Detection and Titration of *Staphylococcus aureus* Agglutinin in Serum.* (25935)

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During studies to demonstrate alteration of metabolism of recently isolated virulent strains of *S. aureus* in presence of serum, an interesting sedimentation pattern suggestive of agglutination was observed. Quantitative differences in capacity of various sera to produce this distinctive agglutination pattern were noted, hence the phenomenon seemed worthy of study. This report presents (a) a description of this phenomenon and its quantitation, (b) evidence supporting specificity of agglutination reaction, (c) staphylococcal agglutinin titers of sera from different species, (d) agglutinin titers of pooled human gamma globulin samples and (e) preliminary data on serologic responses of rabbits and humans to vaccination with an *S. aureus* vaccine.

Materials and methods. *S. aureus* antigen is prepared by inoculating 100 ml of trypticase soy broth (TSB) with a loopful of cells from a trypticase soy agar slant that had been incubated for 16-18 hours at 37°C. The broth culture is incubated for 5 hours at 37°C, then distributed in 1.0 ml amounts into rubber-stoppered vials and stored at −20°C. The standard antigen dilution used is initially determined by pooling the contents of 2 vials and preparing serial 10-fold dilutions in TSB. One-tenth ml of each dilution is added to ten 13 x 100 mm tubes followed by 1.1 ml of TSB. Cultures are incubated overnight at 37°C and antigen titer recorded as the reciprocal of the 50% viability endpoint (VE_{50}) according to the method of Reed and Muench(1). One hundred VE_{50} is the antigen dilution used to determine agglutination response. Once standardized in this manner, titers of stock antigens remain unchanged at −20°C for periods of at least 5 months. In determining agglutination response, the appropriate antigen dilution is made in TSB from pooled contents of 2 vials and viability (VE_{50}) titer confirmed in each test. The strain of *S. aureus* selected as antigen is apparently not critical since a number of *S. aureus* isolates (serotypes I and III) have been used as antigens with good agreement as to agglutinin titers determined. Recently, an antibiotic resistant strain of *S. aureus,* readily cultivated in TSB containing 100 units of penicillin and 100 μg of streptomycin per ml, has been employed as antigen. Use of this organism facilitates the procedure by lessening risk of contamination as test tubes stand open while reagents are delivered with automatic pipettes. Each serum is heated at 56°C for 30 minutes preparatory to testing. Serial 2-fold dilutions (usually starting at 1:100) of serum are made in TSB. One-tenth ml of each serum dilution is placed in a 13 x 100 mm test tube and 0.1 ml of antigen added. Mixtures are shaken and incubated for 1 hour at room temperature after which time 1.0 ml of TSB is added to each tube. For purposes of control, the titer of a standard pool of human serum is determined each time. Variations in titer of the control serum have not been observed in excess of 2-fold on different days. Mixtures are incubated for 16-18 hours at 37°C, then read by inspection. The ease with which the end-point is distinguished is illustrated in Fig. 1. The agglutinin titer is expressed as the reciprocal of highest dilution of serum causing complete agglutination of the antigen.

Results. *Antigen specificity.* To validate the specificity of this reaction, a number of adsorption procedures were carried out. If the reaction is specific, adsorption of antibodies by cells of *S. aureus* should cause a quantitative decrease in titer of adsorbed serum. Accordingly, a cell suspension was prepared and aliquots were mixed with (a) human serum; (b) a prepared pool of commercial human gamma globulin; and (c) rabbit serum, from animals in which antibody for-

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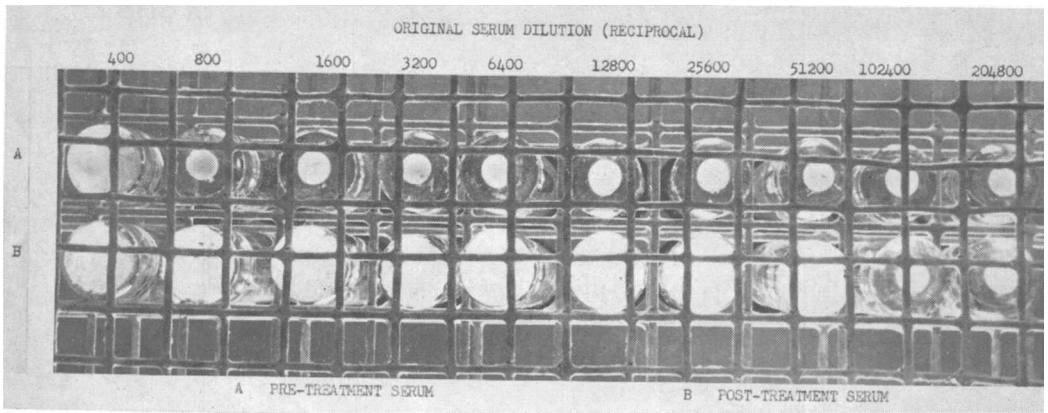

FIG. 1. Agglutination pattern of *S. aureus* in presence of rabbit serum.

mation had been induced by injection with antigen. These mixtures were placed in a refrigerator at –4°C and allowed to stand overnight. Cells were removed by Seitz filtration and sera tested for agglutination capacity by the procedure described above. Reduction of titers, 8 to 32 fold, indicates that adsorption occurred. Adsorption tests carried out with aliquots of the same sera and *S. albus* cells showing no reduction in titer offer further evidence of specificity of this reaction. Use of *S. albus* as antigen in the agglutination test was not successful. The distinctive sedimentation pattern was not observed: all tubes in the series had the same type of diffuse growth.

Agglutinin titers of animal sera. It has been shown in the past that certain animal sera show antibodies to staphylococci, hence it seemed purposeful to determine whether agglutinin titers could be demonstrated in randomly selected animal sera† by this test. Table I indicates presence of measurable agglutinin titers and also a wide range of titers within each species. It is open to speculation whether these titers reflect differences in exposure to staphylococcal infection or differences in capacity to form antibodies. Similarly there is a range of titers in human sera, and preliminary data indicate no difference in titers between persons who have been free of recognized staphylococcal infection for long periods and those who have had recent skin infections due to staphylococci. Of particular interest was the specimen of human serum which had a titer of less than 200. This serum was submitted as an unknown‡ but after decoding it was found that this specimen had been obtained from a patient with agammaglobulinemia. In light of this observation it is intriguing to speculate about the cause of the value of less than 200 in the specimen of goat serum.

Agglutinin titers in human gamma globulin: The finding of insignificant titers of agglutinin in the specimen of agammaglobulinemic serum suggested the testing of pooled human gamma globulin. Use of a living staphylococcus as the antigen made it requisite to obtain gamma globulin specimens prior to addition of preservatives.§ Variation in titer of agglutinins in the various lots is shown in Table I. These values so distinctly higher than those encountered in single samples of human sera that have been tested, suggested that the agglutinins measured are concentrated in the fraction of blood containing the presently recognized antibodies, thus indirectly supporting the view that it is an antibody that is being titered.

Serologic response of rabbits to treatment with staphylococcal vaccine. Rabbits naturally have agglutinin titers as measured by

† The authors wish to acknowledge their thanks to Samuel V. Scheidy, D.V.M., Univ. of Pennsylvania School of Vet. Med. for these specimens.

‡ Kindly forwarded to us by George G. Jackson, M. D., Univ. of Illinois, Chicago.

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TABLE I. Staphylococcal Agglutinin Titers of Sera of Various Species.

Agglutinin titer (reciprocal)	Rabbit	Horse	Cat	Goat	Cow	Sheep	Man	
							Sera	γ-globulin
>409600			1	1	1			
409600								
204800			1					2
102400					1	1		1
51200		2		1	2			
25600				1		1	3	
12800		1	1				3	2
6400		2	1		1	2	17	
3200	1	1			1		11	1
1600	2					1	17	
800	4						5	
400	4			2		1	2	
200								
<200				1			1	

this test (Table I). It seemed of interest to determine whether these titers could be increased by treatment of rabbits with a staphylococcal vaccine. Accordingly 4 albino rabbits were bled, then treated intravenously with a series of injections of bacteriophage-lysed *S. aureus* vaccine. In graded doses at intervals of approximately 4 days over a period of 33 days each rabbit received 80 ml of staphylococcal preparation. Post-treatment sera were obtained from the rabbits one week after completion of treatment. All serologic responses were determined in a single test and post-treatment sera showed a 2 to 64-fold increase in titer.

Serologic response of patients to vaccination. Twenty-one male patients, all of whom had suffered from recurrent furunculosis for a period of many months, were vaccinated subcutaneously with a bacteriophage-lysed *S. aureus* vaccine.‖ The arbitrary schedule of vaccination was 0.05 ml initially, 0.1 ml 4 days later and thereafter increments of 0.1 ml at 4-day intervals, up to 0.5 ml per injection. Treatment at this level was then continued weekly. Local reaction at sites of injection as minimal, the usual reaction being redness less than 3 cm in diameter, with little or no induration. Serum was obtained from each patient immediately before and 4 weeks after beginning of vaccination. Serologic responses of these patients were determined simultaneously in a single agglutination test (Fig. 2). Although there was a therapeutic response in all of these patients, only 13 of 21 patients (62%) responded with at least a 4-fold increase of titer. A therapeutic and serologic comparison of staphylococcal vaccines and toxoids will be presented later.

Discussion. It is well known that the staphylococcus is rich in antigenic substances although there is no ready means of determining the exact number(2). However, there has been a significant lack of evidence to correlate any of the demonstrated antibodies with protection or immunity against staphylococcal infection. Elek(2) pointedly emphasizes the shortcomings of previous work in correlating staphylococcal diseases of animals or man

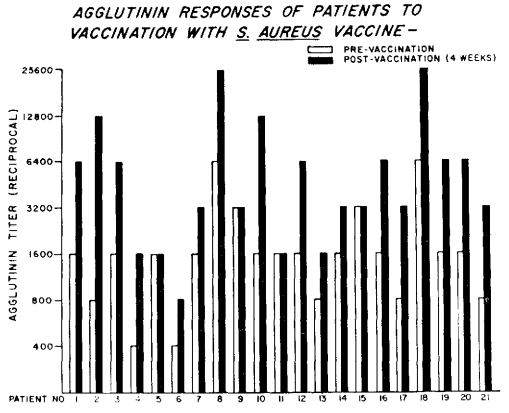

FIG. 2.

‖ This preparation was supplied by Delmont Labs., Swarthmore, Pa.

with the immunologic findings and states that "Staphylococci isolated from lesions are by no means always agglutinated by the patient's serum and the demonstration of agglutinins is of no clinical importance." Without in any way wishing to challenge this statement this paper presents what we believe to be a new,** technically simple and exquisitely sensitive agglutination test, accidentally discovered in our laboratory.

The basis of the test described here is alteration of the characteristic growth pattern of coagulase-positive staphylococci in fluid medium in presence of serum from either human or animal source. It would appear that the agent responsible for this alteration of growth possesses certain generally accepted properties of antibody: a) stability in serum heated at 56°C for 30 minutes; b) semi-quantitative removal by adsorption with homologous (coagulase-positive) but not with heterologous (coagulase-negative) staphylococci; and c) an increase in titer following treatment with a staphylococcal vaccine.

The antibody measured appears to be present naturally in sera of animals and man in varying amounts. Since staphylococci are ubiquitous and there is increasing documentation of staphylococcal infections in animals it is suggested that the antibodies reflect previous experience with this organism. This statement is not necessarily in conflict with the common failure of anti-hemolysin, anti-leucocidin or agglutinin titers to distinguish patients who have staphylococcal infection. This seeming paradox may be due to the unique histopathology of staphylococcal infections.

It has, however, been possible to show an increase of titer in rabbits and in man following treatment with a staphylococcal vaccine. The vaccine employed was a bacteriophage-lysed preparation without preservative and certainly contained a) components of culture medium, b) metabolites of staphylococci, c) soluble residues of lysis of cell walls and d) living bacteriophage particles. It has been impossible to characterize the specific antigen or antibody controlling the altered agglutination pattern which is the basis for the test presented here. The serum factor does not appear to be an antibody to alpha hemolysin or bacteriophage.

The finding of insignificant amounts of the agglutinin in a single agammaglobulinemic serum and the variable, but high titers, of agglutinin in pooled, human gamma globulin support the concept that the factor we are measuring is found in that fraction of blood which contains all meaningful antibodies as we know them today. However, the antibody or agglutinin measured by this new test has not been studied extensively enough to permit an opinion as to clinical significance or its identity with a "protective" antibody. The agglutination test is so simple in its performance, so exquisite in sensitivity and reproducibility that it is presented for trial by other investigators.

Summary and conclusions. A technically simple agglutination test for detection and titration of an unidentified anti-staphylococcal factor, presumably antibody, in human and animal sera is described. By this test variable titers have been found to occur naturally in sera of animals and of man. Agglutinins are present in large amount in pooled human gamma globulin and in insignificant amounts in a single agammaglobulinemic serum. Titers of this factor can be increased by treatment of both animals and man with a staphylococcal vaccine. The suggestion is made that this factor is an antibody on the basis of a) its presence in antibody bearing fraction of blood b) its heat stability c) ability to adsorb it semiquantitatively with coagulase-positive staphylococci and d) ability to elevate titers in rabbits and man by use of staphylococcal vaccine as antigen. Preliminary observations do not enable us to identify the factor being measured by the agglutination test nor assess its clinical significance.

** We have not found any similar test in our search of the literature.

1. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

2. Elek, Stephen D., *Staphylococcus Pyogenes,* Livingstone Ltd., Edinburgh and London, 1959.

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