ELECTRONMICROSCOPICAL OBSERVATIONS ON THE PERITON-EAL MACROPHAGES OF NORMAL MICE AND MICE IMMUN-ISED WITH *LISTERIA MONOCYTOGENES**

I. STRUCTURE OF NORMAL MACROPHAGES AND THE EARLY CYTOPLASMIC RESPONSE TO THE PRESENCE OF INGESTED BACTERIA

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EXPERIMENTAL evidence has been put forward supporting the view that the increased resistance of animals following infections with certain bacteria is due to an acquired capacity of certain phagocytic cells to destroy micro-organisms (Lurie, 1942 ; Suter, 1953 ; Fong, Schneider and Elberg, 1957).

Recent work carried out by Mackaness (1962) has shown that in mice, acquired resistance to reinfection with *Listeria monocytogenes* is associated with a change in the properties of the peritoneal macrophages. Macrophages taken from resistant animals and kept *in vitro* kill the bacteria with astonishing efficiency when compared with similar cells from normal mice which, although they phagocytose the organism permit its rapid intracellular multiplication. Serum antibodies appear to play no part in the acquired resistance to Listeria.

The present study was undertaken to determine whether the differences in antibacterial properties between macrophages from normal and immune animals could be correlated with differences in structural features detectable by electron-microscopy.

This paper deals with the ultrastructure of macrophages from normal (non-immunised) mice prior to, and after the ingestion of Listeria.

In the paper which follows the ultrastructure is described of macrophages from mice immunised with this bacterium.

MATERIAL AND METHODS

Six-week-old female mice of an out-bred strain (Walter and Eliza Hall Institute, Melbourne) were used as a source of peritoneal macrophages. The method for obtaining virulent cultures of Listeria has been previously described (Mackaness, 1962). The mice were injected intraperitoneally with 10^7 virulent bacteria suspended in Hanks' balanced salt solution and the bacteria were allowed to remain in the peritoneal cavity for 10 min. The peritoneal cavity was then washed out with 2 ml. of Hanks' balanced salt solution and the washings gently centrifuged to form a pellet. This procedure took approximately 15 min. An examination of a peritoneal smear revealed that about 40 per cent of the macrophages contained ingested bacteria, and that a large number of bacteria were still free within the peritoneal fluid. The pellet was gently teased away from the centrifuge tube and placed in 1 per cent

osmium tetroxide (cold) dissolved in a balanced salt solution (Zetterquist, unpublished) and buffered at pH 7·6 according to Palade (1952). Fixation was allowed to proceed for 2 hr., after which the pellet was placed in a 1 per cent solution of uranyl acetate for 1 hr. (Ryter and Kellenberger, 1958). It was then briefly washed in distilled water and dehydrated in 70 per cent and absolute ethanol respectively. Araldite was used as an embedding medium and silver sections were cut on a Porter-Blum ultramicrotome with a diamond knife and collected onto a carbon film supported by copper specimen grids. Electron microscopy was performed with a Siemens' Elmiskop I electron microscope using a 50 μ objective aperture. The peritoneal macrophages from 4 mice were examined and many blocks were sectioned.

RESULTS

The plasma membrane

The peritoneal macrophage possesses a well defined plasma membrane about 80 Å thick. The membrane is 3 layered and is similar to the unit membrane described by Robertson (1959).

The plasma membrane displays many surface projections or microvilli. These are 0·1–0·2 μ in diameter (Figs. 1 and 2) and radiate in many directions from the general cell surface.

Invaginations of the plasma membrane are also common and these extend appreciable distances into the cytoplasm. They are more often seen sectioned transversely and may be confused with cytoplasmic vacuoles (Figs. 1, 5 and 7).

The small outpushings and inpushings of the plasma membrane serve to increase the surface area of the cell. Furthermore, their presence indicates that the surface of the cell normally displays a high degree of activity.

The cytoplasm

There is no sharp structural differentiation of the cytoplasm into endoplasm and ectoplasm. However, the peripheral cytoplasm is usually devoid of elements of the endoplasmic reticulum and the cytoplasm of the microvilli does not contain membranes of the reticulum and only rarely possesses the 200 Å ribonuclear-protein particles (RNP). Instead, it consists of a fine granular material which stains slightly.

The more internal cytoplasm contains a variety of structures most of which have membranous components. Membranes of the endoplasmic reticulum are usually well represented (Fig. 1) and enclose sacs and cisternae which may contain fibrous material. The degree of development of the reticulum shows much variation and in some cells it is poorly represented (Fig. 2).

The membranes of the reticulum are characteristically dotted with RNP particles. However, certain parts of them are devoid of particles and this is associated with the formation of " bulbs " at the ends of cisternae (Fig. 5).

The RNP particles are not all attached to membranes, in fact, most of them are free within the cytoplasm. They have a multiple structure and consist of dark staining granules about 30 Å in diameter which are embedded in an amorphous matrix.

Cytoplasmic vesicles

Vesicles enclosed by a unit membrane are very common throughout the cytoplasm. They range from 300 Å to 0·5 μ in diameter and for the sake of convenience are grouped into 3 main types, according to their size and density : (a) pinocytotic

vesicles, (b) vesicles which are slightly stained and (c) large vesicles with dark staining contents.

(a) Pinocytotic vesicles are small (0·1 μ in diameter) and are common in the peripheral cytoplasm. They may occur in chains which extend from the plasma membrane into the cytoplasm. Chain formation is most commonly observed in surface projections (Fig. 7). Pinocytotic vesicles do not appear to possess stainable internal contents, their density is similar to that of the extracellular space.

Many small vesicles in the peripheral cytoplasm appear to be pinocytotic vesicles but their identity cannot be decided on for certain. Furthermore, it is quite possible that the small vesicles deeper within the cytoplasm may also be derived from the plasma membrane, but these have stainable contents and are grouped in the second category.

(b) Vesicles containing a fine granular material which stains only slightly with osmium are very abundant throughout the cytoplasm. These range in size from 300 Å to 0·5 μ in diameter and commonly occur in groups. They may be confused with vesicles of the Golgi apparatus, particularly when a continuous population of vesicles extends from the Golgi apparatus into the cytoplasm (Fig. 3).

(c) The remaining type of vesicle is usually large and contains denser staining material. Such vesicles are absent from most cells but when present they may occur in large numbers. They are illustrated in Fig. 2.

The nucleus

The nucleus tends towards an oval shape, with one or more conspicuous indentations. Two unit membranes enclose the nucleus and these are frequently interrupted to form pores. The external nuclear membrane has RNP particles attached to it and in some places it has been observed to be continuous with the membranes of the endoplasmic reticulum.

The nucleus contains 200 Å particles which are usually distributed marginally. Sometimes nucleoli are observed and these are usually possessed by cells which display only a meagre endoplasmic reticulum.

The mitochondria

The mitochondria are cylindrical in form and are about 0·3–0·5 μ in diameter and 1·5–2 μ in length. They are enclosed by a pair of unit membranes, the internal one invaginating regularly to form the cristae.

The number of mitochondria seen per cell section is relatively small and does not approach the number observed in adult liver (North and Pollak, 1961). However, the number of cristae per mitochondrion is comparable with the number in liver.

The centrosome

The cell centrosome is situated opposite the nuclear indentation and its ultrastructure is easily distinguishable from the remainder of the cytoplasm. It is seen as a finely granular area of the cytoplasm roughly circular in section (Fig. 4). Closely associated with the centrosome and surrounding it are seen groups of Golgi membranes. Many sections reveal that the centrosome is a sphere and occupies about 5 cu. μ of the cell.

One or more centrioles are present in the centrosome and these have a fibrous appearance in longitudinal section (Fig. 4). This study does not reveal a system of parallel tubules within the centriole and this may have been due to the fixative procedure employed.

Intracytoplasmic channels

Parts of the cytoplasm are partitioned off from the remainder by concentric layers of membranes. A study of many sections reveals that these membranes form long intracellular cylinders (Figs. 5 and 6). The cytoplasm within the membranes is peculiar in being almost entirely composed of RNP particles. The degree of permanency of these cylinders is not known but they are a common feature in the macrophage. For this reason they are thought to serve an important function and it is tempting to regard them as channels through which the cytoplasm flows during locomotion.

The Participation of Cytoplasmic Vesicles in reponse to the presence of Ingested Bacteria

Listeria are seen inside macrophages enclosed by the unit membrane of a phagocytic vacuole. The phagocytic vacuole is more than large enough to accommodate the bacterium so that a space exists between the vacuolar membrane


EXPLANATION OF PLATES

FIG. 1.—A survey micrograph showing the conspicuous indentations of the nucleus, a well developed endoplasmic reticulum and large numbers of cytoplasmic vesicles (LL). Projections of the cell can be seen and profiles of surface invaginations (IN) are present within the cytoplasm.

FIG. 2.—Micrograph showing a younger cell with a poorly developed endoplasmic reticulum (ER). The nucleus contains a nucleolus (NO) and numerous cytoplasmic vesicles are present (LL). A bacterium within a phagocytic vacuole is shown (LM). Two of the denser variety of vesicles are also present (DV).

FIG. 3.—Part of the Golgi apparatus showing the characteristic vesicles and lamellae. Note the continuous population of vesicles extending from the Golgi region into the cytoplasm.

FIG. 4.—A centrosome (CS) containing a centriole (CT). Note the granular nature of the centrosome and the fibrous nature of the centriole.

FIGS. 5 and 6.—Transverse and longitudinal views of a cytoplasmic channel (CC). Note the multiple layer of membranes enclosing the channel and that its contents consist of RNP particles. In Fig. 5 a cytoplasmic vesicle appears to be dividing (LL). ER points to a "bulb" at the end of a cisterna of the endoplasmic reticulum.

FIG. 7.—Listeria enclosed by phagocytic vacuoles. These vacuoles contain no other material but the bacteria. Cytoplasmic vesicles are seen in close proximity to the vacuolar membrane (LL). A chain of pinocytotic vesicles at the end of a cytoplasmic projection is present (PV).

FIG. 8.—A phagocytic vacuole containing a bacterium. A "cloud" of amorphous material partly surrounds the vacuole (AM). Cytoplasmic vesicles (LL) are present near this region. Arrows point to parts of the phagocytic vacuolar membrane which appears to be breaking down and amorphous material seems to be entering the vacuole at these points.

FIG. 9.—An enlarged portion of Fig. 8 showing the remains of disintegrated membranes (MR) within the amorphous material.

FIG. 10.—A similar situation to that seen in Fig. 8. Membrane remnants (MR) are again shown. The phagocytic vacuole to the right has accumulated a large amount of amorphous material.

FIG. 11.—A phagocytic vacuole containing amorphous material. This vacuole appears to contain vesicles (arrows). The membrane of the vesicle (LL) below the vacuole is difficult to visualise.

FIG. 12.—The phagocytic vacuole is completely filled with material. Note the identical appearance of the material inside the vacuole and that contained by the vesicles (LL). The vesicles appear to be fusing with the vacuole (arrows).
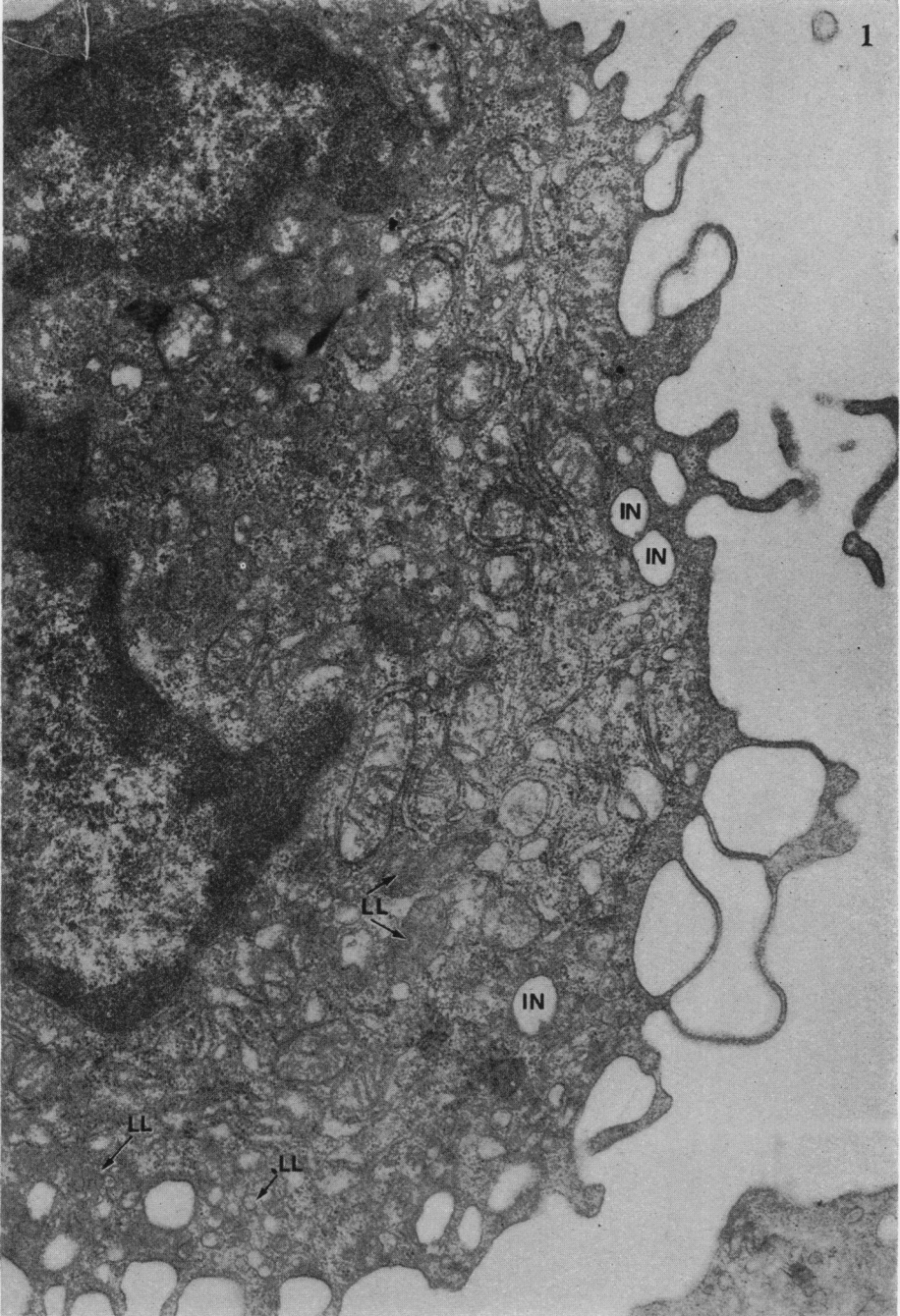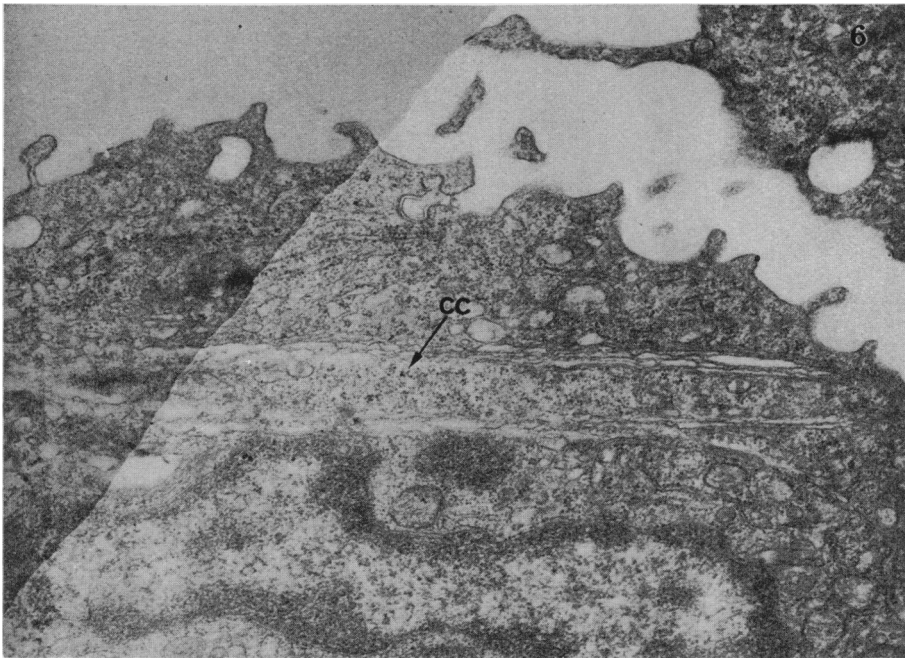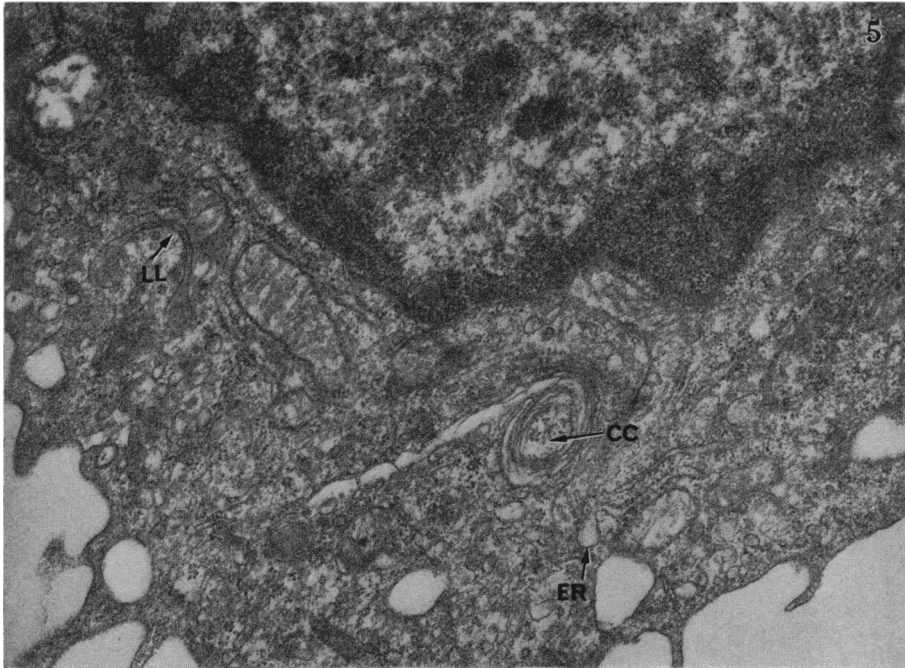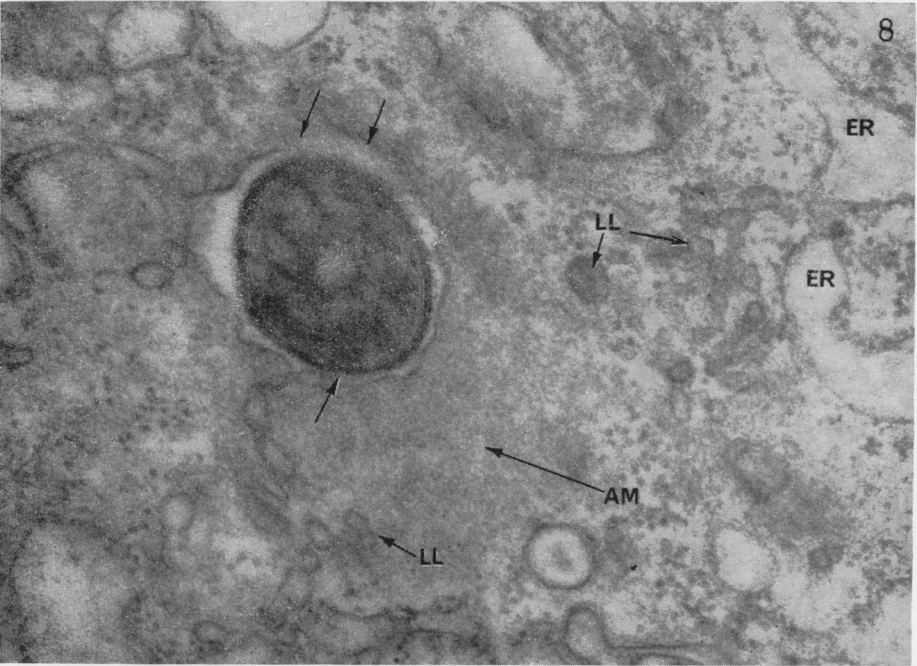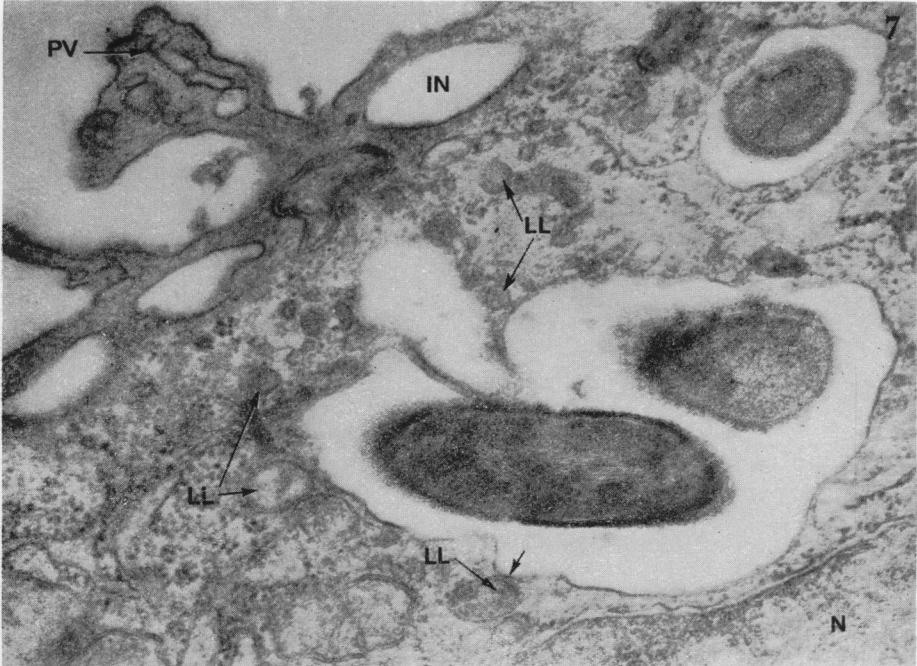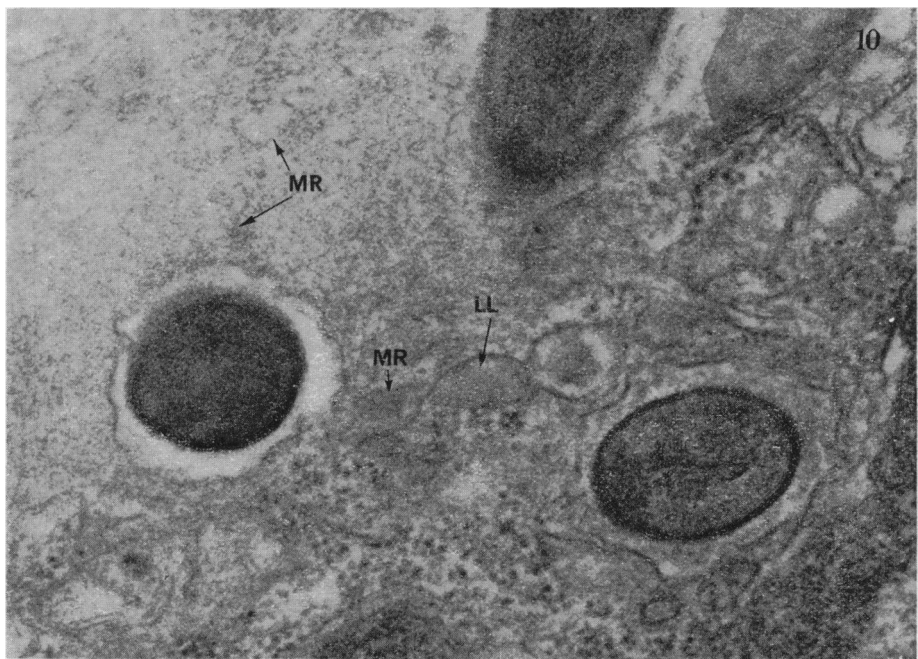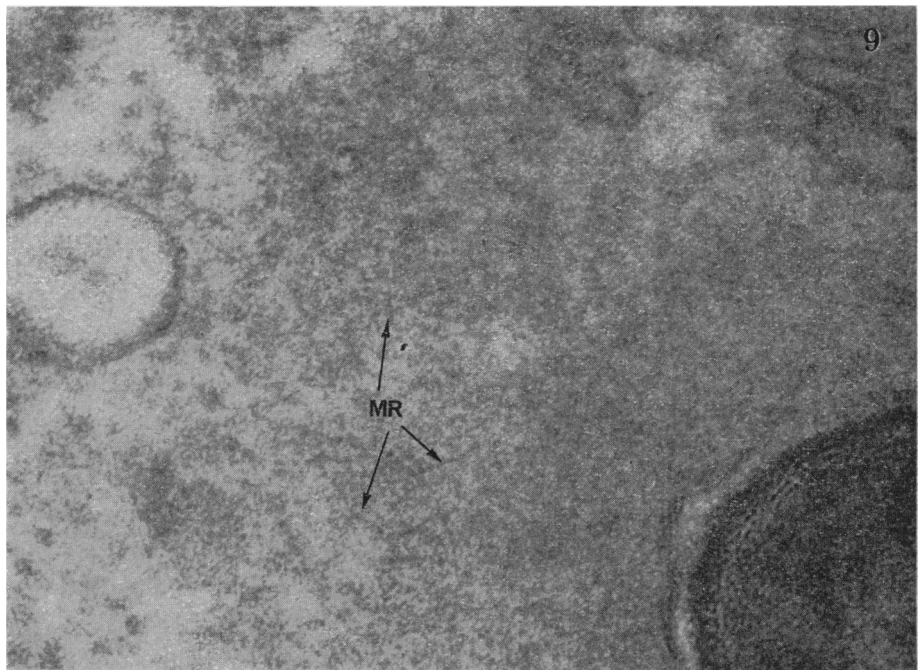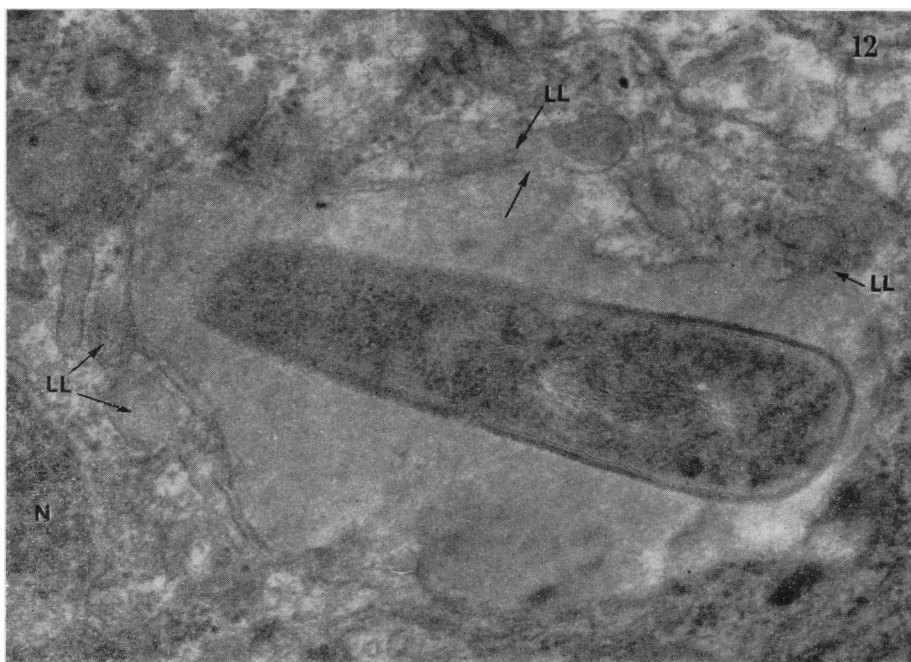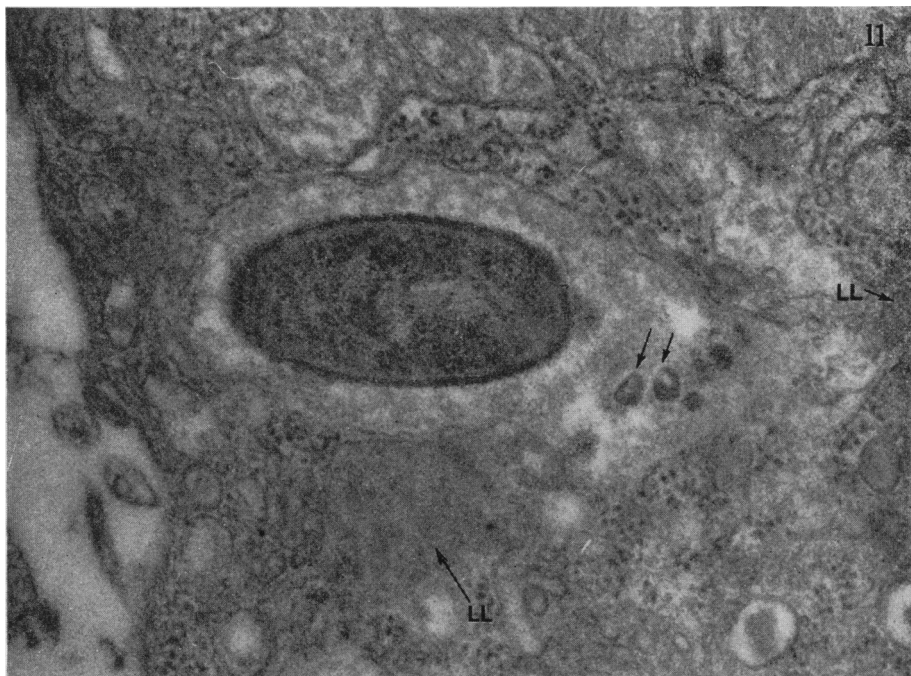
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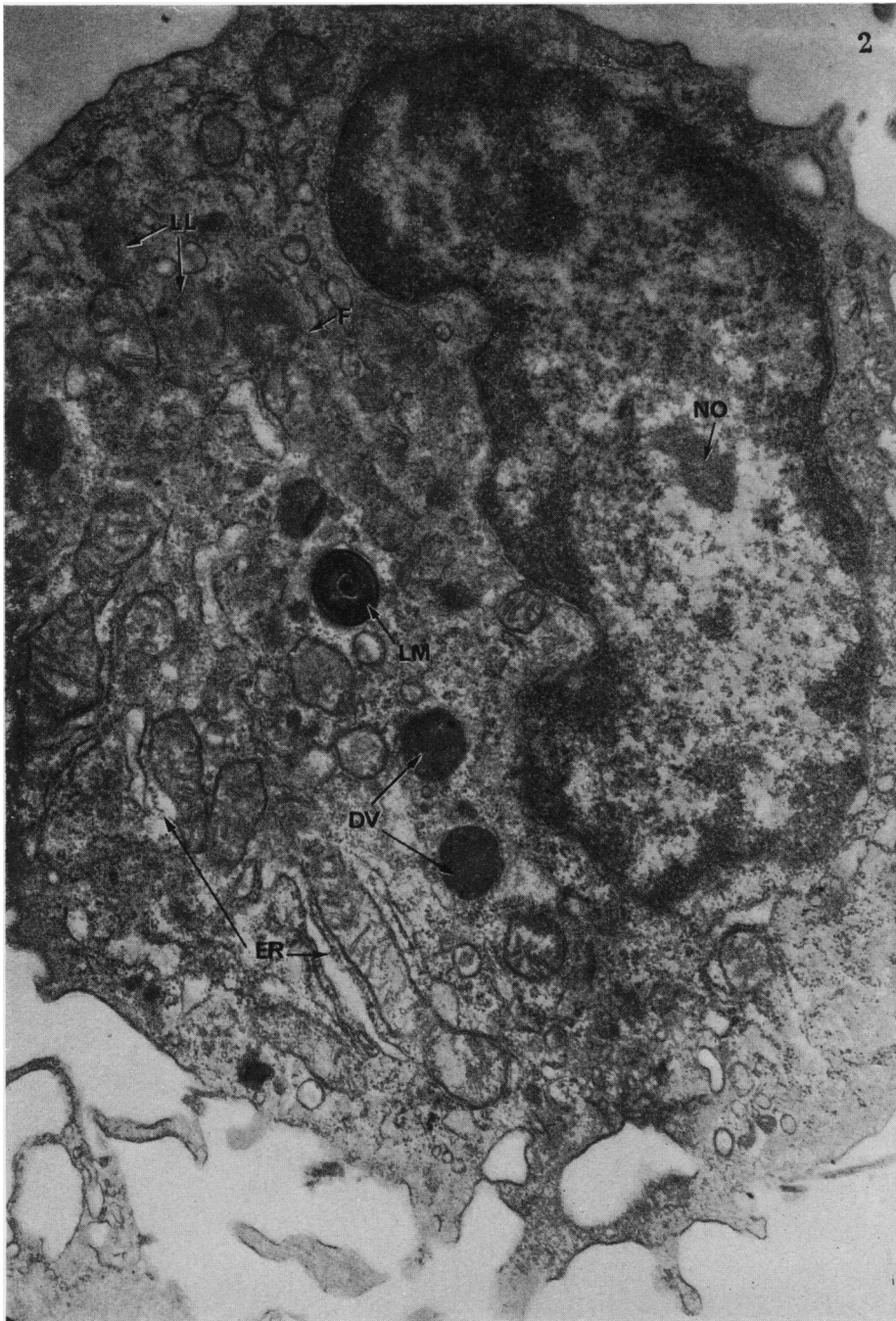
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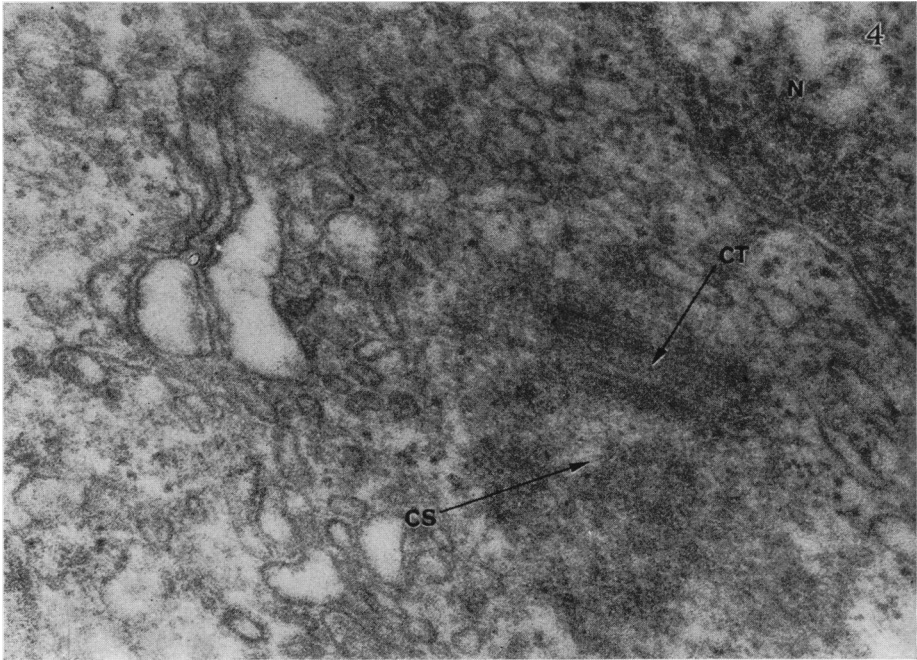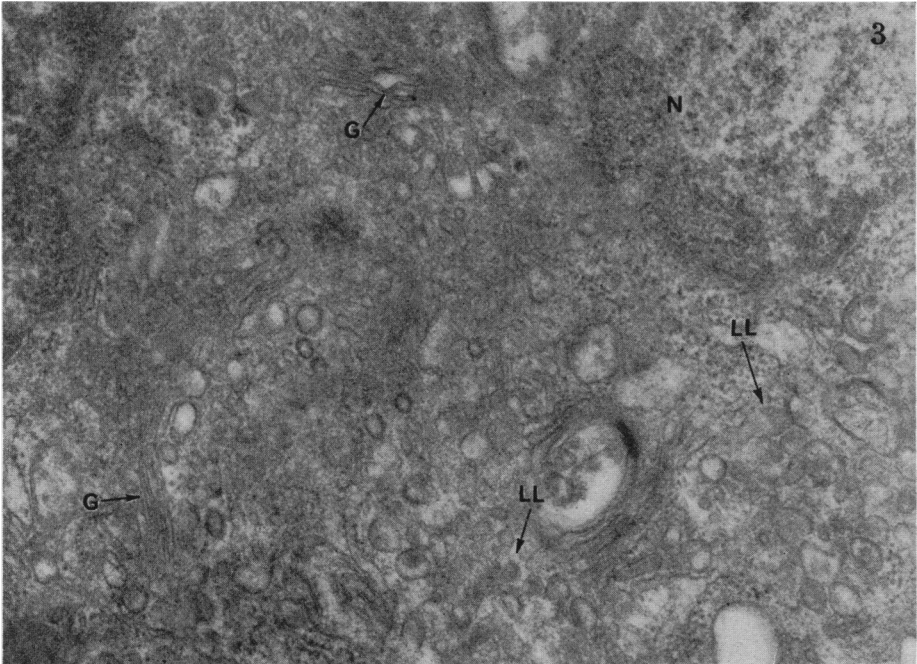
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and the bacterial cell wall. This space may appear " empty " in some vacuoles (Fig. 7), but in others it contains varying amounts of an amorphous, moderately stained material (Figs. 10, 11 and 12). This material may sometimes occupy all of the available space in the vacuole (Fig. 12). An examination of many micrographs suggests that the possession of this material by vacuoles is time dependent in that newly formed vacuoles will contain little or none of this material.

The amorphous vacuolar material is similar in appearance to the contents of cytoplasmic vesicles. In fact, this study suggests that vesicles transfer their contents to phagocytic vacuoles. Fig. 12 serves to illustrate both of these points. It can be seen that cytoplasmic vesicles are in close proximity to a phagocytic vacuole and that some vesicles have fused with the vacuole. Furthermore, the material inside the vacuole is identical in appearance with that inside vesicles.

Some phagocytic vacuoles are observed to be partly surrounded by a " cloud " of structureless material which also resembles the contents of vesicles (Figs. 8, 9 and 10). In this situation the phagocytic vacuoles contain similar material. Furthermore, the material within vacuoles is only separated from identical material outside by parts of the vacuolar membrane which appears to be breaking down. This gives the impression that material has flowed into the vacuoles from the outside.

Within the " cloud " of extravacuolar material are seen small fibrous elements about 30 Å in thickness (MR in Figs. 9 and 10). These structures are of the same thickness as the outer layers of unit membranes and appear to consist of similar material. It is therefore possible that they represent the remains of the disintegrated membranes of vesicles, and that the amorphous material partly surrounding phagocytic vacuoles originate from vesicles which have ruptured.

DISCUSSION

The peritoneal cavity of mice used in this study contains approximately 5×10^6 macrophages. These cells show detailed variations in ultrastructure but share some common characteristics which serve to distinguish them from other types of cells found in this situation.

The structural variations existing among macrophages are probably a reflection of the fact that the population contains individual cells at various stages of differentiation, either from a primitive stem-cell or by division of a parent macrophage. This could explain differences in the degree of development of the endoplasmic reticulum, the relative abundance of free RNP particles in the cytoplasm and the presence or absence of nucleoli. It could also explain differences in the number of mitochondria seen per cell section. In chick liver this number increases as the embryo becomes older (North and Pollak, 1961).

Cells which actively synthesise and secrete protein invariably possess a well developed endoplasmic reticulum (Birbeck and Mercer, 1961). If this is a criterion of " secretory " cells then it is possible that mature macrophages also secrete protein.

The peritoneal macrophage contains RNP particles which display a multiple structure. Alveolar macrophages have also been shown to possess this type of particle (Karrer, 1958) and it is interesting to note that the ribosomes isolated from *Escherichia coli* are divisible into subunits (Huxley and Zubay, 1960).

The presence of a large number of cytoplasmic vesicles is characteristic of the peritoneal macrophage. The majority of them are small and they are not obvious at low magnifications. They undoubtedly serve an important function.

The affinity of cytoplasmic vesicles for phagocytic vacuoles appears to result in either a direct fusion of these two structures or a release of vesicular material outside the phagocytic vacuole. Such observations strongly suggest that cytoplasmic vesicles are in some way concerned with intracellular digestion. The striking similarity between the contents of vesicles and those of vacuoles gives the impression that there has been a transfer of material from the former to the latter.

The participation of definite cellular organelles in a response to the presence of material ingested by cells has been investigated by other workers. Rose (1957), using phase optics, was able to observe the movement of cytoplasmic granules towards incoming fluid droplets in HeLa cells. These were not mitochondria. Similarly, Hirsch (1962) has shown that the characteristic granules of polymorphonuclear leucocytes move towards ingested bacteria and burst. The bursting of granules is followed by a change in the refractive index of the zone immediately surrounding bacteria. Hirsch interprets this as meaning that the phagocytic vacuoles have acquired the material of granules. The accumulation of granules around food vacuoles in amoeba was noted much earlier by Horning (1926) and high concentrations of acid phosphatase activity surrounding engulfed material in macrophages has been demonstrated (Weiss and Fawcett, 1953).

It is possible that the cytoplasmic vesicles of the peritoneal macrophages described in this study are lysosomes. The lysosome concept originated from the work of de Duve, Pressman, Gianetto and Applemans (1955) who obtained a cell fraction from liver homogenates which proved to be particularly rich in hydrolytic enzyme including acid phosphatase. This fraction when examined electronmicroscopically was seen to consist mainly of small membrane-bound vesicles. De Duve (1959) regards lysosomes as being concerned in the mechanisms of intracellular digestion.

Novikoff (1959) is of the opinion that most of the cytoplasmic vesicles seen by electronmicroscopists are lysosomes and future investigations may prove this opinion correct. For example, polymorphonuclear leucocyte granules on isolation have been shown to contain enzymes identical with those present in lysosomes (Cohn and Hirsch, 1960). Straus (1959) has presented evidence which suggests that cells throughout the reticulo-endothelial system contain lysosomes in high numbers. The direct demonstration that acid phosphatase activity is confined to small cytoplasmic vesicles is due to the work of Essner and Novikoff (1960, 1962) who adapted histochemistry to electronmicroscopy.

The above evidence is in accord with the view presented here, i.e. that the cytoplasmic vesicles of peritoneal macrophages transfer their contents into phagocytic vacuoles and that this event leads to the concentration of digestive enzymes around ingested material. It is not clear whether this transfer is brought about by a direct fusion of vesicles to vacuoles or by a movement of " naked " vesicular material into the vacuoles from outside. The first mechanism would be more acceptable since it would enable digestive enzymes to act directly upon the target material without first being diluted in the surrounding cytoplasm. It is possible that the disintegration of vesicles outside phagocytic vacuoles is a fixation artifact. This could be due to a change in the physiological state of

vesicular membranes induced by the ingestion of Listeria which in turn might make them more susceptible to fixation stresses.

No structural changes could be detected in the ingested bacteria.

This study covers only the early stages of the cytoplasmic response of macrophages to the presence of ingested bacteria. The majority of macrophages subsequently die as a result of the rapid intracellular multiplication of the bacteria and the electron-microscopy of these stages will form the subject matter of future publications. In the following paper the early stages of the response of macrophages from mice immunised against Listeria are described.

SUMMARY

Some aspects of the fine structure of peritoneal macrophages are described. These cells possess a large number of cytoplasmic vesicles which have an appearance similar to published illustrations of lysosomes.

The cytoplasmic response resulting from the ingestion of bacteria consists of an accumulation of amorphous material within phagocytic vacuoles. The micrographs presented in this study suggest that the amorphous material originates from the cytoplasmic vesicles which either fuse with or expel their contents outside the vacuoles. This process is thought to represent the way in which the digestive enzymes of the cell are concentrated in phagocytic vacuoles.

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