FATE OF ANTIGEN-BINDING CELLS IN UNRESPONSIVE AND IMMUNE MICE*

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The biological events that occur in cells after contact with tolerogen are still largely unknown, and Burnet's thesis (1) that the deletion of such specific cells may be a prerequisite for a state of immunological unresponsiveness remains without substantial experimental evidence. Binding of highly radioactive labeled antigen to specific lymphocytes as visualized by means of autoradiography has provided a method of searching for specific antigen-binding cells (ABC)[1] in immunologically unresponsive animals. On the one hand, Ada et al. (2) and Cooper et al. (3) found a normal complement of ABC to flagellin and hemocyanin in animals unresponsive to these antigens; and Humphrey and Keller (4), using hemocyanin and the synthetic polypeptide TIGAL as antigens, also concluded that unresponsive animals possessed a number of ABC similar to that seen in normal mice. On the other hand, Naor and Sulitzeanu (5) and Katz et al. (6) found a decrease in specific ABC in animals unresponsive, respectively, to bovine serum albumin (BSA) and the dinitrophenol (DNP) hapten.

In the present report, the fate of ABC was investigated kinetically in a system of immunologic unresponsiveness in which a complete, long-lasting, and specific tolerant state can be induced, namely, unresponsiveness in adult mice to monomeric gamma globulin. Since the cellular events functional in the induction process of this unresponsive state have been described (7), they provide a basis on which to evaluate the morphological data obtained by the technique of antigen binding.

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[1] *Abbreviations used in this paper:* ABC, antigen-binding cells; AHGG, aggregated human gamma globulin; B cell, bone marrow-derived cell; BM cell, bone marrow cell; BSA, bovine serum albumin; DHGG, deaggregated human gamma globulin; DNP, dinitrophenol; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin; MEM, minimal essential medium; T cell, thymus-derived cell; TGG, turkey gamma globulin.

Materials and Methods

Animals.—A/J male mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. They were 8–10 wk of age at the time of use.

Antigen: Tolerogen and Immunogen.—Human gamma globulin (HGG, Cohn fraction II) was obtained through the courtesy of the American Red Cross. In a manner previously described (8), DEAE-purified HGG was employed for the preparation of either deaggregated HGG (DHGG) for use as the tolerogenic material or aggregated HGG (AHGG) for use as the immunogenic material.

Labeling of Antigen.—HGG was iodinated using the chloramine-T method (9) in microvolumes (10). 2–4 mCi of ^{125}I (carrier-free [^{125}I]Na, Cambridge Nuclear Corp., Cambridge, Mass.) in 50 μl was neutralized to pH 7.0 by 25 μl of 0.1 M NaH_2PO_4. 40 μl of a solution of HGG (250 μg/ml) and 5 μl of chloramine-T (5 mg/ml) were added to the ^{125}I solution, and the mixture was allowed to react for 5 min. After this, 5 μl of sodium metabisulfite (5 mg/ml), 1 drop of 1% potassium iodide, and 0.5 ml of fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) were added to the mixture. In order to remove free iodine, the solution was passed through a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).

Preparation of Cells.—Spleen, bone marrow, and thymus were collected in minimal essential medium (MEM) containing 10% fetal calf serum and prepared as single-cell suspensions as described elsewhere (8).

Autoradiography.—The technique of Davie and Paul (11) was used, with minor modifications. Lymphoid cells (20 × 10^6) were suspended in MEM (200 μl) containing 10% fetal calf serum and sodium azide (15 mM). [^{125}I]HGG (100 ng in 10 μl, with a specific activity of 50–70 μCi/μg) was added to the cell suspension for 30 min. The cells were then layered over 5 ml of fetal calf serum and harvested by centrifugation at 180 *g* for 10 min. This last step was repeated three times. All the preceding operations were performed at 4°C.

The cells were smeared on methanol-cleaned microscopic slides, fixed in 1% glutaraldehyde, washed in water, and air dried. The smears were dipped in a solution of NTB_2 nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for 1 mo before developing. Before examination, the cells were stained with Giemsa. Only morphologically intact lymphoid cells with 15 silver grains were considered to be ABC.

Inhibition of [^{125}I]HGG Binding by Unlabeled Antigen.—The lymphoid cells were incubated with unlabeled proteins at 4°C for 30 min, harvested by centrifugation through 3 ml of fetal calf serum, suspended in 200 μl of medium, and then incubated with [^{125}I]HGG and processed as described above.

RESULTS

Binding of [^{125}I]HGG to Lymphoid Cells; Specificity.—The number of ABC to HGG found in the spleen of normal A/J mice varied from 9 to 14 in 5,000 cells counted. In the bone marrow this number varied from 10 to 14. The thymus was found to contain a very low number of ABC to HGG. In some experiments no positive cells were found, whereas in others one positive cell was found in 10,000 cells counted.

In order to demonstrate that this binding was specific for HGG, unlabeled HGG or unlabeled non-cross-reacting antigens, such as turkey gamma globulin (TGG) or keyhole limpet hemocyanin (KLH), were incubated with the lymphoid cells before the reaction with [^{125}I]HGG was undertaken. The results of one such inhibition experiment performed with spleen cells are given in Table I. It can be seen that only previous incubation of spleen cells with unlabeled HGG

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was able to inhibit the [^{125}I]HGG binding and that no inhibition was observed with immunologically unrelated and unlabeled proteins.

Table II shows the results of such an inhibition experiment performed with bone marrow (BM) cells. Unlabeled HGG was also found to decrease the [^{125}I]HGG binding by BM cells, but the dose required to inhibit these ABC (2.4 mg) was 6 times greater than that needed to inhibit ABC obtained from spleens. Furthermore, the same amount of TGG, i.e. 2.4 mg, was able to decrease partially the [^{125}I]HGG binding in bone marrow. The paucity of ABC to HGG

TABLE I

Inhibition of [^{125}I]HGG Binding by Lymphocytes from Normal Spleens by Preincubation of the Cells with Unlabeled HGG, TGG, or KLH

Inhibitor	Amount	Inhibition of [^{125}I]HGG binding
	mg	%
HGG	0.3	22
HGG	0.6	67
HGG	1.2	89
TGG	1.2	0
KLH	1.2	11

TABLE II

Inhibition of [^{125}I]HGG Binding by Lymphocytes from Normal Bone Marrow by Preincubation of the Cells with Unlabeled HGG, TGG, or KLH

Inhibitor	Amount	Inhibition of [^{125}I]HGG binding
	mg	%
HGG	0.6	18
HGG	1.2	36
HGG	2.4	76
TGG	2.4	38
KLH	1.2	18

observed in the thymus made such inhibition experiments with cells from this tissue impossible.

Effect of Tolerogen and Immunogen on the Number of ABC in Lymphoid Tissues of Mice.—To study the kinetics of ABC to HGG in lymphoid tissues after various antigenic treatments, mice were injected on day 0 either with a tolerogenic dose of 2.5 mg of DHGG intraperitoneally or with an immunogenic dose of 0.4 mg of AHGG. A group of saline-injected mice served as a control. At various times after injection, the animals were killed by cervical dislocation; and their spleens, bone marrow, and thymuses were assayed for ABC to HGG. Each ABC determination was made four times, each one with a different cell

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pool coming from two mice; and a minimum of 5×10^3 cells were counted unless otherwise specified.

The results obtained with spleen cells from these experimental groups are presented in Fig. 1. In the spleens of saline-injected mice, the number of ABC in 5,000 cells counted varied from 9 to 14. In contrast, the spleen of tolerogen-injected animals displayed a lower number of ABC. As early as 12 h after the injection of 2.5 mg of DHGG, the number of ABC decreased to approximately half the amount seen in normal animals. 5 days after tolerogen injection, the decrease in ABC was essentially maximal. At day 20 the spleens of unresponsive animals contained 10 times less ABC than that found in the spleens of the control mice.

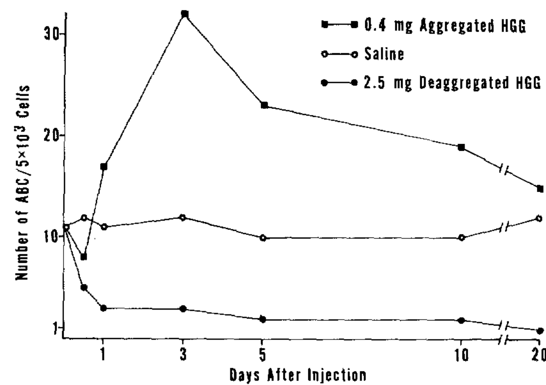

FIG. 1. Kinetics of ABC to HGG in the spleen of mice after injection of either tolerogen (deaggregated HGG), immunogen (aggregated HGG), or saline. Each point represents an average of four counts of ABC, each done a with different cell pool of two spleens. A minimum of 5×10^3 spleen cells were counted.

The number of ABC to HGG in the spleens of immunogen-treated mice began to increase as soon as 24 h after the injection of 0.4 mg of AHGG, reaching a peak on day 3 of three times that number found in normal animals. After this day, the number of ABC in the immunogen-injected group began to decrease; and on the last experimental day, day 20, the number of ABC was only slightly higher than that seen in the spleens of normal mice. However, when 20×10^6 spleen cells obtained from animals primed with AHGG 20 days earlier were incubated with unlabeled HGG before reaction with [^{125}I]HGG, it was found that as little as 0.3 mg of unlabeled HGG was able to abolish completely the binding of [^{125}I]HGG by these cells, whereas the same amount of unlabeled HGG incubated with normal spleen cells did not diminish the number of ABC found in this population.

The kinetic pattern of ABC to HGG in bone marrow cells in the three experimental situations is seen in Fig. 2. In the saline-injected mice, the number of ABC in 5,000 cells counted varied from 10 to 14. In the DHGG-injected mice,

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the number of ABC seen in the bone marrow was similar to that seen in normal mice until day 20, when there was a decrease of ABC in tolerogen-treated BM cells to two-thirds of the number seen in the bone marrow of normal animals. In contrast to that observed in the spleen, the number of ABC in the bone marrow of immunogen-injected mice was the same as that found in normal bone marrow. The paucity of ABC to HGG displayed by the thymus (T) cells did not allow a difference between the three experimental conditions (Table III).

DISCUSSION

The present data demonstrate that the induction of an unresponsive state to HGG in mice is concomitant with the disappearance of specific HGG-binding cells normally located in the spleen and in the bone marrow. In the spleen, the diminution of these ABC begins as soon as 12 h after the injection of tolerogen, reaching an optimal reduction by the 5th day. In the bone marrow, there is a lag

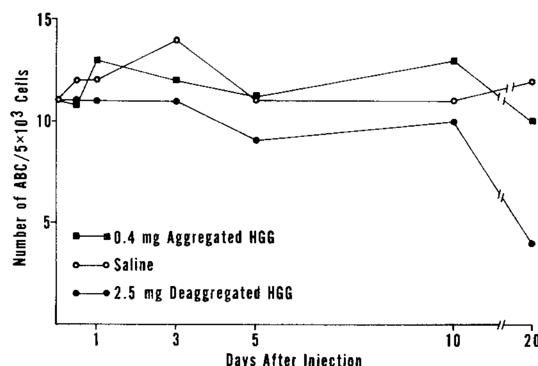

FIG. 2. Kinetics of ABC to HGG in the bone marrow of mice after injection of either tolerogen (deaggregated HGG), immunogen (aggregated HGG), or saline. Each point represents an average of four counts of ABC, each done with a different cell pool coming from the bone marrow of two mice. A minimum of 5×10^3 BM cells were counted.

TABLE III

Quantitation of ABC to HGG in T Cells Obtained from Mice after the Injection of Either Tolerogen (Deaggregated HGG), Immunogen (Aggregated HGG), or Saline

Injection at day 0	No. of ABC/10^4 thymus cells Day after injection					
	0.5	1	3	5	10	20
0.4 mg aggregated HGG	1*	1	<1	<1	1	0
Saline	<1*	0	1	1	1	<1
2.5 mg deaggregated HGG	<1‡	0	<1	<1	1	<1

* Each number in the series is an average of three counts of ABC, each one done with a different cell pool of two thymuses. A minimum of 10^4 thymus cells were counted.

‡ Each number in the series is an average of four counts of ABC, each one done with a different cell pool of two thymuses. A minimum of 10^4 thymus cells were counted.

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period before a diminution of ABC is observed in that a significant reduction does not occur until between days 10 and 20 after tolerogen injection. Although an attempt was made to quantitate differences of ABC in the thymus, significant data were not obtained because of the paucity of ABC to HGG in the thymuses of normal A/J mice.

The kinetic pattern in the reduction of ABC during induction of unresponsiveness demonstrates a remarkable parallelism with previous observations in which the response capacity of various cell populations after the injection of tolerogen (7) was monitored; that is, the reduction in functional immunocompetence of the B cells of the spleen[2] corresponds to the maximal reduction of ABC observed in the spleen, and the 15–21-day latent period required for specific BM cells to become tolerant (7) is the same as that required for specific BM cells to lose their capacity as ABC.

The observed disappearance of specific lymphocytes in animals rendered unresponsive is a phenomenon that may be explained by at least four alternative mechanisms. First, specific cells may have been eliminated after contact with tolerogen; second, the cells may have left the tissues scanned in the present study; third, receptors of specific antigen-sensitive cells may have been saturated with the tolerogen; and fourth, receptors may have been stripped off the specific cells as a result of contact with tolerogen.

The first mechanism, i.e. clonal elimination of specific cells after contact with tolerogen, is a concept that gains support from the data obtained by Azar and Good (12). They reported that the development of tolerance to deaggregated bovine gamma globulin is inhibited in mice depleted of complement and suggested that complement-mediated cytolysis eliminated specific cells. In addition, the fact that thymectomy (13) and bursectomy (14) prolong and sometimes abrogate the termination of tolerance supports the conclusion that termination is dependent on the differentiation of new clones of immunocompetent cells.

The second mechanism, i.e. that tolerogen directs an exodus of specific cells from tissues examined in the present study, is unlikely since it would predict the relocation of these specific cells to other lymphoid tissues, a prediction not substantiated by the experimental findings. However, if antigen-directed exodus of cells occurs, its induction would be restricted to a tolerogenic form of the antigen since, after the injection of immunogen, a similar disappearance of ABC was never observed.

The third possible explanation for the present data, i.e. that the receptors of specific cells are saturated with antigen, is a possiblity that gains support from the recent findings that, in vitro, the state of tolerance may be reversed during the early phase of the induction process by trypsinization (15), a treatment that presumably removes bound antigen from the surface of the cell. However, the reversal of tolerance by this apparent enzymatic stripping of receptors can be affected only as long as 36–48 h after the initial exposure of

[2] Chiller, J. M., and W. O. Weigle. Manuscript in press. *Cell. Immunol.*

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cells to antigen, after which time the tolerant state is refractive to enzyme treatment[3] (15). It appears, therefore, that the cell membrane events that occur during the induction of tolerance may be divisible into two phases: an early phase reversible by trypsinization, and a later phase irreversible by trypsinization. From this standpoint, the initial disappearance of ABC in tolerant lymphoid tissues could be interpreted as a reflection of antigen saturation of receptor sites; but continued reduction of ABC for as long as 20 days after the injection of tolerogen may reflect yet another cellular manifestation. It may be that at this time either clonal elimination or the fourth proposed mechanism, i.e. receptor shedding, is operational.

The finding of a decrease in specific ABC in mice made unresponsive to HGG is in agreement with a similar reduction in ABC observed in the tolerant state in guinea pigs to 2,4-dinitrophenol and glutamic acid-lysine copolymers (6) and in mice to BSA (5), but contrasts with other reports demonstrating a normal level of ABC in states of tolerance in rats to flagellin (2) and hemocyanin (3) and in mice to the lipopolysaccharide of *Escherichia coli* (16). These apparent contradictions should be viewed as cellular manifestations of divergent mechanisms by which tolerance to various antigens may be induced.

In the case of thymus-dependent antigens, it has been shown that unresponsiveness at either T or B cell levels is sufficient to render the whole animal tolerant (7). Tolerance to flagellin and to hemocyanin, both thymus-dependent antigens, may be restricted to T cell tolerance under the experimental conditions used, respectively, by Ada et al. (2) and Cooper et al. (3). Inasmuch as ABC detected in the spleens to flagellin and hemocyanin are B cells (17), the level of ABC in a state of tolerance that did not involve unresponsiveness at the B cell level should not be expected to deviate from that observed in a normal state. Cells binding radioactive HGG are also B cells, a conclusion derived from the observation that spleens from mice thymectomized, irradiated, and reconstituted with anti-θ-treated BM cells displayed a normal level of ABC.[4] The observed disappearance of specific ABC in tolerance to HGG is in accord with the fact that, under the conditions presently used, the state of tolerance to this antigen is known to induce unresponsiveness in both specific T cells and B cells (8).

The presence of ABC in a tolerant state that involves a thymus-independent antigen, such as the lipopolysaccharide of *E. coli*, requires yet another explanation. It is probable that mice other than germfree animals have been stimulated by this antigen, since it is present as part of the normal intestinal flora. Thus, the induction of tolerance to this antigen may be a model for what occurs in primed B cells, and the cellular and subcellular mechanisms by which this phenomenon occurs may be strikingly different from those that take place in

[3] Katz, D. H., T. Hanaoka, and B. Benacerraf. 1972. Immunological tolerance in B lymphocytes. I. Evidence for an intracellular mechanism of inactivation of hapten-specific precursor of antibody-forming cells. Manuscript submitted for publication.

[4] Louis, J., J. M. Chiller, and W. O. Weigle. Manuscript in preparation.

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unprimed cells. Certainly, the ease with which reversibility of the tolerant state occurs in cell transfer experiments to an antigen-free environment with antigens such as *E. coli* polysaccharide (16), pneumococcal polysaccharide (18), and sheep erythrocytes (19) appears to be unique to antigens that induce a state of immunity before achieving tolerance. This is in contrast to the irreversible state of unresponsiveness to HGG, whose induction in normal mice does not include a phase of antibody formation (20).

The numbers of specific ABC in the spleens of mice injected with immunogenic HGG began to increase as soon as 24 h after the injection of antigen, peaked at 3 days, and by 20 days rescinded to a level not significantly different from that present in the spleens of normal animals. Although normal and immune cell populations are quantitatively similar at day 20, they are apparently qualitatively different. This conclusion is based on the observation that the [^{125}I]HGG binding by cells from immune mice was inhibited by much smaller amounts of unlabeled HGG than that needed to inhibit binding by cells from normal mice. These data confirm those obtained by others (21, 22) and suggest that, compared with normal spleens, immune spleens have a restricted spectrum of specific cells, with greater affinity receptors.

SUMMARY

Antigen-binding cells (ABC) to the antigen human gamma globulin (HGG) were quantitated in lymphoid tissues of A/J mice at various times after the injection of deaggregated HGG (tolerogen), aggregated HGG (immunogen), or saline. The reaction of lymphoid cells with highly labeled HGG was specific to that antigen since binding could be inhibited by excess unlabeled HGG, but not by unrelated non-cross-reacting proteins. Compared with normal mice, there was a marked decrease in the numbers of ABC in the spleens of unresponsive animals evident as early as 12 h after the injection of tolerogen. A marked increase in ABC was observed in the spleens of immunogen-injected mice, beginning at 24 h and reaching a peak at 3 days. In bone marrow, no difference in the number of ABC was found among the three experimental groups until day 20, when a reduction in ABC was observed only in tolerogen-injected mice. No quantitative difference in the thymuses in the experimental groups could be determined because of the paucity of ABC displayed by normal thymus cells.

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