TERMINATION OF TOLERANCE TO HUMAN GAMMA GLOBULIN IN MICE BY ANTIGEN AND BACTERIAL LIPOPOLYSACCHARIDE (ENDOTOXIN)*

BY JACQUES M. CHILLER† AND WILLIAM O. WEIGLE‡

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

(Received for publication 26 October 1972)

The antibody response to a number of antigens requires the cooperation of at least two specific cell types, namely, thymus-derived lymphocytes (T cells), which have helper functions necessary for the stimulation of bone marrow-derived lymphocytes (B cells), which are the direct precursors of antibody-producing cells (1). Each of the cell types possesses specificity for a given antigen since each can be made specifically tolerant (2) and since cellular suicide resulting from treatment with radioactively labeled antigen (3) leads to the abolishment of specific immunological activity in each cell type (4-6).

Although both T cells and B cells can be rendered specifically unresponsive to an antigen, the kinetic pattern of induction, maintenance, and spontaneous loss of tolerance for each is essentially different (7). T cells become tolerant rapidly and remain so for a prolonged period whereas the induction of tolerance in B cells requires a greater latent period, and the state is maintained for a shorter time. B cells, in addition, require a higher dose of antigen for the induction of tolerance than do T cells. Therefore, in the case of an antigen for which the immune response requires T-B cell cooperation, unresponsiveness in either one of the two cell populations is sufficient to make the whole animal appear tolerant.

These data may be extrapolated to form a model for the cellular basis of the naturally unresponsive state to those self-antigens present in low concentration within the circulation. The hypothesis would be that in these situations, specific T cells are unresponsive whereas specific B cells are not, and antibody formation cannot occur because of the functional T cell deficiency. Accordingly, any event or agent capable of rendering the immune response to an antigen independent of the required T cell coop-
eration would be expected to allow the induction of antibody formation to the tolerant antigen. Under certain conditions, such an event might even be expected to result in autoimmunity, since this phenomenon may be viewed as the termination of a state of natural tolerance to self-antigens (8).

Bacterial endotoxins are substances whose biological effects on immunological processes may be ideally suited to permitting a response to a variety of antigens in the absence of a specific T helper function. This prediction is based on experimental evidence demonstrating that endotoxins (a) have a mitogenic effect that is restricted to the B cell population (9, 10), (b) can substitute for T cell function in the antibody response to sheep erythrocytes, obtained both in vivo (11, 12) and in vitro (13), and (c) can bypass the requirement for T cells in a hapten carrier system (14).

The present study will detail that, in mice, endotoxin (ET) also possesses the capacity to terminate a state of induced tolerance to human gamma globulin (HGG). This phenomenon can be observed only at a time when B cell tolerance has been predictably lost. Moreover, it appears that during the spontaneous return from an induced unresponsive state, there exists in the tolerant animal a heightened antibody-forming potential, revealed by the present experimental approach.

Materials and Methods

Mice.—A/J male mice, 5 wk old, were purchased from the Jackson Laboratory (Bar Harbor, Maine). They were housed in groups of five each in covered cages and maintained on Purina Chow pellets (Ralston Purina Co., St. Louis, Mo.) and chlorinated acidified water (15) ad libitum.

Antigens.—HGG was obtained through the courtesy of the American Red Cross and prepared as Cohn Fraction II by E. R. Squibb & Sons (New York). Before use, IgG was purified on DEAE-cellulose chromatography by elution with 0.01 M phosphate buffer, pH 8.0. DEAE-purified IgG was rendered monomeric by ultracentrifugation (DHGG) at 116,000 g for 150 min in a swinging bucket rotor (16). The material so prepared will be referred to as a tolerogen and its immunological behavior, as tolerogenic (17). To induce tolerance, 6-wk-old mice were injected with 2.5 mg of the tolerogen given intraperitoneally in a volume of 1 ml. This protocol assures the induction of a complete, specific, and long-lasting state of unresponsiveness in A/J mice (18).

DEAE-purified IgG was aggregated (AHGG) by heating at 63°C for 25 min and precipitating with Na2SO4 (16). The material so obtained evokes an excellent antibody response without the need of an adjuvant. It will be referred to as an immunogen and its immunological behavior, as immunogenic.

Endotoxin.—The ET used was the polysaccharide of Escherichia coli 0111:B4 (lot B35527) obtained from Difco Laboratories (Detroit, Mich.). It was diluted to the desired concentration in 0.15 M NaCl.

Preparations of “B Cell Mice.”—Adult mice were thymectomized (19) and 2-4 wk later were lethally irradiated (1,000 R) using a GammaCell 40 irradiator (Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada) with a cesium-137 source that emitted a central dose of 115 R/min. 4-6 h later, each animal was given either 15 X 106 or 50 X 106 bone marrow cells that had been pretreated with anti-0 serum and guinea pig complement (20). The animals were used for experimental purposes a minimum of 30 days after reconstitution.

Hemolytic Plaque Technique.—Antibody-forming cells to HGG or ET were enumerated using modifications of the Jerne plaque assay (21) in which either protein antigens (22) or ET
(23) were coupled to goat erythrocytes (Colorado Serum Co., Denver, Colo.). Indirect plaque-forming cells (PFC) were developed with squirrel monkey antimouse gamma globulin used at a concentration previously determined to be optimal in this assay.

RESULTS

Effect of ET on the Thymic Dependency of the Antibody Response to HGG in Mice.—In mice, the antibody response to HGG is exquisitely dependent on the cooperation between T and B cells. This is best illustrated by the following experiment. Mice that as adults were thymectomized, irradiated, and reconstituted with T cell-deprived syngeneic bone marrow did not respond to an immunogenic challenge of AHGG unless they were given a source of syngeneic thymocytes (Table I). On the other hand, mice that were similarly deprived of T cells did respond to HGG if, in addition to the antigen, they also received ET (Table II). It should be pointed out that although the response obtained in Table I is greater than that seen in Table II, a quantitative comparison between the two is not valid, inasmuch as each set of animals received a different inoculum of bone marrow cells: 50 × 10⁶ and 15 × 10⁶, respectively. Nevertheless, these experiments clearly demonstrate that the thymus dependency of the response in mice to HGG can be overcome by the use of ET.

Termination of Tolerance with Immunogen and ET.—The capacity of ET to render the response to HGG independent of T cells should allow the termination of a tolerant state when tolerance is maintained solely by the unresponsiveness of a specific T cell population, a cellular state that exists in animals 50 or 60 days after the single injection of a tolerogenic preparation of HGG. This prediction was fulfilled, as seen in the following experiment. Normal

| Treatment | No. of animals | PFC/10⁶ spleen cells |
|-----------|----------------|---------------------|
| ATX*      | 10             | 0.1                 |
| 50 × 10⁶ BM‡ |                | 0.2                 |
| AHGG§     |                |                     |
| ATX       | 10             | 0.6                 |
| 50 × 10⁶ BM|                | 169.3               |
| 90 × 10⁶ T‖|                |                     |

* Adult thymectomy, followed 3 wk later by 1,000 R whole body irradiation.
‡ Syngeneic bone marrow cells treated with anti-δ serum and complement.
§ Aggregated HGG given intravenously (i.v.) on day 0, intraperitoneally (i.p.) on day 10; PFC determined 5 days after the last injection.
‖ Syngeneic thymocytes.
mice or mice injected with 2.5 mg of tolerogen 90 days previously were each given one of the following three regimens: (a) 50 μg of ET intravenously on day 0, 25 μg of ET intravenously on day 6; (b) 400 μg of AHGG intravenously on both days 0 and 6; (c) 400 μg of AHGG + 50 μg of ET intravenously on day 0 and 400 μg of AHGG + 25 μg of ET intravenously on day 6. 5 days after the second injection, each group was assayed for PFC specific to HGG.

It can be seen in Table III that the combination of both the immunogen AHGG and the ET does in fact terminate the tolerant state, although either one of these substances given alone does not. It is both striking and significant that the response so obtained in tolerant mice is higher than that observed in normal animals that had received AHGG, either alone or with ET.

The response to HGG obtained in the tolerant mice is specific to HGG and is not due to an immunological response to ET producing a cross-reaction in the assay system used to detect anti-HGG PFC. This is made clear by the data seen in Table IV, in which the response of tolerant mice given AHGG and ET was assayed for PFC to HGG or to ET and the specificity of antibody-forming cells was verified by inhibition experiments. In all three examples, spleens from tolerant mice contained both PFC to HGG and PFC to ET. However, PFC to HGG were inhibited with HGG, but not ET, when either was incorporated as free antigen in the agar. Similarly, PFC to ET were inhibited with ET, but not with HGG.

**Termination of Tolerance with Immunogen and ET. Demonstration of Hyper-immune Potential in Tolerant Animals.**—A more striking example of the heightened response obtained in tolerant animals given AHGG and ET is seen in Table V. Mice that had received 2.5 mg of DHGG 143 days previously were injected twice with either AHGG or AHGG and ET, by the protocol described in Table IV. The response of tolerant animals that received both AHGG and ET was some eightfold greater than that obtained in normal animals that had been similarly challenged. On the other hand, tolerant animals given only HGG were fourfold less responsive than their normal counterparts.
TABLE III

Termination of Tolerance in Mice with a Combination of AHGG and ET

| Group          | Treatment* | No. of mice | Indirect PFC/10^6 spleen cells |
|----------------|------------|-------------|-------------------------------|
| Normal         | ET‡        | 6           | 0.2                           |
| Normal         | AHGG§      | 6           | 44.5                          |
| Normal         | AHGG + ET  | 6           | 54.1                          |
| Tolerant†      | ET         | 6           | 0.3                           |
| Tolerant       | AHGG       | 6           | 9.3                           |
| Tolerant       | AHGG + ET  | 6           | 93.8                          |

* Each group received the first injection (0.3 ml) on day 0 and the second injection (0.15 ml) on day 6; both were given i.v., and PFC specific to HGG were determined 5 days after the last injection.
‡ 50 and 25 µg in the first and second injections, respectively.
§ 400 µg for both first and second injections.
|| Mice used 90 days after the injection of 2.5 mg of DHGG.

TABLE IV

Specificity of PFC Obtained to HGG in Tolerant Mice after the Injection of AHGG and ET

| Animal no.* | Inhibitor† | Indirect PFC/spleen |
|-------------|------------|---------------------|
|             |            | To HGG   | To ET    |
| 1           | None       | 14,450   | 19,484  |
|             | HGG        | 0        | 20,428  |
|             | ET         | 14,922   | 0       |
| 2           | None       | 12,790   | 1,635   |
|             | HGG        | 96       | 1,694   |
|             | ET         | 12,743   | 93      |
| 3           | None       | 15,608   | 4,394   |
|             | HGG        | 0        | 4,518   |
|             | ET         | 16,228   | 0       |

* Mice treated 90 days previously with 2.5 mg of DHGG were injected i.v. with 400 µg of AHGG + 50 µg of ET on day 90 and 200 µg of AHGG + 25 µg of ET on day 96. Splenic PFC to HGG or ET were determined 5 days after the last injection.
† Added at a final concentration of 7.5 µg/ml in the agar.

In addition, the data presented in Table V demonstrate that the ability of the dual treatment with immunogen and ET cannot terminate a state of tolerance in mice 25 days after the injection of the tolerogen. These data are in keeping with the fact that at this time B cells are tolerant, so that the ability

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of ET to allow a bypass of T cell helper activity is inconsequential in the absence of specific B cell function.

Before and during the experiments presented above, it had been observed that the immunogenic challenge of adoptively transferred spleen cells obtained from mice long after tolerogen injection (> 100 days) sometimes resulted in responses twofold to sixfold higher than those obtained with the similar transfer of spleen cells from normal animals. One such experiment is represented in Table VI. Spleen cells (75 × 10⁶) obtained from either normal mice or mice treated with 2.5 mg of DHGG 120 days previously were injected intravenously into lethally irradiated (1,000 R) syngeneic hosts. At this time, and again 10 days later, each recipient was challenged with 400 μg of AHGG given intravenously and intraperitoneally, respectively; and 5 days after the second injection, they were assayed for splenic PFC specific to HGG. It can be seen

| Table V |
| Comparison of the Effect of ET on the Response of Normal Mice 25 and 143 Days after Tolerogen Injection |
|---|---|---|---|
| Group | Treatment* | No. of mice | Indirect PFC/spleen |
| Normal | AHGG‡ | 7 | 7,314 |
| Normal | AHGG + ET§ | 7 | 8,856 |
| Day 25 tolerant¶ | AHGG | 7 | 502 |
| Day 25 tolerant | AHGG + ET | 7 | 788 |
| Day 143 tolerant¶ | AHGG | 6 | 1,803 |
| Day 143 tolerant | AHGG + ET | 6 | 60,480 |

* Each group received the first injection (0.3 ml) on day 0 and the second injection (0.15 ml) on day 6; both were given i.v. and PFC specific to HGG were determined 5 days after the last injection.

‡ AHGG, 400 μg for first injection and 200 μg for second injection.
§ ET, 50 μg for first injection and 25 μg for second injection.
¶ Mice used 25 days after the injection of 2.5 mg of deaggregated HGG.
¶ Mice used 143 days after the injection of 2.5 mg of deaggregated HGG.

| Table VI |
| Hyperresponsiveness in Adoptively Transferred Spleen Cells Obtained from Mice 120 Days after the Injection of Tolerogen |
|---|---|---|---|
| Group | No. of mice | Indirect PFC/10⁶ spleen cells | Ratio (tolerant/normal) |
| Tolerogen-treated spleen cells* (75 × 10⁶) | 11 | 206 | 6.1 |
| Normal spleen cells (75 × 10⁶) | 9 | 34 | |
| Tolerogen-treated donors | 8 | 31 | 0.14 |
| Normal donors | 8 | 227 | |

* 2.5 mg of DHGG.
that under those experimental conditions, spleen cells from tolerogen-treated
animals responded six times better than did normal spleen cells. In contrast,
animals from the same donor groups challenged twice with immunogen be-
haved as might be expected, that is, their response was seven times less than
that of normal mice.

**DISCUSSION**

The ability of bacterial ET to induce responsiveness to antigens in the ab-
sence of a specific T cell helper function also allows the immunological circum-
vention of a state of tolerance for which the cellular basis is restricted to spe-
cific T cell unresponsiveness. In the present studies it has been demonstrated
that in mice, the T cell-dependent antibody response to HGG can be made T
cell independent if, in addition to immunogen, ET is part of the injection
protocol. In the same manner, the state of induced tolerance to HGG, present
at a time when B cells have lost their unresponsiveness, can be terminated by
the injection of both aggregated HGG and ET, but by neither given alone.
Furthermore, there is no effect on HGG tolerance when this regimen is followed
at a time when B cells are still tolerant.

The mode of action by which ET can substitute for T cell helper function is
not known. It may be that the B cell mitogenic activity of ET (9, 10) coupled
with the specific interaction of antigen to B cell receptors is sufficient to initiate
cellular differentiation and proliferation, processes that normally antigen
alone cannot mediate without T cell help. The adjuvant action of antibody
formation attributable to ET (24) may be partly dependent on this mitogenic
effect. The potent immunogenicity of *Salmonella* O antigen, which, as Nossal
and Ada (25) point out, is 11 orders of magnitude greater than that of serum
protein antigens and 7 orders greater than that of pneumococcal polysaccha-
drides, may also be related to the presence of a mitogenic moiety that, if coupled
to an antigenic determinant on the same molecule, is capable of specific cell
stimulation at an extremely low threshold of antigen concentration.

The present data, therefore, provide evidence for a mechanism by which
induced tolerance maintained by the functional absence of specific T cells
may be readily terminated. Other pathways for the early termination of
tolerance have been described previously; all appear dependent on the ability
to bypass the block created by specific T cell tolerance. For example, the ability
to terminate tolerance with either altered (26) or cross-reacting albumins (27)
is most probably due to the participation of nontolerant T cells stimulated by
unrelated determinants on the altered or cross-reacting molecule. The same
phenomenon can be more directly demonstrated by the ability to obtain a
specific response in a tolerant animal when the tolerated antigen is coupled to a
nontolerated carrier (28). The cellular basis for these phenomena is probably
identical with that operational in the ability to overcome genetic unrespons-
siveness to a variety of determinants by coupling these to functional carrier
molecules (29). It is of interest that antibody formation to HGG in a low-responding strain can be elevated by the use of ET to the level obtained in a high-responding strain, a phenomenon in accord with the finding that the frequency of specific antigen-binding cells (B cells) is identical in both strains.

The hyperresponsiveness to HGG observed in spleen cells after the loss of tolerance in B cells is not clearly understood, although the paradox of heightened reactivity to antigenic challenge after recovery from tolerance has been well documented previously (30, 31). Theoretical considerations of the present studies must incorporate the observations that during the recovery from tolerance (a) the injection of antigen plus ET can terminate tolerance and even produce hyperresponsiveness, (b) the injection of syngeneic cells and/or antigen can do neither, (c) the adoptive transfer of tolerant cells and antigen into irradiated recipients sometimes results in a much greater response than do transferred normal cells, and (d) thymus cells remain unresponsive (7).

It may be that in the animal, a level of tolerogen below the threshold needed to induce tolerance in newly differentiating clones of B cells may be able to prime those cells by a molecular model analogous to that proposed by Diener and Feldmann (32). They postulated that the difference between the immunogenic and tolerogenic properties of antigenic determinants is a function of epitope density. For example, the degree of substitution of the dinitrophenol (DNP) hapten per unit of monomeric flagellin affects the in vitro response to DNP, in that lightly substituted flagellin induces antibody formation whereas heavy substitution induces a specific unresponsiveness (33). A priming effect of precursor cells by subtolerogenic quantities of HGG may induce differentiation and/or proliferation of specific cells whose commitment to antibody formation would still require a T cell helper function (34). However, the antibody-forming potential could be revealed by the ability of ET to circumvent the T cell requirement. When such primed cells along with antigen are transferred to a syngeneic irradiated host, their immunological potential may be expressed if T cell tolerance wanes as a result of transfer to a neutral environment or if the recipient animal can provide a minimal quantity of helper function whether it be cellular, cellular factors (35), or even ET released from a gram-negative bacterial infection after postirradiation stress. Since, in this irradiation environment, a selective advantage for the replication of specifically stimulated cells (36) relative to nonprimed cells could be expected, activated primed cells might be expected to be amplified, providing the data presented above.

Bacterial ET could theoretically play a role in the circumvention of natural tolerance to self-antigens if the cellular basis for the naturally unresponsive state to self-antigens present in low circulating levels is the same as the cellular basis of tolerance obtained using low doses of antigen to induce tolerance (7). It seems that under these conditions, specific tolerance can be induced and

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maintained in T cells, but not in B cells; and expression of immunity cannot occur only because of functional T cell deficiency. Thus any means that renders the response independent of the cooperation with those specific T cells would be expected to permit specific antibody formation to a self-antigen.

As in those studies described above for the termination of induced tolerance to serum albumins, the natural tolerance to rabbit thyroglobulin in the rabbit can be terminated by injecting immunologically cross-reacting thyroglobulins (37), chemically modified thyroglobulins (38), or complexes of heterologous antibody and rabbit thyroglobulin (28). These data support the hypothesis that thyroiditis may be induced because the circulating concentrations of thyroglobulin that reach immunocompetent B cells are too low to induce a tolerant state in those cells, although they are sufficient to render specific T cells tolerant. The presence of immunocompetent B cells specific for autologous thyroglobulin in mice (39) is compatible with this conclusion.

Inasmuch as the physiological state of restricted cell tolerance to certain self-antigens mirrors what occurs after the spontaneous loss of B cell tolerance in mice to HGG, it may be subject to the same ET-mediated termination described in the present studies. This is not to imply that the immunological consequences of a gram-negative infection will always result in autoimmunity, but rather to suggest that under the appropriate temporal release of sufficient self-antigen and bacterial products, the homeostatic balance of self-tolerance could be altered.

**SUMMARY**

Bacterial lipopolysaccharides (endotoxin) allowed the circumvention of the thymus-derived (T) cell helper function otherwise required for the antibody response in mice to human gamma globulin (HGG). In an analogous fashion, the state of tolerance to HGG, existing at a time when bone marrow-derived (B) cells had lost their unresponsiveness, could be terminated by the injection of both immunogenic HGG and endotoxin, but by neither given alone. However, no effect on tolerance to HGG could be observed when this regimen was followed at a time when B cells were tolerant. After the spontaneous recovery from tolerance in B cells, it seemed that specific priming was occurring in that population. This phenomenon was observed either by the injection of endotoxin and HGG or by the adoptive transfer of cells into irradiated hosts. These data have been discussed in the light of potential autoimmune manifestations that could theoretically follow a simultaneous gram-negative bacterial infection along with a release of self-antigen.

We thank Ms. Sandra Cossentine for her excellent technical assistance and Dr. Gail S. Habicht for helpful criticism of the manuscript.

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