FATE OF STAPHYLOCOCCI WITHIN HUMAN LEUKOCYTES*

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In 1894 Van de Velde reported studies from which he concluded that "virulent" staphylococci were not readily destroyed by rabbit polymorphonuclear leukocytes, while "attenuated" strains were killed rapidly following phagocytosis (1). Studies from our laboratory and elsewhere have supported this observation and have suggested that pathogenic strains survive for long periods within the cytoplasm of phagocytic cells obtained from different animals and man (2–5). In more recent work, Cohn and Morse demonstrated that pathogenic strains of staphylococci are ingested by rabbit granulocytes only in the presence of specific antistaphylococcal opsonins (6). These studies, in contrast to our own, also suggested that pathogenic strains were destroyed as rapidly as non-pathogenic strains once phagocytosis had taken place.

Review of the different methods used in studies of staphylococcal–phagocytic cell interactions suggested that these conflicting findings might result from the use of large populations of human or animal leukocytes and staphylococci in the conduct of experiments on phagocytosis. To date, the fate of individual staphylococci ingested by individual leukocytes has not been systematically explored.

The techniques developed by Dr. Armine Wilson in his studies on the phagocytosis and intracellular behavior of streptococci seemed ideally suited to give direct answers to certain questions relating to the phagocytosis of staphylococci (7). These questions were:

1. Are there detectable differences in the intracellular fate of pathogenic and non-pathogenic staphylococci?

2. If pathogenic staphylococci survive within cells, is this property related to their ability to damage leukocytes?

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3. Can pathogenic staphylococci multiply within active, motile phagocytic cells?

The present paper reports results of direct visual studies of the phagocytic process and partial answers to these questions.

Materials and Methods

Cultures.—Two prototype strains of staphylococci were employed in these experiments. The Smith "diffuse" variant, a coagulase-positive *Staphylococcus aureus*, was used as a "pathogenic" strain and was passed through mice at appropriate intervals to maintain virulence. Mendita, a coagulase-negative *Staphylococcus albus*, was used as a representative "non-pathogenic" microorganism. The characteristics of these strains have been described in previous publications (5). Both cultures were preserved on agar slants at 4°C. Two to 3 hour subcultures in Todd-Hewitt broth (Difco) were used to provide young, actively multiplying organisms of maximum viability. In such cultures cocci were rather uniformly dispersed in pairs, although single bacteria, triads, and tetrads were occasionally seen.

The Phagocytic System.—Slide preparations were made as described by Wilson (7). Blood was obtained from a single donor. Equal proportions of human fingertip blood, Todd-Hewitt broth containing 0.05 per cent heparin, and an appropriate amount of the young culture of staphylococci were mixed on a sterile glass slide. A small loopful of this mixture was placed on a sterile coverslip and inverted on a slide so that a thin layer of fluid spread to the edges. The two lateral sides of the coverslip were sealed with melted paraffin. In the original experiments salt agar blocks were used to seal the two opposite ends so that contact was made with the fluid, but during the course of prolonged observations at 37°C., the agar blocks tended to shrink away from the sides of the coverslip, thus breaking connection with the fluid layer. In the experiments reported here, redux® electrode paste[1] was employed after control experiments demonstrated that this material in concentrations as high as 10 per cent did not inhibit bacterial multiplication or leukocyte activity.

Electrodes were inserted into the electrode paste and led through a switch to opposite poles of a heathkit power supply[2] capable of delivering a 500 volt potential.

The completed preparation was placed in a microscope stage incubator (Fisher) maintained at 37°C., and observations were made using a Zeiss photomicroscope. Phase contrast lighting with an interference filter was employed. Magnifications of 1250 were obtained with a 100 × neofluar oil immersion objective, an optovar setting of 1.25 ×, and 10 × oculars.

Within a few minutes after preparation, the polymorphonuclear cells settling on the glass began to show characteristic ameboid activity. An active granulocyte approaching cocci was selected for study. In most experiments the individual leukocyte was observed to ingest one pair of cocci, the time recorded, and the granulocyte then kept under continuous observation without further ingestions. In certain experiments to be described, the leukocyte was permitted to ingest an additional pair or group of cocci in one or two further phagocytic operations. Observations for periods of over 30 minutes were often complicated by extracellular bacterial multiplication which increased the numbers of cocci present in groups available for ingestion. Under these circumstances it was not possible to trace the fate of ingested microorganisms with certainty, and only experiments in which small numbers of cocci were ingested have been used for analysis. The maximum period of intracellular residence suitable for study to date has been 90 minutes.

[1] Redux electrode paste, obtained from the Sanborne Company, Waltham, Massachusetts, contains NaCl, H_2O, quartz, $NaHCO_3$, methyl parasept, propylene glycol, and carboxyl methyl cellulose in unknown concentrations.

[2] Model PS-2, The Heath Company, Benton Harbor, Michigan.

At appropriate intervals the leukocyte which contained cocci was disrupted by passing current through the preparation in brief shocks lasting approximately 0.5 to 1.0 second. Leukocyte motility usually halted abruptly and the cell swiftly rounded. Often a small rupture appeared in the leukocyte membrane which allowed cytoplasmic granules to escape into the surrounding fluid. The red blood cells migrated abruptly toward the anode but were otherwise not visibly altered. Extracellular bacteria usually remained stationary and were able to multiply freely after shock. Intracellular staphylococci remained in the debris of the neutrophil or were extruded with cell rupture. Multiplication of cocci released from cells appeared to take place equally well in either locus. Preparations were observed for 3 to 24 hours. Growth of bacteria was ordinarily apparent within 3 hours.

RESULTS

The Phagocytic Process.—Active polymorphonuclear leukocytes under observation sent out clear homogeneous pseudopods into which granules streamed as the cell proceeded along the glass. At first leukocytes exhibited random movements and pseudopods appeared from any part of the cell membrane. As time went on phagocytes often developed what appeared to be real orientation, and a motionless, more opaque tail-like structure dragged behind the advancing cell (see Figs. 1 through 7). Although the majority of experiments were performed at body temperature, results obtained at room temperature seemed in every way similar. In general, however, the granulocytes appeared more active at the higher temperature.

Although many of the contacts between phagocytes and cocci appeared to be chance occurrences, within a certain distance from the microorganism granulocytes often seemed to move directly toward the cocci, pushing aside red blood cells blocking a direct path.

While no differences in the actual ingestion process were discerned, cocci of the coagulase-negative strain were ingested more swiftly than cocci of the coagulase-positive strain in this system containing human serum. In 63 separate experiments the time elapsing between the mixing of blood and bacteria and the observed ingestion of cocci of the Mendita strain was 17 minutes $\pm$ S. E. 1.8 minutes. In contrast, 26.4 minutes $\pm$ S. E. 1.7 minutes was required for the phagocytosis of cocci of the Smith strain. These differences were statistically significant with a t value of 3.79 and a p value of 0.0003. This observation was in keeping with the findings previously reported with large populations of the same strains (8).

When either pathogenic or non-pathogenic staphylococci were engulfed, the neutrophil often halted and remained stationary for several minutes. Under these circumstances thin, terminally hooked pseudopods often erupted from multiple sites on the cell surface (see Fig. 9). These were thrown out and withdrawn without advancing the cell. The cell would then regain its usual ameboid activities and proceed onward.

As extracellular growth took place in prolonged experiments, pairs of cocci were replaced by larger clumps of microorganisms. In certain experiments,

single granulocytes were allowed to successively engulf several dozen microorganisms to assess the effect of extensive phagocytic action on cellular activity. Granulocytes appeared capable of ingesting large groups of cocci, although phagocytosis of such groups was slow. While leukocytes ingesting massive numbers of cocci became rounded and sluggish, this phenomenon was noted during phagocytosis of either the Smith or Mendita strains, and the occasional

TABLE I

The Fate of Pathogenic and Non-Pathogenic Staphylococci after Increasing Periods of Residence within Human Polymorphonuclear Leukocytes

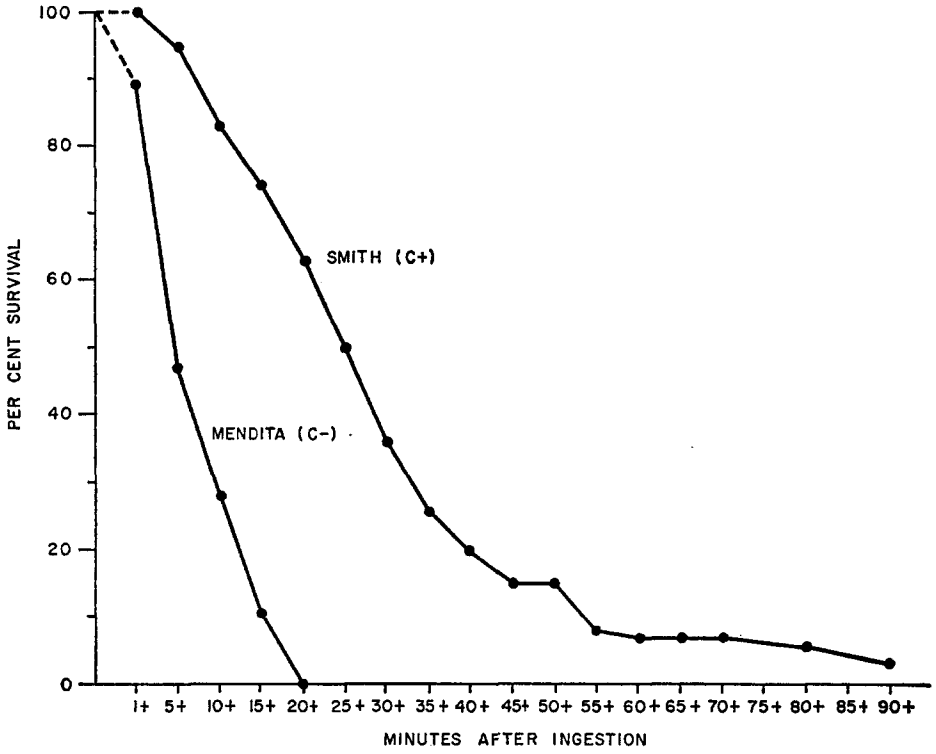
Length of intracellular residence	Mendita (50 experiments)					Smith (50 experiments)				
	Survived	Died	Total survived	Total died	Survival	Survived	Died	Total survived	Total died	Survival
min.					*per cent*					*per cent*
0–4	1	1	8	1	89	2	0	20	0	100
5–9	2	7	7	8	47	2	1	18	1	95
10–14	3	5	5	13	28	2	2	16	3	84
15–19	2	3	2	16	11	2	2	14	5	74
20–24	0	8	0	24	0	2	2	12	7	63
25–29	0	4	0	28	0	2	3	10	10	50
30–34	0	5	0	33	0	2	4	8	14	36
35–39	0	3	0	36	0	1	3	6	17	26
40–44	0	2	0	38	0	1	3	5	20	20
45–49	0	2	0	40	0	0	2	4	22	15
50–54	0	1	0	41	0	2	0	4	22	15
55–59						0	1	2	23	8
60–64	0	1	0	42	0	0	2	2	25	7
65–69						0	1	2	26	7
70–74						0	1	2	27	7
75–79										
80–84						1	2	2	29	6
85–89										
90–94						1	1	1	30	3
95–99										

failure of leukocytes to recover vigorous motion could not be related to pathogenicity. As time went on a network of tiny strands, presumably fibrin, often surrounded the clumps of extracellular staphylococci and appeared to prevent ready phagocytosis by the leukocyte.

Ingested Staphylococci.—Ingested staphylococci were moved into the interior of the cell and either appeared in a vacuole or disappeared among the cytoplasmic granules. Over long periods of observation vacuoles often appeared, vanished, and reappeared. Microorganisms were sometimes clearly visible in the center of vacuoles, sometimes barely apparent at the vacuole edge. Groups

of microorganisms were generally broken up into pairs which migrated around the leukocyte separately as incubation proceeded. (See Figs. 6 through 10).

Occasionally cocci of either strain were observed to divide inside the granulocyte during the first few minutes after ingestion. (See Figs. 13 and 14.) This phenomenon usually occurred within neutrophils which did not fully recover



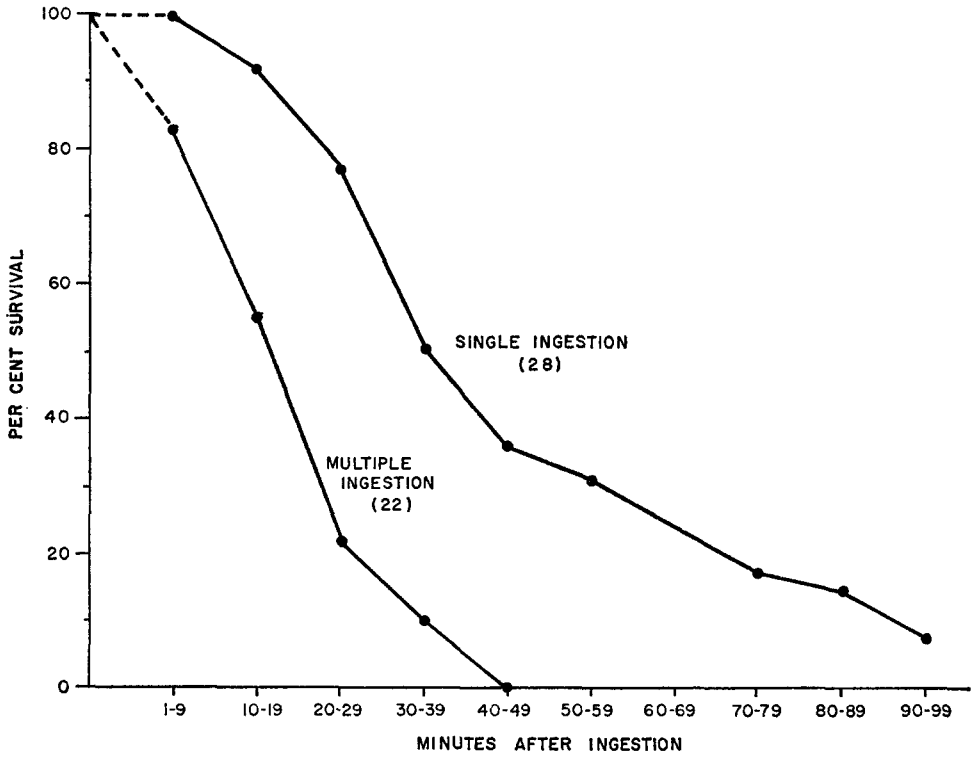
TEXT-FIG. 1. The survival of pathogenic and non-pathogenic staphylococci in human leukocytes. A small but significant number of cocci of the Smith strain survived for periods of 90 minutes, the longest period of intracellular residence studied.

normal motility after phagocytosis. In a few instances intracellular multiplication of the pathogenic cocci occurred as the leukocyte began to lose motility 1 hour or more after phagocytosis. Such intracellular multiplication has been clearly recorded in a series of time lapse motion photomicrographs utilized to better evaluate the characteristics of leukocyte activity during phagocytosis.

On rare occasions cocci were egested. These organisms subsequently were left behind, were reingested, or were dragged along as they stuck to the exterior of the granulocyte.

Survival of Intracellular Staphylococci.—Using this technique, it was possible

to watch the entire process of phagocytosis, intracellular residence, leukocyte disruption, and the subsequent ability of ingested bacteria to multiply *in situ*. (See Figs. 1 to 20). By disrupting different leukocytes at varying intervals after ingestion, the speed of intracellular destruction or the length of intracellular survival could be determined by multiple experiments. The results of 100 experi-



TEXT-FIG. 2. The effect of multiple ingestions on the survival of pathogenic staphylococci. Multiple ingestions markedly reduced the survival of the total intracellular population of cocci.

ments are tabulated according to the method of Reed and Muench (9) in Table I and graphed in Text-fig. 1.

As noted here, impressive differences in the intracellular survival of the Smith and Mendita strains of staphylococci were obtained in such direct experiments. The non-pathogenic strain was rapidly rendered incapable of multiplication after leukocyte ingestion. Following periods of intracellular residence as short as 5 to 9 minutes, less than 50 per cent of the Mendita strain survived, while none was capable of multiplying after 20 minutes within the cell. In contrast, 63 per cent of cocci of the Smith strain survived 20 minutes within leukocytes.

Cocci most commonly remained viable in leukocytes which seemed less active after the ingestion of staphylococci, but this was difficult to establish with certainty. In general, intraleukocytic survival could not be accurately predicted on the basis of the appearance of the contained cocci or the behavior of the leukocyte which ingested them. Of particular interest was the fact that small numbers of Smith strain cocci were capable of surviving periods of 90 minutes, the maximum period of intraleukocytic residence employed in these studies.

The Effect of Multiple Ingestions on the Outcome of Phagocytosis.—Early in the course of these observations it was noted that multiple ingestions of cocci by a single leukocyte appeared to act as a stimulus to bacterial destruction. When more than one group of pathogenic cocci were ingested in two or three separate phagocytic episodes, the survival time of the *total* intracellular population of cocci was greatly reduced.

Data have been regrouped to illustrate this phenomenon in Text-fig. 2. In a series of 28 experiments, the ingestion of a single pair of cocci yielded 50 per cent survival of phagocytized cocci after 30 minutes of intracellular residence. In contrast, in 22 experiments in which an additional pair or additional two pairs of cocci were phagocytized, only 10 per cent of the intracellular bacteria survived similar periods of incubation within the cell.

DISCUSSION

In the present experiments, the ingestion and subsequent fate of staphylococci within individual human leukocytes were observed directly. These studies indicate that a significant number of coagulase-positive staphylococci survive for long periods within living human granulocytes. Leukocyte activity appeared similar following the ingestion of cocci of the non-pathogenic strain which were generally destroyed, or cocci of the pathogenic strain which often survived. Intracellular survival could not be related definitively to leukocyte damage, although cells in which staphylococci survived often seemed less vigorous than leukocytes which killed ingested cocci. Both pathogenic and non-pathogenic cocci were seen to divide in active leukocytes soon after ingestion. Only pathogenic staphylococci underwent obvious late multiplication in viable leukocytes, but this was seen only in granulocytes approaching death.

It seems of particular interest that the phagocytosis of more than one group of pathogenic staphylococci by the process of multiple ingestions appears to result in more efficient destruction of intracellular cocci than the single ingestion of small numbers of microorganisms.

Cohn and Morse have recently shown that rabbit leukocytes undergo a rapid increase in oxygen consumption, glucose utilization, and lactic acid production during the process of phagocytosis (10). These investigators have further demonstrated that polymorphonuclear leukocytes which are allowed to ingest dead bacteria or other par-

ticulate substances prior to exposure to living staphylococci are capable of destroying more bacteria than control leukocytes from the same exudate (11).

These investigators have explained such heightened bacterial destruction on the basis of enhanced phagocytic activity of such cells. The present experiments suggest an alternative hypothesis. It seems possible that the single ingestion of small numbers of staphylococci is well tolerated by leukocytes and does not effectively mobilize the bactericidal capabilities of the cell. In contrast, multiple ingestions of greater numbers of staphylococci may fully activate intracellular bactericidal mechanisms which then result in the destruction of all the ingested bacteria.

Evidence to support this thesis is derived from other studies by Cohn, Hirsch, and Morse. Recent experiments indicate that many intracellular enzymes are located within the leukocyte granules and are not freely released without granule disruption (12). They have shown that intracellular granules disappear from the cytoplasm of rabbit leukocytes during the process of phagocytosis and have tentatively suggested that the lysis of cytoplasmic granules or their incorporation in the wall of vacuoles surrounding bacteria releases bactericidal substances which then act on ingested microorganisms (13). These investigators have also shown that the cytoplasmic granules in rabbit leukocytes disappear during the process of phagocytosis. Degranulation appears more complete when more particles are ingested (14).

It thus appears reasonable to suggest that multiple ingestions may provoke more extensive lysis of granules and release of intracellular enzymes to the cytoplasm of the leukocyte. This process may promote more effective intracellular destruction of pathogenic staphylococci as observed in the present experiments. Attempts to correlate the degranulation of leukocytes with the efficiency of intracellular killing of staphylococci are in progress.

SUMMARY

Direct observations by phase microscopy have demonstrated that small numbers of pathogenic staphylococci survive prolonged periods of time within living human polymorphonuclear leukocytes. Non-pathogenic microorganisms are rapidly destroyed in similar preparations.

Leukocytes in which staphylococci remained viable often appeared less vigorous after ingesting microorganisms, but intracellular survival could not be correlated with obvious leukocyte damage with any consistency.

Both pathogenic and non-pathogenic cocci were seen to divide within living granulocytes during the first few minutes after ingestion. Occasionally pathogenic staphylococci multiplied in dying cells after long periods of intracellular residence.

Phagocytosis of more than one pair of staphylococci by a single leukocyte appeared to act as a stimulus to bacterial destruction. Multiple ingestions of pathogenic staphylococci reduced the incidence of survival of the total microbiol population contained within the cell.

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EXPLANATION OF PLATES

Photomicrographs were taken with a Zeiss photomicroscope on 35 mm. adox film and enlarged to a final magnification of approximately 1200. The entire process of phagocytosis, intracellular residence, leukocyte disruption by electrical shock, and subsequent multiplication of intracellular cocci is illustrated in serial photographs of a single leukocyte.

PLATE 92

FIG. 1. A human neutrophil approaches a group of eight coagulase-positive staphylococci. Note the clear advancing pseudopod and small opaque "tail."

FIG. 2. The rapidly advancing leukocyte pushes between red blood cells toward the cocci.

FIGS. 3 to 5. Process of ingestion.

FIG. 6. Ingestion is complete.

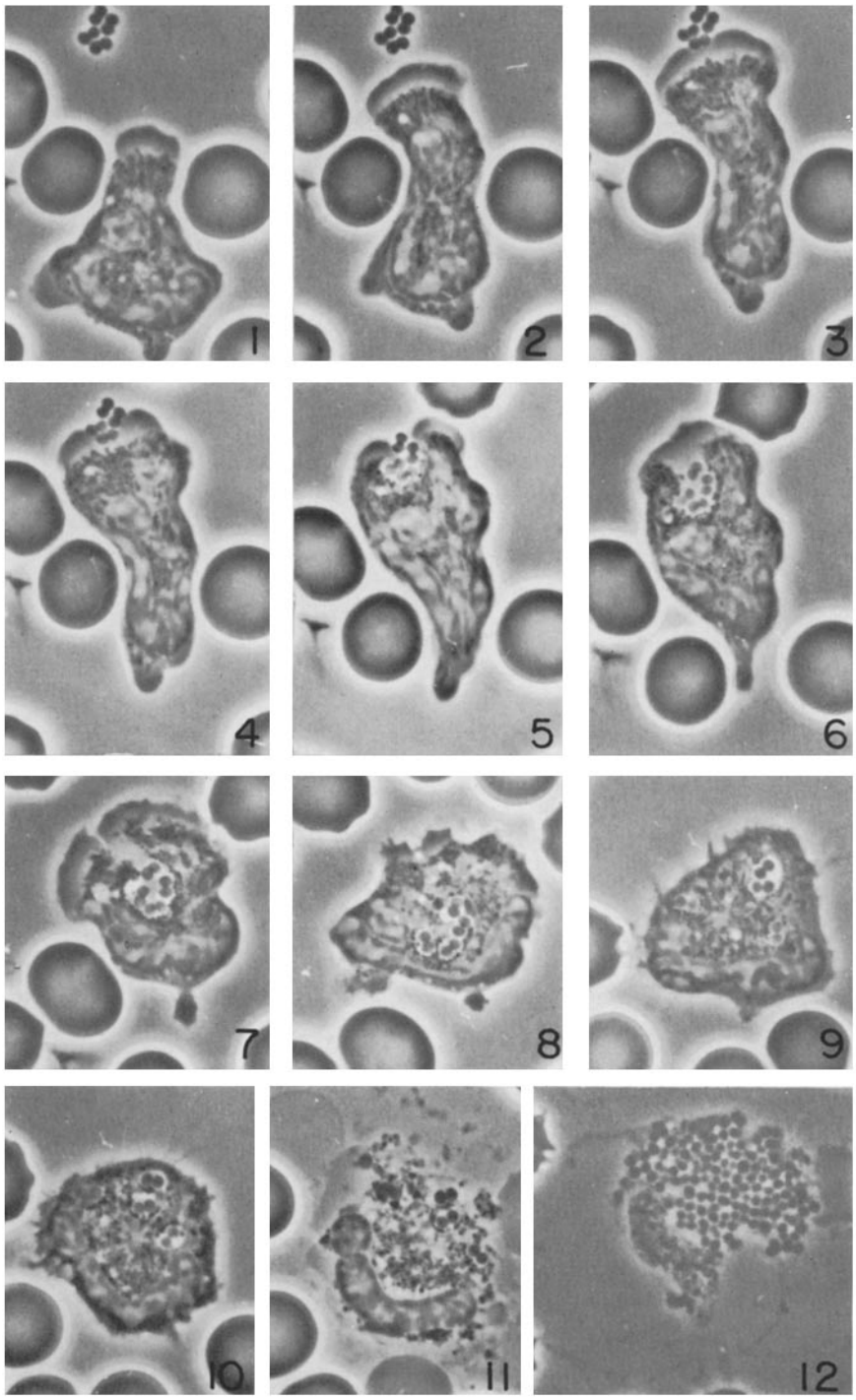
FIGS. 7 and 8. Cocci are moved into interior of cell and appear in vacuoles.

FIG. 9. Pairs of cocci separate within the cytoplasm. Two pairs are clearly within a vacuole, two pairs are only faintly visible. The leukocyte is radiating tiny disorganized projections.

FIG. 10. Cocci separate into pairs within the leukocyte 40 minutes after ingestion.

FIG. 11. The leukocyte 45 minutes after completion of phagocytosis. This picture was taken immediately after granulocyte disruption by passage of electric current.

FIG. 12. Seventeen hours after shock. The ingested staphylococci have undergone extensive multiplication. The nucleus of the leukocyte has swelled and the cell boundary is only faintly visible.



(Melly *et al.:* Fate of staphylococci within human leukocytes)

PLATE 93

FIG. 13. A living neutrophil 15 minutes after ingesting two pairs of coagulase-positive staphylococci. Within 2 minutes after phagocytosis one pair of cocci divided within the living cell, while the second pair appears about to split.

FIG. 14. Same living cell 80 minutes after phagocytosis. The second pair of cocci have divided.

FIG. 15. Immediately after shock; 80 minutes after completion of phagocytosis.

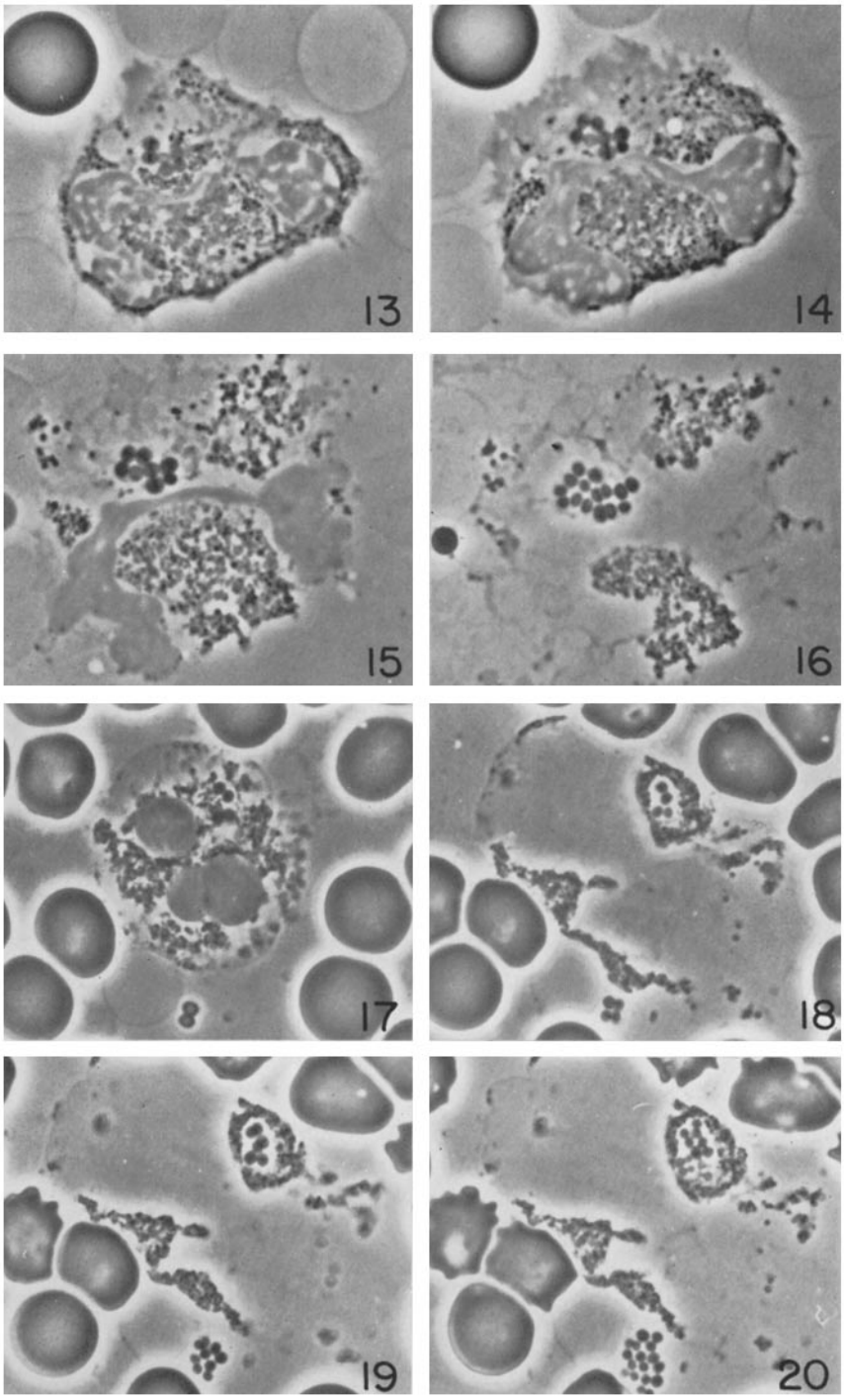
FIG. 16. Twenty hours after shock. The intracellular cocci have undergone multiplication. At least 15 cocci are visible.

FIG. 17. A different leukocyte immediately after shock. Two pairs of pathogenic staphylococci had been ingested 25 minutes before. Note pair of extracellular cocci at bottom of photograph.

FIG. 18. Thirty minutes after shock. Both intracellular and extracellular cocci have each divided once.

FIG. 19. Two hours after shock. Cocci are continuing to multiply.

FIG. 20. Three and one-half hours after shock. Cocci have multiplied extensively.



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