TOLERANCE INDUCTION IN B LYMPHOCYTES BY THYMUS-DEPENDENT ANTIGENS

T Cells May Abrogate B-Cell Tolerance Induction but Prevent an Antibody Response*

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Although recent work has suggested that different mechanisms are responsible for the state of specific immunological unresponsiveness that can be induced by either thymus-dependent or thymus-independent antigens (1, 2), it has been shown that B-cell tolerance to a thymus-dependent antigen can be induced in the absence of functional T cells both in vivo and in vitro (1, 3). Thus, while a complete antibody response to a thymus-dependent antigen such as fowl gamma globulin (FGG) requires the presence of specific T cells (4), there is no obligatory requirement for T cells in the interaction that leads to B-cell tolerance. In fact there is suggestive evidence that activated T cells may counteract the tolerogenic process, since B-cell tolerance induction occurs readily in instances where T-cell activation does not occur because haptens have been presented on nonimmunogenic synthetic copolymers (5), or on autologous proteins (6-8) or erythrocytes (9). Additionally, the physical state of aggregation of the antigen does not seem critical for tolerance induction by thymus-dependent antigens in the congenitally athymic mouse (1, 3), suggesting that the reason that aggregates often prevent tolerance induction in euthymic mice (10, 11) involves the T cell.

This paper approaches two questions raised by the experiments demonstrating the induction, in vivo (1) or in vitro (3), of B-cell tolerance in spleen cells from congenitally athymic mice. The first is that since the responsiveness of spleen cells was studied in both cases in the absence of T cells, there was a theoretical possibility that IgM antibody-forming cell (AFC) precursors had not been tolerated, but rather had been switched to IgG AFC precursors that were more thymus dependent and thus were not activated in the absence of T cells. The

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1 Abbreviations used in this paper: AFC, antibody-forming cells; Agg-FGG, aggregated FGG; De-FGG, deaggregated FGG; FGG, fowl gamma globulin; HGG, human gamma globulin; LPS, lipopolysaccharide; PFC, plaque-forming cells; POL, polymerized flagellin.
second question concerns apparently contradictory proposals for the T-cell role in tolerance induction. While there has been much interest in the role of suppressor T cells (12, 13) in producing tolerant states, as discussed above, there is considerable evidence that activated T cells actually counteract tolerance induction in the B cell.

In this study, partially inbred congenitally athymic nu/nu mice were reconstituted with near-syngeneic thymocytes either before, during, or after a putative tolerogenic treatment. Additionally, nu/nu mice were compared with euthymic mice of a similar genetic background. Results will be presented to demonstrate firstly, that the B-cell tolerance induced in nu/nu mice by thymus-dependent antigens persists when these mice are challenged after reconstitution with thymocytes. Secondly, it will be shown that the presence of T cells prevents the induction of B-cell tolerance by nondeaggregated thymus-dependent antigens and instead priming occurs, both at the level of the T cell and the B cell. However, coexistent with T-cell and B-cell priming there is also a T-cell-dependent suppressive influence which may mask the priming effect and produce an overall hyporesponsiveness.

Materials and Methods

Mice. CBA/H WEHI mice and BALB/c mice of either sex were employed as euthymic strains. Two lines of partially inbred congenitally athymic (nu/nu) mice were used, the nu/nu gene being on either a BALB/c or a CBA background. The nu/nu mice used were the progeny of the 4th-6th backcrosses.

Antigens. FGG was prepared, and ultracentrifuged as previously described (14). Freshly deaggregated material was termed "deaggregated FGG" (De-FGG), while deaggregated material stored in solution at 4-8°C was termed "soluble FGG". Nonultracentrifuged FGG that was stored frozen and thawed several times contained many easily visible aggregates and was termed "aggregated FGG" (Agg-FGG).

Human gamma globulin (HGG) was obtained from Commonwealth Serum Laboratories, Parkville, Australia. DNP-HGG was prepared as in the accompanying paper (3) as was DNP-FGG. The HGG used in some experiments, had been held under alkaline conditions and dialized in parallel with HGG that was undergoing dinitrophenylation. The average conjugation ratios of the preparations used ranged from 4 to 16 DNP groups per molecule. DNP-HGG was ultracentrifuged and stored, as for FGG.

Lipopolysaccharide (LPS) was obtained from Difco Laboratories, Detroit, Mich. Polymerized flagellin (POL) and DNP-POL were prepared as described elsewhere (14).

Tissue Culture. A modified Marbrook system was used as in the preceding study (3).

AFC Assay. Modified hemolytic plaque assays, as described in the preceding study (3), were used to detect anti-DNP or anti-FGG in suspensions of spleen cells, either freshly prepared or after tissue culture. Results are expressed as the arithmetic mean of the response of individual spleens or tissue culture flasks ± SEM. Indirect plaque-forming cells were detected using a polyvalent rabbit antimouse Ig serum at an appropriate dilution (15).

Treatment of Cell Suspension with Mitomycin C. Spleen cells were incubated for 30 min at 37°C with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 40 μg/ml, and were afterwards washed twice through fetal calf serum.

Treatment with Anti-θ Serum. This was performed as described elsewhere (16), using an AKR-anti-C3H-θ serum. Preliminary experiments showed that this treatment had no effect on the ability of CBA spleen cells to respond in vitro to DNP-POL, a thymus-independent antigen in vitro (16, 17), indicating that there was no effect on B cells. The same treatment reduced the in vitro response of CBA or BALB/c spleen cells to sheep erythrocytes by approximately 90%.
Results

The Effect of Pretreatment with Aggregated Hapten-Protein Conjugates on the Hapten-Specific Response in Either nu/nu or Euthymic Mice. BALB/c type nu/nu mice were pretreated with 5 mg of aggregated DNP\textsubscript{10}-HGG intraperitoneally (i.p.). 2 days later these mice, together with a control group of normal nu/nu mice, were injected intravenously (i.v.) with near-syngeneic BALB/c thymocytes, the addition of thymocytes being designed to allow the expression of any potential for IgG production (18). Both groups of mice were challenged with DNP on a heterologous carrier FGG (DNP-FGG) together with POL as an adjuvant.\textsuperscript{2} As shown in Fig. 1, the anti-DNP responses of the mice pretreated with DNP-HGG were significantly less than the responses of the control nu/nu mice that had not been pretreated with DNP-HGG, but only reconstituted with thymocytes. Thus pretreatment of the nu/nu mice with aggregated DNP-HGG had induced a hapten-specific B-cell unresponsiveness that was still apparent when challenge was made in the presence of T cells.

Also shown in Fig. 1 are the results of a second part of the same experiment, involving euthymic BALB/c mice that were injected with DNP\textsubscript{10}-HGG and later challenged with DNP-FGG plus POL in parallel with the nu/nu mice. In contrast to the situation in the nu/nu mice, pretreatment with 5 mg DNP-HGG did not induce unresponsiveness but instead had a marked priming influence on the DNP-reactive B-cell population. Thus the small anti-DNP response seen when BALB/c mice were given a single injection of DNP-FGG (100 \(\mu\)g) was increased strikingly. A similar priming effect on the DNP-reactive B-cell population was evident when BALB/c mice from the DNP-HGG-pretreated and untreated control groups were challenged with a different carrier DNP ovalbumin, again together with POL (data not shown).

This experiment, together with others of similar design, showed a clear difference in the effect of nondeaggregated DNP-HGG between nu/nu mice and euthymic mice, and supported the hypothesis that the presence of T cells in euthymic mice protected the B cells from tolerance induction, in fact favoring priming of the B cells. However, similar experiments to be described below, using FGG rather than a hapten-protein conjugate, were at first sight inconsistent with this framework.

The Paradoxical Effect of the Presence of T Cells on the Induction of Unresponsiveness by FGG. When nu/nu mice were given soluble or aggregated FGG, and subsequently reconstituted with T cells and challenged with FGG, the results were consistent with the conclusions reached in the experiments with hapten-protein conjugates described above. As shown in Fig. 2, the anti-FGG responses in group B, where nu/nu mice were given 10 mg Agg-FGG and reconstituted with thymocytes 2 days later, were much lower than the responses of the control nu/nu mice in group A which had not been pretreated with Agg-FGG.

However, in contrast to the experiments with aggregated DNP-HGG described

\textsuperscript{2} Schrader, J. W. 1975. The adjuvant action of thymus-independent antigens such as polymerized flagellin, lipopolysaccharide, and pneumococcal polysaccharide. Manuscript submitted for publication.
FIG. 1. The contrasting effect on the B-cell population of injection of euthymic and athymic mice with aggregated DNP-HGG. BALB/c mice or BALB/c nu/nu athymic mice were injected i.p. with 5 mg of aggregated DNP+HGG. 7 days later the nu/nu mice were injected i.v. with 75 \times 10^6 BALB/c thymocytes and all mice were injected i.p. with DNP-FGG (100 \mu g) plus POL (10 \mu g). 6 days later spleens were harvested and anti-DNP AFC assayed. Direct PFC numbers are represented by solid columns and indirect by open columns, with bars representing the S.E.M.

above, the presence of T cells during pretreatment with FGG did not seem to prevent the induction of unresponsiveness. Thus the anti-FGG responses of euthymic mice treated with Agg-FGG (group E, Fig. 2) were markedly less than those of nonpretreated mice (group D, Fig. 2). Furthermore, nu/nu mice that were treated with 10 mg Agg-FGG i.p. and at the same time were given 75 \times 10^6 thymocytes i.v. (group C, Fig. 2) responded less well to FGG than did mice receiving thymocytes alone (group A, Fig. 2).

Clearly the presence of T cells did not prevent the induction of an unresponsive state by non-De-FGG. However there was no evidence to indicate whether this unresponsiveness was due to tolerance of either of the FGG-reactive T or B cells, or to a separate suppressive influence, and the following experiments were performed to elucidate this situation.

The Effect of Pretreatment with Agg-FGG on the Subsequent Specific Responsiveness of Euthymic Mice. In further experiments designed to clarify the unexpected tolerogenic effect of non-De-FGG, euthymic mice were injected with varying doses of Agg-FGG. Fig. 3 shows typical results demonstrating the effect of such pretreatment of CBA mice on their subsequent responses to FGG (100 \mu g) plus POL (10 \mu g). Two facts are apparent: (a) at each dose of Agg-FGG, the response of pretreated mice was diminished relative to that of control mice, the decrease being proportional to the dose of FGG used in pretreatment and (b)
The suppressive effect of pretreatment with Agg-FGG in the presence of T cells. Groups designated A-E from left to right. CBA nu/nu mice were compared with nu/+ littermates in the following groups. A control group of nu/nu mice group A were given $50 \times 10^6$ CBA thymocytes i.v. On day 3 they were immunized with $500 \mu g$ FGG (alum precipitated together with $2 \times 10^9$ killed pertussis organisms), given a further injection of $100 \mu g$ FGG on day 8, and the spleens were harvested on day 11. Group B nu/nu mice were pretreated with $10 mg$ of Agg-FGG 2 days before reconstitution with thymocytes (day 0) and were challenged as in group A. Group C nu/nu mice were treated with $10 mg$ Agg-FGG i.p. at the time of reconstitution with the thymocytes and were challenged as in group A. Group D were control LM mice which were challenged with FGG as for group A. LM mice in group E were pretreated with $10 mg$ Agg-FGG at the same time as group C, and were challenged with the other groups on day 3.

in all pretreated animals there was pronounced increase in the proportion of indirect to direct plaque-forming cells (PFC), in fact hardly any direct PFC being detected. These latter results suggested that a “priming” effect was occurring, despite the overall decrease in the response upon challenge.

The Unresponsiveness Induced by Pretreatment of Euthymic Mice with Large Doses of Nondeaggregated Proteins is not at the B-Cell Level. Several possible mechanisms existed to explain the suppression induced when euthymic mice were pretreated with a protein and challenged with that same molecule. Effects of the pretreatment regimen on the B-cell population were considered first.

The result of challenge with the hapten on a heterologous carrier (Fig. 1) suggests that the B-cell population in euthymic mice was being positively affected or “primed” by pretreatment rather than tolerized. The shift to a largely IgG response seen in the small response upon challenge with the homologous
Fig. 3. The effect on a subsequent response to FGG of pretreatment of euthymic mice with Agg-FGG. Groups of CBA mice were injected i.p. with the indicated doses of Agg-FGG. 8 days later all mice were challenged with 100 µg FGG plus 10 µg POL. Spleens were harvested on day 13 and anti-FGG AFC assayed. Direct PFC are shown by black columns, indirect by open.

molecule (Fig. 3) was also consistent with such a view. To show that the suppression caused by pretreatment with FGG was not due to an interference with the B cell, and furthermore that it could act upon a B-cell population that was already primed, the following experimental design was employed. BALB/c were immunized with DNP-POL to provide a population of DNP-reactive memory B cells and were later injected i.p. with varying doses of Agg-FGG. These mice were challenged after 13 days with DNP-FGG, together with POL as an adjuvant. Fig. 4 shows that the suppressive effect of pretreatment with large amounts of carrier protein, applied also to the response of a primed B-cell population. In this experiment, the hypersponsiveness was clearly due to an interference with the activation of the B cells during the challenge, since the DNP-reactive B cells could not have been rendered tolerant by treatment with aggregated FGG.

Evidence for a Nonspecific Suppression by Carrier-Reactive Cells. The two mechanisms likely to account for the unresponsiveness seen when mice were treated with nondeaggregated antigen and challenged with the homologous carrier involved either a lack of carrier-reactive cells, implying that the carrier reactive cells had been tolerized by the pretreatment, or the T-dependent generation of a population of carrier-reactive suppressor cells.

Evidence in favor of the latter possibility was obtained in experiments that indicated that the suppression was nonantigen specific. Fig. 5 shows the results of
The suppressive effect of pretreatment with a large dose of aggregated carrier on the subsequent activation of hapten-primed B cells by the homologous hapten-carrier conjugate. BALB/c mice primed 6 mo previously with DNP-POL (20 μg) were injected i.p. with 0.1 or 10 mg Agg-FGG. 13 days later both groups of mice received 100 μg DNP-FGG plus 10 μg POL i.p. Spleens were harvested after 6 days and the anti-DNP PFC determined. Direct PFC are represented by the black columns.

In Vitro Challenge of Spleen Cells from Euthymic Mice Pretreated with FGG in Various States of Aggregation. It was previously shown that spleen cells from donor nu/nu mice that had been pretreated with high (5–10 mg) doses of soluble thymus-dependent antigens, were specifically unresponsive to in vitro challenge (1). It was therefore of interest to explore the effect of similar pretreatment of euthymic spleen cell donors, in particular seeking evidence for (a) B-cell tolerance and (b) T-cell-dependent helper activity and/or T-cell-dependent suppressor activity. CBA mice were injected i.p. or i.v. with FGG in various
FIG. 5. Nonantigen-specific suppression by carrier-reactive cells in pretreated euthymic mice. The results of an additional group of mice included in the experiment shown in Fig. 1 are depicted. Thus a group of BALB/c mice, treated 7 days previously with aggregated DNP₄HGG (5 mg), was injected with DNP-FGG (100 μg) plus 10 μg POL together in this case with 100 μg HGG (group B). These mice were killed 6 days later together with the groups shown in Fig. 1 and the results of the challenge of mice pretreated with DNP-HGG with DNP-FGG plus POL alone are repeated for the purpose of comparison.

states of aggregation. At varying intervals these mice were sacrificed and then spleen cells were cultured in vitro. Two forms of in vitro antigenic challenge were used, FGG (100 μg) in the presence of POL (10 μg), to enable the gauging of the function of FGG-reactive B cells even though T-cell-function might be ablated, and FGG (1 μg) without POL, the latter challenge resulting in an in vitro anti-FGG response in the present tissue culture system, only in the presence of primed T cells (e.g. Table VI, below).

The situation was shown to be complex. Table I shows the results of one typical experiment, in which CBA mice were injected i.p. with various doses of FGG, either freshly deaggregated or in a grossly aggregated form, and challenge was made either in vivo or in vitro 2 or 9 days later.

2 days after pretreatment of spleen cell donors, in vitro challenge with FGG plus POL revealed a definite depression of the IgM (direct PFC) response to FGG (100 μg) in the presence of POL. However this was accompanied by the appearance of indirect PFC, indicating IgG production. This was so in the case of pretreatment with either Agg- or De-FGG. In each case the depression of the IgM response was greatest with the highest (10 mg) dose used for pretreatment, and the total (direct plus indirect) PFC response was greatest (and significantly higher than the total response of control cultures) with the 2-mg pretreatment regimen. At day 2, in vitro challenge with FGG (1 μg) gave only small or undetectable responses in all groups. In vivo challenge on day 2 of mice from the same
CBA mice were injected i.p. in groups of 10 with either freshly De-FGG or Agg-FGG at the indicated doses. 2 days later, two mice from each group together with a group of control nontreated mice were sacrificed and the pooled spleen cells from each group challenged in vitro in four cultures with 100 μg FGG plus 10 μg POL, plus 0.1 μg DNP-POL (challenge a), and in four cultures with 1 μg FGG alone (challenge b). After 4 days the cells were harvested and the anti-FGG direct and indirect AFC response was determined. The anti-DNP AFC response was also assayed in cultures given challenge (a). Responses to challenge (b) are shown in parentheses. On day 2, three mice from each group were challenged in vivo with FGG (100 μg) plus POL (5 μg) plus DNP-POL (5 μg) all given in a single i.p. injection. Spleens were assayed on day 6.

Evidence for B-Cell Unresponsiveness where CBA Euthymic Spleen Cell Donors were Pretreated with FGG or DNP-HGG. In some experiments there was evidence compatible with a degree of B-cell unresponsiveness in that there was some depression of the in vitro response to FGG plus POL early after pretreatment of spleen cell donors with an i.v. injection of De-FGG (Table III). However, despite the partial reduction of the overall response, there was a definite shift to IgG production in the response to 100 μg FGG given with POL, which together with the acquisition of the ability to mount a significant response.

### Table 1

| Pretreatment with FGG | In vitro response | In vivo response |
|-----------------------|------------------|-----------------|
|                        | Anti-FGG | Anti-DNP, direct | Anti-FGG | Anti-DNP, direct |
|                        | Direct   | Indirect      | Direct   | Indirect      |
| De-FGG                |          |               |          |               |
| 0.2 mg                | 129 ± 52 (0) | 300 ± 96 (6) | 503 ± 76 | 200 ± 115 | 860 ± 4,450 | 4,400 ± 529 |
| 2 mg                  | 107 ± 20 (212 ± 66) | 845 ± 88 (0) | 387 ± 39 | 200 ± 115 | 8,600 ± 4,450 | 4,400 ± 529 |
| 10 mg                 | 30 ± 16 (0) | 421 ± 37 (0) | 379 ± 29 | 0          | 1,670 ± 848 | 4,100 ± 1,010 |
| Agg-FGG               |          |               |          |               |
| 0.2 mg                | 330 ± 104 (41 ± 21) | 120 ± 77 (0) | 676 ± 116 | 150 ± 100 | 15,700 ± 4,680 | 3,233 ± 120 |
| 2 mg                  | 294 ± 103 (0) | 572 ± 167 (0) | 385 ± 88 | 0          | 6,467 ± 3,590 | 3,767 ± 745 |
| 10 mg                 | 126 ± 58 (74 ± 35) | 327 ± 60 (0) | 756 ± 296 | 88 ± 30 | 433 ± 311 | 1,533 ± 390 |
To 1 μg of FGG given alone, indicated that a priming effect was also occurring. When cells from FGG-pretreated donors were challenged in vitro with FGG, it could not be excluded that suppressor T-cell effects, rather than B-cell tolerance, were involved. Thus a more critical test of the state of the B-cell population was to challenge with a different carrier from that used in the pretreatment. Table IV shows the results of such an experiment. Euthymic mice were pretreated with DNP-HGG, and their spleen cells challenged in vitro with DNP-POL, POL being
TABLE IV

B-Cell Unresponsiveness Induced in Euthymic Mice by Various Forms of DNP-HGG

| Donors injected with DNP-HGG (5 mg) | Anti-DNP AFC/culture in response to: |
|------------------------------------|-------------------------------------|
|                                   | DNP-POL | LPS |
| 1 i.v.                             | 25 ± 8  | 80 ± 23 |
| 3 i.v.                             | 69 ± 23 | 291 ± 23 |
| 3 (56°C, 10 min) i.p.              | 110 ± 23| 544 ± 96 |
|                                   | 407 ± 70| 1,290 ± 90 |

Groups of CBA mice were injected i.v. or i.p. as indicated with 5 mg DNP-HGG in phosphate-buffered saline immediately after ultracentrifugation, no. 1 representing the upper 1/3, and no. 3 the lower 1/3 of the solution. In the case of one group, (a), DNP-HGG in no. 3 was held at 56°C for 10 min and injected i.p. After 4 days spleen cells pooled from each group of donors were cultured in quadruplicate with either 50 µg LPS or 0.02 µg DNP-POL plus 10 µg POL, in each case together with 100 µg FGG as a control antigen. Cultures were harvested on day 3. A third set of cultures was set up containing LPS and HGG, 1 µg, but the anti-DNP responses, which did not differ from those to LPS alone, are not shown.

Evidence for the Presence of Helper Activity in Spleens of Mice Pretreated with FGG. The demonstration in the previous experiments that the spleen cells from FGG-pretreated mice, unlike spleen cells from normal mice, were able to respond to a small dose of soluble FGG and to readily produce IgG in this in vitro system, indicated that the donors were equivalent to deliberately immunized mice (see also Table V). The appearance of an IgG response in particular suggested that T-cell as well as B-cell priming was involved (18). This was confirmed in in vitro experiments demonstrating that the ability of spleen cells from mice primed with FGG to respond to FGG, 1 µg, and to produce IgG, was sensitive to treatment with anti-θ serum plus complement (C) (e.g., Table VI). Furthermore, it was shown that mitomycin C-treated cells from FGG-pretreated mice enabled spleen cells from normal mice to make IgG (Table V). Since T cells recently activated are known to function in vitro after treatment with mitomycin C (20) while antibody production is abolished (data not shown), this confirms the presence of FGG-activated T cells in the pretreated mice and their positive influence on IgG production (18).

Evidence for T-Cell-Mediated Suppressor Activity. When 3 x 10⁶ spleen cells from FGG-pretreated mice were added to cultures of spleen cells from mice
TABLE V
The Effect of the Addition to Cultures of Normal Cells, of Mitomycin C-Treated Cells from FGG-Pretreated Mice

CBA mice were injected i.v. with 10 mg of a “deaggregated” preparation of FGG that had stood at 4-8°C for 15 days. 1 wk later these mice were sacrificed and their spleen cells cultured in quadruplicate in the presence of FGG (100 µg) plus POL (10 µg) (group B), in parallel with cells from untreated mice (group A). (*) A third group (C) of cultures each contained 15 x 10⁸ cells from untreated mice plus 3 x 10⁸ mitomycin C-treated cells from the mice injected with FGG used in group B. For comparison, cells from mice immunized 8 days before with alum-precipitated FGG (500 µg) plus pertussis (2 x 10⁸ killed organisms) were cultured in parallel (group D). Cultures were harvested on day 4, and direct and indirect PFC assayed.

TABLE VI
T-Cell-Mediated Suppression of an In Vitro Secondary Response

Spleen cells from CBA mice injected 3 wk previously with 100 µg FGG in CFA were cultured in quadruplicate with FGG (1 µg/ml per 15 x 10⁶ viable nucleated cells). In one group cells were treated with anti-θ-serum plus C before culture. Also included were groups of cultures containing in addition 3 x 10⁸ spleen cells from CBA mice injected 5 days before with 10 mg soluble FGG*, either untreated or incubated with anti-θ-serum and then C. Spleen cells from normal CBA mice, 3 x 10⁸/culture were added to another control group. Cultures were harvested on day 4.

* Significantly different by Student’s t test.

previously immunized with alum-precipitated FGG and pertussis, there was a partial suppression of the response to 1 µg of FGG ranging in separate experiments from 70 to 50% (Table VI). This suppression was abolished by treatment of the spleen cells from the FGG-pretreated mice with anti-θ serum and C. Normal spleen cells had no such effect (Table VI).
Discussion

Firstly, it was shown that the B-cell tolerance induced by thymus-dependent antigens in congenitally athymic mice was still evident after reconstitution with T cells. Secondly, these experiments clarify the relationship between T cells and B-cell unresponsiveness. The salient points are firstly that in euthymic mice the injection of large amounts of nondeaggregated heterologous serum proteins resulted in a priming effect on the B-cell population (Figs. 1 and 3), an observation consistent with a large body of work linking aggregation of antigen with immunogenicity (10, 11). However, the B-cell-priming effect of large doses of nondeaggregated antigens in euthymic mice was masked if the mice were challenged using the same carrier as was used for pretreatment. Even in this case there was evidence of a priming effect in that there was a shift to IgG production, despite the overall reduction in the response (Fig. 3). Thirdly, at least a part of the effector phase of this T-dependent suppressive effect of carrier-reactive cells was not antigen specific, although the activation of the suppressor effect was antigen specific (Fig. 5). Fourthly, it was shown in an in vitro system that T-helper activity was generated when CBA mice were pretreated with large doses of FGG, even when the FGG had been freshly deaggregated, (Tables II–IV). Finally, only early after treatment with De-FGG was there evidence for a reduced in vitro response by spleen cells from pretreated animals (Table IV). Deaggregated DNP-HGG was quite effective in inducing B-cell unresponsiveness, while preparations of DNP-HGG containing aggregates were less so (Table V).

The interpretation offered for these results is that in the presence of T cells, pretreatment of mice with soluble serum proteins results in a strong tendency for immunization to occur, in the case of FGG even when FGG is freshly deaggregated. The activation of T cells is associated with a halt to the tendency for B cells to be tolerized as they would be in nu/nu mice, and instead with an expansion of a population of B cells with the characteristic qualitative property of memory B cells, namely that they are able to produce IgG antibody in the presence of helper T cells (18). The expression of this immunizing effect may be masked by the generation of a T-cell-dependent suppressive influence as shown by Basten et al. (21) who demonstrated in an adoptive transfer situation that spleen cells from mice pretreated with De-FGG possessed both helper and suppressor T-cell activity.

This interpretation is consistent with the results of Miller et al. (18) who have previously reported the difficulty of inducing B-cell tolerance in euthymic mice using FGG. In the present study, evidence consistent with B-cell tolerance in euthymic mice was found only when deaggregated DNP-HGG or De-FGG was used, and in the case of the latter, only early after pretreatment by i.v. injection. While the possibility that this suppression of B-cell responsiveness was due to T-cell-mediated suppression was not excluded, it seems likely that these results reflect a tolerogenic effect on the B-cell population occurring before T cells have been activated. This is clearer in the case of the tolerance to DNP induced by DNP-HGG, since it was demonstrable by in vitro challenge with the heterologous conjugate DNP-POL. Additionally, no suppressor effect has been shown in preliminary experiments where cells from DNP-HGG-treated mice have been added together with HGG to cultures of normal spleen cells challenged with DNP-POL.
It is important to note that both FGG and to a lesser degree DNP-HGG are prone to aggregation, and this may be relevant in the generation of a suppressor T-cell-governed tolerance rather than the T- or B-cell deletion type of tolerance that was demonstrated by Weigle using deaggregated unconjugated HGG (22). Preliminary results suggest that the presence of substances such as POL or LPS which can prevent the induction of pure B-cell tolerance in nu/nu mice (1) may, if anything, enhance the development of the T-cell suppression type of tolerance, again suggesting a parallel between those conditions normally thought to favor T-cell activation and immunity such as the presence of aggregates, and those favoring T-cell-dependent suppression. The present experiments emphasize that while a sufficient dose of antigen can readily tolerize B cells in the absence of T cells in athymic mice, without any stringent requirements for deaggregation of the antigen, deaggregation is of much more importance in euthymic mice.

The nonspecificity of the effector phase of the T-cell-dependent suppressor activity demonstrated in this study is of interest. Gershon and co-workers have demonstrated the effect of pretreatment of donor animals with antigen on phytohemagglutinin responsiveness (23) and have linked such a nonspecific effect with certain types of antigenic competition. It is of interest that, as in the present study, aggregated antigen produced the nonspecific suppressor effect, in fact being more effective than soluble antigen. Likewise, a nonspecific effect of activated T cells may be involved in the immune suppression which can be associated with allogeneic interactions (24). Recently, direct evidence has been reported for a nonspecific suppressor substance that was released from carrier-primed cells upon contact with the carrier (25). This suggests that the suppressive effects of concanavalin A-activated T cells (26, 27) may be a useful model for studying this one type of T-cell-mediated negative influence. As with T-cell "help", antigen-bridging or -focusing concepts may be invoked to explain why responses to homologous carriers are affected more than are those to different antigens given in the presence of the first carrier. Although suppressor T cells may be responsible for both antigenic competition effects and the suppression of a homologous response, other mechanisms may sometimes be involved. For example, some types of antigenic competition involve antigen-handling mechanisms (28), while there is evidence that some forms of T-cell-dependent suppression are mediated by antigen-specific factors (29, 30).

Summary

Thymus-dependent protein antigens such as fowl gamma globulin (FGG) and dinitrophenylated-human gamma globulin (DNP-HGG), readily induced tolerance of the B cell in the absence of T cells even when these antigens were not deaggregated. However, when the same doses of antigen were given in the presence of T cells, the B-cell population was shown to be protected from tolerance induction, especially when the antigen was not in a deaggregated form. In this case, there was in fact evidence of a priming effect, manifest in both the B-cell and T-cell populations. The priming effect on the B-cell population was demonstrated by an increased response of mice pretreated with DNP-HGG, upon challenge with DNP conjugated to a heterologous carrier. The priming effect on the T-cell population was evident in a helper effect demonstrated in vitro. However, when euthymic mice which had been pretreated with large doses of
FGG or DNP-HGG were challenged with the homologous carrier, the results were different. In this case, there was a profound suppression of the response against the carrier or the hapten on that carrier. Suppressor activity was also demonstrated in vitro and was shown to be sensitive to treatment with anti-θ-serum plus complement. Additionally it was shown that the effector phase of the suppression had a definite nonantigen-specific component. Thus, in pretreated euthymic mice, provided the homologous carrier was present, the response to a heterologous carrier was also suppressed.

To account for the observation that nondeaggregated antigens can induce B-cell tolerance in athymic mice, but B-cell priming and T-cell-mediated suppression in euthymic mice, it is proposed that B-cell tolerance occurs when antigen at some critical dose interacts with the B cell in the absence of some second signal. This second signal is normally provided by the macrophage, probably with the assistance of the T cell, and its effect is to divert the result of the interaction of the B cell with antigen towards immunization and away from tolerance induction. When a large dose of an antigen that tends to form aggregates is given to an animal possessing functional T cells, both T-dependent helper and T-dependent suppressor activities are generated, thus accounting for a situation where the B-cell population is immunized, but B-cell activation is suppressed in the presence of the original carrier.

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