observations quite consistently.

It has also become clear that our other interpretation concerning reversibility of tolerance by trypsin treatment, namely that cells susceptible to reversal by trypsin

were unresponsive as a result of surface receptor blockade but had not yet undergone irrevocable intracellular inactivation, is most likely incorrect. This is based on very recent observations of Ault and Unanue[3] which deomonstrate that DNP-KLH-primed B cells which bind metabolizable molecules such as DNP-OVA clear these molecules from their surface membranes quite rapidly and resynthesize their specific immunoglobulin receptors within hours. Nonetheless, as shown by our earlier observations with DNP-OVA (3) and by the observations with DNP-L-GL in the present report, such treated cells are functionally unresponsive unless an additional manipulation, such as enzymatic treatment with trypsin, is performed. The fact that trypsin is so capable of reversing unresponsiveness in certain circumstances, even when there is no overt surface receptor blockade by antigen, coupled with the observations cited herein on the significantly increased responsiveness of control cells to homologous antigen challenge following trypsinization (Figs. 5 and 7) raises important questions concerning the operational influence of trypsin on lymphocyte activation.

In summary, then, although our previous reasoning on the reversibility of B-cell tolerance by enzymatic treatment with trypsin was incorrect in the assumption that trypsin was acting by clearing off "blocked" receptors, the conclusion drawn from this reasoning, however, was nevertheless apparently correct. Thus, B-cell tolerance appears to be distinguished into at least two categories based on the systems employed: the first category, exemplified by perhaps nonphysiological molecules such as DNP-D-GL, involves rapid irreversible tolerance most likely generated initially as a cell surface signal but ultimately resulting in extremely rapid and irrevocable inactivation via events at the intra- or subcellular level. The second category, exemplified by physiological molecules and their analogues (such as DNP-OVA), involves cell surface binding and subsequent unresponsiveness, but the state is clearly reversible by certain maneuvers at least for a finite time, and may or may not either spontaneously reverse itself or develop into irrevocable inactivation. It is with this second category, and the relationship of T-cell function to it, that the following paper will be concerned (18).

SUMMARY

The studies presented here have focused on the important question of reversibility of inactivation of DNP-specific B lymphocytes induced by the DNP derivative of the copolymer of D-glutamic acid and D-lysine (D-GL). In so doing, we have analyzed the capacity of a strong T-cell stimulus, such as that provided by the allogeneic effect, and of gentle enzymatic treatment with trypsin to alter, prevent, or reverse the tolerance induced by DNP-D-GL. Under experimental conditions in which DNP-specific B lymphocytes were exposed first to the tolerogenic molecule, and rendered markedly unresponsive by such exposure either in vitro or in vivo, subsequent exposure to an allo-

[3] K. Ault, and E. R. Unanue, 1974. Events following the binding of antigen to lymphocytes. Removal and regeneration of the antigen receptor. *J. Exp. Med.* **139**:1110.

geneic effect failed to appreciably reverse or alter the tolerant state. This contrasts directly with the capacity of DNP-D-GL to serve as a stimulus for DNP-specific B lymphocytes when the critical moment of specific binding occurs subsequent to the development of an allogeneic effect. In another series of experiments, the effects of enzymatic treatment with trypsin on the tolerant B-cell population were found to vary depending on the stage of tolerance at which such treatment was performed. Thus, when exposure of cells to DNP-D-GL for a relatively short time in vitro is carried out at low temperature (4°C), the development of tolerance can be interceded by immediate trypsinization. In contrast, cells exposed to DNP-D-GL for longer periods of time and/or at 37°C were not reversed to responsiveness by trypsinization. These data were interpreted to indicate that: (*a*) the effect(s) of trypsin in reversing (or preventing) tolerance at the cellular level does not depend necessarily on the susceptibility of the tolerogenic moiety to the action of the enzyme, and (*b*) the generation of the tolerance-inducing signal involves metabolic cellular processes that can be delayed somewhat by low temperature leaving such cells relatively more susceptible to intercedent manipulations such as trypsinization. Taken collectively, therefore, the evidence obtained in these studies reinforces the concept of central tolerance in B cells induced by DNP-D-GL as reflecting sub- or intracellular inactivating events.

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