TERMINATION OF ACQUIRED AND NATURAL IMMUNOLOGICAL TOLERANCE WITH SPECIFIC COMPLEXES*

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The termination of a specifically induced unresponsive state can be achieved by immunizing tolerant animals with antigens which cross-react with the tolerated antigen. For example, rabbits made tolerant to bovine serum albumin (BSA)[1] by the injection of large amounts of BSA during the perinatal period are refractive to immunogenic challenges of aqueous preparations of BSA when tested at 3 mo of age. However, when similarly treated animals are challenged with aqueous preparations of certain cross-reacting albumins such as human serum albumin (HSA), they produced circulating antibody that reacts with both the cross-reacting albumin and BSA although none of the antibody is specific for BSA (1). Furthermore, the antibody response to BSA is quantitatively and qualitatively indistinguishable from the antibody response to BSA elicited by the injection of HSA into normal rabbits. These data suggest that at 3 mo of age, rabbits made tolerant at birth possess a normal anti-BSA antibody-forming potential, that is, a normal complement of B cells specific for the tolerated antigen.

Considered within the framework of cellular cooperation necessary for antibody formation to a number of antigens (2), a cellular interpretation for this phenomenon can be formulated whereby tolerance to BSA at the time of testing (3 mo) is maintained by the functional absence of the BSA-specific carrier recognition mechanism (T cells) in spite of the presence of B cells capable of responding to the antigen. The response manifested by the injection of HSA could then be viewed as the antigen-mediated cooperation between BSA-specific B cells recognizing on HSA those determinants which cross-react with BSA, and HSA-specific T cells recognizing on HSA determinants noncross-reactive with

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; BSS, balanced salt solution; BTg, bovine thyroglobulin; GPGG, guinea pig gamma globulin; HGG, human gamma globulin; HSA, human serum albumin; LPS, bacterial lipopolysaccharide; PFC, plaque-forming cells; RTg, rabbit thyroglobulin.

BSA. The temporal aspects of such a model are compatible with the kinetics which characterize the spontaneous loss of tolerance in T cells and B cells in mice (3).

Predictably tolerance could be terminated at a time when unresponsiveness to BSA is restricted to specific T cells by using a complex composed of the tolerogen (BSA) conjugated to a carrier molecule which itself is immunologically recognizable. Inasmuch as a gross alteration of the BSA molecule can itself lead to specific termination (4), the system chosen to test this prediction for this study was antibody which was coupled to the antigen by immunological complexing in order to minimize alteration of the antigen (BSA). The results to be reported will demonstrate that, in accord with the predictions, such complexes can terminate tolerance not only in a state of experimentally induced unresponsiveness but also in a state of natural unresponsiveness.

Materials and Methods

Antigens. Commercial preparations of BSA and HSA were obtained from Armour Pharmaceutical Co., Chicago, Ill., lot F 71703, and from E. R. Squibb & Sons, New York, lot 591R (prepared from material supplied through the courtesy of the American Red Cross), respectively. Rabbit thyroglobulin (RTg) and bovine thyroglobulin (BTg) were isolated and purified using a modification (5) of the technique described by Edelhoch (6). Rabbit thyroids were obtained from the trachea of New Zealand White rabbits obtained fresh (unfrozen) from Pel-Freez Biologicals, Inc., Rogers, Ark., and bovine thyroids were obtained from a local slaughterhouse.

Animals. All rabbits used in these studies were of the New Zealand White strain. Hartley strain guinea pigs weighing approximately 400 g were used for the production of antisera to HSA and to BTg.

Induction of Immunological Tolerance to BSA. Rabbits were made tolerant to BSA by the subcutaneous injection of 100 mg BSA during the first 24 h of life followed by two injections of 200 mg each before the 5th day of life. This procedure renders all rabbits unresponsive to an intravenous challenge of 20 mg BSA for as long as 6 mo (7).

Antibody Assays

HEMOLYTIC PLAQUE ASSAY. Lymphoid cell suspensions were prepared from the popliteal lymph nodes and spleens of immunized rabbits. Recovered cells from single spleens ($1–5 \times 10^8$ cells) or lymph nodes ($4–9 \times 10^8$ cells) were suspended in balanced salt solution (BSS) (8).

Protein, either BSA or normal guinea pig gamma globulin (GPGG), was conjugated to washed goat erythrocytes using a water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl] according to the method of Golub et al. (9). The antigen-coupled erythrocytes were washed three times in phosphate-buffered saline and then diluted 1:15 in BSS for use in the hemolytic plaque assay. The assay used was the Jerne plaque assay (10) as modified by Mishell and Dutton (8).

INHIBITION OF PLAQUE-FORMING CELLS (PFC) WITH SOLUBLE ANTIGENS. The amount of BSA conjugated to the erythrocytes was estimated and a 1,000-fold molar excess of either soluble BSA or HSA was added to the agarose-cell mixture for plating.

PRECIPITIN ANALYSIS. Measurement of antibodies for injection was made using a quantitative precipitin technique employing ^{125}I-labeled antigen (11).

PREER TEST FOR PRECIPITATING ANTIBODY. The presence of precipitating antibodies to RTg was determined using the one dimensional diffusion analysis described by Preer (12).

HEMAGGLUTINATION ASSAY. Hemagglutination (13) was performed using a 2.5% suspension of tannic acid-treated sheep erythrocytes sensitized with 0.5 mg RTg/ml. Before testing, sera were absorbed with an equal volume of sheep erythrocytes and heated at 56°C for 20 min.

Preparation of Antibodies. Guinea pig anti-HSA was prepared by giving three injections each of 0.2 mg HSA in 1 ml of complete Freund's adjuvant which contained 0.2 mg *Mycobacterium tuberculosis* distributed in four subcutaneous sites. The level of precipitating antibody in individual sera was estimated using varying concentrations of HSA or BSA in the Ouchterlony double-diffusion

analysis (14). High titer antisera were pooled and quantitated. This pooled antiserum contained 6.5 mg anti-HSA antibody/ml of which 1 ml bound 0.7 mg BSA.

Rabbits were immunized with HSA in complete Freund's adjuvant using a series of six injections over a 4-mo period. Each 1-ml injection contained 10 mg HSA and 3.4 mg mycobacteria and was distributed between foot pads and subcutaneous sites. Antisera showing a strong reaction in the Ouchterlony double-diffusion analysis were grouped into two pools. Pool I contained 14.2 mg precipitating anti-HSA/ml of which 1 ml bound 0.3 mg BSA. Pool II had 6.8 mg precipitating anti-HSA antibody/ml of which 1 ml bound 0.4 mg BSA.

Guinea pig anti-BTg was prepared by injection of BTg incorporated into complete Freund's adjuvant containing 0.2 mg mycobacteria/ml. On days 0 and 14, 5 mg BTg in 1 ml complete Freund's adjuvant was divided between the hind foot pads and two subcutaneous sites. The third injection (day 21) was made in four subcutaneous sites. The animals were bled on days 42, 49, and 56 and the resulting sera were grouped in two pools. Pool I contained 1.1 mg precipitating anti-RTg/ml. Pool II contained 0.5 mg precipitating anti-RTg/ml. The antibody to antigen ratio of these antisera was one.

Preparation of Complexes for Injection. All antigen-antibody complexes prepared for injection were precipitated at the optimal proportions of antibody and cross-reacting antigen. The antigen-antibody mixtures were incubated at 37°C for 30 min and then at 4°C for at least 24 h. The resulting precipitates were centrifuged and washed three times in cold saline. The washed precipitates were resuspended in cold saline and then incorporated into incomplete Freund's adjuvant for injection.

For both the serum albumin and thyroglobulin experiments, control groups were challenged with GPGG in amounts equivalent to the amounts of guinea pig antibody received by the experimental groups as calculated from the known combining ratios of the antigens with their antibodies. Other control groups received injections of antigen alone in the same amounts used in complex form. All challenge antigens or complexes were incorporated into Freund's adjuvant and injected in equal volumes in the two hind foot pads.

Complexes of BSA with guinea pig anti-HSA were prepared as described above so that each animal received 36 μg BSA for the first challenge injection (day 0) and 179 μg BSA for the second (day 7). Complexes of BSA with rabbit anti-HSA were also prepared so that each animal received 36 μg BSA and 179 μg BSA in complex form for the first and second challenge injections, respectively.

Animals challenged with RTg complexed to guinea pig anti-BTg received three injections of 400, 800, and 1,200 μg RTg in complex form on days 0, 7, and 15, respectively. In another experiment, rabbits received 266 and 800 μg RTg in complex form on days 0 and 7, respectively.

Histology. Thyroid tissue was taken when the rabbits were sacrificed. The tissue was fixed in Bouin's solution, embedded in paraffin, and sections cut through the long axis. The sections were mounted and stained with hematoxylin and eosin.

The grading of thyroiditis was determined by the degree of cellular infiltration. The lesions were graded + if at least five foci (the size of one follicle or more) of infiltrating cells were present in the longitudinal section of one lobe. Lesions were graded ++ if 10–20 foci were present, each of which occupied the area of several follicles. Lesions were graded +++ if either numerous foci were present in each section, each of which occupied areas the size of a number of follicles or the entire lobe was involved with a more diffuse infiltration.

Results

Termination of Tolerance to BSA using Heterologous Antibody-Antigen Complexes. Rabbits were made unresponsive to BSA by injection of BSA at birth. At 3 mo of age, the tolerant rabbits were divided into three groups. Group A was challenged with two injections of complexes of guinea pig anti-HSA plus BSA which had been precipitated at equivalence. Groups B and C were challenged with either BSA or GPGG, respectively. 8 days after the last challenge, all rabbits were sacrificed and their popliteal lymph nodes and spleen were assayed for PFC to BSA. The results shown in Table I indicate the extent of the response of such treated animals as expressed by the number of indirect anti-BSA PFC per 10^6 popliteal lymph node cells. No direct PFC were detected. It can be seen that BSA in complex with guinea pig anti-HSA (group A)

TABLE I
Termination of Immunological Unresponsiveness to BSA using Immune Complexes Made with Heterologous Antibody

Group	Challenge	No. of animals	Indirect PFC/10^6 LN* cells (range)	Inhibition of PFC by:	
				BSA	HSA
				%	%
A	GP anti-HSA‡ + BSA	8	296 (71-609)	100	14
B	BSA	8	6 (0-31)	—	—
C	GPGG‡	5	0	—	—

* LN, lymph node.
‡ All groups challenged with GPGG made a PFC response to GPGG in the draining lymph node.

terminated the tolerant state while BSA alone (group B) did not. Furthermore, 100% of the BSA-specific PFC in the animals challenged with complexed BSA were inhibited with soluble BSA but only 14% were inhibited by soluble HSA. Thus, the specificity of the PFC response was directed to the previously tolerated antigen. The weaker PFC response obtained in the assays of the splenic PFC (data not shown) led nevertheless to conclusions similar to those made using lymph node cells. Finally, draining popliteal lymph nodes from all animals injected with GPGG alone or in complex (groups C and A, respectively) made a PFC response to GPGG.

Termination of Tolerance to BSA using Homologous Antibody-Antigen Complexes. 3-mo old BSA tolerant rabbits were challenged with two injections of complexes of rabbit anti-HSA plus BSA which had been precipitated at equivalence. The results in terms of numbers of PFC per 10^6 popliteal lymph node cells are shown in Table II. Rabbits challenged with the homologous

TABLE II
Termination of Immunological Unresponsiveness to BSA using Immune Complexes Made with Homologous Antibody

Group	Challenge	No. of animals	Indirect PFC/10^6 LN* cells (range)	Inhibition of PFC by:	
				BSA	HSA
				%	%
A	Rabbit anti-HSA + BSA‡	5	130 (74-245)	100	4
B	Rabbit anti-HSA + BSA‡	5	4 (2-7)	—	—
C	BSA	5	9 (0-31)	—	—

* LN, lymph node.
‡ Groups A and B received exactly the same experimental treatment but are reported separately because of the bimodal distribution of the results.

antibody-antigen complex fell into two groups. One-half of the animals (group A) showed some termination of unresponsiveness to BSA (71–275 PFC/10^6 lymph node cells) while the others (group B) remained tolerant (2–7 PFC/10^6 lymph node cells). BSA alone did not terminate the tolerant state (group C).

Termination of Natural Unresponsiveness to RTg using Heterologous Antibody-Antigen Complexes. Normal rabbits, 3 mo of age, were divided into three groups. Group A was challenged with three injections of complexes made of guinea pig anti-BTg plus RTg mixed at equivalence. This group showed a termination of the natural tolerant state to RTg as shown by the appearance of hemagglutinating antibody in 9 of 10 animals and precipitating anti-RTg in 6 of 10, as well as the development of inflammatory lesions of the thyroid in 6 of 10 (Table III). The groups challenged with RTg (group B) or GPGG (group C) alone did not make an immune response to the RTg.

Discussion

Both experimentally induced and naturally acquired states of immunological unresponsiveness (tolerance) can be terminated in rabbits injected with complexes composed of heterologous cross-reacting antibody and the tolerated antigen. In this manner, tolerance to BSA experimentally induced by the injection of 500 mg BSA into neonatal rabbits was terminated when complexes composed of guinea pig anti-HSA plus BSA were injected into the rabbits at 3 mo of age. Similarly, it has been demonstrated that rabbits tolerant to GPGG lost their experimentally induced unresponsive state when challenged with GPGG as specific antibody complexed to ovalbumin or BSA (W. O. Weigle, unpublished observation). Analogous to these situations in which an induced unresponsive state was abrogated, natural tolerance to a self-antigen, namely RTg, was terminated by challenging with complexes composed of guinea pig anti-BTg plus RTg. This loss of self-tolerance was manifested by both the formation of humoral antibody specific to RTg as well as by the histological demonstration of an inflammatory, cellular infiltration of the thyroid.

TABLE III

*Termination of Natural Immunological Unresponsiveness to RTg using Immune Complexes Made with Heterologous Antibody**

Group	Challenge	No. of animals	Anti-RTg		Thyroid lesions
			Hemagglutination (range)	ppt (preer test)	
A	GP anti-BTg + RTg	10	9/10 (16-5, 120)	6/10	6/10
B	RTg	11	2/11 (16)	0/11	0/11
C	GPGG	6	0/6	0/6	0/6

* These results represent data pooled from two different experiments. No differences were observed between the two experimental sets although one set received three injections of RTg totalling 2,400 μg RTg while the other set received two injections of RTg totalling 1,066 μg RTg (see Materials and Methods).

The interpretation of these data must be considered within the context of those cellular events which have been shown to delineate the state of tolerance to serum protein antigens. For example, it has been shown that in mice tolerance induced to human gamma globulin (HGG) is a function of the immunological state of both the T and B cells (15). However, tolerance restricted to specific T cells is sufficient to maintain an apparent state of unresponsiveness in the intact animal. This situation is observed in tolerant mice beginning some 2 mo after the administration of tolerogen, that is, after B-cell tolerance has waned (3). Thus, it could be assumed that BSA tolerant rabbits at 3 mo of age similarly have a normal anti-BSA antibody-forming potential but lack the T-cell helper capacity for recognizing BSA. This putative cellular status is in accord with data recently reported by Benjamin (16) in which he was able to terminate tolerance to BSA in rabbits by injecting normal thymocytes plus antigen at 3 mo of age. In that light, termination of tolerance using cross-reacting antigens (17), altered antigen (4), or heterologous antibody-antigen complexes can be seen as occurring in a cellular state where tolerance is maintained by unresponsiveness at the level of specific T cells in spite of the presence of a normal complement of B cells with the potential to produce antibody specific for the tolerated antigen. In the case of the observed termination of tolerance by BSA complexed to guinea pig anti-HSA, it might thus be postulated that the unresponsive state was bypassed by using the T-cell helper activity specific for determinants on GPGG in order to provide the B-T cell collaboration necessary for the production of anti-BSA. Guinea pig anti-HSA was chosen so that within the antigen-antibody complex, antigenic determinants specific for BSA would remain exposed and thus be free to interact with the BSA-specific B cells. In accord with this hypothetical mechanism, it would be further predicted that the homologous immunoglobulin in complexes made of rabbit anti-HSA plus BSA should not serve as an appropriate carrier inasmuch as no helper T cells should be capable of recognizing naturally occurring self-components. However, although rabbit anti-HSA plus BSA did not regularly terminate tolerance, one half of the animals tested were shown to display positive responses to the previously tolerated antigen. PFC responses in the half of the animals which did show termination using homologous antibody-antigen complexes were significantly ($0.025 < P < 0.05$) lower (130 PFC/10^6 lymph node cells) than those achieved using heterologous antibody-antigen complexes (296 PFC/10^6 lymph node cells). No termination was achieved in the remaining half of the animals. That some animals did show termination of tolerance while others did not may reflect immunoglobulin allotypic differences between the complexed antibody and the challenged individual. Retrospective analysis of the allotypes of the sera involved was not able to resolve this point.

In the case of natural tolerance to the rabbits' own thyroglobulin, low circulating levels of thyroglobulin (10 ng/ml) although likely insufficient to produce unresponsiveness in B cells should be capable of producing unresponsiveness in T cells (18) so that the natural tolerant state in this case would be maintained at the T-cell level only. This situation would be analogous to that which is obtained in mice injected with low doses of the tolerogen HGG (3). Very low doses of tolerogen (0.1 mg) produced unresponsiveness at the T-cell level only. Normal human and murine lymphoid tissues are known to contain B cells

with receptors which recognize their own thyroglobulins (19, 20). Thus, the use of heterologous antibody complexed to RTg which bypassed the requirement for helper T cells specific to GPGG was able to terminate the tolerant state as manifested by circulating antibody and thyroiditis. Although it is possible to terminate the natural tolerant state to self-antigens which occur in low concentrations in the circulation, such as thyroglobulin, termination of tolerance to antigens occurring in high concentrations, such as serum albumin, would not be expected to occur.

Termination of the tolerant state which is being maintained at the T-cell level only is achieved not only by bypassing the specificity of the T cell or replacing the T cells as described above, but also by using agents such as bacterial lipopolysaccharide (LPS) or polyanions whose mode of action may obviate the necessity for a full complement of specific T-cell helper activity. For example, it has been shown in mice that when tolerance to HGG is being maintained at the T-cell level only, injection of HGG and LPS terminates the tolerant state (21). Similarly, it has been reported that an unresponsive state to heterologous erythrocytes can be terminated by polyanions such as dextran sulfate (22).

Nonspecific T-cell stimulation may also bypass the requirement for specific T-B cell interaction for the termination of tolerance. Thus, tolerance to HGG in $B6AF_1$ mice, at a time when unresponsiveness was presumably maintained with tolerant T cells, was terminated by the administration of the immunogen along with semiallogeneic spleen cells (A strain) (23). On the other hand, injection of syngeneic spleen cells along with immunogen did not terminate the tolerant state. Allogeneic cells have been shown to replace the requirement for specific T-cell helper effect in rats even when the allogeneic cells came from a donor tolerant to the antigen in question (24). Presumably, the transferred parental (semiallogeneic) or allogeneic T cells are stimulated by histocompatibility antigens on host cells and these stimulated cells provide the helper effect nonspecifically. Alternatively, the host B cells plus antigen may direct their facilitation by allogeneic T cells.

Antibody in antigen-antibody complexes has been demonstrated either to inhibit or to enhance immunological responsiveness to the complexed antigen. Antibody-mediated suppression of specific responsiveness has been reviewed (25), and most of the experimental evidence suggests that the suppression is due to an afferent inhibition of the response in which antibody masks recognition of the antigenic determinants in question (26). On the other hand, the experiments reported here are dependent upon an enhanced immunogenicity afforded by antibody-antigen complexes. This may be effected through more than one mechanism. As described here, the addition of heterologous cross-reacting antibody to antigen increases the number of determinants capable of interacting with T cells and thus increases the T-cell helper effect for B-cell antibody formation. That the heterologous antibody be directed against a cross-reacting antigen is mandated by the requirement for exposed antigenic determinants for B-cell stimulation (27). The use of syngeneic antibody-antigen complexes may also result in an enhanced antibody response (G. Terres, personal communication). In this case, the mechanism must be different for there are few new determinants to interact with T cells. Homologous complex stimulation of the

immune response may result from the increased localization of antigen through Fc receptors (28) or other receptors (29) on immunocompetent cells or through increased uptake of antigen by splenic macrophages (30).

Summary

It was possible to terminate the induced unresponsive state to bovine serum albumin (BSA) and the natural unresponsive state to autologous thyroglobulin in rabbits (RTg) by immunization with complexes composed of heterologous cross-reacting antibody and the tolerated antigens. The unresponsive state was terminated in rabbits made unresponsive by neonatal injections of BSA and then 3 mo later injected with complexes composed of BSA and guinea pig antihuman serum albumin. This termination was manifested by the presence of anti-BSA plaque-forming cells. Similarly, the natural unresponsive state was terminated in adult rabbits injected with complexes between RTg and guinea pig antibovine thyroglobulin (BTg) in that thyroid lesions and circulating anti-RTg were produced. The results can be best explained by the presence of unresponsive T cells and competent B cells, where the guinea pig gamma globulin (antibody) activates T cells specific for the guinea pig gamma globulin portion of the complexes and thus permits stimulation of B cells competent to the exposed determinants of the tolerated (BSA or RTg) portion of the complexes. The detailed mechanism for the activation of B cells in tolerant animals is discussed.

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