

Adjuvant Effect on Maintenance and Escape from Tolerance¹ (38955)

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Heterologous serum proteins can provoke a wide range of immunologic responses in experimental animals, depending on the physical state of the protein. Human gamma globulin (HGG), for example, can either stimulate the murine immune mechanism or induce a state of immunologic unresponsiveness (1); injection of aggregated HGG results in a brisk immune response in mice, while administration of the same antigen in a soluble state often produces tolerance (2). Thus, immunogenic or tolerogenic antigen forms can be prepared selectively.

Several agents administered in conjunction with a tolerogenic form of antigen can modify or abrogate inductive events necessary to attain a state of immunologic tolerance in mice. Immunologic adjuvants such as lipopolysaccharides from gram-negative bacteria can modify the induction of tolerance (3); circumvention of a tolerogenic state may be related to the ability of the adjuvant to enhance an immune response. This investigation was conducted to determine the effect of different quantities of mycobacterial components in complete Freund's adjuvant on the induction of a state of tolerance to HGG in several strains of mice.

Materials and Methods. Animals. CBA/H, (A₁ × C57BL/1)F₁, and C3H/b mice, obtained from the Bittner-Martinez mouse colony at the University of Minnesota (4), and CBA/J and C57BL/10J mice, obtained from Jackson Laboratories (Bar Harbor, ME), were used. Animals were maintained according to standard procedures.

Antigen. Human Gamma Globulin (HGG), obtained as Cohn's Fraction II from Squibb and Sons (lot nos. RC75C and RC75D), was further processed by ultracentrifugation in a Spinco model L centrifuge (5).

Adjuvants. Incomplete Freund's Adjuvant

(IFA) lot 564980 and Complete Freund's Adjuvant (CFA) lot 572941 were obtained from Difco Laboratory (Detroit, MI). Complete Freund's Adjuvant contained .025 mg *Mycobacterium butyricum* per dose. In addition, IFA to which was added .025, .125 or .5 mg quantities of *Mycobacterium tuberculosis* (H37 Ra 573037, Difco) were tested on different groups of mice.

Tolerance Induction Dose (TID). HGG was centrifuged at 90,000g for 90 min and the upper third of the tube contents used as tolerogen. Given intraperitoneally on the first day of the experiment, 1 mg of this form of HGG renders tolerant a statistically significant number of mice of all strains studied (6). C3H/b mice received a TID of 0.5 mg HGG intravenously.

Immunizing Dose (ID). Noncentrifuged human gamma globulin was used; the ID consisted of antigen incorporated into one of the adjuvants. Subcutaneous injection of 1/10 mg of aggregated HGG in a water-oil emulsion immunizes most previously untreated mice. This ID was given to experimental mice 5, 7, or 14 days after the TID, or to control mice not receiving a TID.

Immune Elimination Dose (IED). The immune status was evaluated by the immune elimination technique (5, 6). One-tenth milligram ¹³¹I-HGG was administered intraperitoneally 10 days after the ID. Immune elimination curves were plotted from individual whole body count data obtained on days 3, 5, and 7.

Evaluation of the immune response. Counts corrected for radioactive decay of ¹³¹I were obtained for individual animals during the immune elimination phase and group means were plotted on semilogarithmic graph paper. Values were then compared to curves obtained with control-immune and control-tolerant mice. Control-immune mice received no TID, only an ID; control-unin-

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TABLE I. ADJUVANT EFFECT DURING INDUCTION OF TOLERANCE ON CBA/J MICE

Type of adjuvant given with Hgg ^a	No. of tolerant mice ^d		% AG retained ^b					
			1ST IED			2ND IED ^c		
			<u>3</u>	<u>5</u>	<u>7</u>	<u>3</u>	<u>5</u>	<u>7</u>
IFA	11/18	61%	29	18	13	24	13	10
CFA - (.025 mg Myc. but.)	8/20	10%	21	14	9	40	34	23
IFA + .5 mg Myc. tub.	8/20	40%	23	16	10	28	18	11
IFA + .25 mg Myc. tub.	7/19	37%	23	15	9	27	11	9
IFA + .025 mg Myc. tub.	7/20	35%	22	14	8	25	12	10
No Tid, Nor Adjuvant	3/3	100%	44	37	24		Not done	
IFA Without Tid	0/10	0%	13	8	6		Not done	

^a Administered 5 days after tolerogenic treatment of soluble human gamma globulin.

^b Antigen retained on days 3, 5, and 7 after first IED; figures are whole group average.

^c Only tolerant mice are shown.

^d Number retaining more than 10% by day 7/total mice per group and percent.

jected mice received only an IED. Tolerance was determined according to variations from control-immune or control-uninjected mean values. Retention of more than 10% antigen on day 7 was accepted as a sign of tolerance (5, 6). Statistical analysis was done using Student's *t* test.

Results. Effect of adjuvants on mice with established tolerance. Seventeen CBA/H mice were pretreated with a TID followed by an ID in IFA on day 7, and an IED on day 17. Fourteen mice were found to be tolerant, three were immune. The 14 tolerant mice were divided into two equal groups and were given a second immunizing dose of HGG in an emulsion prepared with either incomplete or complete Freund's adjuvant. When the subsequent IED was administered, all mice in both groups were still found to be tolerant. The percentage of antigen remaining in the animals by the seventh day was similar (24%) to the tolerant control group.

Effect of adjuvants on the production of tolerance to HGG. All experimental groups received a TID on day 0. In the first experimental series, the ID was given on day 5 and the IED on day 17. The results are summarized in Table I. HGG incorporated into IFA, to which increasing concentrations of mycobacterial adjuvant was added, caused a statistically significant reduction in the number of tolerant mice compared to the group receiving an ID in IFA only. A *P* value of <.001 was found between the IFA groups and those mice receiving .025 mg of *M.*

tuberculosis while a *P* value of <.01 was found between the IFA groups and those mice receiving CFA containing *M. butyricum*. CBA/J Mice receiving .025 mg of *M. tuberculosis* (H37Ra) exhibited the highest incidence (65%) of circumvention of tolerance. Subsequent evaluation of the immune status of mice that were tolerant following administration of the IFA containing different concentrations of mycobacterial components was repeated 37 days after the first IED, confirming the late maintenance of tolerance (Table I).

These findings (Table I) were confirmed with (A₁ × C57BL/1)F₁ hybrids, but since groups of four mice each were used, they were considered unsatisfactory for statistical analysis.

In C57BL/10J mice, IFA containing small amounts of *M. tuberculosis* produced effects similar to the HGG-IFA data cited above. High concentrations of mycobacterium appeared more effective, but not to a statistically significant extent (Table II).

In the next series, CBA/J mice received an ID of HGG 7 days after the TID. CFA containing *M. butyricum* was the most efficacious in circumventing tolerance induction (Table III), while low-dose *M. tuberculosis* (H37Ra) was least effective. In this same series of experiments, adjuvants also were evaluated when the span between the TID and the ID was extended, in some groups, to 14 days. *Mycobacterium tuberculosis* in IFA obviously was most effective in circumvent-

TABLE II. RESPONSE OF C57BL/10J MICE TO HGG IN VARIOUS ADJUVANTS

TID ^a	Type of adjuvant ^b	Tolerant mice ^c		% AG retained ^d		
				<u>3</u>	<u>5</u>	<u>7</u>
Yes	IFA	4/11 ^e	36%	27	18	11
No	IFA	0/5	0%	23	12	8
Yes	CFA (Myc. but.)	2/11	18%	22	13	7
No	CFA (Myc. but.)	0/5	0%	9	6	5
Yes	IFA + .5 mg Myc. tub.	1/11 ^e	9%	17	11	6
No	IFA + .5 mg Myc. tub.	0/5	0%	14	10	6
Yes	IFA + .25 mg Myc. tub.	2/11	18%	17	11	7
No	IFA + .25 mg Myc. tub.	0/5	0%	11	6	5
Yes	IFA + .025 mg Myc. tub.	3/11	27%	20	16	9
No	IFA + .025 mg Myc. tub.	0/5	0%	12	8	6
No	None (¹³¹ I-HGG only)	3/3	100%	48	35	22

^a Tolerance Inducing Dose of 1 mg human gamma globulin.

^b 100 mg HGG in adjuvant 7 days after TID.

^c Number of mice retaining more than 10% antigen/total mice.

^d Group average on days 3, 5, and 7 after radiolabeled antigen injection.

^e Difference was >.1.

ing tolerance (Table III), but showed no statistically significant advantage over IFA above. The use of *M. butyricum* and .25 mg *M. tuberculosis* favored tolerance development.

Effects of adjuvants on C3H/b. In our experience (7), C3H/b mice are more susceptible to HGG-induced tolerance development than other strains. These mice were initially given the TID intravenously. Four groups of eight mice each received HGG along with differing concentrations of mycobacterium incorporated into IFA as the ID on day 7; all were subsequently shown to be persistently tolerant to HGG. No significant difference was found among the groups receiving adjuvants of varying concentrations.

Discussion. Previous studies have shown that tolerance to HGG can be broken using rheumatoid factor (8) or strongly heat-aggregated HGG (9). Data presented in this report clearly show that if the antigen in adjuvant is administered before tolerance has been completely established, the cellular mechanisms leading to long-lasting tolerance can be disrupted and circumvention from tolerance effected. The number of mice retaining >10% of the IED dose, presumably tolerant, was significantly decreased by using HGG incorporated into IFA with different amounts of *M. tuberculosis* present. These data were compared to mice receiving HGG

TABLE III. EFFECT OF VARYING TIME OF ADMINISTRATION OF ADJUVANTS ON CBA/J MICE

Type of adjuvant ^a	Tolerant mice at TID-ID intervals with			
	7-Day spread		14-Day spread	
IFA	4/8 ^b	50%	1/8	13%
CFA (.025 mg Myc. but.)	2/8	25%	3/8	38%
IFA + .025 mg Myc. tub.	5/8	63%	0/6	0%
IFA + .25 mg Myc. tub.	3/8	38%	4/6	66%
IFA + .5 mg Myc. tub.	3/8	38%	1/8	13%
IFA no TID	n.a.		0/10	0% ^c

^a Given to 5 groups either 7 or 14 days after 1 mg centrifuged HGG.

^b Tolerant mice/total mice and percentage.

^c No TID was given to this control group.

in IFA after a TID of HGG. However, once murine tolerance to HGG has been established beyond the period shown in Table I, it cannot be abolished by using adjuvants of increasing strengths. It is likely that the failure of strong adjuvants to terminate a tolerant state can be attributed to their inability to denature the antigen.

Since the optimal adjuvant dosage was dependent on the strain of mice used, it seems likely that host factors are extremely important. Our observations are consistent with studies showing that susceptibility to tolerance induction varies with different

murine strains (7, 10). Studies by Richou *et al.* (11) in rabbits and guinea pigs also have indicated that adjuvanticity varies with antigen as well as host species.

This report clearly demonstrates that tolerance, once established, is prolonged rather than terminated by antigen treatment, regardless of the intensity of the challenge. A period of labile tolerance, however, is observed near the end of the tolerance-induction phase, that is, 5 days after the initial tolerogenic inoculation. During this early stage, adjuvant-induced circumvention of tolerance was observed (Table I).

Summary. When mice are injected with deaggregated human gamma globulin (HGG), an HGG-tolerant state ordinarily is produced and persists despite subsequent challenges with an immunizing dose of HGG in saline or with an immunizing dose of HGG in Mycobacterium adjuvant. Subsequent administration of an immune elimination dose of radiolabeled HGG, at 27 days and 47 days does not break the tolerant state. Of special interest is the observation that when complete adjuvant containing increasing amounts of mycobacterial components was administered in conjunction with antigen very early in the tolerance induction phase 5 days after TID, it appears to prevent tolerance production.

Mice challenged 5 and 17 days after the tolerance-inducing inoculation exhibit a statistically significant increase in circumvention of tolerance when compared with individuals challenged on the 7 and 17 day schedule. This increased circumvention of tolerance, as evidenced by 5 day challenge mice, seems to be related both to the mycobacterial content of the adjuvant and murine strain.

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