THE MECHANISM OF TOLERANCE INDUCTION IN THYMUS-DERIVED LYMPHOCYTES

I. Intracellular Inactivation of Hapten-Reactive Helper T Lymphocytes by Hapten-Nonimmunogenic Copolymer of D-Amino Acids*

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A substantial amount of literature has appeared in the last decade on immunological tolerance (reviewed in 1). The establishment of thymus-derived (T) and bone marrow-derived (B) cell cooperative interactions in humoral immune responses has necessitated reassessment of conceptual and experimental approaches to the study of tolerance (reviewed in 2). One of the major issues has been to define the site of tolerance induction in a given experimental situation. There is now clear evidence for specific unresponsiveness in both the B- and T-cell lineages, and critical kinetic and dose threshold differences have been elucidated by Chiller et al. (3, reviewed in 4) for tolerance induction in T and B lymphocytes, insofar as thymus-dependent antigens are concerned.

The specific unresponsiveness in the B-cell lineage could be easily induced by injection of a haptenic determinant coupled to a thymus-independent carrier (5–7) or to nonimmunogenic synthetic polypeptides (8–12), and this provided us with a very intriguing experimental system for elucidation of the mechanism of tolerance induction in the B-cell line. In the case of induction of B-cell tolerance by a DNP derivative of a copolymer of D-glutamic acid and D-lysine (D-GL),[1] the maintenance of tolerance of DNP-specific B cells by serial adoptive transfer (9), and the failure to reverse the tolerance by enzymatic treatment with trypsin of the tolerant cells (12), imply that tolerance in this situation reflects deletion of a specific clone of antigen-reactive B cells.

On the other hand, there is also a considerable body of evidence which demonstrates that unresponsiveness could be induced exclusively at the level of the T cells (3, 13–16). This form of tolerance, in contrast to B-cell tolerance, is occasionally associated with normal or even an increased number of antigen-binding cells. Furthermore, it is occasionally infectious in certain situations; thus, T cells from tolerant donors have the ability to inhibit the immune response of nontolerant cells (17–20). The implication of

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; BαA, bacterial α-amylase; CFA, complete Freund's adjuvant; D-GL, copolymer of D-glutamic acid and D-lysine; GPA, guinea pig albumin; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin; MGG, mouse gamma globulin; NMS, normal mouse serum; OVA, ovalbumin; PAB, *p*-azobenzoate; PFC, plaque-forming cells.

these findings is that tolerance of this type is due, not to deletion or elimination of antigen-reactive cells, but to an active suppressor T-cell effect in the tolerant cell population (18–20). However, in another type of T-cell tolerance model, such as unresponsiveness to tissue-associated antigens, the lymphoid cells obtained from unresponsive animals exhibit a responsiveness to that antigen when transferred into another milieu in vivo or in vitro, and this unresponsiveness was easily reproduced by the administration of serum factor from unresponsive animals (21–24). Thus, on this occasion, the unresponsiveness on the T-cell level seems to be induced by a blocking factor in serum which may inhibit the reaction of T cells with the corresponding antigen (21–24). However, another widely accepted explanation for tolerance is that tolerance reflects deletion of a specific clone of antigen-reactive T cells (25–27). In this case, the tolerance is neither infectious nor inducible by the administration of serum from tolerant animals, and is stable on adoptive transfer. Thus, in contrast to tolerance induction in B lymphocytes, the mechanism of induction of unresponsiveness to T cells is still a point of debate (28). A great deal of this confusion stems from the apparently contradictory observation described above; this is a crucial issue since on it rests the correct interpretation of the fate of tolerant T cells. For this and related reasons, the establishment of a model in which unresponsiveness is restricted to the T-cell population may be crucial for elucidation of some of these problems.

The present studies were undertaken to establish a condition of specific unresponsiveness of *p*-azobenzoate (PAB)-reactive helper T lymphocytes in mice by administration of a PAB derivative of a copolymer of D-GL. A unique feature of this model was the relative ease with which specific unresponsiveness of PAB-reactive helper T lymphocytes could be induced in an animal previously primed to PAB. It was of particular interest to obtain PAB-specific unresponsiveness on the helper T-cell level in an adoptive transfer system in mice, since such a model would offer considerable advantage in further experimentation designed to approach questions concerning the mechanism of cellular events responsible for tolerance induction on the T-cell level. Utilizing this system, we have found that the unresponsive state induced in helper T lymphocytes was not broken by serial adoptive cell transfer, and such tolerance was not mediated by suppressor cells. Moreover, the tolerance induced in the T cells does not reflect simple coverage of tolerogen on T-cell receptors in this PAB-D-GL model. Evidence gathered from the experiment utilizing trypsin treatment of tolerant helper T cells provides a forceful argument for the concept of central tolerance in T lymphocytes as a reflection of sub- or intracellular inhibitory events.

Materials and Methods

Proteins and Chemical Reagents. The copolymer of D-GL was obtained from Pilot Chemicals, Inc., Watertown, Mass. The polymer had an average mol wt of 27,000 and a ratio of glutamic acid to lysine residues of 60:40. Crystalline bacterial α-amylase (BαA) derived from *Bacillus subtilis* was purchased from Nagase Sangyo Co. Ltd., Osaka, Japan, and keyhole limpet hemocyanin (KLH) was obtained from Calbiochem, San Diego, Calif. Mouse gamma globulin (MGG) was prepared from Ehrlich tumor ascitic fluid by precipitation with ⅓ saturated ammonium sulfate and was further purified through the use of DEAE-cellulose column chromatography with 0.01 M sodium phosphate buffer, pH 8.0. Human gamma globulin (HGG), Cohn fraction II was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio and was further purified through a DEAE-cellulose column as above. Bovine serum albumin (BSA) was purchased from Armour Pharmaceutical Co., Chicago, Ill., and hen ovalbumin (OVA) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. These proteins are not immunologically cross-reacting.

Preparation of Hapten-Carrier Conjugates

DINITROPHENYL [DNP] PROTEIN CONJUGATES. The following DNP conjugates were prepared using sodium 2,4-dinitrobenzene sulphonate: DNP_5-BαA, DNP_6-KLH, DNP_5-MGG, DNP_{15}-BSA, and DNP_8-OVA. Subscripts refer to the average number of DNP groups per molecule of the proteins, and this was calculated from the absorption readings at 360 and 280 nm in alkaline solution. In these calculations, the mol wt of BαA, KLH, MGG, BSA, and OVA were taken as 50,000, 100,000, 160,000, 68,000, and 45,000, respectively.

PAB-CARRIER CONJUGATES. PAB-D-GL was prepared according to a modification of the method of Tabachnick and Sobotka (29): the D-GL was dissolved in borate-buffered saline, pH 9.0, at a concentration of 5 mg/ml, and a 200-fold excess in molar ratio of diazotized *p*-aminobenzoate was then added dropwise over a period of 15 min, with constant stirring, to the protein solution maintained in an ice bath. During the addition the pH was maintained at 9.0 with 1 N NaOH. After coupling was allowed to proceed at room temperature for 4 h, the reaction mixture was extensively dialyzed at 4°C with continuous stirring for at least 7 days, with daily changes of the dialysis fluid. The average number of moles of PAB per mole of D-GL was calculated from the absorption reading at 460 nm in alkaline solution. In the calculation, the molar extinction coefficient of PAB-lysine was tentatively assumed to be 1,650 at 460 nm based on that of bis(*p*-azobenzenearsonic acid)diazo-ϵ-aminocaproic acid (29). 26 molecules of PAB groups were found to bind covalently with 1 molecule of D-GL in the conjugate by this calculation.

PAB_7-MGG, DNP_5-MGG-PAB_7, and PAB_8-HGG were prepared in a similar manner except that a 100-fold excess in molar ratio of diazotized *p*-aminobenzoate was reacted with MGG, DNP_5-MGG, or HGG. Subscripts refer to the average number of moles of PAB per mole of protein, and this was calculated from the absorption reading at 460 and 500 nm in alkaline solution, assuming PAB had coupled to histidine and tyrosine residues of those molecules (29).

Assay System for Measuring the Activity of PAB-Reactive Helper T Lymphocytes. The ddO albino mice, supplied by the Central Breeding Laboratory of Experimental Animals of Osaka University, Osaka, Japan, were primarily immunized by intraperitoneal injections of 100 μg of PAB-MGG in complete Freund's adjuvant (CFA). At various times (2–3 mo) thereafter, the single-cell suspensions in Eagle's minimal essential medium (MEM) were prepared from the spleens of these primed mice and used as the source of PAB-primed helper T cells. As a DNP-primed B-cell source, single-cell suspensions of spleens from mice which had been immunized intraperitoneally with 100 μg of DNP-BαA or DNP-KLH in CFA were used and transferred intravenously to syngeneic, irradiated (550–600 R) recipients together with PAB-primed helper T cells. In general, antigenic stimulation was performed in vitro by adding 100 μg of DNP-MGG-PAB to those mixtures of primed cells immediately before the cell transfer. PAB-reactive helper cell activity in the transferred PAB-MGG-primed cells was measured by the magnitude of anti-DNP antibody responses of the DNP-primed B cells in recipient spleens 7 days after the stimulation of DNP-MGG-PAB. The anti-DNP antibody responses were determined by a modification of the hemolytic plaque technique as described below. The tolerogen (PAB-D-GL) treatment to the PAB-primed helper T cells was performed by intraperitoneal injection of the PAB-primed donor animals 3 or 4 days before the cell transfer. Modifications of this general adoptive transfer scheme and tolerogen treatment are described in appropriate sections in the Results.

Hemolytic Plaque Assay. Plaque-forming cells (PFC) in the spleens of recipients secreting anti-DNP antibodies were enumerated by a modification of Cunningham's hemolytic plaque technique described in detail previously (30). For this assay, sheep erythrocytes (SRBC) were conjugated with DNP-BSA by using $CrCl_3$. Single spleen cell suspensions from individual recipient mice were treated with 0.85% NH_4Cl, and washed three times with MEM before assay. Direct PFC were enumerated after incubation of monolayers of indicator red cells, DNP-BSA-coupled SRBC, and spleen cells using guinea pig serum as a source of complement (C). Indirect PFC were developed with rabbit anti-Fc of mouse IgG antiserum and C in assay preparations. Since the number of direct PFC was negligibly small in all the experiments, only the numbers of indirect PFC are listed in the Results.

Anti-θ Serum Treatment of PAB-MGG-Primed Splenic Lymphocytes. Anti-θ C3H serum was prepared by intraperitoneal immunization of 100×10^6 C3H/He thymocytes into AKR male mice with 2×10^8 *Bordetella pertussis* vaccine. 1 and 2 wk after the last of three consecutive biweekly immunizations, all mice were bled and the sera pooled. The anti-θ sera obtained lysed 95% of thymocytes at a 1:64 dilution and 30–40% of splenic lymphocytes at a 1:9 dilution in the presence of C,

as measured by trypan blue dye exclusion test. Single spleen cell suspensions were treated with anti-θ serum and C as follows: Spleen cells taken from PAB-MGG-primed mice were suspended in 10% calf serum-supplemented MEM at a concentration of 100×10^6 cells/0.5 ml. 1.0 ml of heat-inactivated anti-θ C3H was added to each such aliquot and the mixture incubated at 4°C for 30 min, washed, and then resuspended in 0.5 ml of medium. 1.0 ml of guinea pig C (absorbed with normal mice spleen cells and diluted 1:2 in medium) was added to each aliquot and the mixture incubated for an additional 40 min at 37°C. Viability of recovered cells was 90% and total recovery of viable cells after such treatment was in the range of 50%.

Treatment of PAB-MGG-Primed Splenic Lymphocytes with Trypsin. Enzymatic treatment of PAB-MGG-primed 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 DNase (Worthington Biochemical Corp., Freehold, N. J.) in MEM. After trypsinization, the cells were washed three times in MEM.

Statistical Analysis. The numbers of PFC per spleen were logarithmically transformed and means and standard errors calculated. Group comparisons were made employing the Student's *t* test. In those mice in which no PFC could be detected in the spleen, a value of 240 per spleen was arbitrarily assigned to allow logarithmic transformation of the data, the value corresponding to the minimal number of PFC detectable in our assay.

Results

Characterization of Hapten-Reactive Helper Activity Raised by the Immunization of Hapten-Isologous Carrier Conjugates

It has been previously reported from our laboratory that hapten (DNP)-reactive helper cells could be raised in mice by the immunization of hapten-isologous protein conjugates (DNP-MGG). Thus, DNP-specific B cells in the DNP-MGG-primed spleen cells can easily respond to DNP-heterologous conjugates such as DNP-HGG or DNP-KLH due to the presence of hapten-reactive helper cells (reference 30 and footnote 2). These hapten-reactive helper cells could be also raised to another noncross-reactive hapten such as PAB by priming with PAB-MGG, and in this case, cooperative interactions of PAB-reactive helper activities could be demonstrated against DNP-primed B cells as described below.

DEVELOPMENT OF PAB-REACTIVE HELPER ACTIVITY IN MICE BY IMMUNIZATION OF PAB-MGG. Three groups of mice were immunized intraperitoneally with 100 μg of PAB-MGG, MGG, or PAB-HGG in CFA. 6 wk thereafter, those immunized mice and another group of nonimmunized normal animals were X irradiated (600 R) and then injected intravenously with spleen cells (50×10^6) from mice which had been immunized with 100 μg of DNP-BαA in CFA 10 wk previously. Immediately before the cell transfer, those DNP-BαA-primed cells were stimulated in vitro with 100 μg of DNP-MGG-PAB. 7 days after the cell transfer, the anti-DNP PFC responses of DNP-BαA-primed cells were measured in the recipients' spleens and the results are graphically depicted in Fig. 1.

It can be seen in Fig. 1 that the DNP-BαA-primed cells did not respond to the stimulation of DNP-MGG-PAB in normal X-irradiated recipients, whereas these cells responded to the stimulation of DNP-MGG-PAB in the PAB-MGG-primed X-irradiated recipient mice. Thus, the helper activity for the induction of

[2] Hamaoka, T., T. Inada, U. Yamashita, and M. Kitagawa. 1975. Preventive effect of hapten-reactive thymus-derived helper lymphocytes on the tolerance induction in hapten-specific precursors of antibody-forming cells. *J. Immunol.* In press.

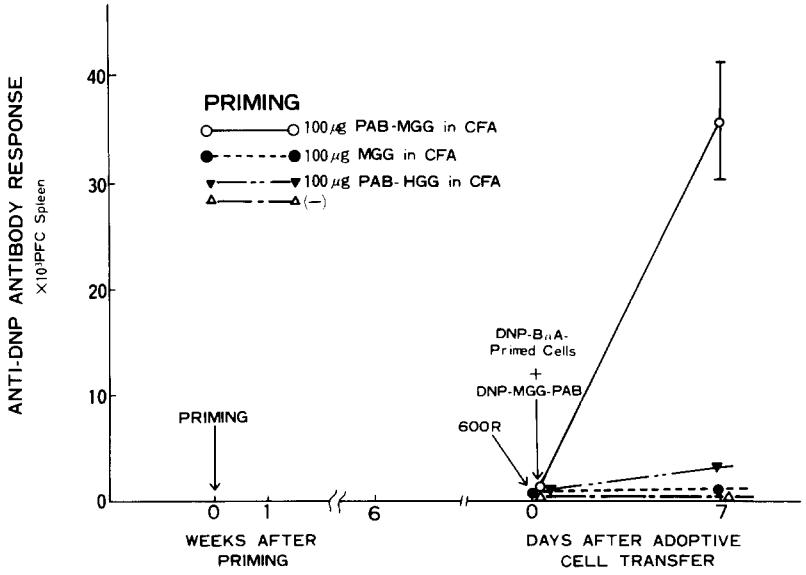

FIG. 1. Development of PAB-reactive helper activity by immunization with PAB-MGG. Recipient mice were primed by intraperitoneal injection of either 100 μg of PAB-MGG, MGG, or PAB-HGG in CFA 6 wk before cell transfer. Those primed mice and other groups of nonimmunized normal mice were X irradiated (600 R) and then injected intravenously with DNP-BαA-primed spleen cells stimulated with 100 μg of DNP-MGG-PAB. The DNP-BαA-primed spleen cells came from mice which had been immunized with 100 μg of DNP-BαA in CFA 10 wk previously. Geometric means and standard errors of anti-DNP PFC responses in spleens of the recipients 7 days after the cell transfer are illustrated.

anti-DNP antibody response to the DNP-primed B cells by the stimulation of DNP-MGG-PAB could be raised by the immunization of PAB-MGG in CFA.

This helper activity may be specific for the PAB portion of DNP-MGG-PAB. The specificity restriction of the helper activity which arose from PAB-MGG immunization may be suggested by the fact that such helper activity in PAB-MGG-primed mice could not be replaced by the MGG immunization. Moreover, PAB specificity of the helper activity was also reinforced by the fact that (*a*) the DNP-primed B cells responded poorly or not at all to the stimulation of DNP-MGG-PAB in recipient mice which had been immunized with MGG conjugates similarily coupled with *m*- or *o*-azobenzoate,[3] and (*b*) the helper activity induced by the immunization of PAB-MGG could be demonstrated to the double hapten conjugate of HGG (DNP-HGG-PAB), which is not cross-reactive with MGG.[4] The possibility that this helper activity was mediated by anti-PAB antibody or B cells themselves was ruled out by the following two observations. (*a*) The effective cell population in PAB-MGG-primed mice was radioresistant (31). (*b*) The helper activity was not observed in PAB-HGG-

[3] Yamashita, U., T. Hamaoka, T. Takami, and M. Kitagawa. Immunological maturation in T lymphocytes: sequential changes in specificities and affinities of hapten-reactive helper T cells during the immune course. Manuscript in preparation.

[4] Yamashita, U., T. Takami, T. Hamaoka, and M. Kitagawa. Termination of immunological tolerance to human gamma globulin in mice by hapten-reactive helper T lymphocytes. Manuscript in preparation.

primed mice at the challenge of DNP-MGG-PAB. The nature and specificity restrictions of helper activity developed in mice by the immunization of PAB-MGG were next explored more definitively.

SUSCEPTIBILITY OF HELPER CELL ACTIVITY DEVELOPED BY PAB-MGG IMMUNIZATION TO THE TREATMENT OF ANTI-θ SERUM PLUS C. In order to assess the nature of helper activity developed by the immunization of PAB-MGG, the classical double-cell transfer system was employed to measure helper activity of lymphoid cells from PAB-MGG-primed donor mice to DNP-primed B cells, with examination of the susceptibility of helper activity of the PAB-MGG-primed lymphoid cells to the treatment of anti-θ serum plus C.

Spleen cells (40×10^6) from mice which had been immunized intraperitoneally with 100 μg of DNP-KLH in CFA 6 wk previously were used as a DNP-primed B-cell source. Spleen cells (50×10^6) from other donor mice which had been immunized intraperitoneally with 100 μg of PAB-MGG 7 wk previously were used as PAB-MGG-reactive helper cells. The PAB-MGG-primed spleen cells were treated with either normal mouse serum (NMS) plus C or anti-θ serum plus C before the cell transfer, mixed together with the DNP-KLH-primed cells, and then transferred into X-irradiated (550 R) recipients after antigenic stimulation with 100 μg of DNP-MGG-PAB.

As a control for the nonspecific effect of anti-θ serum treatment on the primed cells being transferred, the DNP-KLH-primed cells were also treated in a similar fashion with NMS or anti-θ serum plus C and stimulated with 100 μg of DNP-KLH or DNP-BαA in the presence of BαA-primed spleen cells (50×10^6). These BαA-primed cells came from other donor mice which had been immunized intraperitoneally with 100 μg of BαA in CFA 6 wk previously. All the recipients were killed 7 days after secondary challenge. Anti-DNP PFC responses in the recipient spleens are summarized in Table I.

The DNP-KLH-primed cells which were treated with NMS plus C elicited, as expected, high levels of anti-DNP PFC responses after antigenic challenge with DNP-KLH (group VI), whereas the adoptive secondary response was almost completely abolished in the recipients of cells treated with anti-θ serum plus C (group VII). In contrast, the addition of BαA-primed cells restored the capacity of recipients of anti-θ serum-treated DNP-KLH-primed cells to develop an anti-DNP antibody response (groups IV and V). The recipients of DNP-KLH-primed cells which were challenged with DNP-MGG-PAB developed meager responses (group I) unless PAB-MGG-primed cells were administered in addition, in which cases the secondary responses were very significantly augmented (group II). Anti-θ serum treatment of these PAB-MGG-primed cells, however, virtually abrogated this adoptive secondary response to DNP-MGG-PAB (group III). Thus, these results clearly demonstrated that the helper cells developed by immunization with PAB-MGG are θ-positive T lymphocytes.

SUPPRESSION OF HELPER ACTIVITY DEVELOPED BY PAB-MGG IMMUNIZATION BY TREATMENT WITH PAB-D-GL. In order to further assess the specificity restriction of the helper T-cell activity developed by PAB-MGG immunization, an attempt was made to suppress the PAB-MGG-primed helper T cells by treatment with PAB-D-GL. If the reactivity of PAB-MGG-primed helper T cells are suppressed

TABLE I

*Susceptibility of Helper Cell Activity Developed by PAB-MGG Immunization to Treatment with Anti-θ plus C**

Group	Treatment of DNP-KLH-primed cells with:	Helper cell source	Secondary challenge	Anti-DNP antibody response‡ PFC/spleen
I	None	None	DNP-MGG-PAB	376 (1.18)
II	None	NMS-treated PAB-MGG-cells	DNP-MGG-PAB	19,271 (1.06)
III	None	Anti-θ-treated PAB-MGG cells	DNP-MGG-PAB	605 (1.26)
IV	NMS plus C	BαA-primed cells	DNP-BαA	25,538 (1.13)
V	Anti-θ plus C	BαA-primed cells	DNP-BαA	15,866 (1.12)
VI	NMS plus C	None	DNP-KLH	61,766 (1.08)
VII	Anti-θ plus C	None	DNP-KLH	1,807 (1.06)

* 40×10^6 spleen cells from donor mice, primed 6 wk earlier with 100 μg of DNP-KLH in CFA, were used as DNP-primed cells, and they were nontreated (groups I ~ III) or treated in vitro with either anti-θ serum plus C or NMS plus C (groups IV ~ VII). They were then transferred intravenously into 550 R X-irradiated recipients either alone or together with PAB-MGG-primed or BαA-primed spleen cells. The PAB-MGG-primed spleen cells had been also treated in vitro with NMS plus C or anti-θ serum plus C. The PAB-MGG-primed cells and BαA-primed cells came from mice which had been immunized in CFA 7 wk and 6 wk before the cell transfer, respectively. Secondary antigenic challenges were performed in vitro by mixing those primed cells with 100 μg of hapten-carrier conjugates, as indicated, immediately before the cell transfer.

‡ The data are expressed as geometric means of anti-DNP PFC in the recipient spleens 7 days after the cell transfer. Numbers in parentheses represent standard errors. Statistical comparison of the mean PFC responses in various groups yielded the following P values: group II vs. III, $P < 0.001$; IV vs. V, $0.10 < P < 0.20$; and VI vs. VII, $P < 0.001$.

by that treatment, one may conclude that the PAB-MGG-primed helper T cells are specific for PAB groups, since the helper cells will only interact with the PAB portion of PAB-D-GL.

Two groups of animals were immunized intraperitoneally with 100 μg of PAB-MGG in CFA 8 wk previously. One of the groups received 1 mg of PAB-D-GL intraperitoneally and the other received saline. 4 days later, they were then X irradiated (600 R) and injected with 50×10^6 spleens cells from mice which had been immunized with 100 μg of DNP-BαA in CFA 9 wk before, as the DNP-primed B-cell source. Antigenic stimulation to the DNP-BαA-primed cells was given by mixing those primed cells with 100 μg of DNP-MGG-PAB in vitro before the cell transfer. As a control for the background response of DNP-BαA-primed cells to the DNP-MGG-PAB, the same pool of primed cells with antigen was also transferred into nonimmunized X-irradiated mice. Anti-DNP responses in recipients 7 days after the cell transfer are depicted graphically in Fig. 2.

It is evident that the helper T-cell reactivity in PAB-MGG-primed mice was significantly suppressed by pretreatment with PAB-D-GL, as assessed by the reduction of the anti-DNP antibody responses in the PAB-D-GL-pretreated recipients.

This result indicates that the specificity of most of the PAB-MGG-primed helper T cells may be directed against PAB haptenic sites, and this PAB-reactive

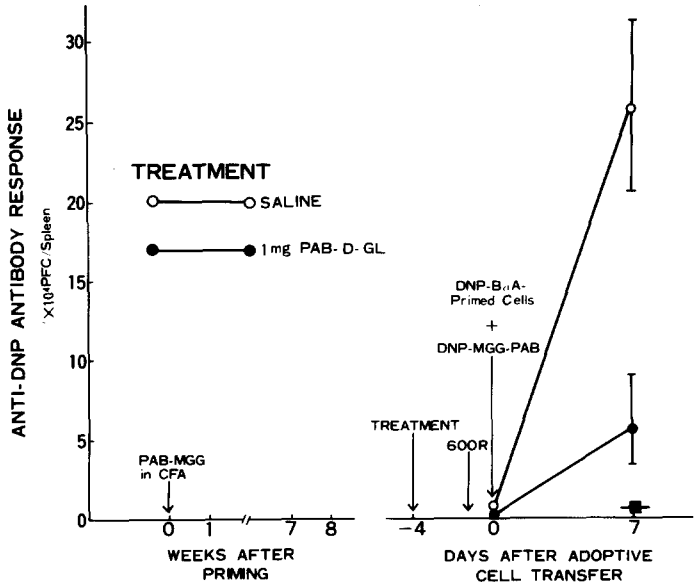

FIG. 2. Suppression of helper activity developed by PAB-MGG immunization by the treatment with PAB-D-GL. 50×10^6 spleen cells from donor mice which had been immunized with 100 μg of DNP-BαA in CFA 9 wk previously were transferred intravenously into three groups of X-irradiated recipient mice together with 100 μg of DNP-MGG-PAB. One of the groups represents normal nonimmunized recipients and the other two represent those immunized with 100 μg of PAB-MGG in CFA 8 wk previously, then treated intraperitoneally with either 1 mg of PAB-D-GL or saline 4 days before the cell transfer. Anti-DNP PFC responses in recipient spleens 7 days after cell transfer are illustrated. Closed squares represent the level of anti-DNP-PFC responses in normal nonimmunized recipients. Statistical comparison of the responses of saline-treated and PAB-D-GL-treated animals yielded a P value of $0.02 < P < 0.025$.

helper T-cell activity could be easily suppressed by interaction with PAB-D-GL.

In the above sections, we have demonstrated that PAB-reactive helper T lymphocytes were developed in mice by immunization of PAB-MGG, and that these helper activities were significantly suppressed by the treatment of PAB-D-GL. If the suppression of the reactivity of PAB-reactive helper T cells by PAB-D-GL treatment, as demonstrated above, had been established on a level of educated helper T lymphocytes, then this PAB-D-GL system may provide a potential advantage over other models for analysis of tolerance induction on the T-cell level since, in general, educated T lymphocytes are difficult to tolerize by the tolerogen treatment. Nevertheless, delineation of cellular events in this phenomenon requires sufficient quantities of specific helper T cells for any such study to be meaningful. In the following section, we have attempted to assess this point directly by using the double-cell transfer system of DNP-specific B cells and PAB-reactive T cells.

Induction of Unresponsiveness of PAB-Reactive Helper T Cells with PAB-D-GL Given to PAB-MGG-Primed Donor Mice

Two groups of donor mice, immunized 10 wk previously with 100 μg of PAB-MGG in CFA, were pretreated with saline or 1 mg of PAB-D-GL

intraperitoneally 4 days before the cell transfer. The spleen cells (50×10^6) from those two groups were mixed with DNP-BαA-primed spleen cells (50×10^6) from other donor mice which had been immunized intraperitoneally with 100 μg of DNP-BαA in CFA 9 wk earlier. They were then stimulated with 100 μg of DNP-MGG-PAB and transferred intravenously into two groups of X-irradiated (550 R) recipient mice. Another two irradiated recipients were also injected with the same DNP-BαA-primed cells alone, and then similarly stimulated with 100 μg of DNP-MGG-PAB or DNP-BαA as a background or positive control response of those primed cells. Anti-DNP antibody responses in the recipient spleens were measured 7 days after the cell transfer.

As shown in Table II, DNP B cells in the DNP-BαA-primed cell population

TABLE II

*Induction of Unresponsiveness in PAB-Reactive Helper T Cells with PAB-D-GL**

DNP-primed cells	PAB-reactive helper T cells	Secondary challenge	Anti-DNP antibody response PFC/spleen
DNP-BαA	None	DNP-BαA	80,974 (1.22)
DNP-BαA	None	DNP-MGG-PAB	240 (1.0)
DNP-BαA	4 days saline-pretreated PAB-MGG-primed cells	DNP-MGG-PAB	13,718 (1.13)
DNP-BαA	4 days PAB-D-GL-pretreated PAB-MGG-primed cells	DNP-MGG-PAB	1,747 (1.32)

* 50×10^6 spleen cells from mice which had been immunized with 100 μg of DNP-BαA in CFA 9 wk previously were transferred intravenously into X-irradiated (550 R) recipient mice either alone or together with PAB-MGG-primed cells from donor mice immunized 10 wk earlier and pretreated intraperitoneally with either saline or 1 mg PAB-D-GL 4 days before the cell transfer. Secondary challenges were performed by mixing them with 100 μg of hapten-carrier conjugates, as indicated, immediately before the cell transfer. Anti-DNP PFC responses in recipient spleens 7 days after the cell transfer are listed. Statistical comparison of the responses of saline-pretreated and PAB-D-GL-pretreated helper T cells yielded a P value of $0.001 < P < 0.005$.

responded nicely to DNP-BαA but did not respond to DNP-MGG-PAB, as expected. When the DNP-BαA-primed cells were stimulated with DNP-MGG-PAB in the presence of PAB-MGG-primed cells from saline- or PAB-D-GL-treated donors, it was found that PAB-reactive helper activities of PAB-MGG-primed cells which were pretreated with PAB-D-GL in the donor mice were significantly lower than the saline-treated group. These results clearly indicate that the responsiveness of PAB-reactive helper T cells was suppressed by the pretreatment of PAB-D-GL in donor mice, and this suppressive state of helper T cells did not revert to normal after adoptive cell transfer into other recipients.

The specificity of suppression induced by PAB-D-GL pretreatment is evident from the results shown in Fig. 3. In this experiment, the protocol for which is shown in the left panel of Fig. 3, two groups of donor mice which had been immunized with 100 μg of DNP-BαA in CFA 6 wk previously, were injected intraperitoneally with 1 mg of PAB-D-GL or not treated. 4 days after such treatment, the spleen cells (50×10^6) from respective donor mice were transferred into two groups of X-irradiated (550 R) recipient mice and stimulated with 100 μg

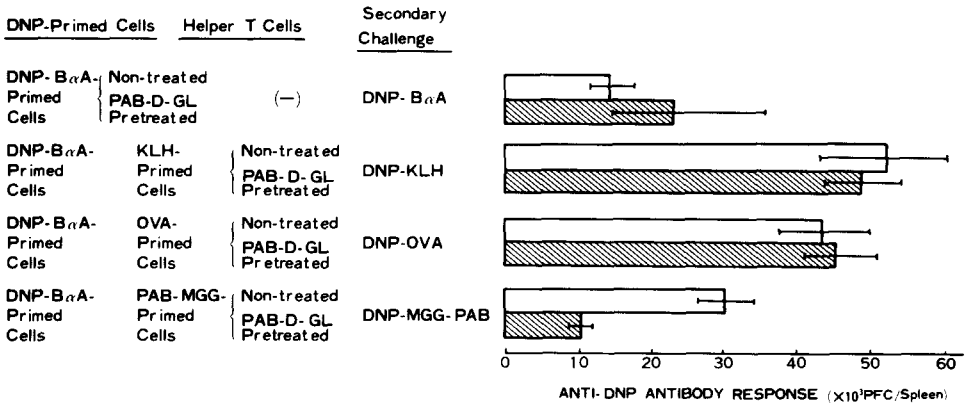

FIG. 3. Specificity of PAB-D-GL-induced tolerance in PAB-reactive helper T lymphocytes. 50 $\times$ 10^6 spleen cells from donor mice which had been immunized with 100 μg of DNP-BαA in CFA 6 wk previously were transferred intravenously into X-irradiated (550 R) recipient mice either alone or together with 50 $\times$ 10^6 KLH-primed, OVA-primed, or PAB-MGG-primed spleen cells. These helper cells came from other donor mice which had been immunized with 100 μg of KLH, OVA, and PAB-MGG in CFA 7, 6 and 8 wk before the cell transfer, respectively. As demonstrated in the left panel, these DNP-primed cells and helper cells were either nontreated or pretreated intraperitoneally with 1 mg PAB-D-GL 4 days before the cell transfer. Secondary antigenic challenges were performed with 100 μg of various hapten-carrier conjugates as indicated. Anti-DNP antibody responses in recipients 7 days after the cell transfer are illustrated. Statistical comparisons of the means among nontreated and PAB-D-GL-pretreated groups in DNP-BαA-primed, KLH-primed, OVA-primed, and PAB-MGG-primed cells yielded, $0.10 < P < 0.20$, $0.70 < P < 0.80$, $0.50 < P < 0.60$, and $P < 0.001$, respectively.

of DNP-BαA. In another six groups of mice, the DNP-BαA-primed cells (50 $\times$ 10^6) from the same nontreated donor pool were transferred together with 50 $\times$ 10^6 KLH-primed, OVA-primed, or PAB-MGG-primed spleen cells which had been either not treated or pretreated intraperitoneally with 1 mg of PAB-D-GL 4 days previously. The recipients were then stimulated with 100 μg of DNP-KLH, DNP-OVA, and DNP-MGG-PAB, respectively. The above carrier-primed cells were obtained from mice which had been immunized 7 wk (KLH), 6 wk (OVA), and 8 wk (PAB-MGG) before the cell transfer, respectively. All the recipients were killed 7 days after the cell transfer and anti-DNP antibody responses of those DNP-BαA-primed cells were measured.

It is immediately evident from the results shown in the right panel of Fig. 3 that the pretreatment of PAB-D-GL specifically suppresses the responsiveness of PAB-reactive helper T cells, while other helper T-cell activities of various specificities such as to BαA, KLH, or OVA, were not suppressed significantly by the pretreatment of PAB-D-GL.

Evidences for an Intracellular Mechanism of Inactivation of PAB-Reactive Helper T Cells by the Treatment of PAB-D-GL

In the above experiments, we were able to induce specific suppression of PAB-reactive helper T cells by administration of PAB-D-GL to the PAB-MGG-primed mice. A unique feature of this model is the relative ease with which the

suppression can be established in the educated T lymphocytes. As possible mechanisms of the specific suppression by treatment with PAB-D-GL, the following possibilities are conceivable: (*a*) During the 4-day period of PAB-D-GL treatment, the suppressor cell activity of PAB specificity may be generated in PAB-MGG-primed animals, with those suppressor cells imparting suppressive influences to the cellular cooperation of DNP-primed B cells with PAB-reactive helper T cells upon stimulation with DNP-MGG-PAB; and (*b*) PAB-D-GL does not suppress the PAB-reactive helper T cells themselves but simply binds to and blocks the T-cell receptor sites, thereby ultimately inhibiting effective cellular cooperation between DNP-primed B cells and PAB-reactive helper T cells. Finally, (3) PAB-D-GL specifically binds to T-cell receptor sites and inactivates the PAB-reactive helper T lymphocyte through some intra- or subcellular mechanism. The following series of experiments were performed to study these possibilities.

ELIMINATION OF THE POSSIBILITY OF CARRY-OVER OF TOLEROGEN OR GENERATION OF SUPPRESSOR CELLS AS THE EXPLANATION FOR THE SUPPRESSION OF PAB-REACTIVE HELPER CELLS BY PAB-D-GL. In order to determine whether suppressor activity can be detected in the PAB-MGG-primed cells which had been exposed to PAB-D-GL in donor mice, PAB-D-GL-pretreated PAB-MGG-primed cells were added to the PAB-reactive helper T cells from nontreated donors and the effect of this addition on helper T-cell activity was studied.

Spleen cells from mice which had been immunized intraperitoneally with 100 μg of DNP-BαA in CFA 12 wk previously were used as a DNP-primed B-cell source and transferred into five groups of X-irradiated (550 R) recipient mice either alone or together with PAB-MGG-primed spleen cells. The PAB-MGG-primed cells came from mice which had been primarily immunized 9 wk previously and boosted 5 wk before, then treated intraperitoneally either with 1 mg of PAB-D-GL or saline 3 days before sacrifice.

As shown in the protocol in Table III, which is substantially the same as in

TABLE III

*Failure to Detect Suppressor Activity in PAB-D-GL-Pretreated PAB-MGG-Primed Cells**

DNP-primed cells	Group	PAB-reactive helper T cells	Secondary challenge	Anti-DNP antibody response PFC/spleen
DNP-BαA	I	None	DNP-BαA	452,894 (1.07)
DNP-BαA	II	None	DNP-MGG-PAB	460 (1.61)
DNP-BαA	III	3 days saline-pretreated PAB-MGG-primed cells (A)	DNP-MGG-PAB	701,878 (1.23)
DNP-BαA	IV	3 days PAB-D-GL-pretreated PAB-MGG-primed cells (B)	DNP-MGG-PAB	776 (1.59)
DNP-BαA	V	(A) + (B)	DNP-MGG-PAB	519,694 (1.08)

* The experimental protocol is substantially the same as Table II. DNP-BαA-primed cells came from mice which had been immunized 12 wk previously and PAB-MGG-primed cells from mice primarily immunized 9 wk previously and boosted 5 wk before. Statistical comparison of the mean PFC responses in various groups yielded the following P values: group II vs. III, $P < 0.001$; III vs. IV, $P < 0.001$· III vs. V, $0.60 < P < 0.70$.

Table II, Groups I and II received DNP-BαA-primed cells alone and were then stimulated with 100 μg of DNP-BαA or DNP-MGG-PAB. Groups III and IV received DNP-BαA-primed cells together with PAB-MGG-primed cells which had been either pretreated with saline or PAB-D-GL 3 days before the cell transfer. Group V received a mixture of equal numbers of both types of PAB-MGG-primed cells. They were then all stimulated with 100 μg of DNP-MGG-PAB. Anti-DNP antibody responses were measured in recipient spleens 7 days after the cell transfer, and the results are summarized in Table III.

In comparison of group II with group III, the PAB-reactive helper (T) cells in the PAB-MGG-primed cell population nicely helped the anti-DNP-antibody response of DNP-primed B cells in the DNP-BαA-primed cell population at the stimulation of DNP-MGG-PAB. On the other hand, as shown in group IV, the pretreatment of PAB-D-GL to those PAB-MGG-primed cells almost completely abrogated such activities. Most importantly, however, when those PAB-MGG-primed cells whose responses had been suppressed by PAB-D-GL pretreatment were added to the above intact PAB-reactive helper T cells (group V), the magnitude of response of those mixtures of PAB-reactive helper cells was almost at the same level as in group III. This result, therefore, argues strongly against the possibility that the PAB-MGG-primed cells which had been exposed to PAB-D-GL afford some suppressive influences either to the PAB-reactive helper cells or to the DNP-specific B cells. This further indicates that, in this tolerance model on the helper T-cell level, PAB-D-GL renders the helper T cells themselves unresponsive. Suppressor factors such as suppressor cells, or the carry-over of some PAB-D-GL with the transferred cell populations seem not to operate in the mechanism of this unresponsiveness of PAB helper T cells.

EVIDENCE THAT TOLERANCE INDUCED BY PAB-D-GL IS NOT MERELY REFLECTIVE OF BLOCKING OF SURFACE RECEPTORS ON T LYMPHOCYTES. In order to test the possibility that PAB-D-GL simply binds to and blocks the T-cell receptor sites and thereby ultimately inhibits cellular cooperation, PAB-D-GL was directly exposed in vitro at 4°C for 1 h to PAB-reactive helper T lymphocytes, conditions in which PAB-D-GL is supposed to bind to the PAB-reactive T lymphocytes. Helper activity of these PAB-reactive T cells after exposure to PAB-D-GL was measured by the double cell transfer system of cellular cooperation with DNP-primed B cells, as described below.

Spleen cells (50×10^6) from mice which had been immunized intraperitoneally with 100 μg of DNP-BαA in CFA 15 wk previously were used as the DNP-primed B-cell source and transferred intravenously into X-irradiated (550 R) recipients alone or together with PAB-MGG-primed spleen cells (50×10^6) from mice which had been immunized intraperitoneally with 100 μg of PAB-MGG in CFA 10 wk previously.

As shown in the experimental protocol in Table IV, DNP-BαA-primed cells were stimulated with 100 μg of DNP-BαA as a positive control of the responsiveness of DNP-primed B cells (group I), and with 100 μg of DNP-MGG-PAB in the absence or presence of PAB-MGG-primed helper T lymphocytes (groups II and III). In group IV, the PAB-reactive helper T cells transferred came from PAB-MGG-primed mice which had been pretreated intraperitoneally with

TABLE IV

*Failure to Suppress Reactivity of PAB-Reactive Helper T Cells when Exposed to PAB-D-GL In Vitro Immediately before the Cell Transfer**

DNP-primed cells	Group	PAB-reactive helper T cells treated with:	Secondary challenge	Anti-DNP antibody response PFC/spleen
DNP-BαA	I	None	DNP-BαA	145,237 (1.05)
DNP-BαA	II	None	DNP-MGG-PAB	2,227 (1.05)
DNP-BαA	III	4 days saline-pretreated in vivo	DNP-MGG-PAB	109,433 (1.16)
DNP-BαA	IV	4 days PAB-D-GL-pretreated in vivo	DNP-MGG-PAB	12,262 (1.12)
DNP-BαA	V	PAB-D-GL mixed in vitro, 0.01 μg	DNP-MGG-PAB	138,723 (1.12)
DNP-BαA	VI	PAB-D-GL mixed in vitro, 0.1 μg	DNP-MGG-PAB	103,595 (1.11)
DNP-BαA	VII	PAB-D-GL mixed in vitro, 1.0 μg	DNP-MGG-PAB	103,299 (1.16)
DNP-BαA	VIII	PAB-D-GL mixed in vitro, 10 μg	DNP-MGG-PAB	70,933 (1.24)

* The experimental protocol is substantially the same as Table II except in groups V ~ VIII, in which various doses of PAB-D-GL were mixed in vitro with PAB-reactive helper T cells (50×10^6) and incubated at 4°C for 1 h before the cell transfer. Donors of DNP-primed cells and PAB-reactive helper T cells came from mice primed with 100 μg of DNP-BαA in CFA 15 wk earlier and PFC responses between group III vs. IV, V, VI, VII, and VIII yielded P values of $P < 0.001$, $0.30 < P < 0.40$, $0.70 < P < 0.80$, $0.80 < P < 0.90$, and $0.10 < P < 0.20$, respectively.

1 mg of PAB-D-GL 4 days before the cell transfer. It is again evident that the reactivity of PAB-reactive helper T cells (group IV) was effectively suppressed by pretreatment with PAB-D-GL, as compared with saline-pretreated helper cells (group III).

In sharp contrast, however, when the PAB-MGG-primed cells (50×10^6) were exposed in vitro to various concentrations (0.01 μg ~ 10 μg) of PAB-D-GL for 1 h at 4°C, mixed together with DNP-BαA-primed cells without washing, and then stimulated with 100 μg of DNP-MGG-PAB (groups V ~ VIII), the PAB-reactive helper T-cell activity in the PAB-MGG-primed cell population was not suppressed within the range of concentrations of 0.01–1.0 μg (groups V ~ VII), and only slightly at 10 μg of PAB-D-GL (group VIII).

Thus, PAB-D-GL when added in vitro to PAB-reactive helper T cells does not suppress the reactivity of those helper T cells. These results suggest that the suppression of PAB-reactive helper T cells induced in vivo by exposure to PAB-D-GL, such as was demonstrated in group IV, may not be due solely to the blocking of T-cell receptors. Although this failure to suppress the PAB-reactive helper T-cell activity when incubated in vitro with PAB-D-GL argues against a predominant surface mechanism, it fails to provide formal evidence on this point. The following experiment was designed and carried out to explore this point more completely.

FAILURE TO REVERSE TOLERANCE INDUCED IN VIVO WITH PAB-D-GL BY TRYPSINIZATION OF CELLS BEFORE ADOPTIVE TRANSFER TO IRRADIATED RECIPIENTS. The second approach to the question of receptor blocking by tolerogen took advantage of capacity of enzymatic treatment of cell surfaces by trypsin to reverse toler-

ance induced by PAB-D-GL. The validity of this approach was based on the previous findings (12) in tolerance induction of B cells by DNP-D-GL that the trypsin treatment of B cells exposed to DNP-D-GL for a relatively short time could clearly reverse the tolerance state to normal, whereas trypsinization of cells exposed to DNP-D-GL for a longer period of time did not reverse the tolerance to responsiveness. This formed a strong basis for our notion that binding of tolerogen on the surface of B lymphocytes for a certain period of time results in functional inactivation of B lymphocytes. In the following experiment, we applied this same reasoning to T-cell tolerance in the PAB-D-GL model.

The protocol of this experiment is schematically depicted in the left panel of Fig. 4. Briefly, two groups of donor mice which had been primed with 100 μg of

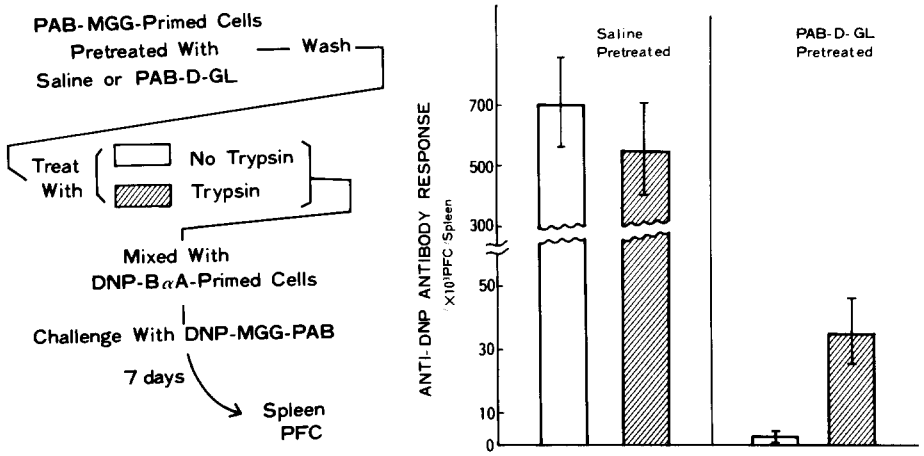

FIG. 4. Failure to reverse tolerance induced in vivo with PAB-D-GL by trypsinization of cells before adoptive transfer. PAB-MGG-primed cells were obtained from mice which had been primed 9 wk earlier, boosted 5 wk earlier, then pretreated intraperitoneally with saline or 1 mg PAB-D-GL 3 days before sacrifice. DNP-BαA-primed cells came from mice immunized 16 wk previously. Relevant statistical comparisons of responses yielded the following P values: (*a*) nontrypsinized saline controls vs. nontrypsinized PAB-D-GL-pretreated cells, $P < 0.001$; (*b*) trypsinized saline controls vs. trypsinized PAB-D-GL-pretreated cells, $0.001 < P < 0.005$; (*c*) nontrypsinized saline controls vs. trypsinized saline controls, $0.50 < P < 0.60$; and (*d*) nontrypsinized PAB-D-GL-pretreated cells vs. trypsinized PAB-D-GL-pretreated cells, $P < 0.001$.

PAB-MGG in CFA 9 wk earlier and boosted with the same dose 5 wk before sacrifice, were pretreated intraperitoneally with 1 mg of PAB-D-GL or saline. After 3 days, spleen cells from these two groups of mice were obtained and 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 the Materials and Methods. Groups of irradiated (550 R) recipient mice were injected intravenously (50×10^6 cells/mouse) with either untreated or trypsinized cells from the respective donor mouse groups; these cells were mixed together with 50×10^6 DNP-BαA-primed spleen cells which came from other donor mice which had been immunized 16 wk earlier with 100 μg of DNP-BαA in CFA. The mixture of those primed cells was then secondarily challenged with 100 μg of

DNP-MGG-PAB. All the recipients were bled 7 days later and anti-DNP antibody responses were measured.

As shown in the right panel of Fig. 4, PAB-reactive helper T cells exposed to saline in the donor mice elicited very good adoptive anti-DNP antibody responses with the DNP-primed B cells upon stimulation by DNP-MGG-PAB; trypsinization barely, and not significantly, reduced or augmented these responses. In contrast, the helper cells which had been exposed to PAB-D-GL failed to induce cellular cooperation to the stimulation of DNP-MGG-PAB, and this inability to induce cellular cooperation was not reversed by trypsinization, even though trypsinization of such cells resulted in a slight increase in magnitude of these responses.

Since trypsin presumably removes surface receptors (and any associated tolerogen) these results indicate that: (*a*) a partial improvement of the reactivity of PAB-D-GL-pretreated helper cells after trypsinization may reflect some degree of unblocking of surface receptor molecules by PAB-D-GL; and (*b*) the ultimate failure of trypsinization to reverse unresponsiveness of PAB-D-GL-pretreated cells to normal levels must, by analogy, reflect more complex inhibitory events not predominantly related to reversible surface membrane factors.

Discussion

Treatment of the PAB derivative of the copolymer of D-GL induces a profound state of unresponsiveness to PAB-reactive helper T lymphocytes present in PAB-MGG-primed mice. A unique feature of the PAB-D-GL tolerance model as presented in these studies is the relative ease with which tolerance can be specifically induced to the PAB-primed helper T lymphocytes. This may provide a potential advantage over other models of tolerance on the T-cell level since, in general, primed T lymphocytes are difficult to tolerize by tolerogen treatment. In the present studies, we have taken advantage of those features to analyse possible mechanisms for tolerance induction on the level of helper T cells.

Thus, spleen cells from PAB-MGG-primed donor mice which contain PAB-reactive helper T cells normally induce very good cooperative adoptive secondary anti-DNP antibody responses to DNP-primed B cells when stimulated with a double hapten-carrier conjugate coupled with mutually noncross-reacting DNP and PAB haptens (Fig. 1 and Table I). However, exposure of such PAB-primed helper T cells to PAB-D-GL completely, or almost completely, abolished the helper activity of those primed cells (Fig. 2 and Table II). This induction of unresponsiveness was specific for the PAB-reactive helper T cells, since such treatment did not have any influence on the responsiveness of BαA-, KLH-, or OVA-primed helper T lymphocytes (Fig. 3).

In view of detailed studies by Chiller et al. (3, 4) on the kinetics of tolerance induction to deaggregated HGG, it is well established that induction of tolerance to thymus-dependent antigens clearly involves the establishment of tolerance in both T and B lymphocytes, the former being rendered tolerant much more rapidly and with a lower concentration of antigen. On the basis of what is known about the operational differences between T and B lymphocytes, establishment of a model in which tolerance is restricted to B or T cells may be very crucial for elucidation of the mechanism of tolerance induction in each cell type. In the previous studies (9, 10, 12), it has been shown that administration of the DNP

derivative of the copolymer of D-GL to inbred mice induces a profound state of tolerance to DNP-primed B cells in such aminals immunized with DNP-heterologous protein conjugates such as DNP-KLH. Both the maintenance of tolerance of DNP-specific B cells by serial adoptive transfer (9) and the failure to reverse tolerance by trypsin treatment of tolerized DNP-specific B cells (12) indicate that the mechanism of tolerance induction on the B-cell lineage is the specific inactivation or elimination of clones by the tolerogen, and cannot be explained solely by simple masking of tolerogen onto the B cells.

On the other hand, concerning the mechanism of tolerance induction in the T-cell lineage, similar mechanisms may conceivably be operating, but this issue is unfortunately still a point of controversy. The following three possibilities may be relevant to consider with reference to the mechanism of induction of unresponsiveness in the T-cell population: (*a*) Tolerance is mediated by a suppressor T-cell effect, since tolerance in certain situations is infectious and the cells from tolerant donors have the ability to inhibit antibody production of nontolerant cells due to an active suppressor T-cell effect in the tolerant cell population (17–20); (*b*) tolerance is mediated by circulating suppressive factors since, in another type of tolerance model such as is observed in unresponsiveness to tissue-associated antigens, the lymphoid cells obtained from unresponsive animals have a capacity to respond to that antigen when tested in a milieu free of serum from tolerant animals (21–24). Especially in this case, this cellular unresponsiveness can be reproduced by addition of blocking serum factor. (*c*) Tolerance implies the specific elimination of clones induced by tolerogen treatment, since some other types of evidence so far reported also imply that tolerance in the T-cell population reflects deletion of a specific clone of antigen-reactive T cells (25–27). In this case, the tolerance is neither infectious nor inducible by the administration of serum from tolerant animals, and is stable on adoptive transfer. Thus, in contrast to tolerance induction in B lymphocytes, the precise mechanism of T-cell tolerance is as yet unresolved. A critical question in any model of T-cell tolerance concerns the mechanism by which suppression of T-cell reactivity occurs. Specifically, what happens to a tolerant T cell? Does it exist in a functionally unresponsive or unrecognizable state, or is it eliminated from the system? Much of this confusion stems from the apparently contradictory observations described above. This is a crucial issue since on its rests the correct interpretation of the fate of tolerant T cells.

In the context of the above considerations, we have tried to analyse the mechanism of tolerance induction and the fate of tolerant T cells in the PAB-D-GL model. One immediate question that arises concerns the possibility that suppression of the reactivity of PAB-reactive helper T lymphocytes in the PAB-D-GL model is mediated by suppressor factors present in tolerant animals. That these suppressor factors exist has been immediately ruled out by the following observations here reported: (*a*) The stability of the unresponsiveness of helper T cells after transfer into other recipients from tolerogen-treated donors where profound unresponsiveness was induced by PAB-D-GL (Table II). If mediation by a blocking factor in serum (or circulating tolerogen) alone is responsible for the helper T-cell tolerance, one might expect that the unresponsiveness of helper T cells from PAB-D-GL treated donors would be reversed after transfer into other recipient mice. (*b*) More importantly, suppression of the reactivity of nontolerant helper T cells by addition of tolerogen-treated helper T cells could not be induced (Table III). In this case, tolerogen-treated helper T cells almost completely lost their reactivity, but nevertheless failed to show any suppressive influences on the reactivity of other nontolerant helper T cells. This observation also rules out the possibility that maintenance of tolerance after adoptive transfer into other recipients was due to carry-over of tolerogenic doses of PAB-D-GL associated with the transferred cell populations. Thus, taken collectively, these observations clearly indicate that suppression of PAB-reactive helper T cells was induced by direct interaction of PAB-D-GL with PAB-reactive helper T cells themselves,

and that the treatment of tolerogen does not generate any suppressor factor responsible for the unresponsiveness of T cells in this PAB-D-GL model. Furthermore, from the experiment of in vitro exposure of PAB-D-GL to the PAB-reactive helper T lymphocytes (Table IV), the possibility of a simple blocking of receptor molecules on the surface of T lymphocytes by tolerogen could be eliminated as an explanation for the suppression of reactivity of helper T cells, and this points emphatically to the existence of a central intracellular mechanism of specific tolerance induction on the T-cell level. The most conclusive evidence that PAB-D-GL tolerance involves more sophisticated events than receptor blockade derives from the failure of enzymatic treatment by trypsin of cells tolerized by PAB-D-GL to reverse, or even diminish, the level of unresponsiveness manifested by such cells (Fig. 4). If these cells still had been capable of responding to PAB but could not do so because all of their surface receptors were competitively blocked by PAB-D-GL, then this situation should have been corrected by trypsinization. Indeed, a slight recovery of responsiveness observed after trypsin treatment may suggest that such a mechanism operates to some extent on the suppression of the activity of helper T-cell function, though the prediction still seems valid that unresponsiveness of this type is probably very transient in nature. Also evident from this result is that the ultimate failure of trypsinization to reverse the unresponsiveness induced by PAB-D-GL clearly supports the conclusion that such cells have been inactivated via sub- or intracellular mechanisms. A tolerant state of this type would be predictably long lasting. We should emphasize, however, that the concept of irreversibility as used here pertains to the individual T cells which have been exposed to the tolerogen and not necessarily to the furture progeny of the stem cell clone-bearing specificity for the tolerogen.

Since, as we have demonstrated, tolerance induced by PAB-D-GL is apparently restricted to the PAB-reactive helper T lymphocytes, this model for tolerance may be very informative with respect to an understanding of the mechanism of tolerance induction in T lymphocytes. However, concerning the tolerance induction of T lymphocytes by hapten D-GL conjugates, there are some previously reported controversial observations. These involve the experiments of hapten-specific tolerance induction in guinea pigs or mice. In guinea pigs, Cohen et al. (32) reported that animals tolerized with DNP-D-GL, although manifesting a marked depression in the humoral response after immunization with DNP-guinea pig albumin (GPA), display a normal delayed hypersensitivity response to DNP-GPA. Furthermore, lymph node cells from those tolerized animals incorporate thymidine into DNA when stimulated with antigen in amounts equivalent to cells from normal immune animals. Thus, cellular immune responses (T-cell-mediated function) to DNP-GPA are normal in guinea pigs pretreated with DNP-D-GL. Basically, the same result was also reported by Benacerraf and Katz (33), in that treatment with DNP-D-GL, which suppresses anti-DNP antibody responses in virgin and primed guinea pigs, does not affect the ability of these animals to develop and to display contact sensitivity to DNCB. Moreover, as will be reported from our laboratory using mice,[2] DNP-reactive helper T lymphocytes generated by immunization with DNP-MGG failed to be tolerized by treatment with DNP-D-GL. These observations confirm the suggestions that important operational differences, such as a basic specificity distinction of receptors, exist in the induction of tolerance in T and B lymphocytes presumed to be concerned with the same DNP specificity.

However, as demonstrated clearly in the present studies, PAB-D-GL treatment

of PAB-reactive helper T lymphocytes induces a profound state of tolerance. The exact reasons for these conflicting results concerning the tolerogenic activity of hapten-D-GL conjugates to different specificities of T lymphocytes are not clear at present. With reference to these discrepancies, the following two possibilities may be worthwhile considering: (*a*) Differences in net charge or hapten-epitome density of tolerogen are crucial for the D-GL conjugate to be highly tolerogenic to T lymphocytes, since the PAB-D-GL molecule used in the present studies was somewhat more acidic in net charge than DNP-D -GL and PAB residues were more highly conjugated on D-GL in mono- or bis-substituted forms. (*b*) Because of some heterogeneity of hapten-reactive helper T lymphocytes with respect to receptor affinity, the induction of tolerance of the hapten-reactive T cells may be both dose and affinity dependent. Under the experimental conditions used to tolerize DNP-reactive helper T cells by DNP-D-GL, the affinity of the receptors on DNP-reactive T cells may be too low to be effectively tolerized with DNP-D-GL. In contrast, PAB-reactive helper T cells such as used in the present studies consist of, on the average, relatively higher affinity populations. In fact, in the model of tolerance induction of PAB-reactive helper T cells with PAB-D-GL, PAB-reactive helper T cells obtained from mice which had been immunized 4 wk previously were found to be rather difficult to render tolerant with PAB-D-GL,[3] whereas primed cells obtained from mice immunized for a relatively longer period of time were easily tolerized, such as demonstrated in the present studies. Furthermore, as shown in data to be presented separately,[3] the increase in susceptibility to tolerance of long-primed PAB-reactive helper T-cell population upon treatment with PAB-D-GL was exactly correlated with the loss of cross-reactivity of those helper cells to various other cross-reactive hapten conjugates such as *o*-azobenzoate-MGG and *m*-azobenzoate-MGG. In addition, those long-primed PAB-reactive helper T cells elicited their full reactivity to the stimulation of smaller amounts of DNP-MGG-PAB when cooperative interaction was elicited with DNP-specific B cells at various doses of second antigen. These preliminary results[3] strongly suggest that specificity restriction and binding affinity of the receptors of PAB-reactive helper T lymphocytes may be more crucial for the induction of T-cell tolerance.

In any case, the precise nature of events at the cellular and subcellular levels that result in specific unresponsiveness with PAB-D-GL on the level of PAB-reactive helper T lymphocytes awaits further investigations. Nonetheless, it may finally be worthwhile pointing out that the establishment of a tolerance model exclusively operating on the T-cell levels, such as described here, may provide certain advantages in studies designed to probe the mechanism of specific inactivation processes of T lymphocytes by tolerogen.

Summary

Treatment of a *p*-azobenzoate (PAB) derivative of a copolymer of D-glutamic acid and D-lysine (D-GL) induced a profound state of unresponsiveness to PAB-reactive helper T lymphocytes generated in PAB-mouse gamma globulin (MGG)-primed mice. This unresponsiveness in T lymphocytes was specific for PAB-reactive cells, since the bacterial α-amylase-, keyhole limpet hemocyanin-,

or ovalbumin-primed helper T lymphocytes were not suppressed by PAB-D-GL treatment. Taking advantage of the relative ease with which PAB-D-GL can induce specific unresponsiveness to helper T lymphocytes in an animal previously primed with PAB-MGG, it was possible to approach certain questions concerning the mechanisms of tolerance-induction and the fate of tolerant helper T lymphocytes in the PAB-D-GL model by utilizing a classical adoptive cell transfer system. Elimination of the possibility of carry-over of the tolerogen with cells or of the generation of suppressor cells as the result of PAB-D-GL treatment as an explanation of the suppression of helper T-cell activity strongly inplicates the existence of a central intracellular mechanism of specific tolerance on the helper T-cell level. The possibility that suppression of the activity of PAB-reactive helper T lymphocytes by PAB-D-GL reflects simple blocking of surface receptor molecules on T lymphocytes was ruled out as it was found that the helper activity of PAB-reactive cells was minimally suppressed even when PAB-D-GL was directly exposed in vitro to helper T lymphocytes. Moreover, the most conclusive evidence on this point derives from the ultimate failure of enzymatic treatment with trypsin to reverse the tolerant state induced by in vivo exposure of primed T cells to PAB-D-GL. It appears, therefore, that specific tolerance induced by PAB-D-GL to PAB-reactive helper T lymphocytes is an example of irreversible inhibition of T-cell reactivity to antigen, reflecting yet to be determined events at the intra- and subcellular levels.

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