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Successive Extraction of Specific Protective Immunoglobulins from Pooled Human Sera

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By use of pooled human sera, specific protective immunoglobulins against the Smith-diffuse strain of *Staphylococcus aureus*, strains SS-620 and SS-619 of types III and II group B streptococci, and strain K-9 of *Klebsiella pneumoniae* were successively extracted from their whole cell and antibody complexes by elution with propionic acid containing 5% sucrose. Injection of 0.14, 0.05, 0.09, and 0.15 mg of these eluates in mice gave protection against lethal infection only with homologous strains. However, no protective effect was observed against these infections, even with considerably higher amounts of a conventional immunoglobulin preparation. The major components of the eluates were the three major immunoglobulins, immunoglobulin G (IgG), IgA, and IgM, although nonspecific proteins were also included. Protective activities of these eluates were absorbed out by their protection-inducing antigens, indicating that they contained specific protective immunoglobulins.

Immunoglobulin preparations have been applied for the treatment of infectious diseases after the appearance of antibiotic-resistant bacterial strains. Although improved preparations include biological activities of the native form of immunoglobulin G (IgG), which participates in phagocytosis by combining with complement, they are complexes of serologically heterogeneous IgG. The therapeutic effects of conventional immunoglobulin preparations are not always reliable, due to the varied contents of specific protective immunoglobulins against infections and to the absence of IgM, which has a significantly higher combining capacity with complement than does IgG. On the other hand, the existence of protective antibodies in normal human sera against several species of pathogenic bacterial strains has been reported. We describe a method to successively isolate immunoglobulins demonstrating protective effects against infections with different species of bacterial strains from pooled human sera.

MATERIALS AND METHODS

Strains. The Smith-diffuse strain of *Staphylococcus aureus*, strains SS-619 and SS-620, representative strains of types II and III group B streptococci, and the K-9 strain of *Klebsiella pneumoniae*, all described elsewhere (4, 10, 12), were used.

Human sera. In a private clinical laboratory, residual patient sera used for clinical examinations were pooled. The number of patients and the diseases were not clear. Sera were stored at −20°C in a freezer for 4 months. They were tentatively designated no. 1 sera. After the antibody in no. 1 sera was absorbed out, primarily with the Smith-diffuse strain by the method described below, they were designated no. 2 sera. The no. 2 sera successively absorbed out by strains SS-620, SS-619, and K-9 were designated no. 3, 4, and 5 sera, respectively.

Determination of relative passive protective activity of human sera against Smith-diffuse, SS-620, SS-619, and K-9 strains in mice. Twofold serial dilutions of no. 1 sera were prepared with saline. A group of five mice received intraperitoneal injections of 0.5 ml. Thirty minutes later, 0.5 ml of 8% mucin (Difco Laboratories) in saline containing 10^4 CFU of the Smith-diffuse strain of *S. aureus* and 0.5 ml of saline containing 10^4 CFU of strains SS-620 and SS-619 and 10^3 CFU of strain K-9 of *K. pneumoniae*, which were the minimum 100% lethal doses, were injected intraperitoneally at a different site. The numbers of deaths were recorded for 7 days, and the maximum dilution of 0.5 ml of no. 1 sera to protect the animals from death due to Smith-diffuse strain was designated as 1.0 U. No. 2, 3, and 4 sera were similarly diluted with saline and injected, and homologous and heterologous strains were injected intraperitoneally into mice at the doses noted above. The minimum amounts providing passive protection against the strains were similarly designated as 1.0 U.

Isolation of protective antibody from human sera. Extraction of specific antibodies to the strains from no. 1 sera was performed by the modified method of Roholt et al. (9) as follows. To 100 ml of no. 1 sera containing 200 U of activity against the Smith-diffuse strain were added 100, 200, and 400 mg (wet weight) of heat-killed Smith-diffuse cells, which were cultured in a brain heart infusion broth (Difco), washed twice with saline, and autoclaved. After maintenance for 30 min in a 37°C water bath, precipitates were obtained by centrifugation at 6,000 × *g* for 30 min. Then 5.0 ml of 1 M propionic acid solution, previously adjusted to pH 3.0 with 0.1 N hydrochloric acid containing a final concentration of 5% sucrose, was added. They were kept at room temperature for 30 min, and centrifuged, and the supernatants were dialyzed overnight against 5% sucrose in 0.067 M phosphate buffer (pH 7.2) at 4°C with a magnetic stirrer. A portion of the contents of the dialysis bag was used for the determination of the bacterial agglutination titer against each homologous strain by regular procedures and animal and immunological experiments (see below), and the remaining contents were combined with the same volume of normal human serum, sterilized by membrane filtration (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and stored at 4°C.

Determination of relative protective activity of eluates against infection with homologous and heterologous strains in mice. The eluates were primarily diluted at 1:10 and then serially diluted twofold with saline. First, 0.5 ml of the diluted eluates were injected intraperitoneally into groups of

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five mice; 30 min later, the Smith-diffuse strain suspended in mucin and strains SS-620, SS-619, and K-9 suspended in saline (see above) were injected intraperitoneally into them. Deaths over a period of 7 days were recorded. With these experiments, the amounts of the eluates administered to the animals were expressed as the protein amounts quantitated by the method of Lowry et al. (7).

Determination of immunoglobulin contents. IgG, IgA, and IgM contents were determined by Immunoplates (Hyland Laboratories, Inc., Costa Mesa, Calif.).

Preparation of the Smith surface antigen of the Smith-diffuse strain and type antigens of group B streptococci. Smith surface antigen was prepared by the method of Morse (8). Type antigens of types II and III group B streptococci were obtained by the method of Lancefield and Freimer (6).

Determination of passive protective activities of the eluates after absorption with homologous protective antigens. To 2.0 activity units of the eluates in 1.0 ml of phosphate-buffered saline were added various amounts of homologous protective antigens and 1.0, 0.3, and 0.1 mg of Smith surface antigen or type antigens of group B streptococci. After being left at room temperature for 30 min, supernatants were obtained by centrifugation at 6,000 × *g* for 20 min, and 0.5-ml volumes were injected intraperitoneally into groups of five mice. Then the animals were challenged with homologous strains by the method described above.

Determination of the passive protective effect of a conventional immunoglobulin preparation against challenge infection. Venoglobulin I (Midori-Juji Pharmaceutical Co. Ltd., Tokyo), a preparation that includes biological properties of native IgG and does not contain isolated Fc fragment, was dissolved in saline, and solutions of 4, 2, 1.0, 0.5, and 0.25 mg/ml were prepared. Intraperitoneal injections of 0.5-ml volumes were made in four groups of five mice each, and 30 min later, Smith-diffuse, SS-620, SS-619, and K-9 strains suspended in mucin or saline, as noted above, were injected intraperitoneally. The numbers of deaths were recorded for 7 days after challenge. The Venoglobulin I administered was also expressed as the amount of protein.

RESULTS

Passive protective effect of eluate from no. 1 sera on mice after lethal doses of Smith-diffuse strain. With the challenge infection of Smith-diffuse strain, all mice receiving 0.5 ml of 1:2 diluted no. 1 sera survived, and 60% of animals receiving 1:4 diluted no. 1 sera survived, indicating that the undiluted sera contained 2 activity units per ml. When eluates obtained by the addition of 100, 200, and 400 mg of the Smith-diffuse organisms to 100 ml of no. 1 sera were examined for their bacterial agglutination activities, the titers were 1:16, 1:160, and 1:8, respectively. The eluate exhibiting the highest titer in bacterial agglutination obtained from a combination of 200 mg of organisms and 100 ml of no. 1 sera was used for animal and immunological experiments. After the administration of 0.14 mg of this eluate, no mouse was killed after the administration of a lethal dose of Smith-diffuse strain. However, no passive protective effect was observed against challenge with heterologous strains, SS-619 and SS-620 of group B streptococci, and K-9 of *K. pneumoniae* (Table 1). Also, with Venoglobulin I, no protection was shown, even at a dose level of 2.36 mg of protein against a similar lethal dose (Table 2). Since the volume of this eluate was 5.5 ml and it contained 5.6 mg of protein per ml, the eluate obtained from 100 ml of no. 1 sera yielded sufficient antibody to protect mice of approximately 4.40 kg body weight from lethal doses of Smith-diffuse strain.

Passive protective effect of eluate from no. 2 sera against lethal doses of strain SS-620 of type III of group B *Streptococcus*. After absorbing out no. 1 sera with Smith-diffuse strain, the bacterial agglutination titer of the sera to strain SS-620 was 1:16. Among eluates similarly obtained by using 200 mg of strain SS-620 to 100 ml of no. 2 sera, the highest bacterial agglutination titer was 1:320; this eluate was used for animal and immunological experiments. Treatment with 0.05 mg of protein of no. 2 sera eluate yielded 100% survival after lethal doses of the homologous strain. With this amount of the eluate, no protection was shown against challenge infections with heterologous strains, the Smith-diffuse strain of *S. aureus*, the SS-619 strain of group B *Streptococcus*, and the K-9 strain of *K. pneumoniae* (Table 3). The eluate volume was 5.6 ml, with a concentration of 2.01 mg of protein per ml; therefore, the eluate obtained from 100 ml of no. 2 sera yielded antibody to protect mice of approximately 4.50 kg body weight against lethal doses of strain SS-620. On the

TABLE 1. Passive protective activity of specific antibody obtained from no. 1 sera against lethal infection with strains Smith-diffuse of *S. aureus*, SS-620 of type III group B *Streptococcus*, SS-619 of type II group B *Streptococcus*, and K-9 of *K. pneumoniae* in mice

Treatment with eluate (mg of protein)	No. of deaths/no. of mice challenged with strain:			
	Smith-diffuse	SS-620	SS-619	K-9
0.14	0/5	5/5	5/5	5/5
0.07	3/5	5/5	5/5	5/5
0.035	5/5	5/5	5/5	5/5
Untreated	5/5	5/5	5/5	5/5

TABLE 2. Passive protective effect of Venoglobulin I against challenge with strains Smith-diffuse of *S. aureus*, SS-620 of type III group B *Streptococcus*, SS-619 of type II group B *Streptococcus*, and K-9 of *K. pneumoniae* in mice

Treatment with Venoglobulin I (mg)	No. of deaths/no. of mice challenged with strain:			
	Smith-diffuse	SS-620	SS-619	K-9
2.36	5/5	2/5	5/5	5/5
1.18	4/5	2/5	4/5	5/5
0.59	4/5	2/5	4/5	5/5
0.30	5/5	4/5	5/5	5/5
0.12	5/5	5/5	5/5	5/5
Untreated	5/5	5/5	5/5	5/5

TABLE 3. Passive protective activity of specific antibody obtained from no. 2 sera against lethal infection with strains SS-620 of type III group B *Streptococcus*, Smith-diffuse of *S. aureus*, SS-619 of type II group B *Streptococcus*, and K-9 of *K. pneumoniae* in mice

Treatment with eluate (mg of protein)	No. of deaths/no. of mice challenged with strain:			
	SS-620	Smith-diffuse	SS-619	K-9
0.05	0/5	5/5	5/5	5/5
0.025	4/5	5/5	5/5	5/5
0.012	5/5	5/5	5/5	5/5
Untreated	5/5	5/5	5/5	5/5

other hand, even 2.36 mg of protein of Venoglobulin I failed to give complete protection (Table 2).

Passive protective effect of eluate from no. 3 sera against lethal doses of strain SS-619 of type II of group B *Streptococcus*. After absorbing out the original pooled human sera with Smith-diffuse and SS-620 strains, eluate obtained from a combination of 200 mg of strain SS-619 and 100 ml of no. 3 sera showed the highest bacterial agglutination to homologous organisms of 1:40, whereas that of no. 3 sera itself was 1:2. It was found that 0.09 mg of protein of the eluate completely protected against homologous challenge infection in mice. However, no protective effect was shown against challenge infections with heterologous strains (Table 4). The original volume of this eluate was 5.3 ml, and it contained 1.8 mg of protein per ml. Therefore, the eluate obtained from 100 ml of no. 3 sera was sufficient to protect mice of approximately 2.12 kg body weight from lethal doses of strain SS-619. However, even 2.36 mg of Venoglobulin I provided no protection against lethal doses of infection (Table 2).

Passive protective effect of the eluate from no. 4 sera against lethal doses of *K. pneumoniae* K-9. After absorbing out no. 3 sera with strain SS-619, the sera were absorbed out with *K. pneumoniae* K-9. The highest bacterial agglutination titer was 1:40 in eluate obtained from a combination of 200 mg of the organisms and 100 ml of no. 4 sera, whose titer was 1:2. Passive protection against challenge infection with homologous strain was shown by 0.5 ml of undiluted eluate containing 0.15 mg of protein. In these cases, no passive protective effect was observed against challenge infections with heterologous strains (Table 5). The original volume of the eluate was 5.4 ml, and it contained 1.20 mg of protein per ml. Therefore, eluate from 100 ml of no. 4 sera was sufficient to protect mice of approximately 0.86 kg body weight against lethal infection with strain K-9. No protection against this strain was shown by Venoglobulin I, even at a dose of 2.36 mg per mouse (Table 2).

Immunoglobulin contents in eluates. When the three major immunoglobulin contents in the four eluates described above were quantitated, the total immunoglobulins of the eluates decreased remarkably after repeated absorption, exhibiting totals of 16.55, 9.12, 7.36, and 5.18 mg, respectively. The decreases in amounts of IgG were especially significant, but no remarkable reduction was observed in IgM after the absorption of Smith-diffuse strain (Table 6). The eluates from sera no. 1, 2, 3, and 4 consisted of 53.7, 81.0, 77.1, and 79.9% immunoglobulin, respectively.

Passive protective effect of the eluates in mice after absorption with the protective antigens against challenge infection with homologous strains. The experiments described above were designed to clarify whether the protective activities of the eluates against homologous challenge infections were due to specific antibodies to their protection-inducing antigens of the organisms. Results indicated that the protective activities of the eluates obtained by strains Smith-diffuse, SS-620, and SS-619 were completely absorbed out with 0.3 mg of Smith surface antigen and 1.0 mg of type III and II antigens, respectively, against challenge infection with homologous strains. In these experiments, the eluate obtained by *K. pneumoniae* K-9 was not included since the protection-inducing antigen in this species of bacteria has yet to be elucidated.

TABLE 4. Passive protective activity of specific antibody obtained from no. 3 sera against lethal infection with strains SS-619 of type II group B *Streptococcus*, Smith-diffuse of *S. aureus*, SS-620 of type III group B *Streptococcus*, and K-9 of *K. pneumoniae* in mice

Treatment with eluate (mg of protein)	No. of deaths/no. of mice challenged with strain:			
	SS-619	Smith-diffuse	SS-620	K-9
0.09	0/5	5/5	5/5	5/5
0.045	4/5	5/5	5/5	5/5
0.022	5/5	5/5	5/5	5/5
Untreated	5/5	5/5	5/5	5/5

TABLE 5. Passive protective activity of specific antibody obtained from no. 4 sera against lethal infection with strains K-9 of *K. pneumoniae*, Smith-diffuse of *S. aureus*, SS-620 of type III group B *Streptococcus*, and SS-619 of type II group B *Streptococcus* in mice

Treatment with eluate (mg of protein)	No. of deaths/no. of mice challenged with strain:			
	K-9	Smith-diffuse	SS-620	SS-619
0.15	0/5	5/5	5/5	5/5
0.07	3/5	5/5	5/5	5/5
0.03	5/5	5/5	5/5	5/5
Untreated	5/5	5/5	5/5	5/5

DISCUSSION

Serum protective antibodies against relatively low-virulence bacterial strains, such as *S. aureus* (14), *Staphylococcus epidermidis* (2), group B streptococci (1), *K. pneumoniae* (3), *Klebsiella ozaenae* (5), and *Serratia marcescens* (11) have been demonstrated in human sera. Also, we previously reported cross-protective antibodies between the Smith-diffuse strain of *S. aureus* and a strain of type Ia group B streptococci (4). Primary human sera used for the preparation of conventional immunoglobulin preparations would contain the antibodies noted above. However, immunoglobulin preparations for practical use should contain sufficient amounts of protective antibodies against causative microorganisms. In particular, opsonic immunoglobulins are required in preparations against opportunistic bacterial infections.

Initially, only IgG antibodies were obtained in the present experiments; however, extremely low protective activities were shown in the eluates against lethal infections with the bacterial strains. Extraction of native IgM has been assumed to be difficult due to the biophysical properties of the macromolecule of this protein. During the process of dissociation of antibodies from antigen-antibody complexes, considerably high protective activities were successfully extracted against four different bacterial strains in the presence

TABLE 6. Total amounts of protein and immunoglobulins in eluates prepared from strains Smith-diffuse of *S. aureus*, SS-620 of type III group B *Streptococcus*, SS-619 of type II group B *Streptococcus*, and K-9 of *K. pneumoniae*

Substance	Amt (mg) in the following strain:			
	Smith-diffuse	SS-620	SS-619	K-9
Protein	30.80	11.25	9.54	6.48
IgG	12.65	7.28	5.83	3.78
IgA	0.74	1.23	0.95	0.86
IgM	3.16	0.61	0.58	0.54

of 5% sucrose, as noted by Yoshida and Nahmias (15). IgM antibodies were labile even in 5% sucrose for maintenance over long periods of time, and normal human serum was used to protect against denaturation of activity. Protection-inducing antigens, such as Smith surface antigen and type antigens of group B streptococci, were employed to extract specific antibodies from human sera. Relatively highly specific immunoglobulins were thus obtained; however, the yields of the antibodies were significantly lower than those obtained by using whole cells. Cross-antigenic substances are known among whole cells of bacterial strains, especially between staphylococci and streptococci. Antibodies to these substances were contained in the eluates obtained in these experiments. Nevertheless, significantly high amounts of specific immunoglobulins were successively obtained, and the protective capabilities of immunoglobulins extracted from 100 ml of pooled human sera against lethal infections with the Smith-diffuse strain of *S. aureus*, strains SS-620 and SS-619 of type III and II group B streptococci, and strain K-9 of *K. pneumoniae* were equivalent to 4.40, 4.50, 2.12, and 0.86 kg of mouse body weight, respectively. According to the description of Yoshida and Ekstedt (13), the minimum amount of specific IgM capable of completely protecting a mouse against lethal infection with Smith-diffuse strain is less than 16.4 μg. In the present experiments, approximately 10 times that amount of protein was required to obtain similar protective effects, indicating that high amounts of protein other than immunoglobulins and nonspecific immunoglobulins are contained in the eluates, even though the protective activity of the eluates was completely absorbed out with the protection-inducing antigen.

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