PHAGOCYTOSIS OF ZYMOSAN BY HUMAN NEUTROPHILS

by

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A rapid cellular response occurs when human neutrophils are challenged with the zymosan particle. Monolayers of neutrophils adhering to glass were incubated with opsonized zymosan at 37 °C for 15 sec to 3 min, fixed and prepared for observation by SEM, immunofluorescence or TEM. At 15 sec zymosan particles are seen by SEM in contact with the cell surface and by 30 sec partial engulfment into open cavities was apparent. The cavity, into which a zymosan particle is engulfed, forms by extension of lamellipodia around and over the particle. The zymosan particle is sequestered in an intracellular vacuole or phagosome by 1 min. At 3 min neutrophils often show bulging profiles of the internalized zymosan and a loss of surface projections. During early phagocytosis, simultaneous staining with contrasting fluorescent antisera to granule constituents, myeloperoxidase and lactoferrin, reveals granule antigens within the cavity of the forming phagosome. Sections through the cells examined by TEM after 1-3 min incubation with zymosan show granule contents in the phagosome peripheral space which retains connection to the extracellular environment.

1. INTRODUCTION

Phagocytosis of bacteria and microorganisms by neutrophilic polymorphonuclear leukocytes (neutrophils) plays an essential role in host defense. The sequential events in phagocytosis including particle recognition, attachment, engulfment and degranulation have been well

studied by light and electron microscopy by numerous investigators (1, 5, 7, 8, 10, 22). Phagocytosis of foreign particles is usually followed by lysis and degradation of the microorganisms through the action of hydrolases and antibacterial proteins contained within cytoplasmic granules (1, 8, 10, 17, 18, 22). Human

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Abbreviations: LF = lactoferrin; MPO = myeloperoxidase; SEM = scanning electron microscopy; TEM = transmission electron microscopy

neutrophils contain two distinct types of granules which have been analyzed morphologically and biochemically (2, 3, 4, 5, 6, 16, 17, 21). Azurophilic or primary granules formed during the promyelocyte stage are dense, round and contain myeloperoxidase (MPO), proteases, lysozyme and acid hydrolases. Specific or secondary granules appearing in early myelocytes are less dense, pleomorphic and contain lysozyme and lactoferrin (LF). Due to the distribution of MPO and LF, they have been designated as specific markers for the azurophilic and specific granules, respectively (13, 16). Using immunocytochemistry with antisera against MPO and LF, it has been possible to investigate neutrophil degranulation and secretion during early phases of phagocytosis of bacteria (12, 14). Degranulation was shown to be rapid and both granule markers, MPO and LF, are observed on the neutrophil surface within 5 sec after phagocytosis of *E. coli* has been initiated.

Most of the studies of phagocytosis in the past have centered on long-term incubation with microbial particles. The present study investigates the morphology and secretory activity of human neutrophils during initial phases of phagocytosis of zymosan. The tools of SEM, immunocytochemistry and TEM were used to probe the early phagocytic events.

2. MATERIALS AND METHODS

2.1. Preparation of cell monolayers

Human venous blood was allowed to clot onto 8 mm glass coverslips as previously described (14). The cells were layered with Gey's tissue culture medium in Tris buffer pH 7.2 (19) containing 10% human AB serum and kept at 37 °C for 10 min. The medium was replaced before incubation with zymosan. Wright's stain was used to estimate the size and composition of the adhering cell population which was about 5×10^5 cells with at least 90% neutrophils. Viability was more than 98% as determined by trypan blue exclusion.

2.2. Preparation of cell suspensions

Heparinized venous blood was allowed to settle at 37 °C for 40-60 min. The buffy coat

of leukocytes was removed, washed with phosphate buffered saline, centrifuged at 600 rpm, and resuspended in Gey's.

2.3. Incubation of neutrophil monolayers with zymosan

Zymosan was opsonized with normal type AB human serum for 30 min at 37 °C and was added to the monolayers at a ratio of 100 per neutrophil at 37 °C with gentle rocking. At intervals phagocytosis was stopped by removal of the medium and simultaneous fixation and washing with 1% paraformaldehyde in 0.072 M-sodium cacodylate buffer containing 0.72% sucrose at pH 7.5. The monolayers were then processed for SEM or immunofluorescence microscopy.

2.4. Incubation of neutrophil suspensions with zymosan

Cell suspensions containing about 1.2×10^7 cells in 1 ml of tissue culture fluid were placed in test-tubes to which was added 2 ml tissue culture fluid containing 1×10^6 opsonized zymosan. Incubation was carried out in a water bath with gentle rocking for 30 sec to 10 min at 37 °C. Phagocytosis was stopped at intervals by addition of an equal volume of fixative containing 3% glutaraldehyde in 0.2 M-phosphate buffer at pH 7.4.

2.5. Preparation for SEM

The monolayers of cells were further fixed for 1-2 hours with 1.5% glutaraldehyde in 0.1 M-phosphate buffer pH 7.4 at 4 °C. Cells were then washed with 0.1 M-phosphate buffer, dehydrated in ethyl alcohol, and critical point dried from CO₂ in a Denton vacuum DCP-I Dryer. The glass coverslips containing the monolayers were mounted on aluminum stubs with silver paint, coated with gold-palladium in a Polaron Cold Sputter Coater, and examined at 20 kV in a JEOL model JSM35.

2.6. Preparation for TEM

Fixation of cell suspensions in 1.5% glutaraldehyde in 0.1 M-phosphate buffer was continued

Figure 1. SEM view of human neutrophil incubated at 37 °C with opsonized zymosan for 15 sec. Four zymosan (z) particles are in contact with its ruffled surface. (Bar is 1 µm.)

Figure 2. At 30 sec two zymosan particles are partially engulfed into open cavities formed by the lamellipodia of the neutrophil. (Bar is 1 µm.)

Figure 3. A zymosan particle is being further engulfed into a forming phagosome at 1 min of incubation. (Bar is 1 µm.)

Figure 4. Several zymosan particles are internalized at 3 min of incubation and appear as bulges on the cell surface (at arrow). (Bar is 1 µm.)

Figure 5. Zymosan particles associated with a neutrophil are shown at 30 sec of incubation. a. Phase microscopy, arrow shows zymosan particle associated with immunofluorescent staining. b. Immunofluorescent localization of fluorescein-labelled antiserum to MPO. c. Immunofluorescent localization of rhodamine-labelled antiserum to LF. The curvilinear staining of MPO and LF indicates secretion of granule contents into the open cavity of the forming phagosome (as seen in Figure 2). Note that some staining for LF is observed on the surface away from the forming phagosome.

for 1 hour at 4 °C. After fixation, the cells were washed twice by centrifugation at 125 g for 10 min. The cells were then dehydrated in increasing concentrations of ethanol and embedded in Epon 812. Thin sections were cut, gathered on copper grids and treated with uranyl acetate and lead citrate before examination on the JEOL 100B transmission electron microscope.

2.7. Immunofluorescence microscopy

Monospecific antisera against human MPO conjugated with fluorescein isothiocyanate and human LF conjugated with tetramethylrhodamine isothiocyanate were used as markers to detect contents of azurophilic and specific granules. The methods used for immunofluorescent staining, specificity of the antisera and procedures used to label the antisera have been described (12, 14, 15). The cells were stained with labelled antiserum for 30 min at 4 °C, washed in phosphate buffered saline, mounted and examined with a Leitz Orthoplan fluorescent microscope using incident light excitation with filter pack H for fluorescein excitation and filter pack N for rhodamine excitation, both with edge filter K480 to minimize quenching. As controls, fluorescent staining was blocked by affinity chromatography of liquid phase adsorption of antisera with its respective immunogen (12).

3. RESULTS AND DISCUSSION

Monolayers of neutrophils incubated with opsonized zymosan for 15 sec show a ruffled cell surface and usually several zymosan particles in contact with the surface ridges and lamellipodia when viewed by SEM (Figure 1). By 30 sec, lamellipodia form a cup around the zymosan and engulf the particle into a large open cavity (Figure 2). At one min the particle being engulfed is further enclosed by the forming phagosome (Figure 3). The leading edges of the lamellipodia eventually meet and fuse to enclose the particle completely. By 3 min the ruffling of the cell surface is decreased considerably and most zymosan particles are well internalized within the neutrophil (Figure 4). The zymosan particles are revealed as smooth bulges on the cell surface, although some particles may remain on the cell surface (Figure 4). If neutrophils incubated for 1 min with zymosan are washed of excess zymosan and incubated for an additional 10 min, the zymosan particles are totally engulfed, and seen as bulges on the cell surface, indicating that the process of internalization was completed.

In order to detect whether phagolysosome fusion takes place before the phagosome is completely formed, cells were stained simultaneously for granule markers MPO and LF using contrasting fluorochromes. The cells were fixed

Figure 6. Electron micrograph of a neutrophil approaching a particle (z); its cell process is almost in contact with zymosan at 1 min after incubation. At this plane, granules appear more numerous in the cytoplasm facing the zymosan particle. (Bar is 1 μ m.)

Figure 7. Neutrophil retains an open channel (at arrow) between the phagosome and the extracellular space after 1 min incubation. (Bar is 1 μ m.)

with 1% paraformaldehyde to detect only antigens associated with the cell surface (14, 15). Zymosan particles associated with a neutrophil 30 sec after incubation are shown by phase microscopy and immunofluorescence staining (Figure 5). Immunofluorescence indicates that degranulation has occurred before the particle is completely ingested. MPO (Figure 5b) and LF (Figure 5c) appear localized around the zymosan particle in the forming phagosome, indicating degranulation from both azurophilic and specific granules. Granule content secretion appears limited to one phagosome (at arrow in 5a). The other cell-associated zymosan particles are unreactive, presumably because phagocytosis has not been initiated or because the antigen is inaccessible to the antibody. Some LF is observed on the cell surface not associated with zymosan. Similar staining is observed after phagocytosis of *E. coli* (14). Staining of this type for MPO was rarely observed. It has been shown

that specific granules are more prone to degranulate than azurophilic granules (20). The curvilinear pattern of fluorescence is coincident with the rim of the open cavity of the early forming phagosome as seen by SEM in Figure 2.

It has been observed by immunocytochemistry that cyclic AMP and cyclic AMP-dependent protein kinase are compartmentalized at the site of particle-cell contact (15). Cyclic AMP may serve as a signal to regulate endocytosis. Before the phagosome is closed, the granule contents may accumulate in the cup, but also may leak out into the extracellular space. Such leakage of the powerful hydrolases and antibacterial proteins may account for some of the injurious effects of inflammation (20). That communication is retained between the forming phagosome and the extracellular space has been pointed out by other investigators (9, 22). From TEM in these zymosan studies, morphological evidence

Figure 8. SEM view of neutrophil in process of internalizing 3 zymosan (z) particles showing the narrowing of the phagosome openings after 3 min of incubation. The opening of a channel (at arrow) appears at the surface between a well-internalized phagosome and the extracellular space. (Bar is 1 μ m.)

Figure 9. Neutrophil with 2 zymosan particles (z) engulfed into phagosomes at 1 min incubation. Nuclear lobes are at n. (Bar is 1 μ m.)

exists for an open channel between the phagosome and the surface after 1 min incubation (Figure 7). By SEM, an opening is seen at the surface of a channel between a well-internalized phagosome and the extracellular space (Figure 8). Phagocytic vacuoles containing internalized particles were reported to retain communication with the external medium for 30 min to 1 hour (9); failure of internal isolation may prevent the vacuole from attaining the proper gradient of H^+ ions required for microbicidal systems.

Further details of phagocytosis and granule secretion into the phagosome can be seen by TEM. In Figure 6, after 1 min incubation with zymosan a neutrophil appears to approach a zymosan particle. Its cell process, almost in contact with the zymosan particle, eventually will form the phagosome. The cytoplasmic granules are most numerous towards the particle being engulfed. The denser granules are most likely the azurophilic granules, while the less dense granules are the specifics (2, 3). After 1

min of incubation other cells are seen having already engulfed zymosan particles (Figures 7 and 9). The cytoplasmic granules appear less numerous and are concentrated at the cytoplasmic periphery of the phagosomes (Figure 9). Figure 10 shows 2 zymosan particles within phagosomes. The peripheral vacuolar space between the phagosome membrane and the zymosan is evident. Several intact granules and some fragmented pieces appear in the vacuolar space around the zymosan particle enclosed within the phagosome membrane, which was previously the plasma membrane of the cell surface. Granule contents enter the phagosome by a continuous passage formed by fusion of the granule membrane with the phagosome membrane (1, 22). This fusion adds to the substance of the phagosome membrane. The loose outline of the phagosome membrane around the granules in the phagosome peripheral space (Figure 10) suggests the phagosome membrane was formed from two sources and now contains former

Figure 10. Neutrophil shows portions of 2 zymosan particles (z) within phagosomes after 3 min of incubation. The granules (g) are within the phagosome membrane (m) now composed of granule membrane fused with the former plasma membrane. Some granules (g') are in the process of dispersing into fragments (f) within the phagosome peripheral space. (Bar is 1 μ m.)

granule membrane inserts. In addition to the intact granules without membranes in the phagosome peripheral space, there is a fine granulation which most likely consists of fragments of cytoplasmic granules. A granule in the process of fragmentation is seen in Figure 10. Presumably it is this granular material which can be detected by surface immunofluorescence before the phagosome is closed. The exact time when the phagosome is completely internalized and closed to the extracellular space is not certain. Some phagosomes may retain connection with the exterior for longer times than others.

Our data show that phagocytosis and internalization of numerous zymosan particles are rapid and occur concurrently with cytoplasmic granule secretion into the neutrophil phagosome. The granule constituents appear at that time to be in free communication by an open

channel between the newly forming phagosome and the extracellular space. Although details of the lytic process within the phagosomes are not clear, the presence of granular contents in the phagosome suggests early initiation of killing or lytic mechanisms in the phagosome and concurrent possible transfer of the same lytic mechanisms into the extracellular environment.

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