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Electron Microscopic Study of Phagocytosis of *Escherichia coli* by Human Polymorphonuclear Leukocytes

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The fate of *Escherichia coli* strains within the polymorphonuclear leukocytes was studied by determining the killing of bacteria, measuring the release of degradation products, and examining the phagocytic bacteria by electron microscopy. When sufficiently opsonized, both unencapsulated and encapsulated *E. coli* strains were rapidly phagocytized by polymorphonuclear leukocytes. Once phagocytized, the two unencapsulated *E. coli* strains (K-12 and O111) were rapidly killed (99% of the bacteria were killed during the first 5 min of phagocytosis) and extensively degraded (about 40% of the radiolabeled material was released from bacteria after 15 min of phagocytosis). Electron micrographs taken after 15 min of phagocytosis revealed extensive structural changes in most of the internalized bacteria. In contrast to the rapid killing and extensive breakdown of these strains, encapsulated *E. coli* O78:K80 was more resistant to killing and withstood degradation by polymorphonuclear leukocytes (only 5% of the radioactivity was released from the radiolabeled bacteria after 1 h of phagocytosis). Electron micrographs of thin sections taken after 1 h of phagocytosis revealed virtually no structural changes. Most of the internalized bacteria were still surrounded by thick capsular material.

The bactericidal activities of serum and phagocytic cells such as polymorphonuclear leukocytes (PMN) and mononuclear leukocytes play an important role in host defense against invading microorganisms (7, 12, 16). Many gram-negative bacteria are sensitive to the bacteriolytic activity of serum. Those which are resistant become opsonized in serum and are recognized by phagocytic cells (12, 19). Once phagocytized, they are usually rapidly killed and degraded (2, 8). The capacity of some gram-negative bacilli to resist phagocytosis is often associated with the presence of cell surface capsular polysaccharides or proteins or of specific sugars in the O antigenic side chains in the lipopolysaccharides (9, 10). These strains are effectively opsonized only in the presence of specific antibodies (13). When bacteria are phagocytized, the phagocytic cell produces a burst in oxygen consumption resulting in the production of toxic oxygen metabolites. Lysosomal granules discharge their contents into the phagosome (1, 14). Most of the proteins present in the lysosomal granules are hydrolytic enzymes (4). Some are able to degrade bacterial targets; others participate directly in killing by the phagocyte. Once phagocytized, most of the gram-negative bacilli are rapidly killed, and the degradation of bacterial components follows (2, 12). Phagocytosis of gram-negative rods has usually been quantitated by measurement of the decrease in viable counts of bacteria, determination under a microscope of the percentage of leukocytes phagocytizing a bacterial load, or measurement of the radioactivity associated with phagocytes after incubation with radiolabeled bacteria. Studies to evaluate the degradation of microorganisms during phagocytosis have mainly been chemical in nature, without a description of ultrastructural changes. We compared the rates of intracellular killing of *E. coli* strains with and without K antigens and studied the fate of these strains within the phagolysosome, with an electron microscope. After adequate opsonization, K-antigen-positive *E. coli* was more resistant to the bactericidal and bacteriolytic activity of PMN than were unencapsulated *E. coli* strains.

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MATERIALS AND METHODS

Bacteria. Unencapsulated *E. coli* K-12 PC2166 and O111 and encapsulated *E. coli* O78:K80 were used (agglutination experiments showed that *E. coli* O111 did not possess a capsule; serotyping was performed by P. A. M. Guinée, Central Institute for Public Health and Environmental Hygiene, Bilthoven, Holland). The presence of K antigen was determined by the hemagglutination inhibition technique of Glynn and Howard (6).

Radioactive labeling of bacteria. Bacteria were inoculated from a blood agar plate into 5 ml of Mueller Hinton broth (Difco Laboratories, Inc. Detroit, Mich.) containing 0.02 mCi of [^{3}H]uridine (29 Ci/mmol specific activity; Radiochemical Centre, Amersham, England). After 18 h of incubation at 37°C, the bacteria were washed three times with phosphate-buffered saline (PBS; pH 7.4) and adjusted to a final concentration of 5×10^8 CFU/ml with a Klett-Summerson photoelectric colorimeter that was standardized by a pour-plate method (17).

Opsonins and opsonization procedure. Normal serum was obtained from 10 healthy donors and pooled. Antiserum was prepared by immunizing rabbits with heat-killed *E. coli* O111 and Formalin-killed *E. coli* O78:K80 (to preserve the K antigen), by a method previously described (11). All sera samples were stored at −70°C, thawed shortly before use, and diluted in Hanks balanced salt solution containing 0.1% gelatin (Gel-HBSS). Bacteria were opsonized by incubation in various concentrations of serum (*E. coli* PC2166 in 2.5% normal serum; *E. coli* O111 and O78:K80 in 10 and 20% antiserum, respectively) at 37°C. After 30 min, bacteria were centrifuged (15 min at 1,600 × *g*) and suspended in Gel-HBSS to a final concentration of 5×10^8 CFU/ml.

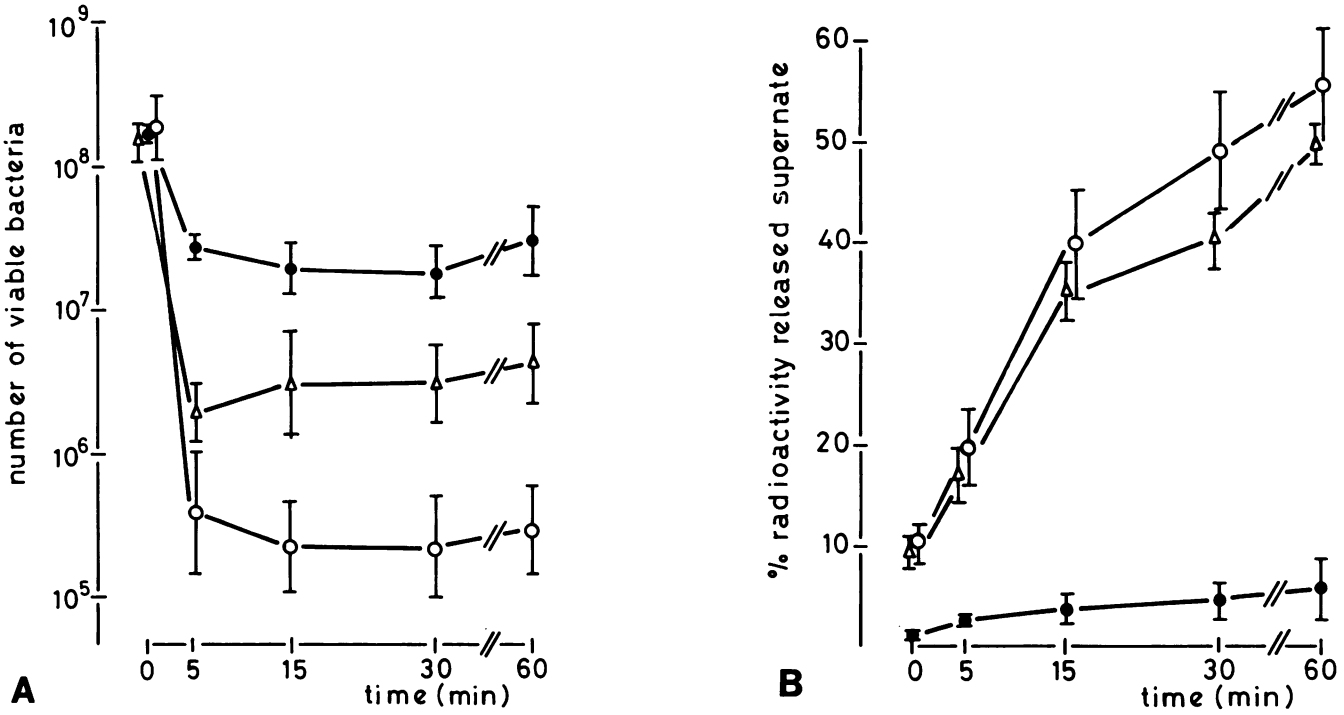


FIG. 1. A. Killing of unencapsulated *E. coli* PC2166 and O111 and encapsulated *E. coli* O78:K80 by PMN. Opsonized *E. coli* PC2166 (○), O111 (△) and O78:K80 (●) were incubated with PMN at 37°C for 5 min. The leukocytes were washed free of bacteria not associated with phagocytes. The PMN with associated bacteria were further incubated for 10, 30, and 60 min. After the indicated times, the PMN were disrupted, and the numbers of viable leukocyte-associated bacteria were determined by a pour-plate technique. Values represent the means plus or minus standard deviations based on at least three experiments. B. Release of radioactivity from [^{3}H]uridine-labeled *E. coli* PC2166, O111, and O78:K80. Opsonized *E. coli* PC2166 (○), O111 (△), and O78:K80 (●) were incubated with PMN at 37°C for 5 min. The leukocytes were washed free of bacteria not associated with leukocytes and were further incubated. After the indicated times, leukocytes were disrupted and centrifuged at 12,000 × *g* for 15 min. The pellets and supernatants were individually suspended in scintillation liquid, and the percentage of radioactivity released into the supernatant was calculated. Values represent the means plus or minus standard deviations based on at least three experiments.

Isolation of PMN. PMN were isolated by a method modified from that of Böyum (3), as previously described (17). Briefly, venous blood samples from healthy adult donors were drawn into heparinized syringes and settled by gravity in 6% dextran (molecular weight, 70,000; Pharmacia, Uppsala, Sweden) in normal saline (10 ml of blood, 3 ml of saline). The leukocyte-rich plasma was withdrawn and centrifuged at 160 × *g* for 10 min. The pellet was suspended in Eagle minimal medium; 16 ml of the cell suspension was carefully layered onto 3 ml of Ficoll-Paque (Pharmacia) and centrifuged at 160 × *g* for 35 min. Mononuclear cells of the interface were removed. Residual erythrocytes in the pellet were lysed with ice-cold NH_4Cl (0.87% in sterile water). After centrifugation at 160 × *g* for 15 min, PMN were washed twice in Gel-HBSS. Leukocytes were adjusted to a concentration of 10^7 PMN per ml of Gel-HBSS (the percentage of monocytes in this suspension was always less than 3%).

Bacterial uptake, killing, and degradation by PMN. Phagocytosis was assayed as previously described (17). Briefly, 0.3 ml of the suspension of opsonized bacteria (5×10^8 CFU/ml) was added to 0.3 ml of PMN (10^7 cells per ml) in each of six polypropylene vials (Biovials; Beckman Instruments, Inc., Fullerton, Calif.). The mixtures were incubated in a shaking water bath at 37°C. After 5 min, phagocytosis was stopped in all vials by adding 3 ml of ice-cold PBS to each of the vials. Vial 1 was used to determine the total added radioactivity. This vial was centrifuged at 1,600 × *g* for 15 min. The pellet representing phagocytized and nonphagocytized bacteria was suspended in 2.5 ml of scintillation liquid. Radioactivity was measured in a Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Vials 2 through 6 were centrifuged at 160 × *g* for 5 min, and the pellets were washed three times with ice-cold PBS to remove non-leukocyte-associated bacteria. The final leukocyte pellet of vial 2 was solubilized in 2.5 ml of scintillation liquid, and the leukocyte-associated radioactivity was measured. Phagocytosis was expressed as the percentage of uptake (after 5 min) of total added radioactivity. The pellets in the remaining vials (3 through 6) were suspended in 0.3 ml of Gel-HBSS and incubated at 37°C for 0, 10, 30, and 60 min, respectively. After the indicated time intervals, 0.7 ml of cold sterile, distilled water was added to the vials to disrupt the leukocytes. Samples (100 μl) from the suspensions were taken to determine the number of viable leukocyte-associated bacteria, with a standard pour-plate technique using brain heart infusion agar (Oxoid Ltd., Basingstoke, England). After 18 h of incubation at 37°C, the number of colonies was counted. The results are expressed below as the numbers of viable intracellular bacteria at the indicated time intervals. The remaining suspensions in vials 3 through 6 were centrifuged at 12,000 × *g* for 5 min. The pellets and supernatants were separated and suspended in scintillation liquid; the percentages of radioactivity released at the different time intervals were calculated and used as measures of the degradation of bacteria.

Electron microscopy. Briefly, 1 ml of the suspension of opsonized bacteria (5×10^8 CFU/ml) was added to 1 ml of the PMN suspension (1×10^7 cells per ml). Mixtures were incubated for 5 min at 37°C. Phagocytosis was stopped by adding ice-cold PBS. Vials were centrifuged at $160 \times g$ for 5 min, and pellets were washed three times with ice-cold PBS, suspended in 0.3 ml of Gel-HBSS, and incubated for 10, 30, and 60 min. The PMN-bacteria interactions were terminated by mixing the suspensions with equal volumes of 0.5% glutaraldehyde in PBS at 0°C. The mixtures were centrifuged at $600 \times g$ for 5 min. The supernatants were removed, and the pellets were suspended in 0.75 ml of human plasma (0°C) and centrifuged again at $600 \times g$ for 5 min. These supernatants were removed, and the pellets were overlaid with additional glutaraldehyde buffer fixative (3% glutaraldehyde, 2% formaldehyde, 0.1% acroleine in 0.1 M phosphate buffer [pH 7.0] at 0°C) for 60 min. The pellets were then washed twice with 0.1 M phosphate buffer at 0°C, postfixed in 1% osmium tetraoxide in phosphate buffer for 60 min, and embedded in Epon. Ultrathin sections were stained with 4% uranyl acetate and lead citrate and were examined in a Carl Zeiss 109 electron microscope (Carl Zeiss, Oberkochen/Württemberg, Federal Republic of Germany).

RESULTS

Uptake, killing and lysis of *E. coli* PC2166, O111 and O78:K80 after phagocytosis by PMN. *E. coli* PC2166 was readily opsonized in 2.5% normal serum, and within 5 min, 70 to 80% of the bacteria was taken up by PMN. *E. coli* O111 and O78:K80 were only adequately opsonized in 10 and 20% antiserum, respectively, and uptake by PMN was 56 and 50%, respectively. To determine the killing of bacteria by PMN, PMN were incubated for 5 min with opsonized bacteria, and the phagocytes were then washed free of nonassociated bacteria and further incubated. The number of viable bacteria within phagocytes was measured at indicated time points (Fig. 1A). The rates of killing of *E. coli* PC2166 and O111 were rapid. After 5 min of phagocytosis, there was a reduction in viable bacteria from the initial number (number of intracellular viable bacteria after 5 min of incubation with PMN) of $2.83 \times 10^8 \pm 2.02 \times 10^8$ CFU/ml to $6 \times 10^5 \pm 3.6 \times 10^5$ CFU/ml for *E. coli* PC2166 and from $1.66 \times 10^8 \pm 1.25 \times 10^8$ CFU/ml to $3 \times 10^6 \pm 2 \times 10^6$ CFU/ml for *E. coli* O111 (more than 99.9% of these stains were killed after 5 min of phagocytosis). *E. coli* O78:K80 was killed less readily. After 5 min of phagocytosis, there was a decrease in viable bacteria from $2.16 \times 10^8 \pm 0.57 \times 10^8$ CFU/ml to $4.33 \times 10^7 \pm 0.57 \times 10^7$ CFU/ml.

To measure the degradation of bacteria within the phagocytes, [^{3}H]uridine-labeled bacteria and PMN were incubated for 5 min, washed free of nonassociated bacteria, and further incubated for 10, 30, and 60 min. After each of these time points, PMN were lysed in distilled water. After phagocytosis of *E.coli* PC2166 and O111 cells, 39.9% ± 5.26% and 35% ± 2.64% of the radioactivity, respectively, was released into the medium after 15 min, and 56% ± 5.52% and 49.3% ± 2.08% was released into the medium after 60 min incubation. After centrifugation of disrupted PMN with phagocytized, encapsulated *E. coli* O78:K80, there was virtually no release of radioactivity into the medium; more than 90% of radioactivity was found in the pellet (Fig. 1B) after 60 min of phagocytosis. When the strains were opsonized in the serum concentrations used and not added to the PMN, no decrease in viability and no release of radioactivity were measured. This indicates that these low serum concentrations were not bactericidal by themselves.

Electron microscopic study of degradation of bacteria. After 15 min of phagocytosis, almost all bacteria were found

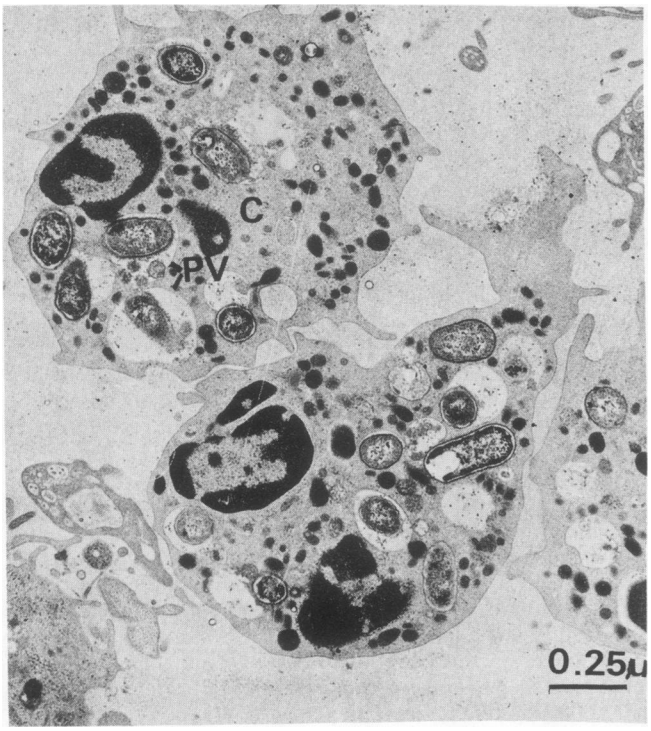


FIG. 2. Transmission electron micrograph of human PMN after 15 min of incubation with unencapsulated *E. coli*. The cytoplasm (C) contained many phagocytic vacuoles (PV) filled with bacteria appearing in different stages of structural change.

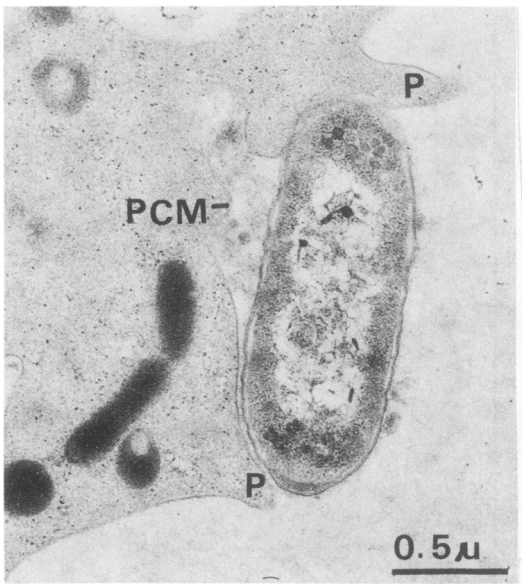


FIG. 3. Attachment and ingestion of bacterium by PMN. The phagocyte cellular membrane (PCM) has been indented, and phagocyte pseudopods (P) extend beyond the bacterium.

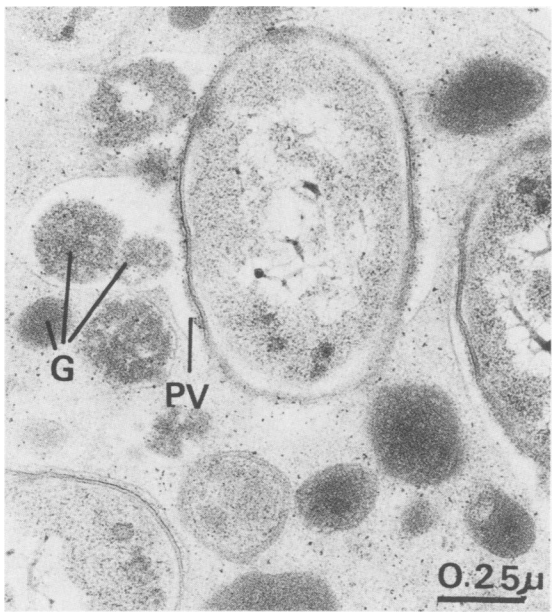


FIG. 4. Engulfment of bacteria and degranulation. Phagocytic vacuoles (PV) fuse with granules (G) forming phagosomes. Shown are a phagocytic vacuole (PV) with phagocytized bacterium and the contents of phagocytic cytoplasmic granules (G) discharged during the fusion process.

intracellularly. Only a few extracellular bacteria were observed. This indicates that radioactivity associated with PMN could be attributed primarily to intracellular bacteria. Examinations of thin sections from samples taken at various intervals during phagocytosis allowed us to study different stages of the phagocytic event. After 15 min of incubation of PMN with *E. coli* PC2166 or O111, PMN were full of bacteria in different stages of destruction (Fig. 2) within phagocytic vacuoles. In a few sections very early stages of phagocytosis, i.e., attachment and engulfment (Fig. 3), could be observed. The attachment of bacteria to the surface of PMN (Fig. 3) resulted in an indentation of the PMN cellular membrane and an extension of PMN pseudopods beyond the bacterium. After the engulfing of bacteria, PMN granules fused with phagocytic vacuoles, formed phagosomes, and released their content into the phagosomes (Fig. 4). Signs of disintegration of the bacterial cells were visible in the cytoplasm of the bacteria (Fig. 5). The cytoplasm appeared to agglomerate and retract from the cytoplasmic membrane. This apparent retraction produced an electron-transparent zone along the inside of the bacterial membrane. Changes in the cytoplasm occurred in the absence of visible damage of either the cell wall or the cytoplasmic membrane. In the next stage, the bacterial cytoplasm became diffuse and electron transparent. The bacterial cell wall lost its rigidity, and its integrity was disrupted, but this event was not necessarily related to simultaneous disruption of the cytoplasmic membrane (Fig. 6A). The disintegration of the cytoplasmic membrane occurred later and led to the formation of membranous vesicles containing bacterial remnants (Fig. 6B). At the end of the phagocytic process, bacterial cells were completely lysed, and only a few bacterial remnants remained visible (Fig. 6C). Interestingly, during phagocytosis different stages of bacterial destruction were found not only within one phagocyte but also within one phagocytic vacuole (Fig. 7). Some of the bacteria seemed to be almost intact, and at the same time other bacteria were completely lysed, and only bacterial debris was found.

E. coli O78:K80 was much more resistant to the degradation process. After 15 min of incubation of adequately opsonized encapsulated bacteria with PMN, bacteria were found intracellularly. Examination of thin sections from the samples taken after 15 min of incubation with PMN showed internalized bacteria surrounded by a thick capsule completely filling the phagocytic vacuoles (Fig. 8). No visible changes were observed. Only after 60 min of phagocytosis did a few of the encapsulated bacteria show some structural changes in the cytoplasm and electron-transparent zones along cytoplasmic membranes (Fig. 9). In some sections, along with intact encapsulated bacteria, a few lysed bacteria without capsular material were observed within the vacuole. When, after 15 min, 50 PMN with about 300 to 500 intracellular *E. coli* PC2166 or O111 cells were evaluated, over 95% of the phagocytized bacteria showed morphologic changes (loss of the integrity of the cell wall and cytoplasm membrane and changes in the cytoplasm). However, when the same number of PMN with intracellular *E. coli* O78:K80 was evaluated after 60 min, only 38% of the phagocytized bacteria showed some changes.

DISCUSSION

This study confirmed previously published data that *E. coli* strains without K antigens are readily phagocytized when opsonized in normal serum (12, 19). However, some strains (e.g., *E. coli* O111) which do not posses capsular antigens resist phagocytosis after opsonization in nonimmune serum, probably due to the presence of specific sugars in the O antigenic side chains in the lipopolysaccharide (13).

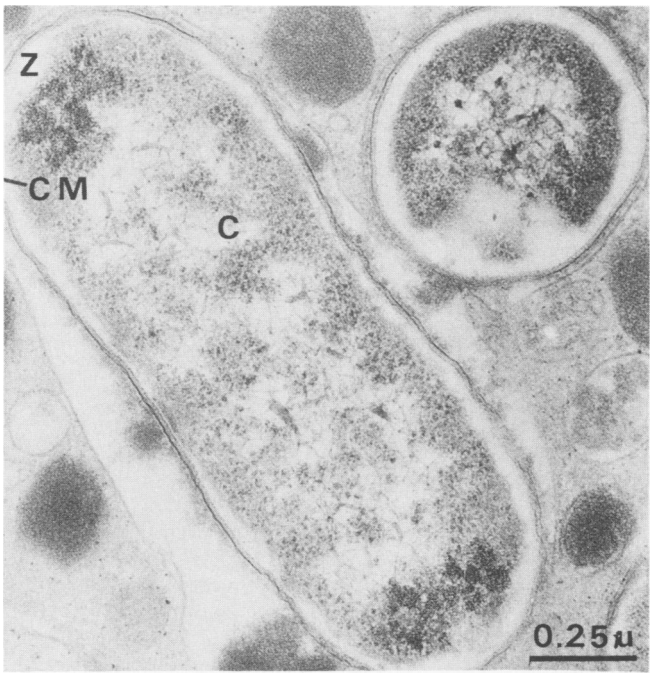
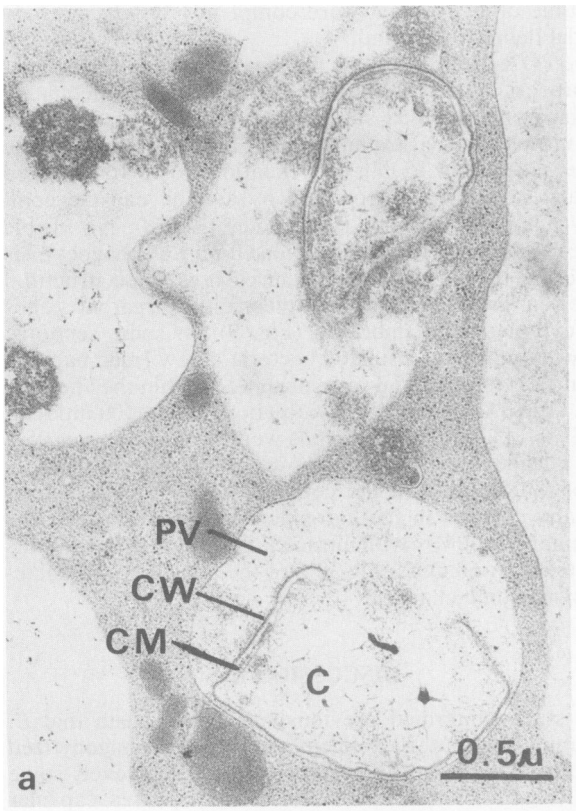
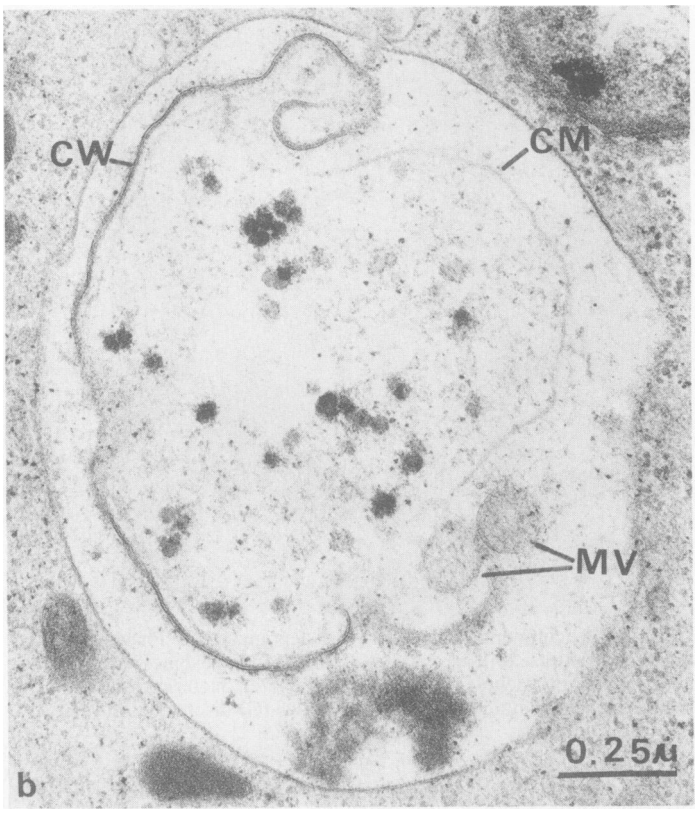


FIG. 5. Early changes in unencapsulated *E. coli*. The cytoplasm (C) appears agglomerated and retracted leaving an electron-transparent zone (Z) along the inside of the bacterial cytoplasmic membrane (CM).





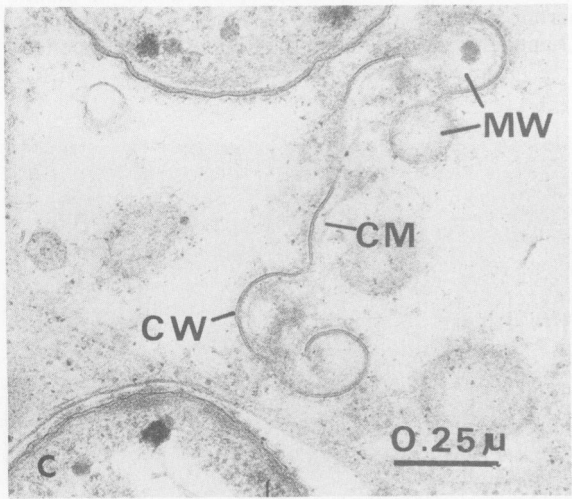


FIG. 6. Late stages of phagocytosis of unencapsulated *E. coli*. (a) Loss of the cell wall rigidity in phagocytized *E. coli* led to the change in bacterial shape. Cytoplasm (C) appeared diffused. In some bacteria, disruption of the cell wall (CW) was visible. (b) Ingested bacterium with the disintegrated cytoplasmic membrane (CM) and the appearance of a membranous vesicle (MV). (c) Presence of the bacterial remnants after complete lysis of bacterial cells.

Such strains require specific antibodies for effective opsonization. Nonimmune serum is also a poor opsonin source for *E. coli* strains with a K antigen. This may be due to inadequate complement consumption by K antigen-positive *E. coli* strains in nonimmune serum (9). Therefore, no C3b is generated, and no opsonization takes place. However, in the presence of antiserum, efficient opsonization and phagocytosis can occur (9, 10). Our study showed that intracellular killing of ingested *E. coli* O78:K80 was less effective than that of *E. coli* O111 and K-12. This suggests that the K antigen rendered the organisms less susceptible to the complex antibacterial activity of the PMN. It is possible that strain O78:K80 is less susceptible to toxic oxygen metabolites or to killing by the oxygen-independent system (such as granule proteins with permeability-increasing and phospholipase A2 activities [5, 18]). Further studies are needed to determine which bactericidal factor of the PMN all of these

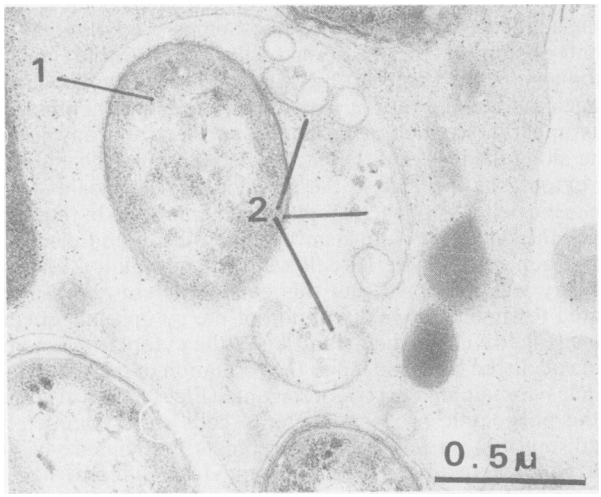


FIG. 7. Different stages of destruction of bacteria present in a single phagosome. 1, Unchanged unencapsulated *E. coli*; 2, completely lysed strains with the presence of bacterial debris.

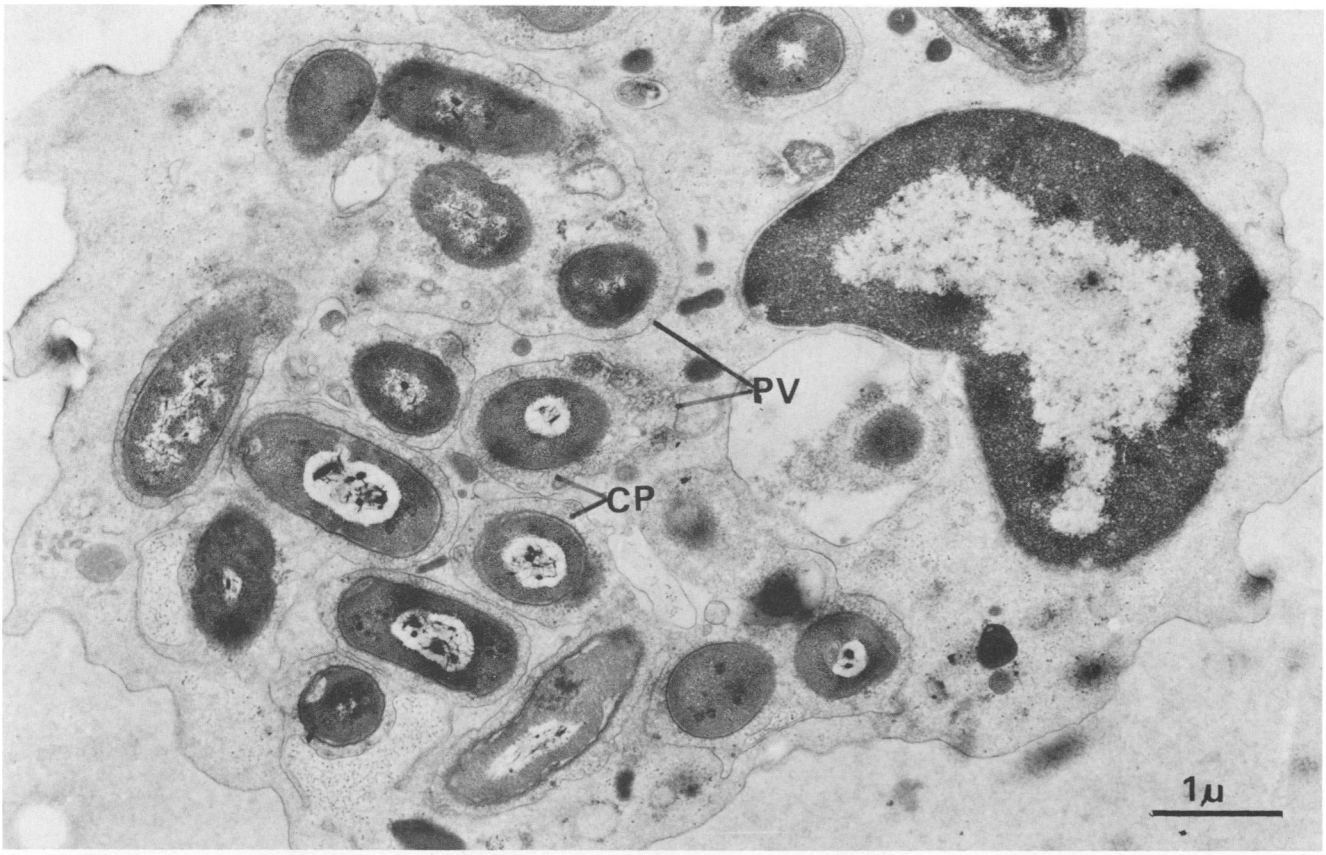


FIG. 8. Encapsulated *E. coli* after 15 min of phagocytosis by PMN. Cytoplasm contained phagocytic vacuoles (PV) filled with bacteria surrounded by thick capsular material (CP).

strains can resist. Strain O78:K80 resisted not only opsonization in normal serum and killing within the PMN but also degradation after ingestion, which did not take place as quickly as it occurred with *E. coli* PC2166 and O111. The degradation of bacteria was measured in two different ways. First, the release of radioactive material from the ingested, radiolabeled bacteria was measured. Of the total radioactivity of the ingested unencapsulated bacteria (*E. coli* PC2166 and O111), 50 to 60% was released within 1 h. Because [^{3}H]uridine labeled only bacterial RNA, either bacterial RNA was broken down to small nucleotides that leaked out of the dead bacteria or bacterial [^{3}H]uridine was released from the intracellular uridine pool. In contrast, no release of radioactive material from the K antigen-positive strain was observed. This is in agreement with our previous finding that a substantial breakdown of bacterial DNA by DNase and a subsequent release of [^{3}H]thymidine are only observed when strains without K antigens are ingested by mononuclear leukocytes (15). Observations about the digestion of *E. coli* were confirmed by electron microscopy. We showed that unencapsulated *E. coli* strains were rapidly internalized and that the bacteria were extensively broken down. Extensive changes were found in most of the phagocytized bacteria. Very often only the debris of bacterial cells was seen within the vacuoles. Interestingly, bacteria in all stages of degradation were observed not only within one PMN but also within one phagocytic vacuole. There could be several explanations for this observation. It is possible that (i) within one bacterial culture some bacterial cells were more resistant to the enzymatic attack by leukocytes than others, (ii) the degradation was more or less a random phenomenon, (iii) some bacteria were ingested earlier than others, (iv) more lysosomes fused with some phagosomes than with other phagosomes, or (v) newly formed phagocytic vacuoles fused with preexisting phagosomes. In contrast to the rapid and extensive breakdown of the unencapsulated *E. coli* strains, virtually no structural degradation of *E. coli* O78:K80 was observed. The capsular material filled almost the whole vacuole. It is likely that the resistance of these bacteria to degradation was due to the presence of the K antigen, although we cannot exclude the possibility that the O type of antigen or the antiserum used contributed to this resistance. Even after 1 h of incubation, many intact encapsulated bacteria were seen, although at the same time some degraded bacteria were observed in the same PMN. Because no capsular material was observed in the vacuoles with degraded bacteria, it is possible that within a population of bacteria with a K antigen, some cells without a capsule could be found and that these bacteria would be more rapidly destroyed.

In conclusion, *E. coli* O78:K80 resisted opsonization in normal serum. Opsonization and subsequent phagocytosis occurred only in the presence of antiserum. This strain was more resistant to killing than unencapsulated strains O111 and PC2166 and resisted degradation by PMN. These differences are likely to have been due to the presence of the K antigens. However, more studies with a variety of strains, especially isogenic ones that differ only in the presence or absence of K antigens, are needed to confirm these findings. Why K-antigen-positive strains of *E. coli* are more resistant

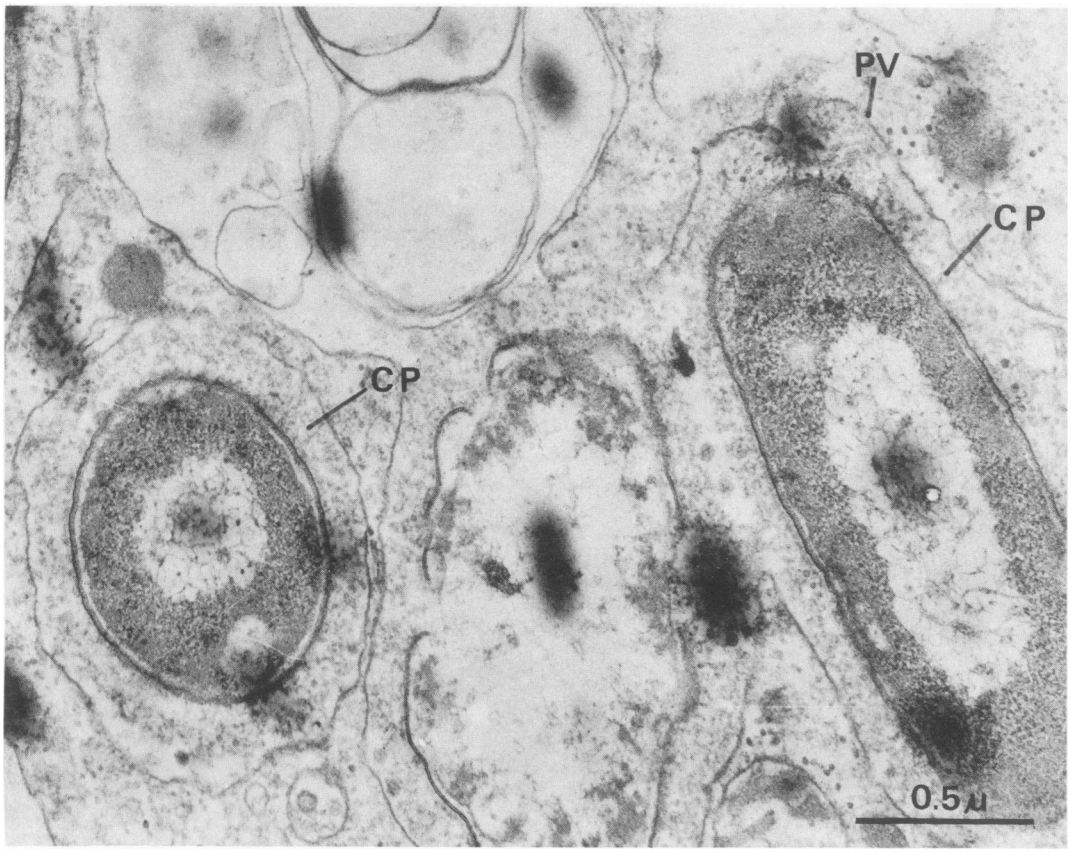


FIG. 9. Encapsulated *E. coli* after 60 min of phagocytosis by PMN. Intact *E. coli* surrounded by bacterial capsular material (CP) still were present in phagocytic vacuoles (PV).

to the bactericidal mechanisms of PMN, a question of great interest for the understanding of the virulence of the strains, is currently under investigation.

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