INTERACTIONS BETWEEN RABBIT POLYMORPHONUCLEAR LEUCOCYTES AND STAPHYLOCOCCI*

By ZANVIL A. COHN, M.D., AND STEPHEN I. MORSE, M.D.

(*From The Rockefeller Institute*)

(Received for publication, May 26, 1959)

The interaction between phagocytes and bacteria, in its simplest terms, consists of two interdependent phases. The first may be considered the ingestion phase in which the leucocyte engulfs the particle and is a prerequisite for the second or intracellular phase. Many of the studies in the past have been confined to the ingestion phase or phagocytosis, and have delineated such important determinants as the nature of the bacterial surface (1), serum components (2), and other constituents of the milieu which influence the functional activity of the phagocyte (3). Little is known, however, about the dynamics of the intracellular phase or even the basic bactericidal mechanisms which are operative in the intact white cell. It is apparent that for effective cellular defense, both phases must operate efficiently and rapidly, before the onset of extensive bacterial multiplication.

The investigation of this process *in vivo* is complicated by a variety of factors which include (*a*) mixed phagocyte populations, (*b*) bactericidal properties of blood and tissue fluids, and (*c*) the inability to evaluate quantitatively the fate and localization of the entire bacterial population. A more precise estimate of the intrinsic properties of the phagocyte-bacterial interaction may be determined *in vitro* under defined conditions. It is apparent that the *in vitro* findings may not parallel the *in vivo* process, nor is it necessarily true that the phagocytes from different species will exhibit identical properties.

This report will deal with the fate and localization of *Staphylococcus aureus* and *Staphylococcus albus* in homogeneous suspensions of rabbit polymorphonuclear leucocytes. Employing low speed centrifugation to separate the leucocyte and bacterial populations, it was possible to estimate and compare the rates of the phagocytic and intracellular bactericidal processes. Supplemental results with other bacterial species, as well as some of the factors which influence the staphylococcal interaction, will be presented.

Materials and Methods

Medium.—The basic medium utilized in all experiments consisted of sterile Hanks' solution as modified by Martin *et al.* (4), 0.01 per cent crystalline bovine serum albumin (Armour),

* This investigation was supported by a research grant, E-1831, from the National Institute of Allergy and Infectious Diseases, Public Health Service.

and 0.0056 M glucose at pH 7.5. This will hereafter be referred to as HBG. The pH of actively metabolizing leucocyte-bacteria suspensions in HBG–10 per cent rabbit serum remained between 7.2 and 7.4 during 4 hours of incubation at 37.5°C.

Peritoneal Exudates.—The procedure for obtaining homogeneous populations of rabbit polymorphonuclear leucocytes was patterned after the method of Hirsch (5). New Zealand rabbits from The Rockefeller Institute colony, weighing 3.5 to 4.5 kg., were used throughout. Employing aseptic techniques, 200 ml. of 0.1 per cent glycogen (C.P., Amend Co., New York) in pyrogen-free saline (0.9 per cent NaCl) was introduced into the peritoneal cavity through a No. 19 short bevel needle. Four hours later 100 ml. of Hanks' solution containing 5.0 mg. of heparin (Connaught Laboratories, Toronto) was injected through a No. 15 perforated needle. The abdomen was gently kneaded and the fluid allowed to drain by gravity into a collecting flask. The average peritoneal drainage was 150 ml. in volume and contained 5 to 13 $\times$ 10^6 leucocytes/ml. In all cases it was possible to utilize the cells from a single animal in any given experiment.

It was observed that the rabbits required two or three "priming" injections before producing leucocyte-rich exudates. Once "primed," the animals were used at 1- to 3-week intervals for up to 14 months, without ill effects or diminution in leucocyte production.

Preparation of Leucocyte Suspensions.—The milky-white exudate fluid was screened through four layers of sterile cheese-cloth to remove clumped cells and fibrin. Duplicate leucocyte counts were performed in a hemocytometer with Türk's solution. Repeated observations on wet chamber preparations and stained smears demonstrated that 99 per cent of the leucocytes were polymorphonuclears. All exudates employed in this study were free of significant numbers of red blood cells.

After screening, appropriate volumes of the exudate fluid were dispersed into sterile, siliconed 40 ml. conical centrifuge tubes. The cells were sedimented at 1,000 R.P.M. for 5 minutes in an International (model V, size 2, rotor No. 240) centrifuge at 25°C. The cell-free supernate was carefully removed with a fine tipped pipette and any residual fluid allowed to drain. The cells were gently resuspended in HBG, pooled, and the concentration adjusted to the desired value. One or two further washes were carried out when cells from immunized animals were used.

Viability of Polymorphonuclear Leucocytes.—The interval between collection and final resuspension of the cells was always less than 30 minutes. Structural and functional integrity of the leucocytes was assayed by the following techniques:—

1. Trypan blue staining: initially less than 5 per cent of the granulocytes exhibited nuclear staining with 1 per cent trypan blue, and at the end of 3 hours of incubation, less than 10 per cent.

2. Phagocytosis: more than 90 per cent of the cells were capable of phagocytosis when sufficient numbers of bacteria were added.

3. Metabolic studies: in experiments to be reported at a later date it was found that the rates of O_2 consumption, glucose utilization, and lactic acid production were linear for at least 180 minutes.

Under conditions in which the leucocytes were incubated with staphylococci, there was no apparent diminution in the above properties.

Bacteria.—All cultures were transferred at monthly intervals. Unless otherwise noted, 16 to 18 hour broth cultures were employed. In the case of staphylococci, no difference in the reactions to be described were evident when younger cultures were substituted.

Staphylococci.—All strains were maintained in Penassay broth (Difco Laboratories, Detroit). Four of the five strains of coagulase negative *Staph. albus* were obtained through the courtesy of Mrs. Mina Ristic of the Presbyterian Hospital, New York. The other was a stock laboratory culture originally isolated from room air.

Coagulase-positive strains of *Staph. aureus* had been maintained in the laboratory of Dr. René J. Dubos at The Rockefeller Institute and, except for one (MAM), were originally isolated from human staphylococcal infections.

Table I lists the properties of the strains of staphylococci employed in the present investigation.

Escherichia coli (K-12), *Salmonella typhimurium* (SR11, RIA), *Serratia marcescens*, *Proteus morganii:* These organisms were kindly supplied by Dr. James G. Hirsch and were maintained in penassay broth.

Mycobacterium smegmatis and *Mycobacterium fortuitum* (Penso): The mycobacteria were propagated for 72 hours before use in tween-albumin liquid medium (6), and were obtained from Dr. Russell W. Schaedler.

TABLE I

Properties of Staphylococcal Strains

Name	Source	Pigment	Coagulase	Hemolysis	Mouse virulence	Phage type
S. albus						
Greaves	Nasal swab	White	0	0	0	—
Prengel	" "	"	0	0	0	—
Mendita	" "	"	0	0	0	—
McGaffrey	" "	"	0	0	0	—
Air	Room air	"	0	0	0	—
S. aureus						
MAM	Skin	Yellow	+	+	±	75
O'Hara	Osteomyelitis	"	+	+	+	52A, 73, 81
Smith	"	"	+	+	+	44A-42E
Stern	—	"	+	+	+	—
Stovall	Blood	"	+	+	+	VA4

Streptococcus pyogenes: We are indebted to Dr. R. C. Lancefield for strains of Groups A, B, D, and G streptococci which were maintained in Todd-Hewitt broth and employed as 4 hour subcultures of an 18 hour growth.

Preparation of Bacterial Suspensions.—Immediately prior to use the broth cultures were centrifuged and the bacterial cells washed twice and resuspended to the appropriate volume in HBG. Viable microbial units assayed by dilution and plating methods were equal to the total number of bacteria present as calculated from Petroff-Hauser chamber counts. Once the growth pattern of each bacterial strain was found to be reproducible, the proper dilutions could be made by means of turbidometric readings.

Serum.—Normal rabbit serum was obtained from animals which were maintained expressly for this purpose. Blood was allowed to clot at room temperature and then placed at 4°C. to permit clot retraction. The serum was stored in small volumes at −20°C. for no longer than 60 days, and thawed just before use. Each lot was screened against the experimental organisms for inherent bactericidal properties.

Leucocyte-Bacteria Suspensions.—The polymorphonuclear leucocytes were dispensed into sterile, siliconed 15 x 150 mm. test tubes. Serum and bacteria were then added and the tubes sealed with white rubber stoppers and thoroughly mixed. The total volume of each tube was 2.0 ml. and contained 30 $\times$ 10^6 leucocytes/ml., 20 to 60 $\times$ 10^6 bacteria/ml., and

10 per cent rabbit serum. The tubes were incubated at 37.5°C. on a reciprocating water bath shaker at 120 cycles/minute (model RW150, New Brunswick Scientific Co., New Brunswick, New Jersey). This degree of agitation maintained the leucocytes and bacteria in suspension and insured adequate mixing.

Enumeration of Viable Bacteria.—The quantitative estimation of the phagocytic and bactericidal activities of the leucocytes required enumeration of the total number of viable organisms and the number in the extracellular and leucocyte fractions. The technique described below for the separation of free bacteria from those associated with polymorphonuclear leucocytes was derived from that originally developed by Maaløe (7).

Total Number of Viable Bacteria.—At prescribed time intervals, from 0 to 180 minutes, 0.2 ml. samples were removed from the leucocyte-bacteria suspensions and placed into cold homogenization tubes containing 1.8 ml. of penassay broth. Homogenization was performed for 1 minute with a teflon pestle driven by a high speed motor (No. 7609-C, Palo Laboratory Supplies, Inc., New York). The homogenate was serially diluted in penassay broth and 0.1 ml. of the dilutions spread on the surface of Felsen plates containing 1.5 per cent penassay agar. The plates were incubated at 37°C. and the bacterial colonies counted after 18 hours. Quadrants containing 10 to 100 colonies were chosen to calculate the number of bacteria/milliliter present in the sample. When streptococci were employed, Todd-Hewitt broth and blood agar plates were substituted.

Viable Extracellular Bacteria.—Simultaneously, 1.0 ml. aliquots were removed and dispensed into 12.0 ml. siliconed conical centrifuge tubes. 4.0 ml. of cold HBG was added and after thorough mixing the suspensions were centrifuged for 3 to 4 minutes at 450 R.P.M. The low speed centrifugation resulted in a firmly packed pellet of leucocytes and a clear supernatant fluid. From the upper third of the tube, 0.2 ml. of supernatant fluid was obtained, homogenized, diluted, and plated as described above. Preliminary experiments demonstrated that free bacteria were not appreciably sedimented under these conditions.

Viable Bacteria Associated with Leucocytes.—The remaining supernatant fluid was carefully withdrawn with a Pasteur pipette without disturbing the packed cells. 1.0 ml. of cold HBG was added, the cells resuspended, and 0.2 ml. removed for homogenization and plating.

The total, supernate, and sediment-viable counts were expressed as bacteria/milliliter of the original suspension.

When stained smears were to be employed, a drop of the leucocyte-bacteria mixture was spread on a glass slide, fixed in absolute methanol, and stained by the Giemsa method.

RESULTS

The Stability of Staphylococci in the Suspending Medium.—Under the present experimental conditions in which the assay of the bacteria-leucocyte interaction depended primarily on the viability of the microorganisms, it was imperative to investigate the effects of the suspending medium. Table II presents the effects of various constituents of the medium on the viability and growth rate of strains of both coagulase-positive and coagulase-negative staphylococci. In the presence of 20 to 50 per cent rabbit serum a slow increase in the number of organisms occurred, with 2- to 4-fold multiplication in 180 minutes at 37.5°C. Similar findings were obtained with 100 per cent serum. These results were characteristic of the 10 or more lots of normal rabbit sera utilized in the course of the investigation. Only one strain of coagulase-positive, mouse virulent, *S. aureus* (Giorgio) was susceptible to the bactericidal action of serum at

concentrations as low as 10 per cent. This activity could be nullified by previous treatment of the serum at 56°C. for 30 minutes. Only those strains which were not affected by serum were employed in the leucocyte system.

The non-serum–containing portion of the suspending medium which was employed for the washing and resuspension of the test organisms was also evaluated. Preliminary experiments in which washed staphylococci were incubated in the balanced salt solution alone at 37.5°C. revealed a striking bacteri-

TABLE II

Stability of Staphylococci in the Presence of Rabbit Serum and Other Constituents of the Medium

Strain	Medium								
	20 per cent rabbit serum in HBG			50 per cent rabbit serum in HBG		Solution HG*		Solution HBG‡	
	Bacteria/ml. after incubation at 37.5°C.			Bacteria/ml. after incubation at 37.5°C.		Bacteria/ml. after incubation at 37.5°C.		Bacteria/ml. after incubation at 37.5°C.	
	Zero	60 min.	180 min.	Zero	180 min.	Zero	180 min.	Zero	180 min.
S. albus									
Greaves	2.1×10^7	4.2×10^7	7.9×10^7	—	—	—	—	—	—
Prengel	6.1×10^7	8.1×10^7	1.1×10^8	—	—	—	—	—	—
Mendita	3.5×10^7	6.2×10^7	8.7×10^7	4.2×10^7	2.3×10^8	8.0×10^7	$<10^4$	8.0×10^7	6.8×10^7
McGaffrey	5.7×10^7	6.3×10^7	9.5×10^7	—	—	—	—	—	—
S. aureus									
O'Hara	4.1×10^7	5.3×10^7	8.9×10^7	2.3×10^8	7.4×10^8	—	—	—	—
Smith	3.2×10^7	4.9×10^7	9.1×10^7	4.5×10^7	3.0×10^8	1.3×10^8	$<10^4$	1.1×10^8	1.4×10^8
Stern	7.4×10^7	9.0×10^7	2.6×10^8	1.6×10^7	5.3×10^8	—	—	—	—
Stovall	3.7×10^7	5.7×10^7	9.8×10^7	3.6×10^7	2.2×10^8	—	—	—	—
MAM	4.6×10^7	6.3×10^7	1.1×10^8	2.2×10^8	8.0×10^8	—	—	—	—

* Balanced salt solution + 0.0056 M glucose.

‡ Balanced salt solution + 0.0056 M glucose + 0.01 per cent crystalline bovine serum albumin.

cidal effect. The addition of 0.01 per cent crystalline bovine serum albumin stabilized the organisms but did not support significant multiplication. Bovine serum albumin as a constituent of solution HBG was therefore used in all subsequent studies.

Although staphylococci were stable in the suspending medium, it seemed possible that the leucocytes could release bactericidal substances into the medium, *e.g.* lysozyme, lactic acid, etc. (8). Experiments were therefore conducted in which cell-free supernatant fluids, obtained from suspensions of leucocytes incubated in 10 per cent rabbit serum, were tested against the two prototype strains of coagulase-positive (Smith) and coagulase-negative (Mendita) staphylococci.

Exudate cells were washed and resuspended to a concentration of 40 × 10^6/ml. in solution HBG containing 10 per cent rabbit serum. An aliquot of 2.0 ml. was dispensed to test tubes and incubated at 37.5°C. for time periods of 10 to 180 minutes. At appropriate intervals the suspension was centrifuged at 450 R.P.M. for 4 minutes and 0.95 ml. of the cell-free supernatant fluid transferred to 12 x 90 mm. tubes. The test organism in a volume of 0.05 ml. was then added, the tubes sealed and incubated at 37.5°C. At 0, 60, and 180 minutes samples were removed and processed in the previously described manner.

Table III presents such an experiment in which no bactericidal action was apparent and shows that under these conditions both strains exhibited growth

TABLE III

Effect of Polymorphonuclear Leucocyte Supernatant Fluids upon the Viability of S. albus "Mendita" and S. aureus "Smith"

Strain	Supernatant fluids obtained from leucocytes incubated at 37.5°C. for:	Bacteria/ml. after incubation in leucocyte supernatant fluid at 37.5°C.		
		Min.		
		Zero	60	180
	min.			
Mendita	10	3.0 × 10^7	3.8 × 10^7	5.3 × 10^7
"	60	2.5 × 10^7	4.6 × 10^7	4.9 × 10^7
"	120	3.8 × 10^7	4.2 × 10^7	8.7 × 10^7
"	180	3.3 × 10^7	4.5 × 10^7	7.7 × 10^7
Smith	60	1.0 × 10^7	1.8 × 10^7	4.7 × 10^7
"	180	3.0 × 10^7	5.1 × 10^7	8.2 × 10^7

rates similar to the growth rate in an equivalent concentration of rabbit serum. It seemed therefore that (*a*) the constituents of the medium were without bactericidal action, (*b*) the growth rates of the bacteria in the suspending medium were not sufficiently rapid to mask a bactericidal effect of the leucocytes, and (*c*) extracellular leucocyte products did not affect microbial viability.

The Interaction of Staphylococci with Polymorphonuclear Leucocytes in the Presence of Normal Rabbit Serum.—In the past the phagocytosis of staphylococci has been evaluated by stained films. In general, the findings have not indicated significant differences between coagulase-positive and coagulase-negative organisms (9). There are, however, exceptions in which coagulase-positive staphylococci were not as readily ingested (10, 11). During the initial phases of the present investigation attempts were made to obtain intracellular populations of *S. aureus* and study their intraleucocytic fate under a variety of conditions. It soon became apparent, however, that appreciable numbers of these organisms were not ingested in the presence of normal rabbit serum.

Because of this finding a careful evaluation of the phagocytosis and subsequent fate of *S. albus* and *S. aureus* was conducted.

Chart 1 represents a characteristic experiment performed with a coagulase-negative *S. albus* strain (Mendita) in the presence of 10 per cent fresh normal rabbit serum. It demonstrates the rapid ingestion and simultaneous intracellu-

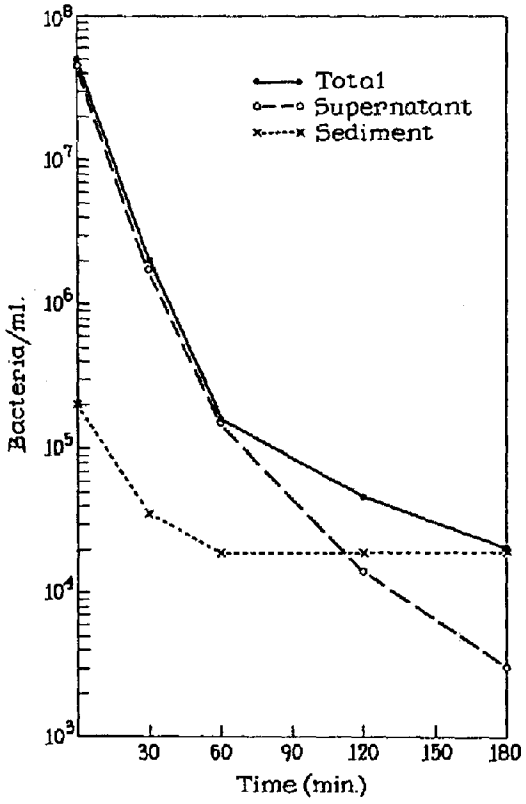

CHART 1. The interaction of *S. albus* "Mendita" with polymorphonuclear leucocytes.

lar bactericidal effect on the organism. As the total number of viable units decreased, there was an identical reduction in the extracellular population for the first 60 minutes. During this period, therefore, the rate of phagocytosis, as determined by the decrease in extracellular "supernatant" bacteria, was identical with the rate at which the total number of bacteria were inactivated by the leucocytes. These results suggest that the rate of intracellular killing of this strain is quite rapid.

As the incubation period progressed there was a gradual dissociation between supernate and total counts so that at 180 minutes all of the remaining viable

organisms were associated with the leucocytes. The sediment counts also reflected the rapid bactericidal action of the intraleucocytic environment since there was no increase in the number of viable units in this compartment. The nature and localization of the small (0.1 per cent) persistent population of bacteria associated with the leucocytes is uncertain. From other experiments it appears that this population is present during the early phases of the experiment and cannot be removed by repeated washing. Although further work is

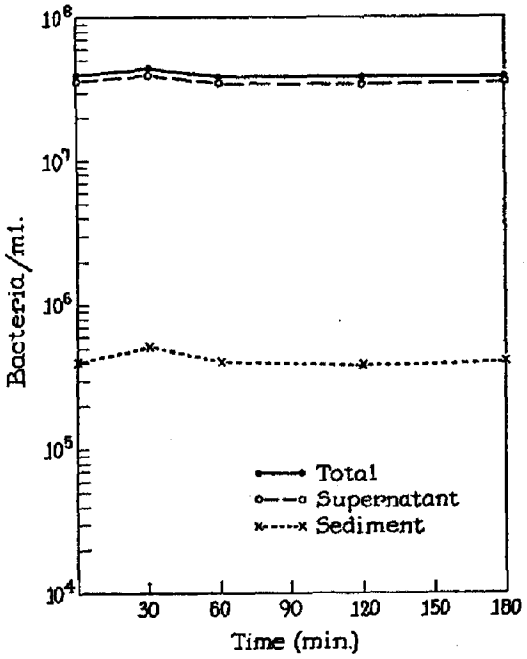

CHART 2. The interaction of *S. aureus* "Smith" with rabbit polymorphonuclear leucocytes.

required, this persistent population may reflect (*a*) resistant bacteria or (*b*) ingestion by leucocytes which have lost their bactericidal properties.

In contrast, Chart 2 represents the interaction of leucocytes with a coagulase-positive, *S. aureus* (Smith) strain. The experimental methods were identical with those described for the results in Chart 1 and the same preparation of leucocytes and serum were employed. During the 180 minutes of the experiment no reduction of viable organisms occurred. From the total counts alone it might be concluded that this coagulase-positive strain survives within rabbit polymorphonuclear leucocytes. It is apparent, however, from supernatant and sediment counts that no appreciable ingestion of this organism had occurred. During the entire experimental period the total number of organisms could

be accounted for as free extracellular bacteria. Similarly, the number of bacteria associated with the sediment showed no appreciable change.

A certain degree of phagocytosis of coagulase-positive staphylococci was discernible by the examination of stained films. When a multiplicity of 1:1 bacteria/leucocyte was employed, not more than 15 per cent of the white cells contained intracellular staphylococci. If the multiplicity was increased to 10 bacteria/leucocyte, more than 90 per cent of the cells showed demonstrable intracellular organisms. It appears, therefore, that a certain *percentage* of coagulase-positive organisms can be ingested in normal rabbit serum, but that

TABLE IV

Effect of Various Concentrations of Rabbit Serum on the Fate and Localization of S. aureus "Smith" and S. albus "Mendita" in the Presence of Leucocytes

Strain	Final serum concentration	Bacterial/ml. after incubation at 37.5°C.			
		Total count		Supernatant count	Sediment count
		Zero	180 min.	180 min.	180 min.
	per cent				
S. albus					
Mendita	100	3.4×10^7	1.1×10^5	9.5×10^3	2.0×10^5
"	50	3.4×10^7	1.1×10^5	8.0×10^3	1.1×10^5
"	10	3.4×10^7	1.4×10^5	8.5×10^3	9.0×10^4
S. aureus					
Smith	100	4.6×10^7	4.5×10^7	3.6×10^7	1.0×10^6
"	50	4.6×10^7	3.2×10^7	3.0×10^7	8.0×10^5
"	10	4.6×10^7	4.2×10^7	2.5×10^7	9.2×10^5

the evaluation of phagocytosis by stained films is critically dependent upon the multiplicity employed. If 10 to 20 per cent of the total bacteria were phagocyted and subsequently killed, this would not produce any detectable bactericidal effect in the leucocyte system. This minimal degree of phagocytosis may account in part for the lack of extracellular multiplication of the coagulase-positive organisms in leucocyte suspensions.

The Effect of Serum Concentration.—Since the efficiency of the phagocytic process might be dependent upon the concentration of normal rabbit serum, analogous studies were conducted with serum concentrations ranging from 10 to 100 per cent.

Exudate cells were prepared in the usual manner and washed with solution HBG. The final resuspension was performed directly in the appropriate volume of 10, 50, and 100 per cent fresh frozen normal rabbit serum. Aliquots of 1.8 ml., containing 60×10^6 leucocytes were transferred to test tubes and 0.2 ml. of freshly washed bacteria added.

Table IV presents in tabular fashion the data obtained with strains "Smith"

and "Mendita." The results suggest that the concentration of serum did not affect either the fate or localization of these two organisms. Subsequent experiments were therefore conducted in the presence of 10 per cent serum.

Although there were differences between the phagocytosis of the prototype strains of *S. aureus* and *S. albus*, additional confirmation with other strains was required. A series of experiments was performed with four other strains

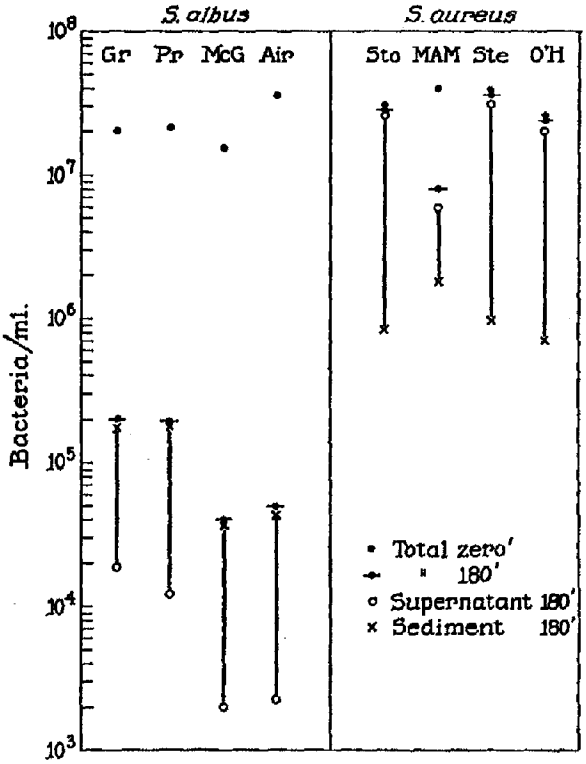

CHART 3. The fate and localization of *S. aureus* and *S. albus* strains in suspensions of polymorphonuclear leucocytes.

of both *S. aureus* and *S. albus*, the results of which are presented in Chart 3. All strains of *S. albus* were promptly killed; at 180 minutes less than 1 per cent of the original organisms remained viable. In addition, more than 90 per cent of the surviving organisms were found associated with the leucocytes.

The coagulase-positive organisms behaved in a fashion similar to the Smith strain. The one exception was the MAM strain which was always killed and phagocyted to a limited extent in four separate experiments. This organism,

although producing a yellow pigment and coagulase, is relatively mouse-avirulent (12, 13).

The Evaluation of Other Bacterial Species.—A variety of other microbial species were studied by the same technique in order to compare the results with those obtained with the staphylococci. Representative data are presented in Table V.

TABLE V

Interaction of Various Bacterial Species with Rabbit Leucocytes

Organism	Bacteria/ml. after incubation at 37.5°C.						
	With PMN leucocytes					Without PMN leucocytes*	
	Total count			Supernatant	Sediment	Total count	
	Zero	60 min.	180 min.	180 min.	180 min.	Zero	120 min.
Esch. coli K-12	5.2×10^7	4.8×10^6	2.0×10^6	2.4×10^6	1.7×10^6	6.0×10^7	1.4×10^8
Sal. typhimurium SR11	3.5×10^7	3.8×10^6	9.2×10^4	2.0×10^6	8.7×10^4	4.0×10^7	1.1×10^8
" " RIA	6.1×10^7	1.3×10^6	1.2×10^6	4.3×10^6	1.2×10^6	1.8×10^7	4.6×10^7
Serr. marcescens	3.7×10^7	7.0×10^6	4.0×10^6	9.5×10^4	3.0×10^6	4.3×10^7	4.8×10^7
Prot. morgani	3.9×10^7	4.1×10^6	1.6×10^6	—	—	7.6×10^7	4.0×10^6
Str. pyogenes							
Group A D58X-11	1.2×10^7	9.2×10^6	1.2×10^7	1.0×10^7	9.3×10^4	7.0×10^6	1.5×10^7
" A D58X	1.3×10^7	2.3×10^7	2.0×10^7	1.5×10^7	3.2×10^5	1.3×10^7	2.3×10^7
" B 040R	1.2×10^7	7.1×10^7	1.0×10^8	1.5×10^8	2.4×10^4	6.0×10^6	1.2×10^7
" D C3	3.8×10^7	3.4×10^6	8.7×10^5	1.8×10^5	6.2×10^5	3.0×10^6	1.4×10^7
" G D166B	2.0×10^7	5.2×10^6	2.3×10^5	4.2×10^4	2.1×10^5	3.3×10^6	4.6×10^7
Myc. smegmatis	4.0×10^7	2.6×10^7	3.4×10^7	2.5×10^8	3.0×10^7	3.2×10^7	2.8×10^7
" *fortuitum* Penso	1.5×10^7	2.2×10^7	1.2×10^7	3.1×10^8	9.8×10^6	5.6×10^7	4.7×10^7

* Bacteria suspended in 12% rabbit serum in solution HBG.

The experimental details were similar to those employed with the staphylococci. The final leucocyte concentration was maintained at 30×10^6/ml. and 12 per cent normal rabbit serum was present. Differences in the composition of the grinding medium, dilution medium, and nutrient agar plates are described in Materials and Methods.

The four species of Gram-negative bacilli were readily ingested and killed by the rabbit polymorphonuclear leucocytes. In general, the rate of phagocytosis was greater with these organisms, most of the bactericidal effect occurring within the first 60 minutes. In all cases the surviving organisms were largely associated with the white cells. It is of interest that *Salm. typhimurium* SR11 and RIA, which are mouse-virulent and mouse-avirulent strains respectively (14), exhibited no significant differences in their susceptibility to the bactericidal process.

Proteus morganii is included to illustrate the differential effect of both a serum and cellular factor on the bactericidal process.

The results with the four groups of streptococci varied markedly depending upon the efficiency of the phagocytic process. The two strains of Group A were poorly ingested as evidenced by the large number of extracellular organisms at 180 minutes and showed no net reduction in viable counts. Strain

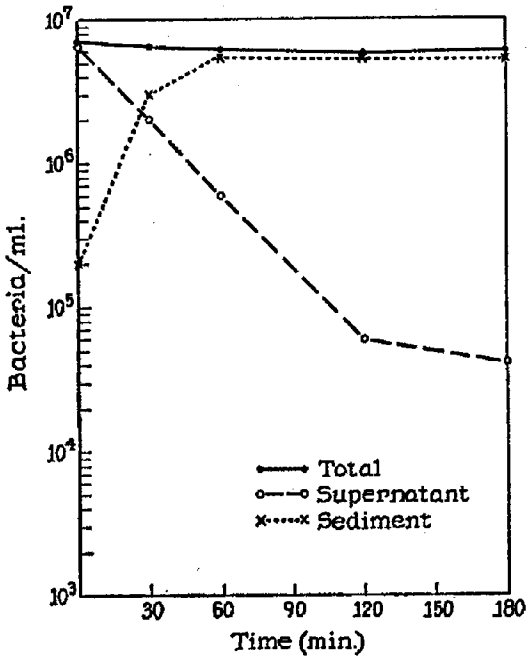

CHART 4. The interaction of *Mycobacterium smegmatis* with rabbit polymorphonuclear leucocytes.

D58X11 contains M protein whereas D58X contains little or no M protein (15). Strain 090R of Group B behaved in a similar fashion but appeared to be capable of more rapid proliferation in the presence of leucocytes. In contrast, the strains of Groups D and G were ingested and killed to varying degrees.

Thus far, all of the bacterial species which were phagocyted were subsequently killed by the rabbit polymorphonuclear leucocyte. It was of interest therefore to examine organisms thought to be readily phagocyted but not killed in the intraleucocytic environment, *i.e.* mycobacteria (16). Two strains were selected which had relatively rapid growth rates on penassay agar and which did not multiply in the incubation medium. *Myco. fortuitum* (Penso) is mouse-virulent and was originally isolated from a human infection (17),

whereas *Myco. smegmatis* is a saprophytic acid-fast bacterium. Preliminary experiments illustrated in Table V suggested that these organisms were phagocyted but not killed by the leucocytes during the 180 minutes of incubation. More detailed studies are shown in Chart 4. Phagocytosis proceeded rapidly, as evidenced by the reduction in supernatant bacteria, and the appearance of a similar number of organisms in association with the cells. Even though more than 90 per cent of the organisms were ingested there was no reduction in total bacterial counts. It appears, therefore, that both strains of mycobacteria are able to withstand the bactericidal action of the leucocytes for periods up to 180 minutes. The experiment is of further interest in that it was possible to recover the entire population of intracellular organisms.

The Effect of Immune Serum on the Interaction of S. Aureus with Leucocytes.—Previous investigators have concluded that *S. aureus* is readily phagocyted and able to survive and multiply within both rabbit and human polymorphonuclear leucocytes (18, 19). These studies have recently been reviewed by Rogers (20). In view of the lack of phagocytosis of *S. aureus* in the presence of normal rabbit serum the susceptibility of this species to the intraleucocytic environment could not be ascertained. The opsonic activity of specific immune serum on the phagocytosis of virulent staphylococci has previously been described by Ecker *et al.* (21). Employing this principle to enhance phagocytosis, it was possible to determine the intracellular fate of *S. aureus* under the present experimental conditions. In all the subsequent studies, the immune serum was obtained from rabbits injected with *S. aureus* "Smith."

The immunizing antigens were prepared in the following manner. Flasks containing 250 ml. of penassay broth were inoculated with 0.3 ml. of an 18 hour broth culture and incubated for 16 hours at 37°C. on a rotary shaker. The bacteria were harvested by centrifugation and washed twice with large volumes of 0.9 per cent pyrogen-free sodium chloride. After the last wash the bacterial pellet was resuspended in 50 ml. of saline and heated at 80°C. for 60 minutes. The heat-killed organisms were then washed twice with saline and resuspended to 50 ml. in the same medium. Phenol was added to a final concentration of 0.25 per cent. Various lots of antigen contained between 5×10^9 and 1×10^{10} bacteria as estimated by direct count in the Petroff-Hauser chamber.

Six adult New Zealand rabbits were immunized according to the following schedule. At 3 day intervals 0.5 ml. of the antigen was injected subcutaneously for a total of seven injections. Rabbits 4 and 5 received two of these injections intravenously and appeared to produce slightly greater quantities of agglutinating antibodies. A consistent finding during the immunization period was the appearance of a delayed type of skin hypersensitivity at the injection site. This was characterized by erythema and induration which first appeared in 24 to 48 hours and lasted 7 to 14 days. The reaction appeared only after the third or fourth subcutaneous inoculation. One week after the last injection the animals were bled by cardiac puncture and the serum was separated and stored by the previously described methods.

Chart 5 presents the results of an experiment in which the fate and localization of *S. aureus* (Smith) were studied in the presence of homologous immune serum.

Each of five test tubes received: (*a*) 1.6 ml. of leucocyte suspension containing 60 × 10^6 cells, (*b*) 0.2 ml. of fresh frozen immune serum, and (*c*) 0.2 ml. of *S. aureus* (Smith) suspended in HBG. The total volume in all tubes was 2.0 ml. and the final concentration of leucocytes and serum was 30 × 10^6/ml. and 10 per cent respectively. At intervals from 0 to 180 minutes a tube was removed from the water bath and sampled for total, supernatant, and sediment counts.

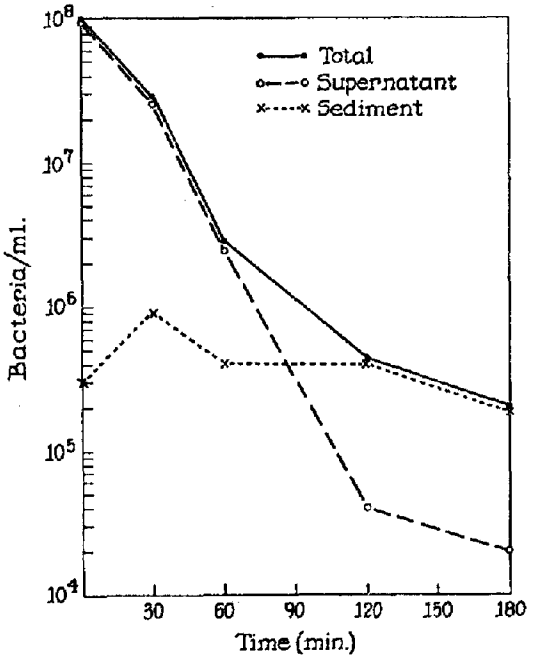

CHART 5. The influence of immune serum on the interaction between *S. aureus* "Smith" and rabbit polymorphonuclear leucocytes.

It is apparent that the addition of immune serum to the leucocyte-bacteria suspension produced a striking effect on the fate of the homologous organism. During the first 120 minutes, the decrease in extracellular bacteria was associated with an identical reduction in the total number of viable organisms, suggesting that the phagocytosis of the Smith strain was rapidly followed by its inactivation. More than 99 per cent of the total bacteria were rendered non-viable during the 180 minutes of incubation and the residual organisms were associated with the leucocytes. The initial increase in sediment counts may reflect a somewhat less rapid inactivation of intracellular cocci as compared with *S. albus* (Mendita). (see Chart 1.)

When Smith immune serum was employed in tests with other strains of

TABLE VI

Effect of Immune Serum on the Fate of Staphylococci in the Presence and Absence of Leucocytes

Test organism	Bacteria/ml.									
	With leucocytes						Without leucocytes			
	Total				Supernatant	Sediment	Total			Supernatant
	Zero	30 min.	60 min.	180 min.	180 min.	180 min.	Zero	60 min.	180 min.	180 min.
S. aureus										
"Smith"	1.1×10^8	8.3×10^6	2.8×10^6	9.0×10^5	5.0×10^4	1.0×10^6	6.3×10^7	6.9×10^7	9.9×10^7	9.2×10^7
Stovall	5.1×10^7	1.4×10^7	9.0×10^6	1.5×10^6	2.1×10^5	2.2×10^6	5.0×10^7	6.2×10^7	8.5×10^7	7.8×10^7
O'Hara	1.3×10^8	1.9×10^7	8.0×10^6	3.0×10^6	6.0×10^5	3.4×10^6	2.4×10^7	4.1×10^7	6.9×10^7	6.3×10^7
Stern	6.3×10^7	1.9×10^6	6.0×10^5	5.0×10^5	2.0×10^4	4.8×10^5	—	—	—	—
Smith*	7.0×10^7	6.3×10^7	4.0×10^7	2.8×10^7	2.5×10^7	1.2×10^6	—	—	—	—
Smith‡	1.1×10^8	1.1×10^8	1.3×10^8	1.3×10^8	1.3×10^8	1.1×10^6	3.2×10^7	4.6×10^7	8.7×10^7	8.4×10^7
S. albus										
Mendita	5.3×10^7	8.0×10^6	7.8×10^5	2.3×10^5	4.0×10^4	2.0×10^5	—	—	—	—
Mendita‡	5.0×10^7	7.6×10^6	7.4×10^5	3.0×10^5	5.2×10^4	3.2×10^5	—	—	—	—

* Smith immune serum inactivated at 56°C. for 30 minutes.

‡ Normal rabbit serum.

S. aureus, a similar opsonic and bactericidal effect occurred (Table VI). This suggested that Smith immune serum was capable of opsonizing the three heterologous strains of *S. aureus* and that these organisms were also readily inactivated within leucocytes. In contrast, the addition of Smith immune serum to Mendita-leucocyte mixtures did not result in a quantitatively greater or more rapid bactericidal effect than that observed with normal rabbit serum.

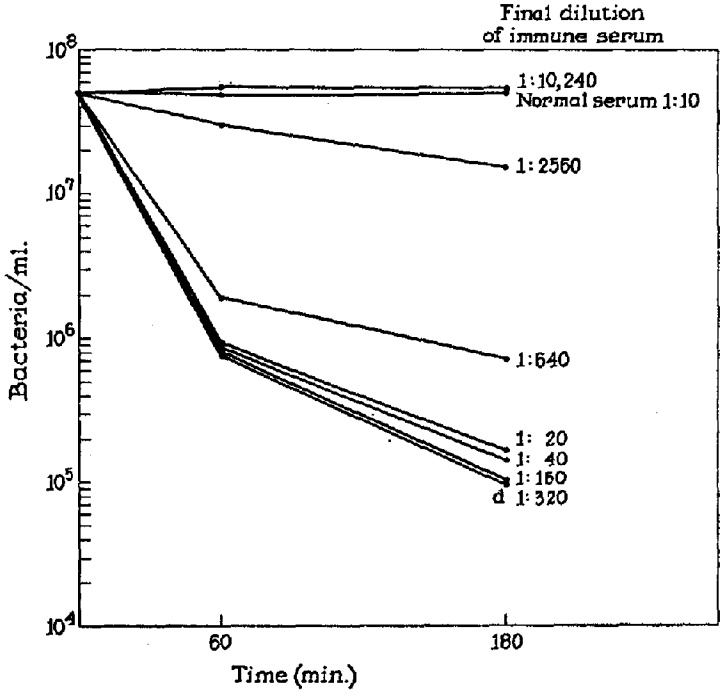

CHART 6. The influence of various concentrations of homologous immune serum on the interaction between *S. aureus* "Smith" and polymorphonuclear leucocytes.

A number of control studies were performed to determine whether bacterial agglutination was a factor in the reduction of total counts. Washed cultures of strains Smith, O'Hara, and Stovall were incubated in 10 per cent immune serum without leucocytes. Samples were removed at 0, 60, and 180 minutes to determine total and supernatant counts. Under these conditions the cocci were able to multiply to an extent similar to that in normal rabbit serum (Table VI). Furthermore, the supernatant counts were identical with total counts, suggesting that large bacterial aggregates were not formed. Moreover, the examination of stained smears prepared from bacteria-leucocyte mixtures revealed the presence of large numbers of intracellular cocci and no significant clumping of extracellular bacteria.

The Properties of the Opsonins in Immune Serum.—The nature of the opsonic effect of Smith-immune serum was next investigated. As shown in Table VI, heating of immune serum at 56°C. for 30 minutes reduced its effect in the leucocyte system. Some phagocytosis occurred but at a much lower rate. This is in keeping with the observations of Ward and Enders (22) that the inactivation of complement in immune serum markedly reduced the rate of phagocytosis of encapsulated pneumococci. The original activity of heated immune serum could be restored by the addition of small quantities of fresh

TABLE VII

Agglutination of Staphylococci in the Presence of Normal and "Smith" Immune Rabbit Serum

Antigen	Normal serum*	Immune No. 1	Immune No. 2	Immune Nos. 4 and 5
S. aureus				
Smith	<5	160	80	320
Stern	<5	160	80	160
Stovall	<5	80	40	80
O'Hara	<5	20	20	20
MAM	<5	—	—	20
S. albus				
Mendita	<5	<5	<5	<5
Greaves	<5	—	—	<5
Prengel	<5	—	—	<5
Air	<5	—	—	<5
McGaffrey	20	—	—	20
E. coli K-12	10	10	10	10

* Reciprocal of the highest serum dilution giving a 2+ reaction.

normal serum. Therefore, both thermolabile and thermostable components were required for efficient ingestion of the Smith strain. Studies on three other strains of *S. aureus* gave similar results.

Additional experiments revealed that the thermostable component could be diluted to low concentrations and still show an appreciable opsonic effect (Chart 6).

Serial dilutions of immune serum (No. 4–5) were prepared in fresh normal serum. The final dilutions of immune serum in the incubation mixture ranged from 1:20 to 1:10,240, whereas the total serum concentration was maintained at 10 per cent.

At dilutions up to 1:320 no significant change occurred in the rate or extent of the bactericidal process. Higher dilutions resulted in progressively less activity and no demonstrable killing took place at 1:10,240.

The Specificity of the Thermostable Opsonin.—The absorption technique was employed to evaluate the specificity of the thermostable opsonin in Smith-immune serum. In addition, the agglutinating antibody content of immune serum was determined as another index of specificity and also to compare the results with the leucocyte system.

Prior to the actual absorption experiments, agglutinin titrations were performed on strains of both *S. aureus* and *S. albus*. Table VII presents the results obtained with three lots of immune serum as well as the characteristic findings with normal rabbit serum.

TABLE VIII

Effect of Absorbed Immune Serum on the Agglutination of Staphylococci

Serum	Absorbed with	Antigen	Titer*
Smith immune No. 4–5	Smith	Smith	<5
" " " "	Stern	"	<5
" " " "	Stovall	"	80
" " " "	O'Hara	"	320
" " " "	Mendita	"	320
" " " "	E. coli	"	320
" " " "	—	"	320
Normal serum	—	"	<5
Smith immune No. 4–5	Stern	Stern	<5
" " " "	Stovall	Stovall	<5
" " " "	O'Hara	O'Hara	<5

* Reciprocal of the highest serum dilution giving a 2+ reaction.

The antigens employed were prepared in the same manner as that described for the immunization of rabbits. Stock antigens were diluted with saline to an optical density of approximately 0.2 at 420 mμ. The agglutination tests were performed in Wassermann tubes which contained 0.5 ml. of twofold serial dilutions of serum and 0.5 ml. of antigen. After mixing, the tubes were stoppered, incubated for 120 minutes at 37°C. and then for 16 hours at 4°C. The degree of agglutination was scored + to 4+ according to standard methods (23), and a 2+ reaction was selected as the endpoint. Saline and normal serum controls were included with each test. The agglutinin titer is expressed as the reciprocal of the highest final serum dilution which gave a 2+ reaction.

None of the strains of *S. aureus* were agglutinated in the presence of normal serum. The immune sera agglutinated all four strains of *S. aureus*. The agglutination (expressed in dilution end points) of the heterologous coagulase-positive organisms exhibited the following pattern: Stern > Stovall > O'Hara and MAM. No agglutination of the *S. albus* strains occurred in either normal or immune serum, except for McGaffrey which agglutinated to a similar extent in both sera. Thus, Smith immune serum agglutinated only the coagulase-positive strains employed in these studies.

The absorption of immune serum was conducted with the six organisms listed in Table VIII.

The bacteria used for absorption were prepared in a manner similar to that described for the immunization of rabbits except for the omission of 0.25 per cent phenol. Immune serum (No. 4–5) was diluted threefold in balanced salt solution at pH 7.5 and 3.0 ml. aliquots dispensed to 12 x 90 mm. screw-capped lusteroid tubes containing 0.5 ml. of packed bacteria. A control sample of immune serum which did not contain bacteria was also processed in an identical manner. After thorough mixing, the tubes were placed at 37°C. for 60 minutes and

TABLE IX

Effect of Absorbed Immune Sera on the Fate of S. aureus in the Presence of Leucocytes

Smith immune serum absorbed with	Test organism	Bacteria/ml.		
		Incubation at 37.5°C. for		
		Zero	60 min.	180 min.
S. aureus Smith	Smith	3.3×10^7	2.3×10^7	3.2×10^7
Stern	"	3.2×10^7	3.5×10^7	3.3×10^7
Stovall	"	3.1×10^7	4.1×10^6	2.1×10^6
O'Hara	"	3.1×10^7	3.1×10^5	1.6×10^5
S. albus Mendita	Smith	3.5×10^7	3.6×10^5	2.3×10^5
E. coli K-12	"	2.4×10^7	3.1×10^5	1.1×10^5
Unadsorbed control	"	3.0×10^7	3.2×10^5	1.2×10^5
Normal serum control	"	3.3×10^7	3.5×10^7	2.6×10^7
S. aureus Stern	Stern	6.1×10^7	6.7×10^7	5.6×10^7
Stovall	Stovall	2.0×10^7	2.1×10^7	1.2×10^7
O'Hara	O'Hara	2.2×10^7	2.3×10^7	1.8×10^7
—	Stern	5.4×10^7	6.0×10^5	2.3×10^5
—	Stovall	1.2×10^7	4.3×10^5	1.2×10^5
—	O'Hara	2.8×10^7	3.0×10^5	1.8×10^5

the absorbing bacteria sedimented at 10,000 R.P.M. for 30 minutes (Lourdes, model AB, rotor 9RA). The supernatant serum was removed and added to new lusteroid tubes containing another 0.5 ml. of packed bacteria. This process was repeated for a total of three absorptions. The absorbed sera were stored at 4°C. until use.

The absorbed sera were first tested by means of the agglutination technique. Table VIII demonstrates that the homologous organism (Smith), as well as Stern, removed all detectable agglutinating antibody. Absorption with the Stovall antigen reduced the titer fourfold whereas O'Hara had no demonstrable effect. *S. Albus* (Mendita) and *E. coli* did not remove anti-Smith agglutinins. It was of interest that sera absorbed with Stovall and O'Hara antigens, although still capable of agglutinating the Smith antigen, no longer agglutinated the respective absorbing organisms.

The same samples of absorbed sera employed in the agglutination test were also used in the bacteria-leucocyte suspensions.

Absorbed sera which had originally been diluted 1:3 were employed in the following manner: 0.1 ml. of absorbed serum and 0.1 ml. of fresh normal serum were added to tubes containing 60×10^6 leucocytes in 1.6 ml. After the addition of 0.2 ml. of washed bacteria, each

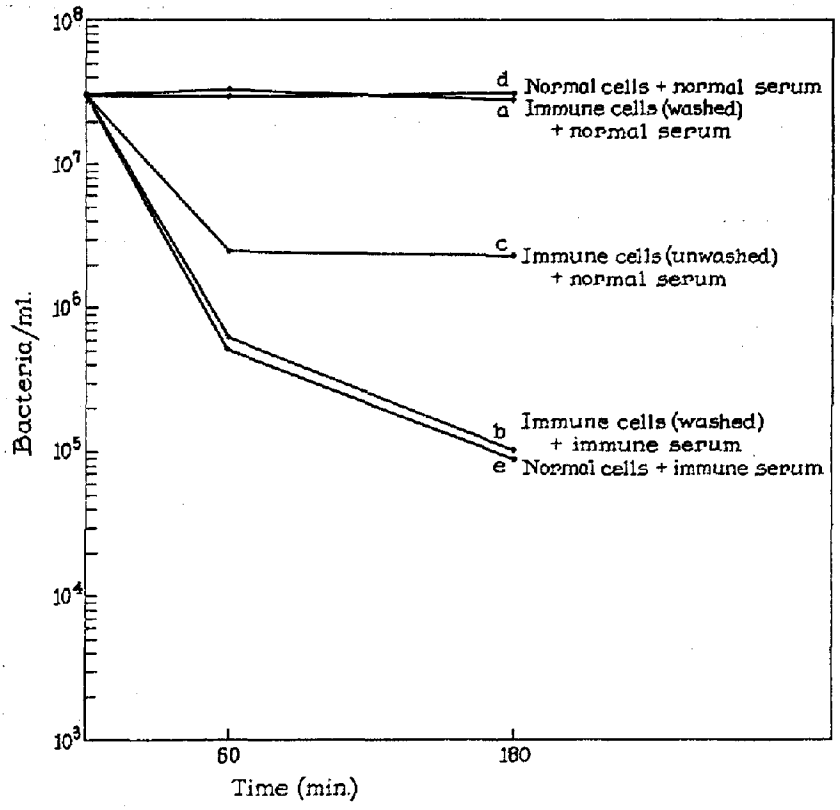

CHART 7. The interaction between *S. aureus* "Smith" and polymorphonuclear leucocytes obtained from normal and immune rabbits.

tube contained a volume of 2.0 ml. with a total serum concentration of 7 per cent. The final dilution of the absorbed immune serum in the test system was 1:60.

Table IX illustrates that the antigens which did not remove agglutinating antibodies for strain Smith also did not affect the opsonic power of the serum. The homologous antigen (Smith), as well as Stern, effectively reduced the opsonic activity of immune serum to that of the normal serum control. Strains Stovall which had an intermediate effect in absorbing anti-Smith agglutinins, had a similar intermediate effect in the leucocyte test. The three heterolo-

gous strains of *S. aureus* were shown to remove all opsonic activity when the respective strains were employed as the test organisms in the leucocyte system.

From the above studies, it appeared that the ability of a given strain to absorb agglutinins was paralleled by its ability to remove opsonizing antibody.

The Role of Leucocytes Obtained from Immune Rabbits.—The altered *in vitro* activity of mononuclear cells from immune animals has been demonstrated with such infectious agents as *Mycobacterium tuberculosis* and the brucellae (24, 25). Less is known concerning the activities of the polymorphonuclear cell under similar circumstances. Although it appeared, under the present conditions, that the opsonizing properties of immune serum were a prime factor in the inactivation of *S. aureus*, it seemed possible that the polymorphonuclear leucocyte might also exhibit distinct properties. This question was evaluated by a comparison of the properties of leucocytes obtained from normal and immunized animals.

Polymorphonuclear leucocytes were obtained from peritoneal exudates prepared in normal and immunized rabbits. The normal cells were processed in the standard fashion except for an additional wash with HBG. One aliquot of immune cells was washed twice with HBG whereas the other was merely centrifuged and resuspended in the final medium (unwashed). After adjusting the cell concentrations to 36×10^6/ml., the following tubes were prepared: (*d*) 1.6 ml. normal cells plus 0.2 ml. normal serum; (*e*) 1.6 ml. normal cells plus 0.2 ml. immune serum; (*a*) 1.6 ml. immune cells plus 0.2 ml. normal serum; (*b*) 1.6 ml. of immune cells plus 0.2 ml. immune serum; (*c*) 1.6 ml. unwashed immune cells plus 0.2 ml. normal serum. A 0.2 ml. aliquot of the Smith strain was then added to tubes (*a*)–(*e*) and sampled as previously described.

The results of one such experiment are presented in Chart 7. Immune leucocytes in the presence of normal rabbit serum behaved in a manner identical with normal cells in the same environment; *i.e.*, *S. aureus* Smith was neither phagocyted nor killed. Immune leucocytes in the presence of immune serum showed no greater efficiency of ingestion and bactericidal activity than normal cells suspended in immune serum. The results of the bacteria-leucocyte interaction in tube (*c*) (unwashed immune cells and normal serum) may reflect the effect of traces of immune serum carried over from the peritoneal exudate fluid. Under these conditions, therefore, there was no apparent functional difference between the immune and normal leucocyte.

DISCUSSION

Utilizing the techniques described in this report, it has been possible to quantitate the fate and localization of various microorganisms in suspensions of rabbit polymorphonuclear leucocytes. In general, three types of interactions were observed, which were distinguished by differences in the phagocytic and/or intracellular processes. The first is illustrated with *S. albus*, the Gram-negative bacilli and certain of the streptococci. Here, rapid phagocytosis

occurred and was shortly followed by intracellular inactivation. In all instances the majority of the ingested bacteria were destroyed, but a small residual population, associated with the leucocytes, persisted throughout the experimental period. When mycobacteria were employed, a second interaction was demonstrated in which phagocytosis took place but the organism was not susceptible to intraleucocytic bactericidal mechanisms. The third type was one in which little or no phagocytosis took place and was illustrated by the behavior of Group A streptococci and *S. aureus* in the presence of normal rabbit serum.

The majority of the experimental results have been concerned with a comparison between virulent, coagulase-positive strains of *S. aureus* and nonvirulent, coagulase-negative strains of *S. albus*. The major distinction between the two groups of organisms *in vitro* was found to be in the efficiency with which they were ingested by rabbit polymorphonuclear leucocytes. In the presence of normal rabbit serum "virulent" staphylococci were not ingested, whereas "avirulent" organisms were readily engulfed. The nature of this difference has not been clearly defined. From past evidence obtained with such bacteria as the pneumococcus (26) and streptococcus (27), it seems possible that the surface antigens of virulent staphylococci may play a deciding role. Although distinct antigenic differences between coagulase-positive and coagulase-negative strains have been demonstrated (28, 29), there is little information concerning the relationship of these materials to the phagocytic process. In this regard, Lyons (30) has reported the presence of capsular material in *S. aureus* and more recently Price and Kneeland (31) have demonstrated capsular swelling with hyperimmune rabbit serum. Such capsular material was present only in coagulase-positive strains which fermented mannitol and produced alpha toxin. The correlation between the presence of a capsule and virulence for the guinea pig has been commented upon by Gilbert (32). In any event, rabbit immune sera specifically enhanced phagocytosis of the coagulase-positive strains, presumably through some combination with a surface component. So far the nature of this component or components is unknown, and its identification will require more detailed knowledge of the antigenic structure of the staphylococci.

At the present time, it has not been possible to detect agglutinins in normal rabbit sera which react with the two prototype strains. In addition, more recent findings in this laboratory have revealed that extracts obtained from both the Mendita and Smith strains did not react with normal serum in the precipitin test. Although not conclusive, these findings suggest that interactions between staphylococci and leucocytes in the presence of normal rabbit serum take place in the absence of specific opsonins. The studies of Jensen (33) support this contention for *S. aureus* strains, in that the majority of rabbit

sera did not react with a Cowan type I antigen. Human sera, however, uniformly contained precipitins against the same antigen. This widespread occurrence of staphylococcal antibody in human sera may explain, in part, the inability of previous investigators to detect differences in the phagocytosis of virulent and avirulent strains (9, 18).

The susceptibility of coagulase-positive and coagulase-negative staphylococci to intraleucocytic bactericidal mechanisms could only be compared under conditions in which a similar degree of phagocytosis occurred; *i.e.*, *S. albus* in normal serum and *S. aureus* in immune serum. During the initial phases of the experimental period both the Mendita and Smith strains were inactivated at a rate which was identical with the rate of ingestion. In both instances a net reduction of more than 99 per cent of the organisms took place during the 180 minute incubation period. It appears, therefore, that both varieties of staphylococci are susceptible to the bactericidal properties of leucocytes. It is of interest that both *S. aureus* and *S. albus* have been reported to be sensitive to the bactericidal properties of leucocyte extracts (34, 35) as well as a variety of leucocyte products (36, 37).

Although the prototype strains did not exhibit any marked differences in either the rate or extent at which they were inactivated by leucocytes, a review of all *S. albus* and *S. aureus* strains tested showed certain distinctions. In a series of twenty or more experiments, it appeared that the absolute number of surviving coagulase-negative organisms was usually lower than that with coagulase-positive strains, by a factor of two- to tenfold. It seems, therefore, that the over-all efficiency of the *in vitro* bactericidal process, which is a reflection of phagocytosis, intracellular inactivation, leucotoxicity, etc., is somewhat greater against the coagulase-negative strains.

SUMMARY

A method has been described for the study *in vitro* of leucocyte-bacteria interactions which permits the simultaneous evaluation of both phagocytosis and intracellular bacterial inactivation. Employing this technique, the fate and localization of staphylococci in homogeneous suspensions of rabbit polymorphonuclear leucocytes have been studied.

Coagulase-positive strains of *S. aureus* were not efficiently ingested in the presence of normal rabbit serum. In contrast, coagulase-negative strains of *S. albus* were rapidly engulfed and inactivated.

Immune sera prepared against a coagulase-positive strain enhanced the the ingestion of the homologous organism as well as of three heterologous strains of *S. aureus*. Following phagocytosis, prompt intracellular killing of *S. aureus* occurred. The thermostable opsonins in immune sera reacted only with strains of *S. aureus*.

A comparison between polymorphonuclear leucocytes obtained from nor-

mal and immune animals revealed no differences in their ability either to ingest or kill coagulase-positive staphylococci.

Studies with other bacterial species are presented to illustrate: (*a*) phagocytosis followed by intracellular inactivation; (*b*) phagocytosis followed by intracellular survival; and (*c*) the absence of phagocytosis.

BIBLIOGRAPHY

1. Dubos, R. J., The Bacterial Cell, Cambridge, Harvard University Press, 1947.
2. Wright, A. E., and Douglas, S. R., An experimental investigation of the role of the blood fluids in connection with phagocytosis, *Proc. Roy. Soc. London*, 1904, **72**, 357.
3. Mudd, S., McCutcheon, M., and Lucké, B., Phagocytosis, *Physiol. Rev.*, 1934, **14**, 210.
4. Martin, S. P., and Green, R., Methods for the study of human leucocytes, *Methods Med. Research*, 1958, **7**, 137.
5. Hirsch, J. G., Phagocytin: A bactericidal substance from polymorphonuclear leucocytes, *J. Exp. Med.*, 1956, **103**, 589.
6. Dubos, R. J., and Middlebrook, G., Media for tubercle bacilli, *Am. Rev. Tuberc.*, 1947, **46**, 334.
7. Maaløe, O., On the Relation between Alexin and Opsonin, Copenhagen, Einar Munksgaard, 1946.
8. Kerby, G. P., A method for detection of leucocyte injury based on release of a lysozyme-like enzyme, *Proc. Soc. Exp. Biol. and Med.*, 1952, **81**, 129.
9. Spink, W. W., Attempts to demonstrate a surface antigen of staphylococci and specific phagocytosis, *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 549.
10. Weinbrenner, K., Untersuchengen uber die Bedeutung der bakteriellen Variabilität für die Phagocytose, *Z. Immunitätsforsch.*, 1934, **83**, 437.
11. Wood, W. B., Jr., and Smith, M. R., Intercellular surface phagocytosis, *Science*, 1947, **106**, 86.
12. Smith, J. M., and Dubos, R. J., The behavior of virulent and avirulent staphylococci in the tissues of normal mice, *J. Exp. Med.*, 1956, **103**, 87.
13. Smith, J. M., Studies on the fate of virulent and avirulent staphylococci in mice, *Ann. New York Acad. Sc.*, 1956, **65**, 67.
14. Schneider, H. A., and Zinder, N. D., Nutrition of the host and natural resistance to infection. V. An improved assay employing genetic markers in the double strain inoculation test, *J. Exp. Med.*, 1956, **103**, 207.
15. Lancefield, R. C., Occurrence of R antigen for group A type 3 streptococci, *J. Exp. Med.*, 1958, **108**, 329.
16. Hotopp, M., and Kahn, M. C., The fate of phagocytized acidfast bacteria as determined by the single cell method, *J. Infect. Dis.*, 1936, **58**, 324.
17. Dubos, R. J., and Schaedler, R. W., Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections. I. Protective effects, *J. Exp. Med.*, 1957, **106**, 703.
18. Rogers, D. E., and Tompsett, R., The survival of staphylococci within human leucocytes, *J. Exp. Med.*, 1952, **95**, 209.

19. Tompsett, R., The survival of staphylococci within phagocytic cells, *Bull. New York Acad. Med.*, 1954, **30**, 480.
20. Rogers, D. E., Observations on the nature of staphylococcal infections, *Bull. New York Acad. Med.*, 1959, **35**, 25.
21. Ecker, E. E., Weisberger, A. S., and Pillemer, L., The opsonins of normal and immune sera. I. Methods. A comparison of normal and immune opsonins on *Staphylococcus aureus*, *J. Immunol.*, 1942, **43**, 227.
22. Ward, H. H., and Enders, J. F., An analysis of the opsonic and tropic action of normal and immune sera based on experiments with the pneumococcus, *J. Exp. Med.*, 1933, **57**, 527.
23. Kolmer, J. A., and Boerner, F., Approved Laboratory Technic, New York, D. Appleton-Century Co., 1945.
24. Lurie, M. B., Studies on the mechanism of immunity in tuberculosis, the fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals, *J. Exp. Med.*, 1942, **75**, 247.
25. Pómales-Lebron, A., and Stinebring, W. R., Intracellular multiplication of *Brucella abortus* in normal and immune mononuclear phagocytes, *Proc. Soc. Exp. Biol. and Med.*, 1957, **94**, 78.
26. Avery, O. T., The role of specific carbohydrates in pneumococcus infection and immunity, *Ann. Int. Med.*, 1932, **6**, 1.
27. Maxted, W. R., The indirect bactericidal test as a means of identifying antibody to the M antigen of *Streptococcus pyogenes*, *Brit. J. Exp. Path.*, 1956, **37**, 415.
28. Julianelle, L. A., and Wieghard, C. W., The immunological specificity of staphylococci. I. The occurrence of serological types, *J. Exp. Med.*, 1935, **62**, 11.
29. Cowan, S. T., The classification of staphylococci by precipitation and biological reactions, *J. Path. and Bact.*, 1938, **46**, 31.
30. Lyons, C., Antibacterial immunity to *Staphylococcus pyogenes*, *Brit. J. Exp. Path.*, 1937, **18**, 411.
31. Price, K. M., and Kneeland, Y., Jr., Further studies of the phenomenon of capsular swelling of *Micrococcus pyogenes* var. *aureus* in the presence of immune serum, *J. Bact.*, 1956, **71**, 229.
32. Gilbert, I., Dissociation in an encapsulated staphylococcus, *J. Bact.*, 1931, **21**, 157.
33. Jensen, K., A normally occurring staphylococcus antibody in human serum, *Acta Path. et Microbiol. Scand.*, 1958, **44**, 421.
34. Fishman, M., and Silverman, M. S., Bactericidal activity of rat leucocytic extracts. I. Antibacterial spectrum and the subcellular localization of the bactericidal activity, *J. Exp. Med.*, 1957, **105**, 521. Fishman, M., Cole, L. J., and Silverman, M. S., Bactericidal activity of rat leucocytic extracts. II. Characterization of the bactericidal substance in leucocyte mitochondrial extracts, *J. Exp. Med.*, 1957, **105**, 529.
35. Tompsett, R., Protection of pathogenic staphylococci by phagocytes, *Tr. Assn. Am. Physn.*, 1956, **69**, 84.
36. Hirsch, J. G., Bactericidal action of histone, *J. Exp. Med.*, 1958, **108**, 925.
37. Dubos, R. J., Effect of ketone bodies and other metabolites on the survival and multiplication of staphylococci and tubercle bacilli, *J. Exp. Med.*, 1953, **98**, 145.