FATE OF NON-VIRULENT GROUP A STREPTOCOCCI PHAGOCYTIZED BY HUMAN AND MOUSE NEUTROPHILS

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The ability of phagocytic cells to destroy pathogenic bacteria is recognized as an important element in host defense. The student of immunity is interested to know whether a particular microorganism is destroyed easily and quickly by phagocytes or whether it can resist injury, surviving and perhaps multiplying within the cells, to escape later and carry on its infectious activities. This subject has recently been reviewed in a valuable paper by Suter (1). The present report presents a new technical approach to this problem and is concerned with the survival of group A streptococci in peripheral blood neutrophils as observed in glass slide preparations.

Materials and Methods

Technic for Estimating Survival of Streptococci in Neutrophils.—The principle involved in this method is that neutrophils, after phagocytizing streptococci, can be disrupted by electricity without killing the cocci. As will be demonstrated later, the cocci will then proliferate in the medium on the slide if they have not been injured by their residence within the phagocyte.

The slide preparations were made as follows: On one end of a clean glass slide were mixed a 2 mm. loopful of sterile heparin solution (50 mg. per 100 ml. of modified Todd-Hewitt broth), a small drop of finger prick human blood or mouse tail blood and a loopful of a light suspension of streptococci grown about 2 hours in modified Todd-Hewitt broth. A loopful of the mixture was transferred to a coverslip, which was inverted and dropped onto the slide. The mixture should spread evenly to all edges of the coverslip without trapping too many air bubbles. Two strips were cut from agar (4.0 gm. of agar to 100 ml. of 0.85 per cent sodium chloride solution, sterilized by autoclaving at 121°C., poured into a Petri dish in a layer 2 to 3 ml. deep and allowed to solidify) measuring approximately 2 × 3 × 15 mm. The strips were transferred to the slide and carefully moved into contact with opposite sides of the coverslip. The edges of the coverslip not adjacent to the agar strips were sealed with vaseline. The slide was then moved to the microscope and the preparation was examined through the dark medium phase contrast 1.8 mm. objective and a neutrophil approaching a chain of cocci was sought. A stop-watch was started at the beginning of phagocytosis and a note was made of the time when phagocytosis was completed.

At any desired time following completion of ingestion, a 500 volt direct current shock

was sent through the preparation by touching electrodes to the two agar strips. The voltage source was the heathkit variable voltage regulated power supply, Model PS-3 (The Heath Co., Benton Harbor, Michigan). The neutrophil almost always disrupted within a few seconds or after several brief shocks and the cocci were seen to lie in the debris of the disrupted cell, or occasionally to lie freely in the medium beside it. The stop-watch was stopped when the cell disrupted and the length of time the cocci were in the intact cell was recorded. The preparation was then watched for 3 hours and by counting the cocci it could be determined whether they had proliferated. In some of the early experiments done with this technic the observation was limited to 2 hours. In most experiments done in the afternoon, the preparations were allowed to stand overnight, and any additional proliferation was noted. Whenever possible an extracellular chain of unphagocytized cocci in the same field or in a nearby field was selected for observation as a growth control. For the purposes of this study, the cocci were considered non-proliferative if the number of cocci had not increased by the end of the observation period, and a chain was considered to have survived if there was any increase in coccal numbers, no matter how small. Thus if 20 cocci were counted immediately after shock and 21 cocci were counted 3 hours later, the chain was listed as having survived the phagocytosis. This obviously weights the results in favor of survival of the ingested cocci, and the error resulting from this arbitrary distinction between survival and non-survival will be considered later. Some difficulty arises in making coccal counts, because there is a stage in the division of a single coccus into two cocci when it is difficult to decide whether to assign a count of one or two. In order to ensure uniformity of treatment, the 3 observers in this study have arbitrarily considered a dividing coccus a single coccus when there is more than the slenderest connection between the two bodies. In some of these experiments the microscopes were enclosed in incubator boxes in which the temperature was maintained at 35°C., but most experiments, including those on proliferation of extracellular chains, were conducted at room temperature, which ranged from 22 to 30°C.

The desire to keep cocci within leukocytes for a predetermined time was sometimes thwarted by egestion of some or all of the cocci. Egestion occurred more frequently in slides kept at 35°C. than at room temperature. Sometimes electric current was applied as soon as egestion was observed and all the cocci were watched for proliferation. Occasionally egested cocci were ignored, and only those remaining continuously in the cell were observed for growth after electric shock. When the egested cocci were immediately rephagocytized by the cell being watched, the experiment was allowed to continue and no adjustment was made for the short time the cocci had been outside the cell. In 6 experiments parts or all of the originally phagocytized cocci were egested, were not rephagocytized, and remained in the field of observation following electric shock. The egested cocci and the cocci liberated by shock were counted separately.

The strains used in these studies, 327W (type 1) and T2/44 (type 2), are laboratory strains that have been maintained on artificial media for many years. They produce little or no M substance and their virulence is low, mice usually surviving intraperitoneal inocula of 10^{-1} ml. of blood broth culture and always surviving 10^{-2} ml. Human peripheral blood neutrophils were obtained from 3 donors whose cells showed no recognizable difference in ability to phagocytize the two test strains.

RESULTS

Failure of Cocci to Multiply in Intact Neutrophils.—In innumerable observations concerned with other aspects of phagocytosis of streptococci by human and mouse neutrophils it was incidentally observed that little or no division of the cocci occurred after they entered the cell, whether a vacuole formed about them or not. At best there was some advance in the degree of constriction in a

dividing coccus, but even this was an uncommon occurrence. In order to obtain more specific information on this point, 3 experiments were performed in which a phagocytosis was observed and the ingested cocci were then observed uninterruptedly for 5 hours. There was no evidence of any multiplication of these intracellular cocci. These observations were not entirely satisfactory because, although there was no evidence of life in the cocci, it could not be assumed that they would not divide if they could be removed from the cell and placed in a favorable environment. This removal happened, in effect, when egestion occurred or when the leukocyte disrupted from leukotoxic action, but these events could not be produced at will. A technic was required in which the leukocyte could be destroyed at any desired time, and the ability of the liberated cocci to multiply could be determined. The electric shock technic was developed to accomplish this.

Effect of Electric Shock on Blood Cells.—When the 500 volt potential is applied to the slide preparations, as described in the section on methods, the erythrocytes and streptococci immediately start to migrate toward the anode and continue to move at a fairly uniform rate as long as the current is flowing. The erythrocytes are not hemolyzed by the amount of current used to disrupt neutrophils, but may do so on prolonged applications, lasting 30 seconds or more. Leukocytes and platelets do not migrate in response to the current. A very short application of electricity usually results in rupture of the surface of neutrophils, and the break is almost always on the anodal side of the cell. Cytoplasmic constituents stream through the break until the current is interrupted. From the time the current first starts to flow until the neutrophil ruptures, the cell often continues in ameboid movement and there is no recognizable attraction of intracellular structures towards anode or cathode. Following rupture, changes occur in the appearance of the cellular detritus that resemble closely the changes following leukotoxic disintegration with cellular rupture (2). Monocytes and eosinophils are somewhat less likely to rupture than are neutrophils, and lymphocytes are even more resistant. The effect of the current on the cocci is discussed later.

Although the voltage used to disrupt the leukocytes is high, the actual amount of current that flows through the very thin layer of fluid in the preparations is small. The resistance between the agar strips varies from 500,000 to 2,000,000 ohms and the current produced by a 500 volt potential is not over 1 ma. (It requires only 4 volts to produce current of similar magnitude between electrodes 22 mm. apart immersed in a tube of Todd-Hewitt broth). We believe that there may be an important mechanical element in the leukocytic rupture because compressed cells disrupt more quickly than cells in thicker parts of the preparation, although more current doubtless flows through the latter areas. The fact that the erythrocytes are not hemolyzed suggests that changes in the medium due to electrolysis are not of great magnitude.

Survival Time of Ingested Cocci.—Using the electric shock technic described

in the section on methods, 187 experiments have been done in which the cocci were allowed to remain within phagocytes for periods varying from 1 to 31 minutes. The phagocytes were human neutrophils in 97 of these experiments (51 with 327W and 46 with T2/44) and were mouse neutrophils in 90 (48 with 327W and 42 with T2/44). The results are presented in Table I, each experiment being entered under the time elapsing between completion of phagocytosis and disruption of the phagocyte by electricity (grouped into 5 minute intervals). The cocci were injured rapidly by the leukocytes. In the group of 16 experiments with human cells in which the cocci were intracellular for at least 20 minutes, only once did the chain survive. None survived longer than 15 minutes in mouse cells. There was no significant difference between the 2 strains of streptococci used, and they have been tabulated together.

The method of Reed and Muench (3) for estimating the LD_{50} of injurious agents is well suited for application to these data, the injurious factor here

TABLE I

Survival of Group A Streptococci in Phagocytes

Source of phagocytes	Time interval in cell				
	0-4 min. 59 sec.	5 min.-9 min. 59 sec.	10 min.-14 min. 59 sec.	15 min.-19 min. 59 sec.	20 min. +
Human	15/17*	11/23	4/21	3/20	1/16
Mouse	13/15	9/24	7/25	0/25	0/1

* Ratio of number of experiments in which the cocci survived to the total number of experiments in the time interval.

being the length of time the cocci have been within the phagocytes; *i.e.*, the LD_{50} is the calculated time at which in 50 per cent of cases the cocci have been rendered non-proliferative. For human blood the LD_{50} was 8 minutes and for mouse blood $6\frac{3}{4}$ minutes.

The validity of these results depends essentially on the correctness of three propositions: (*a*) that streptococci, if they have not been phagocytized, will proliferate in the preparations, (*b*) that phagocytized streptococci on release from the phagocyte by electric shock will proliferate if alive, and (*c*) that if the cocci do not proliferate when released, it is because they have been injured by the phagocyte and not by the electric current used to disrupt the phagocyte.

To test the first of these propositions, observations have been made of 196 unphagocytized streptococcal chains over growth periods of $1\frac{1}{2}$ to 20 hours. 130 were in human blood and 66 were in mouse blood preparations. 172 of the chains were exposed to electric current sufficient to disrupt neutrophils. All the chains in the human blood preparations grew, and all but 3 chains in the

mouse blood multiplied. The generation time varied considerably from chain to chain, and was shorter in fresh preparations than in ones that had stood several hours. The preparations are very thin, about 1 to 2 microns deep, and it is not surprising that growth should not continue indefinitely, because the medium becomes exhausted relatively quickly. Nevertheless, growth has been seen to continue in some preparations as long as 6 hours. The size of the initial bacterial inoculum has a marked influence on the duration of proliferation. The important factor here appears to be the density of chains in a given area in relation to the amount of medium in the area. Small inocula have always been used in these experiments, but there is often an uneven distribution of chains in the slide and exact quantitative regulation of chain-density in a given area has not been possible. The bacterial inoculum cannot be reduced much below the size that we have employed, for if it is, the observer may not succeed in encountering a phagocytosis within a reasonable time.

When the leukocyte is disrupted by electric current, the cocci are usually seen to be lying in the cellular debris. Occasionally they escape from the cell entirely and are found free in the medium adjacent to the debris. When found in this free state it is reasonable to suppose that the cocci should have as good a chance of growing as other cocci in the same environment, unless something has happened to them within the phagocyte. Comparison of these two circumstances makes it possible to say that the debris of the disintegrated leukocyte has little or no inhibitory effect on proliferation of the cocci, for in many observations cocci lying in the debris have multiplied promptly and freely.

The possibility that the non-survival of the ingested cocci is due in some part to electrical injury cannot be eliminated entirely. Against this possibility is the fact, shown above, that unprotected extracellular cocci exposed to the electric shock do not appear to be injured, at least in so far as ability to proliferate is concerned. The amount of electricity used to disrupt leukocytes whether they have contained cocci 1 or 20 minutes is approximately the same, and the cocci have thus been exposed to similar amounts of electric current in these two circumstances. Yet the chances that they will multiply after shock are quite different. It seems to us that this difference can more reasonably be attributed to a direct injurious action of the phagocyte rather than to electric current, unless one postulates a progressive susceptibility to electric injury developing as the cocci remain longer within the cell. Furthermore cocci liberated from phagocytes by egestion or upon leukotoxic disintegration show a similar tendency to multiply if they have been in the intact cell a short time and do not multiply when they have been in the cell a long time. This may be taken as direct evidence of the capacity of leukocytes to injure cocci without ancillary electric injury.

Examples of two different outcomes from residence within neutrophils are shown in the illustrations. In Figs. 1 to 6 strain 327W remained within a leuko-

cyte 55 seconds, and proliferated after disruption of the leukocyte by electric shock. In Figs. 7 to 12 the same strain remained in a phagocyte 21 minutes, 7 seconds, but did not proliferate after shock.

DISCUSSION

Work done on survival of phagocytized streptococci has been limited in extent and has led to equivocal results, so that after reviewing the literature one is unable to say whether streptococci are always killed when phagocytized by a particular cell type, or sometimes survive.

The methods used to study this question have been largely indirect. For example, changes in staining characteristics, described by Bordet in 1896 (4) and studied by many workers subsequently, indicated that streptococcal injury occurred within a few hours of phagocytosis. But the possibility that streptococci had been damaged earlier without change in staining was not ruled out. *In vivo* studies, also by Bordet (5), were thought to indicate prolonged survival in peritoneal exudate cells of guinea pigs, but by the technic used the possibility of persisting unphagocytized cocci was not eliminated. In an extensive study of experimental pleural infections of rabbits, Gay (6) came to the conclusion that the polymorphonuclear leukocyte played a relatively unimportant role in overcoming streptococcal infection, compared to the macrophage. The bactericidal effect of blood *in vitro* on streptococci has been demonstrated by many workers, but when the cocci that had been inoculated were not all destroyed by the blood, it was impossible to be certain whether the cocci that grew out had at one time been phagocytized but had survived within the phagocyte, or whether they had escaped being phagocytized. Several technics that have been used with other bacteria, such as neutral red and tetrazolium vital staining (7, 8) and the technics used by Smith and Wood (9) and Rogers and Tompsett (10) for the pneumococcus, Friedlander's bacillus, and staphylococcus, do not appear to have been applied to the streptococcal problem.

A more direct line of evidence has come from observations in this laboratory, in which it has been shown that cocci egested from leukocytes (11) or escaping upon leukotoxic destruction of leukocytes (2) did or did not divide depending on how long they had been in the cell before liberation. It was stated that cocci egested by human leukocytes within 15 minutes of the phagocytosis might proliferate, but if they had been in the leukocyte 30 minutes they did not proliferate. Compared to studies using bactericidal technics, these observations had the advantage that the cocci studied were definitely known to have been phagocytized; but the observations were few in number, and the findings were suggestive rather than definitive.

Fleck (12) has recently reported an interesting method in which individual leukocytes containing phagocytized cocci were transferred to blood agar plates by micromanipulation and their viability thus determined. The time the cocci had been in the leukocytes was not known and the time the leukocyte survived on the agar was also unknown, and because of the limited number of experiments performed, the author did not emphasize his findings. The method is, however, a promising one. He found that on the agar, colonies grew from 5 of 7 mouse leukocytes but from only 2 of 8

human leukocytes, and he stated that this suggests that human polymorphs may kill ingested streptococci more easily than mouse polymorphs. These results do not agree with those reported here on studies with the electric shock technic. It should be noted, however, that Fleck used a relatively virulent type 18 strain plus antibody and we have used only non-virulent strains.

The variability in survival times of cocci within leukocytes offers possible explanation of the lack of leukotoxic action by certain chains in a culture of a known leukotoxic streptococcus. According to an hypothesis advanced elsewhere (13), the leukocytic injury, which occurs only when living streptococci are phagocytized, results from the elaboration by the cocci of a toxic substance (which we think may be DPNase) within the leukocyte. Since the period of survival of cocci within leukocytes may be quite short, it is conceivable that in some instances the cocci are injured or killed by the leukocyte before they have an opportunity to elaborate enough toxin to injure the leukocyte. This may explain the observation that if the leukocyte is not destroyed within about 10 minutes of the phagocytosis it will survive indefinitely, barring phagocytosis of a new chain.

The question of whether the cocci that fail to proliferate after disruption of leukocytes are actually dead is a difficult one, and involves the nature of the states of life and death in microorganisms, for which no entirely satisfactory definitions can be offered (see discussion by Davis (14)). In this study, cocci are considered to have "survived" if any countable increase in coccal numbers of a chain occurs after leukocyte disruption. It is recognized that this criterion may weight the data in favor of survival, since it is known that a lethal injury may not be manifest immediately, but may become apparent only after one or several cell divisions. On the other hand, it is well known that living bacteria can fail to multiply for prolonged periods owing to the action of bacteriostatic agents or when in the lag phase of their growth curve.

In this connection some work of Eagle and Musselman (15) may be pertinent. They exposed actively growing cultures of group A streptococci to lethal concentrations of penicillin and, after varying periods of time, abolished the drug effect with penicillinase. They observed that the cocci were thrown into a non-proliferative phase by penicillin, and the longer the antibiotic was allowed to act, the longer the stationary growth phase lasted. An exposure to penicillin lasting 4 hours induced a bacteriostatic phase lasting 3.5 hours after the addition of penicillinase. It should be emphasized that the induction of a lag phase by penicillin occurred only when concentrations of the drug were used which, if allowed to act without interruption by penicillinase, ultimately would be lethal.

It is obvious that the non-proliferating cocci that are observed in our preparations may be in a similar bacteriostatic state, and that if ideal growth conditions could be supplied for an indefinite period, some of the cocci we con-

sider "non-surviving" might actually begin to multiply. Under the conditions of the test, we can reliably expect growth conditions to remain good for 3 hours or better. It is conceivable that a bacteriostatic state may have been induced which will last longer than this. In many experiments there has been no lag phase, but the cocci are seen to proliferate as soon as the phagocyte is disrupted. In no case have we observed a non-proliferative phase lasting longer than 1¾ hours. (That is, chains which had failed to multiply by 1¾ hours following the shock have never been seen to start to multiply during the next hour or on standing overnight). The very occurrence of a bacteriostatic phase is doubtless a manifestation of injury to the cocci resulting from residence in the leukocyte.

Compared to the short periods (1 to 31 minutes) the cocci have been allowed to remain in leukocytes in our experiments, the periods of intracellular residence under natural conditions are long (probably for the life of the leukocyte, unless terminated by egestion or leukotoxic action), and it seems reasonable to suppose that many, if not most, ingested cocci are ultimately killed. However, the nature of the experimental conditions does not allow us to maintain that all phagocytized cocci are killed, or that there may not be a rare chain that survives indefinitely in the leukocyte. The fact that in the longest period (20–31 minutes) 1 out of 16 chains survived suggests that this may be the case.

The studies reported here have been limited to human and mouse neutrophils of peripheral blood. It would be unwise to apply these findings to other cell types until analogous studies have been made of them. The occurrence of chronic carriers and of latent foci or deposits of streptococci in tonsils and lymph nodes suggests that living but non-proliferating or slowly proliferating cocci persist in the host, and it is possible that they reside in an intracellular locus. If so, the host cells cannot well be neutrophils, since those cells have a life thought to be no longer than 2 weeks.

The action of electricity on cells has been studied for many years (16), and the ability to disrupt cells (ameba, paramecium) with electric current has been described (17, 18), but the application of that technic to disclose the viability of ingested organisms has not, as far as we are aware, been made previously. The use of the procedure can perhaps profitably be extended to bacterial species other than the streptococci. The method appears to be applicable only to cells in thin slide–cover slip preparations, for exposure of cells in larger volumes of fluid in test tubes to equivalent amounts of current does not produce disruption. Some preliminary explorations have shown that electrical disruption of tissue culture fibroblasts that have been grown on coverslips and used to make thin slide preparations occurs only after much longer application of current than is necessary with peripheral blood leukocytes, and the cellular debris shows less tendency to dissolution.

CONCLUSIONS

The fate of non-virulent group A streptococci phagocytized *in vitro* has been investigated by destroying the phagocyte with electric current and observing whether the liberated cocci multiply.

Human and mouse peripheral blood neutrophils quickly injure ingested cocci, the time required to produce 50 per cent non-survival of chains being 8 and 6¾ minutes, respectively.

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EXPLANATION OF PLATE 96

The photomicrographs were taken with American Optical Company phase contrast microscope on 35 mm. microfile film and enlarged to a final magnification of approximately 900.

(*A*) *Streptococci Multiplied after Short Residence in Cell.*—

FIG. 1. A human neutrophil approached a chain of 10 cocci, one of which had almost divided into two (327W).

FIG. 2. The ingestion, shown in progress here, took 55 seconds.

FIG. 3. 2 minutes and 20 seconds after completion of ingestion, electric current was applied and the neutrophil was disrupted.

FIG. 4. 1 hour after shock. 10 cocci were counted, two of which had almost divided. A fibrin network formed temporarily about cellular debris.

FIG. 5. 2 hours after shock. 20 cocci were counted. The two dark bodies slightly larger than cocci in the upper left are condensations of cytoplasmic material. The four larger bodies in the lower right are condensations of nuclear material.

FIG. 6. 3 hours after shock. 27 cocci were counted.

(*B*) *Streptococci Failed to Multiply after Longer Residence in Cell.*—

FIG. 7. A human neutrophil approached a chain of 23 cocci. (327W).

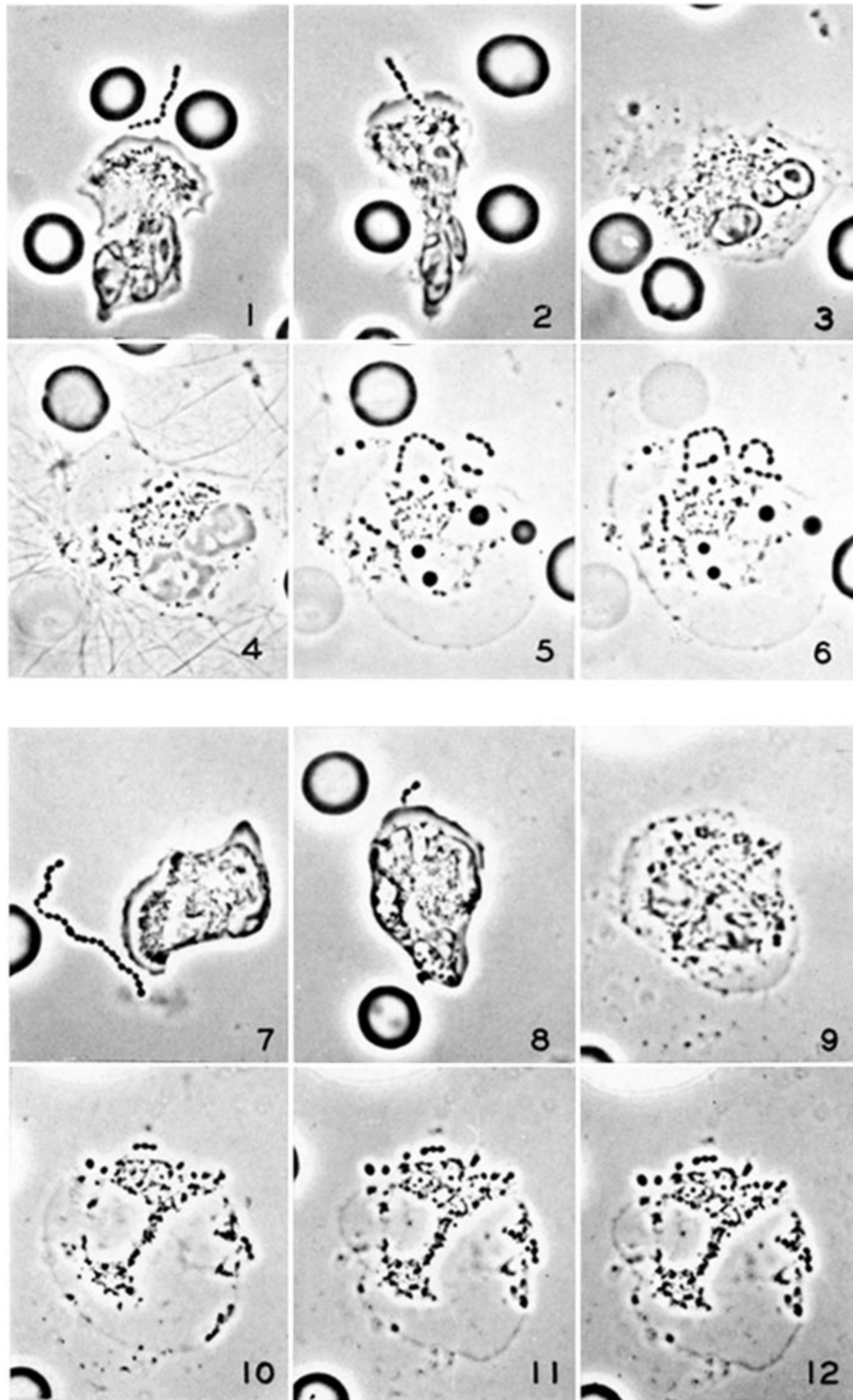
FIG. 8. The phagocytosis, shown almost completed, took 1 minute and 55 seconds.

FIG. 9. The neutrophil was disrupted by electric current 21 minutes and 7 seconds after completion of phagocytosis. Picture taken immediately after shock.

FIG. 10. 1 hour after shock. Some cocci had escaped from cellular debris as neutrophil continued to disintegrate. No multiplication.

FIG. 11. 2 hours after shock. Still no multiplication.

FIG. 12. 3 hours after shock. Still no multiplication. The 23 ingested cocci were countable, but since they are not all in the same focal plane they cannot all be seen in the photograph.



(Wilson *et al.*: Fate of phagocytized streptococci)