

FURTHER OBSERVATIONS ON THE BEHAVIOR OF
STAPHYLOCOCCI WITHIN HUMAN
LEUKOCYTES*

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(Received for publication, December 4, 1959)

Recent studies by Cohn and Morse have indicated that pathogenic strains of staphylococci are not phagocytosed in systems containing rabbit serum and polymorphonuclear leukocytes in the absence of specific, thermostable opsonin (1). These studies have further suggested that both pathogenic and non-pathogenic strains of staphylococci are equally susceptible to rapid destruction within the cytoplasm of rabbit leukocytes once phagocytosis has been accomplished.

Previous studies in our laboratory have demonstrated that there are significant differences in the intracellular disposition of coagulase-positive and coagulase-negative staphylococci following ingestion by human leukocytes (2). Pathogenic, coagulase-positive strains appear to survive within the cytoplasm of human granulocytes while non-pathogenic coagulase-negative strains are rapidly destroyed following ingestion. Observations on the course of staphylococcal bacteremia in rabbits performed in our laboratory have also suggested that the survival of pathogenic strains within rabbit polymorphonuclear leukocytes may play a role in the persistence of staphylococci in the rabbit blood stream (3, 4).

In view of the findings of Cohn and Morse, studies on the behavior of staphylococci within human leukocytes have been extended, and the factors in human serum which promote phagocytosis have been explored. The present paper reports these observations.

This study supports the thesis that a specific opsonin is required for prompt phagocytosis of pathogenic staphylococci in siliconed glass systems. Human polymorphonuclear leukocytes, like rabbit granulocytes, do not phagocytose coagulase-positive staphylococci in such *in vitro* systems in the absence of a specific serum factor. The intracellular fate of staphylococci ingested by human leukocytes appears to differ, however, from the fate of staphylococci within

* Supported by grants E 1078 and 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.

rabbit leukocytes as observed by Cohn and Morse. Under the experimental conditions employed, significant numbers of pathogenic staphylococci survive ingestion by human cells while non-pathogenic staphylococci are rapidly destroyed within the cytoplasm.

Materials and Methods

Cultures.—Various coagulase-positive strains of *Staphylococcus aureus* isolated from human infections and coagulase-negative strains of *Staphylococcus albus* obtained from the air or skin surfaces were employed in preliminary studies. The experiments reported in the present paper were carried out using two representative strains of staphylococci, one pathogenic and one non-pathogenic. These strains were those used extensively by Cohn and Morse and were kindly supplied by these investigators. They are characterized below:—

Smith Strain.—This strain of *Staphylococcus aureus* was isolated in 1930 from a human case of osteomyelitis and has been maintained in the laboratory of Dr. René J. Dubos because of its consistent pathogenicity for mice (5). This strain was coagulase-positive, produced yellow pigment and hemolysins, fermented mannite, and was lysed by bacteriophages 44A and 42E.

Variants of the Smith Strain.—Two variants derived from the Smith strain which have been characterized by Hunt and Moses (6) were kindly supplied by Dr. George Hunt of Bristol Laboratories, Syracuse, New York. Both variants were coagulase-positive. The “diffuse” variant was lysed by bacteriophage 44A. Its high virulence for mouse when administered intraperitoneally, as reported by Hunt and Moses, was confirmed in this laboratory. The “compact” variant was not lysed by the 28 bacteriophages of Blair and Carr (7) and was virtually avirulent for mice when given *via* the intraperitoneal route.

Mendita Strain.—This *Staph. albus* strain was isolated from the nasopharynx of a patient at the Presbyterian Hospital, New York. It was coagulase-negative, did not produce pigment or hemolysins, and did not ferment mannite.

Stock cultures were maintained on agar slants stored at 4°C. The Smith strain was passed through mice at appropriate intervals to insure the maintenance of virulence. Three to 18 hour beef heart infusion broth cultures inoculated from stock strains were utilized in the present experiment.

The Preparation of Human Leukocytes.—Fifty ml. of human blood was obtained from one of eight normal donors early on the morning of each experiment. The blood was delivered to large siliconed tubes containing heparin in a final concentration of 1:10,000. An equal volume of sterile 3 per cent dextran¹ with an average molecular weight of 188,000 was added to promote red blood cell sedimentation.

Sedimentation was allowed to proceed for 20 to 40 minutes at 37°C. The plasma layer containing over 95 per cent of the leukocytes was harvested and the leukocytes were sedimented by centrifugation for 4 minutes at 800 r.p.m. using a No. 2 International centrifuge with a No. 233 head. The majority of platelets remained in the plasma layer and were removed by this procedure.

In a few studies, such leukocytes were used directly by incorporation in 20 per cent human serum in Hanks' solution containing 0.01 per cent sterile bovine albumin and 100 mg. per cent glucose. In the majority of experiments, leukocytes were washed three times in large volumes of Hanks' solution prior to suspension in the phagocytic medium containing the serum under study. Preliminary studies showed that two or more washings effectively removed the serum component promoting phagocytosis from the leukocyte suspension (Table I). Total

¹ Grade H (clinical) lot H 1257. R. K. Laros Co., Bethlehem, Pennsylvania.

leukocyte and differential counts were performed and the quantity of phagocytic medium containing the appropriate serum was adjusted to yield final leukocyte populations of 1.5 to 3.0×10^7 leukocytes per ml.

Studies on leukocyte morphology, motility, phagocytic ability, and nuclear uptake of 1 per cent trypan blue, indicated that over 95 per cent of leukocytes subjected to such procedures were active and viable. Cells obtained by such dextran sedimentation did not appear to differ in their behavior from leukocytes obtained directly from individuals with high sedimentation rates (2).

The Phagocytic System.—Leukocytes suspended in the appropriate medium were divided into aliquots and placed in sterile siliconed 12 x 100 mm. glass tubes. Staphylococci were added in a ratio of 1 to 1.6 staphylococci per polymorphonuclear leukocyte. The contents of the tube were mixed and an aliquot was removed for initial determinations. The tubes were then stoppered with sterile paraffined rubber corks and placed horizontally in an 18 cm. wheel which was rotated constantly at 16 R.P.M. in an incubator maintained at 37°C. The tubes were removed at intervals, the contents mixed, and samples withdrawn for determinations to be described.

TABLE I
The Effect of Successive Washings on the Ability of Human Leukocytes to Ingest Coagulase-Positive Staphylococci in Hanks' Solution

No. of washings	PMN'S containing cocci*
	<i>per cent</i>
0	80
1	3
2	0
3	0

* Determinations of phagocytosis were performed after 3 hours' rotation at 37°C.

Quantitative Determinations of Culturable Staphylococcal Populations.— At each sampling interval, three separate series of quantitative bacteriologic counts were performed to separately determine (a) total culturable staphylococci, (b) culturable extracellular staphylococci, and (c) culturable staphylococci associated with leukocytes. These studies employed a technique originally described by Maaløe (8). This procedure has been utilized in previous experiments to separate leukocytes and extracellular bacteria (3) and was similarly employed by Cohn and Morse.

Following thorough mixing, a 0.1 ml. aliquot was removed, serially diluted in sterile distilled water, and quantitatively plated to determine the total number of culturable "units" remaining in the phagocytic system. Another 0.5 ml. aliquot was immediately delivered to a tube containing 4.5 cc. of 0.85 per cent saline. The contents were mixed and spun in a No. 2 centrifuge at 700 R.P.M. for 5 minutes. Preliminary experiments indicated that such centrifugation effectively sedimented all leukocytes without significantly affecting extracellular microorganisms. The tube was removed from the centrifuge without agitation and the upper layer of supernatant was carefully removed, appropriately diluted, and plated to determine the number of extracellular staphylococci. The supernatant was then completely decanted from the remaining leukocyte button. One-half ml. of sterile distilled water was added to the sediment and the contents of the tube were ground with a teflon tissue homogenizer. The disrupted leukocyte suspension was then serially diluted and plated to determine the numbers of bacteria

associated with leukocytes. In most details, this procedure followed that used by Cohn and Morse (1).

Microscopic Studies of Phagocytosis.—At each sampling interval, multiple coverslip smears were prepared and stained by Wright's or the Gram-Weigert method. Quantitative counts of 100 to 300 polymorphonuclear leukocytes were made, and the numbers of polymorphonuclear leukocytes participating in phagocytosis and the number of staphylococci contained within 100 granulocytes were recorded.

Cultural Studies on the Intracellular Survival of Staphylococci.—Slide microcultures prepared by a technique previously described (2) were used to determine the intracellular survival of staphylococci following various periods of residence within polymorphonuclear leukocytes. In brief, this technique permitted the incorporation of washed leukocytes containing staphylococci in microcultures which would sustain staphylococcal multiplication. The number of polymorphonuclear leukocytes containing multiplying cocci was determined following 4 hours' incubation at 37°C.

Measurement of Antistaphylococcal Antibody.—The antistaphylococcal antibody content of human or rabbit sera was determined using a hemagglutination technique described by Netter (9) as modified by Weld (10). Washed sheep red blood cells were sensitized by 45 minute incubation with antigen prepared from the supernatant of washed, heat-killed staphylococci of the Smith strain. The prepared red cells were then washed three times in large volumes of 0.9 per cent saline and resuspended to a concentration of 2.5 per cent. Test sera were first absorbed with unsensitized red blood cells, then serially diluted in 0.9 per cent saline. Equal volumes of 2.5 per cent antigen-coated red cells were added to each tube and the tubes were incubated for 40 minutes at 37°C. Hemagglutination was read following low speed centrifugation for 1 minute.

RESULTS

The Behavior of Pathogenic and Non-Pathogenic Staphylococci in Phagocytic Systems Containing Human Leukocytes and Human Serum.—The coagulase-positive and coagulase-negative strains of staphylococci under study differed in their behavior in phagocytic systems containing human leukocytes in human serum. During the initial 30 to 60 minutes there was a rapid fall in the total numbers of culturable staphylococci of either strain. Differential cultures indicated that the number of extracellular microorganisms fell rapidly, while the numbers of staphylococci associated with the leukocytes rose to approach, or equal, the total number of culturable units present in the tubes, suggesting that phagocytosis of both strains was taking place.

There was, however, a striking difference in the magnitude of the decline in culturable pathogenic and non-pathogenic microorganisms. In nine different experiments the pathogenic Smith strain showed a seven- to tenfold fall in the total number of culturable staphylococci. In contrast, the coagulase-negative Mendita strain showed a 100- to 200-fold fall in the number of culturable staphylococci during the same time period. A representative experiment is pictured in the upper half of Fig. 1.

Following 30 to 60 minutes of incubation, the rate of disappearance of culturable microorganisms of both strains declined abruptly. Over the next 3 to 5 hours the coagulase-negative strain generally showed a slight fall in culturable

populations, while the number of persisting coagulase-positive staphylococci remained static or increased slightly during the same period.

In general, the number of coagulase-negative staphylococci cultured from the disrupted leukocyte mass equalled the number of microorganisms obtained on simultaneous cultures of the total phagocytic mixture suggesting that staph-

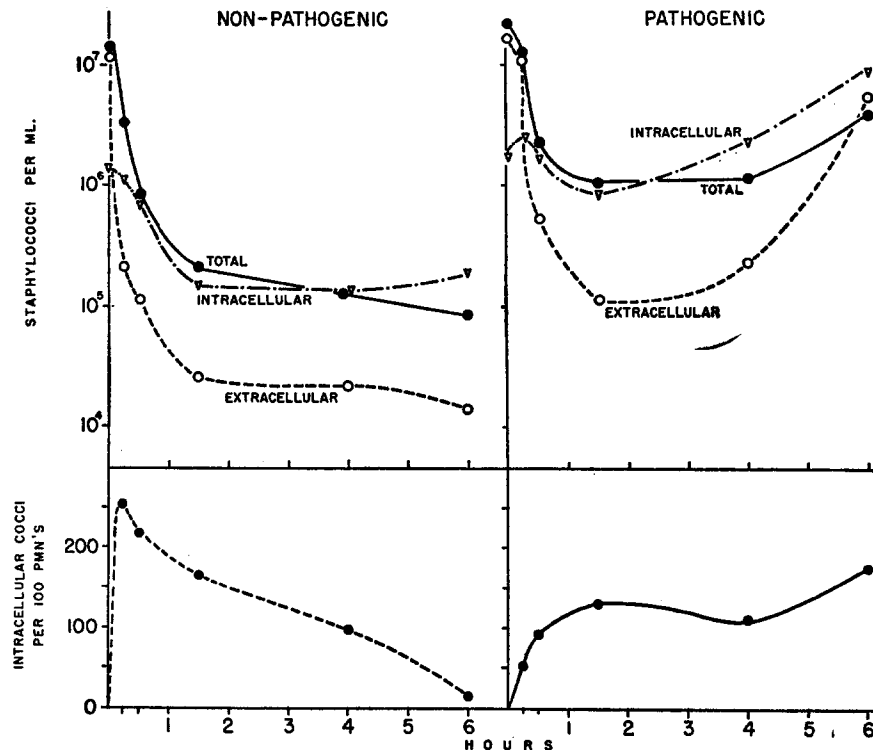


FIG. 1. The behavior of pathogenic and non-pathogenic staphylococci in a phagocytic system containing human serum. Culturable populations of staphylococci during the 6 hour period of incubation are pictured in the upper part of the figure. The speed and magnitude of phagocytosis as determined microscopically are pictured in the lower portion of the figure.

ylcocci were persisting within the leukocytes after the initial phase of destruction. In contrast, the numbers of culturable coagulase-positive staphylococci recoverable from the ground leukocytes often rose to exceed the total counts by three to five times after 90 minutes or more, suggesting that disruption of leukocytes was releasing increasing numbers of cocci from phagocytic cells.

The Rates of Phagocytosis of Coagulase-Positive and Coagulase-Negative Staphylococci in Human Serum.—Serial smears prepared from such experiments indicated definite differences in the rates of phagocytosis of the two strains in sys-

tems containing human serum. In general, the coagulase-negative strain was ingested more swiftly. (See the bottom half of Figs. 1 and 2.) Maximal phagocytosis of coagulase-negative staphylococci obtained from 18 hour cultures commonly occurred within 15 minutes as determined by the percentage of polymorphonuclear leukocytes ingesting cocci or the number of cocci contained within 100 granulocytes. The coagulase-positive strain obtained from similar overnight cultures was phagocytosed more slowly, and maximal phagocytosis was not attained for 45 to 60 minutes. At 1 hour, approximately equal numbers of cocci of either strain were visible within the cytoplasm of polymorphonuclear leukocytes (Fig. 2).

In keeping with previous observations (2) and those of Cohn and Morse (1), the use of young staphylococci obtained from cultures during active growth did not significantly alter the rate or degree of phagocytosis. Indeed, coagulase-positive staphylococci prepared from 4 hour cultures appeared to be ingested more swiftly than older cells, rendering the curve of ingestion more like that of coagulase-negative strains (Fig. 3).

Examination of stained smears prepared after phagocytosis had proceeded for 90 minutes or more showed striking differences in the intracellular appearance of ingested staphylococci. There was a gradual decline in the number of cocci of the non-pathogenic strain detectable within leukocytes, while the number of intracellular coagulase-positive cocci remained relatively constant or increased slightly during experimental periods as long as 6 hours. With the passage of time ingested non-pathogenic staphylococci became paler, smaller, and stained so indistinctly that they were often not identifiable as cocci. In many leukocytes, vacuoles containing tiny granules which appeared to represent staphylococcal remnants were seen as incubation proceeded. In contrast, pathogenic staphylococci continued to stain clearly and sharply within the cytoplasm, and diploid forms were commonly observed within the cell.

The differences in the number of pathogenic and non-pathogenic staphylococci which could be identified within leukocytes are strikingly illustrated in the lower half of Fig. 1. As shown here, the number of visible intracellular staphylococci of the Mendita strain fell from 254 intracellular cocci per 100 leukocytes at 15 minutes to 15 per 100 leukocytes at 6 hours. During the same time interval the number of visible intracellular cocci of the Smith strain remained relatively constant, increasing slightly at 6 hours.

Studies on leukocyte viability performed at 6 hours showed no detectable differences. Approximately 95 per cent of leukocytes in each tube appeared viable as determined by the test procedures previously outlined.

The Behavior of Staphylococci in Phagocytic Systems Containing Human Leukocytes in Rabbit Serum.—That a substance present in the serum of each of the eight human donors was necessary for effective phagocytosis of coagulase-positive staphylococci was confirmed in a series of experiments similar to that illustrated in Figs. 4 and 5.

Washed human leukocytes suspended in systems containing either human or rabbit serum ingested the coagulase-negative strain at similar rates. The fall

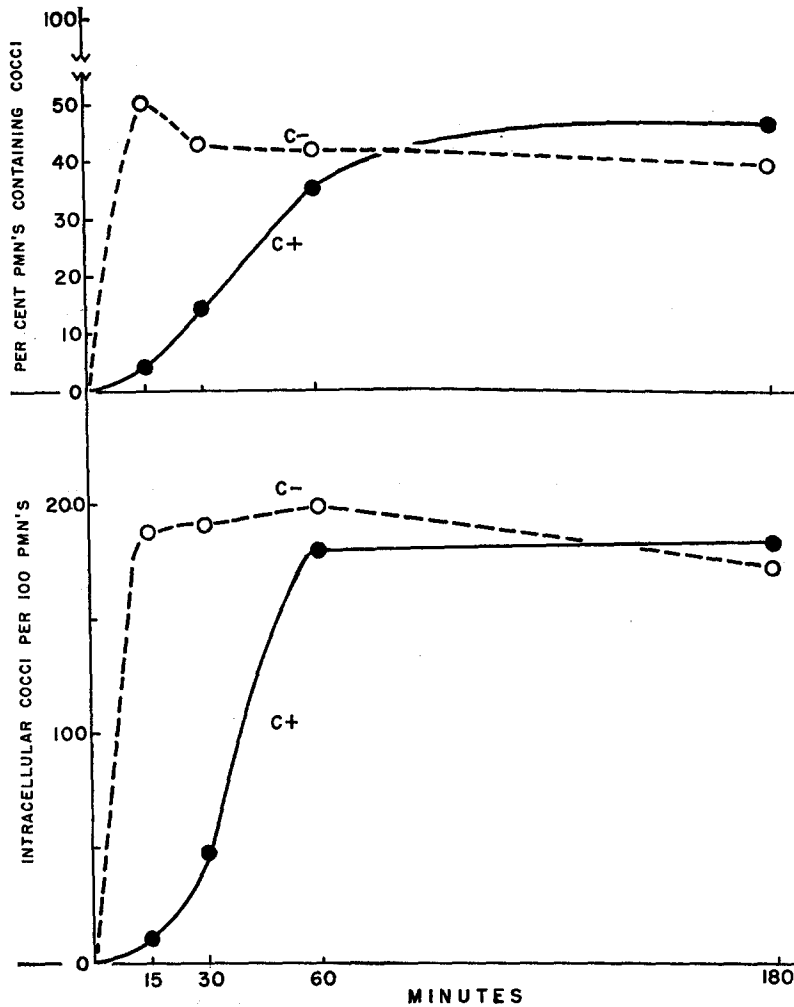


FIG. 2. Comparative rates of phagocytosis of pathogenic and non-pathogenic staphylococci in human serum. 18 hour cultures.

in the number of culturable non-pathogenic staphylococci was similar in both systems, although the total destruction of cocci was less striking when leukocytes subjected to repeated washings were utilized. In contrast, the coagulase-positive strain was phagocytosed in human serum but not ingested by leukocytes suspended in rabbit serum until late in the experiment when extensive multiplication of extracellular staphylococci had produced extremely high

staphylococcus to leukocyte ratios. Cohn and Morse have also shown that phagocytosis of coagulase-positive strains can take place in rabbit serum-cell systems under these circumstances.

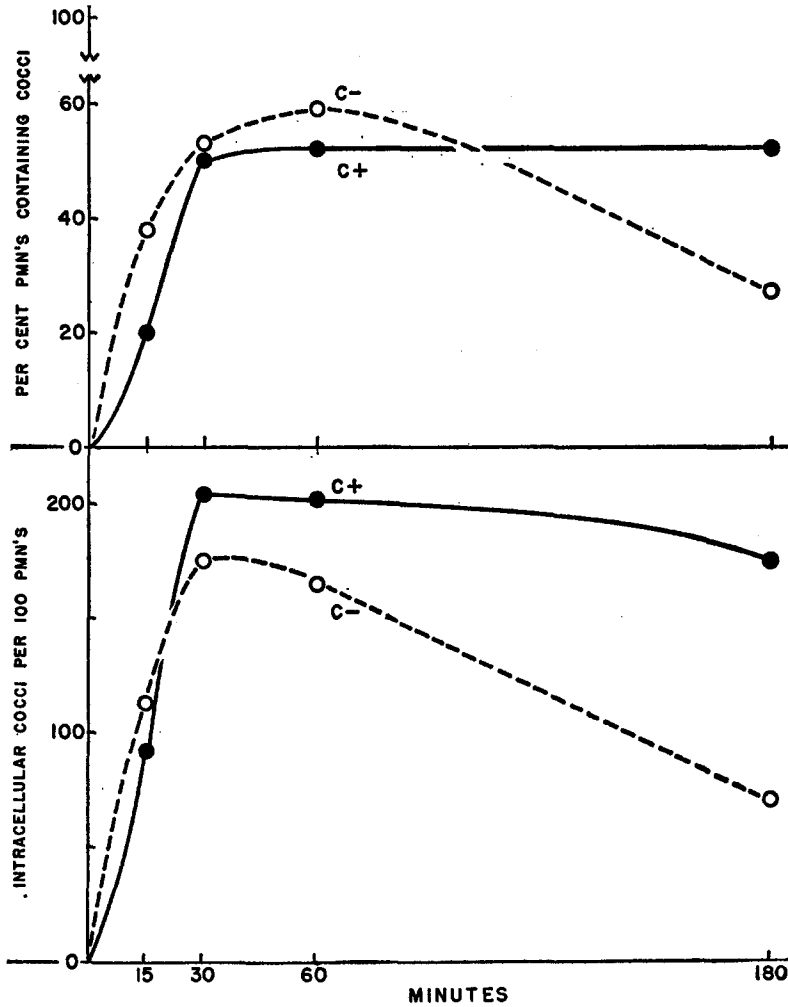


FIG. 3. Comparative rates of phagocytosis of pathogenic and non-pathogenic staphylococci in human serum, 4 hour cultures.

The Intracellular Survival of Staphylococci.—The consistent differences in the speed with which pathogenic and non-pathogenic staphylococci were ingested in systems containing human serum made it seem possible that swifter phagocytosis of the non-pathogenic strain produced greater killing of this microorgan-

ism. To minimize differences in the length of intracellular residence, the following type of experiment was performed.

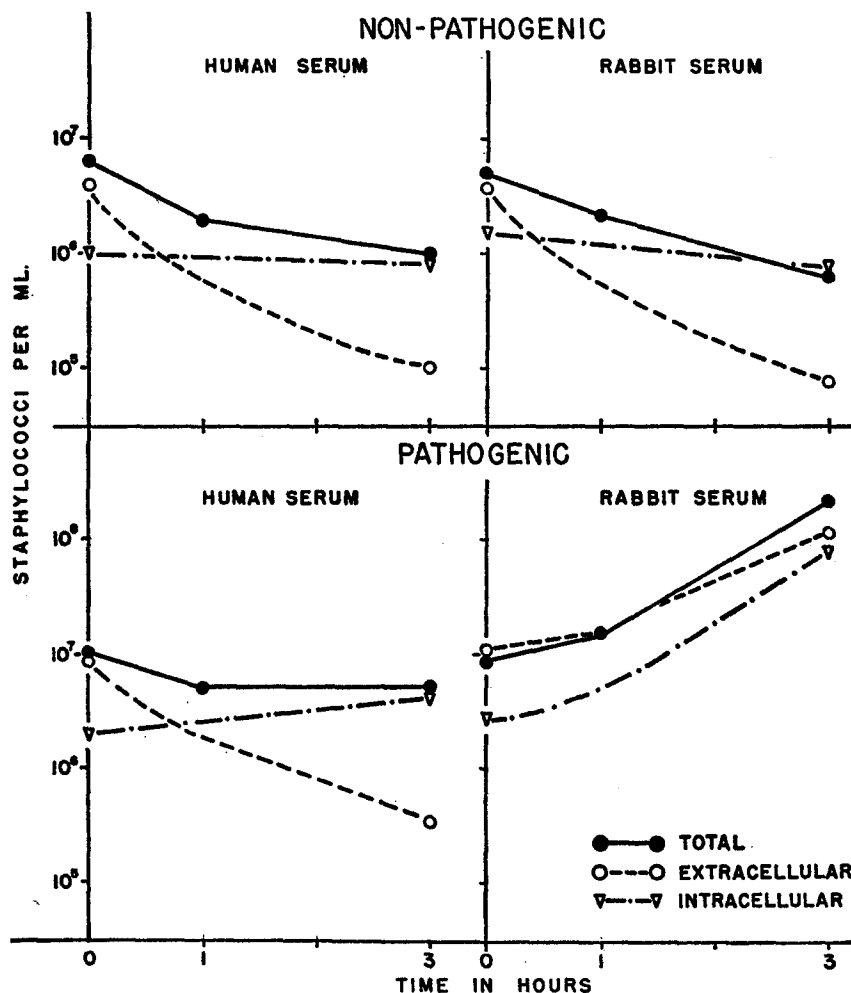


FIG. 4. The behavior of pathogenic and non-pathogenic staphylococci in phagocytic systems containing rabbit serum. The numbers of pathogenic staphylococci increase in the system containing rabbit serum, and culturable extracellular staphylococci remain high, suggesting that phagocytosis is not occurring.

Phagocytosis of both strains was allowed to proceed in the presence of human serum for 1 hour. At this time approximately equal numbers of coagulase-positive and coagulase-negative cocci were visible within leukocytes. The tubes were then removed from the phagocytic wheel and the leukocytes were washed three times in large volumes of Hanks' solution containing 20 per cent human serum and re-

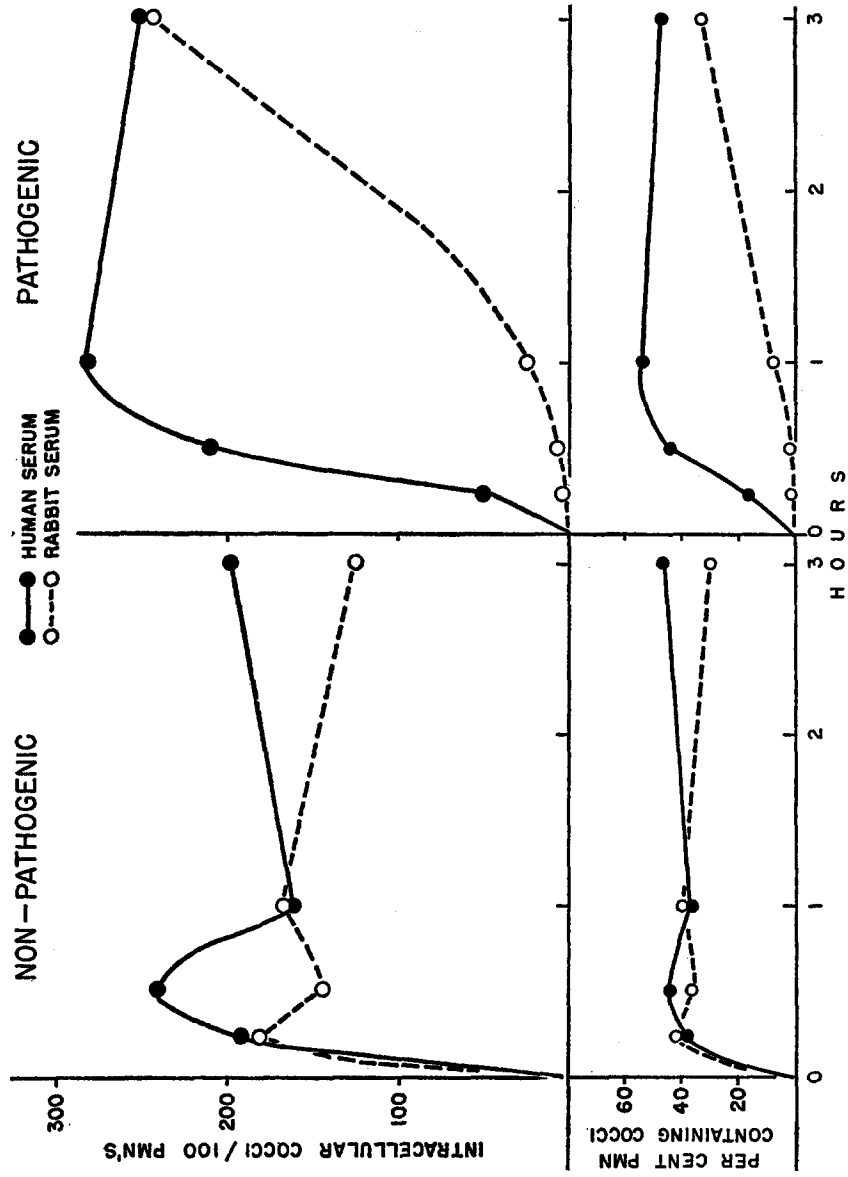


FIG. 5. Microscopic determinations of phagocytosis performed during the experiment pictured in Fig. 4. The pathogenic strain is not phagocytosed in rabbit serum until late in the experiment when extensive extracellular multiplication has occurred.

turned to the incubator at 37°C. Total supernatant, sediment cultures, and stained smears were made at intervals over a 3 hour period. At 3 hours, slide microcultures were prepared and the numbers of leukocytes growing microcolonies were enumerated after 4 hours' incubation. Thus the degree of intracellular survival after not less than 3 or more than 4 hours of residence within leukocytes could be determined for both strains.

As illustrated in Fig. 6, cultural studies performed on the washed leukocytes indicated that virtually all of the culturable staphylococci of both strains were associated with the cells. Extracellular organisms represented less than 2 per cent of the total culturable bacteria and did not multiply significantly during the period of study.

As shown in the lower half of Fig. 6, examination of stained smears indicated that the degree of phagocytosis of both strains was similar. Fifty per cent of polymorphonuclear leukocytes contained coagulase-negative staphylococci within the cytoplasm, while 55 per cent of the polymorphonuclear leukocytes contained coagulase-positive staphylococci. Approximately equal numbers of cocci (174 and 187 cocci per 100 granulocytes) were situated intracellularly. Despite such similar degrees of phagocytosis, simultaneous cultures revealed that 20- to 40-fold more coagulase-positive staphylococci remained culturable, suggesting differences in the viability of the visible intracellular cocci.

Examination of smears prepared during subsequent incubation showed that almost 50 per cent of the visible non-pathogenic cocci disappeared during the 3 hours of incubation. Little change in the numbers of visible intracellular coagulase-positive staphylococci occurred during this period.

Slide microcultures prepared after 4 hours of incubation showed that 30 per cent of the leukocytes containing the coagulase-positive strain harbored cocci growing colonies. In contrast, no colonies were observed emerging from the cytoplasm of over 500 leukocytes containing the coagulase-negative strain.

Studies performed with 1 per cent trypan blue at the end of such experiments failed to show significant differences in the percentage of granulocytes taking intranuclear staining. Over 95 per cent of polymorphonuclear leukocytes appeared to be viable in each system.

Further evidence that differences in the rates of phagocytosis could not alone account for the differences in intracellular survival was fortuitously obtained in studies on the coagulase-positive "compact" and "diffuse" variants of the Smith strain.

As noted in Fig. 7, the phagocytosis of the "diffuse" mouse-virulent variant proceeded slowly and resembled that of the parent Smith strain, while the "compact" mouse avirulent variant was consistently phagocytosed at a rapid rate which paralleled that of coagulase-negative strains. Cultural studies performed simultaneously on aliquots taken from this experiment are shown in Fig. 8.

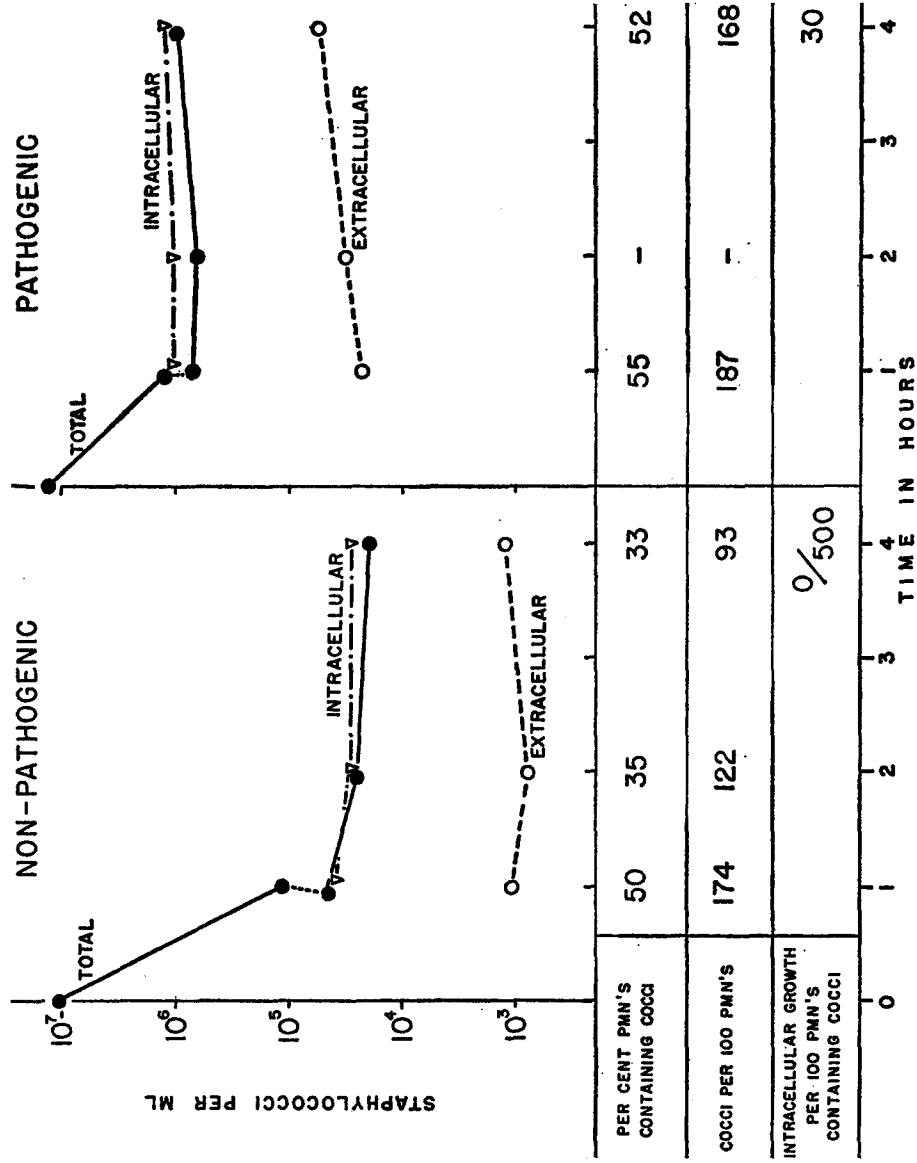


FIG. 6. The behavior of pathogenic and non-pathogenic staphylococci within human polymorphonuclear leukocytes (see text).

Despite the striking differences in the speed of ingestion, culturable populations of both strains of staphylococci remained similar over a 6 hour period. In keeping with other coagulase-positive strains tested, the decline in culturable

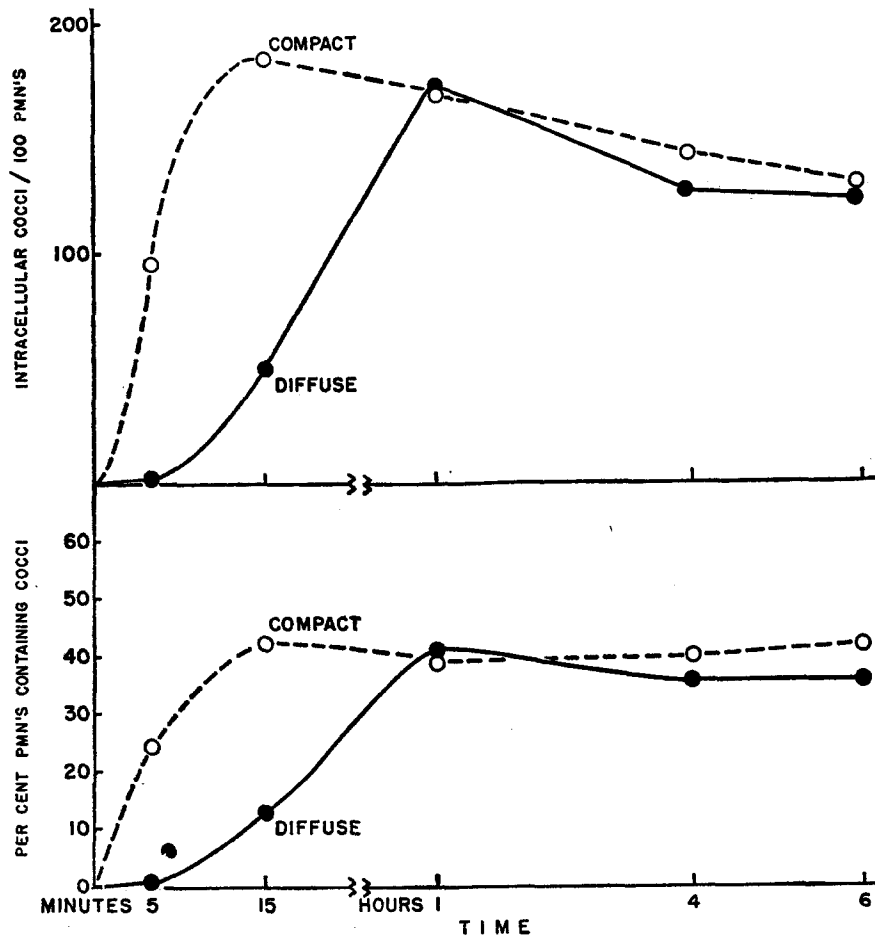


FIG. 7. The phagocytosis of 2 coagulase-positive variants of the Smith staphylococcus. The "compact" variant is ingested at rates similar to those observed for non-pathogenic, coagulase-negative staphylococci.

staphylococci was small, almost 50 per cent of the original population surviving a 6 hour period, despite ingestion of over 99 per cent of the microorganisms.

Slide cultures prepared at the termination of this experiment showed that both variants showed similar degrees of intracellular survival. Thirty per cent of the granulocytes containing the "diffuse" variant and 45 per cent of the cells

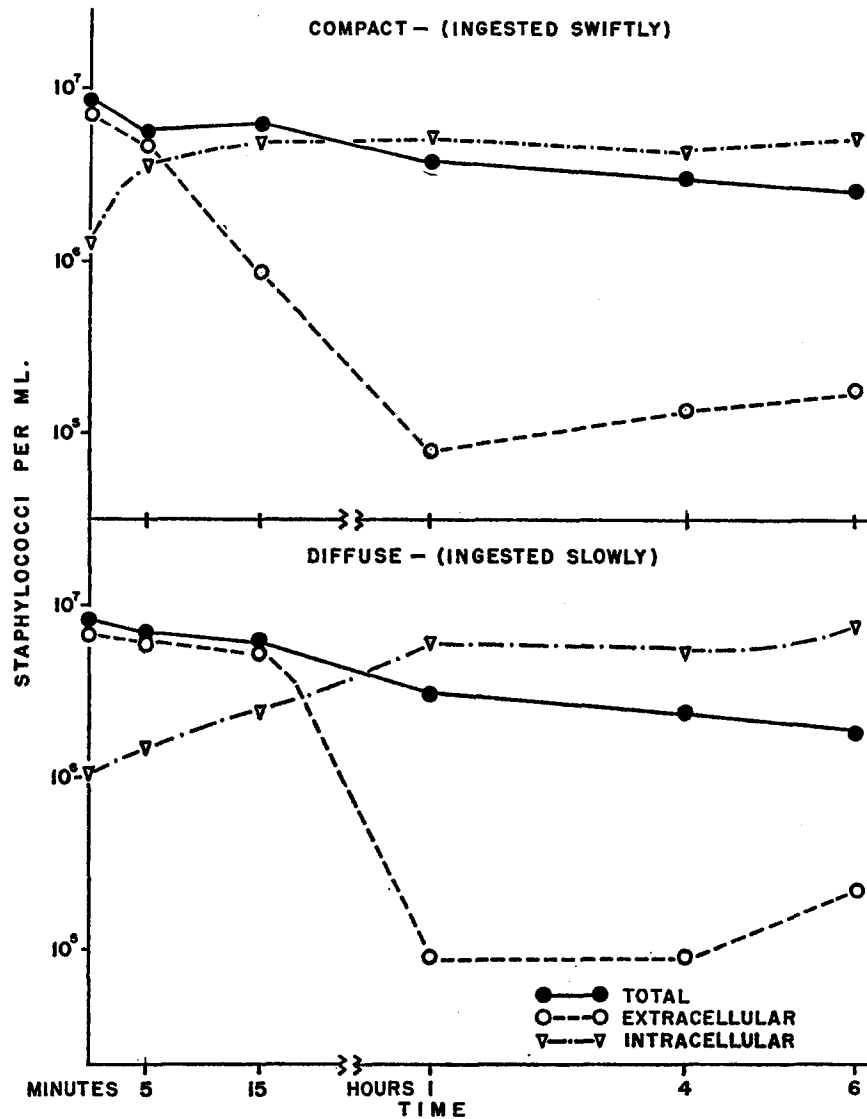


FIG. 8. The cultural behavior of 2 variants of the Smith staphylococcus in the experiment pictured in Fig. 7. Despite differences in the speed of phagocytosis, both coagulase-positive variants survive in ingestion by leukocytes.

containing the "compact" variant were capable of growing microcolonies at this time.

Characterization of the Phagocytosis-Promoting Factor in Human Serum.—Subsequent studies were designed to further characterize the factor or factors

in human sera promoting phagocytosis of coagulase-positive strains of staphylococci in siliconed glass systems.

In the studies to be described, thrice washed human leukocytes and 18 hour cultures of the Smith strain of staphylococcus were employed. Great care was taken to maintain low staphylococcal to leukocyte ratios (0.5 to 1.0 coccus per polymorphonuclear leukocyte) after preliminary studies indicating that serum was not required for phagocytosis when large numbers of staphylococci were present. This phenomenon also tended to confuse prolonged studies of the phagocytic process. It was repeatedly demonstrated that avid phagocytosis could take place late in the experiment in tubes in which minimal or no phagocytosis initially occurred because of the unrestrained multiplication of extracellular staphylococci which were then ingested. (For example see the 3 hour phagocytosis of the coagulase-positive strain in rabbit serum pictured in Fig. 5.) Such multiplication and late ingestion when staphylococcal-leukocyte ratios were high rendered quantitative determinations of phagocytosis after 3 to 4 hours of incubation misleading. Because events occurring during the initial moments of staphylococcal-leukocyte contact were believed to be of fundamental importance, the present experiments were carried out during a 1 hour period of incubation to avoid multiplication of cocci and changing microorganism-cell ratios.

Both quantitative cultures and microscopic determinations of the rate and degree of phagocytosis were simultaneously performed in all experiments. Cultural and microscopic results correlated in all instances. Results obtained from examination of stained smears proved a more sensitive index and allowed more precise quantitation of the early happenings than did quantitative cultural studies. The degree of phagocytosis as determined by the number of leukocytes participating in phagocytosis or by the number of intracellular cocci contained in 100 leukocytes produced virtually identical findings, and only the latter results have been graphically portrayed in subsequent section.

Fresh serum obtained from each of the eight human donors promoted the phagocytosis of the Smith strain by human leukocytes.

In contrast, sera obtained from 28 of 30 normal rabbits used during the course of these studies were incapable of promoting phagocytosis. Sera from two apparently normal rabbits maintained in the rabbit colony for several months opsonized staphylococci in a manner similar to human sera.

Hemagglutination titrations against a Smith strain antigen were performed on representative human and rabbit sera to determine whether the presence of hemagglutinating antibody correlated with the ability of serum to opsonize staphylococci. As shown in Table II, six human sera so studied all showed low titers of hemagglutinating antibody. Five of these six sera had been used in phagocytic studies and all possessed the capacity to opsonize staphylococci. Three of six rabbit sera similarly studied also showed detectable hemagglutininations. Two of these 3 sera were obtained from those animals whose sera proved capable of opsonizing staphylococci. One rabbit serum possessing a significant

hemagglutinin titer consistently failed to promote phagocytosis, suggesting that hemagglutinin- and phagocytosis-promoting factor were not necessarily related.

In keeping with the Cohn and Morse findings on immune rabbit sera, the phagocytosis promoting factor present in human sera appeared specific. A representative absorption experiment is pictured in Fig. 9 and Table III.

Preliminary absorption of serum with the homologous strain of staphylococcus rendered it incapable of promoting phagocytosis. Absorption with a heterologous strain of coagulase-positive staphylococci decreased, but did not eliminate opsonization. Prior incubation with a coagulase-negative strain of staphy-

TABLE II
Serum Antistaphylococcal Hemagglutination Titrations

Serum	Reciprocal hemagglutinin titre	Ability of serum to promote phagocytosis
Human DER	10	+++
MAM	10	+++
NF	10	+++
JTW	2.5	Not done
AM	5	+++
TK	16	+++
Rabbit B ₂ R	10	0
292	5	++
293	2.5	++
60	Less than 2.5	0
61	" " 2.5	0
62	" " 2.5	0

lococci or pneumococcus Type I did significantly change the opsonizing qualities of the serum. While absorption with a strain of *Escherichia coli* apparently reduced the phagocytosis-promoting ability of absorbed serum, microscopic examination showed the leukocytes to be atypical and damaged, suggesting that endotoxin effect on the leukocytes rather than loss of opsonin might account for this finding.

Human serum heated to 56°C. for 30 minutes consistently lost the ability to promote phagocytosis. In contrast to the findings in immune rabbit serum systems (1), 1 per cent, 5 per cent, and 10 per cent fresh rabbit serum failed to restore the phagocytosis-promoting ability of heated human serum. A representative experiment pictured in Fig. 10 is recorded in Table IV. That this did not represent lack of complement in rabbit serum was suggested by the observation that the addition of 2 units of guinea pig complement similarly failed to restore the phagocytosis-promoting activity of heated human serum (Fig. 11).

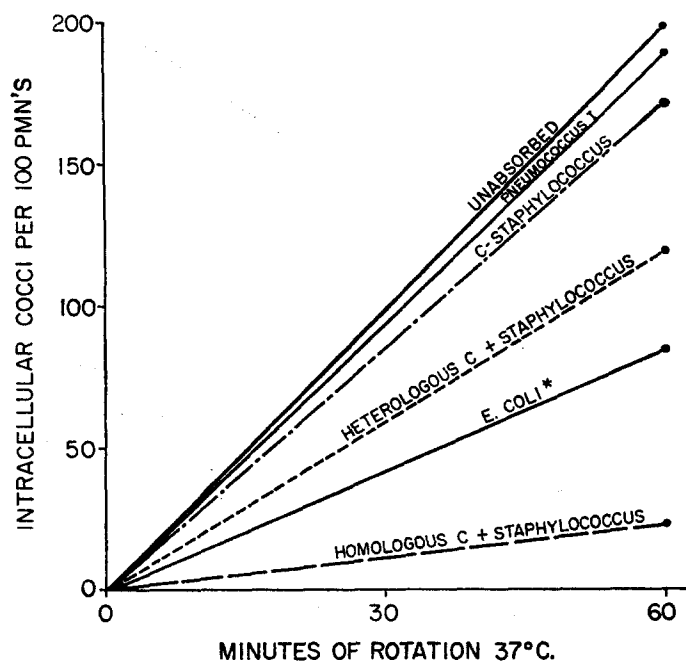


FIG. 9. The effect of prior absorption on the ability of human serum to promote phagocytosis of the Smith staphylococcus.

* Leukocytes appear damaged.

TABLE III

*The Phagocytosis of the Smith Staphylococcus in Sera Absorbed with Different Microorganisms**

Serum absorbed with	PMN's containing cocci	Intracellular cocci per 100 PMN
	<i>per cent</i>	
Control serum	46	198
Smith strain (coagulase-positive)	8	24
Giorgio strain (coagulase-positive)	31	119
Mendita strain (coagulase-positive)	38	172
Pneumococcus I	48	189
<i>E. coli</i> (Besson)	27	84†

* Determinations of phagocytosis were performed following 60 minutes of rotation at 37°C.

† Leukocytes appear damaged.

When increasing increments of fresh human serum were added to heated human serum, a progressive rise in phagocytosis was observed. However, rather similar degrees of phagocytosis were obtained in these concentrations of fresh human serum in the absence of heated human serum (see Fig. 12).

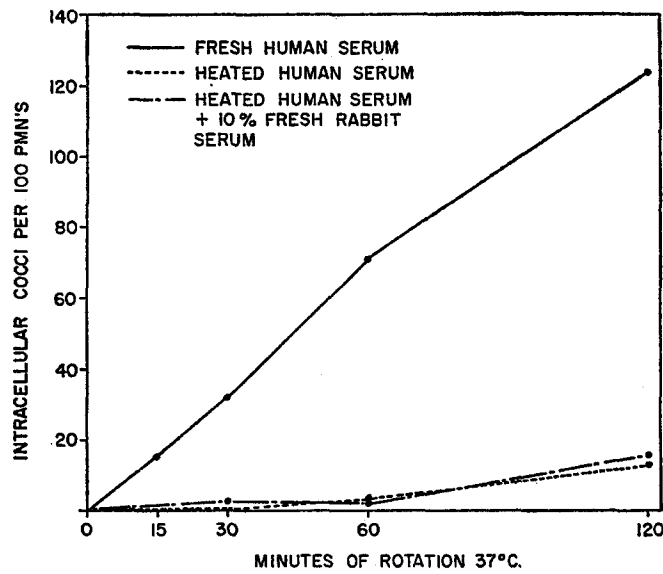


FIG. 10. The effect of heat (56°C. for 30 minutes) on the phagocytosis promoting factor in human serum. Activity is not restored by the addition of 10 per cent fresh rabbit serum.

TABLE IV

Effect of Heat on the Ability of Human Serum to Promote Phagocytosis of Staphylococci

Serum	Time	PMN containing cocci	Intracellular cocci per 100 PMN	Microorganisms per ml.
	min.	per cent		
20 per cent fresh human serum	0	—	—	1.3×10^7
	15	6	15	
	30	10	19	
	60	27	71	4×10^6
	120	40	124	1.3×10^6
20 per cent human serum, 56°C. — 30 min.	0			1.3×10^7
	15	0	0	
	30	0	0	
	60	1	2	1.8×10^7
	120	3	13	3.4×10^7
20 per cent fresh rabbit serum	0			1.3×10^7
	15	0	0	
	30	1	1	
	60	2	13	1.7×10^7
	120	10	58	2.0×10^7
20 per cent heated human serum + 10 per cent fresh rabbit serum	0			1.3×10^7
	15	0	0	
	30	1	2	
	60	0	0	1.5×10^7
	120	5	16	2.8×10^7

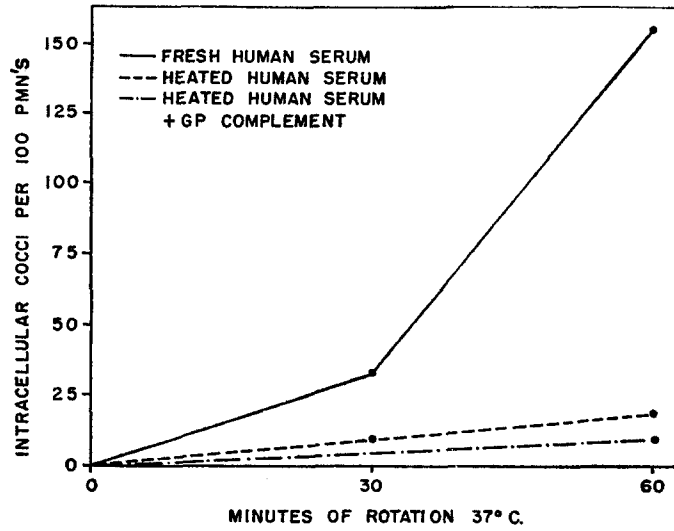


FIG. 11. The effect of heat on the phagocytosis promoting factor in human serum. Activity is not restored by the addition of 2 units of guinea pig complement.

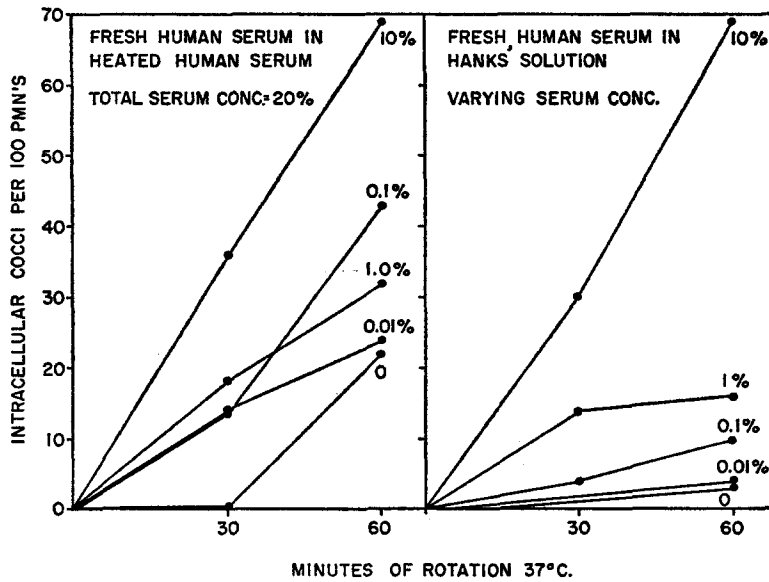


FIG. 12. The phagocytosis of the Smith staphylococcus in human serum. The increasing phagocytosis noted with increasing additions of fresh serum to heated serum appears to be due to the action of fresh serum alone.

TABLE V

Demonstration of the Heat Stability of the Phagocytosis-Promoting Factor in Human Serum

Procedure*	PMN containing cocci	Intracellular cocci per 100 PMN's
	<i>per cent</i>	
Fresh human serum + heated staphylococci	34	91
Fresh human serum + staphylococci 15 min. at 37° then heated	33	153
Heated human serum + heated staphylococci	3	7
Heated human serum + heated staphylococci + guinea pig complement, 24 units	20	44

* "Heated" denotes 56°C. for 30 minutes. Final serum concentration in all tubes was 20 per cent. Washed human leukocytes were added, following the above manipulations and determinations of phagocytosis were performed after 1 hour rotation at 37°C.

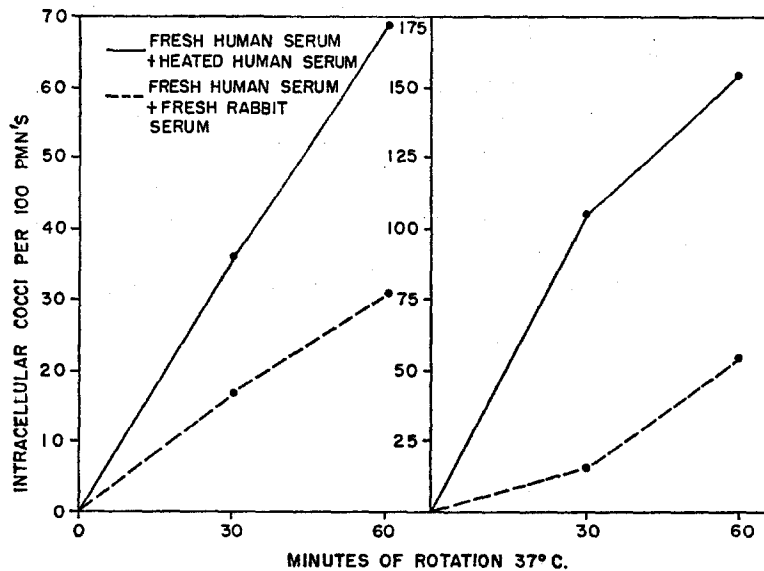


FIG. 13. The inhibition of phagocytosis by the addition of fresh rabbit serum to fresh human serum. Left half of figure:— = 15 per cent fresh human serum, 5 per cent heated human serum;---- = 15 per cent fresh human serum, 5 per cent fresh rabbit serum. Right half of figure:— = 10 per cent fresh human serum from another donor, 10 per cent fresh rabbit serum;---- = 10 per cent fresh human serum, 10 per cent fresh rabbit serum.

Review of the classic studies of Wright and Douglas (11) and the subsequent studies of Dean (12) and Ward and Enders (13) suggested that the restoration of the phagocytosis-promoting activity of heated human serum of low opsonin titer might require large amounts of complement. This possibility was supported by the observation that large amounts of guinea pig complement (24 units) did produce a significant rise in the opsonization produced by heated serum.

That the phagocytosis-promoting factor was probably a heat stable opsonin was supported by experiments in which serum and staphylococci were incubated at 37°C. *prior* to heating to 56°C. for 30 minutes. In this instance, phagocytosis was similar to that observed in equal concentrations of unheated serum (Table V).

Inhibition of the Phagocytosis of Coagulase-Positive Staphylococci by Normal Rabbit Serum.—The ability of fresh human serum to promote phagocytosis was often strikingly depressed by the presence of fresh rabbit serum. Additions of 1, 5, or 10 per cent rabbit serum to fresh human serum produced a striking fall

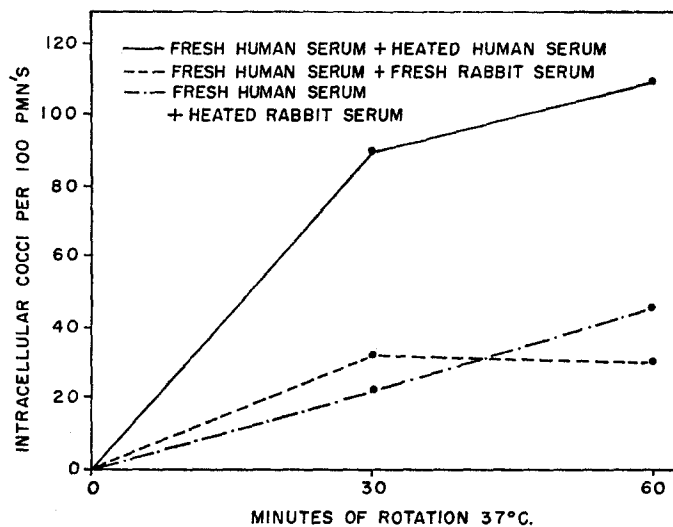


FIG. 14. The ability of rabbit serum to inhibit phagocytosis in human serum is not lost by heating rabbit serum to 56°C. for 30 minutes.

in the degree of phagocytosis obtained in fresh human serum alone, or fresh human serum in the presence of heated human serum. Two of 7 such experiments are pictured in Fig. 13. Rabbit serum subjected to 56°C. for 30 minutes produced the same inhibition of phagocytosis observed with fresh rabbit serum (Fig. 14).

Studies to date suggest that the inhibitory property resides in rabbit serum *per se*. Current experiments indicate that the depression in phagocytosis is not due to the inactivation of complement, leukocyte damage, or abnormal clumping of staphylococci in the presence of rabbit serum. Further studies to define the nature of this inhibition are in progress.

DISCUSSION

The present experiments demonstrate that a specific factor in human serum is required for rapid phagocytosis of coagulase-positive staphylococci by human

leukocytes suspended in fluid systems. It is clear that failure to recognize this fact in previous studies resulted from two factors: one, the presence of anti-staphylococcal antibody (or antibodies) in all of the "normal" human sera or plasma employed; two, the relative ease with which staphylococci are ingested in the absence of serum when staphylococcal-leukocyte ratios are high. The recent studies of Jensen (14) showing that 100 per cent of 500 individuals of varying ages possessed detectable anti-staphylococcal antibody supports the contention that virtually all human beings have had previous immunologic experience with staphylococci. The current investigations, coupled with those of Cohn and Morse, indicate that the concentrations of staphylococci and leukocytes in the test system are critical in detecting significant differences in the leukocyte uptake of coagulase-positive and coagulase-negative strains.

While virulent staphylococci possess the ability to resist phagocytosis in systems in which serum opsonin is absent and surfaces promoting phagocytosis are kept at a minimum, it is clear that such resistance to ingestion is relative, not absolute. In contrast to the striking resistance of virulent pneumococci, Friedlander bacilli, or streptococci to phagocytosis in fluid systems (15, 16), staphylococci can be ingested by human or rabbit leukocytes in such systems as incubation proceeds and extracellular multiplication occurs.

It is our current belief that the factor present in normal human sera which promotes phagocytosis of pathogenic staphylococci may differ only quantitatively from the opsonin present in the serum of immunized rabbits studied by Cohn and Morse. While the phagocytosis-promoting activity of heat-inactivated human sera is *not* restored by the simple addition of fresh rabbit serum or small amounts of human serum or guinea pig complement, the finding that serum-treated staphylococci can be readily phagocytosed after heating suggests that the serum factor promoting phagocytosis is actually thermostable. The studies of Wright and Douglas (11) and Dean (12) characterizing "normal" and "immune" factors which promote phagocytosis of virulent staphylococci agree with this concept. Ward and Enders have indicated that much larger amounts of complement are required to restore the opsonic activity of heat-inactivated normal serum than are required to activate high titer immune sera (13). The partial reactivation of phagocytosis-promoting activity with large amounts of guinea pig complement and the ready phagocytosis of serum-treated staphylococci following heating is in keeping with this thesis. Absorption studies on normal human serum further indicate impressive specificity of the opsonin which is in every way similar to that observed in immune rabbit serum. It thus seems probable that differences in the amount of opsonin rather than its nature may explain the apparent differences in reactivation following destruction of complement.

The finding that the opsonizing activity of human serum can be inhibited by the addition of fresh or inactivated rabbit serum is not yet satisfactorily understood. Ecker, Pillemer, and Kuehn have similarly reported that the addition of

appropriate concentrations of "normal" sera from several animal species to anti-staphylococcal sera often inhibited the degree of phagocytosis of staphylococci observed in immune serum alone (17). It would currently seem possible that both phagocytosis-promoting and phagocytosis-inhibiting factors may be present in the same serum. The nature of this inhibition is receiving continuing study.

The present studies substantiate previous experiments suggesting that there are important differences in the behavior of coagulase-positive and coagulase-negative staphylococci within human granulocytes once ingestion has been accomplished. While large numbers of both coagulase-positive and coagulase-negative staphylococci are destroyed by leukocyte ingestion, virulent staphylococci consistently survive in greater numbers within the cytoplasm.

Cohn and Morse have indicated that many substances known to be present within leukocytes are bacteriocidal to both *Staphylococcus aureus* and *Staphylococcus albus* strains (1). It should be noted, however, that there is considerable evidence to indicate striking differences in the resistance of coagulase-positive and coagulase-negative strains to destruction by such substances. It has been shown, for example, that coagulase-positive strains are considerably more resistant to low pH in the presence of ketone bodies than are coagulase-negative strains (18). Pathogenic staphylococci are relatively resistant to the action of histone when compared with non-pathogenic strains (19). *Staph. albus* strains are more susceptible to the action of lysosyme than *Staph. aureus* strains (20). Acidic extracts of leukocytes have been shown to destroy coagulase-negative staphylococci while coagulase-positive strains survive similar treatment (21). Thus there is considerable ancillary evidence to suggest that coagulase-positive staphylococci may fare better within the cytoplasm of leukocytes than their coagulase-negative brethren.

Our own studies on staphylococcal bacteriemia in rabbits also lead us to believe that the intracellular survival of virulent staphylococci also plays a role in the maintenance of bacteriemia in this species. Table VI illustrates the striking differences in the host management of equal numbers of coagulase-positive staphylococci and a Type III pneumococcus administered intravenously and simultaneously to a single rabbit. As shown here, differential cultures indicate that culturable staphylococci are rapidly associated with the leukocytes and remain so during the phase of persisting bacteriemia. In contrast, viable pneumococci cannot be found in the leukocyte layer and can be cultured only from the plasma. This bacterium disappears completely from the rabbit circulation. These observations are supported by the recent report of Kapral and Shayegani who have also found that virulent staphylococci tend to survive within rabbit leukocytes while non-pathogenic strains are killed by ingestion (22).

It is increasingly apparent that wide variation in results can be obtained by minor variations in techniques used in studying the interaction of staphylococci and phagocytic cells. It thus seems probable that differences in the phagocytosis

and intracellular behavior of staphylococci reported from different laboratories (1-3, 11, 12, 17, 22-26) may be explained in part by differences in the experimental system employed. The concentrations of staphylococci or leukocytes in the test system, the presence or absence of surface factors promoting phagocytosis, the serum employed, the duration of the experiment, and probably other factors as yet unknown may profoundly modify the outcome of such studies.

Under the conditions of the present experiments it emerges that virulent staphylococci possess surface mechanisms which retard or prevent ingestion by human polymorphonuclear leukocytes in the absence of a specific opsonin present in many human sera. Once phagocytosis has been accomplished, pathogenic strains possess the ability to survive in greater numbers within the interior

TABLE VI

The Peripheral Blood Clearance and Plasma-Leukocyte Partitioning of Culturable Staphylococci and Pneumococci in the Rabbit Blood Stream

Time after injection	Staphylococcus (MAM)			Pneumococcus, Type III		
	Bacteria per ml.		Viable bacteria associated with WBC's (calculated)	Bacteria per ml.		Viable bacteria associated with WBC's (calculated)
	Total	Plasma supernatant		Total	Plasma supernatant	
<i>min.</i>			<i>per cent</i>			<i>per cent</i>
1	800,000	250,000	69	950,000	1,060,000	0
5	60,000	4,000	93	460,000	530,000	0
10	6,500	2,000	61	178,000	131,000	?27
20	2,000	100	95	13,000	13,000	0
90	760	50	93	<10	<10	0
150	320	<10	>95	<10	<10	0

of leukocytes than do non-pathogenic strains studied in similar systems.

One must suspect that these intracellular persisters have real biologic significance. Microbial survivors of intracellular residence may be better adapted to existence within human or animal tissues than microorganisms which have not withstood such intracellular passage.

SUMMARY

A specific serum factor was required for rapid phagocytosis of pathogenic staphylococci by human polymorphonuclear leukocytes when the ingestion process was studied in siliconed glass systems and the concentrations of staphylococci were maintained at low levels. In contrast to certain other microbes, the resistance to phagocytosis which characterized pathogenic staphylococci was relative, and phagocytosis was readily accomplished when large populations of staphylococci were present in the test system.

A factor promoting phagocytosis was present in eight of eight normal adult

sera. In contrast, the sera of twenty-eight of thirty normal rabbits did not promote phagocytosis. Serum obtained from 2 rabbits maintained in the rabbit colony for several months acquired the ability to opsonize pathogenic staphylococci.

The phagocytosis-promoting factor was almost completely removed by prior absorption of test sera with the homologous strain. The factor was incompletely removed by absorption with heterologous strains of pathogenic staphylococci and was not significantly reduced by absorption with coagulase-negative staphylococci or unrelated microorganisms.

Present evidence suggests that the factor promoting phagocytosis is a thermostable opsonin. While the activity of heated serum could not be restored by the addition of small amounts of fresh serum or complement, the addition of large amounts of complement partially restored opsonic activity. Incubation of staphylococci in fresh serum prior to heat inactivation did not reduce subsequent phagocytosis, further suggesting the heat stability of the phagocytosis-promoting factor.

Preliminary studies correlating the presence of antistaphylococcal hemagglutinins and phagocytosis-promoting factor in certain sera suggest that the two factors were not necessarily related.

The phagocytosis of staphylococci in fresh human serum was inhibited by the addition of fresh or inactivated rabbit serum. Further studies on the nature of such inhibition are in progress.

Once ingestion was accomplished, coagulase-positive staphylococci consistently survived in significant numbers within the cytoplasm of human granulocytes. Coagulase-negative staphylococci appeared to be destroyed within the leukocyte and could not be recultured from the cytoplasm following 3 to 4 hours of intracellular residence.

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