

# Electron Microscopy of *Listeria monocytogenes*-Infected Mouse Spleen

B. A. ARMSTRONG AND C. P. SWORD

*Department of Microbiology, The University of Kansas, Lawrence, Kansas*

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## ABSTRACT

ARMSTRONG, B. A. (The University of Kansas, Lawrence), AND C. P. SWORD. Electron microscopy of *Listeria monocytogenes*-infected mouse spleen. *J. Bacteriol.* 91:1346-1355. 1966.—Mouse spleen infected with *Listeria monocytogenes* was observed during the acute phase of infection; 72 hr after infection, organisms were usually found within phagocytic vacuoles in the cytoplasm of macrophages. These vacuoles, which resembled phagosomes, often contained several organisms as well as varying amounts of amorphous electron-dense material, ferritin-like particles, membrane fragments, and vesicles of varying density. Breakdown of vacuolar membranes appeared to be accompanied by damage to the host cell cytoplasm. Nuclear membrane damage was occasionally observed when phagocytic vacuoles were close to the nucleus.

Pathologic alterations in listeriosis have been reported by numerous investigators, as reviewed by Seeliger (22). In mouse spleen, during the acute phase of infection, the lesion consists of an area of nuclear debris and neutrophils containing pyknotic nuclei surrounded by a peripheral zone of apparently healthy macrophages. *Listeria monocytogenes* was found within and in association with the mononuclear cells at the periphery of the granuloma (13). The reaction of splenic tissue explants in culture to *L. monocytogenes* was reported by Smith et al. (23). Proliferation of vascular sinus endothelium, resulting in a cell mass with the characteristics of a granuloma, was seen. After at least 4 days, the cell mass was surrounded by large macrophages.

Intracellular multiplication of *L. monocytogenes* within mononuclear exudate cells in vitro was reported (1, 13, 16). Correlation between the intracellular increase of organisms and subsequent cytotoxic effects was observed.

Ultrastructural changes in tissues infected with obligate and facultative intracellular parasites have been reported by several investigators. North and Mackaness (17) employed an electron microscope to study the early cytoplasmic response of mouse peritoneal cells infected with *L. monocytogenes*. Their evidence suggests that material from cytoplasmic granules accumulates in phagocytic vacuoles; however, the short duration of the experiment did not allow them to determine the fate of the organisms.

The purpose of our study was to observe mouse

splenic tissue infected with *L. monocytogenes* and to relate these observations to the mechanisms of pathogenesis of this organism.

## MATERIALS AND METHODS

A virulent human isolate of *L. monocytogenes* A4413, serotype 4b (obtained from the U.S. Army Biological Laboratories, Fort Detrick), was cultured in tryptose broth at 37 C. Female Swiss Webster mice weighing 16 to 18 g were injected intraperitoneally with 0.2 ml of an 18-hr culture washed once and diluted in 1% tryptose. Tissues from moribund mice which had received inocula ranging from  $10^8$  through  $10^5$  organisms, depending on the experiment, were examined. At least  $10^7$  bacteria per gram of tissue were necessary for detection of bacteria in tissue sections. Bacterial numbers during early infection and convalescence were too low for consistent detection of organisms. Therefore, these observations were based only on the acute stage of infection. The results are representative of observations made on many tissue specimens. Spleens were removed and cut into 0.5 mm<sup>3</sup> fragments under cold 1% buffered osmium tetroxide fixative. Tissue fragments were fixed at 4 C for 4 hr, followed by dehydration and embedding in Epon 812 (Shell Chemical Co., San Francisco, Calif.) according to the method of Luft (12). Thin sections were cut with a Porter-Blum MT-1 ultramicrotome with glass knives and picked up on uncoated 400-mesh copper grids. Sections were examined with an RCA EMU-3F2 electron microscope at 50 kv.

## RESULTS

Examination of splenic tissue from mice infected with *L. monocytogenes* revealed or-

ganisms usually within the cytoplasm of macrophages and frequently surrounded by a limiting membrane (Fig. 1). The limiting membrane appeared to be identical with the host cell membrane, and may have been derived from it during phagocytosis. Several organisms were often enclosed within the same limiting membrane, or phagocytic vacuole. The area within the phagocytic vacuole was sometimes electron-transparent, but frequently contained varying amounts of amorphous electron-dense material, membrane fragments, and well-defined vesicles of varying electron density (Fig. 1-6). Electron-dense particles of the ferritin type (19) were also present in some of the phagocytic vacuoles and could be seen in localized areas of the host cell cytoplasm (Fig. 5 and 6).

Cytoplasm surrounding intact phagocytic vacuoles (Fig. 5) did not appear to have suffered any obvious ultrastructural damage. However, in instances where the vacuolar membrane did not appear intact, dissolution of the host cell cytoplasm and vacuolar contents adjacent to the organisms was noted, as evidenced by a clear, or electron-transparent, area (Fig. 2 and 4). Where the phagocytic membranes had not broken down completely, the clear areas were seen only within the phagocytic vacuoles and adjacent to the organisms (Fig. 5 and 6). No limiting membrane could be resolved around the electron-dense material surrounding the dividing organism in Fig. 3, but electron-transparent areas may be seen adjacent to the organism and in the host cell cytoplasm.

Phagocytic vacuoles were seen throughout the host cell cytoplasm and often adjacent to the cell nucleus. Nuclear membrane damage was sometimes observed in the latter instances (Fig. 1 and 4). In Fig. 1, dissolution of the host nuclear membrane and nucleoplasm is seen in an area immediately adjacent to the vacuole-like structure. Evidence of partial damage to the vacuolar membrane is also present in that area. In the same cell, however, another membrane-limited organism is seen in close proximity to the nucleus, with no accompanying damage to either the nuclear membrane or vacuolar membrane. Nuclear membrane damage and dissolution of the nucleoplasm may be observed also in Fig. 4 adjacent to the ends of the dividing organism.

#### DISCUSSION

Our results suggest the following events at the ultrastructural level in listeriosis of mice: (i) organisms enter the cells as a result of phagocytosis; (ii) the phagocytic vacuoles containing organisms assume the appearance of "phagosomes"; (iii) the "phagosome" appears to break down with subsequent release of its contents into

the cytoplasm; and (iv) host cell damage is manifested through less electron-dense zones surrounding the organisms, suggesting cytoplasmic damage, and through loss of integrity of the host nuclear membrane.

Similar observations have been made by other investigators with various intracellular parasites. Electron microscopy of tissues infected with *Mycobacterium lepraemurium* and *M. tuberculosis* revealed, in both cases, a membrane surrounding the intracellular organisms (2, 14). Bacilli enclosed in membrane-bound vesicles within host cytoplasm were also observed in normal guinea pig monocytes infected with *Brucella suis* (18) and *B. abortus* SA-S (10). In each case, these membranes were presumably of host origin, having been taken into the cytoplasm during phagocytosis. Roth et al. (20) and Roth and Williams (21) found no membranes separating virulent encapsulated *Bacillus anthracis* from the cytoplasm of infected mouse splenic cells but did observe them in tissue containing rough, avirulent *B. anthracis*. The absence of a limiting membrane around *M. leprae* proliferating rapidly in lepra cells has also been noted, but membranes were observed surrounding the bacilli in borderline leprosy lesions (8). Using an electron microscope, North and Mackaness (17) observed *L. monocytogenes* in phagocytic vacuoles in infected mouse peritoneal cells in vitro.

Our results showed the majority of intracellular *Listeria* organisms in infected mouse splenic tissue to be present in membrane-limited structures similar to those reported for other organisms and highly suggestive of phagocytic vacuoles. The vacuolar membranes appeared to be identical with host cell membranes.

Merging of cytoplasmic granules, presumably lysosomes, with phagocytic vacuoles has been suggested by cinemicrophotographic and electron photomicrographic observations (5, 11, 26). North and Mackaness (17) observed similar phenomena in the phagocytosis of *L. monocytogenes* by mouse peritoneal macrophages in vitro. In their study, amorphous material resembling that seen in cytoplasmic granules accumulated in the phagocytic vacuoles. The appearance of the structure enclosing the organisms (Fig. 5 and 6) suggests that this structure is a "phagosome" similar to that described by Weissmann (24) and resulting from the union of lysosomes with phagocytic vacuoles that have engulfed foreign material. It would seem, therefore, that phagosomes are formed after phagocytosis of *Listeria*, perhaps with a "digestive" function for ridding the cell of foreign material.

Finely divided electron-dense particles, resembling ferritin, have been observed both in the host cell cytoplasm and within "phagosomes"



FIG. 1. Electron micrograph of *Listeria monocytogenes* in mouse splenic macrophage. Shown are phagocytic vacuole (PV), vacuolar membrane (VM), nucleoplasm (N), nuclear membrane (NM), cell membrane (CM), mitochondrion (M), and *L. monocytogenes* cell (L).  $\times 25,500$ . Insert shows damage to host cell nuclear membrane  $\times 50,500$ .

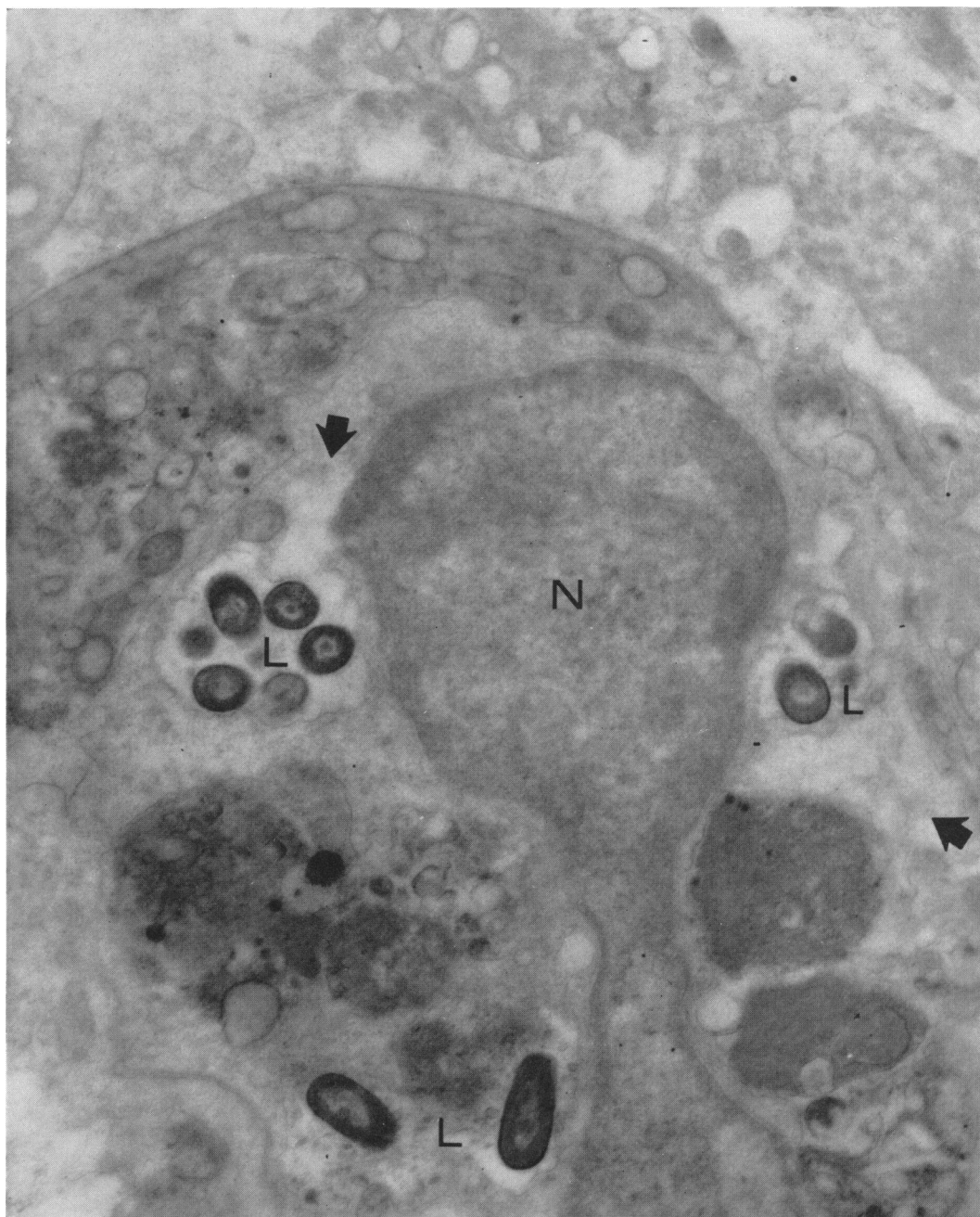


FIG. 2. *Listeria monocytogenes* in mouse splenic macrophage. Arrow indicates cytoplasmic damage.  $\times 27,600$ .

in infected cells. Recent work has indicated that iron may stimulate *in vivo* growth of *L. monocytogenes*. Ferritin within the "phagosome" and the host cell cytoplasm (Fig. 5 and 6) could serve as a source of iron for the organisms, thus enhancing their multiplication. Intracellular

multiplication of *L. monocytogenes* in mononuclear cells *in vitro*, with subsequent destruction of the host cells, has been reported (1, 13, 16). Thus, it may be possible for *L. monocytogenes*, under the stimulation of iron released from ferritin, to multiply rapidly enough within phago-

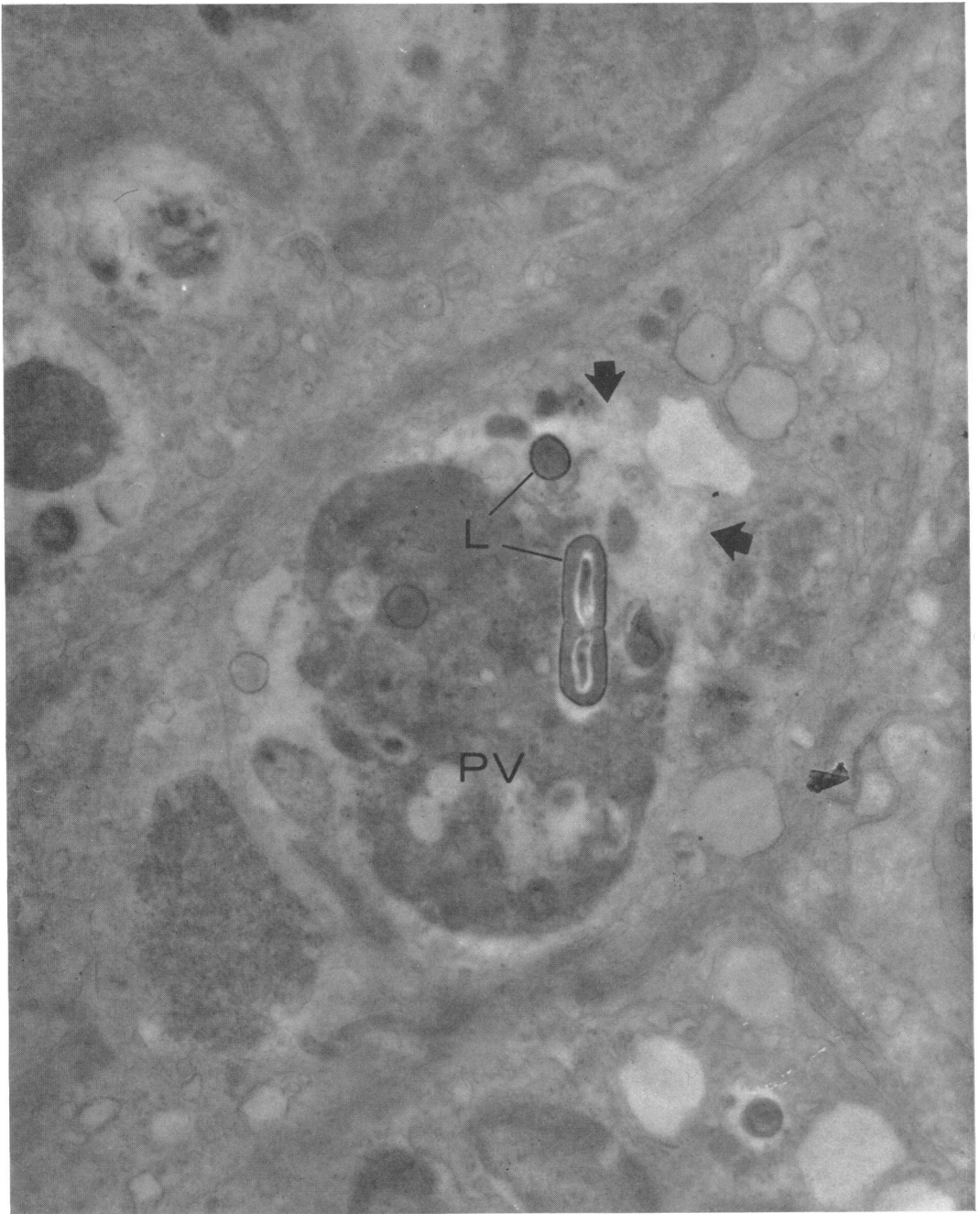


FIG. 3. *Listeria monocytogenes* in phagocytic vacuole of mouse splenic macrophage. Arrow indicates cytoplasmic damage.  $\times 21,100$ .

cytic vacuoles, or "phagosomes," to break through the vacuolar membranes mechanically and to escape the hydrolytic action of the lysosomal enzymes (Fig. 3 and 4).

Another possible explanation for the breakdown of the vacuole membrane may be concerned with hemolysin production by *Listeria*. Girard et al. (4), Njoku-Obi et al. (15), and Jenkins et



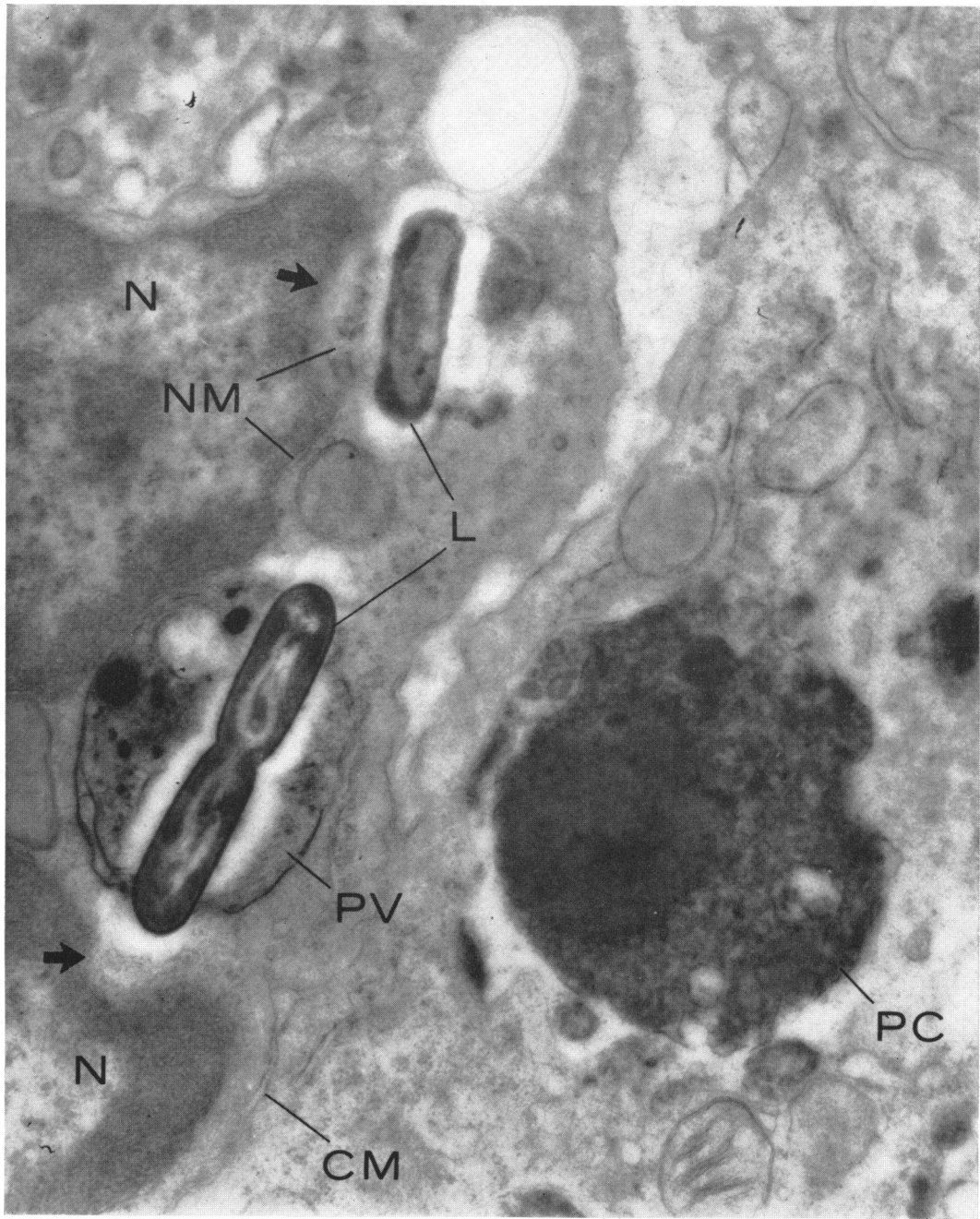


FIG. 4. *Listeria monocytogenes* in mouse splenic macrophage, showing cytoplasmic and nuclear membrane damage. Phagocytized pyknotic cell (PC) within adjacent macrophage.  $\times 33,900$ .

al. (9) reported that the soluble hemolysin behaved in many respects like oxygen-labile streptolysin O, which has been shown to disrupt lysosomes of leukocytes in vitro and in vivo (6,

25). *Listeria* hemolysin was reported by Jenkins et al. (9) to have a lecithinase activity, as indicated by its production of opalescence in serum and lecithovitellin. *L. monocytogenes*, when

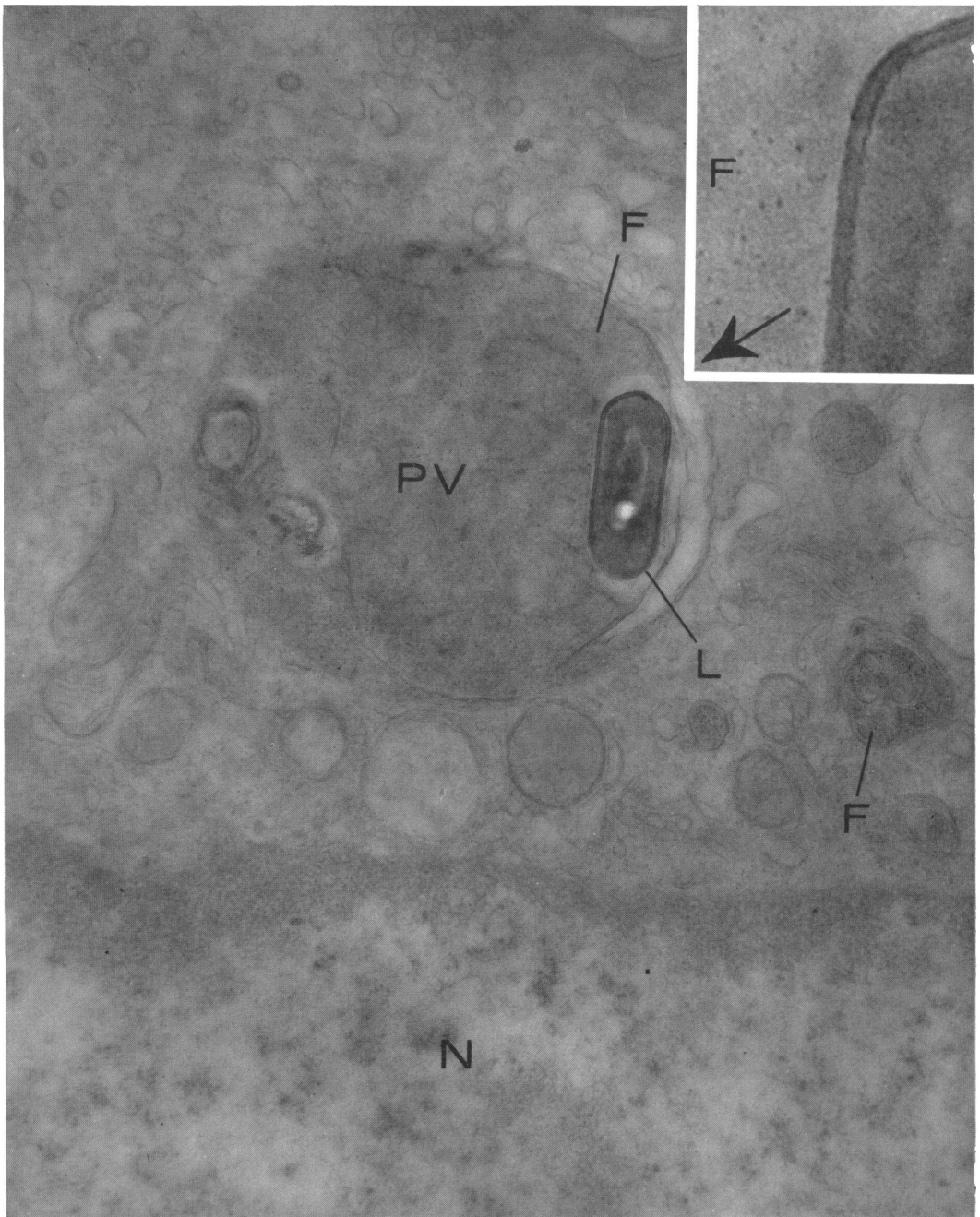


FIG. 5. *Listeria monocytogenes* within phagosome of macrophage.  $\times 30,300$ . Insert shows ferritin-like particles (F) adjacent to *Listeria* cell.  $\times 121,500$ .

grown on egg-yolk medium, caused an opacity in the medium surrounding the colony, an activity often associated with phospholipase activity in other bacterial species (3). Thus, the organism,

or its products, may be capable of acting on the lipoprotein membrane surrounding the lysosomes.

It may be noted that some *Listeria* strains of decreased virulence have been shown to produce

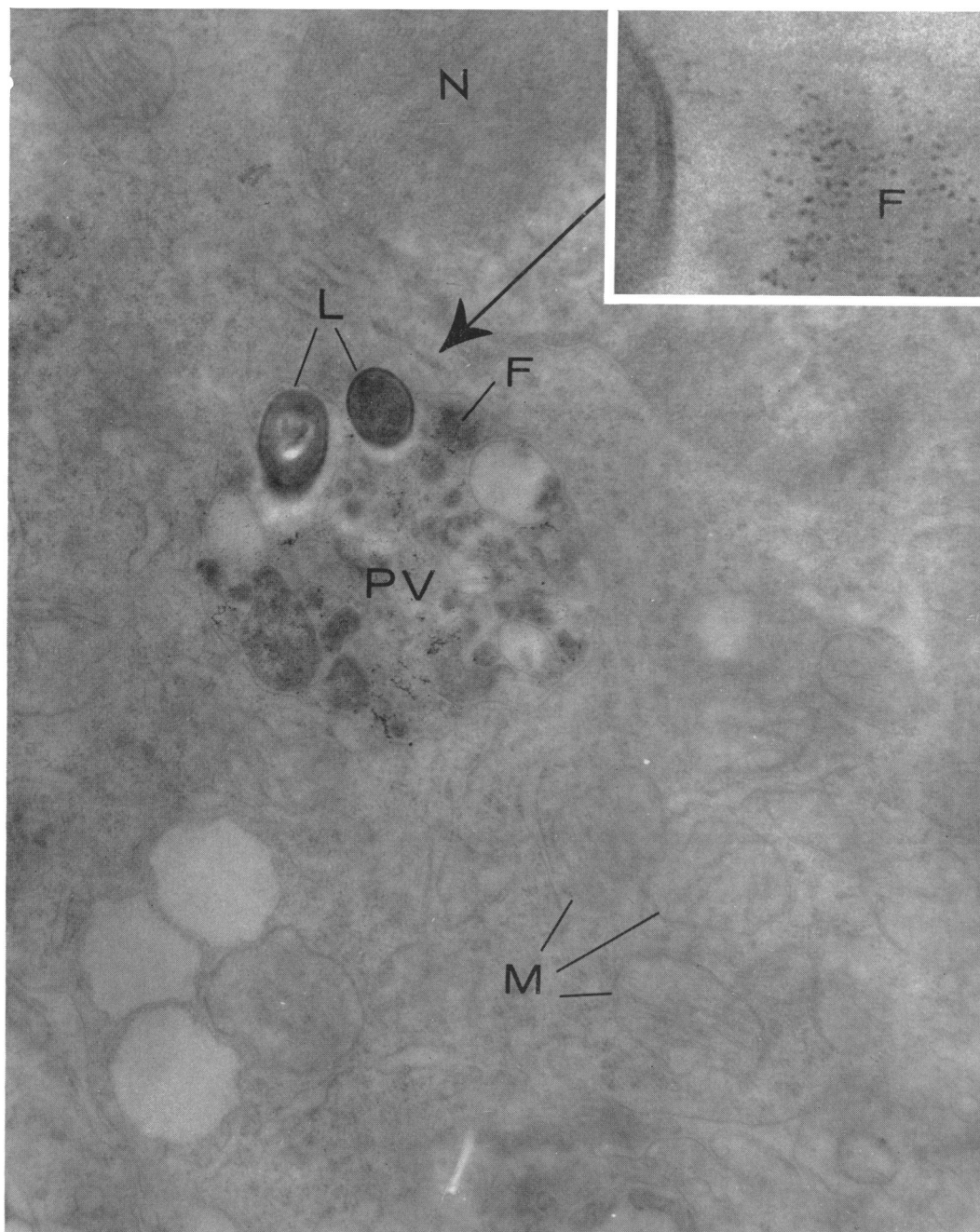


FIG. 6. *Listeria monocytogenes* within phagosome of macrophage, showing amorphous material, vesicles, and ferritin-like particles.  $\times 27,400$ . Insert shows ferritin-like particles adjacent to bacterial cell.  $\times 136,500$ .

high titers of hemolysin (4, 15); however, these strains do not appear capable of optimal growth in tissue, and are possibly eliminated before a damaging level of hemolysin is produced. Thus,

the possibility still exists that *Listeria* hemolysin acts as an accessory factor to the mechanical disruption of the "phagosome" membrane. Immunodiffusion studies have recently suggested



that hemolysin is produced in vivo by *L. monocytogenes* (A. S. Armstrong, unpublished data).

The hydrolytic enzymes in lysosomes are inactive as long as the enzymes remain within lysosomes. Injury to the lysosomes may release the enzymes into the cytoplasm, with subsequent autodigestion of the cell (7, 24). Studying the action of streptolysins on leukocytes, Hirsch et al. (6) observed by phase cinemicrophotography that rupture of the cytoplasmic granules, presumably lysosomes, into the cell sap was followed by liquefaction of the cytoplasm and fusion of the nuclear lobes. Weissmann et al. (25) had previously reported release of acid hydrolases from streptolysin-treated granule fractions from rabbit liver. The existence of some similarities between streptolysin O and *Listeria* hemolysin has been mentioned previously in this paper. In observing interactions of sheep peritoneal exudate cells with *L. monocytogenes* in vitro, Njoku-Obi and Osebold (16) noted a loss of refractile bodies "as the exudate cells lysed from cytotoxic changes." Proliferating bacilli were seen in the resulting cellular debris. In our studies, evidence of damage to the host cytoplasm was seen where the "phagosome" membranes lost their integrity (Fig. 2, 3, and 4). Damage to the nuclear membrane was also occasionally observed (Fig. 1). Presumably, injury to the "phagosome" membrane would result in release of the enzymes contained therein into surrounding host cytoplasm. Electron photomicrographs have frequently revealed intact *Listeria* associated with cellular debris, and occasionally the organisms were seen within the cytoplasmic area of pyknotic cells.

#### ACKNOWLEDGMENTS

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