ROLE OF ALPHA-TOXIN IN LESION FORMATION BY *STAPHYLOCOCCUS AUREUS* ON SUTURES SUBCUTANEOUSLY IMPLANTED IN MICE

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ABSTRACT

TAUBLER, JAMES H. (University of Pennsylvania, Philadelphia), FRANK A. KAPRAL, AND STUART MUDD. Role of alpha-toxin in lesion formation by *Staphylococcus aureus* on sutures subcutaneously implanted in mice. J. Bacteriol. **86**:51–57. 1963.—Evidence was obtained that alpha-hemolysin is essential for the development of purulent lesions by several strains of *Staphylococcus aureus* adsorbed onto sutures subcutaneously implanted in mice. This evidence consisted of findings that active immunization with staphylococcal toxoid afforded significant protection to subcutaneous challenge with this organism and that alpha-hemolysin–negative mutants, derived from strains 18Z and P78, were almost devoid of the ability to produce lesions by this route of infection. However, other unknown factors also appear to be essential for lesion formation since passive immunization of mice with antialpha-toxin, obtained from rabbits immunized with purified toxin, failed to afford protection. A dose-response curve for this type of infection is also presented.

Previous studies (Taubler, Mudd, and Sall, 1962) indicated that administration of human gamma-globulin to mice could effectively reduce the incidence of purulent lesions in mice challenged with a strain of *Staphylococcus aureus* adsorbed onto sutures implanted subcutaneously. This protection could, of course, result from antibodies directed against a wide variety of staphylococcal components. While these studies were in progress, Kapral, Mullarkey, and Taubler (*in preparation*) obtained evidence for the critical role of alpha-toxin as the lethal factor in staphylococcal peritonitis in mice. From these studies as well as many others cited in the literature (Elek, 1959), it was decided to investigate the role, if any, of alpha-toxin in the pathogenesis of lesion formation with subcutaneously implanted infectious sutures.

MATERIALS AND METHODS

Strains. S. aureus strain 18Z, previously described (Kapral and Li, 1960), was employed in the majority of cases in this study. This strain possesses bound and soluble coagulase, hyaluronidase, fibrinolysin, lipase, both components of the Panton-Valentine leucocidin, and alpha- and delta-hemolysin. From the 18Z strain were independently derived two mutants (18Z-G and 18Z-H) that lacked the ability to produce in vitro detectable amounts of alpha-hemolysin.

S. aureus strain P78, described by Kapral and Shayegani (1959), was also used in a small number of experiments. The P78 strain possesses the same measurable characteristics as the 18Z strain, except that it is not phage-typable and lacks fibrinolysin. A mutant (P78-22), lacking the ability to produce alpha-toxin in vitro, was derived from the P78 strain.

Preparation of infectious sutures. The procedure employed was previously described (Taubler et al., 1962).

Active immunization. Staphylococcal toxoid obtained from the Institut Pasteur (a partially purified toxoid not fortified with bacterial lysate) was mixed with sodium alginate (Colab, Chicago Heights, Ill.) and ethylenediaminetetraacetic acid so that each 0.4-ml dose contained approximately 2 units of toxoid. White Swiss male mice, weighing 20 g, were inoculated subcutaneously at 1-week intervals for 6 weeks. Control mice received the same amount of adjuvant with saline in lieu of the toxoid.

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Titration of antialpha-hemolysin in mice. Blood was recovered from each mouse by cutting off a small portion of the tail and collecting the blood in capillary tubes (75 × 1.5 mm).

The tubes were sealed at one end with plasticene and centrifuged in a hematocrit centrifuge. The tubes were broken, the sedimented cells discarded, and the serum expressed with the aid of a small rubber bulb. Titrations were performed with the aid of a microtitrator (Cook Research Laboratories, Menlo Park, Calif.) in the following manner. Titration loops (calibrated to hold 0.025 ml) were charged with serum expressed from the capillary tubes, and serial twofold dilutions of the serum in saline were made. An equal volume of crude alpha-hemolysin (culture filtrate), just sufficient to cause complete hemolysis of the system in the absence of antitoxin, was then added to each serum dilution. After mixing, the serum-toxin mixtures were left at room temperature for 20 min. A 5% suspension of thrice-washed rabbit red blood cells (from selected rabbits) in saline was prepared, and 0.025 ml added to each well. After incubating at 37 C for 1 hr, the titrations were read. The concentration of antitoxin in each mouse serum was determined by comparing the end points of the titrations with that obtained with standard antitoxin (National Institutes of Health, U.S. Public Health Service; 20 units/ml) diluted in the same way.

Purified 12S-free alpha-toxin. Purified 12S-free alpha-toxin (Bernheimer and Schwartz, 1963) was precipitated with 10% potassium aluminum sulfate (alum). New Zealand white rabbits (1.5 kg) received two doses of the alum-precipitated toxin (0.1 mg/dose) 1 week apart intramuscularly in the hind leg. The serum obtained, 7 to 10 days after the second injection, from these rabbits had an antialpha-hemolysin titer of 10 units/ml. This serum was lyophilized and stored at 4 C. Preimmune sera of these rabbits gave no detectable antialpha-hemolysin titer. Pseudo-globulin was removed from these normal sera by the method of Kendall (1937). This was done to reaffirm the absence of antialpha-toxin in the normal serum even after concentration.

Mice were passively immunized with this antiserum by injecting intraperitoneally 0.2 ml of serum (1.5 times concentrated when reconstituted) 18 and 2 hr before suturing and 24, 48, and 72 hr after suturing.

Immunoelectrophoresis of the purified 12S-free alpha-toxin. Electrophorectic runs were carried out in a Buchler Zone Electrophoresis Apparatus using 0.1 M phosphate buffer (pH 7.2). Electrophoretic slides were made of 0.8% Noble agar and were buffered with phosphate buffer to give a final concentration the same as that used in the electrophoretic chamber. All electrophoretic runs were carried out over a 1-, 2-, and 3-hr period at 150 v.

RESULTS

Dose response. The cumulative data with *S. aureus* 18Z are presented in Fig. 1. These data include the control mice infected with strain 18Z as previously published (Taubler et al., 1962) and those data given in Table 1. The numbers, plotted on a logarithmic scale, of organisms initially present on the subcutaneously placed portion of infectious sutures and the percentages of mice developing purulent lesions are related linearly. The regression line and 95% confidence limits are indicated in Fig. 1.

Lesion formation in immunized mice. At weekly intervals for a period of 6 weeks, 50 mice were immunized with staphylococcal toxoid (Institut Pasteur) in sodium alginate. The serum of each mouse was titrated at the end of the fourth, fifth, and sixth weeks. Of the mice, 15 (30%) at the end of 6 weeks had an antialpha-hemolysin level of 1.2 to 2.5 units per ml. Previous studies indicated that the level of antitoxin during the course of gamma-globulin administration was maintained within this range. These mice were then selected for challenge with *Staphylococcus aureus* (18Z) adsorbed onto sutures. Ten control mice that had received only the adjuvant were also challenged. These controls had no detectable antialpha-hemolysin antibody in their sera. Figure 2 demonstrates the percentage of 10 infected control and 12 immunized mice (of the 15 immunized mice, 3 lost sutures), each group being challenged with an equal dose (average dose: 980 organisms per mouse), showing visible lesions over a 5-day period.

Reduction of lesion formation with alpha-hemolysin–negative mutants. Mice (37) were challenged with 18Z-G (alpha-hemolysin–negative) adsorbed onto sutures. Of these mice, 13 received an average dose of 780 organisms per mouse (low dose), and 24 mice received an average dose of 1,870 organisms per mouse

(high dose). Of the animals challenged with 18Z-G, 8% developed lesions. As control animals, 45 mice were challenged with the parent 18Z strain. These mice were given (in four separate experiments) doses in the range of 200 to 1,000 cocci per mouse. At least a sevenfold increase in lesion formation was observed in the control animals (animals challenged with 18Z).

The other independently derived mutant, 18Z-H (in an average dose of 220 organisms per mouse), failed to initiate purulent lesions in 13 animals challenged.

Mice (46) were challenged with the P78 strain in three separate experiments (with an average dose range of 100 to 840 organisms per mouse). Of these animals, 33 to 46% developed purulent lesions.

Mice (23) were challenged with the alpha-toxin–negative mutant (P78-22), derived from P78. Average doses of 140 and 600 organisms per mouse were given. None of the mice tested developed purulent lesions. Although in a preliminary experiment lesions did develop in some mice infected with P78-22 (average dose: 600 organisms per mouse), these results could not be confirmed. These data are presented in Table 1.

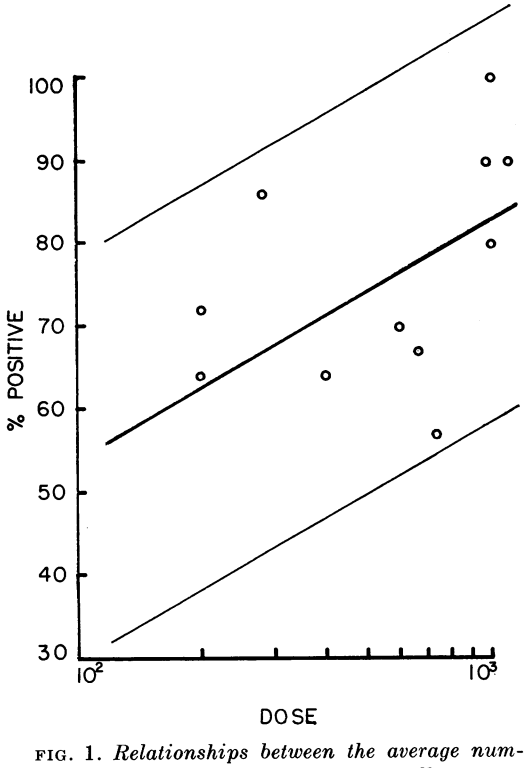

FIG. 1. *Relationships between the average numbers of Staphylococcus aureus 18Z initially present on the portion of suture implanted subcutaneously in mice and the percentage of animals subsequently developing purulent lesions within 5 days after infection. The regression line and 95% confidence intervals are indicated. The standard deviation of the regression line is 12.2% units, and the correlation coefficient is 0.51.*

TABLE 1. *Occurrence of lesion formation in mice subcutaneously infected with hemolytic and nonhemolytic Staphylococcus aureus adsorbed onto sutures*

Strains	Alpha-toxin produced in vitro	Avg no. of colony-forming units/ mouse	No. of mice chal-lenged	Per cent purulent lesions
18Z	+	200	7	72
18Z	+	670	14	67
18Z	+	740	14	57
18Z	+	1,000	10	100
18Z-G	−	780	13	8*
18Z-G	−	1,870	24	8*
18Z-H	−	220	13	0
P78	+	840	10	40
P78	+	250	24	46
P78	+	100	12	33
P78-22	−	600	13	0
P78-22	—	140	10	0

* Organisms could be recovered after 5 days from the pus in three animals that developed lesions when challenged with the 18Z-G. All the organisms recovered lacked alpha-hemolysin as determined by in vitro plate tests.

Passive immunization with purified 12S-free alpha-toxin. Mice (24) were challenged with the 18Z strain with an average dose of 550 adsorbed organisms per mouse. Of these animals, 16 received rabbit antialpha-toxin according to the schedule described. The eight control animals received normal rabbit gamma-globulin, the concentration of which was equivalent to that present in the immune rabbit serum given to the former group of animals. In addition, 12 passively immunized mice and 11 control mice (given normal rabbit gamma-globulin) were infected with an average dose of 1,027 adsorbed organisms per mouse. All those mice receiving antiserum maintained an average antialpha-toxin titer of 2.5 to 5.0 units per ml. This level was at least as high as that obtained by actively immunizing with Institut Pasteur toxoid (see Materials and Methods), or after giving human gamma-globulin according to the previously described

schedule (Taubler et al., 1962). In both these latter cases, partial protection resulted. However, from Table 2 it is evident that the anti-alpha-toxin (against purified toxin) afforded no protection against the challenge dose employed.

In Fig. 3A it can be seen that a single band resulted when purified 12S-free toxin (after electrophoresis) reacted with rabbit antisera produced against this purified toxin. No band of precipitation resulted when normal rabbit gamma-globulin (from preimmunized rabbits) was used in place of rabbit immune serum.

Figure 3B illustrates the development of three precipitation bands resulting from the interaction of crude toxin (after electrophoresis) and rabbit antitoxin (induced by purified 12S-free toxin).

Figure 3C demonstrates the formation of two to three precipitation bands between purified 12S-free toxin (after electrophoresis) and rabbit antisera obtained from animals immunized with crude toxin preparation (ammonium sulfate-precipitated culture filtrate).

TABLE 2. *Effect of normal rabbit gamma-globulin and rabbit antitoxin administration on lesion formation in mice subcutaneously infected with Staphylococcus aureus adsorbed onto sutures*

Mice given	Dose 18Z	Number positive	Number negative	Per cent positive
Normal gamma-globulin	550	5	3	62.5
Antialpha-toxin	550	10	6	62.5
Normal gamma-globulin	1,027	8	3	72.7
Antialpha-toxin	1,027	8	4	67.7

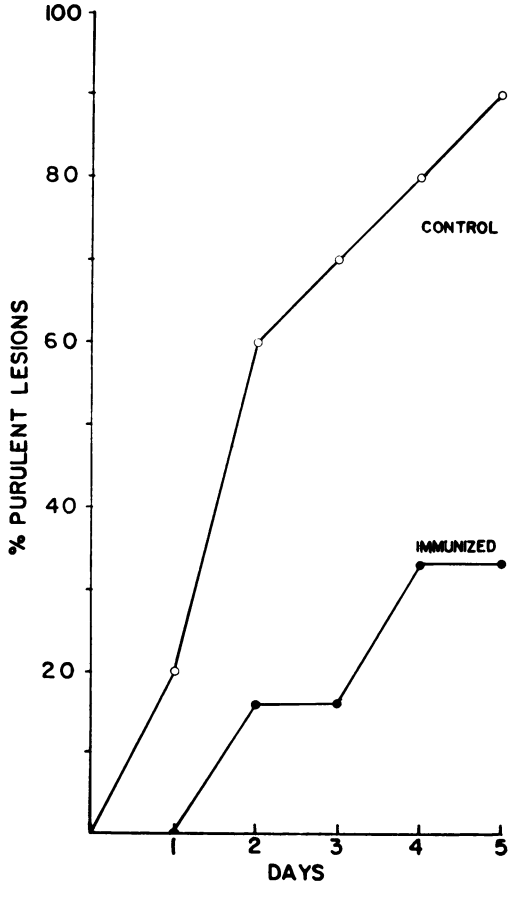

FIG. 2. *Percentage of challenged mice having visible lesions at various times after infection with Staphylococcus aureus 18Z on sutures. Immunized group (12 mice) received staphylococcal toxoid (Institut Pasteur) in adjuvant; 10 control mice received adjuvant only. Average challenge dose was 980 cocci per mouse.*

DISCUSSION

Considering the dose-response curve (Fig. 1), it can be seen that purulent lesions develop in mice challenged by this route with relatively small doses of *S. aureus* 18Z, 100% infection to be expected with a dose in the range of 4,000 organisms per mouse. From the slope of the dose-response curve, it appears that some mice should become positive (develop lesions) with only a few cocci per mouse. This dose response with *S. aureus* 18Z contrasts sharply with that obtained with mice challenged intraperitoneally with the same strain. In the latter case, the dose response is such that no death occurs with a dose of less than 10^8 cocci per mouse, and 100% mortality is attained with a dose in the order of 5×10^9 cocci per mouse (Kapral, Mullarkey, and Taubler, *in preparation*).

That alpha-toxin plays a critical role in lesion formation under these experimental conditions is strongly suggested by the observation that mutants, lacking the ability to produce substantial amounts of this toxin under in vivo conditions, are relatively devoid of the capacity to initiate purulent lesions when compared with their respective parent strains given in similar doses (Table 1). It should be noted that the staphylococci of the infecting strain could be

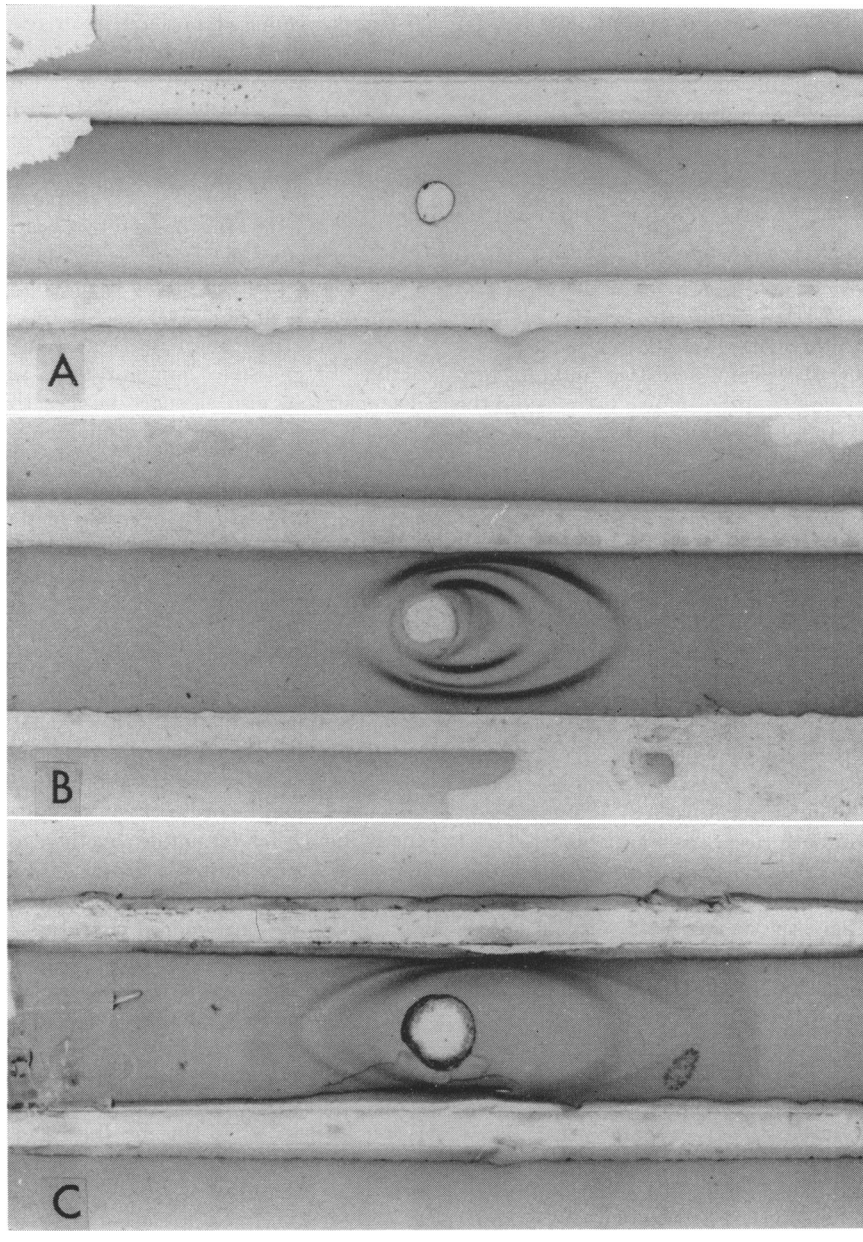

FIG. 3A. *Purified toxin (2 mg/ml) in center well. Electrophoresed for 3 hr at 150 v, 0.1 M phosphate buffer (pH 7.2). Upper antibody trough contains rabbit antitoxin against purified toxin. Lower antibody trough contains normal rabbit gamma-globulin. Migration toward cathode.*

FIG. 3B. *Crude toxin (5 mg/ml) in center well. Electrophoretic conditions same as in 3A. Antibody troughs contain rabbit antitoxin against purified toxin.*

FIG. 3C. *Purified toxin (1 mg/ml) in center well. Electrophoretic conditions same as in 3A. Antibody troughs contain antiserum against crude toxin.*

recovered when the sutures were cultured after being removed from animals which did not develop lesions subsequent to challenge with either the parent or mutant strains.

Although the alpha-toxin-negative mutants employed in these studies produced no measurable toxin when grown in vitro (on various media with or without increased CO_2 tensions), at least two of these, 18Z-G and P78-22, produced small amounts when grown within dialysis sacs implanted in the peritoneal cavity of mice according to the method of Gladstone and Glencross (1960). However, these two mutants produced only one-fifteenth as much toxin as did the parent 18Z and P78 strains when grown under similar in vivo conditions. This amount appears to be insufficient for lesion formation.

Immunication with toxoid (Institut Pasteur) may well stimulate antibody formation against a variety of antigens in addition to alpha-toxin and, therefore, the protection obtained need not necessarily implicate alpha-toxin as a critical factor in this disease. Yet the levels of antialpha-toxin attained by immunizing with this preparation were in the same range as those obtained (*unpublished data*) when mice were given gamma-globulin according to the same schedule reported previously (Taubler et al., 1962). The degree of protection in both cases was comparable.

The fact that passive immunization with rabbit antitoxin against the purified 12S-free toxin failed to reduce the incidence of purulent lesions in challenged mice, even though the antialpha-toxin titers in the sera of these mice were sufficiently high (2.5 to 5.0 units/ml), suggests that other factors are involved. Mice were not actively immunized with purified 12S-free alpha-toxin, since previous studies with crude alum-precipitated toxin indicated that fairly large amounts of antigen were necessary to produce antitoxin levels in the desired range (1.25 to 2.5 units/ml). The amount of purified 12S-free toxin available was not sufficient to actively immunize the numbers of mice required, and smaller doses of purified 12S-free alpha-toxin did not appear to stimulate antitoxin formation.

Although a single precipitation band formed when purified toxin after electrophoresis reacted with rabbit antisera produced against the purified toxin (Fig. 3A), multiple bands formed when: (i) crude toxin (after electrophoresis) reacted with rabbit antiserum against purified toxin and (ii) electrophoresed purified toxin reacted with antisera against ammonium sulfate-precipitated culture filtrate (Fig. 3B and 3C). These results suggest that the purification of the toxin was not complete. A. W. Bernheimer (*personal communication*) is aware of at least one contaminating antigen in the 12S-free toxin preparation. It is doubtful whether this has a direct bearing on the data presented, however, since the antiserum produced against this preparation did not prevent lesion development.

The fact that complete protection either by active or passive immunization was never demonstrated also strongly implies that alpha-toxin is not the only essential factor in lesion formation by this route of infection.

From these data, at least two explanations may be set forth. Alpha-toxin may be a factor essential for lesion formation under these experimental conditions, but not the sole or even the main factor involved. The fact that only partial protection was observed when mice were passively and actively (human gamma-globulin and Institut Pasteur toxoid) immunized could be due to antialpha-toxin antibodies together with antibodies to other factors, whereas passive immunization in mice with rabbit antialpha-toxin produced against purified toxin was not sufficient to inhibit lesion formation by these organisms. However, the results from the parent-mutant combinations seem to indicate that certain levels of alpha-toxin are indeed essential for lesion production. Antibodies to alpha-toxin alone may not reduce the over-all toxin production sufficiently, when other factors are left unchallenged, to give protection.

Another theoretically possible explanation might be that alpha-toxin is not involved in lesion formation. If this be the case, then the parent-mutant studies would have to be explained by a multiple mutation, that is, the simultaneous loss of the ability to produce alpha-toxin as well as other factor(s) involved in lesion formation, or by linked markers. It is the authors' opinion that the former explanation best fits the experimental data.

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Literature Cited

Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**:455–468.

Elek, S. D. 1959. *Staphylococcus pyogenes*. Livingstone Ltd., Publishers, London.

Gladstone, G. P., and E. J. G. Glencross. 1960. Growth and toxin production of staphylococci in cellophane sacs *in vivo*. Brit. J. Exptl. Pathol. **41**:313–333.

Kapral, F. A., and I. W. Li. 1960. Virulence and coagulases of *Staphylococcus aureus*. Proc. Soc. Exptl. Biol. Med. **104**:151–153.

Kapral, F. A., and M. Shayegani. 1959. Intracellular survival of staphylococci. J. Exptl. Med. **110**:123–138.

Kendall, F. E. 1937. Studies on serum proteins. I. Identification of a single serum globulin by immunological means; its distribution in the sera of normal individuals and of patients with cirrhosis of the liver and chronic glomerulonephritis. J. Clin. Invest. **16**:921–931.

Taubler, J. H., S. Mudd, and T. Sall. 1962. Partial protection of mice by human gamma globulin against *Staphylococcus aureus* on subcutaneous sutures. Proc. Soc. Exptl. Biol. Med. **109**:20–23.