DAVID E. ROGERS*
MARIAN ANN MELLY**

Department of Medicine, Vanderbilt University, Nashville

OBSERVATIONS ON THE IMMUNOLOGY OF PATHOGENIC STAPHYLOCOCCI†

Many pathogenic strains of bacteria producing acute infection in man are characterized by capsules or surface factors which resist phagocytosis.[1] Differences in antigenic structure of these surface components have allowed separation of individual pathogenic strains and study of the role of specific antibody in resistance to, or recovery from, infections caused by these microbial species.

To date, no satisfactory serologic procedures have been available for differentiating individual strains of pathogenic staphylococci, and the role of humoral immunity in resistance to staphylococcal infection or the course of straphylococcal disease has remained uncertain.

In 1959, Cohn and Morse showed that certain pathogenic staphylococci resist phagocytosis.[2] In the majority of their studies the Smith staphylococcus was used as a prototype strain. Koenig has recently confirmed and extended these observations in an experimental mouse infection using two variants of the Smith strain.[3] These findings suggested that pathogenicity among staphylococci might relate to possession of specific surface or capsular factors analogous to those characterizing other pyogenic cocci. Subsequent studies showed that phagocytosis resisting components could be extracted from the Smith strain,[4] that specific antibody was required for rapid ingestion of the diffuse variant of the Smith staphylococcus,[2,3,5] that the phagocytosis promoting antibody was present in human sera,[5,6] and that antibody was not removed by absorption with other microorganisms or certain strains of staphylococci.[5]

Because of this suggestive specificity, experiments were undertaken to determine whether systems containing appropriate sera and living leukocytes would permit "typing" of pathogenic staphylococci in the same way the bacteriocidal test is used to detect type-specific antibody directed against Group A streptococci.[7,8]

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* Professor of Medicine.

** Instructor in Medicine.

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

Received for publication 21 December 1961.

The present paper reports certain observations on the immunologic characteristics of the diffuse variant of the Smith strain and other strains of staphylococci producing human infection. Studies on the prevalence and specificity of antistaphylococcal hemagglutinating antibody are included. The factors contained in human and vaccinated rabbit sera which promote phagocytosis of the Smith strain have been partially characterized. The serum factors required for phagocytosis of other strains of staphylococci have also been examined.

These studies indicate that antistaphylococcal hemagglutinating antibody is quite nonspecific, and hemagglutinin techniques do not possess promise for separating different pathogenic strains. Studies on the Smith diffuse strain demonstrate that both specific heat stable antibody and nonspecific heat labile factors are required for its phagocytosis in fluid systems. Fourteen other strains of staphylococci appear to differ in certain important ways from the Smith diffuse variant. These strains are less resistant to phagocytosis and *either* specific antibody or heat labile factors believed to be non-antibody in nature are capable of opsonizing these microorganisms.

MATERIALS AND METHODS

Cultures: *The Smith strain* of staphylococcus has been used as a prototype pathogenic strain in certain studies reported from this laboratory.[5] In the present experiments, the "diffuse" variant, described in a previous publication,[6] was substituted for the parent strain. There is much to suggest that the characteristics of the parent strain derive from the "diffuse" population. This variant is highly virulent for mice and grows in spreading, "diffuse" colonies in plasma soft agar preparations. In contrast, the "compact" variant, although coagulase positive, is relatively avirulent for mice and grows in discrete colonies in plasma soft agar preparations.[9] This variant was also used in certain studies and resembled other strains of coagulase positive staphylococci in its immunologic behavior.

Additional coagulase positive *Staphylococcus aureus* strains were recent isolates obtained from proven human staphylococcal infections.

Other cultures used included a Type 1 pneumococcus, a Type 4 Group A streptococcus, and *Bacillus subtilis,* all maintained in this laboratory.

Stock cultures of staphylococci were maintained on infusion agar slants stored at 4° C. Transplants made in beef heart infusion broth and incubated for 18 hours were used in phagocytic experiments. The pneumococcus and streptococcus were maintained in infusion broth containing five per cent rabbit or human blood.

Measurement of hemagglutinating antibody. Hemagglutinating antibody content of human or rabbit sera was measured using a technique previously described.[10] Sensitized washed sheep red blood cells were added to serially diluted test sera which had been absorbed with normal sheep red blood cells. Hemagglutination titres were read following low speed centrifugation after 40 minutes incubation at 37° C.

Immunization of rabbits. Microorganisms grown on infusion agar plates or in Todd Hewitt broth were harvested and pour plates were made for bacterial counts. After two washes in sterile saline, the bacterial mass was resuspended in appropriate amounts of saline and heated at 60° C. for two hours. The *B. subtilis* suspension was killed by the addition of 0.5 per cent formalin. Vaccines contained 10^{10} organisms per ml. and were checked for sterility before use.

Healthy adult albino rabbits* were immunized with intravenous injections of the heat killed bacterial cells because of the finding that this route promoted maximal antibody response.[10] Four 0.5 cc. intravenous injections were given at three to five day intervals. Immune serum was obtained by cardiac puncture no less than one week after the final injection. Animals used over long intervals of time were given periodic booster injections.

The phagocytic system. Human or rabbit leukocytes were obtained by dextran sedimentation techniques described in earlier studies.[5, 6] For most experiments, leukocytes were washed three times in Hanks' solution containing heparin 1:10,000, 0.1 per cent sterile bovine albumin, and 100 mg. per cent glucose. The cells were counted, then suspended in appropriate numbers in Hanks' solution containing 10 to 20 per cent of the serum under study. All manipulations were carried out in siliconed glassware. Staphylococci were added to the serum-leukocyte mixture in a ratio of one culturable microorganism per polymorphonuclear leukocyte. Tubes were sealed with sterile rubber stoppers and rotated at 7 RPM on an inclined wheel which promoted thorough mixing in a 37° C. incubator.

At intervals, samples were removed from each tube for coverslip smears, and in certain experiments, quantitative cultural determinations. Smears were stained with Wright's stain, 100 polymorphonuclear leukocytes were counted, and the percentage of polymorphonuclear cells participating in phagocytosis enumerated. Cultural determinations were performed according to the technique originally devised by Maaløe[11] used in previous

* Eldridge Rabbitry, St. Louis 11, Missouri and Armstrong Sheet Metal Specialties, North Norwich, New York.

studies.[5, 6, 12] Aliquots of the leukocyte-staphylococcus mixture were removed at intervals, diluted in sterile saline, and divided so that one portion could be centrifuged to separate extracellular bacteria from leukocytes. Total, supernatant, and sediment fractions were homogenized and serially diluted for bacterial plate counts. Thus, the total number of culturable staphylococci, extracellular staphylococci, and staphlococci associated with leukocytes could be separately determined.

Absorption of serum. Absorbed serum was prepared by mixing serum with the bacterial sediment obtained from centrifugation of 40 cc. of an 18 hour culture. After incubation at room temperature for 30 minutes, the bacterial cells were removed by filtration through a Millipore filter* and collected aseptically. In most experiments, absorptions were repeated three times. In some instances, as many as eight absorptions were employed. Absorbed sera were then heated to 56° C. for 30 minutes to destroy any remaining heat labile factors. In most experiments, heat labile factors were replaced by the addition of 12 units of guinea pig complement* or 10 per cent fresh rabbit serum.

Complement titration. Complement was titrated according to the method of Lange.[13] Serial twofold dilutions of serum were made in 0.2 cc. amounts, to which was added 0.2 cc. NaCl, 0.1 cc. hemolysin (containing three units), and 0.1 cc. of a five per cent suspension of washed sheep red blood cells. After incubation for one hour in a 37° C. water bath, tubes were placed at 4° C. overnight. End points were read as the tube showing 50 per cent hemolysis.

Capsule stains. Various techniques were employed to demonstrate capsules. These included India ink preparations, observations under phase contrast using immune serum to detect possible capsular swelling, and different capsular staining methods including Alcian blue[14] and stains described by Muir,[15] Lyons,[16] and Butt.[17]

RESULTS

ANTISTAPHYLOCOCCAL HEMAGGLUTININS

Sera obtained from 175 human subjects were tested for the presence of staphylococcal hemagglutinins. As noted in Figure 1, 110 adult sera all showed detectable hemagglutinating antibody ranging in titre from

* Type HA, Field Monitor, Millipore Filter Corporation, Bedford, Massachusetts.
* Dehydrated Guinea Pig Complement, Texas Biological Laboratories, Inc., Fort Worth, Texas.

1:4 to 1:128. In keeping with the studies of Neter, there was suggestive evidence that this antibody crossed the placental barrier.[18] Twenty-two of 31 cord bloods showed demonstrable antibody in titres of 1:2 to 1:16. The amounts of hemagglutinating antibody appeared to be lower in sera obtained from 20 infants aged from 6 to 12 weeks. In this age group, 8 of 20 showed no detectable antibody. Virtually all infants beyond 12 weeks of life had demonstrable antibody. Hemagglutinin titres on paired serum specimens obtained at four to six weeks intervals from eight infants

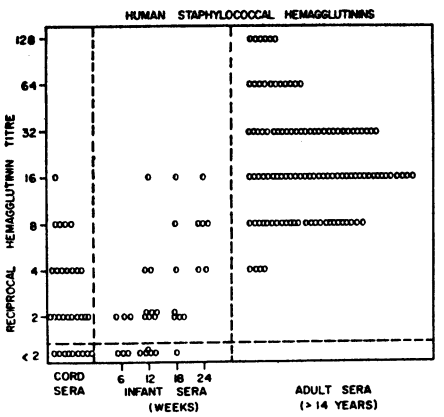

FIG. 1. Titres of hemagglutinating antibody found in 175 persons of varying age.

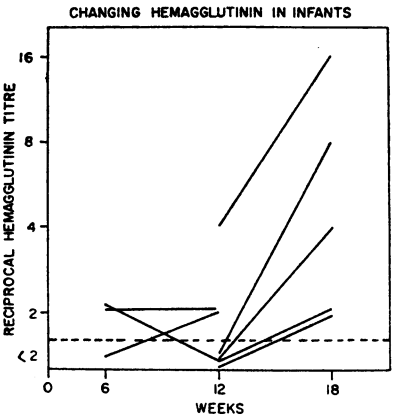

FIG. 2. Hemagglutinin titres in eight infants, showing a rise in titre between 12 and 18 weeks.

between 6 and 18 weeks of age are shown in Figure 2. Three of these infants showed a significant rise in antibody levels between 12 and 18 weeks.

Five adults with serious systemic staphylococcal disease possessed hemagglutinating antibody levels similar to those obtained in normal adults and are included in Figure 1. Vaccination of one normal human subject twice weekly for six weeks with increasing amounts of the heat killed staphylococcal vaccine used to immunize rabbits produced no change in a hemagglutinin antibody titre of 1:16. The maximum single dose used in vaccination contained 1.5 x 10^{10} bacterial cells.

Specificity of antistaphylococcal hemagglutinin. High titre antistaphylococcal hemagglutinating sera was readily produced in vaccinated rabbits. Studies on such sera showed that the antistaphylococcal hemagglutinating antibody resulting from such vaccination could not be completely removed

by repeated absorption with the homologous strain. Furthermore, such sera showed high hemagglutinating titres against red cells sensitized with heat killed Group A streptococci or *B. subtilis*. As noted in Figure 3, a series of rabbits vaccinated with heat killed staphylococci, streptococci or

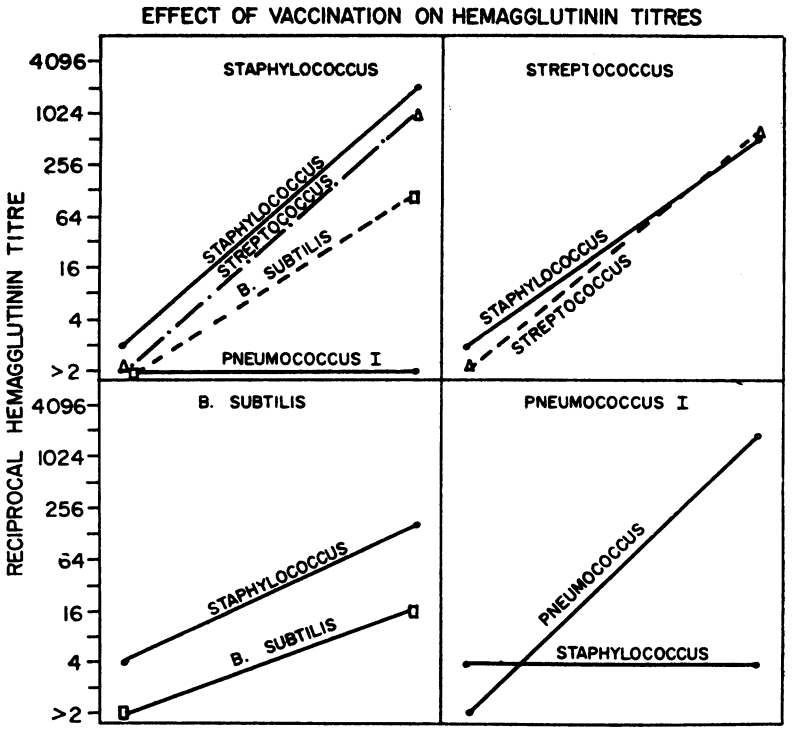

FIG. 3. The nonspecificity of antistaphylococcal hemagglutinins. Vaccination of rabbits with 2 of 3 heterologous microorganisms produced a significant rise in antistaphylococcal hemagglutinins.

B. subtilis all produced hemagglutinating antibody which could be demonstrated with red cells sensitized with any of these microorganisms. A Type 1 pneumococcus vaccine similarly employed did not appear to produce cross reacting antibody. In this instance, hemagglutinating antibody was detectable only when tested with red cells sensitized with the homologous pneumococcal vaccine strain. These studies confirm those of Rantz[19] and suggest that such hemagglutinating antibody is relatively broad and nonspecific.

STUDIES ON SERUM FACTORS REQUIRED FOR THE PHAGOCYTOSIS OF THE SMITH DIFFUSE STAPHYLOCOCCUS

Initial studies on the Smith staphylococcus have been reported.[5] In these earlier studies we were unable to clearly establish dual requirements for heat stable antibody and heat labile complement or complement-like factors which characterize many opsonic systems. In retrospect, insufficient amounts of heat labile factor were used in these earlier studies.

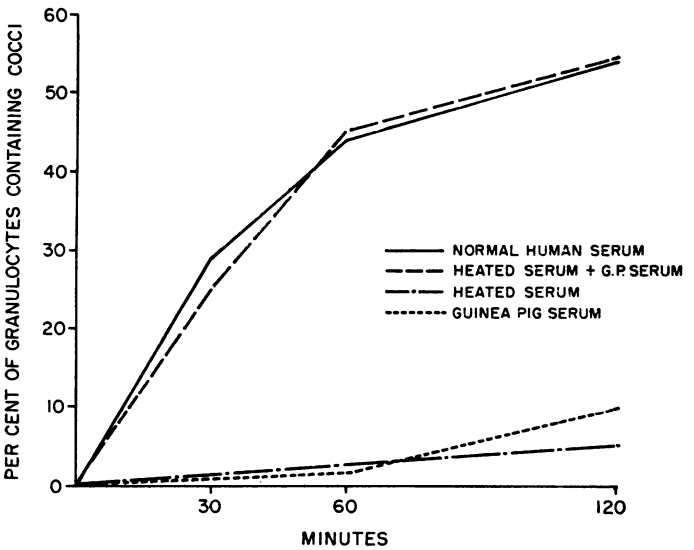

FIG. 4. Serum requirements for opsonization of the Smith diffuse staphylococcus.

The present experiments show that at least two separate factors present in adult human or immunized rabbit sera are required for opsonization. Representative experiments are pictured in Figures 4 and 5.

As noted in Figure 4, fresh adult serum promoted rapid phagocytosis of the Smith diffuse staphylococcus. This property was lost by heating the serum to 56° C. for 30 minutes. Guinea pig complement by itself did not opsonize the Smith diffuse strain. However, when sufficient quantities of guinea pig complement (12 units) were added to heated human serum, phagocytosis-promoting activity was completely restored.

Similar results were obtained when serum from a patient with agammaglobulinemia was substituted for guinea pig complement (see Fig. 5). Fresh unheated serum from this individual did not opsonize the Smith

diffuse strain in 20 per cent concentrations, but small amounts of this sera were capable of restoring the phagocytosis-promoting properties of heated normal human serum.

STUDIES ON HEAT STABLE ANTIBODY

Initial absorption studies yielded inconstant results. Sometimes the opsonizing property of serum was lost following absorption with

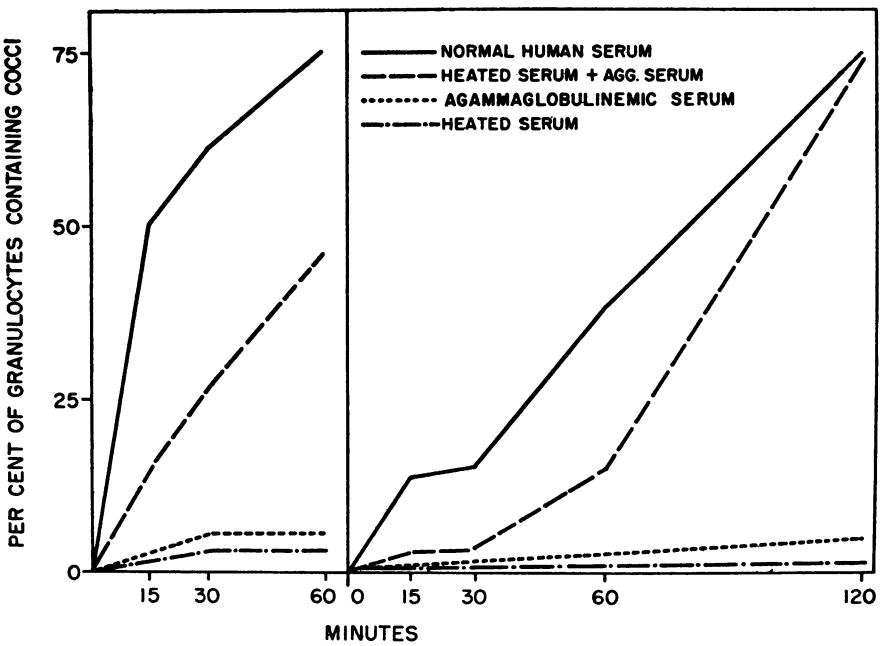

FIG. 5. Serum requirements for opsonization of the Smith diffuse staphylococcus. Agammaglobulinemic serum could serve as a source of heat labile factor.

heterologous microorganisms, sometimes it was not. These variable results suggested that in addition to antibody, heat labile or complement-like substances might be removed by absorption, and, indeed, this appeared to be the case. Certain sera subjected to absorption with heterologous staphylococci at room temperature lost all complement activity as tested in a hemolytic system. The opsonic properties of such sera could be readily restored by the addition of guinea pig complement. Consequently, guinea pig complement was added to all absorbed sera as a source of heat labile factors before testing for opsonic properties.

Human sera absorbed with the Smith diffuse strain were incapable of opsonizing this organism with or without the addition of guinea pig complement. In contrast, sera absorbed with heterologous microorganisms including Group A streptococci, *B. subtilis,* pneumococci and *E. coli* showed no loss in phagocytosis-promoting activity when guinea pig serum was added as a source of heat labile factor (see Fig. 6). Similarly, absorption of human sera with 14 heterologous strains of coagulase positive staphylococci isolated from human infection failed to remove from human

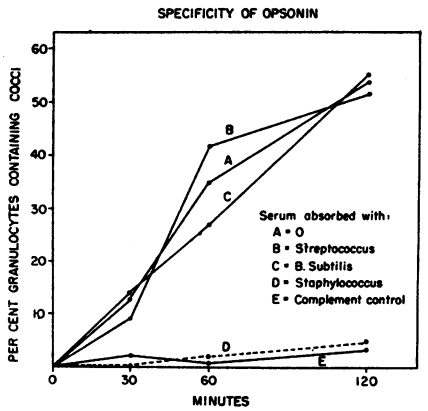

FIG. 6.

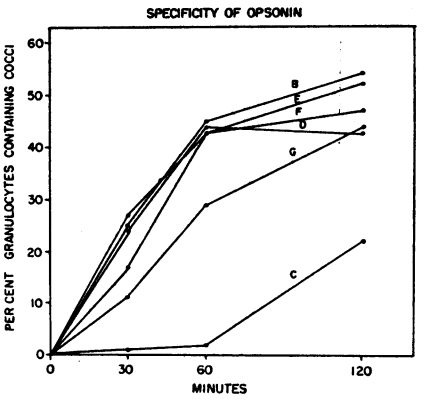

FIG. 7.

FIG. 6. Specificity of the Smith diffuse opsonizing antibody. Absorption of sera with sreptococci or *B. subtilis* did not alter the opsonizing properties of human serum.

FIG. 7. Specificity of the Smith diffuse opsonizing antibody.
B = unabsorbed human serum.
D, E, F, G = same serum absorbed with heterologous strains of staphylococci.
C = same serum absorbed with Smith diffuse staphylococci.

serum the phagocytosis-promoting activity for the Smith diffuse staphylococcus. A representative experiment is pictured in Figure 7. Subsequent studies were designed to better characterize the behavior of specific antibody.

The *rate* of ingestion of the Smith diffuse staphylococcus appeared dependent upon the amount of antibody present in the system. When serum obtained from an immunized rabbit was serially diluted in normal rabbit serum, a steady reduction in the speed of ingestion of the Smith diffuse staphylococcus was observed. Such a study is pictured in Figure 8.

Earlier experiments by Ward and Enders had demonstrated that antibody directed against pneumococci was similarly rate determining, and that slow phagocytosis to the same eventual degree took place with or without specific antibody if "complement" was present.[20] Whether similar

relationships characterize the Smith staphylococcus-serum system was not satisfactorily determined in our studies. To obtain reproducible results, the 1:1 ratio of staphylococci to leukocytes was critical. If studies were prolonged beyond 90 to 120 minutes, extracellular multiplication of staphylococci altered this relationship and phagocytosis took place. (Note the sharp increase in phagocytosis noted in serum absorbed with the Smith diffuse variant at 120 minutes in Figure 7). Attempts were made

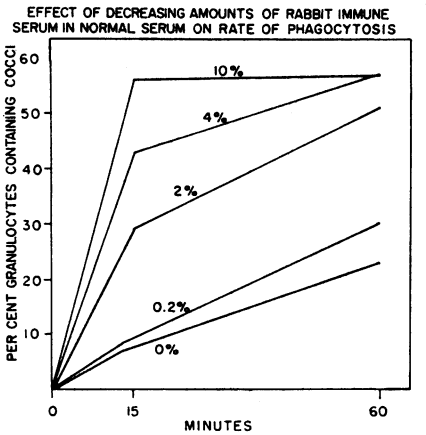

FIG. 8.

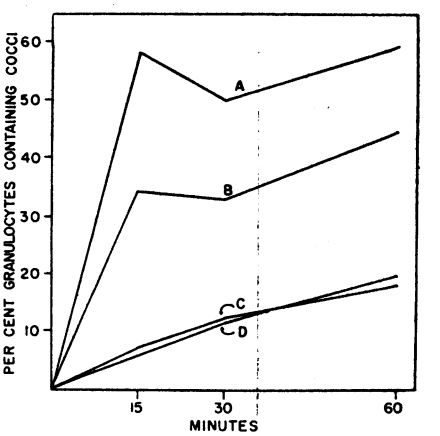

FIG. 9.

FIG. 8. The effect of decreasing concentrations of Smith diffuse immune rabbit serum in normal rabbit serum on the rate of phagocytosis of the Smith diffuse staphylococcus.

FIG. 9. The sequence of opsonizing requirements for the Smith diffuse staphylococcus.
A = Control. Microorganisms suspended in normal unheated human serum.
B = Microorganisms exposed to heated human serum, thrice washed and resuspended in guinea pig complement.
C = Microorganisms exposed to guinea pig complement, thrice washed and resuspended in heated human serum.
D = Control. Microorganisms suspended in heated human serum.

to run prolonged experiments with heat killed staphylococci to avoid this problem, but accurate phagocytic indices could not be obtained with such preparations.

Sequential studies showed that heat stable antibody could be firmly attached to the Smith diffuse variant in the absence of heat labile factor but not vice versa. If staphylococci were exposed to heated human serum, thrice washed and resuspended in systems containing guinea pig complement and leukocytes, rapid phagocytosis took place. In contrast, preliminary exposure of the microorganisms to guinea pig serum followed by similar washing and suspension in heated human serum containing leukocytes

failed to promote greater phagocytosis than that observed in guinea pig serum or heated serum alone. Such an experiment is pictured in Figure 9.

The lack of correlation between the presence of specific opsonizing antibody and hemagglutinin could be readily demonstrated in rabbit sera. Rabbits immunized with the Smith diffuse staphylococcus showed high titres of hemagglutinin and these sera possessed good opsonizing activity.

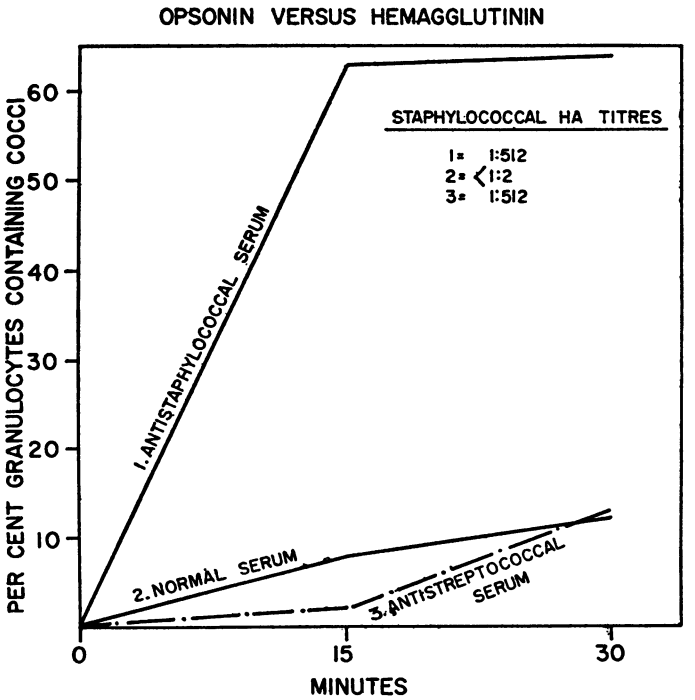

FIG. 10. The lack of identity of hemagglutinin and opsonin. Two rabbit sera with similar hemagglutinin titres show striking differences in opsonizing properties.

In contrast, sera from rabbits vaccinated with other gram positive microorganisms, such as streptococci, showed similar titres of hemagglutinins when tested with red cells sensitized with staphylococcal extracts, but such sera did not promote phagocytosis of staphylococci (Fig. 10).

While we have not attempted to determine the prevalence of this specific phagocytosis-promoting antibody in humans, more than 30 different adult sera used in the past two years have shown good opsonizing activity against the Smith diffuse strain. Studies on the opsonizing activity of

seven paired maternal and cord sera suggested that specific phagocytosis-promoting antibody crossed the placental barrier and was detectable in cord blood. Indeed, it has been difficult to obtain human serum which does not contain antibody, although the sera of a few infants between 6 and 12 weeks of age appeared to lack opsonizing activity. Studies performed on serum from one adult showed that opsonizing antibody could withstand storage at -20° C. or -70° C. for six weeks without loss of

TABLE 1. THE PHAGOCYTOSIS OF THE SMITH DIFFUSE VARIANT AND OTHER STRAINS OF STAPHYLOCOCCI IN DIFFERING SERUM SYSTEMS

	Serum system				*Absorptions*			
Staphylococcal strain	*Fresh human serum*	*Heated human serum*	*Guinea pig complement*	*HHS** + GPC*	*Serum absorbed with:†* (1)	(2)	(3)	(4)
(1) Smith diffuse	++++*	0	0	++++	0	++++	++++	++++
(2) Giorgio	++++	++++	++++	++++	++++	++++	++++	++++
(3) Bowers	++++	++++	++++	++++	++++	++++	++++	++++
(4) Meador	++++	++++	++++	++++	++++	++++	++++	++++

* Indicates degree of phagocytosis at one hour.

** HHS = heated human serum.
GPC = guinea pig complement.

† Following absorption, fresh guinea pig serum was added to all tubes.

its ability to promote phagocytosis if heat labile factor was added, but serum maintained at these temperatures for longer than four months showed an apparent reduction in opsonizing capacity.

OPSONIZING REQUIREMENTS OF OTHER COAGULASE POSITIVE STAPHYLOCOCCI

Because of the apparent specificity of the antibody which promotes phagocytosis of the Smith diffuse strain, it was our hope that cross absorption of sera would yield phagocytic index typing procedures which could separate different pathogenic strains. It became apparent, however, that under the conditions employed all other staphylococci tested were less resistant to phagocytosis and differed in the serum factors required for their ingestion. Fourteen strains were tested under various conditions.

A representative experiment performed in a system containing human serum and human leukocytes is illustrated in Table 1. Strains other than

Smith diffuse were readily ingested in heated human serum or guinea pig complement alone. Repeated absorption of serum with a test organism other than Smith did not alter the opsonizing properties of sera to which guinea pig complement had been added.

Similar results were obtained using unheated normal rabbit serum. All other strains of staphylococci tested were promptly ingested in this

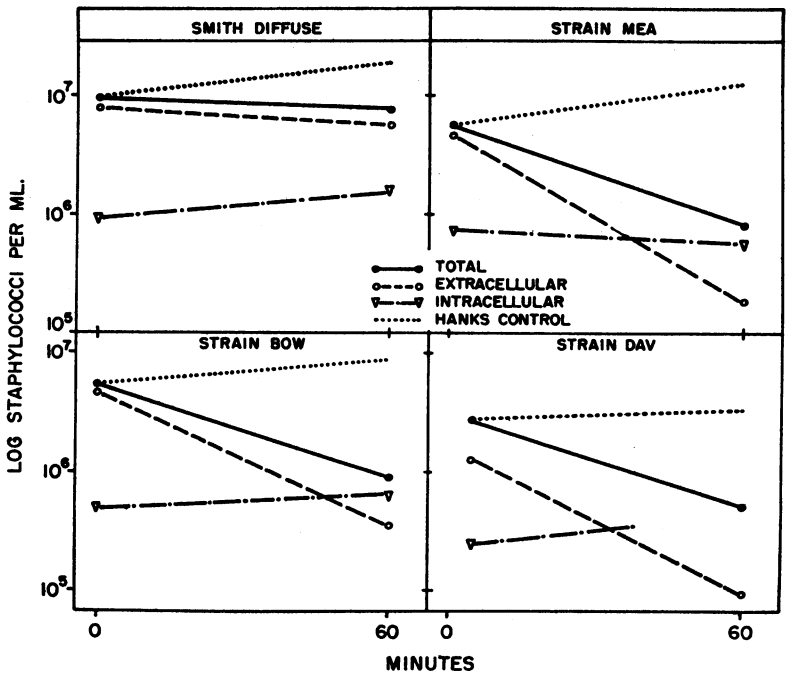

FIG. 11 .Differences in the serum factors required for opsonization of Smith diffuse strain and other staphylococci. Three other strains were readily ingested in normal rabbit serum.

system in which the Smith diffuse strain was not, and quantitative cultural studies confirmed results obtained by phagocytic indices. Experiments conducted with human leukocytes suspended in normal rabbit serum indicated that there was no appreciable ingestion of the Smith diffuse strain and no significant change in the total numbers of viable microorganisms. In contrast, there was a significant drop in staphylococcal populations in tubes containing other strains, and differential cultures indicated that the numbers of intracellular microorganisms rose to approach

TABLE 2. SUMMARY OF THE DIFFERENCES IN SERUM REQUIREMENTS FOR PHAGOCYTOSIS OF THE SMITH DIFFUSE STAPHYLOCOCCUS AND OTHER COAGULASE POSITIVE STRAINS

Human serum			*Rabbit serum*		
Sera employed	*Smith diffuse staphylococcus*	*Other staphylococcal strains*	*Sera employed*	*Smith diffuse staphylococcus*	*Other staphylococcal strains*
Fresh serum (FHS)	++++*	++++	Fresh serum (FRS)	0	+++-++++
Heated serum (HHS)	0	++++	Heated serum (HRS)	0	0
Guinea pig complement (GPC)	0	++++	Smith immune serum (IRS)	++++	++++
HHS + GPC	++++	++++	Heated Smith immune serum (HIRS)	0	++-+++
FHS absorbed with Smith + GPC	0	+++-++++	Smith IRS absorbed with Smith + FRS	0	+++
FHS absorbed with other strains + GPC	++-++++	++++	Smith IRS absorbed with Giorgio + FRS	0	+++
Hanks' solution	0	0	IRS (5 other strains)	++-+++	++++
			HIRS (5 other strains)	0	++-+++
			HIRS absorbed with homologous strain + FRS	0-++	++++

* Indicates degree of phagocytosis at one hour.

the total microbial population, confirming the rapid phagocytosis noted in stained preparations. Such an experiment is shown in Figure 11.

Because these studies were performed with washed human leukocytes, it seemed possible that sufficient antibody might be transferred to the test system with the human leukocytes to opsonize strains less resistant to phagocytosis than the Smith diffuse strain. Simultaneous use of rabbit and

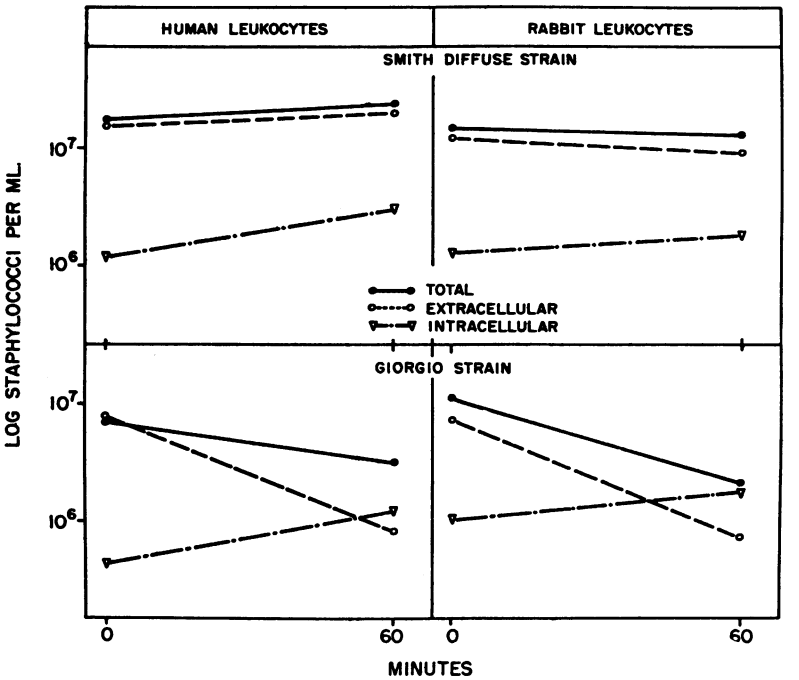

FIG. 12. Differences in the phagocytosis of the Smith diffuse strain and the Giorgio staphylococcus could be demonstrated in normal rabbit serum using either human or rabbit leukocytes. Similar results were obtained with other strains.

human leukocytes indicated that this was not the case. As illustrated in Figure 12, similar results were obtained with leukocytes from either source. The Smith diffuse strain was not ingested, while other strains (in this instance the Giorgio strain) were rapidly phagocytized.

Despite these differences in the opsonic requirements of the Smith diffuse strain and other staphylococci, there were evidences of close antigenic relationships. Sera obtained from rabbits immunized with five other strains of staphylococci proved capable of opsonizing the Smith

diffuse strain. When these sera were absorbed with either the vaccine strains or the Smith diffuse strain, the ability to opsonize the Smith staphylococcus was lost or significantly depressed.

Other studies performed with normal and immune rabbit serum suggested that the simultaneous presence of antibody and heat labile factors

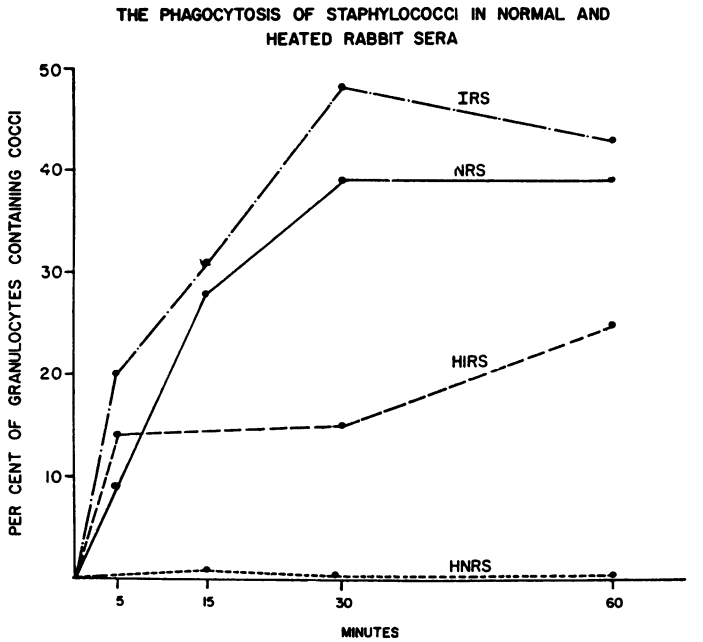

FIG. 13. Sera from immunized rabbits promotes the phagocytosis of other strains of staphylococci despite heating to 56° C. for 30 minutes.
IRS = Unheated immune rabbit serum.
NRS = Unheated normal rabbit serum.
HIRS = Heated immune rabbit serum.
HNRS = Heated normal rabbit serum.

yielded somewhat more rapid rates of ingestion of all strains than observed in the presence of either antibody or heat labile factors alone. Such a study is noted in Figure 13.

As shown here, normal rabbit serum, homologous immune rabbit serum, or heated immune rabbit serum all promoted phagocytosis of strains other than the Smith diffuse at one hour while heated normal rabbit serum did not. Ingestion was more complete and occurred most rapidly in sera containing *both* antibody and heat labile substances.

A variety of studies done with both human or rabbit leukocytes suspended in human or rabbit sera are summarized in Table 2 and 3.

These data justify the following statements:

1. Neither the Smith diffuse strain nor other strains were ingested by washed leukocytes suspended in Hanks' solution in the absence of serum (see Table 3). Thus, serum factors appeared necessary for ready phagocytosis of all strains tested in this fluid system.

TABLE 3. THE PHAGOCYTOSIS OF DIFFERENT PATHOGENIC STAPHYLOCOCCI ON NORMAL AND HEATED RABBIT SERUM AND HANK'S SOLUTION

	Normal rabbit serum		*Heated rabbit serum*		*Hank's solution*	
Staphylococcal strain	*Human WBC*	*Rabbit WBC*	*Human WBC*	*Rabbit WBC*	*Human WBC*	*Rabbit WBC*
Smith "diffuse"	1–7	3–4	0–1	0–2	0-2	2
Davidson	42	36	7	8	5	0
O'Hara	37	44	2	0	2	2
Stovall	28	36	3	12	4	4
Stern	36	24	3	5	1	—

2. Both the Smith diffuse strain and other coagulase positive staphylococci were readily ingested in the presence of fresh human serum. Both specific heat stable Smith antibody and heat labile factors were present in this system.
3. Heated human serum or heated serum from rabbits immunized with Smith diffuse or other staphylococci opsonized strains other than the Smith diffuse strain. These sera contained Smith antibody but lacked heat labile factors.
4. Normal rabbit serum or guinea pig complement opsonized strains other than the Smith diffuse strain. This system contained heat labile factor but lacked demonstrable antibody against the Smith diffuse strain.
5. Heated normal rabbit serum or heated guinea pig complement failed to opsonize either the Smith diffuse strain or other staphylococci. This system contained neither Smith antibody nor heat labile factors.

These observations suggested that the Smith diffuse strain had a surface which, while antigenically similar to other strains, nevertheless rendered it considerably more resistant to phagocytosis than other coagulase positive

staphylococci studied. Repeated attempts to establish the presence of a capsule on this microorganism utilizing various procedures outlined in the section on methods have met with failure. Preparations from cultures of varying age grown in different media, isolates from the mouse peritoneum, or studies on cells exposed to immune sera have not shown a consistently definable capsule or surface which is visibly different from other staphylococcal strains not resistant to phagocytosis.

DISCUSSION

The present studies indicate that characterization of staphylococci by immunologic methods is not a simple problem. In the current experiments, the Smith diffuse staphylococcus was shown to possess readily demonstrable resistance to phagocytosis. Both specific antibody and heat labile factors were required for its rapid ingestion in fluid systems. Other staphylococcal strains could not be so characterized. Other investigators have reported unusual strains of staphylococci which were immunologically distinct and/or resistant to phagocytosis by virtue of unique surface structures or capsules.[16, 21-23] Thus, there is good evidence that certain pathogenic staphylococci may resemble pathogenic strains of pneumococci, streptococci, *Klebsiella pneumoniae,* or *Hemophilus influenzae.* The present experiments make it equally clear, however, that many strains producing human infection are less resistant to phagocytosis and less dependent on specific antibody for ingestion than the unusual Smith strain variant. It thus seems unlikely that surface factors which delay phagocytosis can explain or determine the virulence of certain strains of staphylococci. While antigenic similarities between these other strains and the Smith diffuse variant were demonstrated, the lack of resistance to phagocytosis of these coagulase positive, disease producing staphylococci rendered unsatisfactory typing procedures dependent on phagocytosis in fluid systems containing both antibody and heat labile factors.

The current findings with strains other than Smith diffuse might be explained by the presence of small but significant amounts of opsonizing antibody in normal rabbit serum. Cohen, Cowart, and Cherry have recently suggested that specific pathogen free rabbits may possess antistaphylococcal antibody.[24] While this possibility cannot be definitively eliminated, there is considerable evidence to suggest that this was not the case in the present experiments. In studies performed by Cohn and Morse, no staphylococcal agglutinins could be detected in normal rabbit serum.[3] Similarly, Jensen was unable to find detectable antibody against Cowan Type 1 strains in normal rabbits, whereas adult humans uniformly possessed

such antibody.[25] Recent studies in this laboratory using Ouchterlony techniques have consistently failed to detect antistaphylococcal antibody in normal rabbit serum despite its ready demonstration in serum from immunized rabbits. The absence of staphylococcal hemagglutinins in normal rabbit sera and the differences in the opsonizing capacity of heated normal rabbit serum and heated immune rabbit serum in the present studies add further support to the belief that normal rabbits do not commonly possess opsonizing antibody against staphylococci. It thus appears more reasonable to suggest that there are wider variations in the amounts if not the nature of phagocytosis-resisting surface factors present in pathogenic strains of staphylococci than has been the case with strains of pneumococci or streptococci producing human infection.

Nonspecific heat labile factors, of themselves, appeared to opsonize pathogenic strains of staphylococci other than the Smith diffuse strain in the test system employed. It could be similarly shown that antibody in the absence of the heat labile factors could promote phagocytosis of these other pathogenic strains. Heat labile factors were present in unheated human and rabbit sera and in dehydrated guinea pig complement. They were not present in sera subjected to storage at room temperature or sera heated to 56° C. for 30 minutes. In the few instances in which simultaneous complement titration and opsonization studies were done, loss of heat labile factor activity was associated with loss of complement activity. While it is thus possible that heat labile factors and complement are synonomous, it seems wise to avoid this assumption until the nature of the heat labile factors is more clearly defined. Recent studies have shown that certain heat labile substances other than complement may play an important role in the opsonization of streptococci and this may be true in other phagocytic systems as well.[26, 27]

The number of human sera tested for opsonizing antibody in our studies is not great, but antibody has been present in the majority of normal adult sera tested to date. In a larger study, Jensen has shown that all of a group of 500 adults possessed demonstrable antistaphylococcal antibody against Cowan Type 1 organisms.[25] These findings suggest a possible explanation for the well known immunologic inconsistencies of staphylococcal disease in man.[28, 29] We submit that the immunologic peculiarities of staphylococcal infection may be rendered understandable by viewing the adult human as a host possessing definite antistaphylococcal humoral immunity. The possession of antistaphylococcal antibodies may confer significant protection. The low incidence of clinical infection despite the frequent presence of potentially pathogenic strains in man makes this

thesis attractive. If this be true, overt straphylococcal disease in adults may of itself indicate that humoral "immunity" has been overwhelmed. In this situation, immune mechanisms may have already operated maximally and cannot be further stimulated. The failure to find higher titres of antistaphylococcal antibodies in patients with active infection than in normal control groups[80-82] and the inconstant and unconvincing results of immunizations with staphylococci or staphylococcal products might be explained on this basis. Humoral antibody could not be expected to significantly modify established disease or render more resistant the already immune host under these circumstances.

Despite our failure to develop a serologic system for differentiating staphylococci based on methods used in the typing of streptococci, it is our belief that such procedures can be established. The documented antigenicity of staphylococci and the increasing evidence indicating that surface antigens on some strains resemble those of other gram positive cocci producing human infection, suggest that serologic identification is an approachable goal with appropriately structured test systems.

SUMMARY

The diffuse variant of the Smith staphylococcus was shown to resist phagocytosis in fluid systems. Both antibody and heat labile serum factors were required for opsonization of this strain. Antibody was present in sera obtained from normal adults and could be produced in rabbits by immunization with vaccines prepared from the Smith diffuse variant or other pathogenic staphylococci. Heat labile factors were present in normal human or rabbit serum, guinea pig complement, and serum from a patient with agammaglobulinemia.

The antibody required for opsonization of the Smith diffuse strain appeared specific and was not removed from sera by absorption with heterologous microorganisms or other pathogenic strains of staphylococci. The amount of antibody present in the test system influenced the speed of ingestion of the Smith diffuse strain, and antibody could be attached to diffuse cells in the absence of heat labile factor. A definite capsular structure could not be demonstrated on this microorganism.

Fourteen other strains of coagulase positive staphylococci isolated from human infections differed in their serum requirements for opsonization. *Either* antibody or nonspecific heat labile factor alone could promote brisk phagocytosis of these strains.

Despite these differences in behavior in phagocytic systems, definite antigenic similarities were suggested by immunization experiments. Sera

obtained from rabbits vaccinated with other strains possessed opsonizing antibody for the Smith diffuse strain.

These observations suggest that pathogenic strains of staphylococci may show wider variations in surface antigens resisting phagocytosis than do virulent strains of pneumococci or streptococci. It seems likely that biologic attributes other than resistance to phagocytosis must play a role in determining the virulence of many strains of coagulase positive staphylococci.

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