SENSITIZATION TO DENATURED AUTOLOGOUS GAMMA GLOBULIN*

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(Received for publication, August 4, 1961)

In the course of studies showing that guinea pigs could develop delayed sensitivity to allotypic gamma globulin it was noted that an occasional animal immunized with denatured autologous gamma globulin became sensitized to this material (1). No animals immunized with native autologous gamma globulin developed delayed reactivity to this protein. These observations suggested that if an animal's own gamma globulin were appropriately modified it would become antigenic for that animal.

The recent observations of Milgrom and Witebsky are consistent with such a hypothesis (2). These authors showed that rabbits immunized with autologous gamma globulin prepared by ammonium sulfate fractionation developed antibodies which, surprisingly, were present in much higher titer against human gamma globulin than against the rabbit's own material. The authors postulated that in the course of preparation some of the gamma globulin molecules underwent structural alteration so as to render them antigenic within the same animal.

The present study was undertaken to investigate systematically the possibility that animals could become sensitized to their own gamma globulin, provided it was appropriately altered. For this purpose guinea pigs and rabbits were immunized with autologous gamma globulin which had been subjected to a variety of denaturation procedures. It was found that animals immunized with alkaline denatured autologous gamma globulin regularly developed hypersensitivity to this material; animals injected with autologous gamma globulin modified in other ways occasionally showed reactivity to the immunizing material but more frequently to heterologous gamma globulins. Animals immunized with autologous gamma globulin not subjected to denaturation procedures failed to develop reactivity to any form of gamma globulin.

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* This work was supported by United States Public Health Service Grant A-1395 and by the Health Research Council of the City of New York under Contract I-138.

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Materials and Methods

Animals.—Male and female albino guinea pigs weighing 300 to 500 gm and male and female albino rabbits weighing 1500 to 2500 gm were used.

Fractionation of Gamma Globulin.—The animals were bled by cardiac puncture on alternate weeks. The serum from each bleeding was separately processed in every case; material from the first bleeding was used for immunization, and that from the second was employed in the various testing procedures. In the process of collecting and preparing the various materials great care was taken to avoid contamination. Syringes, glassware, instruments, and needles were thoroughly cleaned and were sterilized at 160°C for 1 hour prior to use. Gamma globulin was isolated by the addition of an equal volume of 32 per cent sodium sulfate to an aliquot of serum. The resulting precipitate was separated by centrifugation at room temperature for 20 minutes at 15,000 RPM. After 2 washings with 16 per cent sodium sulfate the precipitate was redissolved in a small volume of distilled water, centrifuged to remove any sediment, and then reprecipitated with an equal volume of 32 per cent sodium sulfate. A final washing was performed and the resulting precipitate was redissolved in distilled water and dialyzed at 2 to 4°C against pH 7.1, 0.01 M phosphate buffered 0.15 M NaCl until free of sulfate. The globulin fraction was clarified by centrifugation for 60 minutes at 15,000 RPM, and its concentration was determined by ultraviolet absorption spectrophotometry.

Other Antigens.—Bovine gamma globulin was obtained from Armour and Co., Chicago. Purified diphtheria toxoid was supplied by the Department of Public Health, Boston. Human gamma globulin was obtained from the serum of a single healthy donor or from pooled immune gamma globulin and was isolated either by sodium sulfate fractionation or by elution from DEAE cellulose (3). These preparations, as well as the individual guinea pig and rabbit gamma globulin preparations described above, were assayed for purity by cellulose acetate electrophoresis, and by immunoelectrophoresis. In every case gamma globulin was the overwhelmingly predominant component but occasional preparations contained traces of other globulins. For the denaturation procedures solutions were prepared in 0.9 per cent NaCl. They contained 3 mg protein per ml except in the case of diphtheria toxoid, where a concentration of 1 mg per ml was employed and of rabbit gamma globulin, where the concentration was 10 mg per ml. No preservatives were added and materials were stored at 2°–4°C until use.

Freund's complete adjuvant was obtained from Difco, Detroit.

Denaturation of Proteins.—

A. Alkaline denaturation: The pH of the preparation was brought to 11.5 with 1 N NaOH and the preparation was allowed to stand at room temperature for approximately 18 hours. The pH was then adjusted to 7.2 using 1 N HCl.

B. Heat denaturation was achieved by the immersion of the preparation in a constant temperature water bath at 60°C for 20 minutes in the case of mild heat denaturation, and at 80°C for 10 minutes for strong heat denaturation.

C. Urea denaturation involved the addition of either solid urea or a 50 per cent urea solution to the material to make the final concentration 8 M. It was found that urea in such concentration caused tissue damage, and it was therefore necessary to dialyze away the urea from the preparation of test materials. This was not done with materials which were used for immunization.

D. Acid denaturation: The pH of the solution was lowered to 2.0, using 1 N HCl, and the preparation was incubated in a water bath at 40°C for 30 hours. The pH was then adjusted to 7.2.

E. Ultrasound: Ultrasonic denaturation was achieved by the use of magnetostrictive oscillations at 22,000 to 24,000 cycles per second. About 2 ml of the solutions were placed in small bore test tubes, immersed in an ice bath and the vibratory probe was inserted to within 2 mm

of the bottom of the tube. The samples were treated for 15 minutes. In no case did the temperature increase more than 5°C during the procedure (4).

F. Film denaturation: The solutions were placed in a sintered glass filter of medium porosity through which nitrogen gas was bubbled at low pressure. This was continued for 10 to 20 minutes until visible aggregation occurred.

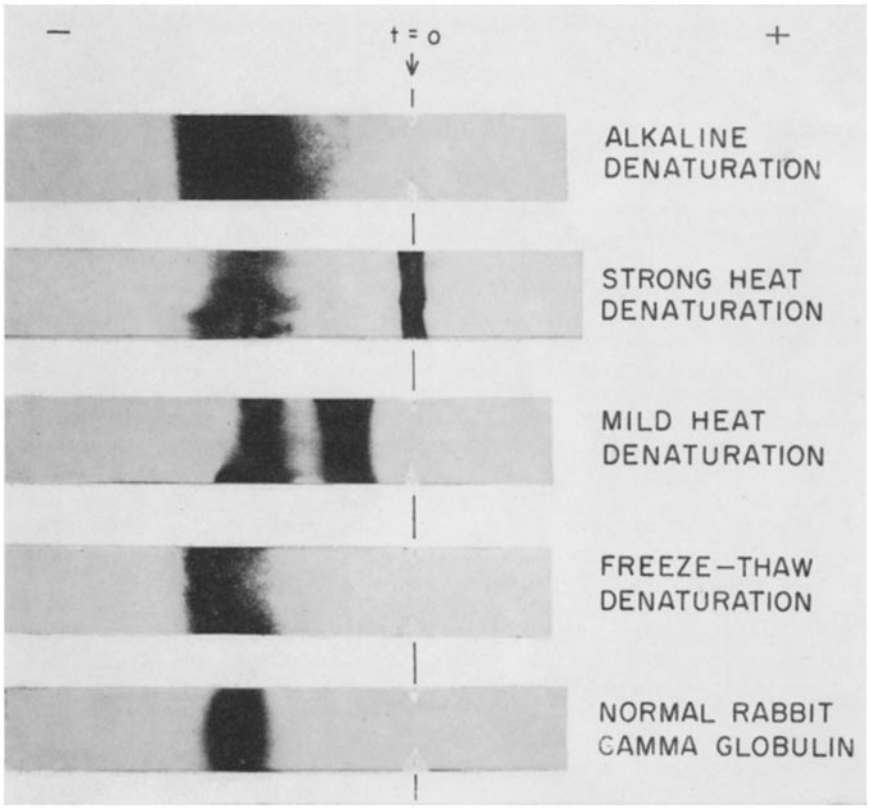

FIG. 1. Cellulose acetate electrophoresis of normal rabbit gamma globulin and several denatured preparations. Five mg. of protein were electrophoresed for 4 hours at a constant current of 0.4 ma per cm strip width in pH 8.6 barbitone buffer 0.07 M and stained with nigrosin.

G. Freeze-thaw denaturation: The solutions were frozen in a deep freeze at −22°C for 2 hours and then permitted to thaw completely at room temperature. This was repeated 5 times.

Samples of several denatured gamma globulin preparations were subjected to electrophoresis(Fig. 1). It can be seen that the denatured preparations exhibit electrophoretic mobilities different from normal rabbit gamma globulin and in every case there is greater spread of the bands, indicating heterogeneity.

Immunization.—Guinea pigs were immunized with an emulsion prepared from a saline solution of the antigen containing 3 mg protein per ml and an equal volume of complete adjuvant. One week after the second bleeding performed for preparation of gamma globulin, 0.25 ml of the emulsion was injected into each rear foot-pad, and the following week into each

front foot-pad. Each guinea pig received a total immunizing dose of 1.5 mg of protein. Animals receiving Freund's adjuvant alone were injected with an emulsion of saline and adjuvant according to same schedule. Diphtheria toxoid was used at an initial concentration of 0.6 mg per ml, resulting in a total immunizing dose of 0.3 mg.

Rabbits were immunized as follows: Once a week for 5 weeks 2 ml of an emulsion containing equal volumes of a 1 per cent solution of the antigen in saline and complete adjuvant were administered to each animal in multiple subcutaneous and intramuscular sites. Then, after a 2 week interval the animals were given 3 intravenous injections at weekly intervals of alum precipitated antigen, each injection containing 5 mg of protein. The total amount of protein used for immunization was 65 mg. Sera were collected and skin tests performed at various intervals during and after the immunization schedule.

Skin Tests.—Skin tests were performed by injecting 50 micrograms of antigen in 0.1 ml saline, intradermally in the flanks. Not more than 4 skin tests were made in any animal at one time. The animals were examined for Arthus type reactivity for several hours after injection; no Arthus reactions were observed in any of the experiments in guinea pigs. Delayed reactions were read at 24 hours and recorded in terms of linear dimensions in millimeters and further qualified as faint or strong on the basis of the intensity of the erythema and induration. In guinea pigs the first skin tests were performed 1 week after the last immunizing injection. In most of the guinea pig experiments autologous, homologous, and heterologous gamma globulins were tested in that order; denatured material was used for testing concomitantly with, or after, the corresponding native preparation. Homologous gamma globulin preparations used for skin tests were obtained from single donors. All testing materials were also injected into normal, unsensitized animals and found to be free of skin-irritating properties that could give rise to false positive reactions.

Serologic Tests.—Antibodies against human gamma globulin in rabbit sera were measured by agglutination titers using human group O Rh-positive red cells sensitized by incomplete human anti-Rh serum employing the methods described by Milgrom and Witebsky (2). Anti-Rh_0 (anti-D) serum was obtained from the New York City Department of Health. The tanned cell hemagglutination technique as described by Stavitsky (5) was used to assay the sera for antibodies against autologous materials, human gamma globulin, and bovine gamma globulin. The concentration of antigen used to coat the tanned cells was 1 mg per ml. Normal rabbit serum was used as the diluent.

Precipitation Tests.—Double diffusion gel precipitation reactions were performed using 0.8 or 1 per cent agar in pH 7.4 to 7.6 buffered saline.

Immunoelectrophoresis was carried out according to the method of Grabar and Williams (6), in a barbital buffer of pH 8.6. In some of the gel diffusion and immunoelectrophoresis studies a rabbit antihuman gamma globulin antiserum was employed. This antiserum was prepared by immunizing rabbits with sodium sulfate fractionated gamma globulin. The pooled antiserum was absorbed with small aliquots of the supernatant of the human serum obtained following gamma globulin preparation until the antiserum showed only a single line corresponding to anti gamma globulin on immunoelectrophoresis.

The guinea pigs immunized with alkaline-denatured autologous gamma globulin which had shown delayed skin reactivity to the immunizing material were also tested for anaphylaxis by intravenous injection of the antigen, to investigate the possibility of antibody production.

RESULTS

I. Delayed Reactivity to Various Gamma Globulins in Guinea Pigs Immunized with Denatured Autologous Gamma Globulin

The first part of the investigation was concerned with a study of the development of delayed reactivity to various forms of native and denatured gamma

globulin in guinea pigs immunized with autologous gamma globulin which had been subjected to one of a variety of denaturation procedures. To serve as controls for these experiments, groups of guinea pigs were immunized with unmodified autologous gamma globulin, with an unrelated protein, diphtheria toxoid, or with Freund's adjuvant alone. These experiments are summarized in

TABLE I

Incidence of Delayed Reactivity to Native and Denatured Gamma Globulins in Guinea Pigs Immunized with Autologous Gamma Globulin, Diphtheria Toxoid, or Freund's Adjuvant Alone

Immunizing antigen	Test materials														
	Aut. GG*	HGG	BGG	Alk.-den. BGG	Heat-den. HGG	Alk.-den. Hom. GG	Aut. GG	Urea-den. BGG	Alk.-den. HGG	Acid-den. HGG	Heat-den. Hom. GG	Aut. GG	HGG	BGG	Alk.-Den. BGG
Autologous gamma globulin	0/14	0/6	0/8	0/2	0/6	0/6	0/2	1/2‡	3/6‡	1/6‡	0/8	0/12	2/8‡	1/12‡	0/6
Day of test	1	2	2	3	3	5	5	5	5	5	5	7	8	8	8

Immunizing antigen	Test Materials													
	Aut. GG	HGG	BGG	Heat-den. HGG	Urea-den. HGG	Heat-den. BGG	Urea-den. BGG	Acid-den. BGG	Alk.-den. HGG	Heat-den. Hom. GG	Alk.-den. Hom. GG	HGG	BGG
Diphtheria toxoid	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/5‡	0/5	0/5	0/5	0/5	0/5
Freund's adjuvant	0/8	0/8	0/8	0/8	0/8	0/8	0/8	3/8‡	0/8	0/8	0/8	1/8	3/8‡
Day of test	1	2	2	4	4	4	4	6	6	7	7	8	8

Test dose: 50 μg protein in 0.1 ml saline.

* The following abbreviations are used throughout this paper: Aut. GG, autologous gamma globulin; Hom. GG, homologous gamma globulin; HGG, human gamma globulin; BGG, bovine gamma globulin; Den., denatured; Alk., alkaline.

‡ Faint reactions.

Table I. In no instance did a guinea pig immunized with these materials show delayed skin reactivity on initial challenge to any of the forms of gamma globulin employed as test materials. However, it was observed that an occasional guinea pig eventually developed delayed sensitivity to heterologous gamma globulin in some form when material from the same foreign species was used for repeated skin testing. The incidence of such reactions was low and they were of a mild character, indicating that although some guinea pigs may become sensitized by skin testing, it is a relatively ineffective method of sensitization.

Delayed Reactivity to Gamma Globulins in Guinea Pigs Immunized with Alkaline-Denatured Autologous Gamma Globulin.—The results of 2 experiments in

TABLE II

Delayed Reactivity to Native and Denatured Gamma Globulins in Guinea Pigs Immunized with Alkaline Denatured Autologous Gamma Globulin

Experiment I

Animal No.	Test materials					
	Alk.-den. aut. GG	Aut. GG	Alk.-den. hom. GG	BGG	Alk.-den. BGG	Alk.- den. aut. GG
1	15 × 15*	0	9 × 10	tr.	0	10 × 10
2	tr.	0	tr.	0	0	5 × 6
3	10 × 10	0	11 × 9	0	0	11 × 10
4	12 × 10	0	8 × 9	0	0	10 × 10
5	15 × 15	0	10 × 10	0	0	
6	10 × 10	0	0	tr.	15 × 15	
7	15 × 11	0		tr.	tr.	
8	10 × 10	0	15 × 12	0	0	
9	15 × 15	0	15 × 12f	0	0	
Day of test	1	4	4	7	7	7

Experiment II

Animal No.	Test materials							
	Alk.-den. aut. GG	BGG	Alk.-den. BGG	Aut. GG	Urea-den. BGG	Alk.-den. hom. GG	HGG	Reagents used for alk. den.
10	15 × 15	0	15 × 15	0	20 × 20	10 × 10	0	0
11	10 × 10	0	0	0	10 ×10f	0	0	0
12	15 × 11	0	0	0				
13	10 × 10	0	0	0	15 × 10	15 × 12	0	0
14	15 × 15	0	0	0	15 × 15	15 × 12f	0	0
Day of test	1	1	1	6	6	6	7	7

Test dose: 50 μg protein in 0.1 ml saline.

* Values refer to reaction diameters in millimeters; reactions of unusual intensity were further qualified as s—strong; f—faint. Minimal reactions were recorded as tr—trace.

which guinea pigs were immunized with their own gamma globulin which had been subjected to alkaline denaturation are shown in Table II. Such animals regularly exhibited delayed skin reactivity to alkaline denatured autologous and homologous gamma globulin. They consistently failed to show sensitivity to native autologous gamma globulin. A few animals showed mild reactivity to

native or denatured bovine gamma globulin on initial challenge. In the second experiment animals failed to react to native bovine gamma globulin on day 1, but on day 6 all animals tested with urea-denatured bovine gamma globulin were positive; the incidence and intensity of this reactivity was greater than that seen in control animals repeatedly skin-tested with bovine gamma globulin (Table I).

At the end of the experiment all of the guinea pigs were again skin-tested with native autologous gamma globulin and found to be negative. In addition, the 9 guinea pigs in Experiment I were injected intravenously with 0.5 or 1 mg

TABLE III

*Delayed Reactivity to Native and Denatured Gamma Globulins in Guinea Pigs Immunized with Heat-Denatured Autologous Gamma Globulin**

Animal No.	Test materials											
	Aut. GG	Heat-den. aut. GG	HGG	BGG	Heat-den. HGG	Alk.-den. HGG	Acid-den. HGG	Urea-den. hom. GG	Heat-den. hom. GG	Heat-den. aut. GG	HGG	BGG
15	0‡	0	0	0	0	10 × 10f	0	15 × 15s	12 × 12§	0	10 × 8	0
16	0	0	0	0	0	12 × 12	15 × 15	10 × 10s	0§	0	15 × 15	0
17	0	0	0	0	0	0	0	0	0	0	10 × 10f	0
18	0	0	0	0	0	7 × 7f	0	0	0	0	0	0
19	0	0	0	0	10 × 6	15 × 15	0	10 × 10s	10 × 10f	0	0	0
20	0	0	0	0	12 × 8	15 × 12	10 × 10	12 × 10s	15 × 10	8 × 8f	12 × 12s	12 × 12
21	0	0	0	0	0	15 × 10	10 × 10	0	0	0	10 × 10	20 × 20s
22	0	0	0	0	0	15 × 15	8 × 8	tr.	0	0	15 × 15	20 × 20s
Day of test	1	1	2	2	4	5	5	5	5	7	8	8

Test dose: 50 μg protein in 0.1 ml saline.

* Strong heat denaturation was employed: 80°C for 10 minutes.

‡ Values refer to reaction diameters in millimeters; reactions of unusual intensity were further qualified as s—strong; f—faint. Minimal reactions were recorded as tr.—trace.

§ These two animals were tested with sonically denatured homologous gamma globulin.

of alkaline-denatured autologous gamma globulin and no signs of anaphylaxis were observed.

In addition, in the second experiment the animals were tested with a neutral solution of the reagents used for denaturation, namely NaOH and HCl, and no skin reactions were elicited.

The results of these experiments show that guinea pigs immunized with alkaline-denatured autologous gamma globulin regularly develop delayed sensitivity to the immunizing material and occasionally to heterologous gamma globulin.

Delayed Reactivity to Gamma Globulins in Guinea Pigs Immunized with Heat-Denatured Autologous Gamma Globulin.—In Table III are shown the results obtained in guinea pigs immunized with autologous gamma globulin which had

been denatured by heating at 80°C for 10 minutes. In contrast to guinea pigs immunized with alkaline-denatured autologous gamma globulin, only one of the present group showed a skin reaction to the material used for immunization (No. 20, day 7). However, 2 animals showed reactions to heat-denatured human gamma globulin on day 4 and all but one reacted to alkaline-denatured human gamma globulin on day 5. In view of the observations with control animals (Table I) it must be concluded that these reactions and subsequent reactions to human gamma globulin were not merely the result of skin testing with native human gamma globulin on day 2 but were essentially due to the immunizing procedure itself. In addition, several of the guinea pigs displayed skin reactivity to denatured homologous gamma globulin on initial challenge. At the end of the experiment all of the animals were again skin-tested with native autologous gamma globulin and failed to show reactions (not shown in Table III).

Delayed Reactivity to Gamma Globulins in Guinea Pigs Immunized with Ultrasound-Denatured Autologous Gamma Globulin.—The observations of 2 experiments on guinea pigs immunized with autologous gamma globulin denatured by ultrasound are recorded in Table IV. Only 2 animals showed skin reactions (and these were mild) to the material used for immunization and in 2 cases there was a trace reaction to native autologous gamma globulin (which were negative on retest). However, a high percentage of the guinea pigs gave positive delayed reactions to human gamma globulin on initial challenge. A smaller number of animals showed delayed reactivity to native or denatured bovine gamma globulin on the first test, and this number increased on repeated testing. In the second experiment a high percentage of animals gave strong delayed reactions to ultrasonically denatured homologous gamma globulin, in striking contrast to the low incidence of reactivity to denatured autologous gamma globulin. It should be pointed out that in the second experiment a new probe of identical design was employed which oscillated at a slightly lower frequency (22,000 *vs.*, 24,000 cycles per second).

At the end of the experiment the animals were again tested with native autologous gamma globulin and found to be negative (not shown in Table IV).

It was thought necessary to rule out the possibility that the reactivity which was observed against heterologous and homologous gamma globulin resulted from contamination of the preparations used for immunization by trace amounts of protein carried on the probe, despite the fact that the instrument was washed thoroughly as routine between runs. Accordingly, the following control experiments were performed. The probe was immersed in a solution containing diphtheria toxoid in a concentration of 1 mg/ml for 15 minutes at 0°–1°C. The probe was then subjected to the usual cleaning procedure which involved acid cleaning followed by rinse with distilled water, wiping, rinse in 6N HCl, wiping, and 2 rinses with distilled water. The probe was then used to ultrasonically denature a solution of homologous gamma globulin (3 mg/ml). This material was used to immunize 6 guinea pigs according to the usual procedure. All of these animals developed delayed hypersensitivity to homologous gamma globulin but none of them to diphtheria toxoid. Following use of the probe for denaturation of the

TABLE IV

Delayed Reactivity to Native and Denatured Gamma Globulins in Guinea Pigs Immunized with Ultrasound-Denatured Autologous Gamma Globulin

Experiment I

Animal No.	Test materials									
	Aut. GG	Sonically den. aut. GG	Sonically den. hom. GG	HGG	BGG	Urea-den. BGG	Acid-den. BGG	Sonically den. aut. GG	HGG	BGG
23	0*	0	0	14 × 10	0	10 × 8	0	0	15 × 15	10 × 10
24	0	0	0	0	0	0	0	0	0	10 × 10
25	0	0	0	0	0	0	0	0	0	0
26	0	0	0	12 × 10	0	0	0	0	15 × 15	0
27	0	0	0	10 × 8	0	0	0	0	12 × 10	15 × 15
28	0	10 × 10f	0	15 × 12	0	10 × 10	0	5 × 5	0	10 × 10
Day of test	1	1	1	2	2	2	2	8	8	8

Experiment II

Animal No.	Test materials							
	Aut. GG	Sonically den. aut. GG	HGG	BGG	Sonically den. hom. GG	HGG	Sonically den. aut. GG	Hom. GG
29	0	0	0	0	20 × 35s	10 × 10	0	0
30	0	0	0	tr.	10 × 12	15 × 15s	0	0
31	tr.	tr.	8 × 10	0	25 × 25s	10 × 15s	10 × 10	0
32	0	0	7 × 10	tr.	0	10 × 10f	0	0
33	0	0	9 × 10	0	25 × 20s	10 × 10	0	0
34	0	0	10 × 10	tr.	25 × 25s	7 × 10f	0	0
35	0	0	10 × 8f	7 × 6f	0	0	0	0
36	tr.	0	10 × 10f	0	20 × 25s	15 × 15s	0	0
Day of test	1	1	1	3	5	5	7	7

Test dose: 50 μg protein in 0.1 ml saline.

* Values refer to reaction diameters in millimeters; reactions of unusual intensity were further qualified as s—strong, f—faint. Minimal reactions were recorded as tr.—trace.

homologous gamma globulin it was again cleaned in the usual fashion and then a saline solution was subjected to oscillations with the probe; an adjuvant emulsion was prepared from this saline solution and used to immunize 6 guinea pigs according to the usual schedule. All of these animals failed to develop delayed reactivity either to homologous gamma globulin or diphtheria toxoid.

Delayed Reactivity to Gamma Globulins in Guinea Pigs Immunized with Urea-Denatured Autologous Gamma Globulin.—The observations on guinea pigs immunized with urea-denatured autologous gamma globulin are summarized in Table V. None of the animals showed a skin reaction when tested with denatured autologous gamma globulin. Once again, however, it was found that some animals reacted to native or denatured heterologous gamma globulin on initial skin test, and that this incidence was seen to have increased when the

TABLE V

Delayed Reactivity to Native and Denatured Gamma Globulins in Guinea Pigs Immunized with Urea Denatured Autologous Gamma Globulin

Animal No.	Test materials													
	Aut. GG	Urea-den. aut. GG	HGG	BGG	Alk.-den. BGG	Urea-den. HGG	Alk.-den. HGG	Acid-den. HGG	Sonically den. hom. GG	Urea-den. hom. GG	Heat-den. hom. GG	Urea-den. aut. GG	HGG	BGG
37	0*	0	0	0		0	10 × 10f	0	0		0	0	10 × 15f	0
38	0	0	0	0		0	0	0	0		0	0	0	0
39	0	0	0	0		0	0	0	0		0	0	0	0
40	0	0	0	0		0	0	0	0		10 × 10	0	tr.	0
41	0	0	0	0		0	13 × 11	8 × 10		20 × 20	10 × 10	0	10 × 10f	10 × 10f
42	0	0	15 × 20	8 × 10		15 × 15	15 × 10	0		0	0	0	6 × 8	10 × 10
43	0	0	0	0		0	14 × 15	8 × 8		0	15 × 20	0	0	6 × 6f
44	0	0	0	0		0	10 × 10	0		0	0	0	0	15 × 15s
45	0	0		8 × 6	8 × 8			7 × 7		0			10 × 12	
46	0	0		4 × 4	4 × 4			0						
47	0	0		0	0			0		0			10 × 10f	
48	0	0		0	0			15 × 15f		7 × 7			10 × 10	
Day of test	1	1	2	2	3	5	5	5	5	5	7	7	8	8

Test Dose: 50 μg protein in 0.1 ml saline.

* Values refer to reaction diameters in millimeters; reactions of unusual intensity were further qualified as s—strong, —faint. Minimal reactions were recorded as tr.—trace.

animals were retested with gamma globulin from that species. In addition, as in the case of guinea pigs immunized with ultrasound-denatured autologous gamma globulin (Table IV, Experiment II), several animals reacted to denatured homologous gamma globulin, although they remained non-reactive to denatured autologous gamma globulin. As in the previous experiments all animals were negative to native autologous gamma globulin when tested at the conclusion of this experiment (not shown in Table V).

Incidence of Delayed Reactivity to Gamma Globulins in Guinea Pigs Immunized with Mild Heat-, Acid-, or Film-Denatured Autologous Gamma Globulins.—In Table VI are summarized experiments in which guinea pigs were immunized with their own gamma globulin which had been subjected to mild heat

TABLE VI

Incidence of Delayed Reactivity to Native and Denatured Gamma Globulins in Guinea Pigs Immunized with Autologous Gamma Globulin Denatured by Mild Heat, Acid, or Film*

Immunizing antigen	Test materials										
	Aut. GG	Heat-den. aut. GG	HGG	BGG	Heat-den. HGG	Alk.-den. HGG	Acid-den. HGG	Urea-den. hom. GG	Heat-den. hom. GG	HGG	BGG
Mild heat–denatured autologous gamma globulin	0/14	0/14	0/8	0/8	0/8	1/7	1/7	0/7	0/7	0/7	2/7
Day of test	1	1	2	2	3	5	5	5	7	8	8

Immunizing antigen	Test Materials														
	Aut. GG	Film- den. aut. GG	Film-den. hom. GG	BGG	HGG	Sonically den. HGG	Urea-den. BGG	Acid-den. HGG	Alk. -den. HGG	Alk.-den. BGG	Heat-den. BGG	Alk.-den. hom. GG	Urea-den. hom. GG	HGG	BGG
Film-denatured autologous gamma globulin	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Day of test	1	1	2	2	2	3	3	4	4	4	4	4	5	7	7

Immunizing antigen	Test materials									
	Aut. GG	Acid-den. aut. GG	Acid-den. hom. GG	HGG	BGG	Urea-den. BGG	Acid-den. BGG	Acid-den. aut. GG	HGG	BGG
Acid-denatured autologous gamma globulin	0/4	0/4	0/4	1/4	0/4	0/4	0/4	1/4	3/4	4/4
Day of test	1	1	1	2	2	2	2	8	8	8

Test dose: 50 μg protein in 0.1 ml saline.

* Mild heat: 60°C for 20 minutes.

treatment, acid, or film denaturation. It was found that animals immunized with mild heat–denatured or film–denatured autologous gamma globulin failed to develop sensitivity to any of the forms of gamma globulin used for testing on initial challenge. Only 2 guinea pigs in the mild heat–denatured group developed reactivity to heterologous gamma globulin seen after repeated skin tests with

human or bovine gamma globulin; this incidence did not exceed that seen in control animals (Table I) indicating that it probably resulted from the skin test without a specific contribution from the immunizing procedure.

The guinea pigs immunized with film-denatured autologous gamma globulin failed to react to any of the materials on initial or repeated testing.

TABLE VII

Antibodies against Denatured Autologous Gamma Globulin in Rabbits Immunized with Denatured Autologous Gamma Globulin

Method of denaturation	Animal No.	Highest dilution of serum giving agglutination*
Undenatured	1	0
	2	0
Freeze-thaw	3	0
	4	0
Mild heat	5	0
	6	0
	7	6400
	8	0
	9	0
Strong heat	10	0
	11	0
	12	0
	13	0
Alkaline	14	320
	15	6400
	16	640

* Agglutination of tannic acid–treated red cells coated with autologous gamma globulin denatured in the same way as the immunizing material.

Some of the guinea pigs injected with acid-denatured autologous gamma globulin showed evidence of sensitization. One of 4 animals reacted to human gamma globulin on first test and one animal reacted mildly to its own acid denatured gamma globulin (day 8). The high incidence of reactivity to heterologous gamma globulins on day 8 probably is the result of the immunizing procedure.

II. Development of Antibodies to Gamma Globulins in Rabbits Immunized with Denatured Autologous Gamma Globulin

The second part of the investigation was concerned with the effects of immunization of rabbits with autologous gamma globulin which had been subjected

to one of several denaturation procedures. In Table VII are shown the titers of antibodies which were produced against the immunizing materials. All the rabbits immunized with alkaline-denatured autologous gamma globulin developed antibodies against this material. With other forms of denaturation only

TABLE VIII

Anti-Human Gamma Globulin Antibodies in Rabbits Immunized with Denatured Autologous Gamma Globulin

Method of denaturation	Animal No.	Days after initial immunization				
		22	34	55	94	94
Undenatured	1	0*	2*	*	40*	10‡
	2	0	1	4		
Freeze-thaw	3	16	400	1280		320
	4	256	1600	2048	8000	320
Mild heat	5	1	0		5	40
	6	0	0		0	0
	7	0	0		0	0
	8	2	0		0	0
	9	0	8		20	0
Strong heat	10	4	16	200	160	0
	11	2	8	160	320	20
	12	4	0		10	0
	13	8	8			
Alkaline	14	128	800	2048	2000	160
	15	1	200		640	160
	16	0	16		40	0

Results recorded as highest dilution of serum giving agglutination.

* Agglutination of human group O Rh positive red cells sensitized by incomplete human anti-Rh serum.

‡ Agglutination of tannic acid–treated sheep red cells coated with human gamma globulin.

one rabbit (No. 7, mild heat) produced detectable amounts of antibody against the denatured autologous gamma globulin used for immunization.

These results present a pattern similar to what had been observed in guinea pigs, where only animals immunized with alkaline-denatured autologous gamma globulin regularly developed delayed reactivity to the immunizing material. Furthermore, as is shown in Table VIII antibodies against human gamma globulin were demonstrated in many of the rabbits; the highest titers were found in rabbits immunized with freeze-thaw– or alkaline-denatured autologous gamma globulin. In the 2 rabbits immunized with freeze-thaw–denatured

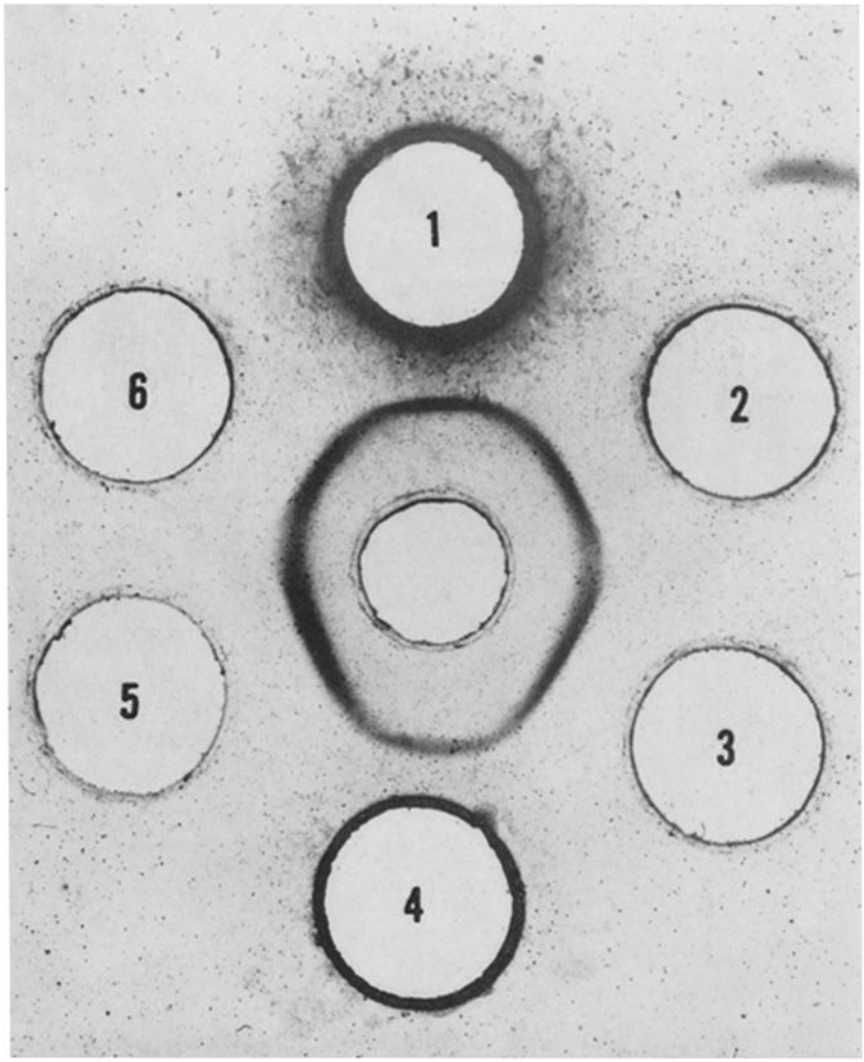

FIG. 2. Ouchterlony plate. Central well: human gamma globulin (0.05 mg/ml). Well 1: serum from rabbit 4, freeze-thaw group. Well 4: serum from rabbit 14, alkaline group. Wells 2 and 3: rabbit anti human gamma globulin diluted 1:8. Wells 5 and 6: rabbit anti human gamma globulin diluted 1:16. A line of identity is seen. Stained with amido black.

autologous gamma globulin and in one of the rabbits (No. 14) immunized with alkaline-denatured material, antibodies against human gamma globulin were demonstrated by double diffusion in agar and by immunoelectrophoresis (Figs. 2 and 3). The pattern seen with immunoelectrophoresis and the line of

identity formed with a known rabbit anti human gamma globulin antiserum demonstrate that the antibodies were specifically directed against gamma globulin rather than against some other constituent of serum.

In addition, both of the rabbits immunized with freeze-thaw–denatured autologous gamma globulin developed antibodies against bovine gamma globulin, demonstrated by agglutination of tanned sheep red cells (titers 1:40). Only one rabbit in any of the other groups (No. 5, mild heat) showed anti bovine gamma globulin antibodies (titer 1:20).

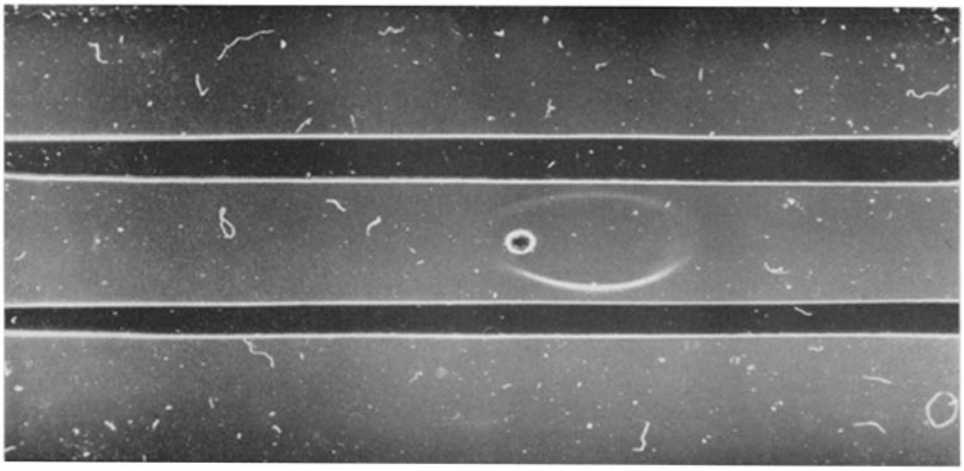

FIG. 3. Immunoelectrophoresis in agar. The central well contained a solution of human gamma globulin 0.4 mg/ml. Electrophoresis 2½ hours at constant current of 0.5 ma per cm width. Upper trough: serum from rabbit 4, freeze-thaw group. Lower trough: rabbit anti human gamma globulin antiserum, diluted 1:64.

The results of skin tests performed in the rabbits with the same form of denatured autologous material used for immunization are shown in Table IX. All of the animals in the alkaline-denatured and freeze-thaw group exhibited reactivity to their own denatured gamma globulin, as did one rabbit immunized with strong heat-denatured autologous gamma globulin. None of the rabbits immunized with mild heat–denatured autologous gamma globulin or undenatured material showed reactivity to their own gamma globulin.

DISCUSSION

The observations presented in this study show that animals can be regularly sensitized to their own gamma globulin, if it is denatured in an appropriate fashion. With the type of alteration produced by alkaline treatment, autologous gamma globulin was so modified that in animals immunized with this material it was always possible to show reactivity to it. When animals were immunized

with autologous gamma globulin modified by other denaturation procedures, such as freeze-thaw, ultrasonic, or urea treatment, only an occasional animal could be demonstrated to react to the immunizing material, but in many instances reactivity or antibodies to heterologous gamma globulin developed.

TABLE IX

Skin Reactions to Denatured Autologous Gamma Globulin in Rabbits Immunized with Denatured Autologous Gamma Globulin

Method of denaturation	Animal No.	Arthus reaction*	Delayed reaction‡
Undenatured	1	0	0
	2	0	0
Freeze-thaw	3	0	+
	4	0	+
Mild heat	5	0	0
	6	0	0
	7	0	0
	8	0	0
	9	0	0
Strong heat	10	0	0
	11	0	0
	12	+	+
	13	0	0
Alkaline	14	+	+
	15	+	+
	16	+	0

Tests performed 39 days after initial immunization.

Test dose: 50 μg protein in 0.1 ml saline.

* Arthus reactions recorded at 4 hours.

‡ Delayed reactions at 24 hours.

The positive Arthus reactions were characterized by erythema and edema but no hemorrhage.

Animals did not develop reactivity or antibodies to their own undenatured gamma globulin. Animals injected with their own gamma globulin which was prepared in such a way as to avoid denaturation almost invariably failed to show evidence of an immune response directed against any type of gamma globulin.

The form of reactivity against gamma globulin which was demonstrable depended upon the species of animal employed. As was to be expected, delayed hypersensitivity to gamma globulin was the type of reactivity exhibited by the

guinea pig, whereas in the rabbit the responsiveness was manifested by the appearance of circulating antibodies.

The possibility that the observations reported here are the result of immunization by extraneous contaminants rather than by the animal's own material can be excluded for several reasons. In the first place, great care was taken to avoid introduction of foreign material during collection, preparation, storage, and immunization. The material used for immunization was obtained and processed separately from that used for testing, so that the likelihood of the same chance contaminant being present in both preparations was extremely slight. Furthermore, guinea pigs immunized with undenatured autologous gamma globulin, diphtheria toxoid or Freund's adjuvant alone did not exhibit delayed reactivity to any form of gamma globulin on initial challenge, thus providing evidence that the immune response observed in animals injected with denatured autologous gamma globulin was indeed the result of the antigenic stimulus provided by the modified gamma globulin.

The possibility that the results with alkaline denaturation could be due to the introduction of antigenic material in the reagents used for denaturation was eliminated by the failure to elicit positive reactions with these substances in immunized animals, as well as by the failure of animals immunized with acid-denatured gamma globulin, in which the same reagents were employed, to react with most of the test materials.

In the case of gamma globulins denatured by ultrasound, the same probe was used to treat each preparation. Since immunization with sonically denatured gamma globulin resulted in a high incidence of reactivity to similarly denatured homologous material, it was necessary to eliminate the probe as a carrier of sufficient contaminating homologous gamma globulin to result in allotypic sensitization. This was done by showing that when the probe was deliberately contaminated by the highly antigenic protein diphtheria toxoid, and then subjected to the usual cleaning procedures, successively treated material did not receive, from the probe, enough toxoid to sensitize guinea pigs.

Further evidence against the possibility that the reactivity resulted from introduction of antigenic material during the denaturation procedure is provided by the fact that animals immunized with heat-denatured material showed a pattern of reactivity similar to that observed with other forms of denaturation. In this case no foreign material at all was introduced during denaturation.

The question arises as to whether the reactivity which developed in animals immunized with their own denatured material was directed against some serum protein present in the preparation in small amounts rather than against gamma globulin itself. The most compelling evidence that the antigen was indeed a form of gamma globulin was the fact that the antibodies produced in the rabbits were specifically directed against some form of gamma globulin, as shown

by immunoelectrophoresis, precipitin reactions in gel and agglutination of red cells coated with human gamma globulin.

The way in which the various denaturation procedures alter protein structure requires comment before an interpretation can be made of how denaturation confers antigenicity on autologous gamma globulin. The mechanism of denaturation of proteins has been recently reviewed by Kauzmann (7) and Putnam (8). From the point of view of the present study several aspects of the subject of denaturation are pertinent. With each of the denaturation procedures a wide range of structural modifications of the molecules takes place. Such changes are evidenced in several ways, as for example, alterations in sedimentation, viscosity, and electrophoretic mobility. Denaturation procedures result in the rupture of intramolecular bonds so that the altered protein molecules contain many potential bonding points. Reformation of bonds may be intramolecular, giving rise to new configurations, or intermolecular, giving rise to aggregates. Such a mechanism serves to explain changes in size and shape in altered molecules. When proteins are denatured, previously masked chemical groups, such as sulfhydryl groups, may become apparent. It has been shown that denaturation is accompanied by alterations in immunologic specificity of proteins. Maurer has recently shown by immunochemical techniques (9) that the properties of denatured proteins depend on the denaturing agent employed and further that there are varying degrees of denaturation.

The observation that animals immunized with alkaline-denatured autologous gamma globulin regularly develop reactivity to the immunizing material indicates that the antigenic form of the gamma globulin is well represented in alkaline-denatured preparations. However, with the other forms of denaturation, reactivity against the immunizing material could only rarely be demonstrated indicating that only a small percentage of molecules were altered in such a way as to confer antigenicity upon them.

The finding that animals immunized with autologous gamma globulin denatured in certain ways, *e.g.* ultrasound, urea, strong heat, or freeze-thaw, developed greater reactivity to heterologous gamma globulin than to the immunizing material appears at first paradoxical. It is reasonable to assume that this observation can be explained in the following way. In the process of some denaturation procedures the structural modifications of molecules can be considered to occur at random. Therefore, only a small percentage of the autologous gamma globulin molecules have new configurations conferred upon them which render them antigenic in the same animal. Since much smaller amounts of antigens are required for sensitization than for elicitation of a skin reaction, a testing procedure utilizing small amounts of the denatured autologous material may contain an insufficient number of the particular molecules necessary to give a positive reaction. In contrast, a heterologous gamma globulin constitutes a relatively homogeneous population with virtually all the molecules exhibiting the same antigenic configuration. If in the course of denaturation of

autologous gamma globulin some molecules have been formed which possess an antigenic configuration characteristic of that normally present in a gamma globulin of a foreign species, for instance human, the pattern of reactivity observed in this study and that reported by Milgrom and Witebsky (2) can be understood.

The significance of the observation that immunization with denatured autologous gamma globulin leads to reactivity to heterologous gamma globulin requires comment from the point of view of the structural features of gamma globulin of various species. From what is known of protein structure (10) it seems likely that mammalian gamma globulins possess similar sequences of amino acids. Species and allotypic differences might therefore result in part from various foldings of these sequences in the secondary and tertiary structure. Denaturation, by disrupting and rearranging the bonds responsible for secondary structures, could then lead to the exposure of similarities that were not previously obvious. In such a way a molecule of rabbit gamma globulin could be altered so that it lost its own identity and resembled a human gamma globulin molecule to the extent necessary to elicit the production of antibodies that would react with human gamma globulin.

Although within each experimental group the responses were within the limits of biological variability, it should be pointed out that by virtue of the many variables affecting the individual molecules in the denaturation procedures, it is not possible to produce preparations with completely reproducible effects. In all instances there are unmeasurable variations in the temperature, local protein concentration, and ionic population. In the case of ultrasonic denaturation, two experiments were performed using slightly different frequencies and there was a significant difference in the reactivity of the two groups to denatured homologous gamma globulin.

A curious phenomenon observed in the present investigation was the reactivity of some of the guinea pigs immunized with certain forms of denatured autologous gamma globulin to denatured homologous gamma globulin even though they failed to react to the corresponding native homologous or denatured autologous preparation. We have no satisfactory explanation for this observation.

It is pertinent to discuss the increased incidence and heightened reactivity to test materials seen in some guinea pigs following intradermal challenge. The fact that occasionally guinea pigs immunized with Freund's adjuvant alone developed mild delayed reactivity to heterologous gamma globulin after repeated skin testing indicates that it is possible to sensitize guinea pigs in this way, but that under normal conditions, it is a very ineffective method. Much more striking is the increase in incidence and severity of reactions seen upon repeated skin testing with heterologous gamma globulin in guinea pigs which had been immunized with certain types of denatured autologous gamma globulin; this together with the fact that many of these animals exhibited reactivity

to heterologous gamma globulin on initial skin test indicate that the immunizing procedure was basically responsible for the sensitization and that the skin test had merely served to enhance the level of sensitivity.

The possibility that these observations may constitute a model for the immune response observed in certain human disease states, such as rheumatoid arthritis, is worthy of comment. It has been suggested that the rheumatoid factor represents an antibody directed against some form of modified autologous gamma globulin (2). The present observations demonstrate that it is possible in an experimental animal to alter its gamma globulin so that this animal produces antibodies against this denatured gamma globulin and that such antibodies have the property of reacting with heterologous gamma globulins. The hypothesis that rheumatoid factor is an antibody to altered gamma globulin has been questioned by Vaughan (11) because of the property of unmodified plasma or serum to effectively inhibit rheumatoid agglutination. Even in so far as such inhibition is not due to allotypic gamma globulin against which the rheumatoid factor is directed, this objection is not necessarily as crucial as might appear, since the possibility exists that some altered gamma globulin is always present in the circulation, possibly representing antibody which was modified by previous combination with antigen. Accepting the hypothesis stated above as to the origin of the rheumatoid factor, the most likely cause for modification of gamma globulin *in vivo* under natural conditions would appear to be its combination as antibody with specific antigen (12). It has indeed been shown that gamma globulin undergoes structural alterations upon combination with antigen (13). In keeping with this interpretation are the findings of Abruzzo and Christian (14) who reported on the appearance of a serum component resembling the rheumatoid factor in rabbits subjected to prolonged immunization with *Escherichia coli*.

Aside from the possible relationship of the present observations to the origin of the rheumatoid factor, it is conceivable that they may serve as a model for a mechanism of tissue damage. In an animal sensitized to its own modified gamma globulin, lesions of hypersensitivity could arise in any location where gamma globulin was similarly altered. In particular, it is not impossible that conditions within joint spaces might lead to structural alterations in proteins.

SUMMARY

Immunization of guinea pigs with denatured autologous gamma globulin results in the development of delayed hypersensitivity to some form of gamma globulin. When the autologous gamma globulin is subjected to denaturation with alkaline treatment as employed in this study, guinea pigs regularly develop reactivity to the immunizing material and occasionally to some form of heterologous gamma globulin. With other forms of denaturation, such as produced by urea, ultrasound, or heat, guinea pigs rarely develop sensitivity to the immunizing material but frequently exhibit delayed reactivity to native or

denatured heterologous gamma globulin. Reactivity against native autologous gamma globulin does not occur. Guinea pigs immunized with undenatured autologous gamma globulin fail to develop reactivity to any form of gamma globulin.

Rabbits immunized with denatured autologous gamma globulin develop circulating antibodies against some form of gamma globulin. Rabbits immunized with alkaline denatured autologous gamma globulin develop antibodies against the preparation used for immunization and against heterologous gamma globulin; rabbits immunized with autologous gamma globulin subjected to freeze-thaw or heat denaturation develop antibodies against heterologous globulin, but antibodies against the immunizing material can only rarely be demonstrated.

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