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Electron Microscopy of Group A Streptococci After Phagocytosis by Human Monocytes

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Group A streptococci were added to cultures of isolated human blood monocytes. The bacteria were readily sequestered within phagocytic vacuoles after being coated with flocculent material, apparently derived from the plasma-containing medium. Progressive lysis of intravacuolar streptococci was observed, characterized by plasmolysis, internal disruption, and eventual plasma membrane dissolution. However, the cell walls remained essentially identical to those of nonphagocytized streptococci, showing no signs of dissolution within the limited in vitro survival time of the monocytes. These results indicate that streptococcal cell walls may persist in migrating human phagocytes in vivo and may be deposited in body tissues. This cell wall material, known to be toxic to animal tissues, may be an important determinant in the pathogenesis of poststreptococcal sequelae in man.

The rate and pattern of degradation of different species of bacteria after phagocytosis are variable. This variability is primarily dependent on the composition of the ingested organism (5). Group A streptococci are degraded to a lesser extent than other bacteria (1). The cell wall is the portion of the streptococcal organism least susceptible to intracellular degradation and is resistant to the action of lysozyme in contrast to some other gram-positive cell walls (8, 25). Streptococcal cell wall material often persists for long periods of time when injected into animals and is associated with various inflammatory organ lesions. A single intradermal injection of cell wall fragments of group A streptococci initiates a chronic granulomatous lesion in rabbits, characterized by remissions and exacerbations (25). Such lesions are associated with the presence of cell wall fragments in vivo (18). Sterile streptococcal wall fragments also produce chronic injury in the joints of rabbits (26) and cardiac lesions in mice (19). Myocardial, hepatic, and diaphragmatic lesions may be induced in rabbits after the intratonsillar injection of living group A streptococci (31). Streptococci and streptococcal mucopeptide, labeled with fluorescein isothiocyanate (FITC) and injected into the rabbit myocardium, persist intracellularly in histiocytes for several months, whereas FITC-labeled group A L-forms, devoid of cell walls, rapidly disappear from the site of injection (22).

The events occurring after ingestion of streptococci by phagocytes have been only partially defined. Wilson (30) showed that the majority of streptococci are killed after residing for 20 min within polymorphonuclear leukocytes (PMN). Ayoub and Wannamaker (1) observed the release of dialyzable components of phagocytized, radioactively labeled organisms and concluded that intraphagocytic digestion occurs slowly. Kantor (15) and Gill and Cole (11), with fluorescent-antibody techniques, studied the disappearance of M protein after ingestion. Electron microscopic studies of phagocytosis of streptococci by human PMN suggest that these phagocytes may completely digest some streptococci in 3 hr (2). The purpose of the present study is to evaluate the degree of morphological degradation of streptococci by isolated human monocytes with particular emphasis on the fate of the cell wall. The products of this degradation may be important in the pathogenesis of poststreptococcal sequelae (12).

MATERIALS AND METHODS

Bacteria. Group A *Streptococcus pyogenes*, M antigen-negative by precipitin tests, was derived from strain T 1/155, obtained from R. Lancefield of Rockefeller University. Cultures were stored on sheep blood-agar slants and cultured as needed in Brain Heart Infusion broth (BHI; Difco). A portion of an overnight culture was added to fresh BHI and grown for 150 min at 37 C. This suspension of log-phase organisms was then washed and suspended in phosphate-buffered saline (PBS), *p*H 7.5, to give a reading

of 200 units in a Klett-Summerson colorimeter with a 560-nm filter (equivalent to 5 $\times$ 10^8 colony-forming units per ml, as measured by pour plate dilutions). For those experiments utilizing heat-killed organisms, the streptococci were heated at 56 C for 45 min.

Monocytes. A modification of the method described by Böyum (3) was used to obtain isolated human monocytes from peripheral blood. A standard Isopaque-Ficoll mixture was prepared accordingly. Hypaque-M (Winthrop Laboratories, New York, N.Y.) was substituted for Isopaque. Blood from normal human donors was heparinized (10 units/ml), layered over the Hypaque-Ficoll mixture, and centrifuged at 400 $\times$ *g* for 40 min. After centrifugation, the top layer of plasma was removed and used to make the medium for the present experiments, consisting of 80% Hanks balanced salt solution (Flow Laboratories, Rockville, Md.) and 20% plasma. The layer of cells at the interface between plasma and the Hypaque-Ficoll, rich in lymphocytes and monocytes, was removed, washed, resuspended in Hanks plasma, counted, and smeared for differential counts. This suspension was poured into plastic tissue culture dishes (60 by 15 mm, Falcon Plastics, Oxnard, Calif.) and incubated at 37 C for 90 min to allow the monocytes to adhere to the surface. Lymphocytes were removed by washing three times with Hanks solution.

Phagocytosis. Log-phase streptococci prepared as above were diluted in Hanks-plasma to give a final concentration of 10 colony-forming units per monocyte and were added to the tissue culture dishes. After 1 hr of incubation at 37 C, most of the remaining extracellular bacteria were removed by washing three times with fresh Hanks solution. Then fresh Hanks-plasma was added to each culture, the cultures were incubated at 37 C, and various dishes were sacrificed at hourly intervals.

Electron microscopy. At the times indicated above, the Hanks-plasma medium was removed from each culture, and 1.5 ml of 2.5% glutaraldehyde, buffered to *p*H 7.2 in 0.2 M sodium cacodylate, was added (24). After 10 min of fixation, the monocytes were gently scraped from the plastic surface with a rubber policeman. Fixation was then continued in glutaraldehyde for as long as 2 hr. After centrifugation, the cells were placed in 0.2 M sucrose, buffered at *p*H 7.2 in 0.1 M phosphate. Secondary fixation at room temperature in acetate-Veronal-buffered 1% osmium tetroxide and subsequent washing, agar embedding, and uranyl acetate treatment were done according to Ryter and Kellenberger (23). Dehydration was performed by passage of agar cubes through graded ethanol concentrations to propylene oxide, from which final embedment was made in Epon 812. Sections were cut on an LKB Ultratome I and were placed on Formvar-coated copper grids. All sections were stained for 3 min with alkaline lead citrate (21). Examination and photography were done with an Hitachi electron microscope (model HU-11C) operated at 75 kv.

Measurements of cell wall thickness were made by the use of a Bausch & Lomb measuring magnifier with a scale marked in 0.1-mm gradations. The dimensions given represent an average of at least 20 measurements on electron micrographs printed at magnifications of 69,000 and 120,000.

Labeling of Bacteria. Bacteria were labeled with fluorescent antibody according to Cole and Hahn (7).

RESULTS

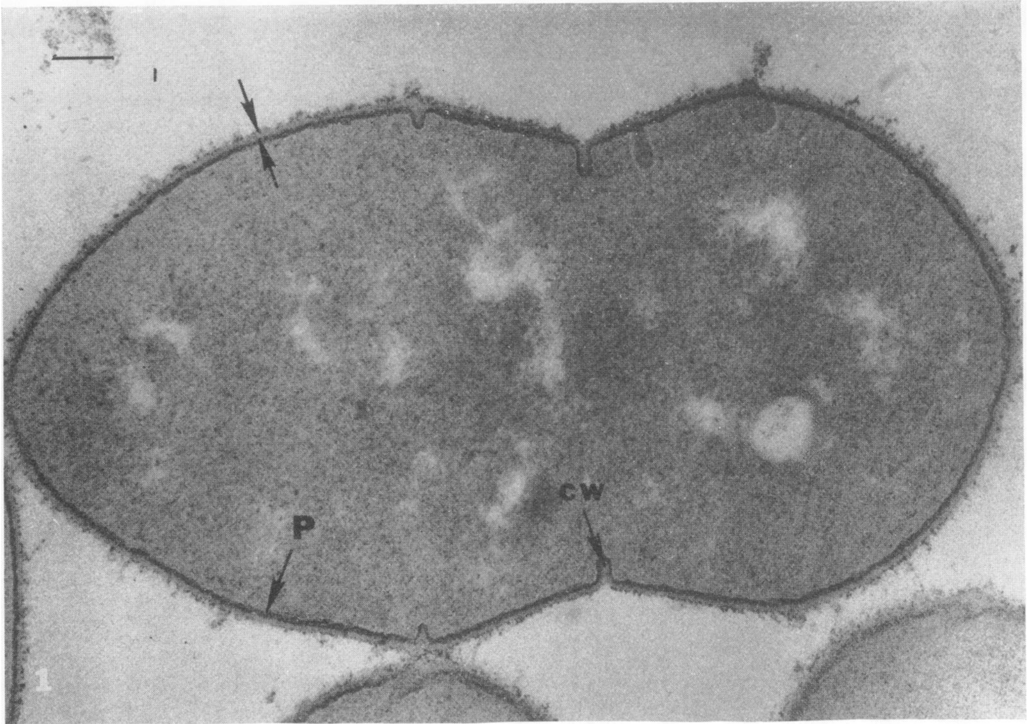
Streptococci. The appearance of log-phase group A streptococci fixed after suspension in PBS is shown in Fig. 1. The cell wall is relatively smooth, indicating that this strain lacks significant amounts of M protein (29). The cell wall appears to consist of a thick outer layer of low density and a thinner inner layer of high density. In intact cells, the inner layer is not clearly differentiated from the periplasm. During plasmolysis, the inner layer becomes clearly visible (Fig. 5).

Log-phase streptococci fixed after suspension in Hanks-plasma are shown in Fig. 2. The only consistent difference from those organisms fixed after suspension in saline is the appearance of a heavy, dense layer of flocculent material surrounding and adhering to the cell wall. Heat-killed organisms also develop this coating when transferred to the plasma-containing medium. Staining with fluorescein-labeled anti-M antibody demonstrates no M protein after incubation for as long as 3 hr in Hanks-plasma. Therefore, it appears that the flocculent material represents protein from the medium.

Most organisms fixed during log phase appear morphologically intact, although there are rare examples of lysing streptococci. Incubation of these organisms in Hanks-plasma for as long as 48 hr does not significantly increase the number of cells undergoing lysis.

Phagocytosis. In those specimens sacrificed after 30 min of incubation, many monocytes contain ingested streptococci; after 60 min, most monocytes contain organisms. Monocyte pseudopods surround the streptococci, resulting in the sequestration of the bacteria within phagocytic vacuoles. These vacuoles contain from one to several organisms. The monocyte cytoplasm surrounding actively forming and peripheral vacuoles usually contains dense, fibrillar material (Fig. 3). This material is usually not present around more deeply situated phagocytic vacuoles close to the nuclear hof, and the material is not observed in monocytes fixed at later stages of incubation.

Occasional membrane-bound vesicles are observed in continuity with phagocytic vacuoles which may represent lysosomes fusing with the vacuoles. Flocculent material of the same density as the contents of some monocyte lysosomes is often present within streptococci-containing vacuoles; however, this material cannot be dis-



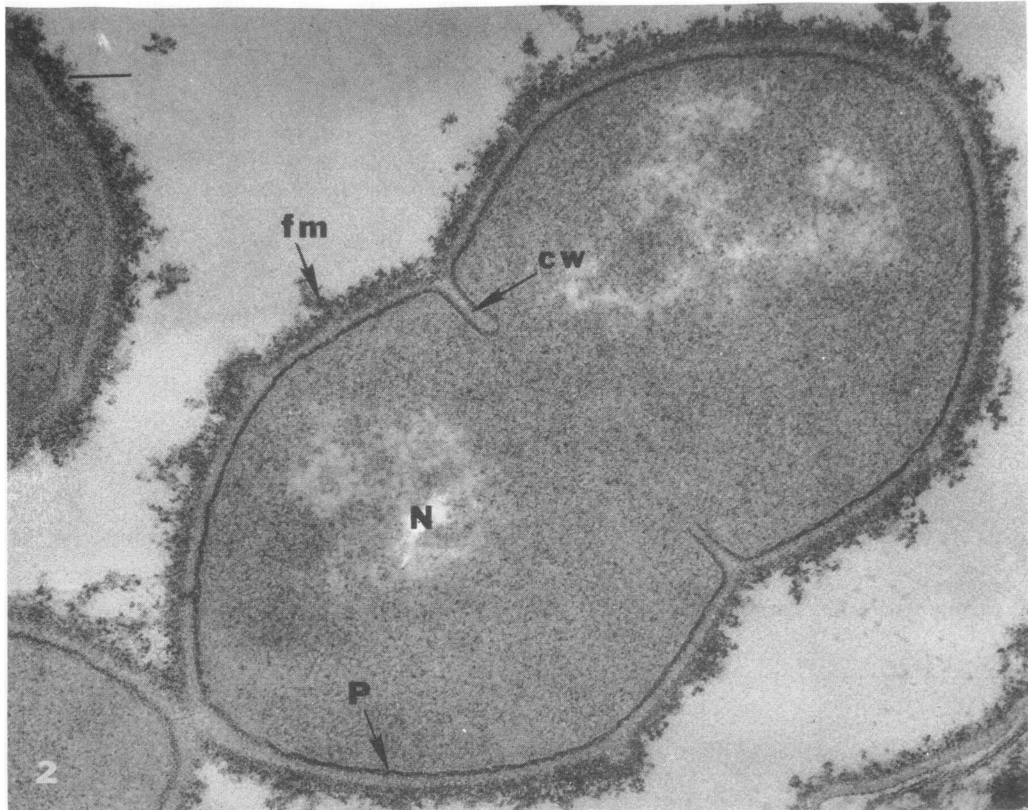


FIG. 1. *Log-phase group A streptococci fixed after suspension in PBS. The cell wall (between arrows) is relatively smooth. P, periplasm; cw, cross wall.* × *80,000; bar, 0.1 μm.*

FIG. 2. *Log-phase group A streptococci fixed after suspension in Hanks-plasma. Dense, flocculent material (fm) coats the cell wall. N, nucleoid; cw, cross wall; P, periplasm.* × *80,000; bar, 0.1 μm.*

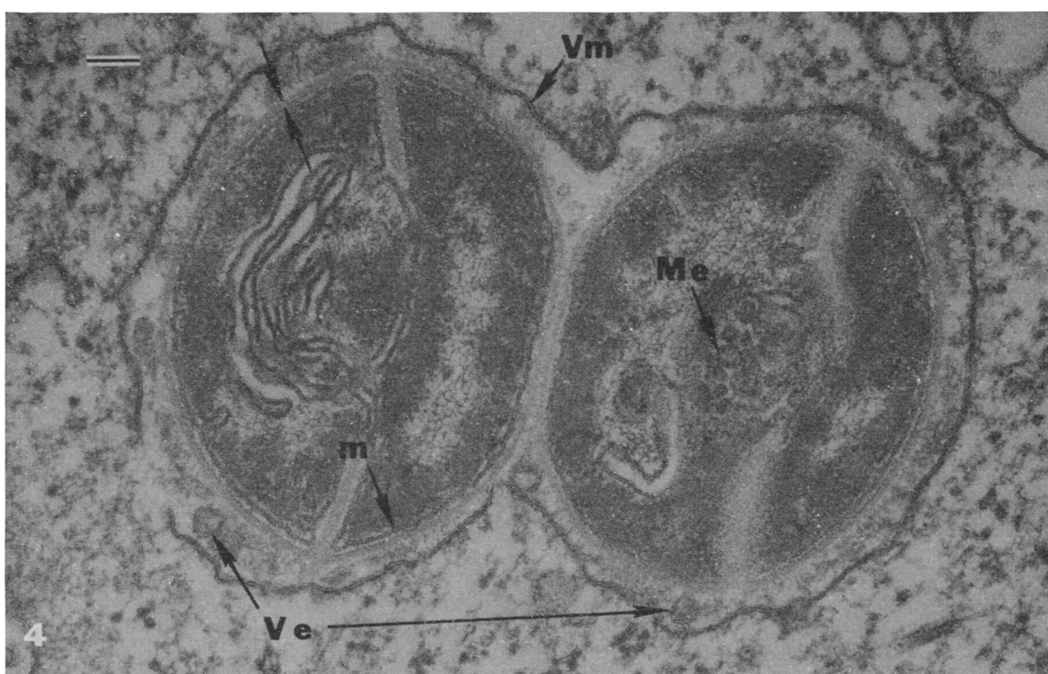


FIG. 3. *Monocyte fixed after incubation for 30 min with streptococci. Pseudopods (Ps) surround extracellular streptococci (S-1). Dense material (dm) is present in the cytoplasm of pseudopods and surrounding an ingested streptococcus (S-2).* × *20,000; bar, 1.0 μm.*

FIG. 4. *Phagocytic vacuole of monocyte after incubation for 3 hr with streptococci. The streptococcal cell wall (between arrows) is separated from the cell membrane (m). Me, mesosome; Ve, mesosome-like vesicles; Vm, vacuolar membrane.* × *69,000; bar, 0.1 μm.*

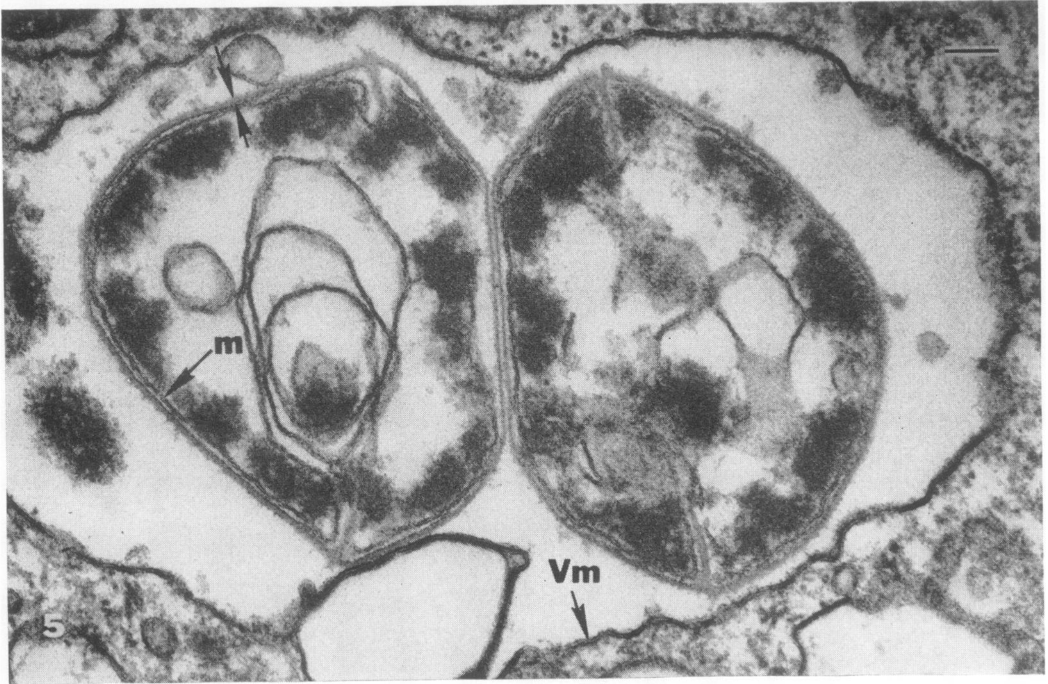


FIG. 5. *Phagocytic vacuole of monocyte incubated for 3 hr with streptococci. The cell wall (between arrows) is separated from the cell membrane (m). Note the disorganized cytoplasm. Vm, vacuolar membrane.* × *69,000; bar, 0.1 μm.*

FIG. 6. *Phagocytic vacuole of monocyte incubated for 6 hr with streptococci. The cell wall (between arrows) maintains its density. The cell membrane has disappeared, leaving disorganized membranous debris within the cell wall shell. Vm, vacuolar membrane; cw, cross wall.* × *120,000; bar 0.1 μm.*

tinguished from the proteinaceous coating described above.

The vast majority of phagocytizing monocytes appear relatively intact and viable when incubated with streptococci for less than 3 hr. However, steadily increasing numbers of degenerating and lysing monocytes are found in specimens incubated for 3 to 6 hr. At 6 hr, very few intact monocytes containing streptococci are present. Virtually no extracellular streptococci are observed in specimens incubated for less than 1 hr. At later times, organisms are found in the extracellular medium. The number of these free organisms appears to increase in parallel fashion with the number of lysing monocytes. These extracellular organisms are often in various stages of lysis, indicating that they most probably were released in a partially degraded state from degenerating monocytes. The death and rupture of monocytes are related to the process of ingestion. Control monocytes incubated for similar periods of time without streptococci do not show degenerative changes. It is unlikely that leukotoxic factors elaborated by the streptococci are responsible for the lysis observed, since phagocytosis of washed, heat-killed organisms produces similar cell damage.

Lysis of streptococci. Lysis of streptococci within phagocytic vacuoles is observed after 1 hr of incubation. Both the number of lysing organisms and the degree of lysis of any one organism appear to increase with increasing incubation time. At 5 and 6 hr, intracellular organisms undergoing lysis are quite common, whereas intact intracellular streptococci are rare.

The earliest morphological changes noted in intracellular streptococci are degenerative cytoplasmic changes, characterized by decreased cytoplasmic density, increased visibility of mesosomes, accumulation of membranous debris, and reticular changes within the nuclear region (Fig. 4). These changes are often associated with plasmolysis. The cell wall separates from the cytoplasmic membrane with simultaneous disappearance of the periplasmic substance (Fig. 4 and 5). The overall shape of the organism remains unchanged. Organisms in this stage of lysis are most numerous at 3 hr of incubation when the majority of intracellular streptococci exhibit plasmolysis.

Membrane-bound vesicular structures (Fig. 4) compatible with mesosomes in size and shape are often observed mixed with proteinaceous debris in phagocytic vacuoles. These structures maintain a close relationship with the outer surface of intravacuolar streptococci.

A minority of streptococci show additional morphological changes. In these cases, bacterial cytoplasm is further decreased in density and becomes completely disorganized (Fig. 5). The cytoplasmic membrane maintains its continuity at this stage.

Organisms exhibiting the most advanced stage of lysis in this study are most numerous at 5 and 6 hr of incubation (Fig. 6). At this stage, there is complete internal disruption with loss of cell membrane continuity. However, the cell wall appears virtually identical to the wall of an organism which has not been phagocytized. There is no change in density. Only minimal reduction, not more than 2 nm, in cell wall thickness is noted. The wall shell often contains a disorganized array of membranes mixed with amorphous material; however, the general shape of the organism is maintained. Cross walls are still present. No definite disruptions in the continuity of the walls are clearly discernible.

DISCUSSION

The ingestion of streptococci by human monocytes is similar to phagocytosis of other organisms by mononuclear phagocytes. The microorganisms are surrounded by cell pseudopods and become sequestered within phagocytic vacuoles. Recently formed phagocytic vacuoles containing streptococci are often surrounded by fibrillar, dense, cytoplasmic material similar to that observed during phagocytosis by Kupffer cells (14), amoebae (4), and peritoneal macrophages (9, 17). Although the significance of the material is unknown, many hypotheses have been offered (4, 17). Dumont and Robert suggested that the material may represent condensed cytoplasmic filaments related to phagocytic mechanisms (9). In the present study, the disappearance of such material is noted as the vacuole approximates the nucleus of the monocyte. This observation suggests that these dense filaments may represent a mechanism by which the phagocyte transports vacuoles from peripheral to central locations within the cell. Both loose and tight vacuoles are observed similar to those seen in phagocytizing macrophages (9); however, no correlation of vacuole type with duration of incubation is noted in the present study.

The proteinaceous material coating streptotococci exposed to the plasma-containing medium may represent adherent, nonspecific, opsonic substances. A radially oriented coating of similar appearance was observed when staphylococci were exposed to serum (14). The absence of this material when the staphylococci were exposed to buffer prompted these investigators to conclude that the coating was derived from serum factors.

Lysis of streptococci is consistently observed within phagocytic vacuoles. The percentage of lysing streptococci increases with increasing time of incubation. The pattern of lysis is similar to that observed with group C phage lysin (2) and with a preparation of *Streptomyces albus* enzymes (28). Initially there are degenerative cytoplasmic changes associated with plasmolysis. The cell wall separates from the cell membrane with a concomitant loss of periplasm. This is followed by progressive loss of protoplasm, mesosome liberation, and eventual loss of continuity of the cell membrane. However, the bulk of the cell wall remains unaltered, maintaining its continuity and shape as a shell. Only slight reduction in cell wall thickness is noted. Streptococci observed in a similar morphological state of lysis caused by enzymes from *Streptomyces albus* were shown to have lost 45% of their rhamnose and 55% of their hexosamine content (28).

Bacterial cell walls may be permeable to large molecules (10). Therefore, lysosomal enzymes might affect the bacterial membrane and cause internal lysis in the presence of an intact cell wall. However, the observation of mesosome-like structures outside the cell wall in phagocytic vacuoles indicates that the wall may have broken in some area, liberating these structures. Because of the relative sensitivity of the cross-wall region (13, 16, 20, 27), it is likely that breaks would occur in this vicinity, releasing protoplasm. There was no direct morphological evidence of this in the present study, although such leaks were observed when streptococci were treated with *S. albus* enzymes (28). Although no definite interruptions in the continuity of the cell wall are visible in the present study, it must be noted that such interruptions cannot be definitely ruled out in regions of tangential sectioning or in the absence of fortuitous serial sections.

Because of the limited survival of monocytes in the phagocytic system, observation cannot be extended reliably beyond 6 hr. At this time, there is no appreciable alteration of the streptococcal cell wall. Monocytes are relatively immature phagocytic cells, containing fewer lysosomal enzymes than polymorphonuclear leukocytes or mature macrophages (6). This may account for the slower and apparently less efficient digestion of streptococci by monocytes as compared to PMN (2). However, in the PMN study, little evidence of cell wall degradation is apparent.

Ohanian and Schwab (18) followed the fate of streptococcal cell wall antigens after intradermal injection of rabbits with intact group A cells. By use of fluorescence and radioautographic methods, these antigens were observed to persist long after morphologically identifiable organisms had disappeared. Based on this observation, these authors suggested that cell wall fragments are released during the degradation of streptococci by phagocytic cells. However, light optical methods did not permit accurate assessment of cell wall fragmentation.

The present ultrastructural study demonstrates that human peripheral blood monocytes do not have the ability to completely degrade group A streptococci within 6 hr in an in vitro system. The streptococcal cell walls remain virtually morphologically unaltered. Therefore, it is possible that cell walls may persist in migrating monocytes or macrophages in vivo and may be sequestered in those tissues in which these phagocytes localize, as suggested by Ginsburg et al. after in vivo studies in rabbits (12). The resulting cell wall material may produce or perpetuate chronic inflammatory lesions through direct toxic effects or hypersensitivity reactions.

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