RESTORATION OF IMMUNE COMPETENCE IN TOLERANT MICE BY PARABIOSIS TO NORMAL MICE*

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In his statement of the clonal selection theory, Burnet proposed that immunologic tolerance is a state that results from the elimination of immunocytes which are specific for antigens recognized, rightly or wrongly, as self-antigens (1). An alternate theory, reviewed recently by Gershon (2) suggests that tolerance is not a defective state but is rather an active state which is caused by lymphoid cells which have antigenically-specific immunosuppressive activity.

In an attempt to determine whether immunologic tolerance is, in fact, an active or defective state, we used the technique of surgical parabiosis to join a normal mouse to a mouse previously rendered immunologically tolerant to human gamma globulin (HGG). It was reasoned that if immunologic tolerance is an active state, the tolerant cells of the tolerant partner of a normal-tolerant parabiotic pair might be expected to convert the normal partner to a tolerant state. If, on the other hand, tolerance is a defective state, the cells of the normal partner might be expected to reconstitute the immune response of the tolerant parabiont. The data presented below indicate that after parabiosis of a normal to an HGG-tolerant mouse, the ability of the tolerant mouse to make antibodies to HGG is restored.

Methods

Animals. (BALB/c × A/J)F_1 male mice ranging in age from 8–20 wk were obtained from Jackson Laboratories, Bar Harbor, Maine. Parabiosis was performed by a modification of the technique of Eichwald et al (3). Briefly, mice were shaved and anesthetized with nembutal; a skin incision was made in the adjacent sides of two mice from ear to hip. The exposed muscle layer and peritoneum of each animal were macerated with toothed forceps and then sutured together with chromic 4-0 gut; a mattress suture of 2-0 silk was used through the adjoining scapula of the two animals and the skin of the mice were then clipped with 9 mm stainless steel wound clips (Clay Adams, Parsippany, N.J.) to close the skin incision. Each partner was given 0.2 mg tetracycline, i.m., and 0.3–0.5 cc saline s.c., daily, for 7 days.

At the time of assay, approximately 1 mo after joining, cross-circulation could be demonstrated by intravenous injection of ^{51}Cr-labeled syngeneic erythrocytes into the tail vein of one partner; 90 min

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later, equal numbers of labeled red cells were found in the circulation of both partners (I. Rivera, R. Nussenzweig, and S. Zolla, unpublished results).

Immunogen and Tolerogen. HGG was purchased from Pentex (Miles Laboratories, Inc., Kankakee, Ill.). Aggregated HGG (AHGG) was prepared by the method of Chiller and Weigle (4). Tolerogen was prepared by a modification of the method of Golub and Weigle (5): A 20 mg/ml solution of chromatographed HGG in 0.01m PO_4 buffer, pH 8, was mixed at room temperature with 1.5 m Na_2SO_4 to a final concentration of 0.81 m Na_2SO_4. After allowing to settle for 10 min, the mixture was spun at 3,400 *g* for 10 min at room temperature. The supernate was then dialyzed in saline, pH 8, for 6 h at room temperature and then against saline, pH 7, overnight at 4°C. The dialyzed fraction was spun at 20,000 *g* at 4° and the material in the top ⅔ of the tube was used immediately as tolerogen.

Immunologic Assays. Antibody-forming cells were enumerated by a modification of the hemolytic plaque assay in which HGG-goat red cells (HGG-GoRC) coupled by the carbodiimide method (6) were used as indicator cells. Both direct and indirect plaque-forming cells (PFC) were enumerated, the latter being developed with rabbit antimouse gamma globulin. Hemagglutinin titers were determined by tube hemagglutination in a total volume of 0.2 cc using HGG-GoRC which were prepared by the method of Avrameas, et al. (7).

Experimental Design. Mice were made tolerant by administration of 1 mg of HGG tolerogen, given twice with an interval of 7–8 days. 6–7 days after the second dose of tolerogen, these mice were given 0.4 mg AHGG, i.v.; 6 days later they were bled (test bleed) and the sera were titrated for antibodies to HGG. Only mice with a hemagglutinin (HA) titer of less than 1:10 were used in further experiments as tolerant mice. Normal mice that had not received the tolerogen but were injected with 0.4 mg AHGG, i.v., and bled 6 days later had HA titers that ranged between 1:320 and 1:1,280.

The protocol for the parabiotic experiments is shown in Fig. 1. Mice were made tolerant by the procedure described above. 6–7 days after the test bleed, mice were parabiosed; normal (untreated) mice were parabiosed to normal mice (N ~ N): normal mice were parabiosed to tolerant mice (N ~ T); and tolerant mice were parabiosed to other tolerant mice (T ~ T). 15–16 days after parabiosis, each partner was given 0.4 mg AHGG, i.v., and 9 days later a booster dose of 0.4 mg AHGG was given to each partner, i.p. 5 days after the boost, the animals were bled and sacrificed to assay for HA antibodies and splenic PFC.

Statistical Analyses. Student's *t* test was used to analyze the data.

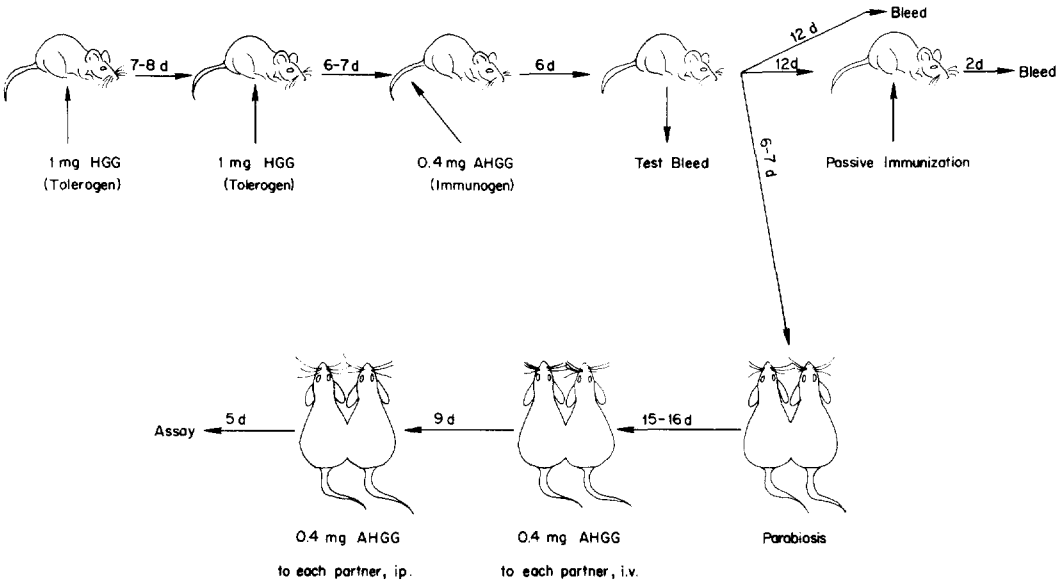

FIG. 1. The protocol for the parabiotic experiments.

Results

The nature of the phenomenon of immunologic tolerance was investigated by studying the ability of three groups of parabiotic mice to make antibodies to HGG. The protocol described above and shown in Fig. 1 was used and the experimental results are shown in Table I. The spleens of N ~ N parabionts contained an average of 9,156 PFC to HGG while the T ~ T parabionts had only 1,722 PFC per spleen. The difference is significant with $P < 0.001$. The average number of PFC per spleen in N ~ T parbionts was 20,611. There was no

TABLE I
Immune Response of Parabiotic Mice to HGG

Pair no.	Immune state of parabiont	Direct + indirect PFC/spleen	Hemagglutinin titer^{-1}	Arithmetic mean ± SE of PFC/spleen	Geometric mean of hemagglutinin titer^{-1}
1	Normal	9,870	ND*		
	Tolerant	7,245	20		
2	Normal	7,350	<10		
	Tolerant	10,290	<10		
3	Normal	10,290	80	20,611 ± 6,175	27
	Tolerant	19,845	ND		
4	Normal	69,510	640		
	Tolerant	26,775	1,280		
5	Normal	34,965	<10		
	Tolerant	9,975	ND		
6	Tolerant	1,050	40		
	Tolerant	1,260	320		
7	Tolerant	630	<10	1,722 ± 715	28
	Tolerant	315	ND		
8	Tolerant	105	160		
	Tolerant	840	160		
9	Tolerant	7,875	ND		
	Tolerant	2,520	ND		
10	Tolerant	1,365	<10		
	Tolerant	1,260	10		
11	Normal	10,815	160		
	Normal	8,295	40		
12	Normal	9,975	ND		
	Normal	18,585	1,280		
13	Normal	8,715	1,280	9,156 ± 1481	254
	Normal	14,490	320		
14	Normal	4,515	320		
	Normal	3,150	80		
15	Normal	5,040	160		
	Normal	7,980	320		

* Not done.

statistical difference between the level of response of the tolerant partner and the normal partner in the N ~ T pairs ($P > 0.3$) indicating that the ability of tolerant partners to respond to HGG was restored after parabiosis to normal mice.

The level of circulating antibodies in the parabionts was measured by hemagglutination of HGG-GoRC. The results shown in Table I indicate that the geometric mean of the serum titers from N ~ N mice was 1:254 while the means of N ~ T and T ~ T mice were 1:27 and 1:28, respectively. These results stood in direct contradiction to the data collected using the plaque assay: the HA titers suggested that both partners of N ~ T pairs were tolerant. An alternate explanation of this data was based on the hypothesis that serum antibody titers would be reduced if HGG were still present in tolerant partners and available to neutralize antibodies released from PFC.

In order to ascertain if HGG was present in tolerant mice at levels capable of absorbing circulating antibodies, tolerant and normal mice (nonparabiotic) were passively immunized with an i.p. injection of 0.15 ml of mouse antiserum to HGG (anti-HGG). The serum had an HA titer of 1:40,960 and it was administered to the tolerant mice 12 days after the test bleeding to which all tolerant mice were subjected (see Fig. 1). 2 days later the mice were bled and the sera titrated. Two passively immunized normal mice had titers of 1:2,560 and 1:5,120. Four passively immunized tolerant mice had HA titers of less than 1:10 indicating that the injected antibody had been absorbed out of circulation in tolerant animals.

Further experiments were performed to determine if the HGG in tolerant mice was found in the serum rather than in a cellular compartment. Sera from normal mice and from tolerant mice which had been test bled 12 days earlier were used (see Fig. 1). Increasing amounts of sera from tolerant and normal mice were incubated with 25 μl of anti-HGG; after 30 min of incubation at 37°C, serial twofold dilutions were made, HGG-GoRC were added and, after further incubation, HA titers of the reaction mixtures were recorded. The results are shown in Table II. They indicate that, wheras the anti-HGG incubated with saline or normal serum had an HA titer of 1:5,120, anti-HGG incubated with greater than 200 μl of pooled tolerant mouse serum had titers reduced to less than 1:20. These results indicate that HGG was present in the serum of tolerant mice and was capable of neutralizing serum antibody.

Discussion

Previous unsuccessful attempts to reconstitute tolerant mice with syngeneic spleen cells from normal mice have been reported (8–10) and form one of the cornerstones of the theory that tolerance is an active immunologic state. The results of the experiments reported here indicate that the immune response of tolerant mice can be reconstituted by parabiosis and, in fact, that the immunologic reactivity appears to be augmented in the N ~ T pair after parabiosis. This heightened response to HGG in N ~ T mice is not statistically significant ($P > 0.05$ when compared to N ~ N), however, it is reminiscent of the "overshoot phenomenon" described by Chiller and Weigle (11). It is also

TABLE II
Hemagglutination Inhibition with Sera from Tolerant Mice

	Hemagglutinin titer^{-1}
25 λ Mouse anti-HGG + saline	1:5,120
25 λ Mouse anti-HGG + 100 λ pooled sera from HGG-tolerant mice	1:160
25 λ Mouse anti-HGG + 200 λ pooled sera from HGG-tolerant mice	<1:20
25 λ Mouse anti-HGG + 300 λ pooled sera from HGG-tolerant mice	<1:20
25 λ Mouse anti-HGG + 300 λ normal mouse serum	1:5,120

noteworthy that although tolerogen must circulate in N ~ T parabionts in quantities sufficient to reduce HA titers, the tolerogen is not effective in rendering the normal partner tolerant.

Parabiosis was also used by Martinez et al. (12) to study the transfer of tolerance to F_1 skin grafts to parental mice after parabiotic union between F_1 and parental mice. Those studies indicated that tolerance to skin grafts could be successfully transferred by parabiosis. However, Brent et al. (13) also used parabiosis to study the transfer of tolerance to an histoincompatible skin graft. In results comparable to ours, they showed that the tolerant parabiont regained its ability to reject a skin graft from the strain used to induce tolerance and that, in fact, the graft survival time from N ~ T pairs was shorter than that from N ~ N pairs.

Our results indicate that tolerance to HGG cannot be transferred by parabiotic union. Indeed, cells from the normal partner were able to reconstitute the immune response of the tolerant partner. These findings support the concept that this type of tolerance is due to an absence of a specifically reactive cell population in the tolerant partner and are consistent with the findings of Naor and Sulitzeanu (14) and Louis et al. (15) that antigen binding cells to the antigen used to induce tolerance are absent from tolerant mice.

Summary

These studies demonstrate that mice tolerant to human gamma globulin (HGG) regain their ability to make antibody to HGG after parabiosis to normal mice. This can be demonstrated by enumeration of PFC in the spleens of both the normal and tolerant partners. Hemagglutinin titers of normal-tolerant parabionts, however, are exceptionally low; serum antibody appears to be neutralized by circulating HGG present originally in the serum of the tolerant partner. These data support the hypothesis that tolerance to HGG in mice is a "defective" state due to the absence of cells capable of responding to this antigen.

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