REGULATORY FUNCTIONS OF HAPTEN-REACTIVE HELPER AND SUPPRESSOR T LYMPHOCYTES

II. Selective Inactivation of Hapten-Reactive Suppressor T Cells by Hapten-Nonimmunogenic Copolymers of d-Amino Acids, and its Application to the Study of Suppressor T-Cell Effect on Helper T-Cell Development*

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The existence of T lymphocytes which suppress antibody responses has been well documented in a variety of systems (1-5). In the preceding study (6), a system was established to detect simultaneously both helper and suppressor T-cell activities with apparent specificity for haptenic determinant in hapten-isologous protein-primed spleen cell population. This was accomplished by immunizing mice with para-azobenzoate (PAB)1-mouse gamma globulin (MGG)-conjugate emulsified in complete Freund's adjuvant (CFA), then reacting their spleen cells with dinitrophenyl (DNP)-specific B cells from DNP-keyhole limpet hemocyanin (KLH)-primed donors in an adoptive transfer system. The stimulation with DNP-MGG-PAB detected PAB-reactive helper T-cell activity, and this was measured by the increment of anti-DNP antibody responses of DNP-specific B cells in comparison with their responses in the presence of nonprimed normal cells. The PAB-reactive suppressor T-cell activity was detected in the above system by stimulation with DNP-KLH-PAB, and the activities were measured quantitatively by the reduction in anti-DNP antibody responses of DNP-specific B cells as compared with their responses in the presence of nonprimed normal cells. The T-cell nature of this hapten-reactive suppressor cell activity was established by its susceptibility to anti-Thy-1 serum

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Abbreviations used in this paper: B cells, precursors of antibody-forming cells; BPO, benzylpenicillloyl; CFA, complete Freund's adjuvant; d-GL, copolymer of d-glutamic acid and d-lysine; DNP, 2,4-dinitrophenyl; GAT, a linear terpolymer of glutamic acid, alanine, and tyrosine; KLH, keyhole limpet hemocyanin; MAB, meta-azobenzoate; MABA, meta-aminobenzoic acid; MGG, isologous mouse gamma globulin; OAB, ortho-azobenzoate; OABA, ortho-aminobenzoic acid; PAB, para-azobenzoate; PABA, para-aminobenzoic acid; T cells, thymus-derived lymphocytes.
plus complement-treatment and higher activities in a B-cell-depleted spleen cell population after anti-Th-B antiserum plus complement treatment. The hapten-reactive suppressor T cells were distinct from the helper T lymphocytes. This conclusion derives from the different properties of the two cell types with regard to: (a) radiation sensitivity, (b) kinetics in developing after priming, and (c) distribution in lymphoid organs. By taking advantage of the hapten-reactive properties of suppressor T lymphocytes, their site of action was investigated in the T-B cell interaction of anti-hapten antibody response. The results suggested that the mechanism of PAB-reactive suppressor T cells might involve the removal or inactivation of helper T-cell functions rather than a direct attack on B cells committed to production of antibody.

The studies presented here extend this hypothesis and further analyze the suppressor T-cell effects on the development of helper T cells in the primary immune response. If we can selectively eliminate hapten-reactive suppressor T-cell population from the system while leaving helper T-cell activity unharmed in vivo, this system may allow us to determine the suppressor T-cell effects on the development of helper T-cell activity. Firstly, we attempted to selectively inactivate the hapten-reactive suppressor T-cell activity by pretreatment with hapten-conjugates of the nonimmunogenic copolymer of d-glutamic acid and d-lysine (D-GL). Then the effect of selective inhibition of suppressor T-cell development on the generation of helper T-cell population was analyzed with respect to hapten-specificity of helper T-cell activity, by assessing their reactivities towards MGG-conjugates of structurally related haptens and by determining their susceptibility to tolerogenesis induced by the hapten-D-GL.

The results show that PAB-reactive helper T-cell activity substantially increased when the suppressor T-cell generation was selectively inhibited by pretreatment with PAB-D-GL, and that the relative cross-reactivity of resulting helper T-cell activity to structurally related determinants, such as meta-azobenzoate (MAB) decreased. Moreover, the PAB-reactive helper T cells generated in the absence of suppressor T lymphocytes were easily made tolerant by PAB-D-GL, in sharp contrast with the helper T cells developed in the presence of suppressor T cells.

Materials and Methods

The proteins, hapten-carrier conjugates, animals, immunization schedules, adoptive transfer system, antibody determinations, and statistical analyses were identical to those described in the preceding paper (6). The following additional hapten-carrier conjugates were employed:

Chemical Reagents and Preparation of Hapten-Carrier Conjugates. 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. PAB2e-D-GL was prepared by reacting a 200-fold excess in molar ratio of diazotized para-aminobenzoic acid (PABA) with D-GL at pH 9.0. Subscript refers to the average number of moles of PAB per mole of D-GL, calculated from the absorption reading at 460 nm. In this calculation, the molar extinction coefficient of PAB-lysine was tentatively assumed to be 1,650 at 460 nm based on that of bis (para-azobenzene-arsonic acid diazo)-α-amino caproic acid. PAB2e-MGG-DNP6, MAB6-MGG-DNP6, and OAB6-MGG-DNP6 were prepared in a similar manner by reacting a 50-fold excess, in molar ratio, of diazotized PABA and meta-aminobenzoic acid (MABA), or a 250-fold excess of ortho-aminobenzoic acid (OABA) with DNP6-MGG. The average number of moles of PAB, MAB, or OAB per mole of DNP-MGG was calculated from the absorption readings at 460 and 500 nm in alkaline solution, assuming azobenzoate compounds coupled to histidine and tyrosine residues of the DNP-MGG molecule.
Benzylpenicilloyl (BPO)-p-GL conjugate was prepared by reacting 300 mg of benzylpenicillin with 50 mg p-GL in 5 ml of 0.2 M carbonate-bicarbonate buffer at pH 9.0. 40 molecules of BPO were calculated to bind covalently with 1 molecule of p-GL as measured by the penamaldate method. BPO-MGG and BPO-KLH were prepared in the same manner by reacting 300 mg benzylpenicillin with 300 mg MGG or KLH in 20 ml borate-buffered saline at pH 9.0 adjusted with 1 N NaOH. BPO-MGG-PAB, or BPO-KLH-DNP, were prepared from BPO-MGG and BPO-KLH by further reacting each conjugate with diazotized PABA as described above, or with sodium 2,4-dinitrobenzene sulfonate as described previously (6).

Results

Selective Inactivation of PAB-Reactive Suppressor T-Cell Activity by PAB-p-GL Treatment. Previously, it was demonstrated that treatment with PAB-p-GL induces a profound state of unresponsiveness in PAB-reactive helper T cells in mice immunized with PAB-MGG 3 mo earlier, whereas the PAB-reactive helper T cells from mice immunized only 3 or 4 wk earlier were rather difficult to render tolerant with PAB-p-GL (7, 8). PAB-reactive helper and suppressor T cells were detected simultaneously by comparing the inactivation of each cell type by the PAB-p-GL treatment after relatively long- or short-term-priming. PAB-reactive T-cell populations were treated with PAB-p-GL for varying periods of time in the donor mice before being assayed with the adoptive cell transfer technique.

In the first experiment, PAB-reactive T-cell populations from PAB-MGG-primed mice which had been immunized 12 wk previously and boosted with PAB-MGG were employed. It was already established that boosting the PAB-MGG-primed mice long after the PAB-MGG-priming was effective for reinducing the suppressor T-cell activity, even when the suppressor T-cell activity had already waned in contrast to the increase in helper T-cell activity. To test the susceptibility difference in inactivation of PAB-reactive helper and suppressor T cells to the PAB-p-GL treatment, the PAB-MGG-immunized donor mice were divided into three groups 14 days after boosting, and the spleen cells from each respective donor group were transferred into 2 groups of 600 R X-irradiated recipients together with DNP-KLH-primed cells. Each of these two groups was given 250 μg of PAB-p-GL 1 (groups III and VII) or 4 (groups IV and VIII) days before cell transfer. The third group remained untreated as a control (groups II and VI). The PAB-reactive helper and suppressor T-cell activities remaining after such treatment were measured by anti-DNP antibody responses of DNP-KLH-primed cells in the recipients 7 days after stimulation with DNP-MGG-PAB and DNP-KLH-PAB, respectively. To test the background response of DNP-KLH-primed cells to the DNP-MGG-PAB or DNP-KLH-PAB stimulation, nonprimed normal spleen cells were transferred in the place of PAB-MGG-primed spleen cells (groups I and V).

PAB-reactive helper T cells and suppressor T cells appear as groups I and II, and groups V and VI, respectively, in Table I. The pretreatment of the PAB-reactive T-cell population with PAB-p-GL for period of 4 days in the donor mice before the cell transfer (groups IV and VIII) resulted in the inactivation of both PAB-reactive helper and suppressor T-cell activities. This is evident from the helper T-cell activity of group IV as compared with that of group II untreated cells, and the suppressor T-cell activity of group VIII as compared with that of
Susceptibility Differences in Inactivation of PAB-Reactive Suppressor and Helper T Cells by PAB-d-GL Treatment

| Responding cells | Exp group | PAB-reactive T cells of longer priming period | 2nd Ag (Activity detected) | Anti-DNP antibody response PFC/spleen |
|------------------|-----------|-----------------------------------------------|---------------------------|-------------------------------------|
| DNP-KLH-primed   | I         | Nonprimed cells                               |                           |                                     |
|                  | II        | PAB-primed cells, Non-treated                 |                           |                                     |
|                  | III       | " treated with PAB-d-GL-1 day                 | DNP-MGG-PAB (Helper)      | 1,016 (1.27)*                      |
|                  | IV        | " treated with PAB-d-GL-4 day                 |                           |                                     |
|                  | V         | Nonprimed cells                               |                           | 18,800 (1.01)                      |
|                  | VI        | PAB-primed cell, Nontreated                   |                           |                                     |
|                  | VII       | " treated with PAB-d-GL-1 day                 | DNP-KLH-PAB (Suppressor)  | 2,597 (1.37)                       |
|                  | VIII      | " treated with PAB-d-GL-4 day                 |                           |                                     |

* PAB-MGG-primed mice which had been immunized 12 wk previously and boosted with PAB-MGG 2 wk before were used as PAB-reactive T-cell donors, and these donors' spleen cells containing both helper and suppressor T-cell activities were treated in vivo with 250 μg of PAB-d-GL 1 or 4 days before cell transfer. The PAB-reactive helper and suppressor T-cell activities remaining after such treatment were measured by transferring them into 300 R X-irradiated recipient mice together with DNP-KLH-primed cells taken from another donor animals immunized 11 wk previously at the stimulation with DNP-MGG-PAB and DNP-KLH-PAB, respectively. Geometric means and standard errors of anti-DNP PFC responses in the spleens of four to five recipients 7 days after cell transfer are listed.

untreated cells in group VI. On the other hand, as shown in groups III and VII, the 1-day pretreatment of PAB-d-GL selectively inactivated suppressor T-cell activities as in group VII, whereas the apparent helper T-cell activity was increased as shown in group III.

Thus, PAB-reactive suppressor T cells were more susceptible to inactivation by PAB-d-GL treatment than the helper T cells since they were easily rendered unresponsive by such treatment for a 1-day period, whereas a 4-day period of treatment was required for helper T cells to be inactivated by PAB-d-GL. The inactivation of long-term-primed helper T cells by PAB-d-GL treatment for a 4-day period is indeed consistent with our previous observation (7). The inability of PAB-d-GL to inactivate PAB-reactive helper T cells by a 1-day period of treatment suggests that suppressor and helper T cells inherently differ in their susceptibility to inactivation by this hapten-nonimmunogenic copolymer.

The higher susceptibility of PAB-reactive suppressor T cells was further demonstrated when the PAB-reactive T-cell populations from relatively short-term-primed donor mice were exposed to PAB-d-GL. Summarized in Table II, the PAB-reactive T-cell populations from PAB-MGG-primed donor mice, which had been immunized for 3 wk, were treated with PAB-d-GL for a 4-day period in donor animals. As previously found (8), the PAB-reactive helper T cells in these PAB-MGG-primed mice were not rendered tolerant by treatment with PAB-d-GL even for 4 days; in fact helper T-cell activity was apparently increased by such treatment (cf. groups II and III). Consistent with the results presented in Table I, the PAB-reactive suppressor T-cell activity was again selectively abrogated (cf. groups V and VI). The apparent increase in helper T-cell activity may be due to the selective elimination of suppressor T-cell activity from the PAB-
Selective Inactivation of PAB-Reactive Suppressor T Cells by the Treatment with PAB-p-GL*

| Responding cells | Exp group | PAB-reactive T cells of shorter priming period | 2nd Ag (Activity detected) | Anti-DNP antibody response PFC/spleen |
|------------------|-----------|-----------------------------------------------|---------------------------|-------------------------------------|
| Nonprimed cells  | I         | Nonprimed cells                               | DNP-MGG-PAB (Helper)      | 360 (1.32)                          |
| PAB-primed cells, Nontreated | II       | "treated with PAB-p-GL-4 day"                | DNP-KLH-PAB (Suppressor)  | 19,742 (1.32)                       |
| PAB-primed cells, Nontreated | III      | "treated with PAB-p-GL-4 day"                |                           | 47,018 (1.17)                       |
| PAB-primed cells, Nontreated | IV       | Nonprimed cells                               | DNP-MGG-PAB (Helper)      | 400,550 (1.11)                      |
| PAB-primed cells, Nontreated | V        | "treated with PAB-p-GL-4 day"                | DNP-KLH-PAB (Suppressor)  | 140,280 (1.33)                      |
| PAB-primed cells, Nontreated | VI       | "treated with PAB-p-GL-4 day"                |                           | 393,311 (1.06)                      |

* The experimental protocol is substantially the same as Table I, except PAB-MGG-primed mice which had been immunized 3 wk previously, were employed as PAB-reactive T-cell donors.

MGG-primed cell populations. Thus, these results clearly indicate that the PAB-reactive suppressor T cells are more susceptible to inactivation by PAB-p-GL treatment than the helper T lymphocytes.

Selective Inhibition of Development of PAB-Reactive Suppressor T Cells by Pretreatment with PAB-p-GL before PAB-MGG-Priming. Based on the distinct susceptibility-differences in PAB-reactive helper and suppressor T cells to treatment with PAB-p-GL, the effect of pretreatment with PAB-p-GL before the PAB-MGG-priming on the generation of respective cell types was next investigated. Two types of experiments were conducted.

In the first experiment, shown in Fig. 1, normal mice were pretreated with 250 µg of PAB-p-GL 3 days before PAB-MGG-priming or not pretreated. 3 wk later, PAB-reactive helper T-cell activity was measured by transferring DNP-primed B cells into these animals after 600 R X-irradiation and then stimulating all mice with DNP-MGG-PAB. Two control groups were either pretreated with PAB-p-GL alone or not pretreated and not immunized with PAB-MGG, and submitted to the same experimental conditions as above. The pretreatment of normal animals with PAB-p-GL (solid circle, Fig. 1) clearly augmented the generation of PAB-reactive helper T-cell activity after PAB-MGG-immunization to approximately three times higher values than the control group similarly primed with PAB-MGG but not pretreated with PAB-p-GL (open circle, Fig. 1). In contrast, as shown in the solid squares, pretreatment with PAB-p-GL without PAB-MGG-immunization did not induce PAB-helper activity.

This augmented generation of helper T-cell activity and concomitant inhibition of suppressor T-cell generation was confirmed by the next experiment. As shown in Table III, pretreatment of normal animals with 250 or 500 µg of PAB-p-GL before PAB-MGG-priming completely inhibited the development of PAB-reactive suppressor T-cell activity as seen when one compares group VI with groups VII or VIII. In contrast to the inhibited development of suppressor T-cell activity, an increase in helper T-cell development was again observed in those animals pretreated with a 250-µg dose of PAB-p-GL as shown in group III. However, pretreatment with 500 µg of PAB-p-GL resulted in only marginal augmentation of PAB-reactive helper T-cell activity (group IV). Thus, pretreat-
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Fig. 1. Augmenting effect of PAB-D-GL-pretreatment on the generation of PAB-reactive helper T-cell activity. Normal mice were either pretreated with 250 μg of PAB-D-GL or not treated, and immunized with 100 μg of PAB-MGG in CFA. PAB-reactive helper T-cell activity generated in the spleen of those animals was measured 3 wk after the PAB-MGG-immunization by transferring the DNP-KLH-primed cells into them after 600 R X-irradiation and stimulating with 100 μg of DNP-MGG-PAB. The DNP-KLH-primed cells came from mice which had been immunized 12 wk previously. Geometric means and standard errors of anti-DNP PFC responses in the recipients' spleens 7 days after the cell transfer are depicted. PAB-D-GL-treatment (+), PAB-MGG-priming (+); •--•, PAB-D-GL-treatment (--), PAB-MGG-priming (+); ○--○, PAB-D-GL (+), PAB-MGG-priming (--); ■--■, PAB-D-GL-treatment (--), PAB-MGG-priming (--); □--□.

Table III
Selective Inhibition of Generation of PAB-Reactive Suppressor T-Cell Activity by PAB-D-GL-Pretreatment*

| Responding cells | Exp group | PAB-D-GL pretreatment 3 days before immunization | Immunization on day | 2nd Ag (activity detected) | Anti-DNP antibody response PFC/spleen |
|------------------|-----------|-----------------------------------------------|--------------------|--------------------------|-------------------------------------|
|                  |           | CFA                                           | DNP-MGG-PAB        | 700 (1.41)               |                                     |
|                  | I         | None                                          | PAB-MGG            | 5,786 (1.21)             |                                     |
|                  | II        | None                                          | 500 μg PAB-D-GL    | 16,090 (1.04)            |                                     |
|                  |            |                                               | PAB-MGG            | 8,585 (1.19)             |                                     |
|                  | III       | 250 μg PAB-D-GL                               | DNP-MGG-PAB        | 702,300 (1.02)           |                                     |
|                  | IV        | 500 μg PAB-D-GL                               | DNP-MGG            | 70,600 (1.21)            |                                     |
|                  |           |                                               | (Suppressor)       | 218,900 (1.26)           |                                     |
| DNP-KLH-primed   | V         | None                                          | DNP-MGG-PAB        | 214,920 (1.30)           |                                     |
| cells            | VI        | None                                          | DNP-MGG            | 214,920 (1.30)           |                                     |
|                  | VII       | 250 μg PAB-D-GL                               | DNP-MGG-PAB        | 70,600 (1.21)            |                                     |
|                  | VIII      | 500 μg PAB-D-GL                               | DNP-MGG-PAB        | 218,900 (1.26)           |                                     |

* Normal animals were either pretreated with PAB-D-GL 3 days before PAB-MGG-immunization or not pretreated. The PAB-reactive helper and suppressor T-cell activities generated in these PAB-MGG-primed animals were measured 3 wk after the PAB-MGG-immunization by transferring them into 600 R X-irradiated recipient mice together with DNP-KLH-primed cells and stimulating with 100 μg of DNP-MGG-PAB or DNP-KLH-PAB. The DNP-KLH-primed cells came from another donor mice which had been immunized 9 wk previously.

ment with PAB-D-GL conjugate was capable of inhibiting selectively the generation of PAB-reactive suppressor T-cell activity, and administration of appropriate dose of PAB-D-GL resulted in an apparent increase in PAB-reactive helper T-cell development.
As will be demonstrated in the following section, the PAB-reactive helper T-cell population developed in the absence of suppressor T cells was far more specific for the haptenic determinant than those developed in the presence of suppressor T cells. In other words, by eliminating PAB-reactive suppressor T cells from the system by PAB-d-GL-pretreatment, the hapten-specificity of helper T cells was increased.

**Hapten-Specificity of PAB-Reactive Helper T Cells Generated in the Absence of Suppressor T Cells.** Next, varying doses of PAB-d-GL were administered to normal animals before PAB-MGG-priming to inhibit suppressor T-cell development and the effect of selective elimination of suppressor T cells from the system on the development of helper T cells was analyzed with respect to their hapten-specificity. Responses of PAB-reactive helper T lymphocytes developed in the presence of suppressor T cells was compared with that developed in the absence of suppressor T cells toward meta-azobenzoate (MAB) and ortho-azobenzoate (OAB) determinants, since it was demonstrated previously (8), that hapten-reactive helper T cells distinguish the structural differences of related haptens and thus respond differently to them.

Four groups of mice were immunized with 100 μg of PAB-MGG in CFA. Three of these groups were pretreated with 125, 250, and 500 μg of PAB-d-GL 3 days before PAB-MGG-immunization to inhibit suppressor T-cell development, and the fourth group was not pretreated. 4 wk after immunization the groups were challenged, respectively, with 100 μg of DNP-MGG-PAB, DNP-MGG-MAB, or DNP-MGG-OAB in the presence of DNP-KLH-primed cells. Helper T-cell activities were expressed as anti-DNP antibody responses in recipient spleens 7 days after antigenic challenge, and the results are summarized in Table IV. Comparing groups IV and V, the helper T-cell activity developed 3 wk after PAB-MGG-immunization cross-reacted very strongly with the structurally related hapten, MAB. In contrast, as shown by groups VII, X, and XI, pretreatment with PAB-d-GL significantly augmented the development of the helper T-cell reactivity to PAB determinants, but less strikingly to MAB determinants (groups VIII, XI, and XIV), thus resulting in a reduction of the ratio of MAB-reactivity to PAB-reactivity. In this experiment, the reactivities of PAB-MGG-primed helper T cells to OAB were less than 3% in all cases (groups VI, IX, XII, and XV).

Thus, the inhibition of suppressor T-cell development by PAB-d-GL-pretreatment resulted in augmentation of helper T-cell activities with more restricted specificities to the primed haptenic determinant (PAB), and this may be due to an increase in the number of hapten-reactive T cells specific for the primed hapten.

**Difference in Susceptibility to Tolerance-Induction in PAB-Reactive Helper T Lymphocytes Generated in the Absence of Suppressor T Lymphocytes.** The change in quality of PAB-reactive helper T cell population developed in the absence of suppressor T cells was further substantiated by the differences in susceptibility to tolerance induction by PAB-d-GL administered a second time 4 days before assay in the adoptive cell transfer.

As seen in Table V, PAB-reactive helper T cells were generated in the presence or absence of suppressor T cells. The inhibition of suppressor cell generation was accomplished by pretreatment with PAB-d-GL 3 days before PAB-MGG-priming as described in Table IV. In comparison between group I
TABLE IV
Responses of PAB-Reactive Helper T Cells Generated in the Absence of Suppressor T Cells to Structurally Related Haptens*

| Exp group | Condition for generation of PAB-reactive helper T cells | 2nd Ag | Reactivity* (anti-DNP FFC/spleen) | Reactivity % |
|-----------|---------------------------------------------------------|--------|----------------------------------|------------|
| I         | DNP-MGG-PAB                                             | 398 (1.35) |                                        |            |
| II        | None CFA                                                | 1,309 (1.34) |                                        |            |
| III       | DNP-MGG-OAB                                             | 302 (1.18)  |                                        |            |
| IV        | None PAB-MGG                                            | 5,439 (1.23) | 100.0                                |            |
| V         | None PAB-MGG                                            | 6,097 (1.23) | 112.0                                |            |
| VI        | DNP-MGG-OAB                                             | 162 (1.10)  | 2.9                                  |            |
| VII       | DNP-MGG-PAB                                             | 20,021 (1.43) | 100.0                                |            |
| VIII      | 125 µg PAB-d-GL PAB-MGG                                 | 4,645 (1.11) | 23.2                                  |            |
| IX        | 250 µg PAB-d-GL PAB-MGG                                 | 214 (1.18)  | 1.0                                  |            |
| X         | DNP-MGG-PAB                                             | 30,702 (1.04) | 100.0                                |            |
| XI        | 500 µg PAB-d-GL PAB-MGG                                 | 7,982 (1.22) | 25.9                                  |            |
| XII       | DNP-MGG-OAB                                             | 281 (1.56)  | 0.9                                  |            |
| XIII      | DNP-MGG-PAB                                             | 11,762 (1.07) | 100.0                                |            |
| XIV       | 500 µg PAB-d-GL PAB-MGG                                 | 6,016 (1.27) | 51.1                                  |            |
| XV        | DNP-MGG-OAB                                             | 162 (1.10)  | 1.3                                  |            |

* Experimental protocol is substantially the same as in Table III, except the reactivity of PAB-MGG-primed helper T cells to the above structurally related haptens were measured 4 wk after PAB-MGG-priming at the stimulation with hapten-carrier conjugates as indicated.

and groups III, V, and VII in exp 1, the inhibition of suppressor T-cell generation by pretreatment with PAB-d-GL resulted in substantial increase in development of PAB-reactive helper T cells, as expected, confirming the results obtained in Table IV. Interesting to note was the susceptibility of these helper T-cell populations to the second treatment with PAB-d-GL. Thus, the PAB-reactive helper T cells generated in the presence of suppressor T cells, resisted the tolerance induction by PAB-d-GL treatment administered 4 days before the assay, and helper T-cell activities increased due to inactivation of coexisting PAB-reactive suppressor T-cell activities (groups I and II, exp 1 and 2). In contrast, as evident from the comparisons between groups III and IV, groups V and VI, or groups VII and VIII in exp 1 and between groups III and IV, exp 2, all the helper T-cell activities developed in the absence of suppressor T cells were highly susceptible to the second PAB-d-GL treatment. This sharply contrasted with the increase in apparent helper T-cell activities in group II. These results indicate, therefore, that the hapten-specificity and tolerance susceptibility of hapten-reactive helper T cells was apparently increased by the inhibition of suppressor T-cell generation. By extension, these results may be interpreted as
### Table V
Higher Susceptibility to Tolerance Induction by PAB-d-GL in PAB-Reactive Helper T Cells Generated in the Absence of Suppressor T Cells

| Exp no. | Group  | Condition for generation of PAB-reactive helper T cells | Indicator tolerogen treatment 4 days before cell transfer | Helper-T-cell activity recovered. |
|---------|--------|---------------------------------------------------------|--------------------------------------------------------|---------------------------------|
|         |        | PAB-d-GL pretreatment 3 days before immunization | Immunizations on day 0 | Anti-DNP PFC/spleen |
| I       | II     | None | PAB-MGG | None | 5,439 (1.23) |
|         |        |     |         | 1 mg PAB-d-GL | 16,898 (1.06) |
| III     | IV     | 125 µg PAB-d-GL | PAB-MGG | None | 20,021 (1.43) |
|         |        |     |         | 1 mg PAB-d-GL | 6,439 (1.39) |
| V       | VI     | 250 µg PAB-d-GL | PAB-MGG | None | 30,702 (1.04) |
|         |        |     |         | 1 mg PAB-d-GL | 11,268 (1.13) |
| VII     | VIII   | 500 µg PAB-d-GL | PAB-MGG | None | 11,762 (1.07) |
|         |        |     |         | 1 mg PAB-d-GL | 1,572 (1.33) |
| I       | II     | None | PAB-MGG | None | 6,080 (1.22) |
|         |        |     |         | 1 mg PAB-d-GL | 30,800 (1.28) |
| III     | IV     | 250 µg PAB-d-GL | PAB-MGG | None | 33,300 (1.48) |
|         |        |     |         | 1 mg PAB-d-GL | 1,360 (1.46) |

* This was conducted in the same experimental system as in Table IV. The susceptibilities of PAB-reactive helper T cells developed in various conditions to indicator tolerogen, PAB-d-GL, were tested 4 wk after PAB-MGG-priming, and this was performed by treatment of PAB-MGG-primed mice with 1 mg PAB-d-GL 4 days before the cell transfer.

† The experimental protocol is substantially the same as exp 1, except the helper T-cell assay was performed 6 wk after the PAB-MGG-priming.

indicating that the suppressor T cells may inhibit the maturation of affinity of hapten-reactive helper T-cell activity during the immune response to the hapten-modified self antigens.

Effect of Elimination of BPO-Reactive Suppressor T-Cell Activity by BPO-D-GL-Pretreatment on the Subsequent Generation of PAB-Reactive Helper T Cells by Immunization with PAB-MGG-BPO. From these results one cannot immediately conclude that the increase in specificity and affinity of PAB-reactive helper T cells generated in mice pretreated with PAB-d-GL merely reflects the absence of suppressor T cells caused by such PAB-d-GL-pretreatment. Alternatively, the increase in helper T-cell generation could derive from the direct actions of PAB-d-GL on the PAB-reactive helper T cells during their development in response to PAB-MGG-immunization. To exclude this possibility, a model system for detecting T-T cell interactions was established to generate hapten-reactive T-cell activity of one specificity (PAB) in collaboration with hapten-reactive T lymphocytes of another specificity (BPO), and the effect of eliminating the suppressor T-cell activity of BPO-specificity with BPO-d-GL on the subsequent generation of PAB-reactive helper T-cell population was investigated.
As a system of T-T cell interactions in the generation of helper T-cell activity, we used a double hapten conjugate, PAB-MGG-BPO, for the generation of PAB-reactive helper T lymphocytes in a manner to allow us to analyze the effect of the preexistence of BPO-reactive T lymphocytes on the generation of PAB-reactive helper T-cell activity.

Three groups of mice were preimmunized, respectively, with either 100 μg of BPO-MGG or BPO-OVA in CFA, or CFA alone. 3 wk later, the animals were further immunized with 100 μg of PAB-MGG-BPO to generate PAB-reactive T lymphocytes. 3 wk later, the spleen cells from each group were then transferred into X-irradiated recipients together with DNP-KLH-primed cells, and the resulting PAB-reactive helper T-cell activities were measured by the anti-DNP antibody responses in the recipient spleens 7 days after stimulation with DNP-MGG-PAB. As shown in Fig. 2, group I is background response of DNP-B cells to the DNP-MGG-PAB stimulation. Comparing groups II and III, when the animals had been preimmunized with BPO-MGG, the generation of PAB-reactive helper T cells was strikingly augmented by immunization with PAB-MGG-BPO (group III). This augmenting effect of BPO-MGG preimmunization could not be replaced by BPO-OVA-preimmunization (group IV), indicating that anti-BPO antibody or BPO-specific B cells may not be responsible for this augmentation. Thus, the pre-existence of BPO-reactive T cells significantly augmented the generation of PAB-reactive helper T lymphocytes upon immunization with the double hapten conjugate, PAB-MGG-BPO. These results therefore represent interactions between T lymphocytes of different hapten specificities in vivo and suggest the physiological interaction of T lymphocytes of one specificity in the generation of helper T cells of another clonal specificity to a complex multideterminant antigen.

In the above experimental system, the inhibition of BPO-reactive suppressor T-cell generation by the pretreatment with BPO-δ-GL resulted in a further increase in PAB-reactive helper T-cell generation as shown in Table VI. As evident from the comparison between groups I and III, pretreatment with BPO-δ-GL before the BPO-MGG-immunization substantially increased the development of PAB-reactive helper T-cell activity. This seemed to be due to the selective inhibition of BPO-reactive suppressor T-cell development, and the resulting BPO-reactive helper T cells efficiently collaborated to generate the PAB-reactive helper T cells upon immunization with the double hapten conjugate, PAB-MGG-BPO. This conclusion was supported by the suppressed generation of BPO-reactive suppressor T-cell activity and increased BPO-reactive helper T-cell activity in the above BPO-δ-GL-pretreated animals. These results derive from a separate experiment involving a protocol similar to that in Table III, except DNP-MGG-BPO and DNP-KLH-BPO were used as the challenging antigens to DNP-KLH-primed cells to detect the BPO-reactive helper and suppressor T-cell activities, respectively (data not listed). Moreover, as shown in groups III and IV (Table VI), the PAB-reactive helper T cells developed in the presence of BPO-reactive helper T cells were highly susceptible to PAB-δ-GL treatment. This again contrasts sharply with the comparison between groups I and II, in which the PAB-reactive helper T cells generated in the presence of BPO-reactive suppressor T cells were not rendered tolerant and the apparent
FIG. 2. Increase in development of hapten-reactive helper T-cell activities by T-T cell interaction. Normal mice were preimmunized i.p. with either 100 μg of BPO-MGG or BPO hen ovalbumin (OVA) in CFA, or CFA alone. 3 wk later, these animals were further immunized with 100 μg of PAB-MGG-BPO. The PAB-reactive helper T-cell activities generated in the spleens of these respective mice were measured 3 wk after the PAB-MGG-BPO immunization by transferring the T cells into 600 R X-irradiated recipients together with DNP-KLH-primed cells and then stimulating them with 100 μg of DNP-MGG-PAB. The DNP-KLH-primed cells came from the animals which had been immunized 12 wk previously. Anti-DNP antibody responses in the recipients' spleens 7 days after the cell transfer are depicted.

TABLE VI
Effect of Elimination of BPO-Reactive Suppressor T Cells on the Subsequent Generation of PAB-Reactive Helper T Cells upon Immunization with PAB-MGG-BPO*.

| Exp Group | Condition for generation of PAB-reactive helper T cells | Indicator tolerogen treatment 4 days before cell transfer | PAB-reactive helper T-cell activity. Anti-DNP PFC/spleen |
|-----------|--------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|
| I         | None BPO-MGG PAB-MGG-BPO                               | None                                                   | 3,652 (1.08)                                           |
| II        | 1 mg PAB-α-GL                                        | 3,292 (1.33)                                           |
| III       | None BPO-MGG                                          | 58,136 (1.34)                                         |
| IV        | 1 mg PAB-α-GL                                        | 11,677 (1.59)                                         |
|           | 250 μg BPO-α-GL                                      | 58,136 (1.34)                                         |
|           | 1 mg PAB-α-GL                                        | 11,677 (1.59)                                         |

* Normal animals were either pretreated with 250 μg of BPO-α-GL or not pretreated, and 3 days thereafter, immunized with 100 μg of BPO-MGG to generate BPO-reactive T lymphocytes. 3 wk after the BPO-MGG-immunization, those animals were further immunized with 100 μg of PAB-MGG-BPO to generate PAB-reactive T lymphocytes through the T-T cell interactions. The susceptibilities of PAB-reactive helper T cells generated in these animals to indicator tolerogen, PAB-α-GL, were tested 4 wk after the PAB-MGG-BPO priming and this was performed by treatment of these donor animals with 1 mg PAB-α-GL 4 days before the cell transfer. The assay protocol of PAB-reactive helper T-cell activities was substantially the same as Table III by using DNP-KLH-primed cell from mice which had been immunized 8 wk previously.

PAB-reactive helper activity was again increased by the indicator tolerogen treatment with PAB-α-GL.

Thus, by pretreatment with hapten-α-GL conjugates, we can selectively eliminate the suppressor T-cell activity and modulate the generation of hapten-reactive helper T lymphocytes in polyclonal fashion, and the result is hapten-reactive helper T lymphocytes with more hapten-specificity. From these results, one can conclude that suppressor T cells inhibit helper T-cell development by suppressing the increase in the functional specificity and affinity of helper T lymphocytes.

Discussion

In recent years, a number of suggestions have been made that suppressor T cells might function by removing or inactivating helper T cells rather than by
Suppressor T-cell effect on helper T-cell development

directly attacking B cells committed to the production of antibody. In an accompanying publication (6), we have presented evidence showing that PAB-reactive suppressor T cells inhibit KLH-reactive helper T-cell activity in collaboration between DNP-specific B cells and KLH-primed T cells after stimulation with DNP-KLH-PAB. In the present study, we have shown that elimination of suppressor T-cell activity from the mixture of suppressor and helper T-cell populations by the treatment with PAB-d-GL resulted in a substantial increase in helper T-cell activity. Thus, the suppressor T cells present in the PAB-MGG-primed spleen cells might reduce or limit the amount of helper T-cell activity available to B cells. The selective elimination of suppressor T-cell activity leaving the helper T-cell activity intact as shown in Tables I and II indicates that hapten-reactive helper and suppressor T cells are functionally distinct T-cell subsets. Furthermore, this result makes highly unlikely the suggestion that the hapten-reactive suppressor T cell exhibits its activity by providing an excess of helper T-cell activity, thereby eliminating the effective antigenic stimulus from the system. Moreover, as shown in Fig. 1 and Table III, the inhibition of PAB-reactive suppressor T-cell generation by pretreatment with PAB-d-GL before PAB-MGG-priming resulted in augmented development of PAB-reactive helper T-cell activity. Thus, these data also make it unlikely that the PAB-reactive suppressor T cells are modified helper T cells or the precursors of helper T cells (9). Instead, these data suggest that hapten-reactive suppressor and helper T cells are distinct, differentiated populations, each with its own role in regulation of the immune response. This is reminiscent of the observation by Cantor et al. (10, 11) that the Ly 2, 3-positive cells, in which suppressor T-cell activity resides, never convert to the Ly 1-positive cells of helper T-cell activity.

The influence of inhibiting suppressor T-cell generation on the development of helper T cells was analyzed with respect to hapten-specificity of the latter's responsiveness, since the functional specificity of helper T-cell activity can be quantitatively measured by the responses of a hapten-reactive T-cell population to varying haptenic determinants of structurally-related compounds. The conclusion that suppressor T cells generated during the primary immune response ultimately exert an effect on helper T-cell development derived from the observation presented here (a) that the development of helper T-cell activity was substantially augmented in the PAB-d-GL-pretreated animals in which the generation of suppressor T-cell activity was completely inhibited; and (b) that the reactivity of helper T lymphocytes developed in the absence of suppressor T cells was more hapten-specific than that developed in the presence of suppressor T cells. The increase in functional specificity of PAB-reactive helper T-cell activity demonstrated in the absence of suppressor T-cell generation may also reflect the increase in functional avidity of helper T lymphocytes to the haptenic determinant. As shown in Tables V and VI, the PAB-reactive helper T cells developed in the absence of suppressor T cells were highly susceptible to tolerance induction upon treatment with PAB-d-GL, whereas the helper T-cell activities developed in the presence of suppressor T cells were not abolished by this treatment. This ample difference in tolerogenesis may be analogous to tolerance induction in B lymphocytes, in which it is generally accepted that the higher affinity population is more susceptible than the lower affinity one to
tolerogenesis (12). Thus, taken collectively, these results clearly indicate that hapten-reactive T-lymphocyte populations developed in the absence of suppressor T cells, have a unique quality, perhaps due to their higher specificity and affinity for haptenic determinants to which they have been primed.

We have previously demonstrated the progressive decrease in cross-reactivity of PAB-reactive helper T cells to structurally related haptenic determinants and increase in susceptibility of tolerogenesis to PAB-d-GL treatment with time after priming (8). These results were interpreted as a reflection of functional maturation of helper T lymphocytes during the immune period. The increase in hapten-specificity and affinity of helper T cells developed in the absence of suppressor T cells thus strongly suggests that the suppressor T-cell function is to inhibit the helper T-cell maturation by lowering the specificity and affinity of functional helper T-cell receptors to a haptenic determinant. In this interpretation, however, the elimination of hapten-specific B cells or anti-hapten antibody must be also taken into account. The possibility that the development of hapten-reactive T-cell population is influenced by the presence of hapten-specific B cells or anti-hapten antibody may be excluded by the results shown in group IV of Fig. 2, demonstrating that the pre-existence of BPO-specific B cells or antibody never interfered with the development of PAB-reactive helper T cells upon immunization with PAB-MGG-BPO in this experimental condition. Thus, the increase in functional specificity of PAB-reactive helper T cells demonstrated in hapten-d-GL-pretreated donor animals before hapten-MGG-priming seemed to be mediated solely by the inhibition of suppressor T-cell development by the hapten-d-GL conjugate.

Our interpretations that suppressor T cells directly inhibit helper T-cell activity may reflect a general immunoregulatory mechanism. Tada (13) has proposed that KLH-reactive suppressor T-cell activity may interfere with helper T-cell function because KLH-reactive suppressor T-cell-derived soluble factors suppress anti-hapten antibody responses only when the hapten is coupled to KLH as a carrier. Kapp et al. (14) also showed that removal of suppressor T cells, which suppress the response to GAT (a linear terpolymer of glutamic acid, alanine, and tyrosine), in GAT-suppressed nonresponder mice did not unmask GAT helper T-cell activity. These authors theorized that helper T cells were missing because GAT did not prime T cells in GAT-suppressed mice, but it is also possible that the GAT-reactive suppressor T cell removed the GAT-reactive helper T cells. More definitively in the same line of evidence, Herzenberg et al. (15) demonstrated in their allotype suppressed mice that allotype suppressor T cells generated in (SJL×BALB/c) F1 mice suppressed production of antibodies marked with the Ig-1b allotype by specifically removing the helper T-cell activity required to facilitate differentiation and expansion of B cells to Ig-1b antibody-forming cells. Specifically, in the allotype suppressed mice, suppressor T cells which suppressed Ig-1b antibody production completely removed the helper T-cell activity capable of helping Ig-1b B cells without impairing helper T-cell activity which helped other IgG B cells. In our hapten-reactive T-cell system, the demonstration of a suppressor T-cell effect on helper T-cell development was reciprocal in that the selective elimination of suppressor T-cell activity from the T-cell populations resulted in the augmentation of helper T-cell
activity. However, our system has many similarities with the suppressor T-cell activities listed above. Thus, the action of suppressor T cells on helper T-cell activity may be a general mechanism of regulation of immune response.

Eichman (16) showed that exposure to antibody determinants (idiotypes) on immunoglobulin molecules resulted in the generation of a suppressor T-cell population that specifically suppressed production of immunoglobulin molecules carrying that idotype. This seems to be closely parallel to the present hapten-reactive suppressor T-cell system, in which the exposure of mice to haptens on mouse immunoglobulin generates the suppressor T-cell activity specific for those haptenic determinants. Analogy between idotype suppressor T cells and hapten-reactive T cells, while not fully established, offers intriguing ground for speculation on how suppressor T cells regulate the immune response. As shown in Table VI, the demonstration that the hapten-reactive suppressor T cells of one specificity suppressed the generation of helper T-cell activity of another hapten-specificity when the animals were immunized with double-hapten conjugate suggests an expanded immune network similar to that postulated by Jerne (17). Here, the suppressor T cells of one specificity affect the helper T cells of another specificity to lessen the specificity and affinity of their functional receptor sites, and this selective force is driven by antigenic stimulation.

Although our knowledge of the molecular mechanism by which suppressor T cells function to lower helper T-cell activity is limited, the implication that both affinity and specificity of hapten-reactive helper T cells increase in the absence of suppressor T cells may relate to future work in manipulating the immune response in vivo. Since the hapten-reactive T lymphocytes are capable of reacting with the haptens coupled on any carrier molecule, such as heterologous proteins or allogeneic and syngeneic tumor cells, one could easily use this T-cell population to regulate the immune response to these antigenic determinants.2

Thus, in summary, in the accompanying paper (6) and present studies, we have demonstrated a system of inciting hapten-reactive helper and suppressor T-cell activities by immunization with hapten-modified, isologous protein conjugates. We have also clarified the means by which suppressor T cells modify helper T-cell activity. For this work, we took advantage of the hapten-reactivity of suppressor T cells and their susceptibility to inactivation by hapten-D-GL conjugates. Moreover, with the aid of basic knowledge of regulatory functions of hapten-reactive suppressor and helper T lymphocytes, we demonstrated the existence of helper to helper and suppressor to helper T-T cell interactions in vivo between the T lymphocytes of distinct clonal specificities. These systems should provide valuable tools for future manipulations of specific immune responses.

Summary

An experimental condition was established in vivo for selectively eliminating hapten-reactive suppressor T-cell activity generated in mice primed with a para-

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azobenzoate (PAB)-mouse gamma globulin (MGG)-conjugate and treated with PAB-nonimmunogenic copolymer of d-amino acids (d-glutamic acid and d-lysine; d-GL). The elimination of suppressor T-cell activity with PAB-d-GL treatment from the mixed populations of hapten-reactive suppressor and helper T cells substantially increased apparent helper T-cell activity. Moreover, the inhibition of PAB-reactive suppressor T-cell generation by the pretreatment with PAB-d-GL before the PAB-MGG-priming increased the development of PAB-reactive helper T-cell activity. The analysis of hapten-specificity of helper T cells revealed that the reactivity of helper cells developed in the absence of suppressor T cells was more specific for primed PAB-determinants and their cross-reactivities to structurally related determinants such as meta-azobenzoate (MAB) significantly decreased, as compared with the helper T-cell population developed in the presence of suppressor T lymphocytes. In addition, those helper T cells generated in the absence of suppressor T cells were highly susceptible to tolerogenesis by PAB-d-GL.

Similarly, the elimination of suppressor T lymphocytes also enhanced helper T-cell activity in a polyclonal fashion in the T-T cell interactions between benzylpenicilloyl (BPO)-reactive T cells and PAB-reactive T cells after immunization of mice with BPO-MGG-PAB. Thus inhibition of BPO-reactive suppressor T-cell development by the BPO-d-GL-pretreatment resulted in augmented generation of PAB-reactive helper T cells with higher susceptibility of tolerogenesis to PAB-d-GL.

Thus, these results support the notion that suppressor T cells eventually suppress helper T-cell activity and indicate that the function of suppressor T cells related to helper T-cell development is to inhibit the increase in the specificity and apparent affinity of helper T cells in the primary immune response. The hapten-reactive suppressor and helper T lymphocytes are considered as a model system of T cells that regulate the immune response, and the potential applicability of this system to manipulating various T cell-mediated immune responses is discussed in this context.

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