FACTORS RELATING TO THE VIRULENCE OF STAPHYLOCOCCI

III. Antibacterial versus Antitoxic Immunity*

By M. GLENN KOENIG,‡ M.D., MARIAN ANN MELLY, and DAVID E. ROGERS, M.D.

(From the George Hunter Laboratory, Department of Medicine, Vanderbilt University, Nashville)

(Received for publication, June 28, 1962)

The relative importance of "antibacterial" or "antitoxic" immunity in resistance to staphylococcal disease has been a subject of prolonged debate based on conflicting evidence (1–12). Recent studies from this laboratory have shown that mice immunized intravenously with heat-killed staphylococci are protected against lethal intraperitoneal doses of several mouse-virulent strains (13, 14). The present study was undertaken to further delineate the importance of antibacterial and antitoxic immunity in this experimental system.

These studies show that although deaths produced by ordinarily lethal doses of alpha hemolysin can be prevented by immunization with crude alpha hemolysin toxoid, only heat-killed staphylococcal vaccine protects mice against death following infection with viable, replicating staphylococci.

Materials and Methods

Cultures. Smith Diffuse Variant.—This previously described mouse-virulent strain of *Staphylococcus aureus* was utilized throughout (13).

Preparation of Staphylococcal Vaccine and Immunization of Animals.—The preparation of heat-killed vaccine has been described (13). Supernatants of these vaccines did not hemolyze rabbit red cells before or after heat inactivation. Vaccines contained between 10^{10} and 10^{11} organisms per ml.

Mice were immunized with 0.1 ml doses given intravenously every 3 to 4 days for a total of 5 injections. Mice were challenged 10 to 12 days after the final immunization. Rabbits received 0.5 ml of vaccine intravenously each week for five doses and were bled 1 week after the final injection.

Preparation of Crude Alpha Hemolysin and Alpha Hemolysin Toxoid and Immunization of Animals.—A modification of Burnet's broth (1) was used to increase alpha hemolysin production. The broth contained beef extract, 3 gm. per liter; proteose peptone, 20 gm. per liter; and

* Supported by Grant E-3082 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, and the George Hunter Laboratory, Vanderbilt University School of Medicine, Nashville.

‡ United States Public Health Service Post-doctoral Fellow, National Institute of Allergy and Infectious Diseases.

0.03 per cent $MgSO_4$. Cultures of the Smith diffuse variant were grown for 6 days at 37°C while 30 per cent carbon dioxide in oxygen was bubbled through the media. The cultures were centrifuged at 2500 RPM, the supernatants removed, Seitz-filtered, and assayed for hemolytic activity using the method previously described (13). The filtrates were "toxoided" by adding formalin to a final concentration of 1 per cent and allowing the mixture to stand at room temperature for several days. The toxoid was rechecked at intervals until no demonstrable hemolysin remained.

Control "toxoid" made from the Smith diffuse culture was prepared in a similar manner with the single exception that no carbon dioxide was bubbled through the media during growth. No hemolysin was demonstrable in these filtrates either before or after the addition of formalin.

The "toxoids" were stored at 4°C and were homogenized with an equal amount of Freund's incomplete adjuvant (Difco Laboratories, Detroit) immediately before use. Rabbits were given five injections of 2 ml subcutaneously at weekly intervals, and bled 1 week after the final injection. Mice were given six injections of 0.2 ml subcutaneously at intervals of 3 to 4 days, and challenge experiments were performed 10 days after the final injection.

Assay of Alpha Hemolysin in the Mouse Peritoneum.—Alpha hemolysin was assayed in peritoneal fluid of mice dying from infection or in mice sacrificed at intervals after challenge. The peritoneal cavity was opened aseptically and 1 ml of sterile saline was added. After washing the cavity gently, the resulting fluid was removed and assayed for hemolysin in the usual manner (13).

Alpha Hemolysin Antitoxin Assay.—Serum was assayed for alpha hemolysin by the hemolytic method. Serial twofold dilutions were made in 0.5 ml of saline, and 0.5 ml of alpha hemolysin in the highest dilution which would cause complete hemolysis in a control assay was added to each tube. After 30 minutes' incubation at 37°C, one drop of a 20 per cent suspension of washed rabbit red cells was added. The tubes were then placed in a 37°C water bath for 1 hour. After initial readings, tubes were placed at 4°C, and final readings were made at 18 hours. The antitoxin titer was read as the highest dilution of serum which remained free of any visible hemolysis. A simultaneous assay was performed using the United States standard staphylococcus antitoxin (20 units per ml)[1] as a reference for computing antitoxin units in the animal specimens.

Quantitative Determination of Culturable Intraperitoneal Staphylococcal Populations.—These studies were performed as described in earlier papers (13, 14). Mice were infected by intraperitoneal injections of 0.4 ml of 18 hour trypticase soy broth cultures of the Smith diffuse variant containing approximately 5×10^8 viable units. At specified time intervals, groups of 3 mice were sacrificed for quantitative determinations and results were averaged in the construction of figures.

In Vitro Assay of the Opsonic Activity of Serum Obtained from Normal and Immunized Rabbits.—The opsonic activity of serum from normal and immunized rabbits was studied in phagocytic systems employing human leukocytes (15). Rabbits were immunized with alpha hemolysin toxoid, control "toxoid" lacking hemolysin, or heat-killed staphylococcal vaccine prepared from the Smith diffuse strain.

RESULTS

Antihemolysin Titers Obtained in Mice and Rabbits Receiving Heat-Killed Diffuse Variant Vaccine or Alpha Hemolysin Toxoid.—As shown in Table I, preimmunization sera obtained from normal mice or rabbits contained no

[1] Obtained through the courtesy of Dr. W. G. Workman, Division of Biologics Standards, National Institutes of Health, Bethesda.

detectable antitoxin. Immunization with heat-killed diffuse vaccine evoked no detectable antibodies to alpha hemolysin despite repeated injections. In contrast, immunization with crude alpha hemolysin toxoid resulted in significant antitoxin titers in both rabbits and mice. Titers ranged from 1:128 to 1:256 in 3 rabbits and 1:64 to 1:128 in the pooled sera of four separate groups of 5 mice.

TABLE I

Anti-Alpha Hemolysin Titers

	Rabbits	Mice
Normal	<1:2* (5 rabbits)	<1:2 (10 mice)
Vaccine-Immunized	<1:2 (2 rabbits)	<1:2 (15 mice)
Toxoid-Immunized	1:128 to 1:256† (3 rabbits)	1:64 to 1:128§ (20 mice)

* Less than 0.02 units of United States standard staphylococcus antitoxin per ml.

† Comparable to 1.2–2.4 units of United States standard staphylococcus antitoxin per ml.

§ Comparable to 0.6–1.2 units of United States standard staphylococcus antitoxin per ml.

TABLE II

Effect of Immunization on Mouse Mortality

	Challenged with living bacteria	Challenged with alpha hemolysin
Normal	43/47 or 91.5 per cent	32/35 or 91.4 per cent
Diffuse immune	2/15 or 13.3 per cent	16/16 or 100 per cent
Alpha hemolysin toxoid–Immune	9/10 or 90 per cent	0/10 or 0 per cent

The Influence of Immunization on Mortality Following Challenge with Alpha Hemolysin or Viable Diffuse Variant Staphylococci.—Mice immunized with the bacterial vaccine or crude alpha hemolysin toxoid were challenged intraperitoneally with either 5×10^8 viable Smith diffuse variant staphylococci, or 0.4 ml of diffuse culture filtrate possessing an alpha hemolysin titer of 1:128.

As noted in Table II, these doses of diffuse variant staphylococci and alpha hemolysin consistently killed normal mice. Mice immunized with the heat-killed vaccine were not protected when given similar doses of alpha hemolysin, but were significantly protected from challenge with viable microorganisms. In contrast, mice immunized with alpha hemolysin toxoid were completely protected from the lethal effects of alpha hemolysin, but showed no protection

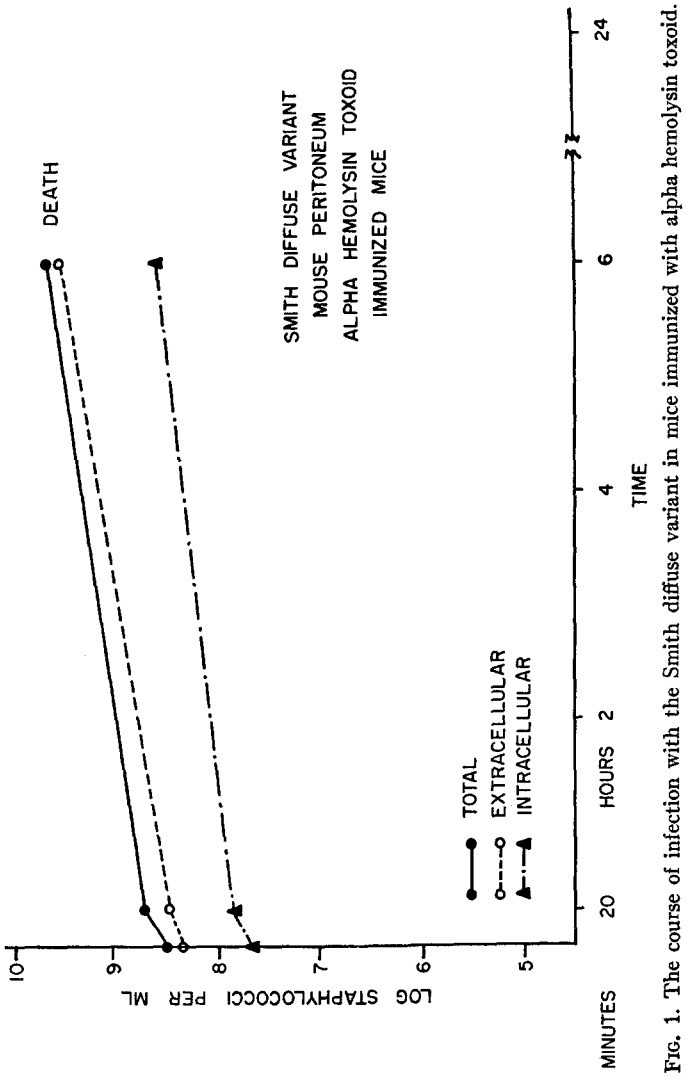

FIG. 1. The course of infection with the Smith diffuse variant in mice immunized with alpha hemolysin toxoid.

when infected with diffuse variant staphylococci. In the latter instance, 9 of 10 mice died between 8 hours and 20 minutes and 22 hours after intraperitoneal infection.

Dynamics of the Intraperitoneal Infection in Mice Immunized with Alpha Hemolysin Toxoid.—Quantitative bacterial studies were performed at appropriate intervals after intrapertoneal infection on mice immunized with alpha hemolysin toxoid. As shown in Fig. 1, intraperitoneal staphylococci multiplied rapidly. Most of the viable microorganisms remained extracellularly, indicating that intraperitoneal phagocytosis was inefficient and ineffective. Thus, in this experimental model, immunity to alpha hemolysin did not result in increased

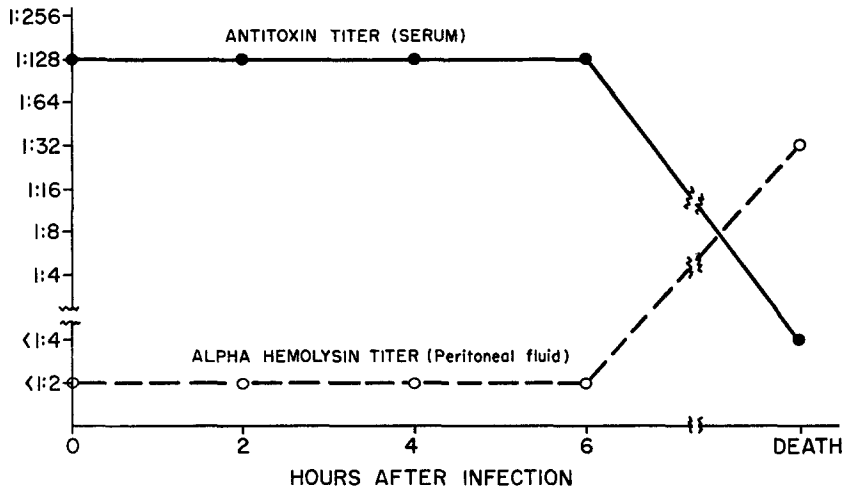

FIG. 2. Antitoxin and alpha hemolysin titers in infected toxoid-immunized mice

phagocytosis of the diffuse variant staphylococci, and such mice resembled normal non-immunized animals reported previously (13).

Assay of Alpha Hemolysin Titers in Mice Dying of Infection.—Previous experiments had shown that mice dying of intraperitoneal infections produced by the diffuse variant staphylococcus had significant intraperitoneal titers of alpha hemolysin at time of death (13). This observation suggested that these animals were killed by alpha hemolysin produced *in vivo* by multiplying microorganisms. In the current studies, alpha hemolysin assays were performed on pooled peritoneal exudates obtained from 2 control or 2 alpha hemolysin toxoid-immune mice at time of death. In such experiments, significant amounts of alpha hemolysin were detected. In 2 experiments, peritoneal titers of 1:32 were found in both normal and toxoid-immunized mice. Alpha hemolysin was not demonstrated in the peritoneal fluid of normal mice given 0.4 ml of heat-killed vaccine sacrificed after 6 hours.

Disappearance of Antitoxin in Alpha Hemolysin Toxoid-Immunized Mice Dying of Infection.—The demonstration of significant titers of alpha hemolysin in the peritoneal cavities of toxoid-immunized mice suggested that the continued production of alpha hemolysin by actively multiplying staphylococci had bound all available alpha hemolysin antitoxin. To test this thesis, mice immunized with alpha hemolysin toxoid were infected with similar numbers of the Smith diffuse staphylococcus intraperitoneally, and groups of 3 to 4 mice were bled and the sera pooled for assay of alpha hemolysin antitoxin at 0, 2, 4, and 6 hours after infection and at time of death. In addition, at each study

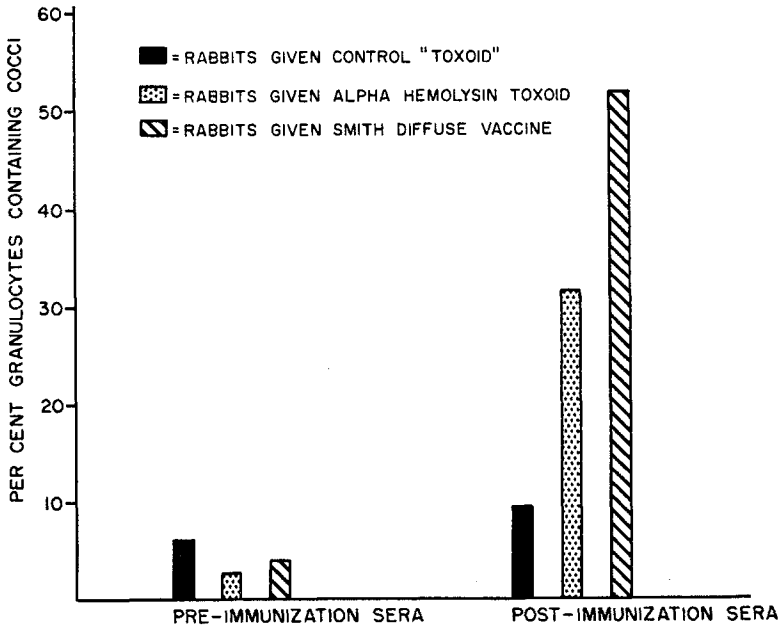

FIG. 3. Opsonic activity of rabbit sera

period the pooled peritoneal fluids obtained from 2 mice were assayed for detectable alpha hemolysin.

The results of such an experiment are shown in Fig. 2. As noted here, serum antitoxin levels persisted at preinfection titers until the time of death when antitoxin dropped precipitously below detectable levels. Simultaneous studies on peritoneal fluid showed that alpha hemolysin did not appear in detectable quantities until the time of death when titers rose sharply to 1:32. This suggested that alpha hemolysin produced late in the infection in amounts sufficient to inactivate all available antitoxin might play a role in the death of these animals.

In Vitro Studies of the Opsonic Activity of Serum Obtained from Normal and Immunized Rabbits.—While immunity to alpha hemolysin did not promote phagocytosis of the diffuse variant staphylococci in the mouse peritoneum, further experiments were performed in an *in vitro* fluid phagocytic system to determine if antibodies to alpha hemolysin could serve as opsonins. In these studies the opsonic activity of the sera of rabbits immunized with heat-killed diffuse variant vaccine was compared to that of sera of rabbits immunized with alpha hemolysin toxoid. Sera obtained from rabbits immunized with a control "toxoid" lacking alpha hemolysin but prepared in the same manner as the alpha hemolysin toxoid, were used as controls. In this manner it was possible to assess the antigenic effect of any diffuse variant surface antigens which might have been present in the crude toxoid preparations. These experiments were performed in a system containing washed human leukocytes suspended in a 20 per cent concentration of the test serum.

The results of these studies are shown in Fig. 3. Each bar represents the average of two experiments on separate animals. As noted, preimmunization sera did not opsonize the diffuse variant. Sera from rabbits immunized with the control "toxoid" lacking alpha hemolysin did not significantly promote phagocytosis. Sera obtained from rabbits immunized with alpha hemolysin toxoid showed a definite increase in opsonins for the diffuse variant in the fluid system employed, though it did not equal the brisk opsonization induced by sera obtained from animals immunized with diffuse variant heat-killed vaccine.

DISCUSSION

The current studies indicate that antitoxic and antibacterial factors can be clearly differentiated in the experimental mouse infection produced by the Smith diffuse staphylococcus. While immunization with toxoid prepared from crude culture supernatants protected animals against lethal doses of alpha hemolysin, it did not influence mortality induced by intraperitoneal infection with living staphylococci. Conversely, animals immunized with a heat-killed whole cell vaccine were readily killed with culture supernates rich in alpha hemolysin, but showed striking resistance to otherwise fatal intraperitoneal infection induced by the Smith diffuse variant. Studies on the dynamics of these intraperitoneal infections yielded an explanation for these differences. Immunization with toxoid did not promote early intraperitoneal phagocytosis and thus failed to prevent progressive extracellular multiplication and death. Previous experiments have shown that staphylococci are rapidly phagocytized in the peritoneal cavities of animals receiving Smith diffuse whole cell vaccines (13, 14).

Studies here and elsewhere have suggested that mice dying of infections produced by the Smith diffuse variant are killed by *in vivo* elaboration of alpha hemolysin (13, 16). The manner in which toxoid immunized mice died following infection with this strain closely resembled that seen in non-immunized infected mice or normal mice receiving alpha hemolysin. This apparent paradox appears to be explained by the current observation that antihemolysin disappears from the serum, and free alpha hemolysin can be found at the time of death in the peritoneal fluid of toxoid-immunized animals. Thus it appears that actively multiplying extracellular staphylococci eventually bind all available antibody, and antitoxic immunity is overwhelmed during progressive infection.

While these studies do not resolve all questions regarding the importance of antitoxic or antibacterial immunity, they do suggest possible explanations for some of the conflicting results. It seems likely that the protection against infection noted in toxoid immunized animals in certain past experiments may have resulted from the administration of significant amounts of preformed alpha hemolysin with the living bacteria at time of challenge (12, 17). Further, the present *in vitro* studies indicating that crude alpha hemolysin toxoid can induce opsonizing antibody suggests that some favorable results following toxoid administration may be explained by their capacity to evoke opsonins rather than antitoxin *per se*. Studies with purified alpha hemolysin (18, 19) may help to settle this problem.

It is now apparent that the Smith diffuse variant, and perhaps certain other staphylococcal strains, possess a surface antigen or antigens retarding phagocytosis (13, 14, 20). Indirect evidence suggests that similar antigens may be produced during *in vivo* growth of other more common strains (14, 21). In the present system, antibacterial immunity which prevented the *initiation* of staphylococcal disease was of more importance in protection than antitoxic immunity directed against an extracellular product of staphylococci produced after infection had been established. Further studies with better characterized substances such as the Smith surface antigen described by Morse (20) may define the role of the cell surface in the *in vivo* virulence of other strains of staphylococci. Such studies might also clarify the part played by antibody directed against these surface antigens in immunity to staphylococcal infection.

SUMMARY

Antitoxic and antibacterial immunity have been clearly differentiated in the experimental mouse infection produced by the diffuse colonial variant of the Smith strain of *Staphylococcus aureus*.

Immunization with crude toxoid protected mice from otherwise lethal doses of alpha hemolysin, but did not alter mortality following intraperitoneal infection with living staphylococci. Conversely, animals immunized with heat killed vaccines were readily killed by culture supernates containing alpha hemolysin, but were strikingly protected from otherwise fatal intraperitoneal infection with viable staphylococci.

Protection was directly related to the ability of the immunizing substance to promote early intraperitoneal phagocytosis of the infecting inoculum. In these studies with the Smith diffuse variant, rapid intraperitoneal phagocytosis was induced by vaccination with whole cell bacterial vaccines but not by alpha hemolysin toxoid.

The technical assistance of Miss Sandra Pickens is gratefully acknowledged.

BIBLIOGRAPHY

1. Burnet, F. M., The exotoxins of *Staphylococcus aureus*, *J. Path. and Bact.*, 1929, **32,** 717.
2. Kitching, J. S., and Farrell, L. N., Staphylococcal immunity, *Am. J. Hyg.*, 1936, **24,** 268.
3. Downie, A. W., A comparison of the value of heat killed vaccines and toxoid as immunising agents against experimental staphylococcal infection in the rabbit, *J. Path. and Bact.*, 1937, **44,** 573.
4. Smith, M. L., Circulating antitoxin and resistance to experimental infection with staphylococci, *J. Path. and Bact.*, 1937, **45,** 305.
5. Forssman, J., Studies in staphylococci. IV. Staphylococcus immunity, *Acta Path. et Microbiol. Scand.*, 1935, **12,** 536.
6. Forssman, J., Studies in staphylococci. VII. On active immunity, against staphylococcal infections and its relation to known antibodies against staphylococci or products of these bacteria, *Acta Path. et Microbiol. Scand.*, 1936, **13,** 459.
7. Forssman, J., Studies in staphylococci. IX. On the mechanism in staphylococcal infection and immunity, *Acta Path. et Microbiol. Scand.*, 1937, **14,** 468.
8. Forssman, J., Studies in staphylococci. XIII. A further contribution to the understanding of the immunity to staphylococci, *Acta Path. et Microbiol. Scand.*, 1938, **15,** 396.
9. Lyons, C., Antibacterial immunity to *Staphylococcus pyogenes*, *Brit. J. Exp. Path.*, 1937, **18,** 411.
10. Flaum, A., Studies in staphylococci and staphylococcal immunity, *Acta Path. et Microbiol. Scand.*, 1938, suppl. **35,** 1.
11. Cowan, S. T., Staphylococcal infection in rabbits: antibacterial and non-specific immunity, *J. Path. and Bact.*, 1939, **48,** 545.
12. Stamp, Lord, Antibacterial immunity to *Staphylococcus pyogenes* in rabbits, *Brit. J. Exp. Path.*, 1961, **42,** 30.
13. Koenig, M. G., Factors relating to the virulence of staphylococci. I. Comparative studies on two colonial variants, *Yale J. Biol. and Med.*, 1962, **34,** 537.
14. Koenig, M. G., Melly, M. A., and Rogers, D. E., Factors relating to the virulence of staphylococci. II. Observations on four mouse pathogenic strains, *J. Exp. Med.*, 1962, **116,** 589.
15. Rogers, D. E., and Melly, M. A., Observations on the immunology of pathogenic staphylococci, *Yale J. Biol. and Med.*, 1962, **34,** 560.
16. Cohn, Z. A., Determinants of infection in the peritoneal cavity. I. The response to and fate of *Staphylococcus aureus* and *Staphylococcus albus* in the mouse, *Yale J. Biol. and Med.*, 1962, **35,** 12.

17. Johnson, J. E., Cluff, L. E., and Goshi, K., Studies on the pathogenesis of staphylococcal infections. I. The effect of repeated skin infections, *J. Exp. Med.*, 1961, **113,** 235.
18. Kumar, S., and Lindorfer, R. K., The characterization of staphylococcal toxins. I. The electrophoretic migration of the alpha hemolytic, dermonecrotic, lethal, and leucocidal activities of crude toxin, *J. Exp. Med.*, 1962, **115,** 1095.
19. Kumar, S., Loken, K. I., Kenyon, A. J., and Lindorfer, R. K., The characterization of staphylococcal toxins. II. The isolation and characterization of a homogenous staphylococcal protein possessing alpha hemolytic, dermonecrotic, lethal, and leucocidal activities, *J. Exp. Med.*, 1962, **115,** 1107.
20. Morse, S. I., Isolation and properties of a surface antigen of *Staphylococcus aureus*. *J. Exp. Med.*, 1962, **115,** 295.
21. Rogers, D. E., Staphylococci and man, *J. Am. Med. Assn.*, 1962, **181,** 38.