

THE SURVIVAL OF STAPHYLOCOCCI WITHIN HUMAN LEUKOCYTES*

BY DAVID E. ROGERS,† M.D., AND RALPH TOMPSETT, M.D.

(From the Department of Medicine, New York Hospital-Cornell Medical Center, New York)

PLATES 8 TO 10

(Received for publication, October 10, 1951)

The mechanisms whereby certain staphylococci are capable of initiating infection in man are poorly understood. Numerous investigations of staphylococci isolated from human infections have characterized certain biologic properties of these microorganisms as related to their pathogenicity. It has been considered that the local initiation of staphylococcal infection is aided by the necrotizing action of exotoxin produced by certain strains (1, 2). It has also been suggested that this process is promoted by the destruction of leukocytes as a consequence of the elaboration of "leukocidin" by staphylococci (3, 4). It is recognized, however, that staphylococci which elaborate no demonstrable exotoxin may produce local lesions indistinguishable from those produced by strains which elaborate a potent toxin (5). Moreover, immunization against toxin and leukocidin fails to prevent the development of staphylococcal abscesses, although the immunity may serve to modify certain of the other manifestations of the experimental infection (6).

A direct relationship appears to exist between the ability of a particular strain of staphylococcus to produce coagulase and the pathogenicity of that strain for man. The precise nature of this relationship, however, has not been clearly delineated. Spink and Vivino have shown (7) that coagulase-positive (*i.e.* coagulase-producing) strains of staphylococcus survive incubation in whole blood (human) whereas coagulase-negative strains fail to survive. The role of the coagulase mechanism in this is difficult to interpret, however, inasmuch as the phenomenon was observed in defibrinated blood which might be expected to provide very little if any fibrinogen as substrate for the action of coagulase.

Hale and Smith (8) have suggested that the action of coagulase may serve to protect staphylococci from phagocytosis and thereby aid in the initiation of infection. In support of this hypothesis, these investigators have reported

* This study was supported in part by grants from the division of Research Grants and Fellowships, Public Health Service; the Lederle Laboratories Division, American Cyanamid Co., Pearl River; and Charles Pfizer and Co., Brooklyn.

† Postdoctorate Research Fellow, National Institutes of Health, Public Health Service.

observations which indicate that in a coagulable medium (*i.e.* a medium containing fibrinogen and an "activator" substance) coagulase-producing staphylococci are less readily phagocytized than strains which produce no coagulase.

In the course of studies in this laboratory of the phagocytosis of staphylococci by human leukocytes, the observations of Hale and Smith were confirmed to the extent that the operation of the coagulase mechanism serves to decrease the phagocytosis of staphylococci which produce this enzyme. Nevertheless, quantitative observations on comparable numbers of coagulase-positive and coagulase-negative staphylococci failed to reveal differences in the degrees of phagocytosis between the two of a magnitude sufficient to appear a factor of major importance in explaining their differences in pathogenicity. In stained smears, however, the appearance of the microorganisms within the phagocytes suggested the possibility that coagulase-producing staphylococci might be able to survive within the leukocytes whereas those strains which do not produce coagulase might lack this ability.

Accordingly an investigation was made of the fate of "pathogenic"¹ (coagulase-positive) and "non-pathogenic" (coagulase-negative) staphylococci when exposed to human leukocytes *in vitro*. The evidence to be presented in this report indicates that pathogenic staphylococci are readily phagocytized *in vitro* by human leukocytes under conditions in which virulent pneumococci, group A streptococci, or Friedländer's bacilli are only rarely ingested. Evidence is also presented that pathogenic staphylococci can remain viable within human polymorphonuclear leukocytes whereas the phagocytosis of non-pathogenic staphylococci results in death of the bacterial cells.

EXPERIMENTAL

Human Leukocytes.—Human leukocytes were obtained from patients with high leukocyte counts and elevated erythrocyte sedimentation rates caused by a variety of diseases for which no antimicrobial therapy had been administered. Blood was obtained from the antecubital vein in sterile, warm (37°C.), silicone-coated syringes and needles containing appropriate amounts of 3 per cent sodium citrate to obtain a final concentration of 0.3 per cent citrate. The blood was then delivered to warm, vertical, silicone-coated glass tubes 12 mm. in diameter by 250 mm. in length which were coned abruptly at the bottom to allow insertion into a small length of rubber tubing which could be clamped. These tubes were placed in an incubator at 37°C. and the blood allowed to sediment for 30 to 45 minutes. The erythrocyte layer was then removed from the plasma by gently releasing the clamp and allowing the erythrocyte mass to drain from the bottom. The supernatant plasma remaining in the tube was rich in leukocytes and platelets and was relatively free of erythrocytes. In most instances, this plasma-leukocyte layer was used immediately for phagocytic studies without further preparation.

¹ In order to simplify the subsequent discussion, unless otherwise specified, the term "pathogenic staphylococci" is used to designate coagulase-producing *Staphylococcus aureus* strains obtained from infections in man. "Non-pathogenic staphylococci" is used to refer to non-producers of coagulase, *i.e.* *Staphylococcus albus* strains obtained from the air and body surfaces.

Total leukocyte counts were performed on such leukocyte suspensions in the routine manner, using standard leukocyte pipettes with Türk's solution as diluent.

Cultures.—The coagulase-positive pathogenic strains of staphylococci employed were derived from various infections in humans. Strains isolated in pure culture from local abscesses as well as strains isolated from blood cultures of patients with staphylococcal bacteremia. Some of the strains had been dried from the frozen state several years previously and preserved as stock laboratory strains. All pathogenic strains produced golden yellow pigment on agar medium, were hemolytic on rabbit blood agar, fermented mannitol, and were coagulase-positive. Repeated determinations of these properties were performed at intervals on all strains to ensure that they were maintained.

Non-pathogenic coagulase-negative strains of staphylococci were isolated from air, normal skin, normal throats, and in one instance from a sebaceous cyst. These strains produced white colonies on plain agar, and were occasionally only slightly hemolytic on rabbit blood agar. None fermented mannitol and all were coagulase-negative.

Other bacteria used in these experiments were the following: (a) *Pneumococcus* Type I (strain PZI, highly virulent for mice); (b) *Streptococcus hemolyticus* (C203 MV); (c) *Klebsiella pneumoniae* (AD, mouse virulent²).

For studies of phagocytosis, 4 hour broth cultures of staphylococci were employed. In studies of intraleukocytic survival of microorganisms, broth cultures incubated 2, 4, and 18 hours were employed, as well as washed suspensions from agar slants. These variations in type of culture were found to have no significant effect on the results obtained.

Coagulase Test.—The coagulase test was performed in the following manner: 0.5 ml. of 18 hour broth cultures of the strains to be tested was added to 0.5 ml. of stock human plasma in serologic tubes. The tubes were thoroughly mixed and incubated at 37°. Any degree of clotting occurring over 18 hours' incubation was regarded as a positive reaction.

Phagocytosis.—Studies of phagocytosis and survival of staphylococci were performed in 12 by 100 mm. warmed, sterile, silicone-coated test tubes. Known numbers of microorganisms were added to plasma-leukocyte mixtures. The contents of the tubes were then mixed by pipette and an aliquot removed for appropriate initial determinations. The tubes were then stoppered with sterile paraffined rubber corks and placed horizontally in holders on 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 and samples obtained, after mixing, for the various determinations.

Stained Preparations.—Duplicate coverslip smears prepared from phagocytic studies were dried and stained immediately with Wright's stain or immediately fixed in methyl alcohol to be subsequently stained by the Gram-Weigert technique.

Enumeration of Bacteria.—In the majority of instances, determinations of the numbers of bacteria were performed with the conventional technique of making pour plates (3 per cent infusion agar) with aliquots of appropriate serial dilutions of the unknown sample. In some experiments, microorganisms were also counted directly in a Petroff-Hausser bacterial counting chamber. For this purpose a 1:2 dilution of the sample was made in 1 per cent agar containing 1 per cent carbolfuchsin, which was kept at 45°C. until used. This procedure was found useful as a check on the dilution pour plates, and for standardization of young cultures before use. The method also permitted accurate counting of the numbers of individual cocci including those situated in small clumps.

Slide Cultures.—In studying the survival of staphylococci within leukocytes, a technique described by Smith and Wood (9) was utilized with minor modifications. Human leukocytes

² Obtained through the courtesy of Dr. Harold White, of the Research Laboratories, American Cyanamid Co., Stamford, Connecticut.

were allowed to ingest organisms over a period of 20 to 30 minutes in the system described above. At this time the plasma-leukocyte mixtures containing bacteria were centrifuged at 1200 R.P.M. for 3 minutes. The supernatant plasma was discarded and the leukocytes were twice centrifuged in warm, cell-free plasma from the same donor in an attempt to remove extracellular bacteria. A drop of the leukocyte mixture was spread between coverslips and allowed to dry for 30 to 60 seconds. Each coverslip was then inverted upon a drop of melted agar (45°C.) containing trypan blue which had been placed on the surface of a warmed, sterile slide. Capillary action caused the agar to spread out rapidly to the edges of the coverslip, embedding the leukocytes against the under surface. The edges of the preparation were sealed with paraffin and the resultant slide culture incubated at 37°C. The suspensions of living leukocytes containing ingested bacteria remaining in the tube were kept at 37°C. without rotation, and samples were removed over a number of hours and incorporated in slide cultures. The leukocytes were rewashed at 3 hours with cell-free plasma from the same donor, in an attempt to remove any extracellular organisms which might have multiplied during this period.

The single modification of the technique as described by Smith and Wood was that of allowing the coverslips to dry momentarily before incorporation in slide cultures. It was found that such drying caused the death of all leukocytes as determined by rounding, loss of motility, and deep staining of the nucleus with trypan blue. In contrast, 50 to 75 per cent of unphagocytized staphylococci survived this momentary drying in control experiments. In such preparations, when observed under the oil immersion lens, leukocytes were readily identified, showing deep blue nuclei and dark blue cytoplasmic granules. The staphylococci were clearly visible within the cytoplasm as refractile, translucent bodies of characteristic shape and size. Control experiments showed that trypan blue in the concentrations used had no effect upon the growth of staphylococci.

The trypan blue agar was prepared by mixing equal parts of 3 per cent agar in double infusion broth with 1 per cent trypan blue. In these concentrations, it is believed that trypan blue does not enter the nuclei of living leukocytes but remains within the cytoplasm (10). Upon injuring the leukocyte, the nucleus immediately stains deeply with trypan blue.

Comparative Phagocytosis of Staphylococci and Other Microorganisms by Human Leukocytes

In a series of experiments performed as described above, the phagocytosis of various strains of staphylococci and other bacteria was studied. The bacteria were harvested from 4 hour tryptose-phosphate broth by centrifugation, resuspended in normal saline solution, and adjusted to equal turbidities, or to equal numbers as determined by direct count in a counting chamber. The suspensions were then added to silicone-coated tubes containing equal numbers of human leukocytes in plasma from a single donor. The tubes were placed on a rotating drum at 37°C., and aliquots removed at stated intervals. Total leukocyte counts were made, and counts of 100 to 300 polymorphonuclear leukocytes were performed on stained coverslip preparations. The percentage of leukocytes containing bacteria as well as the total number of bacteria per 100 leukocytes was recorded.

In experiments with defibrinated plasma, blood was obtained in a silicone-coated syringe which contained no anticoagulant. This blood was delivered to a small Erlenmeyer flask containing glass beads and gently swirled until clotting was complete. Sodium citrate solution was then added to a final concentration of 0.3 per cent and the blood was centrifuged at 3000 R.P.M. for 30 minutes to obtain cell-free defibrinated plasma.

For comparison of whole plasma-leukocyte mixtures with defibrinated plasma-leukocyte mixtures the following procedure was employed: The plasma-leukocyte layer was obtained from citrated blood in the usual fashion and centrifuged at 1200 R.P.M. for 2 to 5 minutes in

silicone-coated tubes. The supernatant plasma was removed and the leukocytes washed twice in normal saline solution containing 0.3 per cent sodium citrate. The leukocytes were then resuspended in a small volume of normal saline solution and equal volumes added to cell-free whole plasma and cell-free defibrinated plasma from the same donor.

The Effect of Fibrin on the Phagocytosis of Pathogenic and Non-Pathogenic Staphylococci.—When human leukocytes were allowed to ingest coagulase-positive staphylococci in plasma, conspicuously fewer cocci were ingested per 100 leukocytes in whole plasma than in defibrinated plasma. As can be seen from the data in Table I, after phagocytosis had proceeded for 20 minutes, the av-

TABLE I
*Effect of Fibrin on the Phagocytosis of Staphylococci by Human Polymorphonuclear Leukocytes in Citrated Plasma**

Strains of staphylococci		Mean No. of cocci per polymorphonuclear leukocyte†		Ratio Defibrinated plasma Whole plasma
		Whole plasma	Defibrinated plasma	
Coagulase positive strains	Po	2.1	3.9	1.8
	Ba	3.9	4.7	1.2
	Ha	3.1	6.9	2.2
	Pe	2.2	4.0	1.8
Coagulase negative strains	Sh	1.8	1.4	0.7
	Sp	1.6	1.0	0.6
	Ad	5.1	5.9	1.1
	On	7.4	4.9	0.6

* All cultures were adjusted turbidimetrically to contain approximately 5×10^7 microorganisms/ml. The period of incubation in each instance was 20 minutes at 37°C.

† Based on counts of 100 to 200 polymorphonuclear leukocytes.

erage number of cocci ingested by each leukocyte was regularly greater in defibrinated plasma than in whole plasma, and at times the average was twice as great in the absence of fibrin as in the whole plasma. In contrast, the phagocytosis of non-pathogenic strains producing no coagulase was approximately the same in both systems, and occasionally even less in the defibrinated system. These observations were in agreement with those of Hale and Smith (8). As also may be seen from the data in Table I, however, the differences in degree of phagocytosis of coagulase-positive staphylococci in the two systems was never so great as threefold and under the conditions of these experiments the absolute numbers of pathogenic staphylococci ingested were impressive even in whole plasma. Moreover, in those experiments in which pathogenic and non-pathogenic strains were employed in equal numbers, the average numbers of each ingested by leukocytes in whole plasma were approximately the same (Table I). Similar

relationships were obtained in determinations made over periods of rotation up to 6 hours and with variations in inocula which ranged between 1 million and 400 million bacteria per ml. These observations differed from the reported behavior of virulent strains of pneumococci or streptococci in glass systems, the phagocytosis of which has been reported to be strikingly less than that of avirulent strains (11, 12).

It was further observed in stained slides prepared both from systems containing fibrin and from defibrinated systems, that ingested pathogenic staphylococci were stained clearly and distinctly regardless of the length of the rotation period. Examination of coverslip preparations obtained after 3 to 4 hours of rotation revealed apparently intact microorganisms in the debris of destroyed leukocytes. In contrast, non-pathogenic staphylococci which had been phagocytized were less distinctly stained than the rare microorganisms which were situated extracellularly. As the time of incubation was increased, many leukocytes containing non-pathogenic staphylococci showed tiny Gram-negative granules within their cytoplasm adjacent to faintly staining microorganisms. This difference between the appearance of the stained pathogenic and non-pathogenic staphylococci can be seen in Figs. 1 and 2.

The Comparative Phagocytosis of Pathogenic Staphylococci and Other Virulent Microorganisms.—In a second series of experiments the phagocytosis by human leukocytes of pathogenic staphylococci was compared with that of virulent pneumococci, group A streptococci and *Klebsiella pneumoniae* in whole plasma under conditions the same as those described above. In all instances, microscopic examination of such preparations taken at intervals up to 6 hours revealed that large numbers of pathogenic staphylococci were ingested by polymorphonuclear leukocytes, monocytes, and in some instances by eosinophiles. Only a few staphylococci could be detected in an extracellular location. In contrast, pneumococci, streptococci, and *Klebsiella* were rarely ingested in this situation but were almost entirely found clustered about the margins of the leukocytes. A representative example of these findings may be seen in Fig. 3.

It was also noted that the occasional pneumococcus, streptococcus, or Friedländer's bacillus which had been ingested by a phagocyte, took the stain poorly and had indistinct cellular outlines, while the stained intracellular staphylococci presented a sharply outlined homogeneous appearance. In preparations examined after phagocytosis had been allowed to proceed for 3 to 4 hours, leukocytes which contained pathogenic staphylococci were frequently broken and disrupted with large numbers of clearly staining microorganisms in the areas of cellular debris (see Fig. 4). This was not seen in preparations containing virulent pneumococci, streptococci, or *Klebsiella*. Attention was thus focussed on the fate of staphylococci within leukocytes and a series of experiments were conducted to study this problem.

The Rate of Disappearance of Culturable Pathogenic and Non-Pathogenic Staphylococci in Human Plasma-Leukocyte Mixtures

The rate of disappearance of pathogenic and non-pathogenic staphylococci from human plasma-leukocyte mixtures was determined by periodic colony counts with the pour plate technique in a series of 15 experiments. Pathogenic and non-pathogenic staphylococci in equal numbers were added to fresh human leukocytes in plasma or defibrinated plasma in warmed silicone-coated tubes. The contents of each tube were mixed by pipette and an aliquot immediately removed for smears, total leukocyte count, and dilution pour plates. In some experiments a measured aliquot was added to an equal amount of trypan blue in normal saline solution to estimate the number of leukocytes killed during incubation.³ The tubes were sealed with paraffined rubber stoppers and placed on the rotating drum in the incubator. Subsequently the tubes were removed at appropriate intervals for sampling and the rotation then resumed.

TABLE II
The Rate of Fall in the Number of Culturable Staphylococci Exposed to Decreasing Numbers of Human Leukocytes

Staphylococcus . . .	Pathogenic	Non-pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Non-pathogenic	Pathogenic
Leukocytes/mm. ³	37,800	17,400	16,500	15,900	13,100	2,300	None	None	None
Time of incubation	No. of culturable microorganisms per ml.								
<i>min.</i>									
0	3.4×10^7	2.7×10^7	2.9×10^7	3.0×10^7	3.4×10^7	3.4×10^7	3.0×10^7	2.7×10^7	2.3×10^7
1		2.0×10^7	2.6×10^7	3.0×10^7			3.3×10^7	2.7×10^7	2.4×10^7
5	1.5×10^7	1.8×10^7	2.3×10^7	3.0×10^7	3.2×10^7	3.2×10^7			2.3×10^7
10	6×10^6	1.7×10^7	1.8×10^7	1.1×10^7	1.5×10^7	1.6×10^7	3.8×10^7	3.4×10^7	2.0×10^7
15	4×10^6	8×10^6	5×10^6	6×10^7	1.0×10^7	1.4×10^7	3.4×10^7	3.6×10^7	2.0×10^7

Following introduction of pathogenic or non-pathogenic staphylococci in human plasma-leukocyte mixtures a rapid fall in the numbers of culturable staphylococci ensued. Results of two representative experiments are presented in Text-figs. 1 and 2. The speed and magnitude of the decline in the number of culturable staphylococci appeared to be directly related to the numbers of leukocytes present in the system. In the absence of leukocytes, the numbers of culturable pathogenic or non-pathogenic staphylococci remained relatively constant, then increased (Table II).

The onset of the decline in the number of culturable staphylococci was gen-

³ It has been reported that in the concentration used in these experiments, trypan blue is taken up in the cytoplasm of living phagocytes but cannot be detected in the cell nucleus until death of the phagocyte has occurred (10). In the present experiments, a drop of trypan blue leukocyte mixture was placed on a warmed slide, a coverslip immediately placed upon it, the edges sealed with paraffin, and the preparation promptly examined under the microscope. One hundred to two hundred polymorphonuclear leukocytes were counted and the percentage with dye within the nucleus was recorded as the index of leukocyte death.

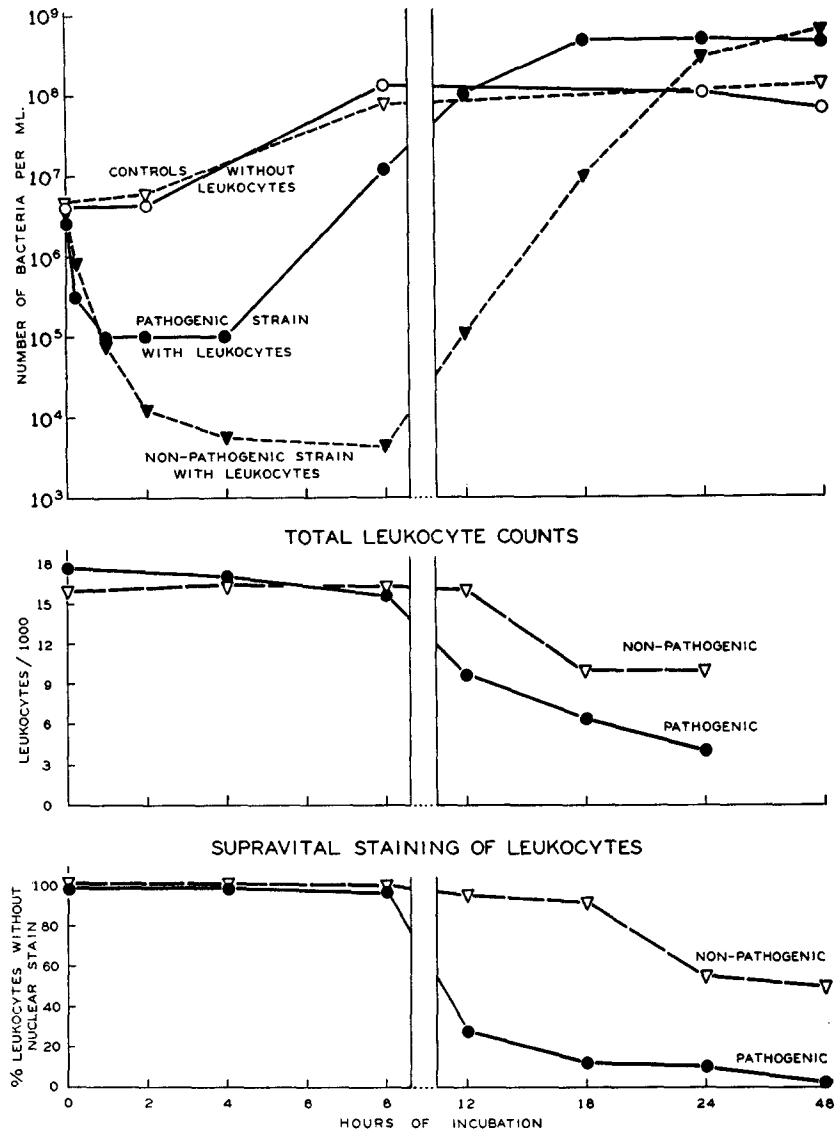
erally evident 10 minutes after their introduction into the plasma-leukocyte mixtures. Observations at 1 minute and 5 minutes after introduction of the microorganisms into the phagocyte suspensions usually revealed no significant or no more than a minimal decline in the bacterial population from the original values (Table II).

The time of onset and the initial rate of decline in the number of culturable staphylococci when exposed to leukocytes was the same for both pathogenic and non-pathogenic strains. The subsequent behavior of the two forms of staphylococci, however, was distinctly different. With the pathogenic staphylococci, the decline in population continued for a period which usually ranged between 2 and 4 hours although in a few experiments the lowest number of culturable microorganisms occurred at 8 hours. At the end of this period there was a rapid increase in the number of viable staphylococci and the growth curves then assumed a logarithmic form (Text-figs. 1 and 2). With the non-pathogenic staphylococci the fall in number of microorganisms continued steadily for as long as 12, 18, and even 24 hours prior to the initiation of logarithmic growth. As a consequence, the total fall in the number of viable non-pathogenic staphylococci was always greater than that of the pathogenic staphylococci. As can be seen in Text-figs. 1 and 2, the magnitude of the fall in numbers of non-pathogenic staphylococci was approximately 20 times that observed for the pathogenic strains, and was at times as much as 400 times that of the pathogenic strains.

Final Growth Attained in Plasma-Leukocyte Mixtures and in Plasma without Leukocytes.—Although no decrease in the number of culturable staphylococci was observed in plasma in the absence of leukocytes, growth was sluggish in this system and the populations increased only moderately during a 24 or 48 hour period (Text-figs. 1 and 2). In contrast, in plasma containing leukocytes, once growth became evident, it proceeded logarithmically, and the population eventually exceeded that of the plasma control tubes.

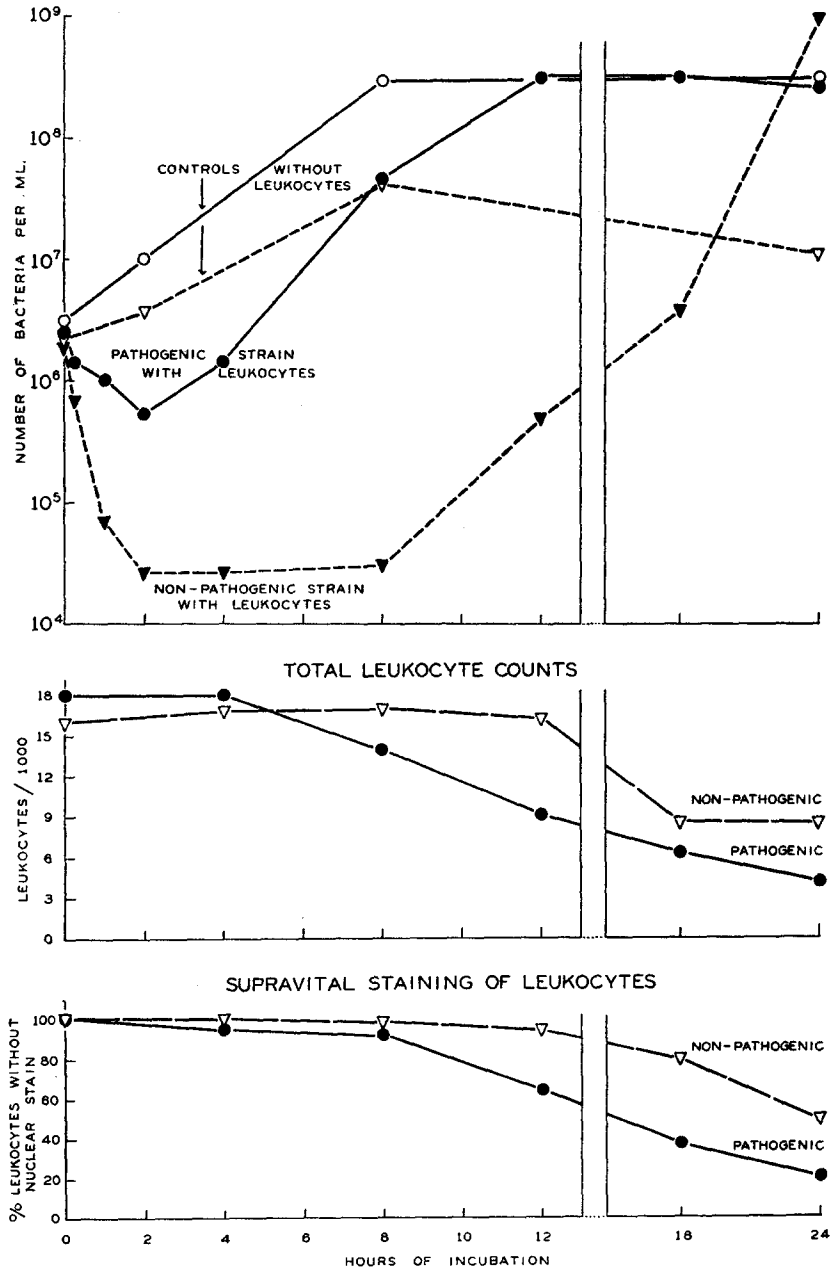
Total Leukocyte Counts.—Total leukocyte counts were performed on aliquots of the leukocyte-plasma mixtures at intervals throughout the period of incubation. In the mixtures to which pathogenic staphylococci had been introduced there was a steady fall in the total leukocyte count. In the plasma-leukocyte mixtures which contained non-pathogenic staphylococci, the total leukocyte counts remained relatively constant during the first 12 to 18 hours of incubation with a relatively rapid fall in the counts thereafter (Text-figs. 1 and 2).

Survival of Leukocytes as Determined by Supravital Trypan Blue Staining.—Aliquots from plasma-leukocyte mixtures were also examined in supravital preparations which contained trypan blue. 100 to 300 polymorphonuclear leukocytes were counted and the percentage of leukocytes containing trypan blue within the nucleus was recorded. 8 to 12 hours after introduction of the pathogenic staphylococci, many of the leukocytes had trypan blue within the nuclei



TEXT-FIG. 1. The comparative survival of pathogenic and non-pathogenic staphylococci during incubation in plasma-leukocyte mixtures, together with the changes in total leukocyte count and in the proportion of the leukocytes damaged, as indicated by nuclear staining with trypan blue.

and at 18 to 24 hours, nuclear staining was visible in 70 to 90 per cent of the small number of phagocytes which were still present. In contrast to these findings, leukocytes which had been incubated with strains of non-pathogenic

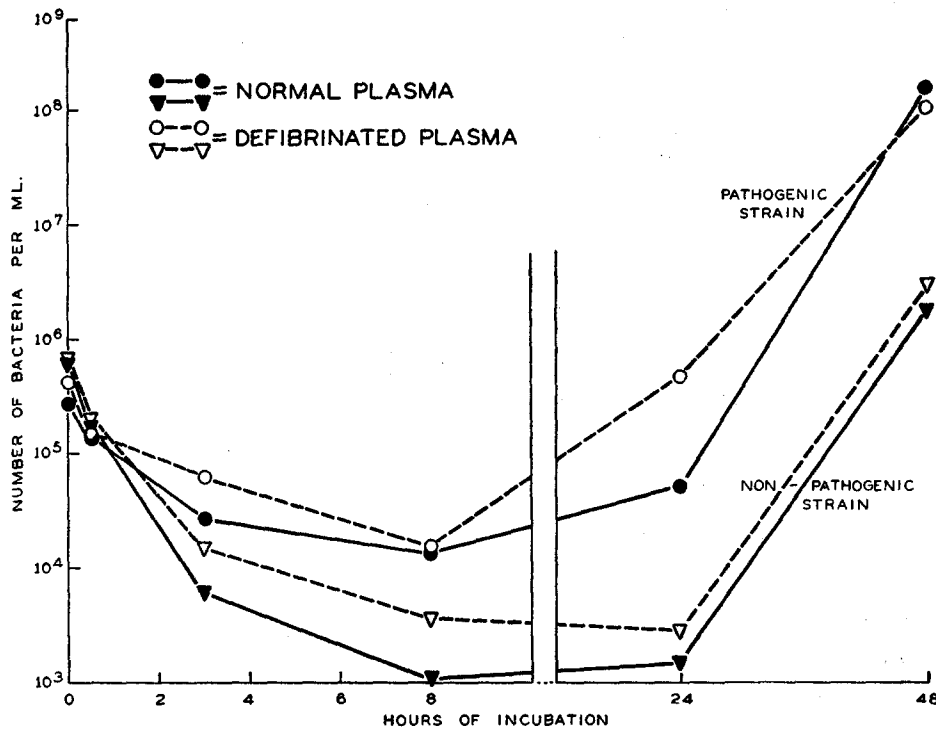


TEXT-FIG. 2. The comparative survival of pathogenic and non-pathogenic staphylococci during incubation in plasma-leukocyte mixtures, together with the changes in total leukocyte count and in the proportion of the leukocytes damaged, as indicated by nuclear staining with trypan blue.

staphylococci were more plentiful and seldom revealed nuclear staining before 18 hours. At 24 hours the dye was visible in the nuclei of 10 to 50 per cent of the cells.

Influence of Fibrin on the Disappearance of Culturable Staphylococci Exposed to Human Leukocytes

It was not possible to demonstrate that the rate or magnitude of the fall in numbers of either pathogenic or non-pathogenic staphylococci was influenced by prior removal of fibrin from the plasma. Studies of survival conducted



Text-Fig. 3. The rate of disappearance of viable staphylococci during incubation in plasma-leukocyte mixtures, and the failure to influence the comparative survival of pathogenic or non-pathogenic strains by removal of fibrin from the plasma.

simultaneously in normal and defibrinated plasma-leukocyte mixtures gave comparable results. The data from such an experiment are presented graphically in Text-fig. 3. As can be seen, a pathogenic strain of staphylococci exhibited a twentyfold fall in number of culturable microorganisms in both fibrin containing and defibrinated systems in contrast to an 800-fold decrease in the number of culturable non-pathogenic staphylococci.

Investigation of Other Possible Mechanisms for the Observed Decrease in Viable Staphylococci in Plasma-Leukocyte Mixtures

The known ability of polymorphonuclear leukocytes to ingest and destroy bacteria made it seem likely that the early decline in the number of viable staphylococci observed was due to their phagocytosis by leukocytes and subsequent death. Among the other possible mechanisms whose operation might account for the observed results, the following two were subjected to study: (a) the possibility that aggregation of cocci occurred in the presence of leukocytes with consequent reduction in the number of viable units; (b) the possibility that intracellular staphylococci though still viable might be unable to initiate growth while situated within the leukocyte.

TABLE III
The Survival of Staphylococci during Incubation with Human Leukocytes
Comparative Values Obtained with Two Methods of Enumeration

Time, min.....		No. of organisms per ml. after indicated period of incubation							
		0		15		45		100	
Strains		Dilution pour plates	Bacterial counting chamber	Dilution pour plates	Bacterial counting chamber	Dilution pour plates	Bacterial counting chamber	Dilution pour plates	Bacterial counting chamber
Pathogenic	1	3.4×10^8	3.2×10^8	3.1×10^7	4.8×10^7	2.0×10^7	1.0×10^7		
	2	*	3.0×10^8	1.0×10^8	1.0×10^8	1.6×10^7	3.2×10^7		
	3	3.3×10^8	5.3×10^8	1.1×10^8	1.1×10^8	6.7×10^7	2.6×10^7	4.0×10^7	2.3×10^7
Non-pathogenic	1	4.2×10^8	5.6×10^8	3.5×10^7	5.0×10^7	1.0×10^7	8×10^6		
	2	3.3×10^8	3.7×10^8	1.2×10^8	1.2×10^8	5.8×10^7	6.8×10^7		
	3	3.5×10^8	4.5×10^8	*	*	*	*	3.4×10^7	2.3×10^7

* No determination made.

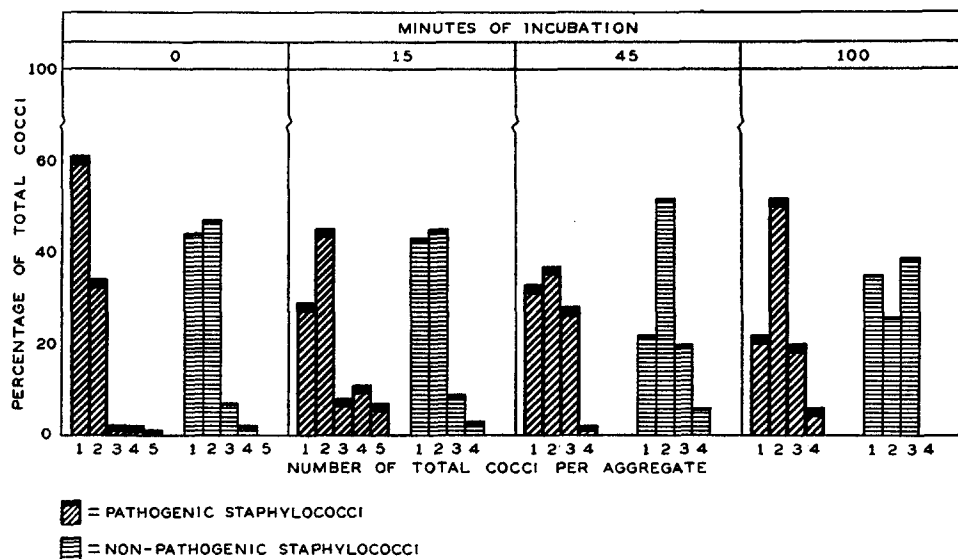
Aggregation of Staphylococci.—The possibility that a significant degree of bacterial clumping was responsible for the apparent rapid reduction in culturable staphylococci was investigated by use of the Petroff-Hausser bacteria counting chamber.

The microorganisms were added to the plasma-leukocyte mixtures as in the previous experiments and aliquots were removed at intervals. The numbers of bacteria in the aliquots were measured both by direct enumeration of individual cells in the counting chamber and by the pour plate technique.

As can be seen in Table III, at all periods of sampling there was close agreement between the number of staphylococci enumerated as individual cells in the counting chamber and the number of colonies which appeared on the pour plates. With both methods of measurement the same rapid ten- to twentyfold decrease in staphylococci upon introduction of the bacteria into plasma-leukocyte mixtures was observed. The great majority of the staphylococci seen in the

counting chamber were present as individual cells or as diplococci although clumps containing four or five microorganisms were occasionally noted. Aggregates consisting of more than six to eight microorganisms were rarely seen. During the incubation period, no significant change in the distribution of organisms in clumps occurred (Text-fig. 4).

Viability of Microorganisms Following Disruption of Leukocytes.—The possibility existed that staphylococci, although viable, might be unable to initiate growth while situated within the leukocytes. When the total number of intra-



TEXT-FIG. 4. The distribution of staphylococci in bacterial units during incubation with human leukocytes. Note that there is no significant increase in bacterial aggregates containing four or more cocci for either strain during incubation for 100 minutes.

cellular cocci at each sampling period was calculated from the total leukocyte count and the number of microorganisms per leukocyte as determined on stained preparations, it appeared likely that phagocytized staphylococci gave rise to colonies on pour plates. In order to investigate this question further, however, a series of experiments were performed in which the leukocytes were intentionally disrupted before the plasma-leukocyte-staphylococci mixtures were cultured.

Staphylococci were added to plasma-leukocyte mixtures as in the preceding experiments and the occurrence of phagocytosis was determined by examination of stained smears. At appropriate intervals aliquots were removed and the leukocytes in one aliquot were disrupted by grinding with cold sterile sand in an iced mortar. In control experiments it was shown that unphagocytized staphylococci survived such grinding and no intact leukocytes could be detected following the grinding procedure. The samples obtained at each interval were cultured

by the pour plate technique and the number of colonies which developed from the ground and the unground specimens were compared.

In a series of five such experiments, no significant difference was found in the number of culturable staphylococci obtained from the ground and the unground samples. The data obtained in such an experiment are presented in Table IV. As can also be seen from these data, both pathogenic and non-pathogenic strains of staphylococci behaved in identical fashion. As in the previous experiments, the non-pathogenic strains showed a decrease in culturable units twenty times greater than that of the pathogenic strains during the 3 hour period of the experiment.

TABLE IV
The Survival of Staphylococci during Incubation with Human Leukocytes
Comparison of plate counts obtained from ground and unground aliquots.

Length of incubation	Plate counts pathogenic staphylococci		Plate counts non-pathogenic staphylococci	
	Control sample	Ground sample	Control sample	Ground sample
<i>min.</i>				
0	2.6×10^6	2.8×10^6	4.5×10^6	4.5×10^6
20	1.9×10^6	1.9×10^6	4.4×10^6	4.0×10^6
60	3.2×10^5	3.5×10^5	5.3×10^4	5.5×10^4
180	3.7×10^5	2.8×10^5	2.8×10^4	2.5×10^4

*Differences in Behavior of Pathogenic and Non-Pathogenic Staphylococci
Subsequent to Phagocytosis*

Comparison of the appearance of pathogenic and non-pathogenic staphylococci within leukocytes had suggested the possibility that pathogenic strains might be able to survive phagocytosis better than non-pathogenic strains. As such a difference between the two forms of staphylococci might afford an explanation for the observed difference in the speed of resurgence of growth following the initial fall, it seemed pertinent to investigate the problem further.

Leukocytes were incubated with staphylococci in tubes on the rotating drum for 20 to 30 minutes to permit phagocytosis to take place. The leukocytes were then washed in cell-free plasma from the same donor to remove as many extracellular microorganisms as possible, and allowed to stand without rotation in plasma at 37°C. Washings were repeated at 3 hour intervals. Stained coverslip preparations were made at appropriate intervals for determination of the numbers of polymorphonuclear leukocytes which contained microorganisms. Unstained, sterile coverslip preparations were made at the same intervals and were allowed to dry for 30 to 60 seconds before incorporation in thin agar slide cultures containing 0.5 per cent trypan blue. This procedure was adopted after it was discovered that the momentary drying of the coverslip caused the death of all leukocytes as determined by rounding, loss of motility, and deep staining of nuclear with trypan blue, whereas in control experiments, 50 to 70 per cent of unphagocytized staphylococci survived the momentary drying. In other control experiments

it was demonstrated that trypan blue in the concentrations used had no demonstrable effect upon the growth of unphagocytized staphylococci.

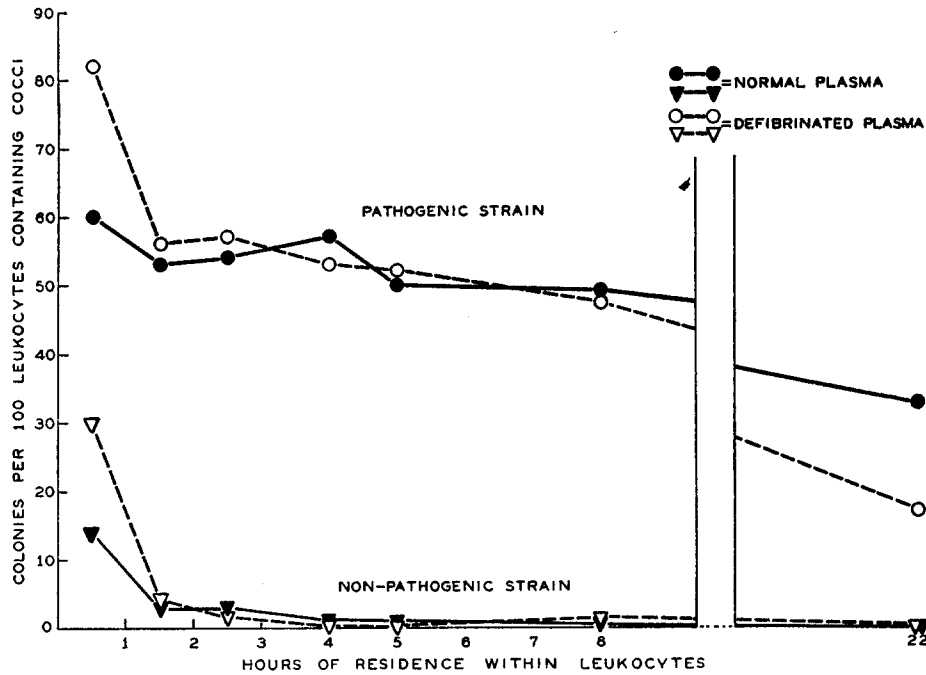
The experiments were thus designed so that staphylococci situated within leukocytes were incubated for progressively increasing periods of time. At the end of each stated preliminary incubation period the leukocytes were then killed abruptly, and the ability of the previously phagocytized bacteria to multiply was tested by observation of their behavior in the slide cultures.

Nineteen strains of hemolytic coagulase-positive *Staphylococcus aureus* isolated from human infections and nine strains of coagulase-negative *Staphylococcus albus* isolated from air, the skin, and mucosal surfaces, were studied in this fashion. Striking differences were observed between pathogenic and non-pathogenic strains of staphylococci. When microorganisms had remained intracellularly for 10 to 30 minutes before incorporation within slide cultures, only moderate differences between the intraleukocytic survival of pathogenic and non-pathogenic strains were noted. In these circumstances, 44 to 100 per cent of polymorphonuclear leukocytes which contained pathogenic staphylococci revealed the presence of colonies of bacteria while 10 to 49 per cent of leukocytes containing non-pathogenic strains were the site of microbial growth. As the time of intracellular residence was increased, the percentage survival of non-pathogenic strains fell rapidly, however, and approached zero when the microorganisms had been subjected to the action of the phagocytes for as long as 2 to 3 hours before incorporation in the slide cultures. In contrast, pathogenic strains of staphylococci continued to display the ability to form colonies in the killed leukocytes despite previous residence within living leukocytes for periods as long as 22 hours. In Text-fig. 5, can be seen the respective percentages of surviving pathogenic and non-pathogenic staphylococci plotted against the time of residence within leukocytes with two of the twenty-eight strains so tested.

In Table V, it can be seen that significant numbers of all strains of staphylococci isolated from human infections survived for periods of at least 4 hours within polymorphonuclear leukocytes. In contrast, only one strain of *Staphylococcus albus* (C.A.) survived in significant numbers beyond 4 hours. This strain was of particular interest. It had been isolated in pure culture from a sebaceous cyst found to be filled with purulent exudate although no signs of acute inflammation had been present. Thus, in terms of the production of lesions in the human, strain C.A. may represent a degree of pathogenicity intermediate between the other two large groups.

Appearance of the Microorganisms in Slide Cultures.—When examined with a warm stage microscope the multiplying cocci appeared as translucent, refractile bodies which could be observed throughout the process of division within the cytoplasm of the leukocytes. The microorganisms rapidly filled the cytoplasm of the phagocyte and eventually extended beyond the visible cell bound-

aries. In leukocytes containing non-multiplying microorganisms the staphylococci appeared to take up the trypan blue and were stained a color which was easily recognizable against the background of the darker, smaller cytoplasmic granules. Only colonies which clearly originated within the cytoplasm of polymorphonuclear leukocytes were enumerated in the comparative studies (Table V). Colonies which had become so large as to obscure cellular outlines



TEXT-FIG. 5. The intraleukocytic survival of staphylococci. These data indicate the marked difference between pathogenic and non-pathogenic staphylococci in their ability to initiate growth in artificial culture medium after residence within human polymorphonuclear leukocytes. Again the results were essentially the same in whole plasma and in defibrinated plasma.

were not included. Representative examples of pathogenic and non-pathogenic staphylococci as observed in the slide cultures can be seen in Fig. 5.

Stained Preparations.—In the stained coverslip preparations, counts of the number of visibly stained microorganisms within polymorphonuclear leukocytes were made at each sampling period. The number of visibly stained microorganisms contained within 100 polymorphonuclear cells remained essentially constant during the period of incubation in tubes containing pathogenic staphylococci. The numbers of stained microorganisms which could be seen within leukocytes fell progressively in tubes containing non-pathogenic strains of staphylococci. Data from a typical experiment are presented in Text-fig. 6.

Supravital preparations stained with trypan blue were made on samples taken after the plasma-leukocyte-staphylococci mixtures had been rotated at 37°C. for 2 to 4 hours. In these, an occasional leukocyte with unstained nuclei

TABLE V
The Intraleukocytic Survival of Staphylococci
Colonies per 100 leukocytes containing organisms.

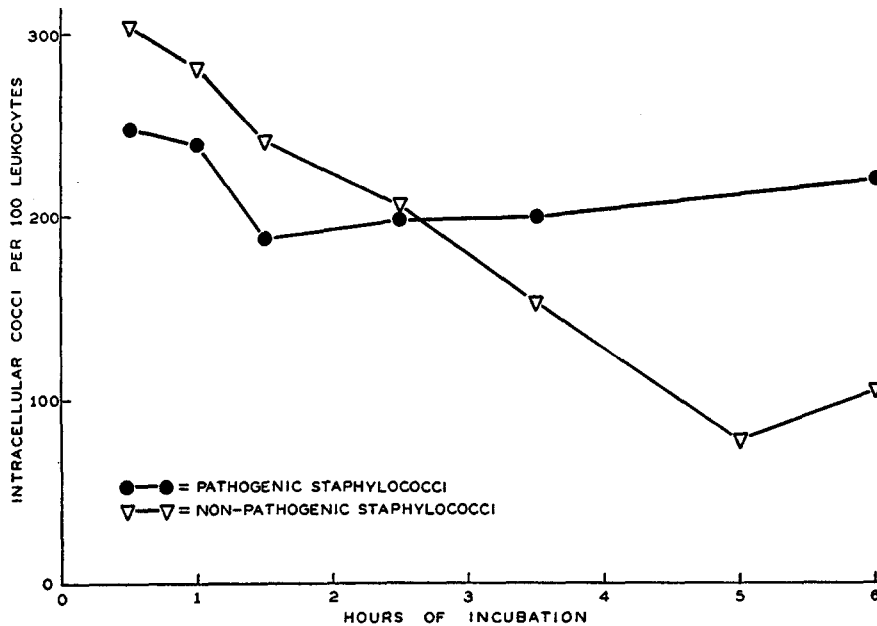
Strain	Source	Coagulase	Time of residence within PMN	
			Less than 1 hr.	4-5 hrs.
Gi	Osteomyelitis	+	60	57
Po	Endocarditis	+	80	47
Pe	Pneumonia-empyema	+	84	52
Hav	Pneumonia-empyema	+	62	56
Ba	Endocarditis	+	50	29
Oh	Osteomyelitis	+	100	44
Glo	Endocarditis	+	77	30
Mi	Empyema	+	96	96
Lo	Osteomyelitis	+	82	35
Sne	Bacteriemia	+	80	33
Row	Osteomyelitis	+	100	100
Row	Osteomyelitis	+	95	71
Haa	Osteomyelitis	+	100	64
Mc	Pneumonia	+	62	39
Sw	Empyema	+	79	70
Wh	Endocarditis	+	95	40
Wi	Bacteriemia	+	87	33
S.A. No. 235*	Osteomyelitis	+	44	35
Sh	Furuncle	+	57	52
Du	Skin	-	18	2
MAM	Skin	-	43	3
Air	Air	-	10	1
CA	Sebaceous cyst	-	43	13
Sp	Skin	-	12	0
Ou	Nose	-	17	4
Ma	Skin	-	49	9
Ca	Skin	-	16	5
Sc	Skin	-	11	0

*Obtained through the courtesy of Dr. W. B. Wood, Jr., Washington University School of Medicine, St. Louis. Although originally obtained from a patient with osteomyelitis, this strain has been maintained in the laboratory for many years because of its high virulence for mice.

and a moderate number of leukocytes with stained nuclei were found to be bulging with microorganisms. In some instances, the phagocytized microorganisms could be observed throughout the process of cellular division.

The possibility could not be eliminated that during the incubation period

leukocytes occasionally ingested an extracellular microorganism not removed in the washings. From the control studies, however, in which moderate numbers of microorganisms (100,000 per ml.), were incubated without rotation in identical tubes with human leukocytes it appeared that such continued uptake of microorganisms was probably negligible. Less than one per cent of polymorphonuclear leukocytes were found to contain staphylococci after 4 hours of incubation under these conditions.



TEXT-FIG. 6. The number of visibly stained intracytoplasmic staphylococci per 100 leukocytes following increasing periods of incubation.

Intraleukocytic Survival of Pathogenic and Non-Pathogenic Staphylococci in Defibrinated Systems

The above experiments on the viability of pathogenic and non-pathogenic staphylococci following various periods of exposure to leukocytes in plasma were repeated in both defibrinated and in normal plasma. As can be seen in Text-fig. 5, neither the presence nor the absence of fibrinogen during the period of exposure to the leukocytes exerted any influence on the capacity of the microorganisms to survive phagocytosis as determined by subsequent slide cultures. In either situation, a pathogenic staphylococcus retained the ability to multiply in significant numbers after 22 hours exposure to leukocytes whereas an intracellular non-pathogenic strain failed to show evidence of multiplication after an exposure period of more than 1 to 2 hours.

Survival of Staphylococci within Monocytes.—No quantitative determinations were made to determine the fate of staphylococci phagocytized by human monocytes. It was observed, however, that these cells ingested staphylococci, and the survival of pathogenic staphylococci within monocytes as determined by slide cultures appeared to parallel the survival of these strains within polymorphonuclear leukocytes.

DISCUSSION

The occurrence of phagocytosis of bacteria in virtually all infections of man has been a conspicuous feature of the host-parasite relationship and one which has been subjected to study by many investigators. In the main, attention has been directed toward the role of the phagocytic cells in the destruction of the invading microorganisms, a role which is generally considered of first importance in the host defense mechanism. It seems clearly established that many bacteria are destroyed by leukocytes in the process of phagocytosis (11). Little is known about the possibility of the survival of bacteria within the leukocytes although it was suggested that phagocytosis may at times exert a protective rather than a destructive effect upon certain bacteria after their ingestion (13, 14).

From the observations reported here it appears that the relationships of pathogenic staphylococci to human leukocytes may differ from those of pneumococci, group A streptococci or *Klebsiella pneumoniae*. Pathogenic staphylococci were found to be ingested in significant numbers by human leukocytes in systems in which pneumococci, streptococci, and *Klebsiella* were rarely ingested. Moreover, appreciable numbers of staphylococci in the pathogenic strains appeared to be able to survive within human polymorphonuclear leukocytes, to multiply within the cytoplasm of the leukocyte, then eventually to escape after death and lysis of the cell had occurred. It was found also that non-pathogenic strains, although taken up in a similar fashion by the leukocyte appeared unable to survive within the white cell and were soon unable to multiply following their ingestion.

The mechanism whereby pathogenic staphylococci are able to survive within human leukocytes has not been clarified in the present studies. The importance of the coagulase mechanism in the process is suggested by the work of Spink and Vivino (7) who observed that strains acquiring or losing coagulase activity concurrently gained or lost the ability to survive in defibrinated blood. In the experiments performed in this laboratory, however, no reduction in the survival of pathogenic strains within human leukocytes was demonstrable in a system from which fibrin and fibrinogen had been removed by standard methods of defibrination. That traces of fibrinogen remain in such a system could not be excluded, however, and could conceivably be sufficient to allow the staphylocoagulase to exert its action.

The rapid disappearance of leukocytes observed in the presence of pathogenic strains suggests that staphylococcal leukocidin which is known to be active against human leukocytes may serve to cripple the metabolic activities of the leukocyte responsible for the destruction and digestion of microorganisms. That injury of the leukocyte may assist intraleukocytic survival is indirectly suggested by the observation that a few non-pathogenic staphylococci may also withstand ingestion for brief periods if the leukocytes are promptly killed by momentary drying after ingestion of the microorganisms.

Other investigators have suggested that intraleukocytic survival of staphylococci may occur. Lyons (4) reported that young cultures of pathogenic and non-pathogenic staphylococci produce a capsule which renders them resistant to phagocytosis. In his experiments, the capsule was lost by both pathogenic and non-pathogenic strains in serum, rendering them susceptible to phagocytosis. Pathogenic strains appeared to reencapsulate within the cytoplasm of the leukocyte, and survived in human defibrinated blood in contrast to non-pathogenic strains. During incubation there was evidence of leukocyte destruction in systems containing pathogenic strains. Lyons thus suggested that pathogenic strains were not killed by the process of ingestion and that the production of leukocidin resulted in the destruction of leukocytes and escape of viable staphylococci. We have been unable to demonstrate such encapsulation of staphylococci during the early growth phase, or to obtain significant differences in the phagocytosis of young and old cultures of pathogenic staphylococci such as are seen with other encapsulated organisms. Furthermore, pathogenic staphylococci survived within human leukocytes in our experiments regardless of the age of the culture used.

Flaum (15) demonstrated that defibrinated rabbit's blood containing leukocytes was not bactericidal for pathogenic strains of staphylococci although great numbers of non-pathogenic cocci were killed in such bactericidal tests. He was unable to demonstrate significant differences in the phagocytosis of pathogenic and non-pathogenic strains, and thus suggested that the survival of pathogenic strains might be due either to the production of leukocidin or to survival within leukocytes.

The possibility that pathogenic staphylococci may possess a phase of existence within the phagocytes of the human host is suggested from the observations in the present study. Microorganisms producing acute human infections are generally considered to be extracellular parasites which are promptly destroyed once phagocytosis has occurred. Many microorganisms which produce chronic infections are readily phagocytized but appear to survive and multiply within cells of the host. It would seem that staphylococci may occupy an intermediate position, possessing the ability to survive and multiply extracellularly, yet withstanding destruction by incorporation within the polymorphonuclear cell and possibly multiplying within the cytoplasm following destruction of the cell.

It should be stressed that while pathogenic staphylococci appear to survive polymorphonuclear leukocyte ingestion, no studies have been performed on the survival within cells of the macrophage system. There is evidence that mononuclear phagocytes and macrophages operate differently in dealing with tubercle bacilli (16) or streptococci (17), and similar differences may exist with regard to staphylococci. It is suggested that intraleukocytic survival may well enhance the ability of certain strains of staphylococci to produce human infection, recognizing that many other factors such as exotoxin and coagulase production, or elaboration of leukocidin are in all probability operative and may contribute to such intracellular survival.

SUMMARY

In a study of the phagocytosis of staphylococci by human leukocytes it has been observed that strains of staphylococci producing human infection were phagocytized by human polymorphonuclear leukocytes *in vitro* under conditions in which virulent pneumococci, streptococci, or *Klebsiella* were rarely engulfed.

In the presence of human leukocytes in plasma there was a rapid fall in the numbers of viable staphylococci of both pathogenic and non-pathogenic strains, the beginning of which was detectable in 10 to 15 minutes. The fall in culturable pathogenic microorganisms was considerably less marked, however, and a rapid resurgence of growth occurred in 4 to 8 hours, whereas the number of culturable non-pathogenic microorganisms remained low for 18 to 24 hours.

These differences appear to be explained by the observation that a significant number of microorganisms of pathogenic strains were able to survive within human leukocytes. Such intracellular survival was found to be associated with evidence of destruction of the leukocytes. In contrast, non-pathogenic strains of staphylococci failed to survive within human polymorphonuclear leukocytes following ingestion.

The technical assistance of Miss Marian Ann Melly is gratefully acknowledged.

BIBLIOGRAPHY

1. Blair, J. E., *Bact. Rev.*, 1939, **3**, 97.
2. Menkin, V., and Walston, H., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1259.
3. Panton, P. N., and Valentine, F. C. O., *Lancet*, 1932, **1**, 506.
4. Lyons, C., *Brit. J. Exp. Path.*, 1937, **18**, 411.
5. Blair, J. E., in *Bacterial and Mycotic Infections of Man*, (R. J. Dubos, editor), Philadelphia, J. B. Lippincott and Co., 1948, 325.
6. Kleiger, B., and Blair, J. E., *Surg., Gynec. and Obst.*, 1940, **71**, 770.
7. Spink, W. W., and Vivino, J. J., *J. Clin. Inv.*, 1942, **21**, 353.
8. Hale, J. H., and Smith, W., *Brit. J. Exp. Path.*, 1945, **26**, 209.
9. Smith, M. R., and Wood, W. B., Jr., *J. Exp. Med.*, 1946, **86**, 257.

10. Evans, H. M., and Schulemann, W., *Science*, 1914, **39**, 443.
11. Wood, W. B., *Trans. Assn. Am. Physn.*, 1947, **60**, 77.
12. Hare, R., *Brit. J. Exp. Path.*, 1931, **12**, 261.
13. Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, **23**, 601.
14. Magoffen, R. L., and Spink, W. W., *J. Lab. and Clin. Med.*, 1951, **37**, 924.
15. Flaum, A., *Acta path. et microbiol. Scand.*, suppl. XXXV, 1938, **1**, 137.
16. Lurie, M. B., *J. Exp. Med.*, 1939, **69**, 579.
17. Gay, F. B., and Morrison, L. F., *J. Infect. Dis.*, 1923, **33**, 338.

EXPLANATION OF PLATES

PLATE 8

FIG. 1. Photomicrographs of staphylococci within leukocytes following 4 hours' incubation at 37°C. Gram-Weigert stain. $\times 690$.

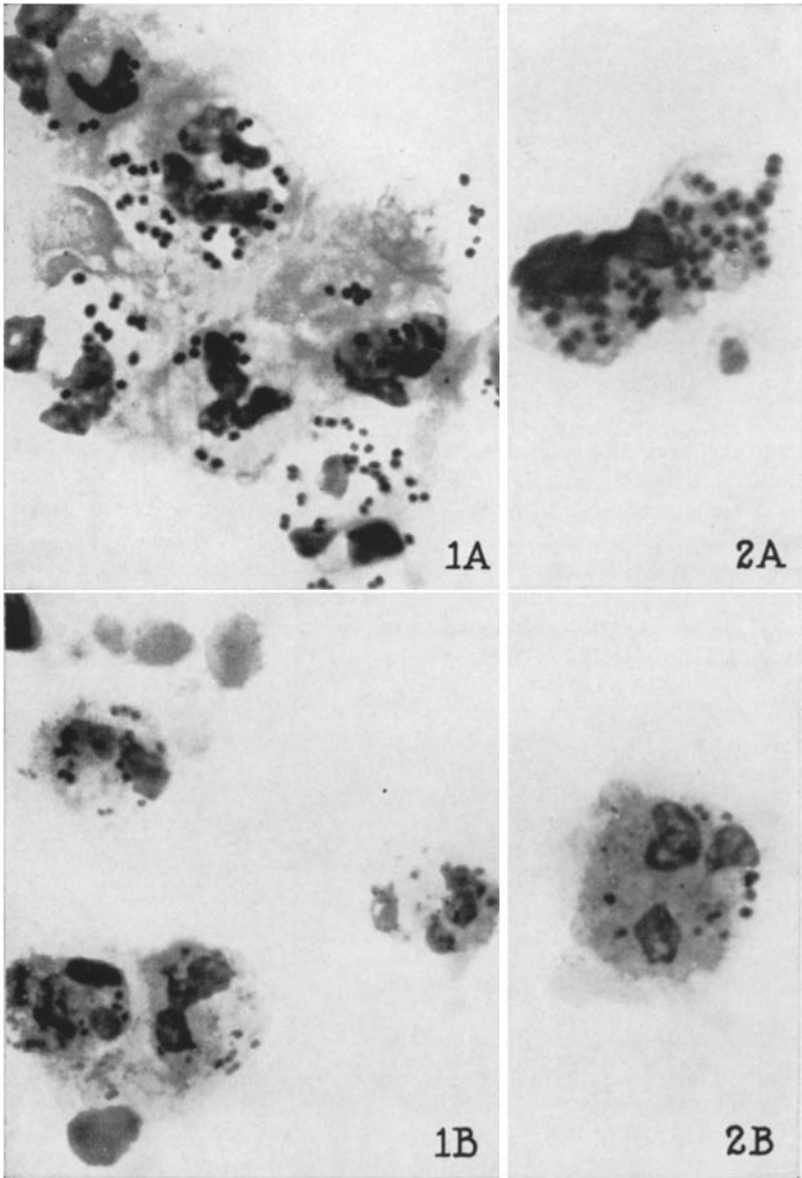
FIG. 1 A. Pathogenic staphylococci. Intracytoplasmic organisms stain sharply and distinctly.

FIG. 1 B. Non-pathogenic staphylococci. The outline of many intracellular microorganisms has become fuzzy and indistinct.

FIG. 2. Photomicrographs of a similar experiment following 6 hours' incubation. Gram-Weigert. $\times 1080$.

FIG. 2 A. Pathogenic staphylococci. Note the clearly staining diploid and tetrad arrangements of intracellular microorganisms suggesting possible division.

FIG. 2 B. Non-pathogenic staphylococci. Tiny faintly staining granules suggestive of cocci undergoing digestion are seen within the cytoplasm of the leukocyte, as well as some intact organisms.



(Rogers and Tompsett: Survival of staphylococci within leukocytes)

PLATE 9

FIG. 3. The comparative phagocytosis of equal numbers of pathogenic staphylococci and other organisms following 30 minutes' incubation at 37°C. Wright's stain. $\times 675$.

FIG. 3 A. Pathogenic staphylococcus.

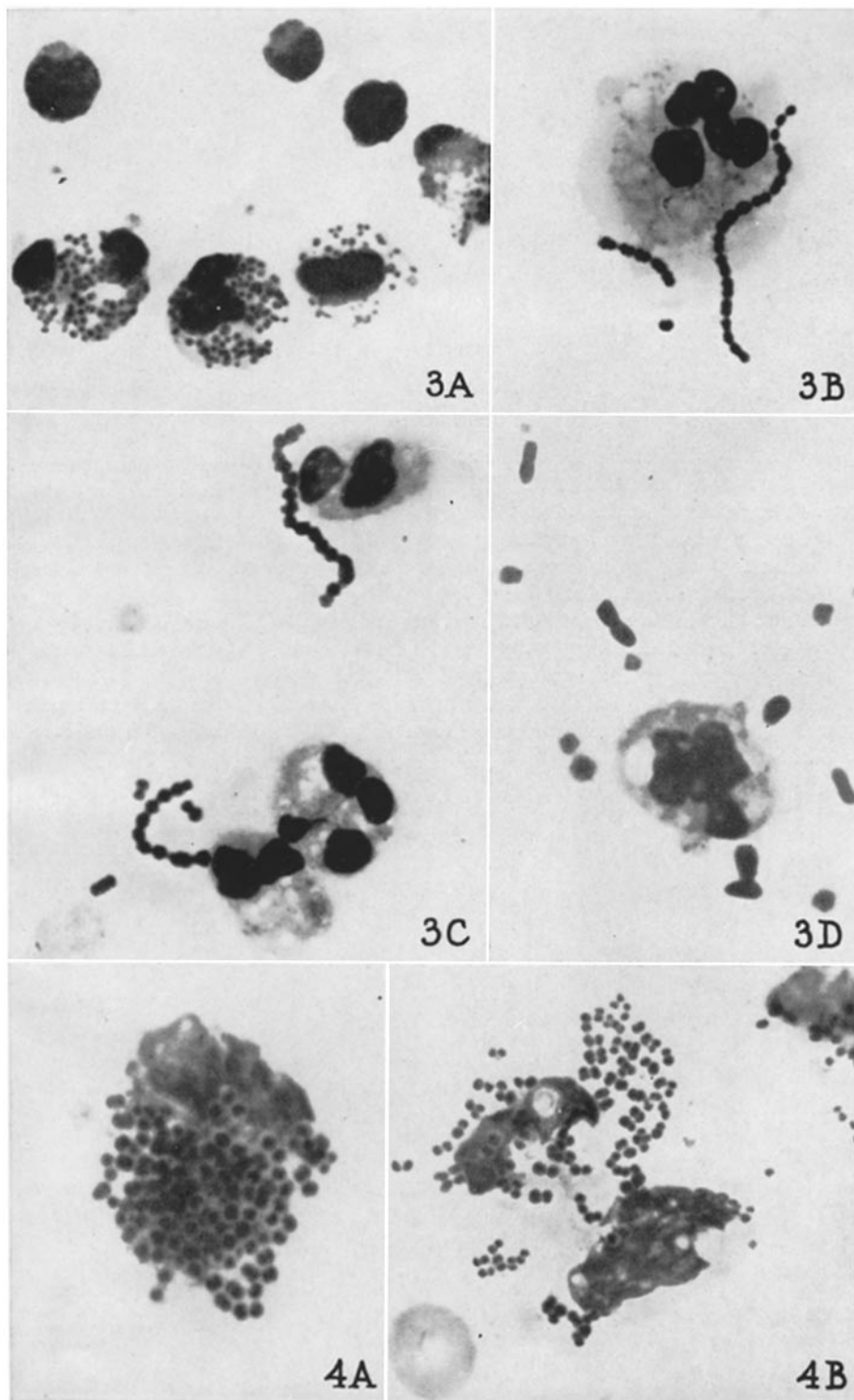
FIG. 3 B. Type I pneumococcus.

FIG. 3 C. Group A streptococcus.

FIG. 3 D. *Klebsiella pneumoniae*.

There are large numbers of staphylococci within the cytoplasm while other microorganisms tested remain extracellular.

FIGS. 4 A and 4 B. Photomicrographs of mixtures containing pathogenic staphylococci and human leukocytes following 8 hours' incubation at 37°C. Sharply staining cocci in diploids and tetrads lie within areas of cellular debris surrounding disrupted leukocytes. Wright's stain. $\times 1000$.



(Rogers and Tompsett: Survival of staphylococci within leukocytes)

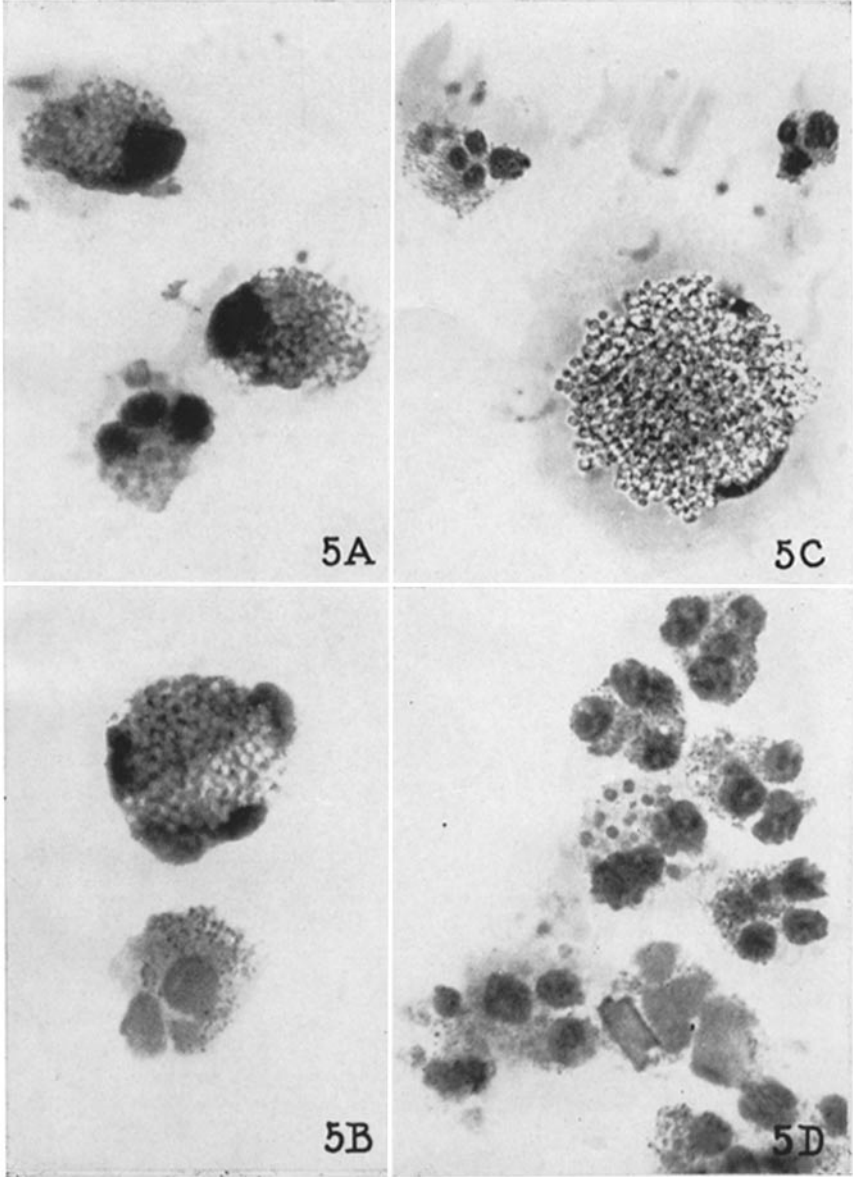
PLATE 10

FIG. 5. Photomicrographs of slide cultures of leukocytes containing staphylococci. Trypan blue agar slides. $\times 1030$.

FIGS. 5 A and 5 B. Leukocytes containing pathogenic staphylococci. Note the refractile, translucent cocci which have actively divided to fill the cytoplasm and distort the nucleus of the leukocyte. There is one leukocyte in each figure not containing dividing cocci. 3 hours' incubation at 37°C .

FIG. 5 C. Similar experiment, 5 hours' incubation at 37°C . Here multiplying intracytoplasmic cocci have completely disrupted the leukocyte, nuclear remnants remaining at the periphery of the colony.

FIG. 5 D. Leukocytes containing non-pathogenic staphylococci. Note the pale staining intracellular organisms which show no signs of multiplication. 5 hours' incubation.



NOTICE: THIS MATERIAL MAY BE PROTECTED BY
COPYRIGHT LAW. (TITLE 17 U. S. CODE.)

(Rogers and Tompsett: Survival of staphylococci within leukocytes)