lenge. The dose in the latter group was 3.8 $\times$ 10^9 organisms as compared to doses also in excess of 10^9 streptococci or staphylococci required to cause fatal infections. In non-fatal infections, positive blood cultures were detected for 14 to 28, 10 to 56 and 10 to 28 days, respectively, after intravenous challenge with streptococci, pneumococci and staphylococci.

Summary. Aerosol challenge with staphylococci produced no demonstrable disease in rhesus monkeys. Intravenous challenge was followed by definite clinical and laboratory evidence of infection. Toxoid immunization did not alter the course of infection. No significant differences were noted between splenectomized and normal monkeys in all parameters measured.

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Termination of Acquired Immunological Tolerance in Mice with Antigen Aggregates.* (31545)

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It has been previously shown that neonatal inoculation of human gamma globulin (HGG) renders mice tolerant to this antigen(1). This tolerant state lasts for about 14 weeks, but if inoculations of antigen are repeated, tolerance is prolonged(2) for as long as the tolerogen is maintained at suitable levels in the tolerant animal.

Acquired tolerance to soluble antigens resembles natural tolerance to self constituents, insofar as there exists in the host a suppression or a lack of antigenic stimulation capable of producing a specific immune response(3). In natural tolerance to self constituents the presence of autologous material in the body seems to exert a suppression of self rejection. In this respect the tolerogen behaves in the body as a self substance(3). On the other hand, in autoimmunity this mechanism of control or self protection is impaired even in the presence of the circulating autologous substances.

A state of acquired immunological tolerance to tumor antigens seems to be present in neoplasm(4). This is the case of tolerance to the

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T antigen of tumors induced by DNA viruses. On the other hand, induction of immune unresponsiveness to the histo-compatibility antigens is the aim for successful organ transplantation.

A great deal of work has been devoted to developing an experimental model of tolerance that can be satisfactorily terminated as a means to study autoimmunity and/or cancer rejection. This report concerns the termination of neonatally acquired immunological tolerance to HGG in mice by inoculation of heated HGG. Heat aggregated HGG was used because of its high antigenic property and its close antigenic relationship with the tolerogen. As it will be demonstrated, heat-aggregated HGG has a more active biologic activity and it is apparently catabolized at a more rapid metabolic rate.

Materials and methods. Animals. Inbred HS Swiss albino mice from the Hale-Stoner colony(1,2-5) were used.

Antigens. Human gamma globulin (HGG) was obtained from Pitman-Moore Co., Lot 172299. IgG was purchased from Mann Research Institute, Lot 4260 1894. The Fc piece of IgG was obtained as described previously (5).

Antisera. Goat anti-human Fc fragment and goat anti-human Fab fragment were purchased from Hyland Laboratories.

Preparation of HGG aggregates. Aggregates were prepared in two ways. A 1.6% solution of HGG was heated at 58°C for 30 minutes. This preparation will be referred to as mild aggregated gamma globulin (MAGG). A second aggregate was produced by heating at 75°C for 15 minutes. This will be referred to as strong aggregated gamma globulin (SAGG).

Assay methods. The hemagglutinating technique and immunodiffusion in agar technique were the ones previously described(5). The latter was assayed using either IgG or the Fc fragment as the antigen. To demonstrate remaining fragments of HGG in the mice circulation, their sera were assayed against goat anti-Fc fragment and goat anti-Fab fragment sera. Mouse sera were collected at the given days by bleeding from the caudal artery.

Immune elimination of radiolabeled antigen. IgG-I^{131} was prepared according to the technique by Rosen(6). At 6 weeks of age tolerant and control mice were inoculated with 1 mg IgG-I^{131} intraperitoneally. Bleeding followed at days 1, 2, 3, 5, and 7. Each sample was drawn in a microtube which was weighed before and after collection to determine radioactivity by weight. The data are summarized in Fig. 1.

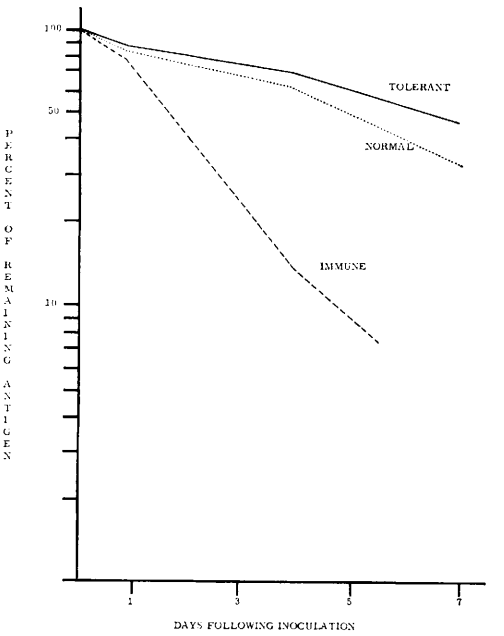

FIG. 1. Graphic representation of immune elimination of IgG-I^{131} at days 1, 3, 5, and 7.

Analytical ultracentrifugation. Ultracentrifugation of HGG preparations was carried out in a Spinco Model E ultracentrifuge through the courtesy of Dr. A. F. S. A. Habeeb. A single sector cell in an AnD rotor was used. After reaching full speed (59,780 r.p.m.) photographic plates were obtained at 16-minute intervals. Since SAGG completely precipitated after centrifugation at 600 r.p.m., no further analysis was obtained.

Experimental. Experiment I was designed to study the effect of HGG and SAGG on normal (non-tolerant) mice. Two groups of 15 mice were used. These mice were 8 weeks old and had received no previous injections, hence were non-tolerant to HGG or SAGG.

Group A was given 1 injection of 2 mg of HGG and group B was given 1 injection of 2 mg of SAGG. Both groups received 1 mg of IgG-I^{131} fifteen days later and the

TABLE I. Immune Response in Tolerant Mice to HGG.

	Antibodies to IgG*					Slow elimination of IgG†				
Groups of tolerant mice	6	9	10	11	12-13	6	9	10	11	12
SAGG challenged 9, 10 wk	0	0	16/32	26/32	28/32	32/32	29/32	12/32	6/32	4/32
MAGG challenged 9, 10 wk	0	0	0	0	0	25/25	25/25	25/25	25/25	25/25
Challenged with HGG 6, 9, 10 wk	0	0	0	0	0	25/25	25/25	25/25	25/25	25/25

* Shown by the hemagglutinating and precipitation in agar techniques.
† Shown by immunodiffusion using specific antisera.

immune elimination followed as it is described above.

Experiment II was designed to study the possibility of terminating tolerance with aggregates of MAGG and aggregates of SAGG. Three groups of 8-week-old mice which were all shown to be tolerant to HGG were used. This tolerance was proven in each instance by the fact that they failed to show an immune elimination of antigen or failed to show hemagglutinating and precipitating antibodies.

Group 1 was composed of 32 tolerant mice. Each mouse received 2 injections of 2 mg of SAGG(IP) 7 days apart.

Group 2 was composed of 25 tolerant mice. Each mouse received 2 injections of 2 mg of MAGG 7 days apart.

Group 3 was composed of 25 tolerant mice and served as the control. These mice all received 2 inoculations of 2 mg of HGG at an interval of 7 days.

Results. Exp. I demonstrated that upon inoculation of either HGG or SAGG, normal mice readily developed an immune response. After the first inoculation of antigen, mice receiving SAGG, had eliminated circulating antigen by the 9th day. Those mice treated with HGG, eliminated their antigen from the circulation after 11 days. After these two groups were challenged with IgG-I^{131}, the immune elimination demonstrated that group A had eliminated the antigen by the 5th day, while group B had eliminated by the 7th day. These differences, though they may not be significant, were consistent following the primary inoculation as well as after the challenging doses.

Experiment II indicated that after inoculation of SAGG, mice previously tolerant to HGG, developed an immune response specific for the Fc fragment of IgG. By immunodiffusion analysis it was demonstrated that mouse antibodies after termination of tolerance gave a reaction of identity against IgG and the Fc fragment. Sixteen of the 32 mice treated with SAGG displayed antibodies by the 10th week. After the second inoculation of SAGG, by the 11th week, 26 of the 32 mice had demonstrable antibodies by immunodiffusion technique. A total of 28 mice were shown to have developed specific antibodies during the entire period of follow up (Table I). The hemagglutination titers of these sera, tested with IgG coated RBC's was 512.

On the other hand, Groups 2 and 3 did not show antibodies to IgG either by the precipitation in agar technique or by the hemagglutination technique. Persistence of antigen in the circulation of these mice was shown by immunodiffusion. The findings are summarized in Table I.

Discussion. Data presented here show that HS mice were rendered tolerant by the neonatal inoculation of HGG and that unresponsiveness was prolonged for at least 20 weeks, provided HGG was reinoculated while the mice were still tolerant. However, when those tolerant mice were treated with the "tolerogen" modified by strong heating, tolerance was terminated in a significant number of mice(29/32). Since the antibodies found in sera of mice that terminated tolerance reacted with both IgG and the Fc fragment, it is very unlikely that the immune response elicited in previously tolerant mice was directed to buried antigenic sites of the native molecule. Weigle and Fudenberg(7) have recently demonstrated that the inoculation of IgG of different genetic types did not induce an immune response in HGG tolerant rabbits. According

to that report, neonatal exposure to human gamma globulin renders tolerance to the entire HGG molecule.

In a study treating non-tolerant mice with both HGG and SAGG it was first established that the HS mice are suitable for this type of experiment. It was then determined that after inoculation of HGG or SAGG, normal mice readily developed an immune response characterized by the presence of precipitating as well as hemagglutinating antibodies. Then it was established that tolerance can be prolonged by repeated inoculations of the antigen for as long as the span of the investigation requires.

The "specific" antigenicity of heat aggregated gamma globulin was studied next. It is widely accepted that antigenicity depends upon the concentration of the antigen in the host(8). It also has been demonstrated that even at the same amount, aggregated gamma globulin is offered to phagocytic cells at a more rapid rate. The results of Gambler(9) and Biro and Garcia(10) indicating a more rapid uptake from the circulation of aggregated HGG have been confirmed.

The biological activity of SAGG has been previously recognized by others(11). Aggregated human gamma globulin was used in this investigation because it was felt that termination of tolerance should be attempted with a related antigenic structure which would be so denatured or different in antigenic and biologic activity as to break down unresponsiveness.

The activity of SAGG resembles the antigenic properties of antigen-antibody complexes. It is likely that SAGG acts both as an antigen-antibody complex and as a related antigen in terminating the unresponsive state. Studies at the cellular level are in progress in an attempt to determine, if possible, the responsible mechanism. Stoner(12) has postulated that the enhancing effects of antigen-antibody complexes may be due to enhanced antigenicity due to either increased molecular size or to tertiary changes in the molecules. Another possibility refers to preferential localization of complexes in antibody producing cells. Gras(13) has found that unresponsiveness due to treatment with small amounts of antigen can be terminated by administration of large amounts of that antigen. SAGG may exert an enhancing effect through either of the above mechanisms. An increased uptake of SAGG and its immunogenicity in HGG tolerant mice can be explained by the presence in SAGG of antigenic determinant not readily detectable in IgG. Hirose and Osler have demonstrated that some rheumatoid arthritis sera reacted with aggregated HGG as well as with turbid H chains but failed to react with the L, S or F chains(14).

Termination of tolerance can be better discussed in relation to an experimental model for study in autoimmunity and cancer rejection. In the autoimmune diseases, natural tolerance to one's own constituents is reversed and the immune system seems to react against self, even when the autologous material persists in the host. In cancer rejection acquired unresponsiveness to tumor antigens must be broken down to elicit an immune response that would reject the tumor cells. When the HS tolerant mice are inoculated with HGG, they do not develop antibody formation, rather they eliminate the tolerogen at an exponential rate. But when SAGG is introduced while mice are still tolerant, a rapid elimination of the antigen follows and an immune response is shown by the presence of specific circulating antibodies. These data suggest that the rate of phagocytosis of HGG is greatly increased after inoculation of SAGG. No data are available at this time to determine the nature of the findings. It may well be due to change in the molecular size of the aggregates (12) or to the change of the antigenic determinants of the denatured molecules(14) or the joint action of both.

The presence in SAGG of new antigenic sites that might increase its uptake by immune cells raises the question whether termination of tolerance has developed to previously tolerant or to non-tolerant sites of the HGG molecule. It is, herein, postulated that tolerance has been terminated against previously tolerant sites. This is supported by the following considerations. First, if the antibodies from previously tolerant mice were developed toward antigenic sites present only in SAGG and probably exposed by the digested Fc fragment, it would have been very

unlikely that these antisera would have reacted with native IgG. The reactivity of mouse antisera with both IgG and the Fc fragment favors the postulation. On the other hand, the possibility of a cross reaction can not be entirely discarded, since it is not known the magnitude of the change undergone in the tertiary structure of the molecule after heating.

Secondly, a previous demonstration from this laboratory showed that active rheumatoid factor terminated tolerance while inactive rheumatoid factor failed to restore immunity (1,2,5). Termination of tolerance was then interpreted as a result of the formation of antigen-antibody complexes at the tissue level of the mouse(5). Since inactive rheumatoid factor and MAGG do not terminate tolerance it can be elaborated that molecular size or preferential localization(12) plays an active role in abolishing tolerance. This will be further tested by creating larger complexes with an inert carrier. Finally, as stated above, Weigle and Fudenberg(7) have demonstrated that rabbits tolerant to HGG did not develop an immune response to various genetic antigenic determinants not previously seen by the host.

Attempts by others to terminate tolerance to gamma globulin by treating tolerant animals with globulin aggregates have not been successful(15). It seems very important to establish as the first contention, that the antigen and the species under study are properly suitable to undertake this type of investigation. In that sense the HS mice readily respond to the antigenic stimulation of aqueous suspension of HGG and SAGG. Furthermore, the neonatal inoculation of 10 mg of HGG rendered mice tolerant and this state of unresponsiveness can be prolonged for at least 20 weeks. Barth *et al*(16) have recently demonstrated that strain differences in the antibody response can occur up to a hundred-fold differences following similar antigenic stimulation.

Summary. Neonatal inoculation of 10 mg of human gamma globulin rendered HS mice immunologically unresponsive to it for as long as inoculation was repeated. Inoculation of heat aggregated HGG was followed by a breakdown of the tolerant state. When tolerant mice were treated with mild heated gamma globulin, tolerance was not terminated but prolonged. Animals that terminated tolerance developed antibodies that reacted specifically with the Fc piece of IgG.

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