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Role of Protein A in Nonspecific Immunofluorescence of *Staphylococcus aureus*

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γG-globulin from nonimmunized rabbits and from rabbits immunized with various bacteria reacted in the immunofluorescence technique with protein A-containing *Staphylococcus aureus*. Pepsin digestion of most immunoglobulin preparations eliminated the reaction, thus showing that the Fc fragment is involved and that the reaction is not a true antigen-antibody reaction. As the specific immunological activity of the immunoglobulin molecules was intact after digestion, it is suggested that the method be used to eliminate reactions with *S. aureus* in the fluorescent-antibody technique.

So-called "normally occurring antibodies" against *Staphylococcus aureus* have been demonstrated with the fluorescent-antibody (FA) technique in sera from different species (1, 3, 4, 17, 22). The well-known fact that many strains of *S. aureus* react with antisera to other bacteria has been a major problem in the FA technique (2, 5, 6, 18).

Protein A has been shown to be associated with the cell surface of most *S. aureus* strains (13, 27). A direct reaction between protein A and rabbit γG-globulin through interaction with structures on the Fc portion of the γ-globulin is now well documented (11, 26).

This paper provides evidence that the FA reaction between *S. aureus* and γG-globulin from nonimmunized rabbits and from rabbits immunized against various bacterial species is not due to an antigen-antibody reaction. Instead it seems to be a reaction between the Fc part of the γG-globulin molecules and protein A from *S. aureus*.

MATERIALS AND METHODS

Strains and culture techniques. *S. aureus* types Cowan I and Wood 46 were used. The other bacteria used were isolated in the routine laboratory of the Institute of Medical Microbiology. All strains were cultivated overnight on conventional solid media before use. In some experiments, *S. aureus* type Cowan I was grown on Mannitol Salt Agar plates (Difco).

Sera. Pooled serum from nonimmunized rabbits and antisera from rabbits immunized against *Neisseria gonorrhoeae*, *N. meningitidis*, *Escherichia coli* type 0111, and *Shigella boydi* type 3 were used. The antisera were obtained by immunization according to the current methods at the Institute of Medical Microbiology (5, 9). The antisera had agglutination titers against the corresponding bacteria ranging from 1/64 to 1/512.

Preparation of γG-globulin. Rabbit γG-globulin was prepared from sera by precipitation at 37% saturation with ammonium sulfate. The precipitate was dissolved in distilled water and the procedure was repeated twice. The precipitated and redissolved material was dialyzed against 0.0175 M Na_2HPO_4 (pH 6.3), further purified by chromatography on a diethylaminoethyl (DEAE)-cellulose column equilibrated and eluted with the dialysis buffer (11), and finally concentrated with an ultrafiltration cell (Diaflo m. 50, Amicon Corp.) to a protein concentration of 18 to 20 mg/ml. The protein concentration was determined by measuring the optical density at 280 nm. Each sample was divided into two portions. One portion was dialyzed against 0.15 M NaCl and diluted with 0.15 M NaCl and carbonate-bicarbonate buffer (*p*H 9.0, 0.5 M) to a final concentration of about 10 mg of protein per ml (23). Carbonate-bicarbonate buffer made up 20% of the final solution. The other portion was dialyzed against 0.1 M sodium acetate.

For two sera, the purification step on DEAE-cellulose was omitted. After precipitation with ammonium sulfate, the crude γG-globulin fraction was dissolved in distilled water, divided into two portions, and dialyzed as described above.

Pepsin digestion. Pepsin digestion of γG-globulin from normal and immunized rabbits was carried out by the method of Nisonoff (24). After the dialysis against 0.1 M sodium acetate, the *p*H was lowered to 4.5. Pepsin (Worthington Biochemical Corp.) dissolved in 0.1 M sodium acetate (*p*H 4.5) was added in the proportion 1 mg of enzyme per 50 mg of protein. The digestion was allowed to proceed at 37 C for 8 hr, after which time the *p*H was adjusted to 8.0. The di-

gestion products were precipitated twice at 18% Na_2SO_4. The final precipitate was dissolved in distilled water and dialyzed against 0.15 M NaCl. The protein content was adjusted to about 6.6 mg/ml with 0.15 M NaCl and carbonate-bicarbonate buffer, which made up 20% of the final solution. The concentration of γG-globulin was thus about two-thirds of that in the undigested portion, but the concentration of the $F(ab^1)_2$ fragments was equivalent on a molar basis.

Conjugation. Under vigorous stirring with a magnetic agitator at 4 C, 0.05 mg of fluorescein isothiocyanate (FITC) was added per mg of protein. After 24 hr, the conjugation was interrupted and nonreactive material was removed by passing the solution through a Sephadex G-25 column (23). The conjugate was divided into several samples and stored at −20 C in small test tubes. When a portion was to be used, it was thawed and kept at 4 C.

Staining smears and recording of reactions. Smears were prepared from overnight cultures (23). A platinum loop was dipped into a colony and the bacteria were smeared onto a slide. After heat fixation, a drop of conjugate was placed on the smear and the reaction was allowed to proceed for 15 min, after which time the smear was rinsed for 5 min in phosphate-buffered saline and mounted under a cover slip with a phosphate-buffered solution of glycerol, *p*H 7.2 to 7.4. The preparations were read under a Zeiss fluorescence microscope by using the following standards: 3+, intensely fluorescent margin, well marked edges; 2+, faintly fluorescent margin, edges usually diffuse; 1+, barely distinguishable fluorescent margin with diffuse edges.

Determinations of protein A. Protein A was extracted from *S. aureus* by the method of Jensen (Thesis, Munksgaard, Copenhagen, 1959). The extract

TABLE 1. *Fluorescent-antibody reactions of γG-globulins or $F(ab^1)_2$ fragments of γG-globulins with Staphylococcus aureus strains Cowan I and Wood 46 and with the bacteria used for immunization*

FITC[a]-labeled antiglobulin to	Reaction with									
	Strain Cowan I						Strain Wood 46 (1/10)	Bacteria used for immunization		
	1/10[b]	1/20	1/40	1/80	1/160	1/320		1/10	1/20	1/40
Escherichia coli 0111										
γG-globulins not pepsin-digested	3+[c]	2+	2+	1+	1+	−	−	3+	−	
γG-globulins pepsin-digested	−						−	2+	−	
Shigella boydi type 3										
γG-globulins not pepsin-digested	3+	3+	2+	1+	−		−	3+	3+	−
γG-globulins pepsin-digested	−						−	3+	3+	−
Neisseria meningitidis										
γG-globulins not pepsin-digested	2+	2+	2+	2+	1+	−	−	2+	−	
γG-globulins pepsin-digested	−						−	2+	−	
N. gonorrhoeae 1										
γG-globulins not pepsin-digested	3+	2+	2+	1+	−		−	2+	2+	−
γG-globulins pepsin-digested	−						−	2+	2+	−
N. gonorrhoeae 2										
γG-globulins not pepsin-digested	3+	2+	2+	2+	1+	−	−	3+	2+	−
γG-globulins pepsin-digested	3+	2+	−				−	3+	2+	−
Normal rabbit										
γG-globulins not pepsin-digested	3+	3+	2+	2+	1+	−	−			
γG-globulins pepsin-digested	−						−			

[a] Fluorescein isothiocyanate.

[b] Titers.

[c] Standards: 3+, intensely fluorescent margin, well marked edges; 2+, faintly fluorescent margin, edges usually diffuse; 1+, barely distinguishable fluorescent margin with diffuse edges; −, no fluorescence.

was diluted approximately twice in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.0) so that 1 ml of the final solution corresponded to 25 mg (wet weight) of the unextracted bacteria.

The hemagglutination technique for quantitation of protein A (26) was used, beginning with a dilution of 1:1, followed by doubling dilutions. The technique is known to be very sensitive.

Precipitation in gel (10, 11) was performed against 1% human γG-globulin in 1% agar (Special Agar Noble, Difco) in 0.05 M Tris-hydrochloride, *p*H 7.2.

RESULTS

Reactions with γG-globulin from nonimmunized rabbits. Table 1 shows the reactions of *S. aureus* types Cowan I and Wood 46 with FITC-labeled γG-globulin from nonimmunized rabbits. *S. aureus* type Cowan I reacted strongly. It is also known to be the strain containing the most protein A of those investigated (K. Jensen, Thesis, Munksgaard, Copenhagen, 1959). No reaction was noticed when staining with pepsin-digested FITC-labeled γG-globulin. *S. aureus* type Wood 46 did not react with nonimmune γG-globulin before or after pepsin digestion. By immunodiffusion against normal human γG-globulin it was not possible to detect any protein A in the extract from Wood 46. However, by the more sensitive hemagglutination technique (26), Wood 46 was shown to contain a very small amount of protein A, less than 1% of the content in Cowan I.

Reactions with γG-globulin from immunized rabbits. FITC-labeled γG-globulins prepared by precipitation and chromatography on DEAE-cellulose from antisera against various bacteria were tested for specific staining of the corresponding bacteria and for nonspecific staining of *S. aureus*. Table 1 shows that the reactions with *S. aureus* types Cowan I and Wood 46 were very similar to the reactions obtained with nonimmune serum. *S. aureus* type Cowan I reacted strongly with undigested γG-globulin but did not react after digestion of γG-globulin with pepsin. However, one antiserum to *N. gonorrhoeae* reacted with *S. aureus* Cowan I in moderate dilutions (≤1:20) even after pepsin digestion. This serum was not further investigated.

Table 1 also shows that pepsin digestion did not interfere with the specific reactions. The molar concentrations of the conjugates of the γG-globulin and of the $F(ab^1)_2$ fragments obtained by pepsin digestion of the γG-globulin were the same.

The two γG-globulin preparations obtained from sera treated by precipitation alone gave the same results as the more highly purified γG-globulins.

Reactions between rabbit γG-globulin and various strains of S. aureus. Table 2 shows that the other staphylococcal strains gave various degrees of reaction with nonimmune γG-globulin, but after pepsin digestion the reaction disappeared. With the specific antisera to various bacteria, the staphylococcal strains tested gave reactions very similar to the reaction with nonimmune γG-

TABLE 2. *Fluorescent-antibody reactions of γG-globulin or $F(ab^1)_2$ fragments of the globulin with various Staphylococcus aureus strains*

S. aureus strain	Normal rabbit γ-globulin						Antiglobulins to								Hemagglutination titer (protein A content)
							Neisseria Meningitidis		*N. gonorrhoeae*		*Shigella boydi* type 3		*Escherichia coli* 0111		
	1/10	1/20	1/40	1/80	1/160	1/10D[b]	1/10[a]	1/10D	1/10[a]	1/10D	1/10[a]	1/10D	1/10[a]	1/10D	
1	3+[c]	3+	2+	2+	−	−	3+	−	3+	−	3+	−	3+	−	1/512
2	3+	2+	2+	2+	−	−	2+	−	3+	−	3+	−	2+	−	1/512
3	2+	2+	2+	2+	−	−	2+	−	2+	−	2+	−	2+	−	1/512
4	2+	2+	2+	2+	−	−	2+	−	3+	−	2+	−	3+	−	1/512
5	2+	2+	2+	−		−	2+	−	2+	−	2+	−	1+	−	1/512
6	2+	2+	1+	−		−	2+	−	2+	−	2+	−	1+	−	1/128
7	2+	1+	1+	−		−	2+	−	2+	−	2+	−	2+	−	1/256
8	2+	1+	1+	−		−	2+	−	2+	−	2+	−	2+	−	1/256
9	2+	1+	1+	−		−	1+	−	1+	−	1+	−	1+	−	1/512
10	1+	1+	1+	−		−	1+	−	1+	−	1+	−	1+	−	1/32
11	1+	1+	1+	−		−	1+	−	1+	−	1+	−	1+	−	1/64
12	1+	1+	1+	−		−	2+	−	2+	−	1+	−	1+	−	1/16
13–17	−					−	−	−	−	−	−	−	−	−	1/2–64

[a] Positive reactions obtained with antiglobulins diluted more than 1/10 are not included in the table.

[b] D indicates that γG-globulins are pepsin-digested.

[c] Standards: 3+, intensely fluorescent margin, well marked edges; 2+, faintly fluorescent margin, edges usually diffuse; 1+, barely distinguishable fluorescent margin with diffuse edges; −, no fluorescence.

globulin and these reactions disappeared after digestion of the immunoglobulin molecules. The smears of *N. gonorrhoeae*, *N. meningitidis*, and nonspecific-staining *S. aureus* were indistinguishable under the ultraviolet microscope. Table 2 also shows the quantity of protein A in the different *S. aureus* strains as determined by the hemagglutination technique (26). There seems to be a good correlation between the protein A content and the immunofluorescent reaction.

Mannitol Salt Agar media have been reported to inhibit the production of protein A (8, 14). *S. aureus* type Cowan I grown on this medium give no nonspecific immunofluorescent reaction in our experiments, which is in accordance with preliminary experiments reported by Lind (19).

DISCUSSION

The problem of nonspecific reaction of *S. aureus* in the FA technique has been studied by many researchers. There have been attempts to eliminate the cross-reactions by digestion of the bacteria with papain and trypsin and by Formalin treatment (7, 16, 20, 25). The results obtained indicated that the reactive component on the bacterial surface is a protein but did not reveal its exact nature.

The reacting antibodies have been studied (15, 19–21). Lind et al. (19–21), by using several immunological methods, suggested that the cross-reactions are due to a nonspecific affinity for the bacterial surface. It was also shown that two absorptions with a *S. aureus* strain giving strong nonspecific immunofluorescence reduced the γG-globulin content of serum to less than $\frac{1}{16}$th of the original. Lind found that *S. aureus* Cowan I reacted strongly in immunofluorescence with nonimmune rabbit γ-globulin, whereas *S. aureus* Wood 46 failed to react, which is in accordance with our results. She also suggested, but did not prove, that the nonspecific reaction is due to the protein A content of *S. aureus* (19, 20).

Our experiments show that it is possible to eliminate completely the nonspecific FA reaction with *S. aureus* by pepsin digestion of γG-globulin prepared from nonimmune rabbit serum as well as for rabbit antiserum against various bacteria. The reaction is thus similar to that observed by Forsgren and Sjöquist (11, 12) in reactions between purified protein A and nonimmune rabbit γG-globulin. The staphylococci having a high content of protein A showed strong nonspecific immunofluorescence. It thus seems very probable that the nonspecific reaction results from the reaction of protein A with the Fc fragment of the immunoglobulin molecules.

In our experiments, serum from one rabbit immunized against *N. gonorrhoeae* gave a reaction with *S. aureus* that was not completely eliminated by pepsin digestion. Digested antiglobulin reacted with *S. aureus* only in moderate dilutions. Since neither Danielsson (6) nor Lind (19) observed a reaction between antistaphylococcal serum and gonococci, there is probably no true antigenic similarity. The most likely explanation would seem to be a *Staphylococcus* infection of the rabbit. There may be a similar explanation for the results reported by Jones and Foster (15), who found that the nonspecific immunofluorescence of *S. aureus* was localized to both the Fab and Fc fragments obtained by papain digestion of rabbit γG-globulin.

Danielsson (6) examined the reaction from a clinical point of view, with the aim of eliminating the nonspecific reaction of antigonococcal sera with staphylococci. He advocated mixing a lissamine rhodamine B-labeled antistaphylococcal γ-globulin with the FITC-labeled antigonococcal γ-globulin, thereby achieving a practical diagnostic method. Our work shows that digestion of the γ-globulin with pepsin does not interfere with its specific reactions. Since for most immunoglobulin preparations the nonspecific reaction with staphylococci disappears completely, pepsin digestion could afford a more straightforward way of eliminating nonspecific reactions in the identification of various bacteria, for example, *N. gonorrhoeae*. Pepsin digestion of crude γG-globulin is as effective in eliminating nonspecific immunofluorescence as is pepsin digestion of γ-globulin purified on ion-exchange chromatography. Thus, the method is technically simple in practice.

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