

## REGULATORY FUNCTIONS OF HAPTEN-REACTIVE HELPER AND SUPPRESSOR T LYMPHOCYTES

### I. Detection and Characterization of Hapten-Reactive Suppressor T-Cell Activity in Mice Immunized with Hapten-Isologous Protein Conjugate\*

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In studies designed to analyze T-B cell interactions in immune responses, hapten-carrier conjugates have been used as a model antigen, since normally B lymphocytes specifically react with the hapten and collaborate with T cells specific for the carrier. With these conjugates one can clearly dissect the responses of B lymphocytes and those T lymphocytes which possess distinct clonal specificities. However, carrier-specific T lymphocytes invariably react with, not one, but many of the complex determinants on the carrier molecule, so that the investigator never deals exclusively with a monospecific T-cell population. This point is especially important when one takes into account the functional diversity of T-cell activities. Consequently, a monospecific hapten-reactive T-lymphocyte system would enable us to analyze regulatory T-cell functions in the initiation of immune responses and to understand better the basic mechanisms of regulatory T-cell interactions. Moreover, since hapten-reactive T lymphocytes theoretically react with the haptenic determinants on any carrier molecule such as a heterologous protein, or allogeneic or syngeneic tumor cells, the system developed for one hapten in a particular immune response should apply equally well to other immune responses involving that hapten regardless of the carrier. Previously, we used hapten-isologous protein conjugates to prime mice whose spleen cells were later analyzed for helper T-cell functions (1, 2). We now describe an assay system used to measure hapten-reactive suppressor T cells in the same population of spleen cells and to determine their means of inducing anti-hapten antibody responses and their site of activity.

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## Materials and Methods

*Antigens and Chemical Reagents.* Keyhole limpet hemocyanin (KLH)<sup>1</sup> was purchased from Calbiochem, San Diego, Calif., and hen ovalbumin (OVA) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Dextran T2000, average mol wt 2,000,000 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Mouse gamma globulin (MGG) was prepared from pooled serum of normal mice by precipitation with 40% saturated ammonium sulfate and purified through diethylaminoethyl (DEAE)-cellulose column chromatography with 0.015 M sodium phosphate buffer, pH 8.0. Human gamma globulin (HGG) (Cohn fraction II) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, and further purified through DEAE-cellulose column chromatography by using 0.01 M potassium-phosphate buffer, pH 8.0. Bovine serum albumin (BSA) was purchased from Armour Pharmaceutical Co., Chicago, Ill.

*Preparation of Hapten-Carrier Conjugates.* The following dinitrophenyl (DNP)-conjugates were prepared by using sodium 2,4-dinitrobenzene sulfonate: DNP<sub>9</sub>-KLH, DNP<sub>6</sub>-MGG, and DNP<sub>15</sub>-BSA. Subscripts refer to the average number of DNP groups per molecule of protein, as calculated from absorption at 360 and 280 nm in an alkaline solution.

$\epsilon$ -Dinitrophenyl-lysine-substituted dextran (DNP-dextran) was prepared by modifying (3) the method of Fielder et al. (4). This preparation had a molecular ratio of DNP-lysine to dextran of 50:1, as measured by absorption at 360 nm.

*Para-azobenzoate (PAB)-carrier conjugates* were prepared by reacting carrier proteins with diazotized *p*-aminobenzoic acid at pH 9.0 as described previously (1). The following PAB-protein conjugates were prepared: PAB<sub>17</sub>-MGG, PAB<sub>9</sub>-MGG-DNP<sub>6</sub>, PAB<sub>10</sub>-KLH-DNP<sub>9</sub>, PAB<sub>15</sub>-OVA, and PAB<sub>12</sub>-HGG. Subscripts refer to the average number of PAB groups per molecule protein, as calculated from absorption at 460 and 500 nm in an alkaline solution. PAB was assumed to have coupled to tyrosine and histidine residues of the protein.

Benzylpenicilloyl (BPO)-KLH conjugates were prepared by reacting 100 mg KLH with 100 mg of benzylpenicillin in 5 ml borate-buffered saline adjusted to pH 9.0 with 1 N NaOH. 13 molecules of BPO-groups were covalently bound per molecule of MGG as measured by the penamaldate method (5). BPO<sub>7</sub>-OVA was prepared in the same manner by reacting 75 mg OVA with 100 mg benzylpenicillin.

For the calculation of hapten units per molecule of carrier, the mol wt of KLH, MGG, HGG, OVA, and BSA were taken as 100,000, 160,000, 160,000, 45,000, and 68,000, respectively.

*Assays of Helper and Suppressor Activities in PAB-Reactive T-Lymphocyte Populations.* DDO albino mice (supplied by the Central Breeding Laboratory of Experimental Animals of Osaka University, Osaka, Japan) were immunized by i.p. injection of 100  $\mu$ g of PAB-MGG in complete Freund's adjuvant (CFA). At intervals from 1 to 20 wk later, the spleens or mesenteric lymph nodes of these primed mice were removed, suspended in Eagle's minimal essential medium (MEM), and used as the source of PAB-reactive T cells. Single cell suspensions from spleens of mice immunized i.p. with 100  $\mu$ g of DNP-KLH or BPO-OVA in CFA 2-3 mo previously were the sources of DNP-primed or BPO-primed B cells. Cell transfers involved injecting DNP-KLH- or BPO-OVA-cells intravenously together with the PAB-reactive T-cell populations into syngeneic mice given 550 R-600 R X-irradiation. As a control, lymphoid cells from normal animals were transferred in the place of PAB-MGG-primed cell populations.

In general, antigenic stimulation was performed by i.p. injection into recipients immediately after the cell transfer. For the assay of helper and suppressor T-cell activities in the PAB-MGG primed T-cell population, 100  $\mu$ g of DNP-MGG-PAB or 100  $\mu$ g of DNP-KLH-PAB was administered to recipients after cell transfer. The magnitude of the anti-DNP antibody responses of transferred DNP-primed B cells in the presence of PAB-reactive T cells was compared with that in

<sup>1</sup> *Abbreviations used in this paper:* B cells, precursors of antibody-forming cells; BPO, benzylpenicilloyl; BSA, bovine serum albumin; C, complement; CFA, complete Freund's adjuvant; D-GL copolymer of D-glutamic acid and D-lysine; DNP, 2,4-dinitrophenyl; FGG, fowl gamma globulin; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin; MGG, isologous mouse gamma globulin; NGS, normal goat serum; NMS, normal mouse serum; OVA, hen ovalbumin; PAB, para-azobenzoate; PFC, plaque-forming cells; SRBC, sheep erythrocytes; T cells, thymus-derived lymphocytes.

the presence of normal cells. Recipients' spleens were assayed 7 days after adoptive cell transfer and antigenic stimulation, techniques that are described in the appropriate sections of Results.

*Hemolytic Plaque Assay.* Plaque-forming cells (PFC) in the spleens of recipient mice were counted by using a modification of Cunningham's and Szenberg's hemolytic plaque technique (6). For the assay of anti-DNP PFC, sheep erythrocytes (SRBC) were conjugated with DNP-BSA by using  $\text{CrCl}_3$  (1). For the assay of anti-BPO PFC, 1 ml packed SRBC was reacted with 900 mg of BPO in 15 ml of 0.14 M glycine-0.14 M NaOH buffer, pH 10, for 1 h at room temperature (7). Single spleen cell suspensions from individual recipient mice were treated with 0.85%  $\text{NH}_4\text{Cl}$  and washed three times with MEM. Direct PFC were counted in cultures of indicator SRBC, spleen cells, and guinea pig serum used as the source of complement (C). Indirect PFC were developed by using rabbit antisera directed against the Fc portion of mouse IgG in the presence of C. Since the number of direct PFC was always negligible, only indirect PFC are listed in the Results, unless otherwise indicated.

*Depletion of B Lymphocytes from PAB-MGG-Primed Spleen Cells.* PAB-MGG-primed spleen cells were treated in vitro with goat antiserum specific for a surface membrane determinant shared by mouse B lymphocytes and some immature thymus cells in the presence of C. This antiserum, known as anti-Th-B, was kindly provided by Dr. M. Yutoku in our laboratory. The details of the antiserum's preparation and specificity have been published (8). To  $5 \times 10^7$  viable PAB-MGG-primed spleen cells in 1 ml of MEM, we added 1 ml of a 1:4 dilution of either in vivo absorbed goat antiserum or in vivo absorbed normal goat serum (NGS), and as a source of C, 1 ml of a 1:4 dilution of rabbit serum absorbed with mouse myeloma, spleen, and thymus cells. The diluent was MEM. The cells were incubated at 37°C for 45 min while shaken, washed twice with cold MEM, and suspended in an appropriate amount of MEM. Approximately 40% of the original number of cells was recovered. B-cell depletion was then evaluated by transferring the recovered spleen cells into X-irradiated recipients, injecting the recipients with 200  $\mu\text{g}$  of DNP-dextran (thymus-independent antigen) (3), and determining the animals' responsiveness to this antigen.

*Depletion of T Lymphocytes from PAB-MGG-Primed Spleen Cells.* The preparation of anti-Thy-1.2 alloantiserum, the determination of its cytotoxicity, and the method for depleting spleen cells of T cells by using anti-Thy-1.2 antiserum plus C have been published (1). Anti-Thy-1.2 antiserum-treated spleen cells were also tested for nonspecific effects on their B-lymphocyte constituents as determined by responses to DNP-dextran.

*Statistical Analysis.* The numbers of PFC per spleen were logarithmically transformed and geometric means and standard errors calculated. Group comparisons were made by employing Student's *t* test. In those mice without detectable splenic PFC, a value of 240 per spleen, the minimal number of PFC detectable in our assay, was arbitrarily assigned to allow logarithmic transformation of the data.

## Results

*Detection of Suppressor Cell Activity in PAB-MGG-Primed Spleen Cells.* Previously (1), hapten-reactive helper T cells were detected after DNP-specific B lymphocytes were stimulated with double hapten-conjugated isologous protein, DNP-MGG-PAB, in the presence of PAB-MGG-primed spleen cells. We have now extended this system to the detection of suppressor T-cell activity in the same hapten-isologous protein-primed cells.

Briefly, the experimental system follows: spleen cells from mice immunized either 3 wk previously with 100  $\mu\text{g}$  of PAB-MGG in CFA or 10 wk previously with 100  $\mu\text{g}$  DNP-KLH in CFA were the sources of PAB-reactive T cells and DNP-specific B cells, respectively. A mixture of spleen cells from PAB-MGG-primed and DNP-KLH-primed donors was adoptively transferred into 600 R X-irradiated mice, and DNP-MGG-PAB was given as the stimulating antigen. Consistent with our previous observations (1), helper T-cell activity was detected by the greater anti-DNP antibody responses of DNP-specific B cells in the presence of PAB-MGG-primed spleen cells than in the presence of unprimed

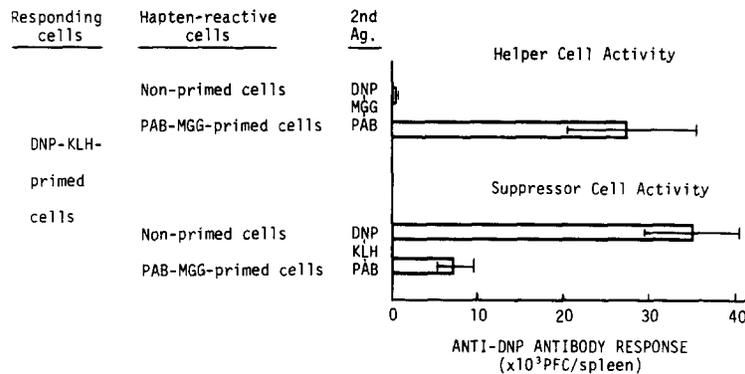


FIG. 1. Detection of both PAB-reactive helper and suppressor cell activity in a single experimental system.  $50 \times 10^6$  spleen cells from donor mice primed 10 wk earlier with  $100 \mu\text{g}$  of DNP-KLH in CFA, were used as responding cells, and transferred intravenously into 600 R X-irradiated recipients together with spleen cells from either nonprimed mice or PAB-MGG-primed mice. The PAB-MGG-primed mice had been immunized i.p. with  $100 \mu\text{g}$  of PAB-MGG in CFA 3 wk before the cell transfer. Secondary antigenic challenges were performed by i.p. injection of  $100 \mu\text{g}$  of hapten-carrier conjugates, as indicated, immediately after the cell transfer. Geometric means and standard errors of anti-DNP PFC responses in spleens of the recipients 7 days after the cell transfer are illustrated.

donor cells (Fig. 1, top). On the other hand, when DNP-KLH-primed cells were stimulated with  $100 \mu\text{g}$  of DNP-KLH-PAB in the presence of PAB-MGG-primed cells, anti-DNP antibody responses were significantly lower than in the presence of unprimed cells (Fig. 1, bottom). This suppressor cell effect was induced through the PAB-portion of DNP-KLH-PAB since no such suppression followed stimulation of DNP-KLH-primed cells (in the presence of PAB-MGG-primed cells) with DNP-KLH (not conjugated with PAB, data not shown).

Two possible mechanisms of this response are: (a) suppression induced by PAB-MGG-primed cells was mediated by suppressor T cells which are reactive with PAB-haptenic groups on the KLH molecule; (b) suppression was induced by a simple elimination of effective antigenic stimulus from the system through the reaction of PAB-reactive cell population with PAB on KLH molecule. To clarify the mechanism of suppression further, the cell population responsible for this suppression was investigated.

#### *Evidence for T-Cell-Mediation of Suppressor Activity*

(a) FAILURE TO DETECT SUPPRESSOR CELL ACTIVITY IN PAB-HETEROLOGOUS PROTEIN CARRIER-PRIMED CELLS. To exclude the possibility that suppressor cell activity in PAB-MGG-primed cell populations is mediated by anti-PAB antibody or PAB-specific B cells, various PAB-heterologous protein conjugates were used to induce anti-PAB antibody and PAB-specific B cells in donors. The double-cell transfer system was again employed to measure suppressor cell activity on the DNP-KLH-primed cells after stimulation with DNP-KLH-PAB. As shown in Table I, suppression was consistently observed only in PAB-MGG-primed cells, but never in PAB-HGG-primed or PAB-OVA-primed cells, although the latter two conjugates probably induce comparable or higher levels of anti-PAB antibody or PAB-specific B-cell activities than the first conjugate. Thus, neither an

TABLE I  
*Failure to Detect PAB-Reactive Suppressor Cell Activity in PAB-Heterologous Protein Carrier-Primed Cells*

Responding cells	Cells for detection of suppressor activity*	2nd Ag	Anti-DNP antibody response PFC/spleen
DNP-KLH-primed cells‡	Unprimed cells	DNP-	63,100 (1.23)
	PAB-MGG-primed cells	KLH-	9,465 (1.12)
	PAB-HGG-primed cells	PAB	41,136 (1.37)
	PAB-OVA-primed cells		89,731 (1.14)

\* These represent  $50 \times 10^6$  spleen cells from mice primed 3 wk earlier with 100  $\mu\text{g}$  of various hapten-carrier conjugates as indicated in CFA.

‡ These came from mice primed 8 wk previously.

anti-PAB antibody-dependent cell-mediated phenomenon or a direct suppressive effect of PAB-specific B cells seems to be involved. Furthermore, the failure to detect suppressor cell activity in PAB-heterologous protein carrier-primed cells is reminiscent of the previous observation (1) that helper T-cell activity could not be raised in mice by immunization with hapten-heterologous protein conjugates.

(b) SUPPRESSOR CELL ACTIVITY IN B-CELL-DEPLETED PAB-MGG-PRIMED SPLEEN CELLS. To further exclude the possibility that B lymphocytes in PAB-MGG-primed spleen cells are responsible for this suppressor cell activity, the PAB-MGG-primed spleen cells from mice immunized 3 wk earlier were depleted of B cells. The efficiency of B-cell depletion in the PAB-MGG-primed spleen cells was determined by their responsiveness to stimulation with 200  $\mu\text{g}$  of DNP-dextran (thymus-independent antigen), after transfer into X-irradiated recipients (without mixing with DNP-KLH-primed cells). The anti-DNP direct PFC responses of B lymphocytes in NGS-treated spleen cells were  $15,373 \pm 1.17$  in the recipient spleens 7 days after DNP-dextran stimulation, whereas those of anti-Th-B-treated spleen cells were  $1,715 \pm 1.41$ , indicating that almost all B-lymphocyte activities were successfully eliminated by this treatment (around 90% reduction). This almost complete reduction in B-cell activity of anti-Th-B-treated PAB-MGG-primed spleen cells contrasts sharply with the intact helper and suppressor cell activities shown below.

The B-depleted and NGS-treated spleen cells were then mixed with DNP-KLH-primed cells and transferred into two groups of X-irradiated (550 R) recipient mice. One group of recipients was stimulated with DNP-MGG-PAB to detect helper cell activity, and the other with DNP-KLH-PAB to measure suppressor cell activity. As a control DNP-KLH-primed cells together with unprimed spleen cells were transferred into another two groups of recipients. The anti-DNP PFC responses in the recipients 7 days after the cell transfer are depicted in Fig. 2.

The PAB-MGG-primed cells treated with NGS plus C elicited, as expected, ample helper and suppressor cell activities after challenge with DNP-MGG-PAB and DNP-KLH-PAB, respectively, as compared with unprimed cells. However, PAB-MGG-primed spleen cells treated with anti-Th-B antiserum plus C exhibited higher helper and suppressor cell activities than the NGS-treated PAB-

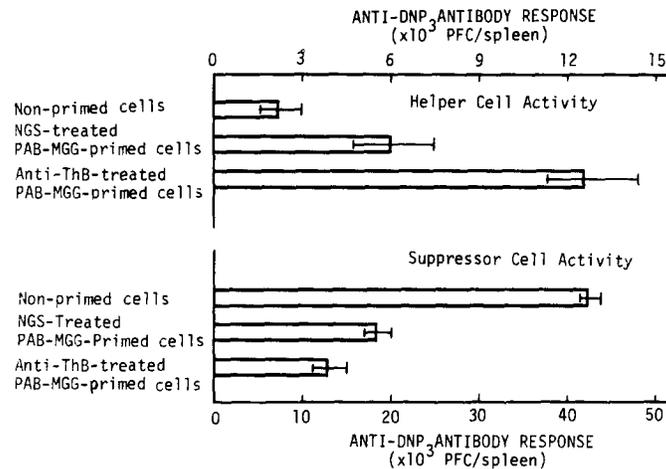


FIG. 2. PAB-reactive helper and suppressor cell activities in B-cell-depleted PAB-MGG-primed spleen cells. The experimental protocol is essentially the same as in Fig. 1, except the PAB-MGG-primed spleen cells were treated *in vitro* before the cell transfer with goat anti-Th-B antiserum plus rabbit C for depleting the B lymphocytes, or with NGS plus C as a control. The functionally-active B lymphocytes were more than 90% depleted by this treatment, as determined by the B-cell responses of respective spleen cells to stimulation with DNP-dextran.

MGG-primed spleen cells. These results clearly indicate therefore, that suppressor cell activity is not mediated by the B lymphocytes present in PAB-MGG-primed spleen cell populations.

(c) SUSCEPTIBILITY OF SUPPRESSOR CELL ACTIVITY IN PAB-MGG-PRIMED CELLS TO TREATMENT WITH ANTI-THY-1.2 ANTISERUM PLUS C. To assess the T-cell nature of suppressor cell activity in PAB-MGG-primed cells, the spleen cells from mice immunized with PAB-MGG 3 wk previously were treated with either normal mouse serum (NMS) plus C or anti-Thy-1.2 antiserum plus C. Helper and suppressor cell activities were measured after double cell transfer into X-irradiated recipients and challenge with DNP-MGG-PAB or DNP-KLH-PAB.

PAB-MGG-primed cells treated with NMS plus C consistently elicited high levels of helper and suppressor activities after antigenic challenges with DNP-MGG-PAB and DNP-KLH-PAB, respectively, compared with unprimed cells (Fig. 3). In contrast, both helper and suppressor cell activities were almost completely abolished by treatment with anti-Thy-1.2 antiserum plus C. The specificity of this anti-Thy-1.2 antiserum treatment was verified by the B-cell responses of such treated spleen cells to DNP-dextran after transfer into another group of X-irradiated recipient mice. 7 days after cell transfer, anti-DNP PFC responses to DNP-dextran were almost intact ( $19,824 \pm 1.25$ ), and comparable to those of NMS-treated spleen cells ( $24,093 \pm 1.28$ ). Thus, helper and suppressor cell activities developed by the immunization with PAB-MGG were both clearly mediated by Thy-1-positive T lymphocytes.

*Properties of Suppressor T Cells in PAB-MGG-Primed Cells.* To exclude the possibility that T cells exhibiting suppressor activity simply eliminated the effective antigenic stimulus by reacting with PAB on the KLH molecule, these T

## HAPTEN-REACTIVE SUPPRESSOR T-CELL ACTIVITY

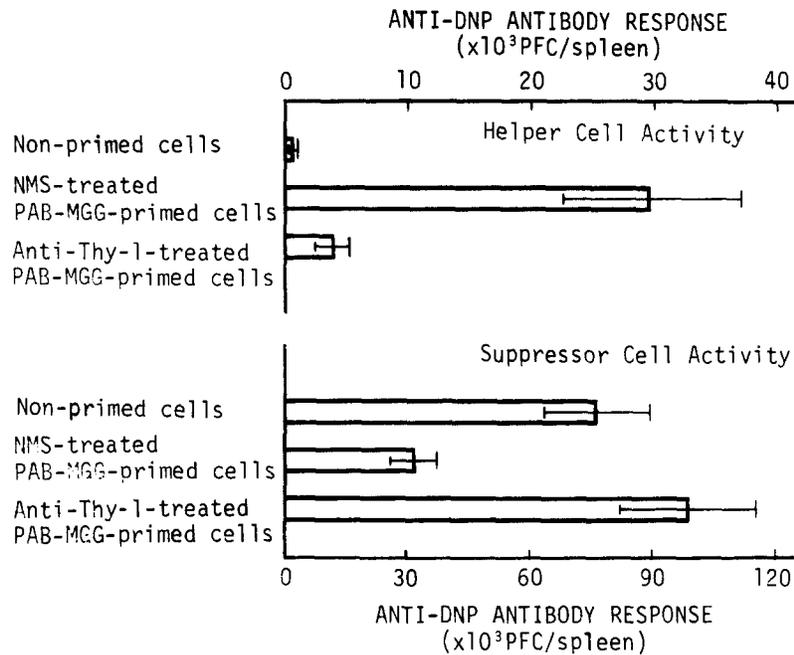


FIG. 3. Susceptibility of PAB-reactive helper and suppressor cell activity developed by PAB-MGG-immunization to treatment with anti-Thy-1 antiserum plus C. The experimental protocol is essentially the same as Fig. 1, except the PAB-MGG-primed spleen cells were treated *in vitro* with NMS plus guinea pig C or anti-Thy-1 antiserum plus C before cell transfer. The B-cell activities of anti-Thy-1-treated spleen cells were almost comparable to those of NMS-treated spleen cells as determined by the responses to DNP-dextran (see text).

cells were characterized and compared with helper T cells with respect to: (a) time of appearance after PAB-MGG-immunization in donors, (b) sensitivity to X-irradiation, and (c) organ distribution.

(a) **TIME OF APPEARANCE.** In the first experiment, four groups of donor mice were immunized *i.p.* with 100  $\mu$ g of PAB-MGG in CFA. 4, 10, 16, and 20 wk later, these animals' spleen cells together with DNP-KLH-primed cells were transferred into four groups of 600 R X-irradiated recipients. Half of each group was stimulated with DNP-MGG-PAB for measuring helper T-cell activity and the other half with DNP-KLH-PAB for measuring suppressor T-cell activity. As a control, another two groups of recipients received DNP-KLH-primed cells and unprimed normal spleen cells and were stimulated with the above antigens.

As evident from the upper panel of Fig. 4, the peak helper T-cell activity was attained at about 10 wk after priming and maintained thereafter. Yet, suppressor T-cell activity developed notably earlier as shown in the lower panel of Fig. 4. Significant suppressor T-cell activity was detected 4 wk after PAB-MGG-priming, and gradually waned thereafter. In another experiment, potent suppressor T-cell activity was detected only 7 days after immunization, and also at 2 and 3 wk. (The data were combined and appear in Fig. 4). These results indicate that the kinetics of suppressor T-cell activity and of helper T-cell activity differ; the suppressor activity is generated and detectable earlier. Although the grad-

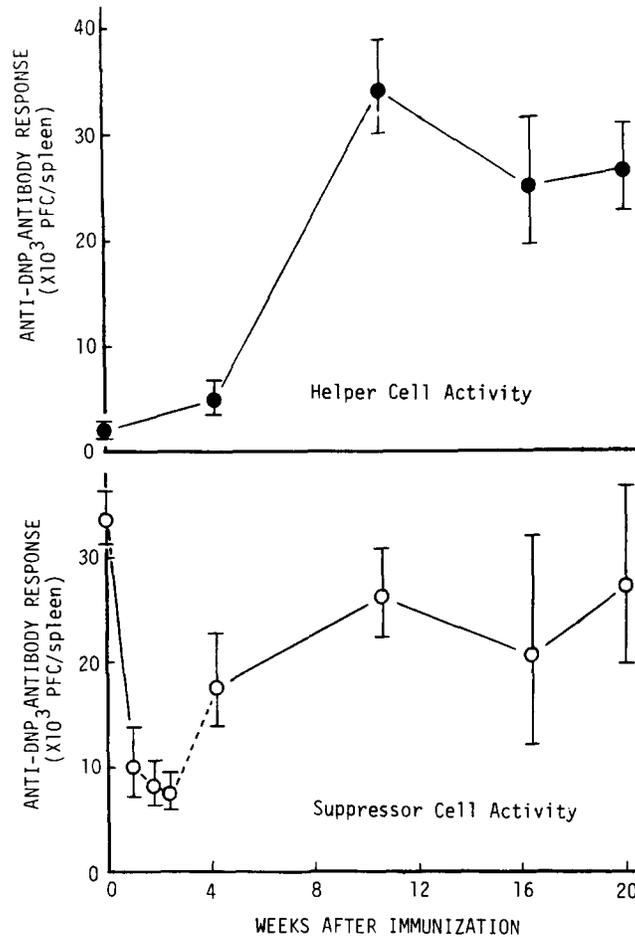


FIG. 4. Earlier appearance of PAB-reactive suppressor T-cell activity than helper T-cell activity after PAB-MGG-immunization. Helper T-cell activities developed in the spleens various times after priming are illustrated in the upper panel, and suppressor T-cell activities in the lower panel. The suppressor T-cell activities of two separate experiments were combined and depicted. The helper and suppressor activities on 4, 10, 16, and 20 wk after priming were measured in one experiment by utilizing DNP-KLH-primed cells from mice primarily immunized 12 wk previously, and the suppressor activities on 1, 2, and 3 wk after priming were measured by utilizing DNP-KLH-primed cells from 9 wk-primed mice in another experiment. The values were normalized to the former experiment by calculating a ratio of suppression to control.

ual waning of suppressor T-cell activity 4 wk after PAB-MGG-immunization may result from masking or interference from the potent helper T-lymphocyte activity in this experimental condition, their distinctly different times of peak activity strongly suggest that the suppression of anti-DNP antibody response of DNP-KLH-primed cells to DNP-KLH-PAB in the presence of PAB-MGG-primed cells is not a simple elimination of effective antigenic stimulus from the system by the reaction of helper T lymphocytes with DNP-KLH-PAB.

(b) **RADIOSENSITIVITY.** Briefly, the experimental protocol for comparing re-

sponses to X-irradiation (9) by suppressor and helper T cells involved removing spleen cells from mice primed 4 wk earlier with PAB-MGG and injecting these cells i.v. into two groups of syngeneic recipients. The first group was X-irradiated (600 R) immediately before and the second group 24 h after receiving PAB-MGG-primed cells. In the latter group, therefore, PAB-MGG-primed cells were exposed to X-irradiation *in vivo* but presumably well after migrating to the lymphoid organs. All recipients were then injected i.v. with DNP-KLH-primed cells 24 h after the first PAB-MGG-primed cell-transfer. Control groups consisted of mice that received DNP-KLH-primed cells but had been injected with unprimed normal cells in the place of PAB-MGG-primed cells. Half of each group was then given a second challenge with DNP-MGG-PAB or DNP-KLH-PAB immediately after transfer of the DNP-KLH-primed cells. The results are summarized in Table II.

Mice in the control group that did not receive PAB-MGG-primed cells failed to respond to DNP-MGG-PAB (Groups I and III). In contrast, mice that were injected with PAB-MGG-primed helper cells developed highly significant secondary anti-DNP antibody responses to DNP-MGG-PAB regardless of exposure to *in vivo* X-irradiation in the adoptive host (Groups II and IV). In contrast, the suppressor T-cell activity detected in nonirradiated PAB-MGG-primed cells (Group VI) was virtually abrogated by *in vivo* X-irradiation in the adoptive host (Group VIII).

We therefore conclude that helper T-cell activity is functionally radioresistant, whereas the suppressor T-cell activity is clearly abrogated by exposure of cells *in vivo* to X-irradiation and consequently that suppressor and helper cell functions are mediated by different T-cell populations.

(c) ORGAN DISTRIBUTION. Mice were immunized i.p. with 100  $\mu\text{g}$  of PAB-MGG in CFA. 3 wk later, spleen and mesenteric lymph node cells were removed and transferred ( $10 \times 10^6$ ) together with  $50 \times 10^6$  DNP-KLH-primed spleen cells into X-irradiated (550 R) mice, which were then stimulated with either DNP-MGG-PAB or DNP-KLH-PAB. In control groups, the DNP-KLH-primed cells were stimulated with these antigens without transferring with PAB-MGG-primed cell population. The anti-DNP antibody responses were measured 7 days after cell transfer and antigenic stimulation.

In the results shown in Table III, two points are worthy of special note: (a) the helper T-cell activity in the mesenteric lymph nodes was more predominant than in the spleen cells, and highly significant helper T-cell activity was detectable even by relatively fewer ( $10^7$ ) PAB-MGG-primed lymphoid cells. In contrast, (b) the suppressor T-cell activity was only detectable in the spleen cells. In other experiments not shown, the suppressive activities of thymocytes from PAB-MGG-primed mice were measured at various times after the PAB-MGG-immunization; however, inconsistent with the observation of others (10), no significant suppressor activity was detected. Nevertheless, the different organ distribution of the helper and suppressor T-cell activities further supports the likelihood that the helper and suppressor cell activities in the PAB-MGG-primed mice are mediated by two distinct cell subpopulations.

*Inhibition of Helper T-Cell Activity by Suppressor T Cells.* The site at which suppressor T cells act in anti-hapten antibody responses was next determined by

TABLE II  
Radiosensitivity of PAB-Reactive Suppressor T Cells after Transfer\*

Activities detected	Exp group	Hapten-reactive T cells	2nd Ag	Anti-DNP antibody response PFC/spleen
Helper	I	Unprimed cell (0R)	DNP-MGG-PAB	533 (1.34)
	II	PAB-MGG-primed cells (0R)		27,458 (1.33)
	III	Unprimed cells (600 R)	DNP-MGG-PAB	1,314 (1.18)
	IV	PAB-MGG-primed cells (600 R)		10,076 (1.02)
Suppressor	V	Unprimed cells (0R)	DNP-KLH-PAB	35,051 (1.21)
	VI	PAB-MGG-primed cells (0R)		7,275 (1.32)
	VII	Unprimed cells (600 R)	DNP-KLH-PAB	49,916 (1.34)
	VIII	PAB-MGG-primed cells (600 R)		76,471 (1.27)

\*  $50 \times 10^6$  spleen cells from donor mice which had been primed with PAB-MGG 4 wk earlier were injected i.v. into four groups of recipients. Recipient mice in groups II and VI were irradiated (600 R) immediately before the cell transfer, while the groups IV and VIII were irradiated 24 h after the cell transfer. Control groups in I and V, or III and VII were given unprimed spleen cells in the place of PAB-MGG-primed cells, and received the same treatment as above. All the recipients were then injected i.v. with  $50 \times 10^6$  DNP-KLH-primed spleen cells, and stimulated with a second antigen as indicated. DNP-KLH-primed spleen cells came from mice primed 10 wk previously.

TABLE III  
Organ Distribution of PAB-Reactive Helper and Suppressor T-Cell Activities\*

Activities detected	PAB-reactive T cells	2nd Ag	Anti-DNP antibody response PFC/spleen
Helper	None	DNP-MGG-PAB	370 (1.30)
	Spleen cells		1,710 (1.13)
	Lymph node cells		7,350 (1.23)
Suppressor	None	DNP-KLH-PAB	20,760 (1.23)
	Spleen cells		7,910 (1.35)
	Lymph node cells		48,560 (1.32)

\* Adjusted numbers ( $10 \times 10^6$ ) of spleen cells or mesenteric lymph node cells from mice primed with PAB-MGG 3 wk earlier were transferred into 550 R X-irradiated recipients together with  $50 \times 10^6$  DNP-KLH-primed spleen cells, and then stimulated with secondary antigens as indicated. DNP-KLH-primed cells came from mice primed 8 wk previously.

using the rationale schematically depicted in Fig. 5. When DNP-KLH-primed cells are stimulated with DNP-KLH-PAB in the presence of suppressor T cells, the anti-DNP antibody response of DNP-specific B cells is consistently suppressed. If one assumes that the effects of suppressor T cells reflects a direct action on DNP-specific cells mediated through the DNP-KLH-PAB molecule, then the addition of BPO-specific B cells, which are concomitantly stimulated with BPO-KLH, should not suppress the anti-BPO response, since the BPO-B

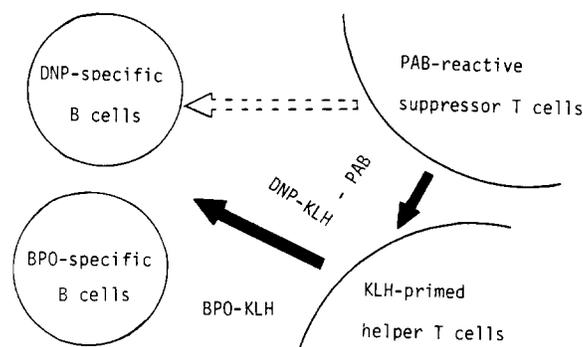


FIG. 5. KLH-primed helper T cells as a target of PAB-reactive suppressor T-cell function. Effect of PAB-reactive suppressor T cells on DNP-specific B cells: broken arrow — mediated by DNP-KLH-PAB, solid arrow — inhibition of KLH primed helper function by PAB-reactive suppressor cells.

TABLE IV  
*Inhibition by PAB-Reactive Suppressor T Cells on KLH-Reactive Helper T-Cell Activity in the Induction of Secondary Responses\**

Exp group	Responding cells	Hapten-reactive T cells	2nd Ag	Antibody response PFC/spleen	
				Anti-DNP	Anti-BPO
I	DNP-KLH-primed cells	Unprimed cells	DNP-KLH-PAB	132,310 (1.41)	1,560 (1.12)
II		PAB-MGG-primed cells		71,010 (1.28)	ND‡
III	+	Unprimed cells	DNP-KLH-PAB + BPO-KLH	100,790 (1.08)	33,710 (1.21)
IV		PAB-MGG-primed cells		33,260 (1.34)	12,190 (1.10)
V	BPO-OVA-primed cells	Unprimed cells	BPO-KLH	960 (1.0)	26,600 (1.15)
VI		PAB-MGG-primed cells		ND‡	34,830 (1.61)

\*  $50 \times 10^6$  spleen cells from either unprimed normal mice or mice primed with PAB-MGG 3 wk earlier were transferred i.v. into 600 R X-irradiated recipients together with the mixture of each  $50 \times 10^6$  of DNP-KLH-primed cells and BPO-OVA-primed cells. The DNP-KLH-primed cells and BPO-OVA-primed cells represent the spleen cells from mice which had been primed with respective antigens in CFA 12 and 10 wk earlier, respectively. The secondary antigenic challenges as indicated were given i.p. into the recipients immediately after the cell transfer.

‡ Not done.

cells would only be innocent bystanders. However, if the responses of BPO-specific B cells are suppressed, one can conclude that suppressor T cells acted by inhibiting KLH-primed helper T-cell functions. The latter occurred as clearly shown in Table IV.

Six groups of 600 R X-irradiated recipients were given a mixture of hapten-specific B cells obtained, first, from donors primed with DNP-KLH and, second, from donors primed with BPO-OVA. As seen in Groups II and IV, after stimulation with DNP-KLH-PAB and transfer of PAB-MGG-primed cells the anti-DNP antibody response of DNP-specific B cells was suppressed. The crucial observation is the comparison between anti-BPO antibody responses of Groups III and IV when the secondary challenge consisted of a mixture of double conjugate of DNP-KLH-PAB and single conjugate of BPO-KLH. In group IV, suppressor T cells produced almost 70% lower anti-BPO responses to BPO-KLH than in Group III, when such T cells were stimulated by simultaneous exposure to DNP-

KLH-PAB. This contrasts with the results shown by Groups V and VI, in which anti-BPO responses were not significantly different after challenge with BPO-KLH alone, whether suppressor T cells had been transferred or not.

Thus, in Group IV, PAB-reactive suppressor T cells, stimulated by the relevant PAB-hapten, could have in turn inhibited the KLH-reactive helper T cells in their capacity of helping the BPO-specific B cells as well as DNP-specific B cells.

### Discussion

The studies described herein clearly demonstrated that suppressor T-cell activity is generated in mice immunized with PAB conjugated with an isologous protein, MGG. Several investigators have reported that suppressor T cells can be induced by antigens (11, 12-18), by mitogens (19, 20), by anti-allotype antibody (21), or by anti-idiotypic antibody (22). The suppressor T-cell effects so far reported are antigen-specific (10-16) or nonspecific (17, 18). The existence of suppressor T cells has been thus well-documented in antibody responses as well as in cell-mediated immunity (23-27) including tumor-specific immunity (25-27). Nevertheless, the major biological and physiological roles of these suppressor T cells and their mechanisms of immunosuppression are not fully understood, although good evidence exists that they may be regulators of the immune response. One of the difficulties in analyzing the physiological role of suppressor T cells lies in the fact that antigen-specific suppressor T-cell activities can be detected only with very sophisticated experimental systems or immunizing protocols that magnify their activities, which may otherwise be masked by the predominant helper T-cell activities in the antigen-primed T-cell population.

We have now devised a system to detect consistently the hapten-reactive suppressor T-cell activity in spleen cells from mice immunized with hapten-isologous protein conjugates. We previously described these cells' content of hapten-reactive helper T lymphocytes (1). The salient feature of the present system is that both helper and suppressor T-cell populations from single donor pool (Fig. 1) were raised against a simple hapten, and their activities were detectable simultaneously. Both subtypes of cells are (*a*) hapten-reactive, (*b*) present in B-cell-depleted cell populations (Fig. 2), and (*c*) sensitive to treatment with anti-Thy-1 antiserum plus C (Fig. 3). In addition, the suppressor T-cell activity compared with helper T-cell activity (*d*) develops earlier after priming (Fig. 4), (*e*) is more radiosensitive (Table II), and (*f*) is distributed in separate lymphoid organs (Table III). Namely, suppressor T-cell activity was found in spleen populations more than those of mesenteric lymph nodes after immunization i.p. with CFA. In sharp contrast, helper T-cell activity was found in both lymphoid organs and was usually stronger in mesenteric lymph nodes than in spleens. Thus, these suppressor T cells are undoubtedly distinct from the helper T cells and have properties consistent with the properties of suppressor T cells reported by others (28).

There has already been a considerable body of evidence that helper and suppressor T lymphocytes are mutually distinct T-cell populations. One can discriminate and obtain one of the T-cell subsets by using Ly-antigen markers; the helper T cells having Ly-1 antigen and the suppressor T cells, Ly-2, 3-

antigen (29-31). However, in at least one system, suppressor T cells expressing the Ly-1<sup>+</sup> phenotype have been recently described (32).

In the present study, we demonstrated kinetically that the hapten-reactive suppressor T cells belong to a distinct T-cell subset from helper T cells. Highly enriched hapten-reactive suppressor T-cell activity was raised in mice in a relatively short time (1-3 wk) after priming (Fig. 4), and a functionally more enriched population of helper T-cell activity was obtained relatively later after priming, even after X-ray inactivation of suppressor T-cell activity *in vivo* (Table II). The use of a selected population of hapten-reactive suppressor or helper T-lymphocyte activity may be thus especially relevant to the establishment of a model system for manipulation of various immune responses in appropriate directions, i.e. suppression or enhancement, to any carrier on which haptens can be coupled. Moreover, as will be described in detail in the accompanying publication (33), the hapten-reactive suppressor T cells and their precursors are highly susceptible to inactivation after treatment with hapten-D-glutamic acid and D-lysine (D-GL) conjugates. This difference in susceptibility between helper and suppressor T lymphocytes is further applicable to a system in which one wishes to arbitrarily modify one of these T-cell functions.

The present observation that the suppressor T cells easily express their functions by reacting with the hapten on a heterologous carrier such as DNP-KLH, and the fact that the suppressor T cells are highly susceptible to inactivation by treatment with hapten coupled on completely heterologous nonimmunogenic carrier, D-GL (33), both suggest that receptor-specificity of the suppressor T lymphocytes may be more hapten-specific than that of helper T lymphocytes. This notion seems to be further supported by the fact that, in general, hapten-reactive helper T-cell activity is hardly detectable after challenges with the hapten coupled on a heterologous carrier other than the primed isologous MGG carrier, in sharp contrast with suppressor T cells. However, only the direct characterization of receptor molecules on both cell types will prove their specificities. Since the receptors on both cell types might bind to hapten coupled to an immunoadsorbent and their receptors may then remain on the immunoadsorbent after incubation (34), one could use the hapten-reactive T-cell system described above to isolate and characterize the respective T-cell receptors. Moreover, one can arbitrarily vary the specificities of the haptens by using structurally-related compounds then, by measuring the responsiveness of a hapten-reactive T-cell population, the specificities of the functional T-cell receptors can be also analyzed. The experimental system described herein, therefore, provides a means of characterizing T-cell receptors and, thereby, the regulatory function of suppressor T cells.

In our model system, hapten-reactivity was used to locate the site where T cells act in T-B cell interactions. PAB-reactive suppressor T cells inhibited the helper function of protein-carrier (KLH)-reactive helper T cells in their capacity to induce anti-DNP antibody response of DNP-specific B cells after stimulation with DNP-KLH-PAB (Table IV). This result is discordant with the observations by Basten et al. (35), who claimed that their HGG-reactive suppressor cells taken from HGG-tolerant mice acted directly on B lymphocytes. This was based on the following two lines of evidence: in their system, the HGG-reactive

suppressor cells substantially decreased the anti-DNP antibody response of DNP-flagellin-primed B cells mixed with KLH- and HGG-reactive helper T cells after stimulation with DNP-KLH plus DNP-HGG. Probably more definitively in their system, the anti-DNP antibody response of DNP-specific B cells induced by DNP-Ficoll (thymus-independent antigen) was similarly suppressed by concomitant stimulation with DNP-HGG in the presence of HGG-reactive suppressor cells.

In our hapten-reactive suppressor T-cell system, however, the direct action of suppressor T lymphocytes on B cells does not appear to be a major pathway for the effective suppressor T-cell functions. Thus, even when the mixture of DNP-KLH-primed cells and PAB-reactive suppressor T-cell population was concomitantly stimulated with DNP-KLH and DNP-fowl gamma globulin (FGG)-PAB, i.e. the DNP- and PAB-haptens being coupled on an inert carrier in the system, and PAB-reactive suppressor T-cell action allowing to act directly on the DNP-B lymphocytes through DNP-FGG-PAB, no significant PAB-reactive suppressor T-cell activity was detected (unpublished observation). This sharply contrasts with the substantial decrease of bystander B-cell responses such as shown in Table IV, in which the PAB-reactive suppressor T cells effectively suppressed the anti-BPO-B-cell response when the mixture of DNP-KLH-primed cells and BPO-OVA-primed cells was concomitantly stimulated with DNP-KLH-PAB and BPO-KLH. Thus, from these results, we concluded that the mechanism of suppressor T-cell function is to suppress the helper T-cell activity.

Since hapten-reactive T lymphocytes are capable of reacting with the haptens coupled on any carrier molecule, one can easily analyze regulation by cells in the response to any carrier antigenic determinant. Therefore, this system may provide an informative model for the analysis of regulatory T-cell effects on the induction mechanism of B lymphocytes of different immunoglobulin classes as well as on T-T cell interactions in cell-mediated immunity to the corresponding carriers.

### Summary

Helper and suppressor T-cell activities were detected simultaneously in the spleen cells of mice immunized with para-azobenzoate (PAB)-mouse gamma globulin (MGG). Dinitrophenyl (DNP)-specific B cells were raised by immunization with DNP-keyhole limpet hemocyanin (KLH) and used as the indicator B-cell population. The helper and suppressor T-cell activities were determined after adoptively transferring spleen cells from PAB-MGG-primed donors and DNP-KLH-primed donors into X-irradiated recipients. Stimulation of these recipients with DNP-MGG-PAB detected helper T-cell activity, which was measured in terms of increased anti-DNP antibody responses of DNP-KLH-primed cells over these responses in the presence of unprimed cells. On the other hand, when DNP-KLH-primed cells were stimulated with DNP-KLH-PAB in the presence of PAB-MGG-primed cells, anti-DNP antibody responses were substantially lower than in unprimed normal cells.

This suppressor cell population was (a) hapten-reactive, (b) present in B-cell-depleted spleen cells, (c) Thy-1 positive, (d) detectable earlier than the helper T-cell activities after priming (e) more radiosensitive than helper cells, and (f)

found in the spleen but not the lymph nodes in contrast to helper T cells. These data indicate that these suppressor T cells are distinct from the helper T cells. PAB-reactive T cells clearly suppressed the antibody response by inhibiting KLH-reactive helper T-cell functions. The hapten-reactive T-lymphocyte system described here should be useful for analyzing and manipulating the immune response and for studying regulatory interactions of helper and suppressor T cells in the induction of antibody responses.

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