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Rabbits Immunized with Mixtures of Staphylococcal Protein A and Autologous IgG Produce Anti-Human IgG Antibodies

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Abstract. The results of this study provide evidence that protein A may render IgG immunogenic in the autologous host. Antibodies to human but not rabbit IgG were detected in sera of rabbits immunized with a mixture of autologous serum and protein A. Anti-human IgG antibodies appeared within 2 weeks at which time the antibodies were of the IgM class. Upon further immunization, both IgM and IgG antibodies were produced with the IgG class predominating. The antibodies elicited by a mixture of protein A with autologous IgG resembled those which arise in response to autologous IgG that has been denatured by physicochemical means, in that they react mainly with foreign species IgG and weakly, if at all, with IgG of rabbit origin.

Protein A, present in virtually all strains of *Staphylococcus aureus,* is a cell wall protein of 42,000 molecular weight that can bind to the Fc portion of IgG molecules of most mammals [reviewed in (1)]. Its interaction with IgG closely mimics that of an antigen–antibody reaction. IgG antibody undergoes molecular transformation in the course of the serological reaction with its corresponding antigen and as a result novel antigenic determinants on IgG may appear (2–4). Similarly, protein A may crosslink IgG through its Fc piece and induce conformational changes in IgG such that determinants are revealed that are not expressed in the native conformation (5, 6).

It has been proposed that human rheumatoid factor (RF), an antibody to IgG, is produced in response to determinants that appear on IgG antibody as a result of immune complex formation with antigen (4, 7, 8). Evidence for this theory is derived from observations that (i) RF is produced in diseases of persistent antigenic stimulation in which immune complexes may form such as autoimmune diseases (9) and chronic infection with bacteria (10), viruses (11) or parasites (12), and (ii) may be elicited by prolonged immunization of animals with potent heterologous antigens (7, 13, 14) as well as autologous IgG that has been denatured by physical or chemical treatment (15, 16). The purpose of this study was to determine whether protein A could similarly denature IgG to stimulate the formation of anti-IgG antibodies in the autologous host.

Materials and Methods. *Rabbits.* New Zealand white female rabbits were obtained from Becken's Research Animal Farm, Sanborn, New York.

Anti-human IgG serum. Rabbit anti-human IgG serum was obtained from Pentex, Incorporated, Kankakee, Illinois.

Anti-RBC sera. Rabbit anti-sheep RBC sera were obtained by intravenous injection with 2 ml of a saline suspension of sheep RBC. Rabbits were injected three times each week for 5 weeks. The first injection contained an equivalent of 0.2 ml of packed RBC; each week thereafter, the dosage was increased by 0.2 ml. Sera were obtained 7 days after the last injection and were decomplemented at 56°C for 30 min before use.

Rabbit antiserum containing IgG antibodies to rabbit blood group G was prepared in our laboratory according to a previously described procedure (17).

Human anti-Rh serum, Ripley, was provided by Dr. Marion Waller, Department of Pathology, School of Medicine, Richmond, Virginia. Other anti-Rh sera were kindly supplied by Dr. James Mohn, Department of Microbiology, SUNY, Buffalo, New York.

Serum proteins. Rabbit and human γ-globulin (Cohn's Fraction II) and human albumin

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(Cohn's Fraction V) were obtained from Pentex, Incorporated.

Protein A. Protein A was obtained as a secreted product from the filtrates of Penassay broth cultures of the Cowan I strain of *S. aureus* (ATCC 12593, American Type Culture Collection, Connaught Laboratories, Rockville, Md.) grown in the absence of human serum proteins as described by Kessler (18). Bacteria maintained on agar slants were inoculated into 10 ml of nutrient broth and incubated overnight at 37°C. The bacterial suspension was then poured into 1 liter of fresh broth and incubated overnight at 37°C with aeration. The supernatants of six 1-liter cultures were dialyzed against water and lyophilized; approximately 4 g of powder was obtained. A crude fractionation of protein A was obtained by precipitation of redissolved protein A powder with 80% ammonium sulfate; the pellet was redissolved in water and dialyzed against PBS for chromatography on Sephadex G-200 (90 × 2.5 cm) (19, 20). Effluent fractions were collected in 4-ml volumes and assayed for protein A by double diffusion in agarose for their ability to precipitate IgG in normal human serum. Fractions positive for protein A were pooled, concentrated by ultrafiltration, and dialyzed against 0.1 *M* ammonium formate for ion-exchange chromatography on DEAE–Sephadex A-50 (20). Elution of protein A from the ion exchanger was accomplished stepwise using 0.1, 0.2, and 0.3 *M* ammonium formate, and 2-ml fractions were collected. Most of the protein A was eluted by 0.2 *M* buffer. The amount of protein A in the purified material was determined by reverse radial immunodiffusion against normal human serum (20).

Immunization protocol. Three groups of five rabbits were immunized with either undiluted autologous serum, or protein A, or mixtures of undiluted autologous serum and protein A. Serum from the 15 rabbits was obtained prior to immunization, sterilized through Millipore filters (0.45 μm) and stored at 4°C as individual samples. Protein A–IgG complexes were prepared by incubating equal volumes of autologous serum and protein A (0.025 mg/ml) at 37°C for 1 hr. Protein A (0.025 mg/ml) and autologous serum controls were diluted with an equal volume of PBS and incubated at 37°C for 1 hr. All three preparations were emulsified in an equal volume of complete Freund's adjuvant (Difco, Detroit, Mich.) for the first injection and in incomplete Freund's adjuvant for the next four injections. Rabbits were injected weekly with 1 ml of immunogen intradermally into several sites on the back. Sera were obtained 1 week after the last injection.

Assay for anti-IgG antibodies. Rabbit antisera were assayed for antibodies to IgG by "indirect hemagglutination" of sheep RBC sensitized with rabbit antibodies (21), rabbit G^+ RBC sensitized with rabbit antibodies (17, 22), and human Rh^+ RBC sensitized with human Rh antibodies (2, 23). All rabbit antisera tested were preabsorbed with sheep and human RBC to remove natural hemagglutinins. For the test proper, to 0.1 ml of antiserum diluted in PBS was added 0.1 ml of 1% sheep RBC, G^+ rabbit RBC, or Rh^+ human RBC sensitized with a subagglutinating dose of rabbit anti-sheep RBC, rabbit anti-G or human anti-Rh serum, respectively. The suspensions were incubated for 2 hr at room temperature and then centrifuged at 500 rpm for 3 min. The reciprocal of the highest dilution that gave definite agglutination was taken as the antibody titer.

For inhibition of agglutination, inhibitors were diluted in 0.1 ml PBS and incubated with 0.1 ml of a constant amount of antiserum for 30 min prior to addition of sensitized RBC.

Mercaptoethanol treatment of rabbit antiserum. This procedure was performed by mixing serum diluted 1:5 in saline with an equal volume of 2-mercaptoethanol at various concentrations. Mixtures were incubated for 1 hr at 37°C and, without further treatment, tested for anti-IgG antibodies by indirect hemagglutination.

Sucrose density gradient fractionation of rabbit antiserum. Sucrose density gradient centrifugation was carried out as described by Fudenberg and Kunkel (24). Briefly, 0.5 ml of undiluted serum was layered upon a 10–40% sucrose gradient in PBS and centrifuged at 30,000 rpm at 4°C in a Spinco L-2 ultracentrifuge using a SW50-1 rotor. Fractions were collected in 0.2-ml volumes and tested in indirect hemagglutination for anti-IgG.

Results. One group of five rabbits was immunized with a mixture of protein A and autologous serum emulsified in adjuvant. As controls, a second group was injected with autologous serum in adjuvant and a third group

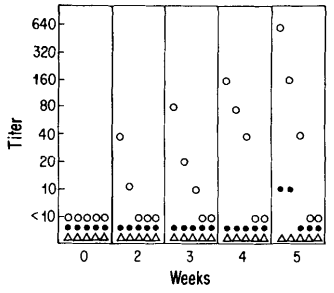

FIG. 1. Production of anti-human IgG antibodies in rabbit injected with autologous serum (●), Protein A (△), or a mixture of autologous serum and protein A (○). Values presented are indirect hemagglutination titers using Rh^+ RBC sensitized with human anti-Rh antibodies.

was immunized with protein A in adjuvant. As can be seen in Fig. 1, antibodies to human IgG were produced by three of five rabbits that were injected with a mixture of protein A and autologous serum. Antibodies to human IgG appeared after 2 or 3 weeks of immunization. After 5 weeks, titers of the three positive anti-"mixture" sera were 640, 160, and 40, as determined by hemagglutination tests using Rh^+ human RBC sensitized by anti-Rh serum, Ripley. Antibodies to human IgG were also produced by two of five rabbits that were injected with autologous serum in CFA. These antibodies, however, had low titers of anti-IgG which were detectable only after 5 weeks of immunization. None of the five rabbits injected with protein A alone produced anti-human IgG antibodies after five weekly immunizations.

None of the sera from any of the three groups of rabbits contained antibodies to rabbit IgG as determined by hemagglutination tests using sheep RBC sensitized by any of 11 rabbit anti-sheep RBC sera, and rabbit G^+ RBC sensitized by rabbit anti-G serum. No reactions were obtained even when sera were tested at dilutions as low as 1:2.

The inhibitory effect of various specimens on the agglutination of sensitized human RBC by an anti-"mixture" serum is shown in Table I. It can be seen that pooled human serum, as well as human Fraction II as a source of human IgG, were strongly inhibitory while human Fraction V was not inhibitory. Similar results were obtained in agglutination inhibition tests with a rheumatoid arthritis (RA) serum and a rabbit antiserum to human IgG. Pooled rabbit serum, rabbit Fraction II, and protein A were incapable of inhibiting the agglutination of the RBC sensitized by human antibodies.

Data presented in Table II show that rabbit anti-"mixture" serum agglutinated Rh^+ RBC sensitized by a variety of anti-Rh sera. Titers obtained with a rabbit anti-human IgG serum and a RA serum are included for comparison. The RA serum showed specificity for certain allotypes in that it agglutinated RBC sensitized by some but not all anti-Rh sera that were tested. In contrast, rabbit anti-human IgG serum and rabbit anti-"mixture" serum demonstrated foreign species but not allotypic specificity in that it agglutinated RBC sensitized by all anti-Rh sera used for sensitization of Rh^+ RBC.

The experiment depicted in Table III shows that 2-mercaptoethanol abolished the hemagglutinating activity of anti-"mixture" serum taken at Week 2 and reduced but did not abolish the activity of the serum taken at Week 5. Activity of the hyperimmune rabbit anti-human IgG serum was hardly affected, whereas activity of the RA serum was abolished.

Density gradient centrifugation analysis

TABLE I. AGGLUTINATION OF SENSITIZED HUMAN RH^+ RBC BY ANTISERUM FROM A RABBIT IMMUNIZED WITH A MIXTURE OF ITS OWN SERUM AND PROTEIN A: INHIBITORY EFFECT OF SERUM, SERUM FRACTIONS, AND PROTEIN A

Inhibitor	Reactions obtained with inhibitor diluted 1 to:							
	1	3	9	27	81	243	729	2187
Pooled human serum	—	—	—	—	—	—	—	2+
1% Human fraction II	—	—	—	—	—	—	—	1+
1% Human fraction V	3+	3+	3+	3+	3+	3+	3+	3+
Pooled rabbit serum	2+	3+	3+	3+	3+	3+	3+	3+
1% Rabbit fraction II	3+	3+	3+	3+	3+	3+	3+	3+
1% Protein A	3+	3+	3+	3+	3+	3+	3+	3+

TABLE II. AGGLUTINATING ACTIVITIES OF RABBIT ANTI-"MIXTURE" SERUM, RABBIT ANTI-HUMAN IgG SERUM, AND RA SERUM FOR HUMAN Rh^+ RBC SENSITIZED BY VARIOUS HUMAN ANTI-Rh SERA

Anti-Rh serum used for sensitization	Indirect hemagglutination titer obtained with:		
	Anti-"mixture" serum	Anti-human IgG serum	RA serum
Ripley	640	5120	1280
M4051	320	5120	640
Palmer	320	5120	<10
No. 4	40	640	<10
No. 31	40	320	<10

showed that most of the anti-human IgG reactivity of an anti-"mixture" serum, obtained after two injections, was confined to the IgM region of the gradient (Fig. 2A), whereas the activity of a serum taken after 5 injections was present in both IgM and IgG regions (Fig. 2B). The fractionation profile of rabbit anti-human IgG serum (Fig. 2C) closely resembled that of the 5-week anti-"mixture" serum, in that the hemagglutinating activity was distributed in both IgM and IgG regions. In contrast, the hemagglutinating activity of RA serum was strictly confined to the IgM region (Fig. 2D), in accord with its complete sensitivity to reduction by mercaptoethanol.

Discussion. The results presented in this study suggest that protein A can cause conformational changes in IgG molecules sufficient to render IgG immunogenic for the autologous host. Rabbits immunized with a mixture of protein A and autologous serum as a source of IgG produced antibodies to human IgG. No antibodies were detected to rabbit IgG.

Activity of these antibodies to human IgG was inhibited by human IgG, but not by rabbit IgG or protein A. Attempts to inhibit these antibodies with protein A–rabbit IgG complexes also were not successful even when excessive amounts of the complexes were used (data not shown). Conceivably, protein A–rabbit IgG complexes bearing the proper determinants that elicited production of antibodies to human IgG were in sufficient concentration to be immunogenic, but were not in high enough concentration to inhibit antigen–antibody interactions *in vitro*.

The above results are in contrast to those obtained by Lind (5), who reported that rabbits injected intravenously with protein A-bearing staphylococci produced antibodies directed against the protein A–rabbit IgG complex. In Lind's experiments, antibody activity was demonstrated only against protein A–rabbit IgG complexes and not against rabbit IgG alone. In our studies, protein A was not needed to show rabbit antibodies to human IgG. This could be due to the fact that Lind injected staphylococci intravenously, whereas in our studies, preformed protein A–rabbit IgG complexes that were emulsified in complete Freund's adjuvant were injected intradermally.

Despite our inability to detect anti-rabbit IgG antibodies in the anti-"mixture" sera, it is possible that the indirect hemagglutination

TABLE III. EFFECT OF 2-MERCAPTOETHANOL (2-ME) ON THE HEMAGGLUTINATING ACTIVITY OF RABBIT ANTI-"MIXTURE" SERUM, RABBIT ANTI-HUMAN IgG SERUM, AND RA SERUM

Concentration of 2-ME in the treated serum	Titer of agglutination of human Rh^+ RBC sensitized by Rh antibodies			
	Anti-"mixture" serum			
	Week 2	Week 5	Anti-human IgG serum	RA serum
None	40	640	5120	1280
0.05 *M*	10	160	640	80
0.20 *M*	<10	80	640	<10

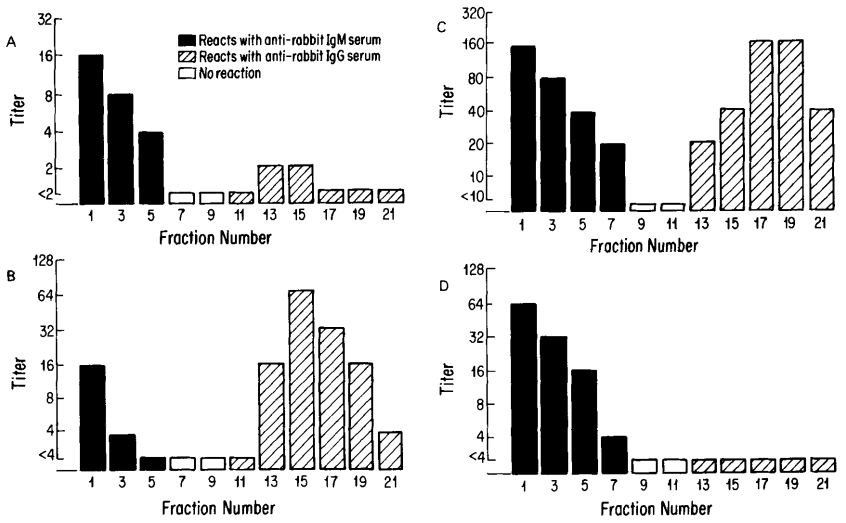

FIG. 2. Sucrose density gradient centrifugation of rabbit antiserum to mixture of protein A with autologous serum. Fractionation profile of anti-"mixture" serum obtained after two (A) and five (B) weekly immunizations. Sucrose density gradient centrifugation of rabbit anti-human IgG serum (C) and rheumatoid arthritis serum (D). Values presented on ordinates are hemagglutination titers obtained with Rh^+ RBC sensitized with human Rh antibodies.

used for their detection may not have been of sufficient sensitivity. If anti-rabbit IgG antibodies were present they would probably be of low avidity and/or occur in very low concentrations compared with the anti-human IgG population. However, an absence of antibodies to homologous IgG in rabbits injected with denatured IgG has been reported by others. In these studies, immunization of rabbits with rabbit IgG precipitated with ammonium sulfate (15) or denatured physically by heating or repeated freezing and thawing or chemically by urea (16) induced antibodies to foreign species IgG but not homologous IgG. In contrast, antibodies to homologous IgG can be elicited using preformed antigen–antibody complexes as the immunogen. In this regard, rabbits injected with immune complexes composed of ferritin and rabbit anti-ferritin antibodies produced antibodies to rabbit IgG (25).

We also observed that autologous serum in adjuvant without protein A induced antibodies to human IgG. These antibodies, however, appeared only after the fifth injection whereas antibodies to the protein A–autologous serum mixture arose after the second injection. Moreover, the former antibodies were present in lower titer than were antibodies in the anti-"mixture" sera. The ability of autologous rabbit serum alone to elicit anti-human IgG antibodies could be due to the denaturation of serum immunoglobulin that might have occurred during the storage of the sera or more likely in the process of emulsification of the serum in adjuvant. It is also out of concern of denaturation that IgG was not purified from serum for preparation of protein A–IgG complexes. Purification, however, of IgG was considered unnecessary since an interaction of protein A with rabbit proteins other than IgG has not been observed (26).

Rabbits injected with protein A in adjuvant without autologous serum did not produce detectable levels of antibodies to human or rabbit IgG. Conceivably, protein A alone might stimulate production of anti-IgG if an interaction could occur in the immunized host between protein A and autologous IgG. The failure of protein A to induce anti-human IgG suggests that, if such complexes did form *in vivo,* (i) there was not a sufficient amount of protein A–IgG to serve as immunogen, and/or (ii) the quality of the complex that would form in tremendous IgG excess in the immunized host was not particularly immunogenic.

Finally, it is interesting to consider the role that denaturation of autologous IgG by protein A may play in the production of anti-IgG in

man. It is known that individuals afflicted with *S. aureus* septicemia contain antibodies to the Fab portion of human IgG (27). Although anti-staphylococcal antibodies denatured in the course of serological reaction with *S. aureus* could induce RF, the data presented here suggest a second possible stimulus in which autologous serum immunoglobulin is rendered immunogenic by interaction with protein A either as a soluble protein in the circulation or possibly as a cell wall protein on the bacterial surface.

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