A previous publication documented certain of the morphological and biochemical events associated with the differentiation of mononuclear phagocytes (1). Under both in vitro and in vivo conditions, it was ascertained that mouse peritoneal cells underwent a temporal sequence of morphological changes. These resulted in a much larger cell which under the light microscope demonstrated increased numbers of (a) phase-dense, acid phosphatase-positive granules, (b) mitochondria and ultimately the formation of refractile lipid droplets. In addition, concomitant biochemical studies revealed the production of large quantities of acid phosphatase, beta-glucuronidase, and cathepsin. These alterations occurred both in a tissue culture environment and in the peritoneal cavity following the induction of a mild inflammatory process.

This article will point out the structural features of macrophage maturation at the electron microscopic level and will serve to complement the prior findings employing the phase microscope.

Materials and Methods

The Stimulation of Cells within the Peritoneal Cavity.—Mice of the NCS strain (pathogen-free) maintained at The Rockefeller University were employed for all studies. At the time of an experiment, 20 μg of a lipopolysaccharide endotoxin from Salmonella abortus-equus (Pyrexal) was administered intraperitoneally in 0.2 ml of physiological saline. This resulted in an inflammatory event in which large numbers of polymorphonuclear (PMN) leukocytes entered the peritoneum within the first 24 hr. By the 4th day the cell population was composed primarily of large mononuclear phagocytes and lymphocytes, whereas PMN leukocytes were rarely seen after the 3rd day. At this time, the mice were sacrificed and the cells harvested for both light and electron microscopy. The procedures for light microscopy and cytochemical observations may be found in a previous reference (1). Mice were anesthetized with chloroform, the skin over the abdomen reflected and 2.0 ml of warm medium 199 (Microbiological Associates, Bethesda, Maryland) injected into the imperforate peritoneum. The abdomen was

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kneaded gently and the cell rich fluid removed immediately with a fine tipped Pasteur pipette and rubber bulb. The peritoneal contents of 5 to 6 animals yielded a sufficiently large yield to satisfy the requirements of electron microscopy. Cells obtained from three separate experiments were examined.

Cells from normal, nonstimulated animals were harvested in an identical fashion.

*The in vitro Cultivation of Mononuclear Phagocytes.*—The cells from normal, nonstimulated animals were harvested in the usual manner (1) with heparinized medium 199. They were then centrifuged lightly at 50 g for 5 min and resuspended to a density of $2 \times 10^6$/ml in a complete medium consisting of 20% newborn calf serum, No. 199, and Penicillin. 10 ml of this suspension was then dispensed to each T flask, gassed with 5% CO$_2$ air and incubated for 60 min at 37°C. After this preincubation the medium was decanted and the cell sheet washed vigorously with 10 ml of warm No. 199. This removed the poorly adherent lymphocytes leaving a homogeneous population of monocytelike phagocytes. 10 ml of the complete medium was then added, the flasks gassed and reincubated for periods of 24 to 90 hr at 37°C. Examination of cells by electron microscopy was usually performed on the pooled contents of two flasks. Alterations in the composition of the complete medium are described in Results.

*Harvesting and Processing of Cells for Electron Microscopy.*—

*Cells obtained directly from the peritoneal cavity:* Cells obtained from the unstimulated peritoneal cavity or following lipopolysaccharide stimulation were pooled in conical tubes and centrifuged at 50 g for 5 min. This resulted in a firm pellet and a clear supernatant fluid which was discarded. 3 ml of ice cold 1% buffered OsO$_4$ was added to the pellet (0.1 to 0.2 ml packed cells), the cells resuspended gently with a pipette and allowed to fix at 0°C for 15 to 30 min.

*Cells obtained from culture flasks:* Cells which were maintained in T flasks for periods of more than 60 min, adhered firmly to the glass surface and could not be removed by either versene or trypsin. This necessitated the use of physical methods on cells which had previously been fixed in situ. At the time of harvest, tissue culture medium was completely removed and 5 ml of cold 1% buffered OsO$_4$ added quickly to the cell sheet. The T flasks were then stoppered and placed in a horizontal position at 4°C for 15 min. The fixative was then removed by means of a fine tipped Pasteur pipette, the monolayer rinsed with 5.0 ml of buffered saline and replaced with 3.0 of saline. The cells were then scraped from the glass surface by means of a rubber policeman and transferred to conical centrifuge tubes.

Because of the small packed volume of cells, a method was required to maintain the pellet during the subsequent dehydration procedures. This was accomplished by the following technique. After collecting the harvested macrophages by low speed centrifugation, the supernatant fluid was decanted and one drop of warm 2% bacteriological agar (Difco Laboratories, Inc., Detroit) was allowed to envelope the pellet. This material gelled rapidly, entrapping the cells and allowed handling without significant loss of cells.

*Embedding, sectioning, and staining:* Osmium-fixed cells were then dehydrated and embedded in Epon (2). Thin sections were cut with a Porter-Blum microtome and a diamond knife. The sections were stained with lead (3) and uranyl ions and viewed with a Siemens Elmiskop I electron microscope.

**RESULTS**

*The Structure of Mononuclear Phagocytes obtained from the Unstimulated Peritoneal Cavity.*—The mouse peritoneal cavity is somewhat unique in that it contains a large, normal population of phagocytic cells which resemble monocytes, as well as smaller nonphagocytes which are indistinguishable from the small lymphocyte. Polymorphonuclear leukocytes are rare and mast cells constitute 1% or less of the total cell population. This presentation will stress the properties of the mononuclear phagocytes normally found in NCS mice.
Fig. 1 illustrates a typical cell immediately after its removal from the peritoneal cavity. Under these conditions, in which the cells are fixed in suspension, the shape is spherical. The cell surface is very irregular and this accounts for the large peripheral lacunae seen on cross-sections. Also typical are the large number of fine, fingerlike projections extending from the surface. These are usually devoid of cytoplasmic organelles and are not as prominent in the more mature cells to be described later. The nucleus is almost always deeply indented and has the same overall appearance as that seen in the peripheral blood monocyte.

The major findings to be described are found in the cytoplasm and will be presented in detail since they serve as a baseline for all subsequent experiments. The unstimulated peritoneal phagocyte contains a moderate amount of rough surfaced endoplasmic reticulum which is peripherally oriented in the cytoplasm (Fig. 1). Occasional clusters of free ribosomes are observed although the vast majority are membrane bound. A small percentage of cells will have a much more extensive rough surfaced endoplasmic reticulum than seen in Fig. 1.

The Golgi complex is always found in the "hof" of the nucleus. In thin sections of the unstimulated cell the Golgi region is composed of a single stack of parallel, smooth membranes or lamellae as well as a small number of electronlucent, smooth vesicles. These vesicles appear to be budding from the ends of the lamellae and are found on both of its faces. No direct connections between the rough surfaced endoplasmic reticulum and the smooth membranes of the Golgi apparatus have been observed in these sections. Smaller profiles containing ribosomes are seen in the cytoplasm adjacent to the Golgi zone and may represent cisternae of the endoplasmic reticulum cut in cross-section.

Moderate numbers of mitochondria are scattered through the cytoplasm without any specific localization. Their size is best appreciated under the phase microscope (see Fig. 1) (1). In the unstimulated cells mitochondria take the form of either very short rods or spheres.

The other major cytoplasmic component consists of electron-opaque granules. These vary in size and density and are preferentially located in the cytoplasm surrounding the Golgi apparatus. Some have a homogeneous matrix whereas others are multivesicular structures. In the usual section, no more than five or six such profiles are visualized in the unstimulated cell.

Frequently one also sees many small vesicles scattered through the cytoplasm. Some of these are electron lucent and resemble pinocytic vesicles whereas others contain dense material and may represent smaller variants of the larger dense granule.

The Structure of Mononuclear Phagocytes obtained from the Peritoneal Cavity of Lipopolysaccharide (LPS)-Treated Mice.—The intraperitoneal injection of 20 μg of LPS results in an inflammatory response with the emigration of peripheral leukocytes and the extravasation of plasma constituents. During this process there is a pronounced sequential alteration in the mononuclear phag-
cytes characterized by an increase in (a) cell size, (b) the number of phase-dense, acid phosphatase-positive granules in the centrosphere, and (c) the apparent number and length of mitochondria. These changes at the light microscope level as well as concomitant biochemical alterations have been documented in a previous publication (1).

The changes in cell ultrastructure have been studied at a time point 4 days after injection, when the acute inflammatory response has ceased but at a period when the mononuclear phagocytes display similar morphological changes. Fig. 2 illustrates a portion of a characteristic cell presented at a magnification similar to that of Fig. 1.

The increase in cytoplasmic mass is evident. No significant change in either nuclear size or structure has been noted. The major alterations in the cytoplasm are associated with the new structures visualized in the perinuclear region or centrosphere. Although not illustrated, moderate amounts of rough surfaced endoplasmic reticulum are found in the periphery of the cytoplasm and on that side of the nucleus opposite to the location of the centrosphere.

The enlarged centrosphere contains numerous electron-opaque granules, surrounded by distinct membranes, which are larger than those observed in unstimulated cells. These granules, which can be defined at the light microscope level, react positively for acid phosphatase (1). The granules vary in density, size, and structure. With osmium fixation, many have a homogenous matrix although some contain small vesicles. Granule counts on thin sections have shown that their number is increased 3- to 15-fold over that seen in the unstimulated cell.

The number of mitochondrial profiles is also increased over that found in the unstimulated cell. No evidence concerning the length of these organelles is obvious from thin sections, nor does the organization of the cristae intramitochondriales seem unusual.

One of the most striking changes occurs in the Golgi apparatus. As mentioned previously, this structure is usually composed of a single stack of membranes in the unstimulated cell. In cells from LPS-treated mice a much enlarged Golgi zone is apparent. This consists of small stacks of lamellae, usually 8 to 9 in number which are scattered throughout the region and arranged about the centrioles. Many, small, electron-lucent vesicles are found scattered between the lamellae.

The only other finding of note has been the presence of nonmembrane bound lipid droplets which are found in the neighborhood of the rough surfaced endoplasmic reticulum.

The Structure of Unstimulated Mouse Peritoneal Phagocytes Cultivated In Vitro.

Following the introduction of peritoneal phagocytes into a tissue culture environment they rapidly adhere to and spread out on the glass surface. The subsequent events in vitro are largely governed by the composition of the
medium (5). In the presence of low concentrations of newborn calf serum, cell spreading, phase-dense granule formation and the production of hydrolytic enzymes occurs at a very slow rate. In contrast, the presence of high concentrations of serum accelerates this process.

Cells were cultured in T flasks with 20 and 50% newborn calf serum medium. Samples were taken for electron microscopy after 24, 48, and 72 hr of cultivation. Cells were also cultured in 50% serum medium for 24 hr, then washed with medium 199 and suspended in 1% serum medium for 20 hr. This results in a reduction of granule size and a loss of hydrolase activity (6).

The in vitro cultivation of the mouse phagocytes in either 20 or 50% serum leads to similar alterations in ultrastructure. The major difference occurs in the rate of formation of granules and in the hypertrophy of the Golgi apparatus. Fig. 3 a illustrates a portion of a cell after 18 hr of incubation in 50% serum. Nuclear morphology remains unchanged during 3 days of cultivation. The number of mitochondrial profiles increases with in vitro residence but the appearance of these organelles is not appreciably altered.

The major changes occur in the perilinuclear zone of the cytoplasm. These are again characterized by an increase in the number of Golgi lamellae similar to that described in cells obtained from LPS-treated mice. Many small, smooth surfaced Golgi vesicles are also present. There is never any formation of large Golgi cisternae. At this time large, electron-opaque, membrane-bound granules are already present and localized in and about the Golgi complex. These granules vary in size from cell to cell but are on the average larger and more numerous in cells cultured in the 50% calf serum medium. Many of the granules contain within them, vesicles of different size and electron opacity.

Also to be seen are both large and small, electron-lucent vacuoles which arise from pinocytotic activity. The larger, clear vacuoles are derived from the fusion of small pinocytotic vesicles and are seen to accumulate in the peri-Golgi region.

During the formation of granules, the endoplasmic reticulum with its attached ribosomes is displaced to the periphery of the cytoplasm and is most prominent on the side of the nucleus opposite to that which contains the Golgi apparatus. This produces a highly polarized cell which is similar in organization to the BCG-induced macrophages described previously (7). An example of the endoplasmic reticulum is seen in Fig. 3 b and is taken from a section of a cell incubated for 48 hr in 50% newborn calf serum. At this time highly refractile lipid droplets are apparent under the phase microscope. These droplets are often surrounded by the ergastoplasm and in close association with its ribosomal surface. They are never bounded by a discrete membrane. No evidence of smaller lipid droplets are seen in the cell and it appears that these larger fat bodies are formed in situ rather than arising from a fusion of smaller structures. In addition, there has been no evidence of fusion of either Golgi vesicles or pinocytotic vesicles with the larger lipid deposits. Accumulation of lipid has not
been seen within mitochondria. The lipid droplets increase in number with continued cultivation, and are more numerous in cells cultured in higher concentrations of serum.

Figs. 4 a to 4 c present other details of cells cultured for varying periods of time. Fig. 4 a demonstrates the appearance of a portion of the Golgi apparatus in a cell cultivated for 72 hr in 20% newborn calf serum medium. Many small Golgi vesicles are present and fill the centrosphere region. Some are relatively electron opaque whereas others are quite lucent. This variation in density is commonly observed and fluctuates with different cells in the same preparation. Many times the Golgi vesicles appear to cluster about the larger, electron-lucent, pinocytic vacuoles.

Fig. 4 b illustrates the variation in granule morphology in a cell cultivated for 48 hr in 50% newborn calf serum medium. Some granules have a homogeneously dense internal structure whereas others contain a complex array of membranes. Within a single granule there may also be a variation in the density of the internal structures. In general, cells cultivated in vitro contain more complex granules than those found after LPS stimulation in vivo.

Fig. 4 c shows a portion of a single large granule in a cell cultivated for 72 hr in 50% serum. It contains a large array of vesicles, many with concentric membranous lamellae resembling the myelin figures associated with macrophages and other cell types (7, 8). This type of granule represents less than 1% of the total population and the presence of typical myelin figures is even less frequent.

Observations on cells cultured for periods of up to 4 days have not revealed the presence of granules which contained portions of cytoplasmic organelles such as mitochondria or ergastoplasm. This suggests that the formation of "autophagic vacuoles" or "cytolysomes" does not occur or is extremely infrequent in these cultured macrophages.

When macrophages which have been cultivated in 50% serum medium for 24 hr are then washed and placed in 1% serum medium for another day, there is a reduction in the size of the dense granules. These structures are \( \frac{1}{2} \) to \( \frac{1}{4} \) the size of the granules in high serum medium, appear more electron opaque and usually have a more homogeneous matrix. In addition, pinocytic vesicles are reduced in number as are the smooth surfaced vesicles and lamellae of the Golgi complex.

**DISCUSSION**

From studies in this and other laboratories (9) the unstimulated mouse peritoneal phagocytes resembles the peripheral blood monocyte in both size and nuclear morphology. However, it appears that the unstimulated peritoneal cell is a more "mature" cell with fewer clusters of free ribosomes and a greater number of electron-opaque granules. These changes may reflect its activities
in the peritoneal cavity prior to cell harvest. Although the origin of the peritoneal mononuclear phagocytes is uncertain, there are indications that similar cells may arise from the circulating blood monocytes. This association with the monocyte is reinforced from studies in which blood monocytes have been shown to acquire the characteristics of macrophages in a tissue culture environment (10) and in areas of inflammation (11).

It appears that the induction of macrophage maturation, occurring either in the peritoneum or the test tube is a similar phenomenon on both morphological and biochemical grounds. In terms of ultrastructure it is associated with the formation of electron-opaque granules, bounded by a discrete limiting unit membrane and displaying different forms of internal organization within the same cell. This type of structural heterogeneity suggests a certain randomness in the formation of these organelles, a process which may perhaps be associated with distinct chemical and enzymatic properties of individual granules. Since these organelles display strong activity for acid phosphatase at the light microscope level (1), they may be considered to be a form of lysosome. In this regard, the lysosomes of other cell types also display marked variations in ultrastructure as well as their density in continuous sucrose gradients (12).

A second change which is worthy of emphasis is the alteration in the size and complexity of the Golgi apparatus. In both the LPS-stimulated and cultivated cell, the Golgi apparatus hypertrophies and makes up a considerable portion of the juxtanuclear region. This is associated with the formation of many tiny vesicles as well as a loosely arranged network of lamellae. Although the serum level of the medium has no clear cut effect either on the number of lamellae or vesicles, it appears that the development of the Golgi apparatus takes place more rapidly in higher concentrations of newborn calf serum. The temporal relationship between the hypertrophy of the Golgi complex and the formation of lysosomes suggests a functional association which will be described in more detail in a subsequent communication.

The progressive development of lipid droplets has been a constant finding. Their association with the ergastoplasm and the lack of pinocytized or phagocytized lipid microdroplets, suggests that they may be synthesized at this site. In this respect, a number of investigators have described lipid droplets in the foam cells of atheromatous plaques (13). It is of interest that batches of newborn calf serum vary in their ability to stimulate the formation of lipid droplets.

One of the interesting questions which arises from this study is the mechanism by which the dense granules are formed. What is the origin of their limiting membrane as well as intragranule matrix? Where are the acid hydrolases formed in the cell and what is the mechanism by which they are transferred to the granules? What is the role of the Golgi complex in this process? Certain of these questions will be discussed in the accompanying communication.
SUMMARY

The structure of unstimulated mouse peritoneal phagocytes has been examined by electron microscopy and compared to cells obtained from the inflamed peritoneum and from cultures maintained in vitro.

The unstimulated cell resembles the blood monocyte and contains a moderate amount of rough surfaced endoplasmic reticulum, a small but well defined Golgi apparatus and a few, small, electron-opaque granules in the cytoplasm.

During in vitro cultivation there are marked changes in cell ultrastructure. Most prominent is the formation of large electron-opaque granules, some of which have a complex matrix containing both electron-opaque and lucent vesicles. In addition, there is an increase in size of the Golgi apparatus with the appearance of new lamellae and tiny, smooth surfaced vesicles. With continued cultivation, large lipid droplets are found in apposition to the rough endoplasmic reticulum. The formation and size of electron-opaque granules as well as the enlargement of the Golgi region is stimulated by high concentrations of serum in the medium.

Cells obtained from the peritoneal cavity of lipopolysaccharide stimulated animals demonstrated changes in ultrastructure similar to those seen in cells cultured in vitro.

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EXPLANATION OF PLATES
PLATE 72

Fig. 1. A typical mononuclear phagocyte obtained from the unstimulated peritoneal cavity of the NCS mouse, demonstrating the indented nucleus (N) and moderate amount of cytoplasm. A Golgi apparatus (Go) is present in the juxtanuclear region and consists of a stack of lamellae and tiny vesicles. Strands of rough surfaced endoplasmic reticulum (RER), with attached ribosomes, are peripherally oriented. A few electron-opaque granules (GR) are present. The cell surface is irregular and contains many fingerlike projections. Some of these are cut in cross-section and appear as acunae. × 33,000.
(Cohn et al.: Macrophage differentiation)
PLATE 73

Fig. 2. A portion of a macrophage obtained from the mouse peritoneal cavity 4 days after the injection of 20 μg lipopolysaccharide. Cell size is greatly increased and is related to an increased cytoplasmic mass. The juxtanuclear zone now contains many stacks of Golgi lamellae as well as smooth surfaced electron-lucent vesicles. Numerous, large electron-opaque granules are scattered throughout the cytoplasm. × 28,000.
PLATE 74

FIGS. 3 a and 3 b. Mononuclear phagocytes from unstimulated animals cultured in vitro.

Fig. 3 a. Juxtanuclear region of a cell cultivated for 18 hr in 50% serum medium. There is an increase in the Golgi apparatus (Go) including both lamellae and vesicles. Dense granules (GR) are numerous. A number of electron-lucent pinocytic vacuoles (Vuc) are also present. × 28,000.

Fig. 3 b. A portion of a cell cultivated in 50% serum medium for 48 hr. Lipid droplets (L) are seen in association with the rough surfaced endoplasmic reticulum (RER). The lipid droplets have no discernible limiting membranes. A few dense granules (GR) are seen on the left. × 24,000.
(Cohn et al.: Macrophage differentiation)
FIGS. 4 a to 4 c. Portions of cells cultivated in vitro.

Fig. 4 a. A cell cultured for 72 hr in 20% serum medium. Two stacks of Golgi lamellae (Go) are seen as well as many smooth surfaced vesicles. A large, electron-lucent, pinocytic vacuole (Vac) is present and contains a number of smaller vesicles. × 30,000.

Fig. 4 b. Cell maintained for 48 hr in 50% serum medium and demonstrating the variation and complexity of the internal structure of granules. × 30,000.

Fig. 4 c. A portion of a granule present in a cell cultivated for 48 hr in 50% serum medium. Many electron-opaque vesicles are within it and some have a lamellated appearance resembling the early stages of myelin figure formation. This type of structure is quite rare in cultivated cells. × 30,000.