JOURNAL OF BACTERIOLOGY, Nov. 1968, p. 1540-1545
Vol. 96, No. 5

Antibody Response to *Staphylococcus aureus* in Rabbits: Sequence of Immunoglobulin Synthesis and Its Correlation with Passive Protection in Mice

KOSAKU YOSHIDA[1] AND RICHARD D. EKSTEDT

Department of Microbiology, Northwestern University Medical School, Chicago, Illinois 60611

Received for publication 16 August 1968

Antibody in hyperimmune rabbit antisera specific for *Staphylococcus aureus* teichoic acid was shown to be associated with the IgM fraction. Treatment of such sera with mercaptoethanol destroyed its activity in passive mouse protection tests, whereas absorption with antirabbit IgG had no effect. Antibody response in normal rabbits immunized by a single or by three daily injections of a killed vaccine of *S. aureus* was followed by a sensitive passive hemagglutination test. Antibody detected during the "primary" response was completely susceptible to reduction with mercaptoethanol. Most of the antibody detected after a secondary antigenic stimulation at 10 weeks was also susceptible to mercaptoethanol. The antibody titers correlated well with mouse protective activity, and this activity of the serum was also shown to be associated with the IgM fraction.

Previous studies in this laboratory (3, 4) have shown that hyperimmune rabbit antisera prepared against heat-killed vaccines of the Smith diffuse strain of *Staphylococcus aureus* will protect mice against challenge with the homologous organisms in appropriate passive protection experiments. It was further shown that absorption of such sera with teichoic acid, extracted from ribonuclease-, trypsin-, and deoxyribonuclease-digested cell walls and purified according to the method of Sanderson, Strominger, and Nathenson (11), would remove the protective antibody.

Experiments reported here have shown that the antiteichoic acid antibody is associated with a persisting IgM antibody in these hyperimmune sera. In view of these results, we examined the immune response of rabbits to immunization with a killed staphylococcal vaccine. We investigated the sequence of immunoglobulin synthesis in relation to the protective activity of the sera against experimental staphylococcal infection in mice. This report describes the results of these studies.

MATERIALS AND METHODS

Preparation of vaccine. The Smith diffuse strain of *S. aureus* was used throughout these experiments. It was obtained from M. G. Koenig, Vanderbilt University Medical School, Nashville, Tenn. The culture was maintained by monthly transfer on Brain Heart Infusion (BHI) agar slants (Difco). At each transfer, the organisms were inoculated into BHI serum-soft agar (5) to confirm the "diffuse" type growth characteristic of this strain. For vaccine preparation, the organisms were subcultured into 100 ml of BHI broth and incubated at 37 C for 18 hr. After incubation, the cells were harvested, washed twice with cold, sterile saline (0.85% NaCl), adjusted turbidimetrically to contain 1 to 3 $\times$ 10^9 colony-forming units, and autoclaved at 121 C for 15 min.

Immunization schedule. Normal adult Flemish Cross rabbits, obtained from a local supplier and weighing between 5 to 8 lb initially, were injected intravenously on 3 successive days each week for 3 weeks. The first week, the animals received 0.5 ml of vaccine at each injection; the second and third weeks, the dose was increased to 1.0 ml per injection. Between 10 and 14 days after the ninth injection, the animals were bled and the serum was separated, dispensed in amounts of 2 to 3 ml in small screw-cap vials, and stored at -20 C until used.

In order to study the antibody response to the staphylococcal vaccine, two groups of two rabbits each were used. The first group received a single intravenous injection of 1.0 ml of the vaccine; the second received three 1.0-ml injections on 3 successive days. All animals were bled for control serum prior to immunization. After their last immunization injection the animals were bled at weekly intervals for a period of 18 weeks. At 10 weeks, both

[1] Present address: Department of Bacteriology, Nippon Medical School, Tokyo, Japan.

groups of animals were given a single intravenous booster injection of 1.0 ml of the same vaccine. At each bleeding, approximately 10.0 ml of blood was taken and the sera were separated and stored.

Preparation of the erythrocyte-sensitizing antigen and the passive hemagglutination test. The staphylococcal antigen used to sensitize normal rabbit erythrocytes was prepared by a modification of the method originally described by Yoshida et al. (14). Figure 1 summarizes the steps used in its preparation. The hemagglutination test was performed as previously described. (14).

Mercaptoethanol treatment and IgG absorption of the sera. Samples of serum (1 ml) were mixed with equal volumes of 0.2 M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.) and allowed to react at 4 C for 18 hr. Each sample was then dialyzed against 0.02 M iodoacetic acid in phosphate-buffered saline for 2 hr and in fresh PBS for an additional 24 hr at 4 C. Control sera were treated in the same manner, except that PBS was used in place of the mercaptoethanol.

In order to remove the IgG from whole rabbit antisera, 1.0 ml of serum was mixed with an equal volume of goat antirabbit IgG (lot no. 866, Immunology, Inc., Glen Ellyn, Ill.) and allowed to react at 4 C for 18 hr. The resulting precipitate was removed by centrifugation, and the supernatant fluid was stored at −20 C. Immunoelectrophoresis of the absorbed sera confirmed the specific removal of the IgG fraction.

Sucrose density gradient fractionation of the sera. Some serum samples were centrifuged through 5.0 ml of 10 to 40% sucrose gradients prepared in PBS at 86,000 × *g* for 18 hr in a model L2 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) with the SW-39 head. Fractions of approximately 0.2 ml were then collected and diluted to 1.0 ml with 10% sucrose; the optical densities were read at 280 nm in a Zeiss PMQ II spectrophotometer.

Passive protection tests. Adult, male mice (CF 1 strain, Carworth Farms, New York, N.Y.) weighing 18 to 25 g were used. The protective activity of the sera was determined as previously described (2, 4). In testing the IgM and IgG fractions for mouse protection, the mucin challenge method of Parker et al. (10) was used, as modified in this laboratory (13). The animals were then observed and deaths were recorded for 2 weeks, at which time the experiments were terminated.

Immunoelectrophoresis. Immunoelectrophoretic analyses were carried out with agar (Difco), at a concentration of 1.5%, prepared in Barbitone-Acetate buffer (Consolidated Laboratories, Chicago Heights, Ill., *p*H 8.6 at 4.4 g per liter). After electrophoretic separation, goat antirabbit whole serum (Immunology, Inc., Glen Ellyn, Ill.) and purified teichoic acid (30 and 100 gμ/ml, respectively) were added to the appropriate troughs. Diffusion was allowed to continue at room temperature for 24 to 48 hr, after which the bands of precipitate were photographed unstained by use of an immunodiffusion camera (Cordis Corp., Miami, Fla.) and Polaroid ASA-3000 film.

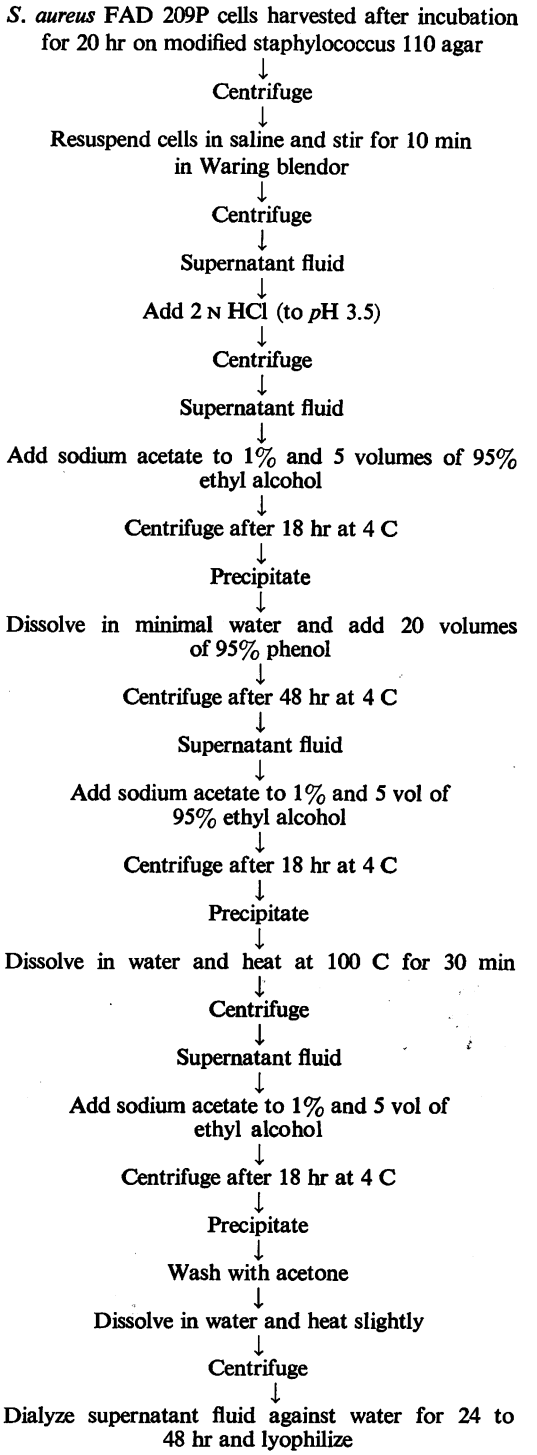

FIG. 1. *Outline of the procedure for preparation of S. aureus erythrocyte-sensitizing antigen used in passive hemagglutination tests.*

Protein was determined by the method of Lowry et al. (8), with crystalline bovine serum albumin (Cohn Fr. II, Armour Pharmaceutical Co., Chicago, Ill.) as the standard.

RESULTS

Rabbit antiserum hyperimmune to S. aureus. Analysis of hyperimmune sera from several rabbits immunized by repeated intravenous injections of an autoclaved saline suspension of *S. aureus* indicated that the antibody specific for the teichoic acid antigen was localized in the IgM fraction. Figure 2 shows an immunoelectrophoretic analysis of a typical serum of this kind. Whereas most samples of sera taken from animals after a single 3-week course of immunization showed similar results, an occasional serum taken from animals that had undergone repeated courses of immunization over a period of several months also showed IgG antibody specific for teichoic acid. Normal rabbit sera never showed a reaction under the conditions used.

Since sera taken after one or multiple courses of immunization all showed strong protection against experimental staphylococcal infection with the homologous strain of *S. aureus*, it was of interest to determine the effect on protective activity of treating such sera with mercaptoethanol. In addition, absorption of protective antisera with specific goat antirabbit IgG was done to determine the effect on protective activity of removal of this fraction. Table 1 shows the results of a typical mouse passive protection experiment in which the challenge organisms were preopsonized in saline suspension with untreated, mercaptoethanol-treated, and IgG-absorbed homologous antiserum prior to injection. It is clear that, whereas IgG absorption had no effect, treatment with mercaptoethanol significantly decreased the protective activity of the antiserum.

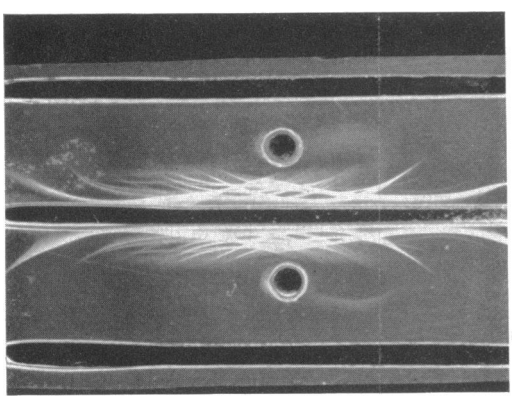

FIG. 2. *Immunoelectrophoretic pattern of hyperimmune rabbit antiserum against S. aureus (Smith diffuse). Wells: antiserum, undiluted. Center trough: goat antirabbit serum. Upper and lower troughs: S. aureus (Smith diffuse) teichoic acid; 100 and 30 ug/ml, respectively. Anode is to the left.*

TABLE 1. *Effect of mercaptoethanol and IgG absorption on mouse passive protective activity of hyperimmune homologous rabbit antiserum*

Serum treatment	Mortality of animals challenged with preopsonized organisms	
	No. dead/no. challenged	Per cent mortality
None	3/20	15
0.2 M mercaptoethanol plus iodoacetamide	12/20	60
Antirabbit IgG	1/20	5
Normal serum (control)	17/20	85

Antibody response to immunization with S. aureus. Although a number of studies have been reported in recent years describing the sequence of specific immunoglobulin synthesis in animals injected with bacteriophage (12) or with classical antigens such as bovine gamma-globulin or hemocyanin (1), no comparable studies have been reported of the synthesis of protective antibody against an experimental bacterial infection.

With the development of a sensitive hemagglutination method using a staphylococcal antigen described by Yoshida et al. (14), it has been possible to study in rabbits the immune response to minimal antigenic stimulation with a killed vaccine of *S. aureus*. The sequence of immunoglobulin synthesis in response to the vaccine and the development of mouse protective antibody were followed. Figures 3-R31 and 3-R23 show the antibody titers observed in two rabbits that were given a single intravenous injection of approximately 10^9 killed *S. aureus* cells in saline suspension. These animals were given a single booster injection of the same vaccine 10 weeks after the first injection. The effect on the hemagglutination titers of treatment of the sera with mercaptoethanol is also shown. Figures 3-R28 and 3-R27 show the titers in two rabbits that were given three intravenous injections of the same vaccine on successive days and the effect of mercaptoethanol on these titers. In both groups of animals, the titers reached a peak 2 weeks after the immunizing injections, declined over the next 2 to 4 weeks, and stabilized at a level above the preimmunization titers through the initial 10-week period. The hemagglutinating activity of the sera from these bleedings was destroyed by treatment

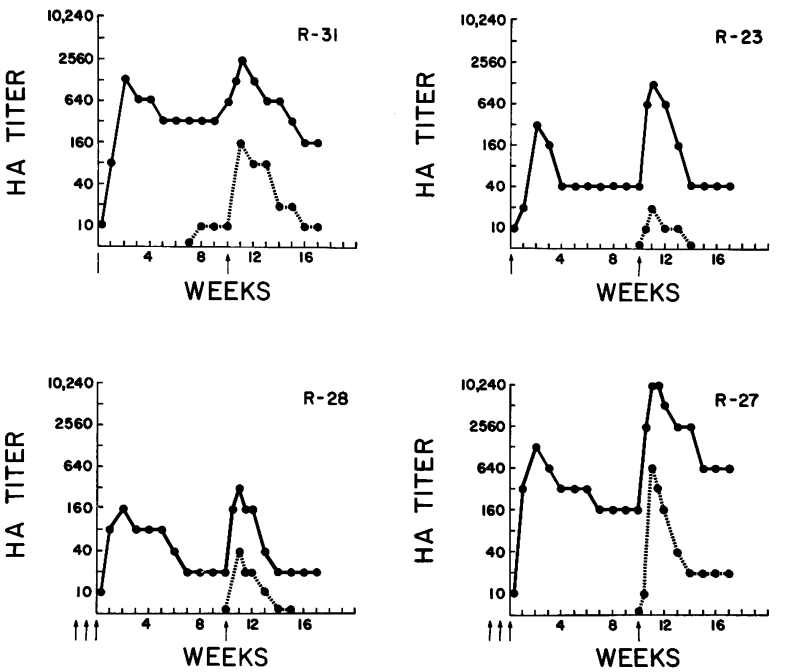

FIG. 3. *Hemagglutination titers of serum from rabbits immunized with S. aureus (Smith diffuse) killed vaccine. R-31 and R-23: single immunizing injection, 10 week booster. R-28 and R-27: three daily immunizing injections, 10 week booster. Symbols: solid lines, untreated serum; broken lines, mercaptoethanol-treated serum. Titers are expressed as reciprocal of serum dilution.*

with mercaptoethanol, suggesting that the antibody was of the IgM class. After the booster injection, all animals showed a significant rise in titer 1 week later, which again declined over the next 4 weeks to the level observed after the initial immunization. Treatment of the sera from this period with mercaptoethanol, however, showed that at least part of the antibody was resistant and probably of the IgG class.

Serum obtained each week from rabbit no. 27 was tested for its mouse protective activity by the preopsonization of various dilutions of saline-suspended homologous organisms (Table 2). It is clear that the hemagglutination titers and the passive protection activity of the sera correlated well. Serum taken at 2 weeks could be diluted 1:30 and still show protective activity, whereas serum taken at 11 weeks was still active at a dilution of 1:100. Intermediate sera showing lower titers were less effective in protecting mice against challenge infection with the homologous strain of *S. aureus*.

Sera taken at 2 weeks and at 11 weeks from rabbit no. 27 were also subjected to sucrose density gradient fractionation, and the distribution of the hemagglutination titers was determined (Fig. 4). The hemagglutination titer of the serum taken at the peak of the initial response was found completely in the IgM fraction. The serum taken 1 week after the booster injection of the vaccine also showed the majority of the titer in the IgM fraction, although a low level of hemag-

TABLE 2. *Mouse passive protective activity of sera taken from rabbit no. 27 at weekly intervals after immunization with S. aureus*

Weeks after immunization	Reciprocal HA titer	No. dead/no. challenged at various dilutions of serum used to preopsonize organisms			
		1:10	1:30	1:100	1:300
0[a]	10	10/10	10/10	9/10	9/10
1	320	5/5			
2	1,280	0/5	1/5	4/5	5/5
3	640	0/5	2/5	3/5	5/5
4	320	2/5	4/5		
6	320	4/5			
8	160	4/5			
10[b]	320	5/5			
11	10,240	0/5	1/5	0/5	3/5
12	5,120	0/5	0/5	1/5	5/5
13	2,560	1/5	3/5	5/5	
14	2,560	3/5	5/5		
16	640	4/5			

[a] Control.
[b] Booster

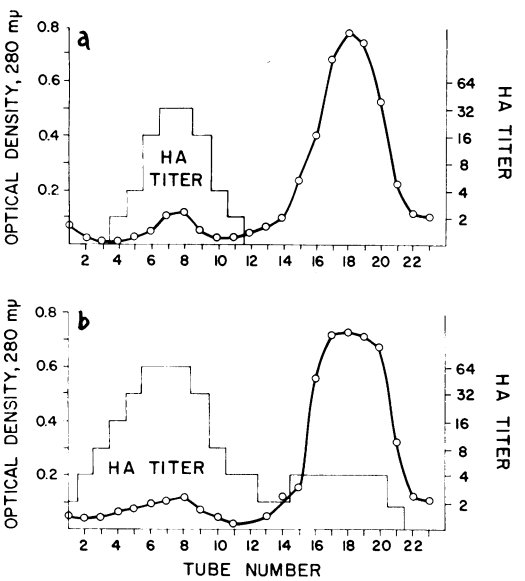

FIG. 4. *Density gradient fractionation and hemagglutination titers of serum from rabbit no. 27. (a), Serum taken at 2 weeks; (b), serum taken at 11 weeks. Titers are expressed as reciprocal of serum dilution.*

glutinating activity was also found in the IgG fraction.

The passive protection activity of the IgM and IgG fractions obtained by sucrose density gradient centrifugation of serum taken at 11 weeks from rabbit no. 27 was also studied. Pooled fractions from tubes 3 through 11 and from tubes 15 through 22 were concentrated by ultrafiltration through collodion membranes of <5 nm porosity (Schleicher & Schuell, Keene, N.H.), and the protein concentration was determined. Groups of mice were treated with either 50 or 16.7 μg of protein of either the IgM or IgG fractions and challenged within 30 min with the Smith diffuse strain of *S. aureus* that was grown on modified 110 agar and suspended in 5% hog gastric mucin at a dose of approximately 10^2 colony-forming units per ml (13). The protective activity of the serum was located in the IgM fraction (Table 3). In other experiments, as much as 70 μg of the IgG fraction showed no protective activity under the same conditions.

TABLE 3. *Mouse passive protective activity of immunoglobulins separated by sucrose density gradient centrifugation of serum taken from rabbit no. 27 at 11 weeks*

Treatment given to normal mice[a]	Mortality of animals challenged with *S. aureus* (Smith diffuse) in mucin[b]	
	No. dead/no. challenged	Per cent mortality
IgM, 50 μg	1/9	11
IgM, 16.7 μg	2/16	12.5
IgG, 50 μg	9/10	90
IgG, 16.7 μg	9/10	90
Control (untreated)	18/20	90

[a] Injected intraperitoneally <30 min before challenge with 0.25 ml of fraction in PBS.

[b] Animals were challenged intraperitoneally with organisms grown in modified staphylococcus 110 medium mixed with 5% mucin at a dose of approximately 10^2 organisms per 0.5 ml.

DISCUSSION

Because most of our earlier studies (3, 4) on immunity to staphylococcal infection in mice had been directed toward elucidating the protection-inducing antigens of these organisms, we investigated the antibody response of animals to immunization with *S. aureus*. In view of our earlier results, the antibody response to the teichoic acid antigen was of particular interest.

Rabbits that had been hyperimmunized with a killed staphylococcal vaccine show a persisting IgM antibody response specific for teichoic acid. Treatment of such sera with mercaptoethanol and iodoacetamide reduced its mouse passive protective activity significantly, whereas specific absorption of the IgG had no effect. The localization of teichoic acid antibody in the IgM fraction of these sera was not particularly surprising, because it is well known that many polysaccharide antigens tend to induce a persisting IgM response (7).

As an extension of these studies, the antibody response of normal rabbits injected with minimal doses of killed staphylococcal vaccine was also followed by use of a sensitive passive hemagglutination test. The response detected in this manner correlated well with the mouse protective activity of the sera taken at various times after immunization. It should be made clear, however, that the antigen used in the hemagglutination assays, although shown to be contaminated with teichoic acid (*unpublished data*), was not dependent on its content of teichoic acid for its erythrocyte-sensitizing activity. The hemagglutination titer of antisera absorbed with purified teichoic acid remained unchanged. Several attempts have been made without success in this laboratory to sensitize normal, tanned, or glutaraldehyde-treated sheep, rabbit, and human erythrocytes with purified teichoic acid. Oeding (9) reported similar findings.

The correlation between the hemagglutination titer and mouse protective activity of the sera

from immunized rabbits would appear to be fortuitous. It is conceivable that, since the erythrocyte-sensitizing antigen and the teichoic acid antigen of *S. aureus* both are polysaccharide in nature, the immune response in rabbits to both antigens would parallel each other.

The antibody observed during the primary response was completely susceptible to reduction with mercaptoethanol, whereas low levels of mercaptoethanol-resistant antibody were detected during the secondary response. The titers attained in the secondary response were in general higher than in the primary response, as might be expected, but they dropped again in 2 to 3 weeks to approximately the same levels that were seen after the primary response and during the 10-week interval prior to the secondary stimulation. In other words, the persistence of a higher titer for a longer period of time in the secondary response was not seen in this system. Whether this represents a lack of memory in restimulated animals as a result of their having undergone an exclusively IgM response after initial stimulation, as has been demonstrated in other systems (12), remains to be determined.

Density gradient centrifugation of sera taken at the peak of the primary and secondary responses at 2 and 11 weeks, when tested by hemagglutination, showed the major portion of the titer to be associated with the faster sedimenting fraction in both cases. Since IgM antibody is known to be considerably more efficient than IgG in hemagglutination reactions (6), these results could be a reflection of the method used to assay the sera.

Of even greater interest was the observation that serum taken at the peak of the secondary response, when separated by gradient centrifugation and tested in passive mouse protection tests, showed that the protective antibody was again localized in the IgM fraction. To our knowledge, this is the first demonstration of this phenomenon reported in this host-parasite system. It is conceivable that, in addition to being more efficient in hemagglutination and complement fixation, IgM antibody is also more efficient in opsonization of staphylococci in the experimental system described.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-07716 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Bauer, D. C., and A. B. Stavitsky. 1961. On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens. Proc. Natl. Acad. Sci. U.S., **47**:1667–1680.
2. Cohn, Z. A. 1962. Determinants of infection in the peritoneal cavity. I. Response to and fate of *Staphylococcus aureus* and *Staphylococcus albus* in the mouse. Yale J. Biol. Med. **35**:12–28.
3. Ekstedt, R. D. 1965. Mechanisms of resistance to staphylococcal infection: natural and acquired. Ann. N.Y. Acad. Sci. **128**:301–334.
4. Ekstedt, R. D. 1966. Studies on immunity to staphylococcal infection in mice. IV. The role of specific and nonspecific immunity. J. Infect. Diseases **116**:514–522.
5. Finkelstein, R. A., and S. E. Sulkin. 1958. Characteristics of coagulase positive and coagulase negative staphylococci in serum-soft agar. J. Bacteriol. **75**:339–344.
6. Grey, H. M. 1964. Studies on changes in the quality of rabbit-bovine serum albumin antibody following immunization. Immunology **7**: 82–90.
7. Lospalluto, J., W. Miller, B. Dorward, and C. W. Fink. 1962. The formation of macroglobulin antibodies. I. Studies on adult humans. J. Clin. Invest. **41**:1415–1421.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
9. Oeding, P. 1965. Antigenic properties of staphylococci. Ann. N.Y. Acad. Sci. **128**:183–189.
10. Parker, S. E., P. Warner, and W. K. Slipetz. 1966. Estimation of virulence of *Staphylococcus aureus* for mice: the exceptional virulence of the Smith strain. Can. J. Microbiol. **12**:753–762.
11. Sanderson, A. R., J. L. Strominger, and S. G. Nathenson. 1962. Chemical structure of teichoic acid from *Staphylococcus aureus*, strain Copenhagen. J. Biol. Chem. **237**:3603–3613.
12. Uhr, J. W. 1964. The heterogeneity of the immune response. Science **145**:457–464.
13. Yoshida, K., and R. D. Ekstedt. 1968. Relation of mucoid growth of *Staphylococcus aureus* to clumping factor reaction, morphology in serum-soft agar, and virulence. J. Bacteriol. **96**:902–908.
14. Yoshida, K., S. Mizunari, and H. Hattori. 1964. Immunological studies on "surface antigen" extracted from *Staphylococcus aureus*, I. Isolation of the antigen and properties of the immune rabbit serum. Japan. J. Microbiol. **8**: 67–74.