THE LEUKOTOXIC ACTION OF STREPTOCOCCI*

By ARMINE T. WILSON, M.D.

(From the Alfred I. du Pont Institute, Wilmington, Deleware)

Plates 33 to 41

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This is the third study in a series on the interactions between streptococci and host cells. Previous reports have concerned the egestion of streptococci by phagocytic cells (1), and the failure of streptococci to lose their capacity of resisting phagocytosis after being killed with gentle heat or ultraviolet radiation (2). The present work is concerned with one type of outcome following phagocytosis; namely, the destruction of the phagocytizing leukocytes which occurs when certain strains of streptococci are ingested. This injury will be called here "leukotoxic action," and the pertinent attribute of injurious streptococci will be called "leukotoxicity." It was described by Levaditi in 1918 (3), but has received no attention since that time as far as we have been able to discover.

The leukotoxic effect is seen only after intact cocci have been phagocytized. It must not be confused with the action of streptococcal leukocidin, a soluble substance elaborated into the medium by growing cocci, which destroys leukocytes even when all coccal cells have been removed by filtration. Todd has presented impressive evidence indicating that leukocidin and streptolysin O are identical (4).

In the present report consideration will be given to the biological characteristics of leukotoxicity, to its distribution among streptococci, its relation to other known streptococcal products, its relationship to virulence and its possible significance in streptococcal disease.

Materials and Methods

Strains.—Streptococci of the several serological groups and of types within group A have been accumulated from numerous sources, chiefly from Dr. Rebecca C. Lancefield, Dr. Charles H. Rammelkamp, Jr., Dr. Floyd Denny, and by primary isolation in this laboratory. Strains in current use are maintained in blood broth culture at 4°C. Strains used as stock reserves are kept in the dried state or frozen in blood broth at −20°C. Group and type of the strains were determined by the capillary precipitin method (5) using sera kindly supplied by the Communicable Disease Center, Chamblee, Georgia, or by Dr. Lancefield, and by slide agglutination with sera kindly supplied by Dr. R. E. O. Williams of the Streptococcal Reference Laboratory, Colindale, England.

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Special mention whould be made of four strains which have been used as representative leukotoxic and non-leukotoxic cultures. These are *AD238B*, a highly leukotoxic strain, originally isolated in 1952 at the Warren Air Base, Wyoming, by Dr. Rammelkamp. The strain belongs to Type 3, but gives only a ++ reaction with anti-M serum. Even when grown in serum broth it produces no capsule, and its virulence for mice following intraperitoneal inoculation is low, as will be discussed below. It is readily phagocytized *in vitro*. Strain T2/44 is Dr. Lancefield's representative of Griffith's original Type 2 strain, and has received 44 serial passages in mice. It is typable by slide agglutination but produces little M substance. It has a very small capsule if any, and is of low mouse virulence. It is non-leukotoxic. *327W* was originally isolated in a food-borne epidemic in the United States Navy in 1944. It belongs to Type 1 but produces very little M substance. It produces a good capsule in serum broth, but is of low mouse virulence. It is fairly readily phagocytized and is non-leukotoxic. *D58/47* is a descendant of the well known strain "Richards." It has received 47 intraperitoneal mouse passages, produces considerable M substance, belongs to Type 3, is highly mouse-virulent (as few as 10 cocci almost always kill mice), highly resistant to phagocytosis and is leukotoxic.

Estimation of Leukotoxicity.—The capacity of an unknown strain to produce leukotoxic injury is determined microscopically by direct observation of the fate of living leukocytes following phagocytosis. The strain to be tested for leukotoxicity is grown in blood-broth overnight at 37°C. 0.2 ml. of this culture is inoculated into 2.0 ml. of medium made by combining 1.0 ml. of normal serum with 1.0 ml. of autoclaved modified Todd-Hewitt broth (Difco). This culture is incubated for 1 to 2 hours, at which time, since the initial inoculum was large, growth is moderately heavy. The most suitable bacterial density for the test is approximately half that of a fully grown culture. Because various strains grow at different rates, an exact time for growth cannot be stated, but is usually between 1 and 2 hours. Visual estimation of this degree of growth is adequate, and more precise methods of measurement have been avoided, since the distribution of cocci in various areas of the final slide preparation is variable and it was thought wise not to give a false impression of quantitation. A loopful of this culture is mixed on one end of a clean glass slide with a loopful of heparin solution (50 mg. heparin dissolved in 100 ml. Todd-Hewitt broth, sterilized by filtration) and a small drop of human blood, and slides are prepared as described in an earlier report from this laboratory (1). When mouse blood was used in place of human blood, it was obtained by cutting off the extreme tip of the tail. Rabbit blood was obtained from the marginal ear vein.

For some studies, the slides were immediately transferred to the incubated phase contrast microscope (kept at 35°C.) and observed continuously. In surveys of the leukotoxicity of series of strains, preparations were made as described, but were placed in a 37°C. incubator for 90 minutes. Each slide was then transferred to the microscope and rapidly scanned, counts being made of the number of neutrophils that had failed to phagocytize, the number that had phagocytized and had suffered a characteristic leukotoxic injury, and the number that had phagocytized without undergoing injury. Occasionally in a normal preparation, containing no bacteria, a neutrophil will be seen having a morphologic appearance indistinguishable from that produced by leukotoxic action. This is quite rare, and when seen has always occurred in preparations that have stood many hours. Nevertheless, to avoid error from this source, a strain was not considered leukotoxic unless more than 10 per cent of the phagocytizing cells had been injured. It can easily be seen that this type of analysis does not lend itself to precise quantitation, but it does allow a reasonably accurate division of strains into leukotoxic and non-leukotoxic classes. A serious difficulty in estimating leukotoxicity arises when the test streptococci are highly resistant to phagocytosis, so that at the end of the period of preliminary incubation, few or no neutrophils are met that have accomplished ingestion. Sometimes in this situation the preparations have been allowed to stand an hour or so longer, and by that time phagocytosis may have occurred sufficiently to allow enumeration. This procedure is

satisfactory unless excessive extracellular proliferation has caused all cellular elements on the slide to be injured. A few strains have been retested after a lapse of over a year, but no change in leukotoxicity was noted.

Many studies of phagocytosis by other workers have been done with leukocytes washed free of plasma and suspended in salt solutions or salt-gelatin solutions. These suspensions are well suited to the investigation of factors such as inorganic ions, blood and plasma constituents, surface active agents, and others. In the present work the leukocytes have not been washed, and the preparations contain the constituents present in the fresh blood (or occasionally the buffy coat) of the human or animal donors. By supplementing blood with an approximately equal volume of Todd-Hewitt broth it was thought that the best possible medium for streptococcal growth was provided, and, indeed, excellent proliferation of the cocci is the rule in the slide preparations. (See Figs. 79 and 82). It is thought further that the conditions in these preparations are closer to those obtaining in natural infections than would be supplied with washed leukocytes and artificially compounded supplements.

Photographic Analysis.—Still photographs were taken by the technics and with the apparatus described in a previous paper (1). Cinemicrophotography has proved invaluable in providing material for dynamic morphologic analysis, since a single leukotoxic episode may be viewed many times and at several speeds, and observations made at leisure of what happens to the various cellular characters (cell wall, cytoplasmic granules, apparent cytoplasmic viscosity, nucleus, vacuoles, and pseudopods, and other processes extending from the cell surface) as well as to the ingested streptococci. The initial disintegration of the leukocyte often occurs so rapidly that a viewing of the process at slow projector speeds is an advantage, while the slow changes that occur in the cell later and, particularly the fate of the ingested streptococci, can best be seen by accelerating events through time-lapse photography.

The pictures were taken with the cine-Kodak special II, to which was fitted a Bausch and Lomb beam splitter, making it possible to view events, center the field, and adjust focus while pictures were being taken. The "normal" speed used for photographing relatively fast events was 8 frames per second. Continuously uniform speed was obtained with an electric camera motor. A kodaslide 35 mm. projector, aircooled and with heat filters, using a 1000 watt tungsten filament lamp was used as light source. A second heat filter was mounted where the light beam entered the plastic incubator box that was built around the microscope. The box was heated with heating tapes, and even distribution of heat was achieved with a small electric fan. Temperature in the box was regulated with a red-top thermoregulator and mercury relay (H-B Instrument Co., Philadelphia), and for most of the experiments reported here was kept at 30°C. In spite of the two heat filters, when the lamp was turned on a quick rise of 4°C. occurred in the microscope field. This was determined with a thermistor telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio), using a hypodermic needle probe, the temperature-sensitive tip of which was placed in a drop of immersion oil between glass slide and microscope objective. But even with continuous illumination the temperature quickly reached equilibrium and did not rise more than 4°C. above the ambient temperature in the box. Time-lapse pictures were taken by means of a tandem recycling timer (Industrial Timer Corp., Newark, New Jersey) which was continuously adjustable to take single frames at rates of 1 per second to 1 per 30 minutes. The secondary impulse from this timer sent current simultaneously to the microscope lamp and to a time-delay relay (agastat). After 0.5 second (the time required for the lamp to reach peak illumination) current was sent from the time-delay relay to a solenoid, which tripped the single frame advance of the camera. Thus the microscope lamp was on only about 0.7 second to take each frame. Pictures were taken on duPont microcopy 16 mm. film, which, although of low sensitivity to light, had the advantages of high contrast, fine grain, and high resolution.

American Optical Co. phase contrast optics were used throughout. Most of the photographs

were made with the bright H 1.8 mm. oil immersion objective. Negatives so obtained were used as projection positives, and projected objects (such as streptococci and certain cellular structures) appeared dark on the screen although they appeared bright through the microscope ocular while the pictures were being taken.

Projection for analysis was done with a Bell and Howell "Time and Motion Study" projector, which had variable projection speeds, a hand crank for slow advance of the film, and a frame-counter.

EXPERIMENTAL

When a leukotoxic streptococcus is phagocytized by a leukocyte the latter usually undergoes a rapid and dramatic series of morphologic changes which ends with the cocci lying in the debris of a thoroughly disrupted cell. In order to convey better the significance of this phenomenon, brief descriptions will be given first of the fate of leukocytes in preparations free of bacteria and in preparations containing non-leukotoxic streptococci.

Fate of Leukocytes in Normal Blood Preparations.—For these studies, glass slide preparations have been made by mixing normal human blood, heparin solution, and sterile broth, the intention being to determine how long the cells, particularly the neutrophils, with which we are chiefly concerned, remain viable, active and suitable for microscopic observations and to ascertain when injured cells appear and what they look like so that they may be compared to cells injured by leukotoxic action.

In glass slide preparations there is a very thin zone in which the cells have been crushed. Crushed neutrophils consist of masses of neutrophilic granules lying around or among the nuclear remnants (Figs. 1 and 2). The granules are individually distinct and are not agglutinated. They appear to be held in more or less well defined masses by persisting cytoplasmic material, and even when the cell membrane has been ruptured, they do not become freely dispersed throughout the surrounding medium. The nuclear lobes are hazy, swollen, and in the course of time their phase density becomes reduced almost to that of the surrounding medium (Fig. 3, from a preparation 24 hours old). The granules, however, persist indefinitely with relatively little change in appearance. The crushed cells are quite different in appearance from neutrophils that have been injured by leukotoxic action, as will be shown later.

Except in the very thin areas just described, the neutrophils are uninjured. Initially they are rounded, non-motile and lacking in vacuoles (Fig. 4), but within a few moments they start ameboid movement, and this movement continues for several hours without cessation. (Fig. 5). Clear, watery vacuoles of several types, which have been described by Bessis (6), form and increase in number. Beginning at 3 or 4 hours the cell contains several very dense bodies which increase in number and in size over the next 4 or 5 hours. They reach the size of cocci, with which they may be confused unless one has learned to recognize them. By the dark medium phase contrast objective these bodies appear to have a light center. There is marked variation in the number of clear vacuoles and dense bodies found within individual cells. By 7 or 8 hours most of the neutrophils are still in good condition and move about actively (Fig. 6 and 7), but about this time (and in occasional preparations somewhat earlier) seriously injured cells begin to appear. The injury sometimes takes the form of a general contraction and thickening of the cell associated with cessation of ameboid movement and loss of distinctness of the internal structures of the cell. A different type of reaction to injury may be called the "reaction of agitation." In this the cell sends forth several fluid snake-like processes, which move ac-

tively in many directions, but do not lead to a change in position of the cell on the slide (Fig. 9). This motion may last from several minutes to more than an hour and the cell finally ends up as a round object with several dense circular structures representing the nuclear lobes (lower cell in Fig. 10). Occasionally a neutrophil on injury assumes an appearance similar to that of leukotoxic action (Fig. 11) but this is rare in bacteria-free preparations and occurs only after the preparation has been set up for several hours. Although injured cells begin to appear within 7 or 8 hours, in some preparations most of the neutrophils are still in good condition after 24 hours (Fig. 8); often, however, by that time no living neutrophils will be found. A common appearance of dead cells at 24 hours is shown in Fig. 12. In this cell the nuclear lobes have contracted to a single rounded mass without internal structure, the phase-dense bodies are conspicuous and the neutrophilic granules are well preserved and show little tendency to be agglutinated.

Lymphocytes and monocytes assume ameboid movement several minutes to several hours after the neutrophils have become active, but they persist in active motion and are apparently uninjured long after the neutrophils have succumbed: usually for several days.

Thus it can be seen that when there are no living bacteria in the preparation, the neutrophils remain in good condition for several hours, and the mononuclear cells for one to several days.

Fate of Leukocytes in Preparations Containing Non-Leukotoxic Streptococci.— When a small inoculum of a readily phagocytized, non-leukotoxic strain is used, the neutrophils quickly ingest the chains and few or no unphagocytized bacteria remain in the medium to proliferate. The cells in such a preparation remain in good condition about as long as in bacteria-free preparations, including cells that have phagocytized cocci.

The neutrophils in Figs. 13 and 14 were living, healthy cells even though the preparations were 5 and 6 hours old. A small inoculum of the readily phagocytized strain *T2/44* was used and extracellular proliferation in this preparation was minimal because most chains were phagocytized promptly. The cell in Fig. 15, also from the 6 hour preparation, shows definite signs of injury. The proportion of injured cells increased rapidly during the next few hours.

It is apparent that the phagocytosis of non-leukotoxic bacteria and the presence of such bacteria within the cells seem to have little or no deleterious effect on the leukocytes for many hours. The ingested cocci do not multiply in the cells, or at best accomplish only part of a cell division. Sometimes the phagocytized cocci are egested, as described previously (1).

In the test for determination of leukotoxicity of unknown strains (see above under Materials and Methods), a heavier inoculum is used than in the preparation described immediately above.

In Figs. 25 to 36 are shown representative cells from two preparations, one made with a non-leukotoxic strain (Fig. 25 to 30) and the other with a leukotoxic strain (Fig. 31 to 36) and photographed after the customary 90 minute incubation period. The cells that had phagocytized non-leukotoxic streptococci were all in active ameboid movement, even when the number of cocci ingested was very large (Fig. 30), whereas the cells that had phagocytized leukotoxic cocci had disintegrated. On continued incubation of these preparations, however, all leukocytes were ultimately killed as a result of growth of extracellular bacteria. This

included the polymorphonuclears and monocytes, whether they had phagocytized cocci, as well as the lymphocytes, which are never seen to phagocytize. Hemolysis occurred in varying degree, depending on the strain of streptococcus.

When a strain of streptococcus with a marked ability to resist phagocytosis was used, extracellular proliferation on the slide occurred rapidly, and in a few hours the preparation was swarming with bacteria. In spite of this, for an hour or so, the leukocytes remained in good condition, moving among the proliferating cocci as if the latter were not there. However, after a period that depended on the size of the initial inoculum, the cells of all types were killed, as in the preparation containing a large inoculum of the readily phagocytized strain.

It is apparent that very heavy extracellular growth on the slide, even of non-leukotoxic streptococci, ultimately destroys the white cells. The agents responsible for this delayed destruction are not known, but probably inciude leukocidin and other soluble secretions of the cocci, metabolic by-products such as lactic acid, and altered physical conditions, such as the O-R potential, resulting from streptococcal growth. In any case, this injury, since it occurs in the absence of phagocytosis, is quite different from the leukotoxic process.

Fate of Leukocytes in Preparations Containing Leukotoxic Streptococci.—

When a leukotoxic strain of streptococcus injures a human neutrophil, the following changes may be observed: For a variable time following ingestion, the leukocyte shows no abnormality, but continues in ameboid movement. Within 30 seconds to 8 or 10 minutes, the cell rather suddenly stops moving and becomes rounded. The neutrophilic granules, which normally show some degree of brownian movement as well as movement resulting from cytoplasmic streaming, become less motile, indicating that the cytoplasm has become more solid. This quieting of motion of the granules may involve only part of the cell, and is of short duration. It is succeeded by a marked increase in brownian movement of the granules, accompanied by an increase in the diameter of the cell and a decrease in the phase density of the cytoplasm. These changes are interpreted as resulting from imbibition of fluid by the cell. The fluid does not always dilute the entire cytoplasmic area of the cell, but often a dense zone of solidified, possibly coagulated, cytoplasm (corresponding to the cell center) persists, and in this area many of the neutrophilic granules are trapped, remaining motionless. They sometimes are irregularly distributed in this relatively solid protoplasm, and appear to be agglutinated (Fig. 53). Similarly, in the peripheral parts of the cell, agglutination of the granules occurs, and the agglutinated masses often stick to the cell wall (Fig. 35 and 55). Sometimes while these changes are going on, the cell wall will send out innumerable protoplasmic processes, which may be filamentous (Fig. 40) or may be blunt and rounded (Fig. 77). Pieces of protoplasm may pinch off and become free-floating in the medium (Fig. 44). When this happens, the detached fragments usually become spherical, and they may contain a few neutrophilic granules. By time-lapse photography these spheres are sometimes seen to form as an eruption from all aspects of the cell simultaneously.

Sometimes there is actual rupture of the cell wall with release of some of the granules to the surrounding medium, in which they persist indefinitely in brownian movement (Figs. 16 to 24). Nuclear lobes and streptococci may also be released (Fig. 20). It is possible that the compressed state of the cell between slide and coverslip plays a role in the rupture. When rupture occurs, subsequent changes in the cell are greatly accelerated. The cell usually forms a new boundary at the site of rupture, and later the released elements may be seen lying adjacent to an apparently whole cell that has thus reconstituted its borders (Fig. 24).

Shortly after imbibition of fluid by the cell or rupture of the cell membrane, changes occur

in the nucleus. These are highly variable, both as to form and rate of occurrence. The nuclear lobes may individually become rounded (Fig. 55) or may coalesce into a single rounded (Fig. 31) or irregular mass (Fig. 68). The internal structure of the lobes becomes indistinct and the whole nucleus may suddenly or slowly lose most of its phase density; and this may be accomplished by a marked swelling and spreading out of the nuclear mass (Fig. 24).

When the cell has reached the end of the changes it undergoes, it is usually noticed that the granules have become inconspicuous, being represented only by some faintly punctate debris in the area corresponding to the cell center and by the dark masses previously mentioned along the remnant of the cell membrane. Sometimes some very dense bodies appear in the cell (Fig. 36). By time-lapse photography they are seen to form by the condensation of diffuse material in the cell. They do not correspond in number to the nuclear lobes and their origin is obscure.

The preceding description is typical of the commonest changes that occur in the leukotoxic reaction and is based on many direct observations through the microscope and on the analysis of motion picture sequences of 17 leukotoxic episodes. There is considerable variation, however, in the reaction.

At times imbibition of fluid does not take place, but rather the cytoplasm appears to solidify. The cellular outline, instead of being circular, is highly irregular and the cellular membrane rather than being a distinct hairline structure, as in the imbibition reaction, is represented only by the junction of the dense cytoplasm and the surrounding medium (Fig. 31). Sometimes the injured cell will send forth several slowly motile processes from several aspects of the cell. These rather closely resemble the pseudopods of normal ameboid movement, but they do not cause the cell to move on the slide. They may continue in a rhythmical, pulsating motion for from several minutes to an hour or more. During one observation by time-lapse photography an extraordinary process developed from the cell, extending further than a cell diameter from the main cell border, and engaged in a flame-like motion for about 30 minutes before rejoining the cell.

Often when a long chain is phagocytized the ingestion stops when the phagocytosis is still incomplete. An instance of this is illustrated in the photographic sequence in Figs. 37 to 44. The cessation of phagocytosis in such a case is the first visible indication that the leukotoxic process has started. By contrast, the phagocytosis of a long non-leukotoxic chain almost invariably continues until the entire chain is ingested, although this may cause extreme filling of the expandable cell. Rarely, however, phagocytosis of a non-leukotoxic chain may cease for reasons that are not apparent, although the cell remains healthy and actively motile.

Disintegration of the leukocyte that has phagocytized a leukotoxic chain may start within a half minute or may not start for 8 to 10 minutes, as mentioned previously. If it has not occurred within that time it can safely be assumed that the particular chain phagocytized will not injure the cell, and indeed in most preparations some phagocytoses are not followed by leukotoxic disintegration. Two examples of this are shown in Figs. 45–56. A cell that has survived the ingestion of one chain will usually encounter and phagocytize other chains and sooner or later will succumb. This suggests that there is a variation among individual chains in leukotoxicity. The number of non-leukotoxic chains in serum-broth cultures of *AD238B* is relatively small, but in other strains may be much greater. With *AD238B* it almost never happens

that there are intact neutrophils left in preparations that have been incubated for 1½ hours. This suggests that all neutrophils are susceptible to leukotoxic injury. As few as three cocci have been observed to destroy a neurophil; and on the other hand, neutrophils have been seen to survive the phagocytosis of chains containing 20 or 30 cocci of a leukotoxic strain.

Review of the morphologic changes taking place in the injured cells suggests that the primary site of injury is the cytoplasm and possibly the cell wall. Because mitochondria in neutrophils are obscured by the specific granules it has not been possible to see if and when they are injured. The earliest signs of injury appear to involve changes in the apparent viscosity of the cytoplasm as manifested by arrest of cell movement, a reduction followed by an increase in brownian movement of the granules, often followed by a decrease in phase density of the cytoplasm. These changes may be accompanied or followed by the formation of several types of protoplasmic process from the cell borders and sometimes by actual rupture of the cell wall. Nuclear changes are definitely secondary to cytoplasmic changes in time.

The appearance of individual cells that have undergone the leukotoxic reaction is not specific to that injury. As mentioned above, cells of similar appearance are encountered, although rarely, in old, bacteria-free preparations, and they are seen more often in old preparations made with non-leukotoxic streptococci. But in practice differentiation between preparations made from leukotoxic and non-leukotoxic strains is readily made because of the early appearance and high incidence of injured cells in the former (Figs. 25 to 36). Certain types of cellular injury are readily distinguished from any of the usual appearances of the leukotoxic reaction: particularly the crushed cells, cells that are having or have had the reaction of agitation, and the small dense rounded cells with well preserved granules shown in Fig. 12.

Fate of Phagocytized Leukotoxic Streptococci.—Following the disintegration of the cells, the ingested cocci are seen to lie in the cellular debris. Occasionally a vacuole containing cocci may persist after disruption of the cell, and the cocci are seen to lie in a circle or sphere of fluid surrounded by a very thin line, representing the vacuolar wall. In time all evidence of the vacuole disappears. In one motion picture sequence, the most recently ingested part of a long, incompletely phagocytized chain escaped from the neutrophil as it disintegrated. This phenomenon must be distinguished from the egestion phenomenon, which is a function of healthy, intact cells.

Lying in the cellular debris, the cocci may multiply (Fig. 57 to 68) or they may not (Fig. 69 to 80). In a general way, the more rapid the leukotoxic disintegration of the cell, the more likely the proliferation of the cocci. In many cases there is a mutual destruction, the cocci destroying the neutrophil and the neutrophil killing the cocci before it disintegrates. It has not been possible to accumulate many data on the time elements involved here, because except in the most rapid and abrupt disintegrations it is difficult or impossible to be certain within many seconds when the cell first shows the leukotoxic effect.

The cellular debris following leukolysis has a strong chemotactic attraction for other leukocytes on the slide. If the density of extracellular chains is low (so that leukocytes do not find chains in their immediate neighborhood to be attracted to), many neutrophils may accumulate around and in the debris. Usually the cocci that caused the initial neutrophil to disintegrate are phagocytized by subsequent neutrophils that are attracted to the scene, and the latter may or may not in turn suffer leukotoxic disintegration. As many as 18 neutrophils have been seen in a group of cells, most of them being uninjured, not having phagocytized cocci. They do not phagocytize remnants of the injured cells. This phenomenon is illustrated in Fig. 81.

Distribution of Leukotoxicity among Streptococcal Strains.—Using the assay procedure described under Materials and Methods, a total of 101 strains of streptococci belonging to several serological groups and types have been examined. Human neutrophils were the test cells. The results from testing 87 group A strains are presented in Table I. Although the data are somewhat limited in scope, it can be seen that leukotoxicity has an irregular, but not

TABLE I

Leukotoxicity of Group A Streptococci for Human Neutrophils

Serological type	Non-leukotoxic strains	Leukotoxic strains
1	3	0
3	2	7
4	2	6
5	19	1
6	0	5
12	0	23
14	3	0
30	3	0
Others	10	3

a random distribution among the serological types. There is a distinct tendency for leukotoxicity to be associated with certain types of group A streptococci (6, 12) and non-leukotoxicity to be characteristic of other types (5, 14, 30) whereas in some types both leukotoxic and non-leukotoxic variants occur (3, 4).

A few strains of the other serological groups have been tested with human leukocytes. Leukotoxicity was not encountered in 2 group B strains, 2 group D strains, or 1 group L strain. However, 2 of 7 group C strains and 1 of 2 group G strains were leukotoxic. The number of strains studied is too small to permit generalizations about the incidence of leukotoxicity in the groups, but it can be concluded that leukotoxic strains are not limited to group A.

The large number of type 12 strains tested here reflects interest in the association of this type with acute glomerulonephritis (7). When it was first discovered that all the type 12 strains available for testing in this laboratory were leukotoxic, other strains of this type and of other types isolated from nephritic patients were obtained through the kindness of Dr. Charles H. Rammelkamp, Jr., and Dr. Floyd Denny. The results of this survey are shown in Table II. All the type 12 strains, whether isolated from nephritis patients or not, were leukotoxic.

However, only 2 of 5 type 4 strains isolated from patients having nephritis were leukotoxic. Another strain from nephritis (not included in Table II), was obtained through the kindness of Dr. Lewis Wannamaker. It also was leukotoxic. The strain was isolated from an epidemic of nephritis and has been found by Updyke *et al.* (8) to belong to a provisional new serological type, called Red Lake.

Types of Cells Susceptible to Leukotoxic Injury.—Most of the studies done here on leukotoxin have been made with peripheral blood cells and with neutrophils because they are faster in ameboid movement and phagocytize more readily than the other cell types. Several observations, however, including two motion picture records, have been made of leukotoxic action on human monocytes.

The morphologic changes induced are quite similar to those occurring in neutrophils and consist of cessation of motion of the ruffle pseudopods, changes in apparent cytoplasmic viscosity, swelling and spreading of the cells, and loss of normal architecture of the nucleus.

TABLE II

Incidence of Leukotoxicity in Strains Isolated from Nephritics and Non-Nephritics

	Total	Leukotoxic	Non-leukotoxic
Type 12 strains			
Isolated from nephritics	11	11	0
Not from nephritics	12	12	0
Type 4 strains			
Isolated from nephritics	5	2	3
Not from nephritics	2	2	0

These changes occur somewhat more slowly than with neutrophils. The end product of leukotoxic action on the two is so similar in appearance that when a late survey is made of injured cells, one cannot usually be certain which variety of cells has been injured. One instance has been observed of injury to an eosinophil. Limited observations of exudate leukocytes from the mouse peritoneum have shown that they also are susceptible to the toxin. Studies of other cells, such as tissue macrophages, fibroblasts, and other cells in tissue culture or suspensions of cells from organs have not been undertaken.

In Table III are shown the reactions of polymorphonuclear leukocytes from three species to a selected group of leukotoxic and non-leukotoxic strains. They were tested by the usual microscopic assay method and it is seen that, on a qualitative level, the cells of the three species are entirely similar in susceptibility to leukotoxic action. Studies have also been made using mouse blood, but too little phagocytosis of some of the test strains occurred to permit analysis of leukotoxic susceptibility and inclusion in the table. The special problem of phagocytosis by mouse cells will be the subject of a later report. When phagocytosis did occur, the mouse cells were often injured by leukotoxic strains. Injured cells from rabbit and mouse blood are shown in Figs. 83 to 88.

Leukotoxic Action in Ingestion Tests.—The studies described so far have been limited to events happening in glass slide preparations. The question arose whether cellular injury would occur and be detectable when cells and cocci were allowed to interact in rotated glass tubes, such as are used in ingestion tests, in which accomplished phagocytosis is recognized by staining material withdrawn from the tubes at suitable intervals.

For these studies two readily phagocytized strains were used: 327W, non-leukotoxic and AD238B, leukotoxic. Ingestion tests were performed as described elsewhere (2), except that

TABLE III

Leukotoxicity of Selected Group A Streptococci for Neutrophils or Pseudo-Eosinophils of Several Species

Strain	Human	Rabbit	Guinea pig
Type 2/44/9	−	−	−
AD254	−	−	−
Type 1	−	−	−
AD469	+	+	+
AD238B	+	+	+
AD459	+	+	+
AD480	+	+	+

TABLE IV

Differential Counts in Ingestion Tests of Leukotoxic Strain AD238B and Non-Leukotoxic Strain 327W

Amount of rotation	Ingestion (200 neutrophils counted)		Intact neutrophils in 400 intact leukocytes	
min.	*per cent*	*per cent*	*per cent*	*per cent*
	AD238B	*327W*	*AD238B*	*327W*
15	90	97	38	51
30	100	100	27	43
60	—	—	9	42
120	—	—	10	46

50 per cent donor's serum broth was used and the organisms were not resuspended. Material was withdrawn for stained smears at the times indicated in Table IV, and Figs. 89 to 97 show representative cells of the stained preparations. Within 15 and 30 minutes, phagocytosis was extensive with both strains. Stained smears of the 327W preparation showed no morphologic abnormalities of the phagocytizing neutrophils, except for a rare disrupted cell such as will be present in most blood smears, until the 60 minute preparation. At this time, most of the neutrophils appeared normal, but some showed an even, diffuse staining of the nuclear lobes and a few showed a disruption of the cellular membrane with a few irregular pieces of cytoplasm adhering to the nuclear lobes. Extracellular proliferation was extensive on the slides, and the chains were diffusely distributed. In contrast, with strain AD238B, even at 15 minutes many of the neutrophils appeared injured. Many masses of cellular debris were seen in which the only recognizable remnants of cells were nuclear fragments and irregular pieces of cytoplasm associated with streptococci. Some neutrophils and their phagocytized cocci showed

no evidence of injury, however. As rotation was continued, the incidence of cellular deterioration increased. At 30 minutes a striking finding was the occurrence of small, dense masses of streptococci, and some of these masses had nuclear fragments attached to them. The masses that survived the shearing force of making double coverslip preparations for staining may have represented cocci that had grown out in the cellular debris of neutrophils that they had killed, but they may also have resulted from agglutination of extracellular organisms. By 1 hour these coccal masses had increased in number and size. Most neutrophils on the slide at that time were unrecognizable or disrupted. However, there were a few neutrophils in the 327W preparation which had a similar appearance at the same time, and the leukotoxic action cannot be said to have produced a specific morphologic alteration in the cells. Valentine (9) has noticed somewhat similar changes in cells injured by staphylococcal leukocidin (culture filtrate).

It was thought the best index of cellular injury in preparations of this type would be a differential count in which the percentage of total intact cells due to surviving, undisrupted neutrophils would be determined. (It was inadvisable to make a direct count of the disrupted cells because of the difficulty of being certain whether a fragment of material taking cytoplasmic or nuclear stain actually represented a disrupted cell and if so, whether it was the only countable structure on the slide derived from that cell.) The results of the differential counts are given in Table IV and show that with 327W a moderate drop in the percentage of neutrophils occurred between 15 and 30 minutes, but no significant change occurred thereafter, whereas with the leukotoxic strain AD238B, there was a fall in recognizable neutrophils from 38 per cent at 15 minutes to 10 per cent at 2 hours. Lymphocytes did not appear to be injured in either preparation. The author is indebted to Dr. Grove Wiley of this laboratory for performing the ingestion tests.

Characteristics of Leukotoxic Action.—The distinctive feature of the leukotoxic reaction as observed in glass slide preparations is that it occurs only after a phagocytosis, and that it is a dramatic and rapidly developing process which may begin as early as 30 seconds after completion of phagocytosis or, when a long chain is involved, even before the phagocytosis is completed, and in any case starts within 10 minutes of the phagocytosis.

Heat-killed streptococcal cells do not produce the leukotoxic reaction.

A culture of AD238B in 50 per cent serum broth was divided into two parts. One part was untreated and the other part was immersed in a water bath at 56°C. for 5 minutes to kill the cocci. Slide preparations were made of the two cultures and examined after standing at 37°C. for 90 minutes. In the preparation made with living cocci, over 90 per cent of the neutrophils had been killed. In the preparation made with heat-killed cocci, not a single cell was encountered that had suffered the leukotoxic injury, although more than 90 per cent of the neutrophils had phagocytized large numbers of organisms.

The leukocytic injury is not produced when culture supernates or filtrates are added to blood in the slide preparations. The filtrates used for these studies were obtained from the short term cultures of leukotoxic strains (AD238B

and D58/47) in 50 per cent serum broth, strains which are known to produce the injury when the cocci themselves are present and are phagocytized.

It was also noticed that material liberated from disrupted leukocytes on the slide does not have an injurious effect on leukocytes that move through the detritus, although if streptococci lying in the debris are phagocytized, the cells may disrupt.

To investigate this phenomenon further, the leukotoxic reaction was allowed to proceed in a test tube by rotating a mixture of human buffy cells and AD238B for an hour. The material was then centrifuged, and the supernate mixed with fresh cells of whole blood as in the usual test for leukotoxicity. No leukocyte injury resulted. From these observations, it is apparent that the leukotoxic injury results only from the phagocytosis of intact, living cocci.

Based on a tentative assumption that the leukotoxic reaction may be due to a specific chemical substance elaborated by the cocci and entering the medium (but unable to injure leukocytes because unable to penetrate the cellular membrane) attempts were made to introduce the hypothetical substance into the leukocytes by adsorbing it from broth onto non-leukotoxic cocci before phagocytosis.

Strains AD238B and 327W were grown in 50 per cent serum broth in the usual way for 2 hours. The cultures were centrifuged and the supernates withdrawn and saved. To the bacterial sediment of the non-leukotoxic 327W was added the culture supernate of the leukotoxic AD238B, the two were thoroughly mixed and allowed to stand at room temperature for ½ hour and a slide preparation was made with the mixed culture. Phagocytosis occurred rapidly, but the white cells were not injured. It was apparent that the non-leukotoxic cocci were unable to adsorb and carry into the cell any postulated leukotoxin present in the broth of a leukotoxic strain.

When washed leukocytes were mixed with culture filtrate of a leukotoxic strain there likewise was no injury. Any postulated serum inhibitors should largely have been removed by the washing.

The leukotoxic injury is produced most extensively when young, actively dividing cultures growing in 50 per cent serum broth are used. When one uses old cultures, for example ones that, after growing 2 hours at 37°C., have been allowed to stand at room temperature overnight, or when one uses cultures that have grown in serum-free broth (or in blood broth), no matter what the age, few leukotoxic reactions occur during the first 30 minutes or so, even though extensive phagocytosis takes place.

The slide preparations are excellent culture media and the inoculated cocci start to grow promptly. The mixture on the slide contains in the neighborhood of 25 per cent serum. Phagocytoses occurring after 30 minutes are more and more likely to be followed by the leukotoxic reaction, so that in time the cocci growing on the slide seem to have reached a state similar to those grown in serum broth. Rabbit and horse sera can be used in place of human sera in the growth media with similar effects. The fact that cultures of non-leukotoxic strains grown in broth containing 50 per cent rabbit serum fail to produce leukotoxic injury in human leukocytes indicates that the leukotoxic reaction is not due merely to the introduction of heterol-

ogous serum into the leukocytes from the medium adsorbed onto the cocci. This is also borne out by the fact that 50 per cent human serum broth cultures (the serum being obtained from the cell donor) are as leukotoxic as cultures made from 50 per cent rabbit or horse serum.

The microscopic experiments reported here were all done with microscopes enclosed in incubators providing an environmental temperature of 35°C., (except the ones for motion pictures, as discussed above), or on slides given a preliminary incubation at 37°C. in a bacteriological incubator. To discover whether temperature affects leukotoxicity, parallel preparations were maintained at 35°C. and at room temperature (22°C.).

Leukocyte motility and phagocytosis occurred more quickly in the incubated preparation; but even though the room temperature preparation lagged somewhat in these respects, nevertheless when phagocytosis did occur it was followed by leukotoxic disintegration about as frequently as at the higher temperature.

Although this type of investigation does not lend itself to quantitative analysis, an impression was gained by the observer that the effect of temperature, within the limits studied, was primarily on the phagocytosis rather than on the capacity of the ingested cocci to produce injury.

Relation of Streptococcal Leukotoxicity to Known Streptococcal Products.— The type-associated distribution of leukotoxic strains suggests that leukotoxicity is not due to formation of such products as erythrogenic toxin, the streptolysins, streptokinase, leukocidin, protease, desoxyribonuclease and the like, since these substances do not have particular type-associations. The strains in certain serological types of group A streptococci have a fairly uniform pattern of sugar fermentations, but no attempt has been made to correlate these patterns with the distribution of leukotoxicity.

Additional information indicating the lack of relation between leukotoxicity and leukocidin production was obtained by testing a series of 7 strains for the two activities.

Todd's method for leukocidin (4) is based on the fact that uninjured mouse peritoneal exudate cells when suspended in physiological sodium chloride solution with kaolin and rendered alkaline are clumped, whereas cells injured by adding leukocidin (as culture filtrates) remain unclumped. There was no correlation between leukotoxicity and leukocidin production. For example the highly leukotoxic strain AD238B had no detectable leukocidin by Todd's method.

Parallel tests were made on 47 strains for leukotoxicity and for streptolysin O production by the method of Slade and Knox (10).

The results are shown in Table V. Although in general leukotoxic strains produced more streptolysin O than did non-leukotoxic strains, the fact that seven leukotoxic strains produced too little of the hemolytic toxin to be measured by the technic employed makes it highly unlikely that the two activities are due to the same streptococcal product.

Relation of Leukotoxicity and Virulence.—It is commonly believed that phagocytosis plays a key role in the host's defense against streptococcal infections, and that the virulence of streptococci may reside largely in their ability to resist destruction by the phagocyte. It would seem reasonable to suppose, therefore, that the possession of leukotoxicity by a streptococcal strain should confer on it a measure of virulence. Such, however, is not necessarily the case.

The strain that has been used most frequently in these studies, because of its high leukotoxicity, AD238B, has very little virulence for mice. If 10^{-1} ml. of blood broth culture, containing approximately 30,000,000 chains is injected intraperitoneally into each of 6 mice, only 1 or 2 mice will die. With 10^{-2} ml. and smaller inocula, all mice survive. This same strain when tested *in vitro* with peripheral blood of mice, or when tested *in vivo* by intraperitoneal inoculation in mice, causes death in a high proportion of phagocytic cells. There can be no question

TABLE V

Relation of Leukotoxicity and Streptolysin O

Streptolysin O titre*	Leukotoxic strains	Non-leukotoxic strains
>0.25	7	13
0.25	5	1
0.125	4	2
0.06	11	0
0.03	3	0
0.015	0	0
0.007	1	0

* Smallest quantity of culture filtrate in milliliters causing complete hemolysis.

therefore of the simultaneous possession of high leukotoxicity and low virulence for mice by this strain.

An explanation which the author believes is adequate to resolve the paradox is offered by observations made in the slide preparations. This lies in the fact, as stated above, that the phagocytizing leukocyte sometimes kills the streptococci before it succumbs itself (defining death of the cocci in this connection as failure to proliferate in the cellular debris), and if the cocci are not killed they may still be phagocytized by other cells with the result that ultimately the cocci lie in the intact internal cellular environment long enough to be destroyed. Examination of Figs. 81 and 82 shows that even though many leukocytes have been destroyed by cocci, growth of the bacteria is markedly inhibited compared to free growth of a nearby chain In experimental streptococcal inflammations the number of phagocytes that appear in the lesion is greatly in excess of the number required to phagocytize the injected bacteria, as pointed out many years ago by Bordet (11), so that

after many cells have been killed by leukotoxic action there will be others to phagocytize any cocci that may survive.

Estimation of leukotoxicity in virulent streptococci is hindered by the small amount of phagocytosis that occurs with virulent strains.

For example, D58/47, a highly mouse-virulent strain of group A Type 3, possessing a large capsule and abundant M substance, is not phagocytized at all for several hours in preparations made with a small initial inoculum. However, when the preparation becomes overcrowded from proliferation on the slide, phagocytosis slowly begins to occur. Most of the phagocytizing neutrophils suffer the leukotoxic disintegration. One gets the impression that as bacterial outgrowth on the slide has occurred, a change has taken place in the cocci rendering them more susceptible to phagocytosis. It is well known that old cultures are more readily phagocytized than young ones (12). Nevertheless, the ability of leukotoxic strains to injure leukocytes is not lost by this aging and crowding of the culture on the slide.

All virulent strains are not leukotoxic. For example, strain AD242/23, group A, Type 14, is almost as virulent for mice as D58/47, but it is not leukotoxic. Leukotoxicity is not, therefore, an essential element in the ability of a streptococcus to survive, multiply, and produce disease in the host.

Antigenicity.—Sera from two patients convalescent from streptococcal infections were tested for the possible presence of antibodies that would inhibit leukotoxic action. In this test, microscopic preparations were made in the usual way for demonstrating leukotoxicity except that a loopful of convalescent serum, undiluted or diluted 1:10, was added to the mixture. The two patients had had Type 12 infections followed by acute glomerulonephritis, and the strain isolated from the patient's throat was used in the test. One serum (obtained through the courtesy of Dr. Rammelkamp) was drawn 3 weeks after the onset of the nephritis. Sera from the other patient (obtained through the courtesy of Dr. Denny) were drawn during the acute throat infection and 5, 7, 10, 14, and 17 weeks later. None of the above sera prevented leukotoxic disintegration in the slide test.

Four rabbits were immunized to discover whether neutralizing antibodies would be produced by leukotoxic streptococci. A preliminary course of injections of heat-killed vaccine was given, followed after a 2 months' rest by four courses of whole living culture (50 per cent normal rabbit serum broth), the same material that is used in leukotoxin assay tests. Each course consisted of the injection of 1.0 ml. of the whole culture on the first 3 days of the week. The antisera were tested with the same technic used for the convalescent human sera, but they did not inhibit leukotoxicity.

Failure of antisera to prevent the leukotoxic injury could result from any one of a number of circumstances. The leukotoxic injury may not be produced by an antigenic substance, or for that matter by any specific chemical substance. Or it may be produced by an antigenic substance, but the injury may result only from formation of the substance within the cell (or elution from the coccal bodies of preformed substance after entering the

cell) where antibodies are not present. Or the hypothetical agent may combine with antibody without having its leukocyte-injuring capacity inactivated. Or the particular sera studied may have come from subjects who failed to respond with antibody production. At present it is not possible to say which of these possibilities applies.

DISCUSSION

The fact that streptococcal leukotoxicity is operative only after phagocytosis places it in a distinct class of cytotoxic actions by bacteria. The only streptococcal products having a demonstrated injurious effect on isolated living cells are the streptolysins O and S. Streptolysin O has been shown to injure leukocytes (leukocidin) and cardiac muscle cells (13) as well as erythrocytes, and streptolysin S is said to injure lymphocytes (14); but both of these substances exist or can be obtained in solution, and in such state act on the cells. So far as the author is aware, no other bacteria have been shown to have a toxic effect after phagocytosis only. Injury produced by extensive proliferation of intracellular bacteria appears to be different from the rapid destruction produced by leukotoxic streptococci, which are not seen to multiply inside the cell between ingestion and disruption of the cell.

The leukolytic activity of streptococcal leukocidin is quite different from leukotoxicity. Leukocidin is demonstrable in culture filtrates, it is antigenic (antisera produced against it neutralizing its leukocidal action), it is hemolytic and it is active only under reduced oxygen tension. It is likely that the leukocyte-injuring effects studied by Weld (15), Nakayama (16), Gay and Oram (17), and Channon and McLeod (18) were due to leukocidin since they used culture filtrates. However, the leukocyte destruction described by McLeod (19) when whole streptococcal cultures were injected intravenously may in part, at least, have been due to leukotoxicity.

With respect to Levaditi's study, however, there can be no doubt that he was dealing with leukotoxic action (3). As in the present work, leukotoxic injury was measured by direct microscopic observation. Levaditi grew his strains in serum broth, recognized that the injury occurred only after phagocytosis and was not produced by killed cocci. He was unable to obtain an injurious agent dissociated from the coccal bodies. Our studies agree with Levaditi's observations on these characteristics, but in one further respect we do not agree. He believed that leukotoxicity was related to virulence. He found that streptococci isolated from wounds early were more leukotoxic than those isolated later; and he found the cocci isolated from the blood stream to be more leukotoxic than those isolated from wounds of the same patient. While we have not examined strains similar in origin to these, we feel that our demonstration that highly mouse-virulent strains may be either leukotoxic or not and that strains having very low virulence for mice may

be leukotoxic or not indicates that leukotoxicity and virulence are not associated. Todd reached a similar conclusion with respect to leukocidin (4).

The method by which leukotoxic streptococci injure white blood cells has not been disclosed by these investigations, and, indeed, it is not possible to be certain whether the leukotoxic reaction follows elaboration of a specific chemical substance by the cocci, or results from some other type of cellular activity, such as competition with the host leukocyte for materials required for growth or energy. The peculiar type-distribution of leukotoxicity makes it unlikely that any general metabolic activity of streptococci, such as lactic acid production, could be responsible because those activities are common to streptococci of all serological classes. The unusual features of leukotoxic activity, namely its occurrence only after phagocytosis and only when living cocci are ingested, makes it reasonable to suppose an unusual mechanism of injury may be involved; and these features are in keeping with the concept that the injury results from an activity of the cocci rather than from a specific substance. But the latter possibility is by no means eliminated and all the observations could be accounted for by postulating the elaboration of a specific toxin which is unable in free form to penetrate the leukocyte membrane, but causes injury only when it is produced within the cell by the ingested cocci or is liberated from the coccal bodies within the cell.

The necessity of demonstrating leukotoxicity by laborious microscopic examination of the reaction of phagocytizing cells, a technic that is strictly qualitative, makes it difficult or impossible to conduct certain types of studies that would be highly desirable. Some recent observations by Bernheimer, Lazarides, and Wilson (20) have given some insight into the possible nature of leukotoxicity and will be reported later.

The role of leukotoxicity in streptococcal disease is unknown. Its peculiar type distribution makes it unlikely that it plays an important role in the pathogenesis of such streptococcal diseases as acute sore throat, purulent infections, scarlet fever, or rheumatic fever, since these are known to result from infection with most types of streptococci. The occurrence of type-association in streptococci possessing leukotoxicity and in streptococci isolated from acute glomerulonephritis makes it tempting to speculate on a causative relation of leukotoxicity to that disease, but leukotoxicity appears to have a wider type distribution than nephritis, it has been encountered in group C and G strains, which there is no reason to believe are associated with nephritis, and it was not detected in three Type 4 strains isolated from mephritic patients.

However, the impressive and dramatic injury produced in leukocytes suggests that some pathogenic role may exist if cell types other than leukocytes are destroyed, but as yet no information is available on this subject. It is conceivable that in the balance between the capacity of the invading organ-

ism to proliferate and the capacity of host cells to dispose of the bacteria, a leukotoxic capacity might at times make enough difference to allow disease to become established.

SUMMARY

Following phagocytosis of certain streptococci human neutrophils undergo a rapid disintegration: the leukotoxic reaction. Monocytes and eosinophils are similarly injured, as are polymorphonuclear cells of rabbit and guinea pig blood.

The leukotoxic injury is not produced by culture filtrates of leukotoxic cocci nor does it follow phagocytosis of heat-killed cocci. The leukotoxic effect does not appear to be due to action of any presently known streptococcal product.

The distribution of leukotoxicity among streptococci is not random, for it was found in all strains tested of certain types of group A (6, 12), and was absent from almost all strains of other types (5, 14, 30). Still other types (3, 4) had both leukotoxic and non-leukotoxic representatives. The injury was also produced by some group C and G strains.

Often the streptococci that cause leukocyte death remain alive and proliferate in the cellular debris, but sometimes they are injured by the phagocyte before the latter disintegrates and are unable to proliferate on the slides. The capacity of a strain of streptococcus to injure leukocytes does not necessarily confer virulence on it. This is thought to be because a chain of streptococci, having survived its sojourn in a leukocyte it has killed, is still susceptible to phagocytosis by a fresh leukocyte, and serial phagocytoses may continue until the chain has been exposed sufficiently to the unfavorable intracellular environment to be, itself, killed.

Whether leukotoxicity plays a role in naturally occurring streptococcal disease is unknown. The high incidence of leukotoxicity in Type 12 strains suggested that it might be involved in acute hemorrhagic nephritis, but if so there must be other factors since leukotoxic strains are present in types and groups not now known to be associated with nephritis.

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EXPLANATION OF PLATES

The photographs were taken with technics and equipment described earlier (1). Figs. 1 to 88, inclusive, are contact prints of phase contrast photomicrographs having a magnification of approximately 1000.

PLATE 33

Normal human blood cells. The preparations were made by mixing a loopful of heparin solution, a loopful of uninoculated Todd-Hewitt broth (Difco) and a small drop of blood obtained by finger prick. Preparations were incubated at 35–37°C.

FIGS. 1 and 2. Two crushed neutrophils in thinnest part of preparation, photographed immediately after preparation was made.

FIG. 3. A crushed neutrophil in a preparation 24 hours old.

FIG. 4. An uncrushed neutrophil immediately after preparation was made, showing rounded form, cell center, and lack of vacuoles.

FIG. 5. A neutrophil in ameboid movement, 1 hour after preparation was made.

FIG. 6. A neutrophil in ameboid movement in a preparation 8 hours old, showing the phase-dense bodies that have formed.

FIG. 7. A neutrophil in ameboid movement in preparation 8 hours old showing extensive vacuole formation.

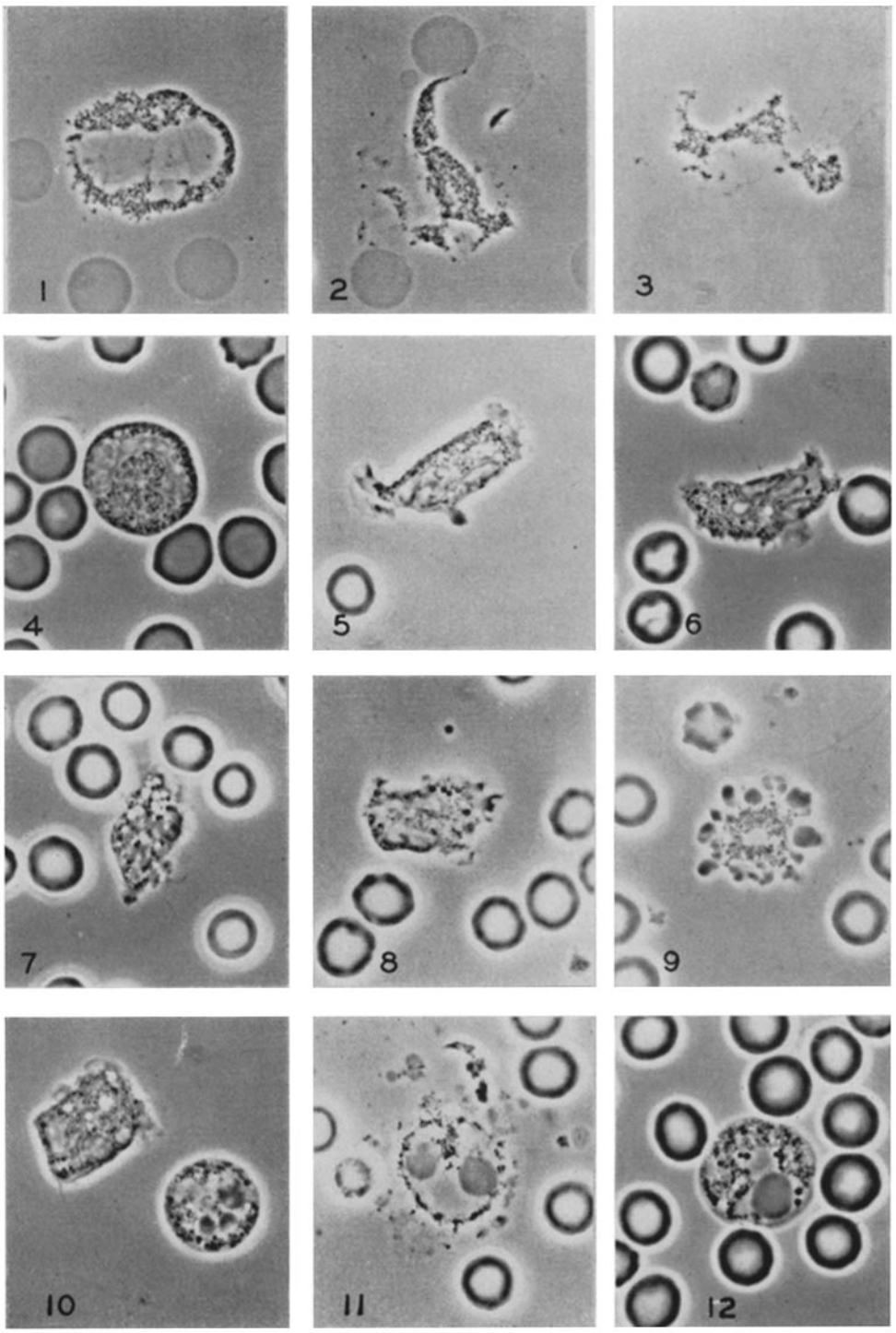
FIG. 8. A neutrophil in ameboid movement in preparation 24 hours old showing phase-dense bodies, but having few vacuoles.

FIG. 9. A neutrophil showing reaction of agitation. Preparation 24 hours old.

FIG. 10. A healthy neutrophil in 24 hour preparation showing phase-dense bodies and vacuoles, and a dead neutrophil that has undergone the reaction of agitation.

FIG. 11. A dead neutrophil in a 24 hour preparation. The cell has strong resemblance to cells that have been injured by leukotoxic action.

FIG. 12. A dead neutrophil in 24 hour preparation. Note the phase-dense bodies and the lack of agglutination of neutrophilic granules.



(Wilson: Leukotoxic action of streptococci)

PLATE 34

Survival of human neutrophils that have phagocytized a non-leukotoxic strain (Type 2/44).

FIG. 13. The preparation has been set up 5 hours. The neutrophil is healthy and in active ameboid movement although it has phagocytized many streptococci. This cell is representative of most others in the preparation.

FIG. 14. The same preparation, 6 hours old. A healthy, although somewhat sluggish, neutrophil.

FIG. 15. The same preparation, 6 hours old. A neutrophil showing injury. The cell is immobile and has formed several bud-like processes.

Leucotoxic action of AD238B on human neutrophil, showing rupture of cellular membrane.

FIG. 16. A neutrophil approaches a chain of 18 cocci 6 minutes after preparation was set up.

FIG. 17. 7 seconds after beginning of ingestion.

FIG. 18. 1 minute 5 seconds after beginning of ingestion. Cell has become immobile. 2 cocci are not ingested.

FIG. 19. 3 minutes.

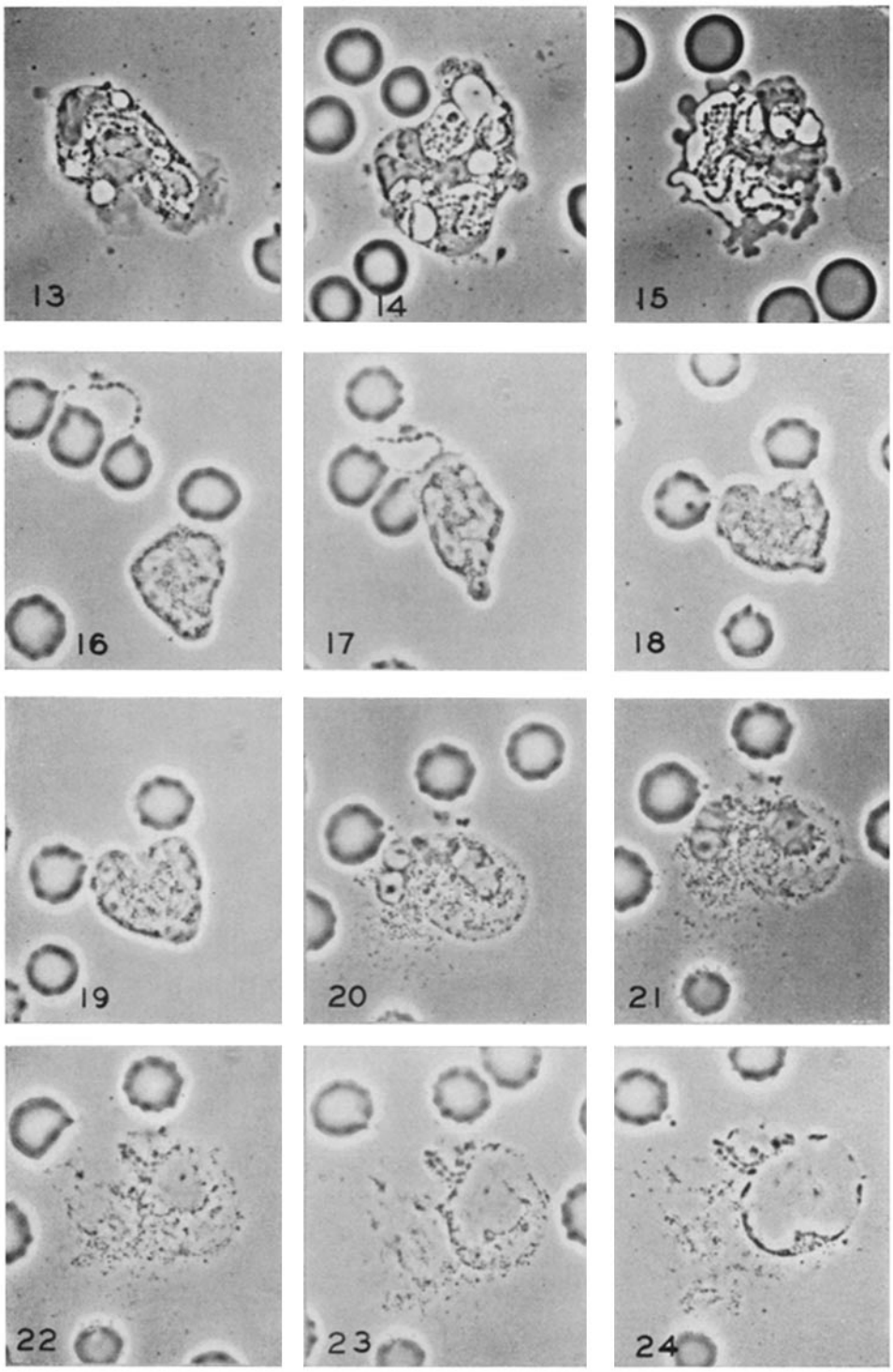
FIG. 20. 4 minutes 40 seconds. Cell membrane has ruptured, releasing specific granules, two nuclear lobes and the streptococci.

FIG. 21. 5 minutes 6 seconds. The nuclear lobes are beginning to swell and become hazy. Some of the released granules are drifting away. The streptococci are more distinctly shown.

FIG. 22. 6 minutes 16 seconds. The nuclear lobes are swollen and indistinct.

FIG. 23. 8 minutes 22 seconds.

FIG. 24. 17 minutes 30 seconds. Many of the released granules persist. Granules within the main cell mass are agglutinated and aligned along the cell membrane. Nuclear remnants are indistinct.



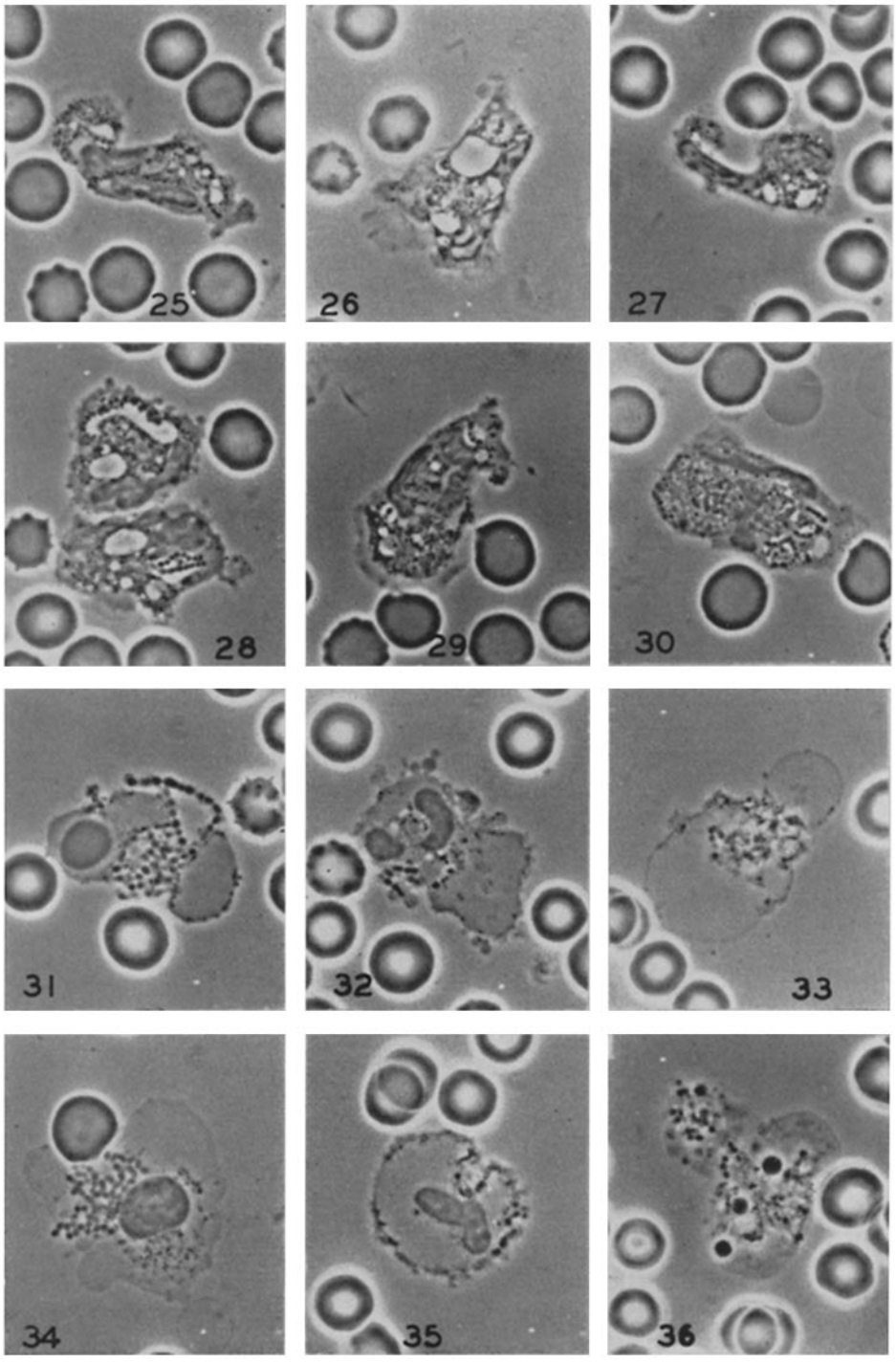
(Wilson: Leukotoxic action of streptococci)

PLATE 35

Comparison of action of non-leukotoxic and leukotoxic streptococci on neutrophils. The technic is that used for estimation of leukotoxicity of unknown strains. (See Materials and Methods).

FIGS. 25 to 30. Non-leukotoxic strain (T2/44). Representative cel's from a preparation 1½ hours old.

FIGS. 31 to 36. Leukotoxic strain (AD238B). Representative cells from a preparation 1½ hours old.



(Wilson: Leukotoxic action of streptococci)

PLATE 36

Arrest of ingestion before phagocytosis is completed, due to leukotoxic injury to leukocyte. Strain AD238B and human blood.

FIG. 37. A neutrophil approaches a long chain of cocci 37 minutes after the preparation was made.

FIG. 38. ½ minute later phagocytosis is in progress.

FIG. 39. 1 minute 5 seconds after start of phagocytosis cell is in good condition, but phagocytosis is incomplete.

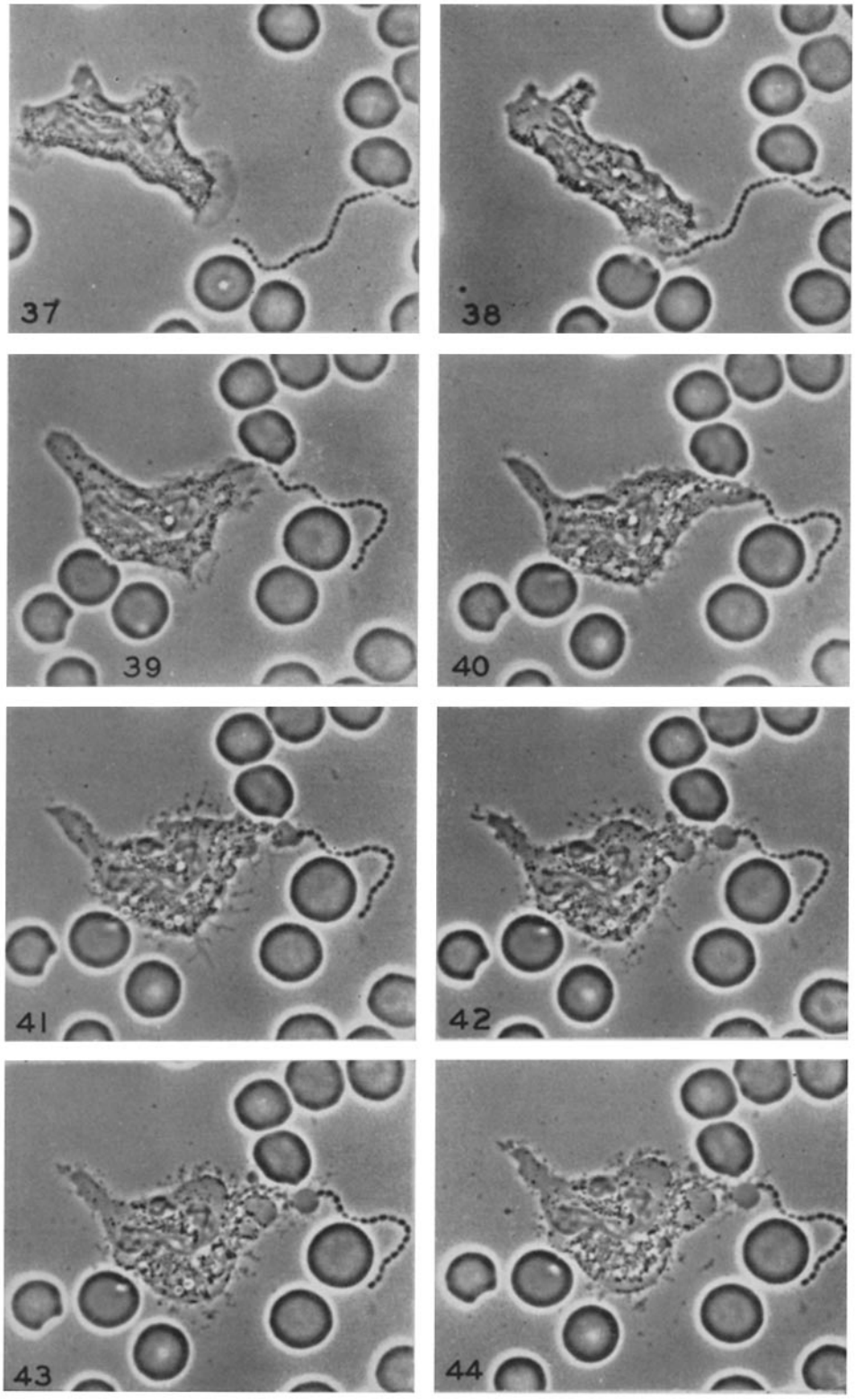
FIG. 40. 2 minutes 18 seconds after start of phagocytosis cell has stopped phagocytosis, is immobile, and has formed numerous filamentous processes. Vacuoles have formed around some of the ingested cocci.

FIG. 41. 2 minutes 45 seconds.

FIG. 42. 3 minutes 15 seconds.

FIG. 43. 3 minutes 45 seconds.

FIG. 44. 4 minutes 30 seconds. Figs. 41 to 44 show progressive stages in disintegration of cell, with spreading, loss of density of cytoplasm, pinching off of pieces of cytoplasm, and beginning agglutination of neutrophilic granules.



(Wilson: Leukotoxic action of streptococci)

PLATE 37

Three ingestions by two neutrophils, not leading to injury of leukocyte, followed by a final ingestion that destroys the cell. AD238B and human blood.

FIG. 45. Beginning of phagocytosis of a chain of 11 cocci 15 minutes after preparation was set up.

FIG. 46. The leukocyte is still in good cond tion 20 minutes later. The streptococci are seen with difficulty and are not distinguishable in the photograph.

FIG. 47. Another similar preparation. A neutrophil approaching a chain of 6 cocci 21 minutes after preparation was set up. Phagocytosis was completed 1 minute later.

FIG. 48. The same cell 52 minutes later. It has phagocytized another chain of 12 cocci (not shown) without suffering injury. Vacuole formation is conspicuous and some of the cocci are distinctly seen.

FIG. 49. 25 minutes later the same cell approaches a chain of 24 cocci.

FIG. 50. 1 minute after start of phagocytosis.

FIG. 51. 3 minutes after start of phagocytosis. The cell is immobile and has formed several delicate cytoplasmic processes.

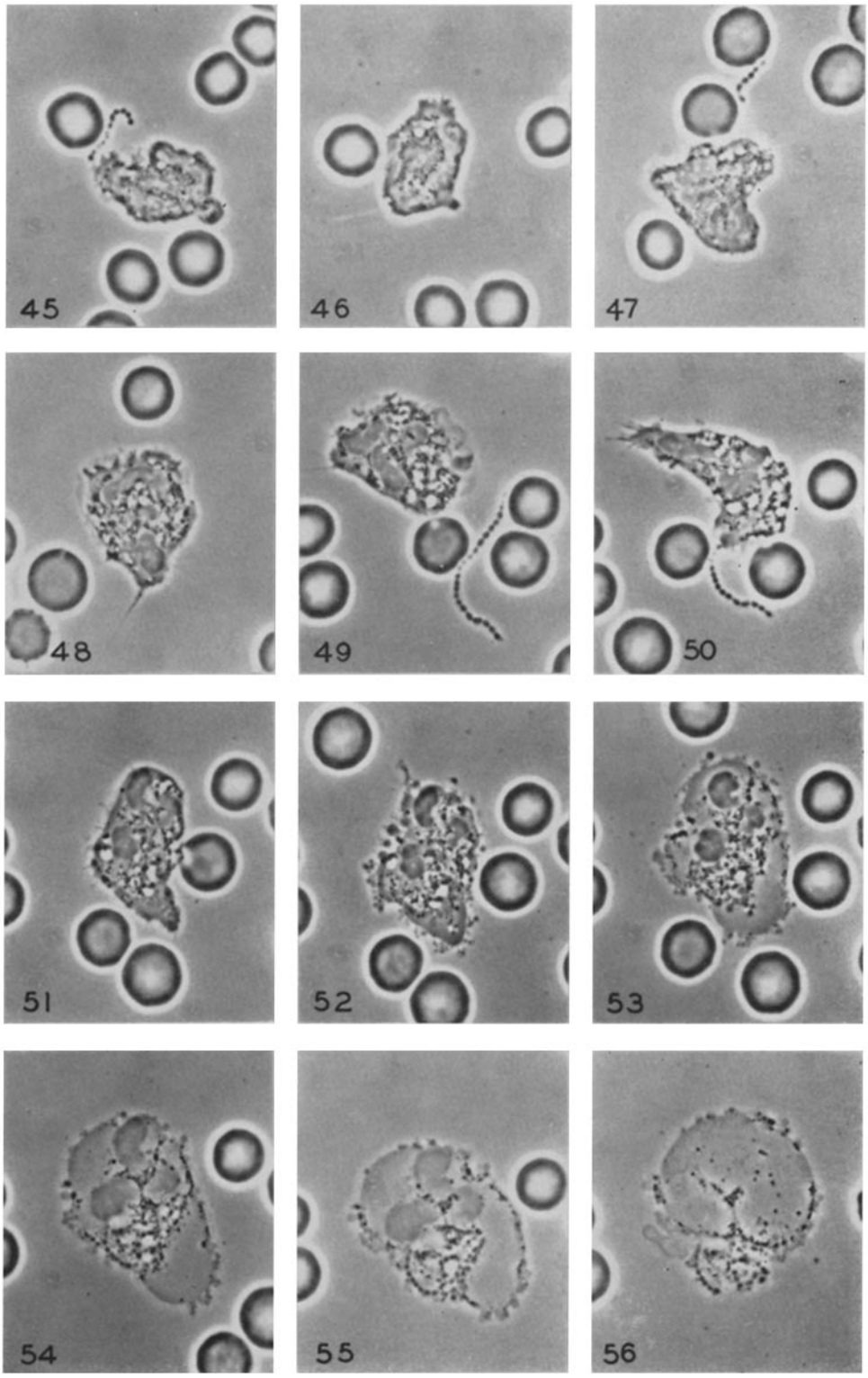
FIG. 52. 4½ minutes, the cell has started to spread. Cytoplasmic processes are more marked and some have pinched off and are free in the surrounding medium.

FIG. 53. 5½ minutes. The cell has spread further, cytoplasm is less dense. Pinched-off cytoplasmic fragments are seen around cell. Nuclear lobes have started to enlarge.

FIG. 54. 9 minutes. Further spreading of cell. Nuclear lobes enlarged and hazy.

FIG. 55. 28 minutes

FIG. 56. 1 hour 45 minutes after start of final phagocytosis. Cell is much spread out. Nuclear remnants cannot be distinguished. Many of the cocci are clearly seen although all that were ingested cannot be counted in the photograph. Most of them are lying in a mass of debris that represents an extruded part of the cell.



(Wilson: Leukotoxic action of streptococci)

PLATE 38

Leukotoxic action followed by proliferation of cocci. AD238B and human blood.

FIG. 57. A neutrophil approaches a chain of 12 cocci lying near a mass of debris, 34 minutes after preparation was set up.

FIG. 58. 7 seconds after onset of phagocytosis.

FIG. 59. 1 minute 43 seconds. The phagocytosis is complete. The cell is immobile and has formed some filamentous processes.

FIG. 60. 3 minutes. The processes are more conspicuous.

FIG. 61. 4 minutes. The processes are more numerous and small pieces have pinched off. The cellular granules have started to agglutinate.

FIG. 62. 5 minutes.

FIG. 63. 6 minutes. Granules are strongly agglutinated. Nucleus is becoming hazy.

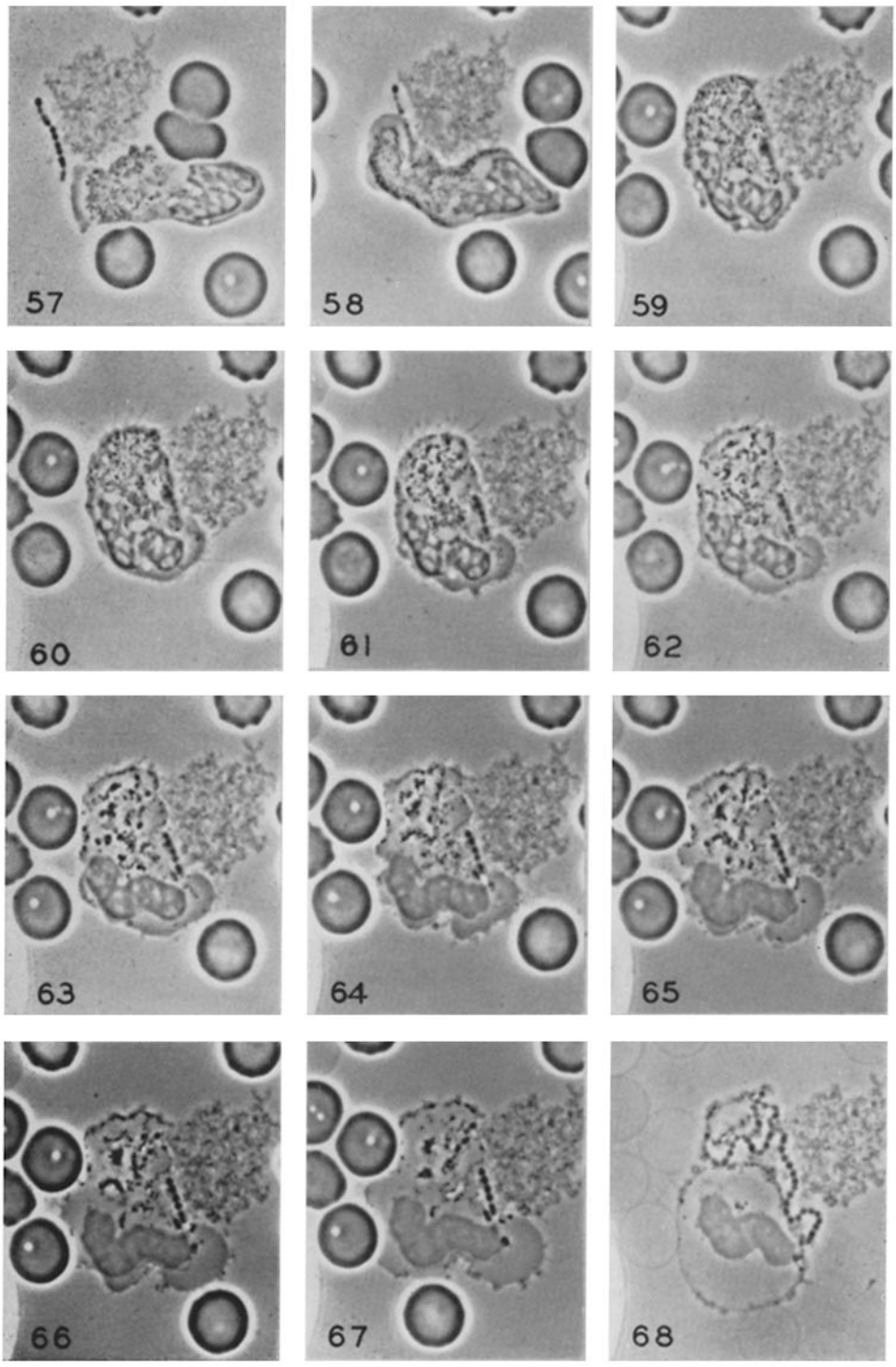
FIG. 64. 8 minutes 30 seconds. Cell is spreading.

FIG. 65. 10 minutes.

FIG. 66. 15 minutes.

FIG. 67. 30 minutes. The twelve cocci can be counted.

FIG. 68. 3 hours. Proliferation of the cocci has occurred. The paranuclear part of the cell has reconstituted a boundary after the cocci escaped. Cellular granules have largely disappeared. Red blood cells are hemolyzed.



(Wilson: Leukotoxic action of streptococci)

PLATE 39

Leukotoxic action not followed by proliferation of the ingested cocci. AD238B and human blood.

FIG. 69. Beginning of phagocytosis of a chain of 24 cocci, 21 minutes after preparation was made.

FIG. 70. 30 seconds later. Phagocytosis completed.

FIG. 71. 4 minutes after start of phagocytosis. Cell is immobile. A vacuole has formed around segment of chain. Other cocci are difficult to see.

FIG. 72. 6 minutes 30 seconds. Cell has put forth numerous cytoplasmic processes from borders. Nuclear markings are prominent.

FIG. 73. 9 minutes. Processes more marked. Cytoplasm is very fluid as judged by brownian movement of granules.

FIG. 74. 18 minutes. Cell has formed many clear watery vacuoles. Continues to extrude protoplasmic processes, some of which pinch off and float away. Is putting forth broad pseudopods like those of normal ameboid movement, but these do not cause cell to change position on slide.

FIG. 75. 25 minutes. Cell continues to form pseudopod-like and slender processes. Clear vacuoles have largely disappeared.

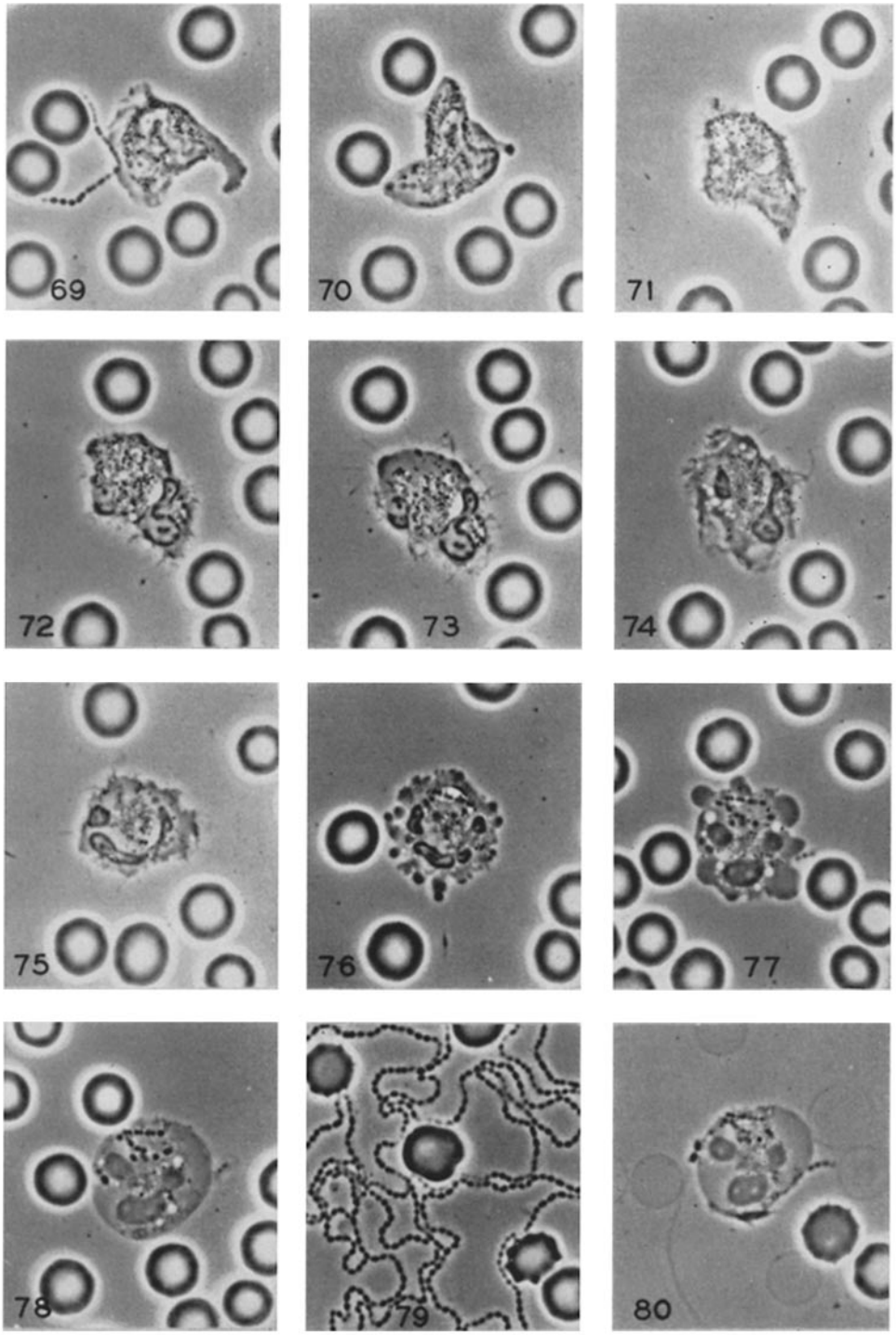
FIG. 76. 1 hour. Cell is forming many fluid, active bud-like processes. Nuclear lobes are dense, and one appears to have segmented.

FIG. 77. 3 hours. Cell continues to form active, blunt processes. Clear vacuoles have appeared again. A chain of 4 cocci (three are in focus in the photograph) has escaped from the cell. Nuclear lobes have enlarged.

FIG. 78. 6 hours. Cell is rounded and inactive. Nuclear lobes are enlarged and hazy. Some clear vacuoles persist. Neutrophilic granules have largely disappeared. Some streptococci are clearly shown in focus-plane of picture. There has been no proliferation of cocci in 6 hours following phagocytosis.

FIG. 79. 6 hours. Part of a microcolony that has grown out, having escaped phagocytosis, in a nearby field.

FIG. 80. 24 hours after phagocytosis. Cellular granules have largely disappeared, being represented by vague punctate debris in center of cell. Nuclear remnants have changed little from state at 6 hours. Streptococci have not proliferated. Most of the erythrocytes have been hemolyzed.



(Wilson: Leukotoxic action of streptococci)

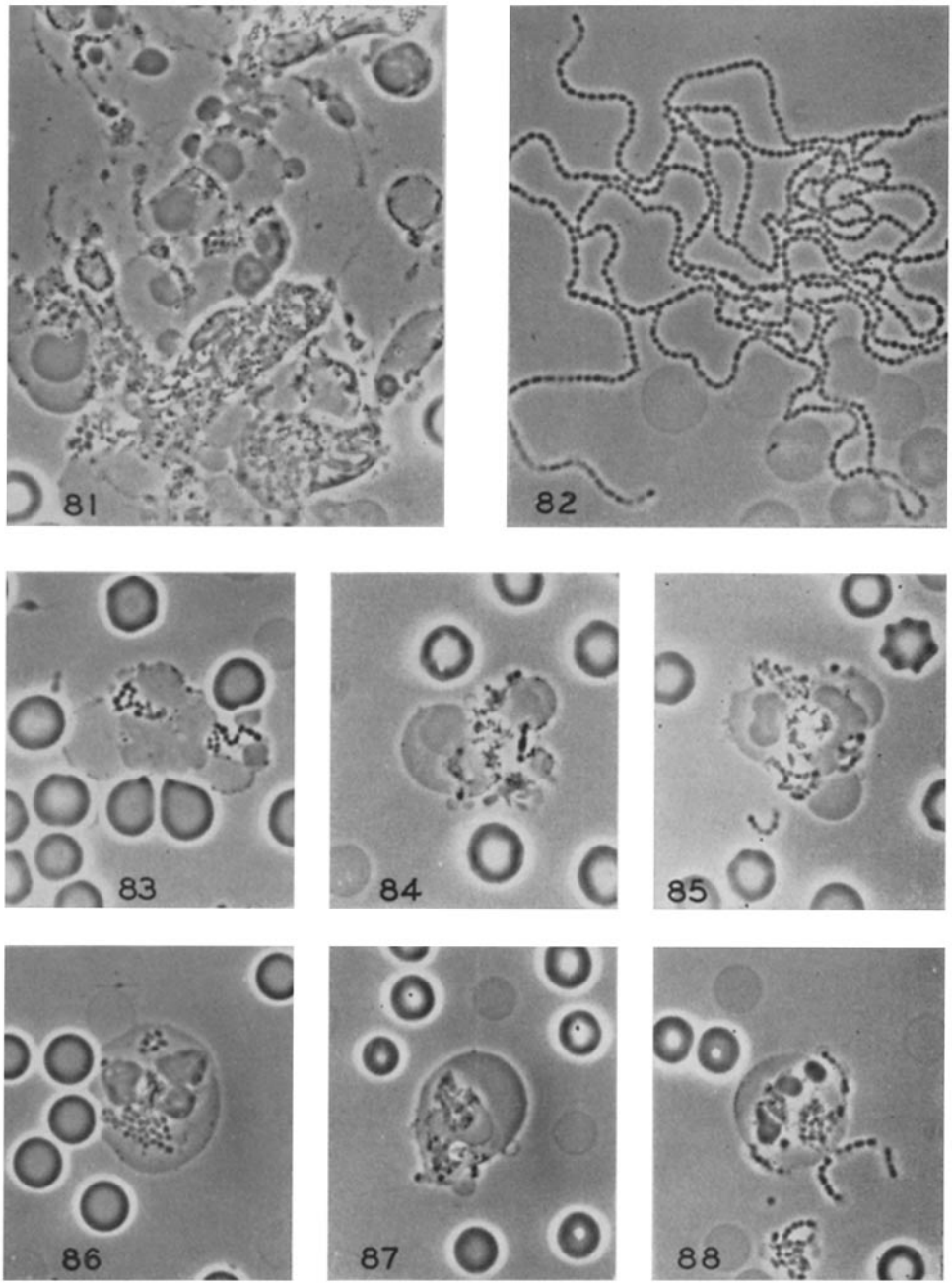
PLATE 40

FIG. 81. Part of a large mass of cellular debris from many neutrophils that have been attracted to debris of cell injured by leukotoxin. Part of an uninjured eosinophil is seen at the top and 2 uninjured neutrophils are in the lower part of the picture. The preparation was set up 2½ hours before the photographs were taken. Strain AD238B and human blood. Although many leukocytes have been destroyed, the streptococci have not grown out.

FIG. 82. Another field of the same preparation, taken immediately after the preceding picture. Part of a microcolony that has grown out on the slide. It represents uninhibited growth of streptococci compared to the arrested growth of cocci in Fig. 81.

FIGS. 83 to 85. Polymorphonuclear leukocytes of rabbit blood that have been injured by leukotoxic action following phagocytosis of AD238B. The appearances are quite similar to corresponding human cells.

FIGS. 86 to 88. Polymorphonuclear leukocytes of mouse blood that have been injured by leukotoxic action following phagocytosis of AD238B. Compared to human blood, the cells of mouse blood spread less and show less tendency to imbibe fluid. Disappearance of or agglutination of granules is similar in cells of the two species.



(Wilson: Leukotoxic action of streptococci)

PLATE 41

Appearance of stained cells from ingestion tests of non-leukotoxic (327W) and leukotoxic (AD238B) streptococci and human blood. Wright stain. Magnification approximately 1000.

FIGS. 89 to 90. Neutrophils from 327W preparation that has been rotated for 60 minutes, showing phagocytosis without leukocyte injury.

FIG. 91. Neutrophils from corresponding preparation of AD238B, showing streptococci and nuclear remnants.

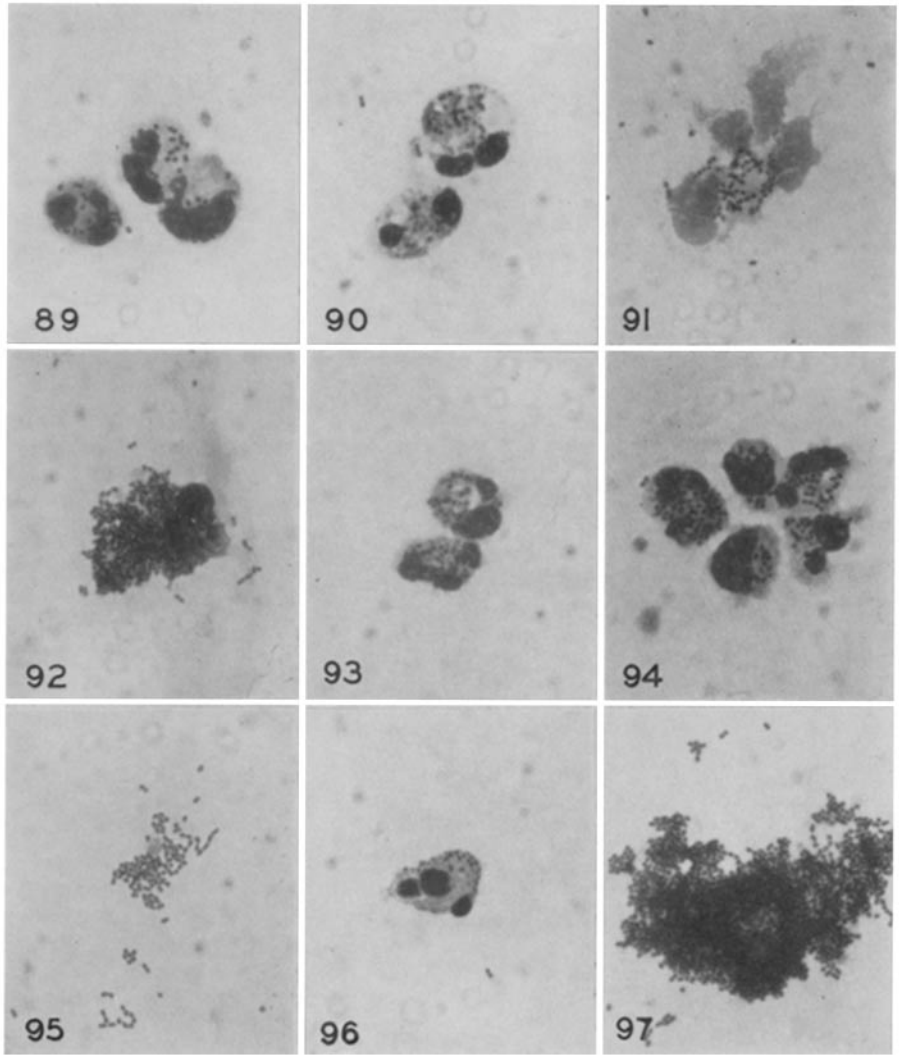
FIG. 92. From same preparation, showing microcolony in cellular debris.

FIGS. 93 to 94. Neutrophils from 327W preparation that has been rotated 2 hours, showing phagocytosis without leukocyte injury.

FIG. 95. Corresponding preparation of AD238B, showing cocci in cellular debris.

FIG. 96. Same preparation. Intact surviving cell.

FIG. 97. Same preparation. Large mass of streptococci and cellular debris.



(Wilson: Leukotoxic action of streptococci)