An understanding of the mechanism of immunological tolerance remains a major unsolved problem. The establishment of two main classes of lymphocytes, the bone marrow-derived B cell and the thymus-derived T cell and the delineation of their role as obligatory collaborators in many humoral responses (1) have directed theories of tolerance to considerations of whether similar mechanisms of tolerance operate at both the B- and T-cell level (2), or whether indeed tolerance exists at the B cell level at all (1). Recent work has shown that T cells may have an active suppressor role in tolerance at the level of the whole animal by preventing B-cell activation (3, 4).

An experiment of nature excluding T-cell-governed tolerance as a unique explanation for self-tolerance situations, is the example of the "nude," congenitally athymic (nu/nu) mouse that is deficient in detectable T-cell function (5, 6), but which as observed by Ada and Cooper shows no obvious stigmata of autoimmunity (7). The nude mouse would seem to be an ideal model then for the elucidation of non-T-cell-dependent mechanisms of tolerance induction at the B cell level. Since an athymic environment is compatible with immunological harmony these mechanisms are probably important in physiological self-tolerance. Bretscher and Cohn (8) proposed a mechanism of discrimination between activation and inactivation in terms of two signals, antigen-receptor interaction alone being an obligatory signal for paralysis, antigen-receptor interaction plus a second signal given by associative antibody being required to produce activation. In the case of the B lymphocyte, their theory states that activation is via antigen-receptor interaction plus interaction with a T-cell-derived associative antibody (8). Recently, evidence has been presented that the activation of the B lymphocyte may indeed be by a two-signal mechanism (9). However, the experimental situation, since nude mice were used, would seem to preclude the obligatory role of the T cell in providing such a signal (9). Furthermore, short term exposure of B lymphocytes in vitro to antigen alone, a situation equivalent to the sole paralyzing "signal one" of Bretscher and Cohn, did not produce tolerance, although no immune response was seen unless a form of "signal two" was added subsequently (10).
In the experiments reported here the possibility was therefore examined that interaction in vivo of the B lymphocyte with antigen alone in the absence of T cells, and for a variety of time spans, might induce tolerance.

**Materials and Methods**

**Animals.**—Congenitally athymic (nu/nu) nude mice were used at 6–8 wk of age. The mice were from a closed but not inbred colony (6). CBA/H/Wehi mice were used at 8 wk of age.

**Antigens.**—Fowl gamma globulin (FGG), a thymus-dependent antigen (11) was prepared and deaggregated as described previously (9). Flagella (FLA) or polymerized flagellin (POL) were prepared from *Salmonella adelaide* by a standard method (12).

Dinitrophenylated-POL (DNP-POL) and dinitrophenylated human gamma globulin (DNP-HGG) were prepared using dinitrobenzene sulphonylic acid (Eastman Kodak Co., Rochester, N. Y.) (13). The average conjugation ratio of the DNP-POL was 1.5 DNP groups per unit of monomeric flagellin (40,000 daltons). The preparations of DNP-HGG used in this study had conjugation ratios of 4.5 and 20 DNP groups per HGG molecule.

**Antibody-Forming Cell (AFC) Assays.**—Anti-FGG AFC were assayed by a modified hemolytic plaque technique, using sheep erythrocytes (SRC) coated with an anti-SRC antibody raised in the domestic fowl, as described previously (11, 9). Anti-DNP AFC were assayed by a modified hemolytic plaque assay using SRC coated with dinitrophenylated rabbit anti-SRC-Fab fragments (13). Indirect plaque-forming cells (14) were revealed using a polyvalent rabbit anti-mouse immunoglobulin serum, donated by Dr. Pam Russell, and shown in preliminary experiments to efficiently reveal IgG-secreting AFC, generated in secondary responses both in vivo and in vitro.

**Tissue Culture.**—A modification of the Marbrook system was used (15). The medium employed was Eagle's Minimal Essential Medium, with added nonessential amino acids (Cat. no. F-15, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum (Commonwealth Serum Laboratories, Parkville, Australia). Cultures were incubated for 3 or 4 days in 10% CO₂ and humidified air.

**Clearance of FGG from the Blood.**—Groups of mice were injected i.v. with 5 mg of FGG, containing trace amounts of 131I-labeled FGG (16). 90 min later the first blood sample was taken from the tail vein using a microhaematocrit tube (Yankee, A-2930, Clay-Adams, Inc., Parsippany, N. J.). The length of the blood column was measured and counts per millimeter were calculated from the total disintegrations per 10 s per haematocrit tube, as determined in a deep-well scintillation counter.

**RESULTS**

The Effect of In Vivo Injection of i.v. Deaggregated FGG on the In Vitro Response to FGG of nu/nu Spleen.—Nude mice were injected i.v. with 10 mg of deaggregated FGG in saline. After 9 days the mice were sacrificed and the ability of the spleen cells to respond to FGG in vitro was tested. As described previously nu/nu spleen cells respond to deaggregated or normal FGG in vitro if polymerized flagellin (POL) or flagella (FLA) are present (9). Accordingly the spleen cells to be challenged were cultured with 100 µg FGG plus 10 µg POL or FLA, together with a second antigen DNP-POL. The response to

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1 Abbreviations used in this paper: AFC, antibody-forming cell; B cell, bone marrow-derived lymphocyte; DNP-HGG, dinitrophenylated human gamma globulin; DNP-POL, dinitrophenylated polymerized flagellin; FGG, fowl gamma globulin; FLA, flagella; POL, polymerized flagellin; SRC, sheep erythrocytes; T cell, thymus-derived lymphocyte.
DNP-POL is thymus independent (16) and served as a specificity control. In some experiments, 3 or 4 days later the cells were harvested and assayed for anti-FGG antibody-forming cells (AFC) and anti-DNP-AFC. It was found that the anti-FGG AFC response was markedly reduced in cultures of spleen cells from FGG-treated animals, while the response to an unrelated antigen, the DNP determinant of DNP-POL, was not significantly different from that of spleen cells from control animals (Table I). In no experiment did the use of enhancing serum in the anti-FGG AFC assay reveal any indirect plaque-forming cells (14).

The Effect of In Vivo Injection of DNP-HGG on the In Vitro Response to DNP-POL.—In the reverse experiment to the above, soluble DNP-HGG was injected into nu/nu mice in vivo in doses from 5 to 10 mg. 1–9 days later, the spleens were removed and challenged in vitro with DNP-POL, this time with FGG, in the presence of POL, being the control antigen. Table II shows

| Donor of cells | AFC/culture |  |
|---------------|-------------|---|
|               | Anti-FGG    | Anti-DNP |
| Untreated     | 558 ± 96    | 300 ± 33 |
| Treated with FGG in vivo | 72 ± 66 | 321 ± 45 |

Athymic (nu/nu) mice were injected i.v. with 10 mg of freshly deaggregated FGG. 9 days later the spleens were removed and the cells cultured for 4 days in the presence of FGG (100 μg/ml), DNP-POL (1 μg/ml), and POL (10 μg/ml). Spleen cells from untreated athymic (nu/nu) mice were cultured in parallel as a control.

| Pretreatment of donor | AFC/culture |  |
|----------------------|-------------|---|
|                       | Anti-DNP    | Anti-FGG |
| Exp. I —              | 459 ± 60    | 465 ± 102 |
| DNP_{4.8}-HGG, 5 mg*  | 0           | 540 ± 102 |
| DNP_{4.8}-HGG, 5 mg†† | 0           | 450 ± 26  |
| Exp. II —             | 122 ± 60    | 333 ± 133 |
| DNP_{20}-HGG, 10 mg†† | 10 ± 4      | 322 ± 58  |

Groups of nu/nu mice were injected i.v. with two preparations of soluble DNP-HGG with the indicated conjugation ratios. The DNP-HGG had been deaggregated and stored at 4–8°C for 1 wk (DNP_{4.8}-HGG), and 26 wk (DNP_{20}-HGG), respectively. After 4 days (†), or 3 days (‡), mice were killed and the spleens cultured with DNP-POL (1 μg) (different batches were used in exps. I and II), FGG (100 μg), and POL (10 μg). Cultures were harvested after 3 days and each was assayed for anti-DNP and anti-FGG AFC.
representative results with two preparations of DNP-HGG indicating that the induction of nonresponsiveness was not peculiar to FGG.

**Route of Injection and State of Aggregation of FGG.**—The use of freshly deaggregated material was not critical. Thus, solutions of FGG that had been deaggregated and then kept sterile at 4–8°C were as effective as freshly deaggregated FGG (e.g., Table III, exp. I). I.p. injections were found to be regularly effective, although often the degree of unresponsiveness induced was less than that obtained by an i.v. injection. I.p. injection of FGG that contained readily visible aggregates gave the most variable results, on some occasions the AFC response to FGG of cultures of cells from mice thus treated, reaching 50% of control values. It was noted that the anti-DNP response tended to be higher in cultures of spleens from mice injected with some preparations of aggregated FGG, as suggested in the results shown in Table III, exp. II.

**TABLE III**

| Experiment number | Treatment of donor mouse | AFC/culture |
|-------------------|--------------------------|-------------|
|                   |                          | Anti-FGG    | Anti-DNP    |
| I                 | 7.5 mg FGG i.v.*         | 0           | 231 ± 35    |
|                   | Control                  | 276 ± 52    | 196 ± 21    |
| II                | 7.5 mg FGG i.p.†         | 93 ± 60     | 400 ± 65    |
|                   | Control                  | 350 ± 85    | 290 ± 65    |

Nu/nu mice in groups of three were injected with (*)FGG from a deaggregated preparation that had stood in the refrigerator (4–8°C) for 2 wk, or (†) FGG which was from the bottom fraction of a solution of FGG after ultracentrifugation and which had been stored frozen. In exp. I, the spleens were taken 24 h after injection of FGG and the cells cultured for 3 days with FGG (100 μg/ml), POL 10 (μg/ml), and DNP-POL (1 μg/ml). In exp. II, the spleens were taken on the 3rd day after injection and cultured with antigens as above for 4 days. Similar results were obtained in other experiments where these variables were tested using periods between injection and culture of from 1 to 9 days.

The Kinetics of the Induction of Unresponsiveness In nu/nu Spleen.—To determine how long was needed after the in vivo injection of FGG for unresponsiveness to become apparent, and to investigate the duration of the effect, groups of mice were injected i.v. with 7.5 mg of deaggregated FGG at varying times before sacrifice and culture of spleen cells. Fig. 1 demonstrates the pooled results of several experiments, in each of which two to four time points were examined. It is apparent that unresponsiveness is induced relatively rapidly, not being evident at 6 h but being reproducing completely by 24 h. The persistence of this effect is still under study; however, at least until 11 days, unresponsiveness is complete.

The Dose of FGG Required for the Induction of Unresponsiveness.—The effect of reducing the amount of FGG injected i.v. was investigated. Basically it was
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found that 5 mg was required to consistently induce unresponsiveness, amounts less than 1 mg being ineffective. Fig. 2 shows the effect of varying the dose of FGG, given i.v. 5 days before in vitro challenge.

Susceptibility of the Unresponsive State to Breakage by Allogeneic Confrontation.—There are several reports of states of specific unresponsiveness being terminated by confrontation with allogeneic cells (17). Therefore, the effect of adding to unresponsive spleen cells a source of T cells which recognize the nu/nu cells as allogeneic was investigated. Spleen cells from CBA mice contain T cells that recognize as allogeneic the nu/nu spleen cells obtained from members of the Hall Institute nude mouse colony (6, 18). Previously it has been shown that the release of factors amplifying AFC responses is not affected by pretreating the CBA spleen cells with doses of mitomycin C that inhibit thymidine uptake (18). As would be expected the mitomycin C-treated CBA spleen cells do not themselves contribute to an anti-FGG AFC response (18). Fig. 3 shows a typical result. In no experiment was there any evidence that the unresponsiveness was abrogated by confrontation by allogeneic cells, while in control, nontolerant cultures, the expected amplification effect was noted.
TOLERANCE INDUCTION IN ATHYMIC MICE

Fig. 2. The dose of FGG required for the induction of unresponsiveness. Deaggregated FGG was injected i.v. in the indicated doses. 5 days later spleen cells were challenged in vitro with FGG (100 μg), POL (10 μg); and DNP-POL (1 μg). The data from two experiments is shown; each point represents the mean responses of four or eight cultures. The anti-DNP responses were equal in all groups.

The Effect on the Induction of the Unresponsive State of the Simultaneous Injection of POL with FGG.—It has been proposed that POL provides a "second signal" required for the activation of B cells by FGG when T cells are not present (10). Therefore, it was of interest to see whether the induction of the unresponsive state in vivo could be prevented by giving POL together with the deaggregated FGG. Fig. 3 illustrates the results of one typical experiment that demonstrated that giving POL with i.p. injected FGG, blocked the induction of unresponsiveness. In other experiments of similar design POL, 10 μg i.p., also substantially reduced the specific unresponsiveness induced by the i.v. injection of FGG.

The Effect of Delaying the Injection of POL after Pretreatment with FGG.—To determine how long an exposure to antigen in vivo was required before unresponsiveness was no longer reversible by the addition of POL, a large group of nu/nu mice was injected with FGG, 7.5 mg i.v. Mice were then injected in groups of three with POL, i.p. 10 μg, either within 5-10 min or at intervals thereafter. After 2–6 days the mice were killed and the spleen cells cultured with FGG, POL, and DNP-POL. As shown in Table IV, the injection of POL 24 h after the injection of FGG did not reverse the unresponsiveness. This ob-
Fig. 3. The abrogation of tolerance induction by the administration of POL with FGG and the lack of effect of allogenic confrontation on the unresponsiveness to FGG. Groups of nu/nu mice were injected i.p. with 5 mg FGG or with 5 mg FGG i.p. together with 10 μg POL i.p. 5 days later the mice were sacrificed and portions of spleen cell suspensions were challenged with FGG, POL, and DNP-POL. The anti-FGG responses of these groups together with that of spleen cells from untreated mice are shown by the solid columns. Anti-DNP responses were equivalent in these groups. Portions from each of these three spleen cell suspensions were also cultured with $3 \times 10^6$ mitomycin C-treated (40 μg/ml for 30 min at 37°C) CBA spleen cells together with FGG, POL, and DNP-POL. The anti-FGG responses in the presence of CBA spleen cells are shown by the open columns. Anti-DNP responses were equivalent in these groups.
The Effect of the Injection of POL on the Induction of Unresponsiveness by FGG

| Pretreatment of donor | Time to POL injection | Anti-FGG AFC/culture |
|----------------------|-----------------------|----------------------|
| -                    | -                     | 212 ± 24             |
| +                    | -                     | 10 ± 35              |
| +                    | 10 min                | 183 ± 60             |
| +                    | 4 h                   | 213 ± 70             |
| +                    | 10 h                  | 264 ± 85             |
| +                    | 24 h                  | 63 ± 25              |

Groups of three nu/nu mice were injected i.v. with 7.5 mg of FGG. At the indicated intervals, POL (10 μg) was injected i.p. 4 days later the mice were killed and the spleens cells cultured for 3 days with FGG (100 μg), POL (10 μg), and DNP-POL (0.1 μg). The data shown represent the pooled results of three experiments.

Clearance of FGG in the Presence of POL.—One possible explanation for the effect of POL in abrogating tolerance induction by FGG was that it enhanced removal of FGG from the extracellular fluid, perhaps by stimulating the reticuloendothelial system. This point was approached by investigating the clearance from the blood of 125I-labeled FGG, in the presence or absence of an i.p. injection of POL. Fig. 4 shows that the clearance of FGG in the first 24 h in these two situations. It can be seen that there was no significant difference between the two groups. Thus POL did not alter the amount of FGG present in the extracellular fluid during the first 24 h period that is critical for tolerance induction. It should be noted that in both cases the FGG is cleared rapidly, its half-life being about 7 h, comparable with the 11 h reported for another avian gamma globulin in the mouse (19).

In Vivo Challenge of FGG-Injected nu/nu mice.—Nu/nu mice respond in vivo to FGG provided that POL is injected also (unpublished data). Therefore it was possible to challenge the capacity of nude mice to mount an anti-FGG response in vivo after pretreatment with large doses of FGG. Nu/nu mice were treated with FGG, 7.5 mg i.v., and after 3-17 days were challenged with FGG, 100 μg, plus POL, 10 μg. After 3 or 4 days at which time the anti-FGG response in nude mice thus challenged is maximal (unpublished data), the mice were killed and the direct and enhanced anti-FGG plaque-forming cells per spleen were assayed. Table V shows the results of a typical experiment demonstrating that the deficit in anti-FGG response in FGG preinjected nu/nu mice is apparent on in vivo challenge also.
FIG. 4. The lack of effect of POL on the clearance of FGG from the blood. Nu/nu mice in two groups of three were injected i.v. with 5 mg of $^{35}$S-trace-labeled FGG. One group was injected i.p. with POL, 10 μg (○), 5 min later. The data shown represent counts per 10 s per unit of blood, as described above.

TABLE V
The Induction of Unresponsiveness by i.v. FGG to In Vivo Challenge with FGG Plus POL

| Pretreatment interval | Direct anti-FGG AFC/spleen |
|-----------------------|-----------------------------|
| days                  |                            |
| 8                     | $42 \pm 40$                |
| 17                    | $350 \pm 100$              |
| —                     | $3050 \pm 600$             |

Nu/nu mice in groups of three were injected i.v. with FGG 7.5 mg at the indicated intervals before they were challenged together with a group of untreated mice (*) with FGG (100 μg) plus POL (10 μg) i.p. 3 days later the mice were killed and direct and indirect anti-FGG AFC per spleen were assayed. No indirect AFC were detected in this or in other similar experiments.

DISCUSSION

The present results show that B-cell tolerance to a thymus-dependent antigen can be induced in the absence of T cells. The mechanism of the functional
elimination of specific B-cell clones is not clear. A simple blockage of the receptors by the high concentration of antigen would seem to be unlikely. If this were so the addition of POL at either the in vivo or in vitro stage of the experiment would be expected to produce B-cell activation, since, in the presence of POL, B cells respond in vitro to deaggregated FGG at a concentration up to at least 1 mg/ml (9). The fact that the addition of POL in vivo prevented the induction of unresponsiveness only at an early stage in tolerogenesis (Table IV) points to the B cell undergoing some definite irreversible change. The lack of reversal of the tolerance by allogeneic confrontation (Fig. 3) is a further indication that the FGG-specific B cells may have disappeared. This would be consistent with the situation reported by Louis et al. (20) where B-cell tolerance that had been induced by a high dose of deaggregated antigen was accompanied by a loss of antigen-binding cells in the spleen.

Much interest has centered on the importance of epitope density in tolerance induction, a high density of determinants putatively favoring tolerance induction (21). In the case of simple protein antigens, it has been proposed that antigen-antibody complexes may supply the required epitope density (21). This mechanism is unlikely to be relevant in the present model, firstly, since there is no detectable antibody formation with the injection of FGG alone into the nude mouse (unpublished data); secondly, since the amount of antigen injected is relatively large; and thirdly, since the tolerance is established within 24 h, before antibody synthesis would be underway.

As FGG is a globulin, there is the possibility that binding to Fc receptors on lymphocytes (22) or macrophages (23) could be producing a matrix of determinants, presumably those on the Fab end of the molecule. However the anti-FGG antibodies detected in the plaque assay are most likely directed against the Fc end of the molecule, the Fab portion being bound to the indicator SRC. In addition DNPl-bovine serum albumin is effective in this system, in inducing unresponsiveness to DNP (unpublished data). A final point, with regard to the physical configuration required for tolerance induction, is that FGG is made up of two identical heavy chains and two identical light chains, and therefore it is possible that a given B cell could recognize two identical epitopes on the one molecule. On the evidence available then, a simple interaction of the B cell with antigen may suffice for tolerance induction; the present experiments however cannot exclude that two determinants on a molecule need bind to the B cell.

What are the implications of the basic observation that B-cell tolerance to a thymus-dependent antigen can be induced in the absence of T cells? Firstly these experiments point to an alternative to T-cell-dependent tolerance mechanisms (3, 4) at the level of the B cell. Secondly, they illustrate a simple mechanism for the maintenance of self-tolerance of the nude mouse to its serum proteins. Teleologically it would seem very desirable that B cells, as well as T cells (24), be self-tolerant since it is clear that ubiquitous substances such as endotoxin (25, 26) and products from activated macrophages (18), which could
in theory be stimulated by infection or inflammation, allow B cells to respond to thymus-dependent antigens in the absence of activated, specific T cells. Thus tolerance existing solely at the T cell level could be broken (27).

Miller et al. have reported that they were unable to tolerize B cells using a high dose of FGG in normal CBA or C57 X CBA mice (28). A comparison with the ease of B-cell tolerance induction in athymic mice that was demonstrated in the present study, suggests that T cells may in fact protect B cells from tolerance induction. A difference is especially obvious where there is some degree of aggregation of the antigen, in which case CBA mice are primed while nude mice tend still to be tolerized (J. W. Schrader, manuscript in preparation). One explanation is that the T cell is normally involved in the provision of a second signal (8, 9, 18), that diverts the B cell from becoming tolerant upon encounter with antigen. Tolerization of the B-cell population might await a virtually complete functional deletion of specific T cells, an hypothesis compatible with the observations of Weigle and colleagues on the relative kinetics of induction of tolerance in T and B cells (2). Experiments demonstrating B-cell tolerance to determinants on molecules or homologous erythrocytes which cannot activate specific T cells (29, 30), support the concept that the interaction of antigen with the B cell in the absence of a second signal may lead to tolerance. Thus the results of Miller et al. who found no evidence for B-cell tolerance using FGG in CBA mice (28), may be the result of the incompleteness of tolerance at the T-cell level which can be seen in the ability of thoracic duct lymphocytes from "tolerant" mice to collaborate with FGG-primed B cells (28).

The present model does not explain tolerance to thymus-independent antigens like POL, endotoxin, or pneumococcal polysaccharide. Rather it makes tolerance to them paradoxical since these substances seem to be good adjuvants and B-cell stimulants (9, 25, 26, 31), and endotoxin (32), and now POL, have been shown to prevent the induction of tolerance to thymus-dependent antigens. It is necessary to postulate that a different mechanism is involved in tolerance to thymus-independent antigens. Recently we have reported new evidence to suggest that highly multivalent, thymus-independent antigens such as POL or DNP-POL may cause a substantial diminution of the rate of antibody secretion by single AFC, a phenomenon we have termed effector cell blockade. We have suggested that some forms of nonreactivity mediated by multivalent antigens or antigen-antibody complexes might really represent effector cell blockade rather than the suppression of the potential for B-cell activation.

SUMMARY

Specific immunological unresponsiveness was induced using thymus-dependent antigens in congenitally athymic (nu/nu) mice, in which no T-cell

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8 Schrader, J. W., and G. J. V. Nossal. 1974. Effector cell blockade: a new mechanism of immune hyporeactivity induced by multivalent antigens. \textit{J. Exp. Med.} \textbf{139}: in press.
function has been demonstrated. The tolerance was induced in vivo by the
injection of 5–10 mg of either FGG or DNP-HGG.

Spleen cells from treated mice were tested in vitro for the ability to mount
thymus-independent immune responses against FGG in the presence of polymer-
erized flagellin POL, and the DNP determinant conjugated to POL. A specific
deficiency in either the in vitro anti-FGG or anti-DNP response was demon-
strated, depending on the antigen used for treatment of the spleen cell donor.
Athymic mice treated with FGG were also tested by in vivo challenge with
FGG given with POL as an adjuvant and were found to be hyporesponsive.

Unresponsiveness to in vitro challenge was established by 24 h after
the in vivo injection of FGG. It was found that the injection of POL with the
FGG prevented the development of unresponsiveness, but not if the POL was
given 24 h or more after the FGG.

The unresponsiveness could not be overcome by confrontation with allogeneic
spleen cells from CBA mice, although the presence of allogeneic spleen cells had
a large amplifying effect on the response of control spleen cells. These experi-
ments demonstrate a mechanism for the tolerization of bone marrow-derived
cells by thymus-dependent antigens in the absence of the thymus.

Mr. John Pye prepared the POL and performed the radioiodinations. Professor G. J. V.
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