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ANTIPHAGOCYTIC ACTIVITY OF *STAPHYLOCOCCUS AUREUS* ANTIGENS*

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ABSTRACT

CAMERON, C. M. Antiphagocytic activity of *Staphylococcus aureus* antigens. *Onderstepoort J. vet. Res.* (1969), 36 (2), 199-206.

Systematic fractionation of a capsulated strain of *Staphylococcus aureus* has led to the isolation of two antigens which would specifically absorb opsonizing antibody from immune rabbit serum. One of these antigens was shown to be serologically identical to teichoic acid.

Teichoic acid is considered to be an important antigen for mediating phagocytosis and killing of staphylococci, but other antigens may also play a role in immunity depending on the strain involved, the route of infection, and possible deleterious effects of hypersensitivity reactions.

INTRODUCTION

Identification of the antigen(s) which are responsible for inducing immunity to *Staphylococcus aureus* infection would have great practical value. This would afford a rational basis for the preparation of vaccines, and by using such an antigen in a test system, a reliable serological test indicative of immunity could be developed.

Although antitoxic immunity may mitigate the deleterious effects of an established staphylococcal infection, it is generally accepted that antibacterial immunity and phagocytosis are more important in preventing the primary infection (Tompsett, 1962; Koenig, Melly & Rogers, 1962b; Ekstedt, 1963a). This realization has led to the intensive study of the chemical nature and antigenic composition of staphylococcal cells and cell walls (Morse, 1965). Numerous specific antigens have been identified and attempts have been made to define their biological role.

The Smith surface antigen is found in certain rare strains which have an exceptionally high virulence for mice. It has also been shown to be antiphagocytic (Morse, 1960; Koenig, Melly & Rogers, 1962a) and immunization with it protects mice against lethal challenge with the homologous strain (Fisher, 1960; Morse, 1962a; Fisher, Devlin & Erlandson, 1963).

Two cell wall polysaccharides have been isolated (Haukenes, Losnegard & Oeding, 1961; Hofstad 1965a) which are identical with the cell wall teichoic acid (Haukenes, Elwood, Baddiley & Oeding, 1961). The serological difference between these antigens is due to α- and β-N-acetyl glucosamine linkages respectively (Torii, Kabat & Bezer, 1964; Hofstad, 1965b). Large amounts of teichoic acid will absorb opsonins from immune serum (Mudd, Yoshida, Li & Lenhart, 1963).

The common protein agglutinogen, which is probably identical to protein A (Yoshida, Mudd & Lenhart, 1963; Grov, Myklestad & Oeding, 1964), does not absorb opsonizing antibody from immune serum (Lenhart, Mudd, Yoshida & Li, 1963), but it has been reported to have some immunizing activity (Stamp & Edwards, 1964).

Apart from these well recognized antigens, heat labile and heat stable proteins with immunizing activity have been found (Ekstedt, 1963b). Certain cellular aggressins (Fisher, 1963, 1965), abscess-forming factors (Lam, Sweeney, Witmer & Wise, 1963a, 1963b) and endotoxinlike substances (Higgingbotham & Bass, 1964) have also been described. They may all play a role in the pathogenesis of infection and act as protective antigens. Finally, at least 30 type specific agglutinogens (Oeding, 1952, 1965) and a cell wall peptide (Hisatsune, De Courcy & Mudd, 1967) have been identified. No biological activity has as yet been ascribed to these antigens.

It is clear that virtually every major staphylococcal somatic antigen has been endowed with some degree of protective activity, but because various models have been used in these studies and the purity of some of the preparations has not been beyond doubt, it is difficult to assess the relative importance of any one antigen. Moreover, in most instances, immunity was tested by administering lethal doses of bacteria to immunized animals. This is not the normal type of infection and immune mechanisms which would protect against septicaemia may be quite different from those effective in chronic purulent infections.

It was therefore proposed to examine various antigen preparations on a comparative basis for their ability (a) to absorb opsonizing antibody from immune serum, and (b) to protect against purulent infection. The results of the first phase are reported here, while the second aspect is still under investigation.

MATERIALS AND METHODS

Strains

S. aureus strain 68 V5 is a mutant obtained from a virulent strain (#24276) which was isolated from a case of acute bovine mastitis. It possesses Smith surface antigen (SSA), is of low mouse virulence, and has been previously described in detail (Cameron, 1966). It resembles the Smith compact variants (Hunt & Moses, 1958).

The Smith diffuse strain was obtained from Dr. R. D. Ekstedt* and the Wood 46 strain from Dr. R. K. Lindorfer**.

Large quantities of bacterial cells were produced by 24 hour shake cultures in P & M broth (Pattison & Mathews, 1957). The cells were collected by cen-

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trifugation, washed twice with distilled water and stored at −20°C.

Serological methods

Immune serum to strain 68 V5 was produced in rabbits by repeated intravenous injection of phenol killed bacteria (Wiley, 1961). Each animal received two series of injections comprising a total of 10 mg dry bacteria. They were bled 7 to 10 days after the last injection.

Serum was absorbed with whole cells or antigen fractions (Oeding, 1957). Ouchterlony gel diffusion tests were done in petri plates or on microscope slides using 1.0 per cent Ionagar #2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) in saline (pH. 7.5) with 0.1 per cent sodium azide as preservative (Oeding & Haukenes, 1963). Haemagglutination tests were performed as described by Morse (1962b).

In vitro phagocytosis

Opsonin activity of serum was tested by adding it to a mixture of rabbit peritoneal polymorphonuclear phagocytes (PMN's) and live bacteria (virulent parent strain #24276), and determining the rate of phagocytosis and killing by periodic plate counts.

Rabbit PMN's were collected as described by Kumar & Lindorfer (1962), except that Hanks' balanced salt solution (BSS) containing 10 units of heparin/ml was used instead of saline. The cells were counted with a haemocytometer, deposited at 1 000 rpm for 10 min, washed twice with BSS (without heparin) and resuspended in BSS to give the required concentration.

The phagocytosis tests were done essentially as described by Cohn & Morse (1959). It was found, however, that a ratio of bacteria to leucocytes of 10:1 gave better differentiation between normal and immune serum (Fig. 1). The final reaction mixture was composed as follows:—

Serum		0.3 ml (10%)
BSS		0.3 ml
Bacteria	(5×10^6 or 10^7)	1.2 ml
Leucocytes	(5×10^5 or 10^6)	1.2 ml

Samples (0.5 ml) were collected at the onset of the experiment and at 40, 80 and 120 min thereafter. The samples were immediately mixed with 4.5 ml cold distilled water, shaken by means of a vortex mixer for 30 sec, diluted and plated in duplicate. This method of rupturing the leucocytes and liberating living intracellular bacteria gave the most consistent results (Morello & Baker, 1965).

Chemical extracts

Five crude extracts from whole cells of strain 68 V5 were prepared using procedures that would ensure the presence of any of the recognized antigens in at least one of the extracts.

Preparation 1. Trichloracetic acid extract (TCA): Twenty g wet cells were extracted with 100 ml hot TCA (Morse, 1962b). After removal of the insoluble residue by centrifugation, the supernatant fluid was precipitated with two volumes of cold acetone, washed twice with acetone and dried *in vacuo*.

Preparation 2. Acetic acid extract: Twenty g wet cells were extracted twice with 100 ml 1 N acetic acid for 20 min in a boiling water bath. The supernatant fluid was freed of insoluble material by centrifugation, precipitated with four volumes of cold ethanol containing 0.5 per cent potassium acetate, washed twice with ethanol, and dried *in vacuo*.

Preparation 3. Sodium dodecyl sulphate extract (SDS): Ten g wet cells were suspended in 100 ml 2 per cent SDS in 0.1M phosphate buffer, pH 7.5, and extracted for 18 h at room temperature by gentle stirring. This procedure was repeated twice. After clearing by centrifugation at 30 000 *g* for 30 min, the pooled extracts were dialyzed against distilled water for 3 days (3 × 100 vols) at 4°C. It was then precipitated by dialysis against ammonium sulphate at pH 7.5 to give a final concentration of 80 per cent saturation (Löfkvist & Sjöquist, 1963). The precipitate was collected by centrifugation at 10 000 *g*, washed twice with 80 per cent saturated ammonium sulphate solution and dissolved in distilled water. The solution was freed of residual ammonium sulphate by dialysis, concentrated by perevaporation and lyophilized.

Preparation 4. Dornase extract: The procedure of Yoshida *et al.* (1963) was followed and repeated five times to obtain the maximal yield. The pH of the cleared extract was adjusted to 3.5 with 0.1 N HCl and the resulting precipitate removed by centrifugation and discarded. The supernatant fluid was then precipitated with ammonium sulphate, dialyzed, concentrated and lyophilized as for the SDS extract.

Preparation 5. Protoplasm: Twenty g wet cells were suspended in 30 ml distilled water mixed with 20 g washed 120 μ diameter glass beads (Minnesota Mining & Manufacturing Co., St. Paul, Minnesota) and homogenized in a Mini Mill (Gifford-Wood Co., Hudson, New York) at maximum speed for 30 min. The mixing chamber was kept cold by submerging it in an ice bath. The homogenate was centrifuged at 2 000 *g* for 30 min to deposit the beads and unbroken bacteria. This sediment was resuspended in 30 ml distilled water and the homogenization procedure repeated twice. The supernatant fluid (containing protoplasm and cell walls) was cleared by two centrifugations at 30 000 *g* for 60 min. The protoplasm thus obtained was treated further as for the SDS extract.

Smith surface antigen was extracted from the Smith strain with hot acetic acid (Fisher, 1963) and purified as described by Perkins (1963).

Teichoic acid was extracted and purified from the Wood 46 strain with cold TCA by the method of Morse (1962b).

Column chromatography

Gel filtration, using Sephadex G 100 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was done as described by Kim (1965) except that the gel was washed and equilibrated with 0.02 M phosphate buffer, pH 7.0, instead of saline. One hundred mg of the extract to be fractionated was dissolved in 10 ml buffer and allowed to stand overnight at 4°C in order to obtain maximal solubility. It was then centrifuged at 2 000 *g* for 60 min to remove all insoluble material. The supernatant fluid was applied

to a 2.5 × 75 cm column and eluted with 500 ml buffer at a flow rate of approximately 25 ml per h. Fractions (5 ml) were collected and the absorbancy read at a wavelength of 280 mμ. Tubes comprising the different peaks were pooled, dialyzed against three changes of distilled water, concentrated by perevaporation and lyophilized.

After lyophilization, some of the fractions were poorly soluble. In later experiments, once the active fraction had been identified, it was no longer lyophilized but the concentrated material used as such for the next step in the fractionation procedure.

Ion exchange chromatography with diethyl-aminoethylcellulose (DEAE, 0.7 meq/g, Bio-Rad Laboratories, Richmond, California) was also done according to Kim (1965). Five hundred mg of pooled preparations of the active fraction obtained by gel filtration were dissolved in 10 ml starting buffer and applied to a 2.0 × 40 cm column. In preliminary experiments the material was eluted with 600 ml of a linear gradient phosphate buffer from 0.02 M pH 7.1 to 0.5 M pH 4.5 + 1 M NaCl. In later experiments fractions were recovered by stepwise elution with 250 ml of the following buffers:

(a) 0.02 M pH 7.1,
(b) 0.15 M pH 6.2 + 0.3 M NaCl, and
(c) 0.5 M pH 4.5 + 1 M NaCl.

Fractions (10 ml) were collected, pooled, dialyzed and lyophilized as for the gel fractions. The dry material was stored at 4°C under vacuum in a desiccator.

Results

The efficiency of *in vitro* phagocytosis is greatly influenced by the total numbers of bacteria and phagocytes as well as by the ratio of bacteria to phagocytes and, unless a precise experimental system is used, it is difficult to demonstrate any significant difference between a system containing "normal" serum and a system containing immune serum. A system employing a final concentration of 2—5 × 10^6 bacteria per ml and 2 × 10^5 or 5 × 10^5 leucocytes per ml was found to give the best results and was used in all the subsequent experiments. A typical experiment is shown in Fig. 1.

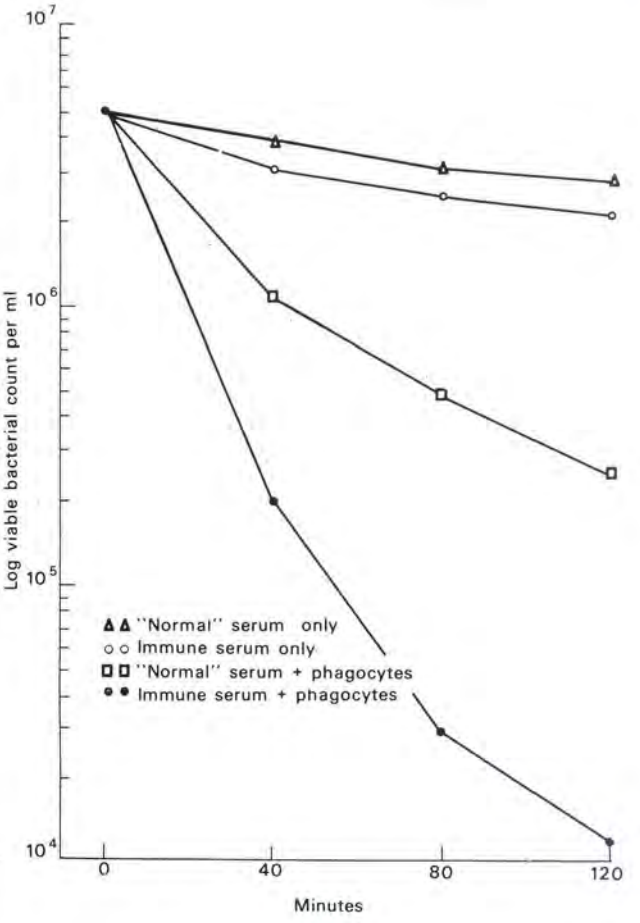


FIG. 1.—Phagocytosis and killing of *S. aureus* strain 24276 by rabbit peritoneal PMN's in the presence of normal and hyperimmune rabbit serum. The reaction mixture consisted of serum, 0.3 ml; bacteria (10^7/ml), 1.2 ml; PMN's (10^6/ml), 1.2 ml; Hanks' 0.3 ml. Viable counts were made after 40, 80 and 120 min incubation at 37°C.

It has been shown by Li, Mudd & Kapral (1963), that phagocytosis of staphylococci is slower in the presence of heated serum than with fresh serum and that the factor concerned is probably complement (Li & Mudd, 1965). Preliminary experiments were therefore done to determine whether reduction in opsonizing activity of absorbed serum is in fact due to removal of specific antibody and not just due to inactivation of complement. Fresh and inactivated serum, either absorbed with whole bacteria (5 mg dry weight/ml) or unabsorbed, were compared. The results in Fig. 2 show that the reduction in opsonizing activity is partially due to inactivation of complement by antigen-antibody complexes, but that specific opsonizing antibody is also removed. Even after restoration of the phagocytic activity by the addition of 5 per cent fresh guinea pig serum, the difference between absorbed and unabsorbed serum is still detectable. These findings are in agreement with earlier reports (Li & Mudd, 1965; Craig & Suter, 1966; Shayegani & Mudd, 1966) according to which heat labile serum factors promote phagocytosis and killing but are not essential.

Ouchterlony diffusion tests showed that the different chemical extracts had many antigens in common but in different proportions.

Immune rabbit serum was absorbed with whole dry bacteria and the five chemical extracts (2 mg/ml). When tested for reduction in opsonizing activity, it was found that except for the dornase extract all the other preparations reduced the opsonizing activity to some extent. However, only the acetic acid extract and the protoplasm fraction were superior to whole cells. A typical result is shown in Fig. 3. This figure also shows that heating the serum for 2 h at 37°C during the absorption procedure does not influence its opsonin activity.

The protoplasm and the acetic acid extracts were further fractionated by gel filtration. Both preparations gave a similar elution profile but with grossly different proportions of the various peaks. The acetic acid extract was not readily soluble but, because it was consistently superior in reducing the opsonizing activity of serum, it was used for further experiments.

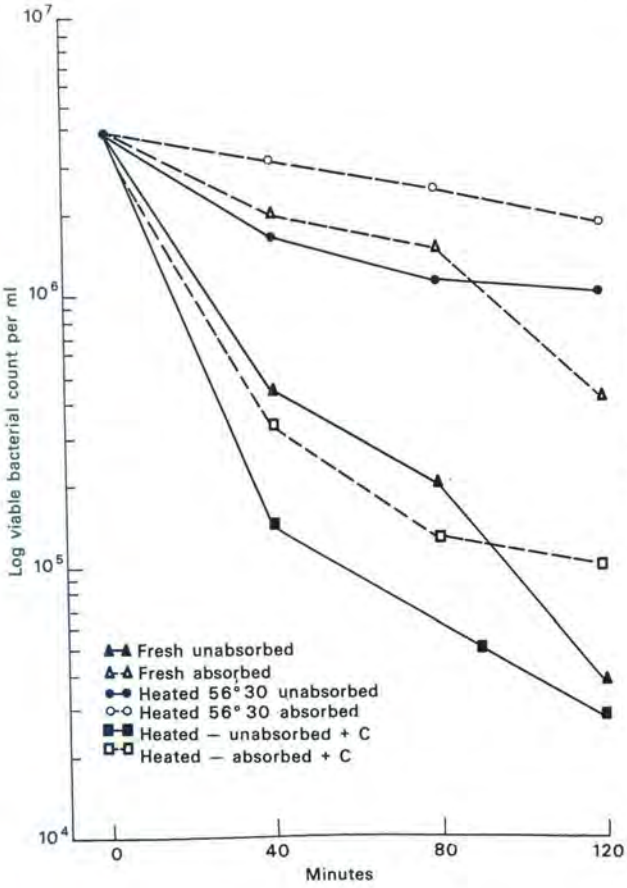


FIG. 2.—Effect of inactivation and absorption of serum with bacteria on opsonizing activity. Sera were inactivated at 56°C for 30 min and absorbed with 5 mg dry bacteria per ml. Complement activity was restored by the addition of 5% fresh guinea pig serum to the reaction mixture.

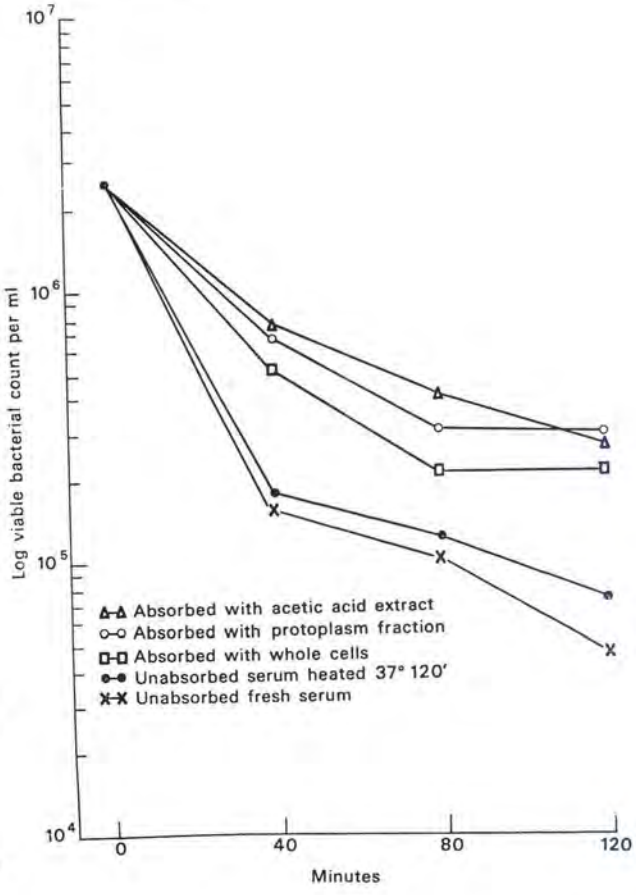


FIG. 3.—Reduction of opsonin activity of immune serum after absorption. Serum aliquots were absorbed with whole bacteria, acetic acid extract or crude protoplasm fraction (all 2 mg/ml) and tested for their ability to promote phagocytosis.

A typical elution pattern is shown in Fig. 4. The tubes comprising sections A, B and C from a number of experiments were pooled.

The three fractions thus obtained were tested for their ability to reduce the opsonizing activity of immune serum and as shown in Fig. 5, most of the active material was present in fraction A.

This fraction was then subjected to further fractionation by ion exchange chromatography. Three clearly defined peaks ("x", "y" and "z") were consistently eluted with the appropriate buffers (Fig. 6).

Fig. 7 shows the combined results of three different experiments when immune serum was absorbed with these fractions. Both fractions "y" and "z" had anti-opsonin activity.

In an attempt to identify the antigens present in these fractions, they were compared with teichoic acid and SSA using Ouchterlony's double diffusion technique. Although pooled immune serum had an anti-teichoic acid haemagglutination titre of 1/128, only serum from some individual rabbits would produce precipitin lines in agar gel and in order to obtain clear precipitin lines it was necessary to

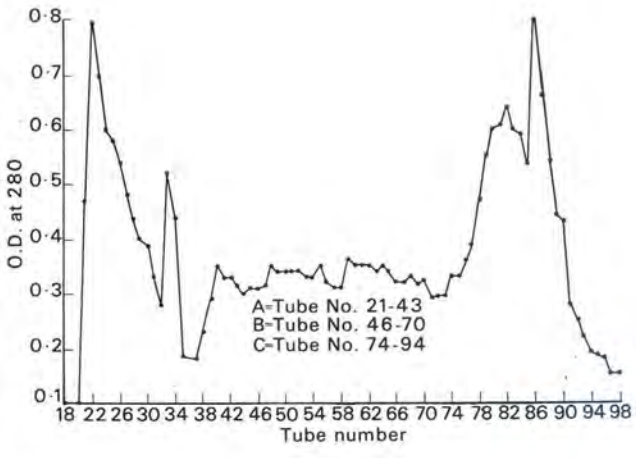


FIG. 4.—Column chromatography of acetic acid extract on Sephadex G 100. Buffer 0.02 M phosphate (pH 7.0); flow rate, 10 ml/h; fractions, 5 ml/tube.

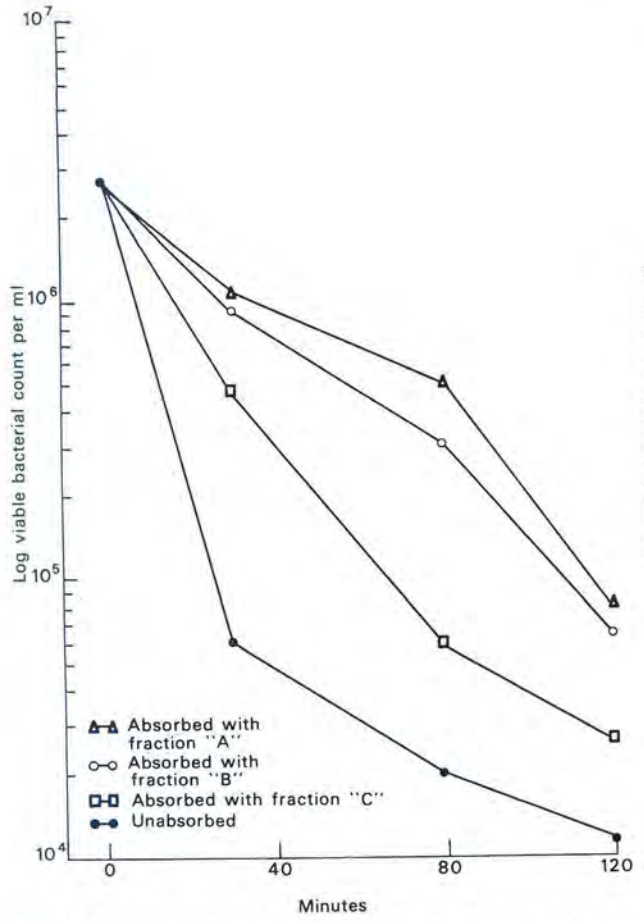


FIG. 5.—Reduction of opsonizing activity of immune rabbit serum after absorption with fractions obtained by Sephadex chromatography. Serum aliquots were absorbed with fractions A, B and C (2 mg/ml) and tested for reduction in their ability to promote phagocytosis and killing of *S. aureus* 24276.

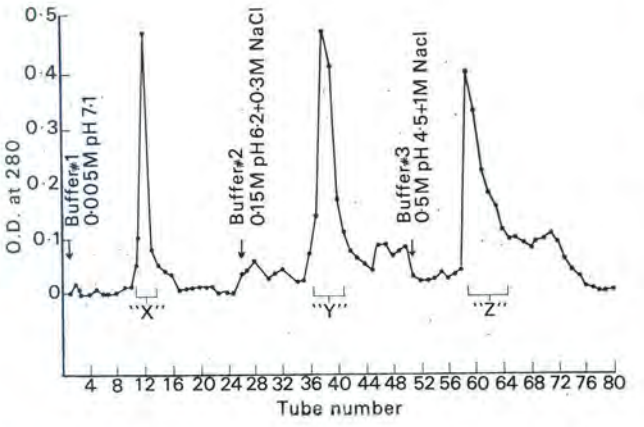


FIG. 6.—Chromatography of Sephadex fraction "A" on DEAE cellulose (0.7 meq/g).
Column size, 2 × 40 cm; flow rate, 10 ml/h; fractions collected, 10 ml/tube. Fractions "x", "y" and "z" were eluted stepwise with 250 ml phosphate buffers 0.02 M (pH 7.1), 0.15 M (pH 6.2) + 0.3 M NaCl and 0.5 M (pH 4.5) + 1 M NaCl, respectively.

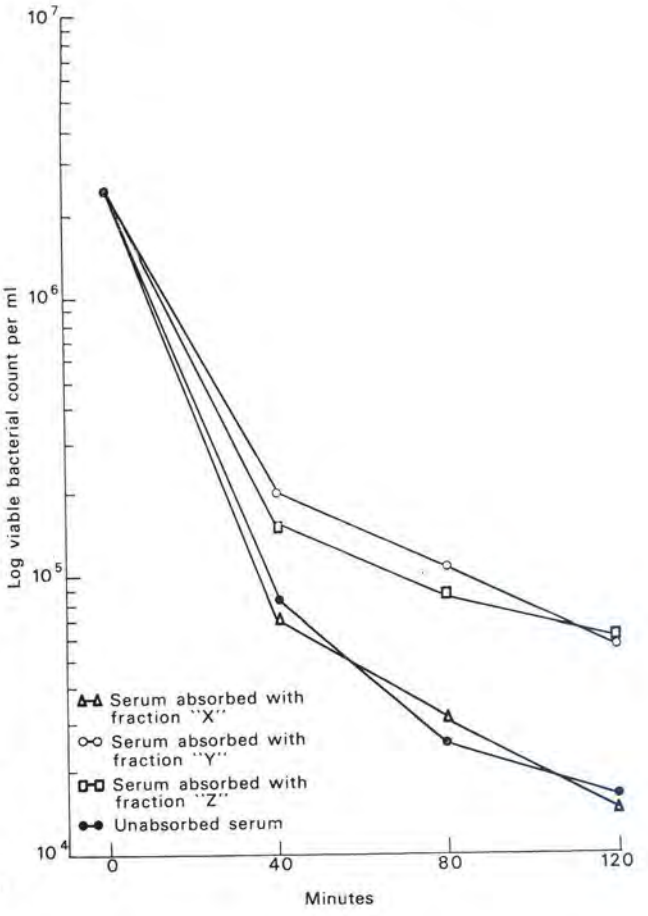


FIG. 7.—Reduction of opsonizing activity of immune rabbit serum after absorption with DEAE fractions "x", "y" and "z" (2 mg/ml.)

concentrate it four-fold. As demonstrated in Plate 1, fraction "y" showed complete identity with teichoic acid. Fraction "x" was similarly shown to be primarily teichoic acid but had no anti-opsonin activity.

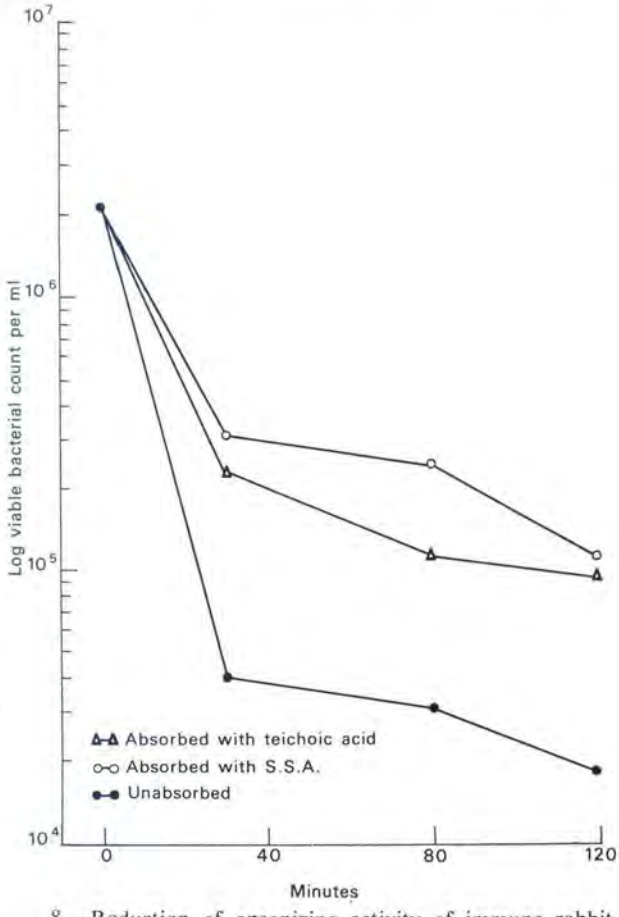
It was not possible to produce a precipitin line in agar gel with fraction "z" and consequently it could not be identified by this method. It nevertheless produced a slight precipitate in tube tests.

The ability of teichoic acid to absorb opsonizing antibody was confirmed in an experiment in which purified teichoic acid prepared from the Wood 46 strain was used (Fig. 8).

In the same experiment it was also shown that SSA prepared from the Smith strain could also absorb opsonizing antibody.

DISCUSSION

The results presented here show that anti-teichoic acid antibodies are important in promoting phagocytosis and killing of staphylococci. It was, however, also found that another unidentified antigen as well as SSA could reduce the opsonizing activity of immune rabbit serum. The activity of the SSA preparation could possibly be partially due to the presence of a small amount of teichoic acid, as is evident from Fig. 8.



8.—Reduction of opsonizing activity of immune rabbit serum after absorption with teichoic acid (2 mg/ml) and Smith surface antigen (2 mg/ml.)

PLATE 1.—Immunodiffusion showing identity of fraction "y" and teichoic acid. Wells 1 and 4 contain fraction "y" (20 mg/ml), wells 2 and 5 contain teichoic acid (2.5 mg/ml), wells 3 and 6 contain Smith surface antigen (2.5 mg/ml) and well 7 contains four times concentrated rabbit antiserum to whole bacteria.

The fact that fraction "x", which contained teichoic acid, would not absorb opsonizing antibody could be explained on the basis that it may be a different polymer. Various polymers of teichoic acid are known to exist (Burger, 1966) and it is conceivable that they may vary in their serological activity.

Although teichoic acid plays a prominent role in phagocytosis, it is not the exclusive factor involved in staphylococcal immunity. Other antigens contribute to staphylococcal immunity as well (Ekstedt, 1965).

It is very difficult to assess the relative immunizing importance of any specific antigen. One of the main obstacles is that purified antigens often lose some of their immunogenicity. Moreover when injected in this form they no longer benefit from the possible adjuvant activity of other substances with which they are normally associated in the intact bacterial cell.

Furthermore, an antigen which is important for one strain may not necessarily be important for another. Despite evidence that immune serum will promote the phagocytosis of heterologous strains (Cohn & Morse, 1959), considerable discrepancy between strains has also been reported (Rogers & Melly, 1962). These differences are especially marked when *in vivo* immunity tests are used (Ekstedt, 1963a; Warner, Slipetz & Kroeker, 1966). The question now arises whether these differences represent distinct antigenic differences between the strains or whether the differences are simply quantitative. Except for differences in minor agglutinins (Oeding, 1952), most staphylococcal strains share all the recognized antigens and in instances where certain antigens cannot be detected *in vitro*, it has been proposed that they are nevertheless produced *in vivo* (Koenig & Melly, 1965). It is also well known that staphyloccocal strains vary greatly in their ability to produce different exotoxins (Smith, 1962) and marked quantitative differences have also been reported in respect to the somatic antigens (Barber & Taga, 1965).

It would appear that serological and immunological differences between staphylococcal strains are more quantitative than qualitative. Consequently, it is reasonable to expect that certain virulence factors would be more prominent in some strains than in others and that antibodies directed at different antigens will be of greater or lesser importance depending on the strain involved.

Another aspect that has to be considered when attempts are made to assess the immunizing importance of different antigens, is the influence of the route of challenge or natural infection (Frappier, Sonea & Panisset, 1955). In different situations, different virulence factors or antigens may be more or less important in establishing and promoting infection, and consequently the degree of protection afforded by antibodies directed against different specific antigens will vary accordingly.

Finally, it is well established that hypersensitivity reactions play a prominent role in the pathogenesis of staphylococcal infections (Cluff, 1965) as well as in the development of acquired immunity. The consequences of such reactions should be carefully assessed when the immunizing properties of an antigen are evaluated.

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