# MACROPHAGE-LYMPHOCYTE CLUSTERS IN THE IMMUNE RESPONSE TO SOLUBLE PROTEIN ANTIGEN IN VITRO

## II. Ultrastructure of Clusters Formed During the Early Response

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Morphological studies have shown that lymphocytes interact physically with macrophages during the immune response (1–6). Thus, contact between macrophages and lymphocytes has been observed in antigen-stimulated lymphoid tissues from nonimmune (2, 3) and immune (3–5) animals and bridge formation and cytoplasmic flow between macrophages and lymphocytes has been described in vivo (3) and in vitro (7).

Functional studies have shown that lymphocytes and macrophages cooperate during the immune response (8–19). Both antigen-induced lymphocyte proliferation (8–17) and the differentiation of antibody-forming cells from lymphocyte precursors during the primary response in vitro (15, 20) require the assistance of macrophages, although it is clear that the specificity for antigen in these reactions resides in the lymphocytes. Some of these studies indicate that functional cooperation requires physical interaction between the two cells (8–10, 20), while others indicate that it may be mediated through soluble substances released from the macrophage to the extracellular environment (21–25).

In the companion paper (1) we have described a phenomenon of antigen-specific formation of macrophage-lymphocyte clusters in cultures of lymph node cells and peritoneal macrophages from guinea pigs immunized with tubercle bacilli. Seen in the light microscope these clusters typically contained a single macrophage which adhered to the bottom of the culture vessel and several lymphocytes, usually 7–20, which were apparently attached as a bunch to a small spot on the macrophage surface. Our observations (1) indicate that in this reaction the macrophages play the role of antigen-binding or -processing cells, while the lymphocytes play the role of antigen-specific cells.

The structure of the macrophage-lymphocyte cluster provides for an intimate contact between the participating cells through which functional cooperation

might conceivably occur, and it was therefore felt that further morphological investigation was warranted to throw light on the nature of macrophage-lymphocyte interaction. The purpose of the present study was to examine the ultrastructure of antigen-induced macrophage-lymphocyte clusters, employing transmission and scanning electron microscopy.

## Materials and Methods

*Cell Cultures.* Suspensions of peritoneal exudate cells (PEC)[1] and autologous immune lymph node cells (LNC) were prepared from guinea pigs immunized with *Mycobacterium tuberculosis* as previously described (1). PEC and LNC were mixed in the ratio of 1:9 and diluted to a cell concentration of approximately $4 \times 10^6$ cells/ml.

The cells were grown on sterile glass cover slips for 20 h at 37°C in Leighton tubes containing 5 ml completely supplemented minimum essential medium with 15% heat-inactivated fetal calf serum with purified protein derivative of tuberculin (PPD) (10 μg/ml) added, in a 5% $CO_2$ air atmosphere.

*Fixation and Embedding.* The cells were fixed by adding 1 ml 7.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 to the culture flask. After 5 min of fixation at 37°C the medium was replaced by 3% glutaraldehyde in cacodylate buffer, pH 7.3 with 5 mM $CaCl_2$ added, and the cells were fixed for 25 min at 4°C. They were then washed in cacodylate buffer, pH 7.3 containing 0.15 M sucrose and fixed for 90 min at room temperature in 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3. After osmium fixation the cover slips were washed in cacodylate buffer, dehydrated in alcohol, followed by 1,3-epoxypropane, and left overnight in a mixture of vol/vol Vestopal W (Martin Jaeger, Geneva, Switzerland) and 1.3 epoxypropane in an open vessel. Next day they were changed to Vestopal W and left for 24 h. No. two gelatin capsules with cut-off bottoms were placed—bottom up—on the cover slips. One drop of Vestopal W was added to each capsule and the cover slips were placed at 60°C for 1 h. They were then filled up with Vestopal W and polymerization allowed to proceed for 24 h at 60°C. The Vestopal-embedded cells were separated from the cover slip by placing it on a block of carbon dioxide ice. Cell clusters were selected by light microscopy of the Vestopal blocks and sectioned on a LKB-Ultrotome 3 (LKB Instruments, Inc., Bromma 1, Sweden), in most cases parallel to the plane defined by the surface on which the clusters had grown. Ultrathin sections were collected on Formvar-covered copper grids (TAAB Laboratories, Emmer Green, Reading, England) and stained with magnesium uranyl acetate (26) and lead citrate (27). Electron micrographs were taken with a Siemens Elmiscope 1 (Siemens, Corp., Berlin, Germany).

*Ruthenium Red Staining of the Cell Coat.* The fixation procedure was modified as follows: ruthenium red to a final concentration of 0.1% was added to the 3% glutaraldehyde fixative, osmium tetroxide fixative, sucrose buffer, and to the first change of alcohol. The fixation time in 3% glutaraldehyde and osmium tetroxide was extended to 1 h and 3 h, respectively.

*Scanning Electron Microscopy.* 18.5, or 20 h cultures were fixed with glutaraldehyde as described above and by 1% osmium tetroxide for 18 h at 4°C. The specimen was then washed in sucrose buffer, dehydrated in alcohol, and transferred to vol/vol alcohol/benzene for 15 min. After two changes of 100% benzene, 15 min each, the specimen was freeze-dried, and finally covered with gold on a rotary stage. Microscopy was performed with a Cambridge 600 scanning electron microscope.

## Results

*Macrophage-Lymphocyte Cluster.* The macrophage-lymphocyte clusters consisted of one or a few macrophages, and from a few to more than 20 lymphocytes. Two morphological types of lymphocytes were involved. One with a diameter of approximately 9.1 μ × 6.5 μ had a spherical shape (CL, Figs. 2–5) with usually one flat side. The remaining lymphocytes measured 7.1 μ × 5.4 μ

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[1] *Abbreviations used in this paper:* LNC, lymph node cells; PEC, peritoneal exudate cells; PPD, purified protein derivative of tuberculin.
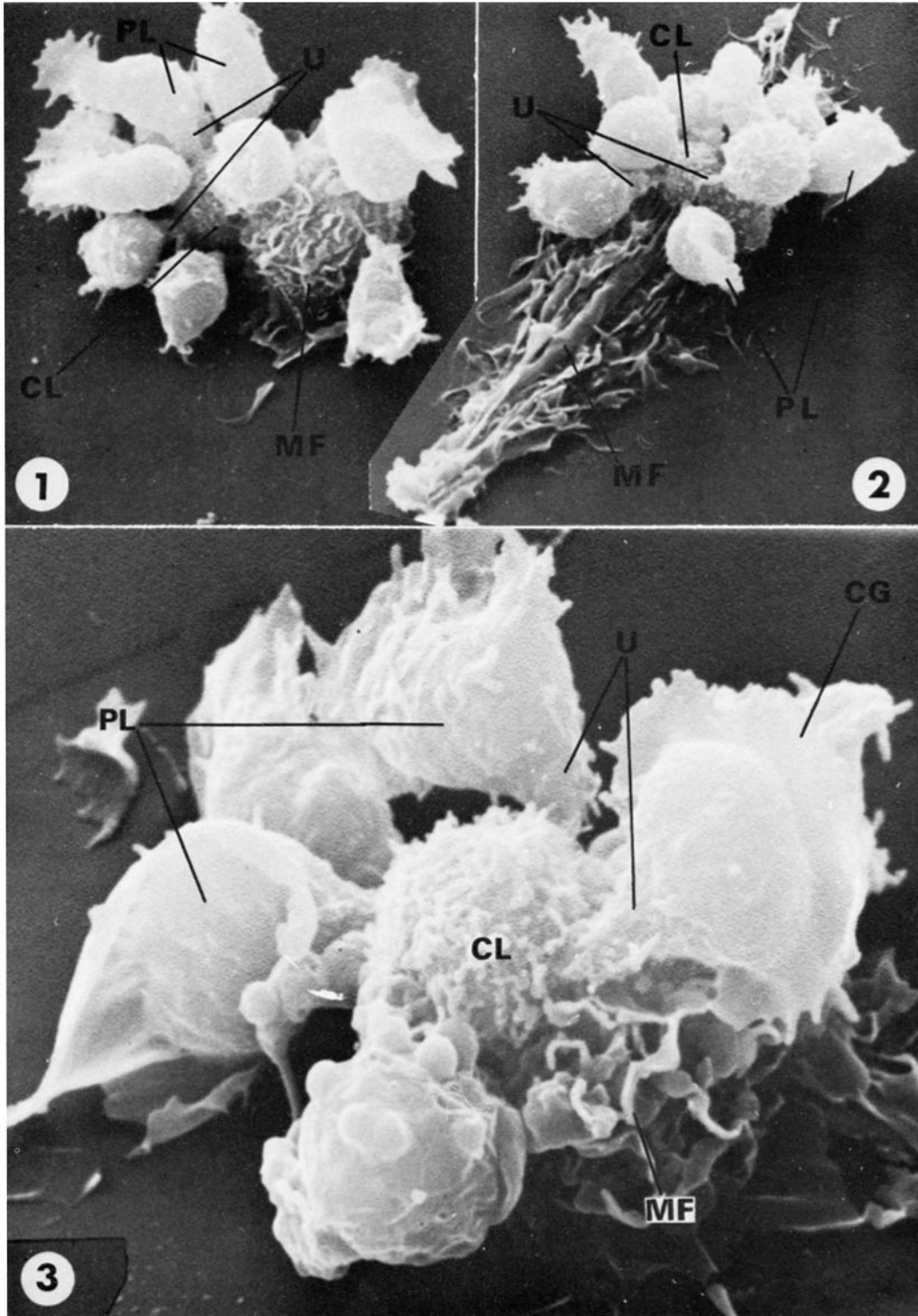
FIGS. 1 and 2. Scanning electron micrograph of lymphocyte-macrophage clusters. The peripheral lymphocytes (PL) are attached to the central lymphocyte (CL) by slender uropods (U). The folded surface of the macrophage is marked (MF). × 2,000.

FIG. 3. Peripheral lymphocyte (PL) attached to a central lymphocyte (CL), which rests on the folded surface of a macrophage (MF). Uropods (U) and crista galli-shaped cytoplasmic extensions (CG) are visible. The smooth surface of the peripheral lymphocytes form a sharp contrast to the microvillous surface of the central lymphocyte. × 4,300.

and were pear-shaped (PL, Figs. 1-5). They displayed one uropod at the narrow end (U, Figs. 1-5) and often a flat crista galli-shaped pseudopodium at the other (CG, Figs. 3, 4).

The smaller lymphocytes were located at the cluster periphery, while the large lymphocyte had a central position. They are therefore in the following referred to as peripheral and central lymphocytes, respectively. The peripheral lymphocytes were attached, by means of a uropod, to the surface of the central lymphocyte (Figs. 1-5), which on its side had a broad surface contact with a macrophage (Fig. 7). The most simple clusters thus consisted of one macrophage, one central lymphocyte, and several peripheral lymphocytes (Figs. 1 and 2). Some clusters, however, displayed two or three macrophages or one macrophage with two central lymphocytes attached to the surface. Even in these clusters of a more complex type, each of the peripheral uropod-bearing lymphocytes was attached to one of the central lymphocytes only.

*Peripheral Lymphocytes.* Except for a uropod and a few slender cytoplasmic projections the peripheral lymphocytes had a smooth surface (Figs. 1-3). The nucleus displayed one or a few deep indentations and high electron density due to large masses of peripheral heterochromatin. The nucleolus was small and often obscured by the heterochromatin. The cytoplasm was scant and intensely stained. The cytoplasmic organelles were few and mostly confined to the cytoplasmic area between the nuclear indentations and the base of the uropod (Fig. 5). The organelles were: a few mitochondriae with distinct cristae and a rather electron-dense matrix (MT, Fig. 5), a few cisterns of granular endoplasmic reticulum, a small Golgi apparatus and many free single ribosomes (RI, Fig. 6). Spherical vesicles, some with a trace of electron-dense material, were usually present in this part of the cytoplasm (VE, Figs. 4 and 5) and sometimes also in the proximal part of the uropod. The overall high electron density of the cytoplasm in the peripheral lymphocytes was due to a tight network of microfilaments. These were preferably located in the uropod (FI, Fig. 6) and toward the cell periphery. The uropods varied in length from 0.7 $\mu$ to 2.8 $\mu$. Some were almost regularly cone-shaped (U, Fig. 5), others displayed an irregular shape (U, Figs. 4, 6), but common to all was a smooth surface, normally with only few microspikes or microvilli (MS, Fig. 6). The tip of the uropod which was in contact with the central lymphocyte was usually flat. The two cell membranes were separated by a space 160 Å wide (Fig. 6). A few mitochondria, spherical vesicles, and occasionally Golgi saccules were located in the proximal part of the uropod. The distal part had no large cytoplasmic organelles except for an occasional flat vesicle, which was layered in parallel with the flat tip of the uropod—and a single mitochondrion.

*Central Lymphocyte.* The central lymphocyte was covered with short microvilli or microspikes all over the free surface (CL, Fig. 3). The nucleus of the central lymphocyte was lighter than the nucleus of the peripheral lymphocytes; it contained less heterochromatin but one or two enlarged nucleoli which displayed a reticulum of coarse granular material around small islands of homogenous texture. The nucleus was deeply indentated (arrow, Fig. 7) and residual-bodies with myelinlike contents were often present in the indentations (RB, Fig. 5). The nuclear indentations usually faced the surface contact area of the macrophage

and the part of the cytoplasm, which contained most of the organelles. The cytoplasm was rather abundant, but less intensely stained than the cytoplasm of the peripheral lymphocytes. The mitochondria had a light matrix and distinct cristae (MT, Fig. 6). The Golgi apparatus was prominent (GO. Fig. 7), often duplicated, and was always surrounded by numerous small vesicles, some of which were coated vesicles. The free ribosomes of the central lymphocyte were typically polysomes (PR, Fig. 7) and only few cisterns of the granular endoplasmic reticulum were present. The cytoplasm contained only few microfilaments.
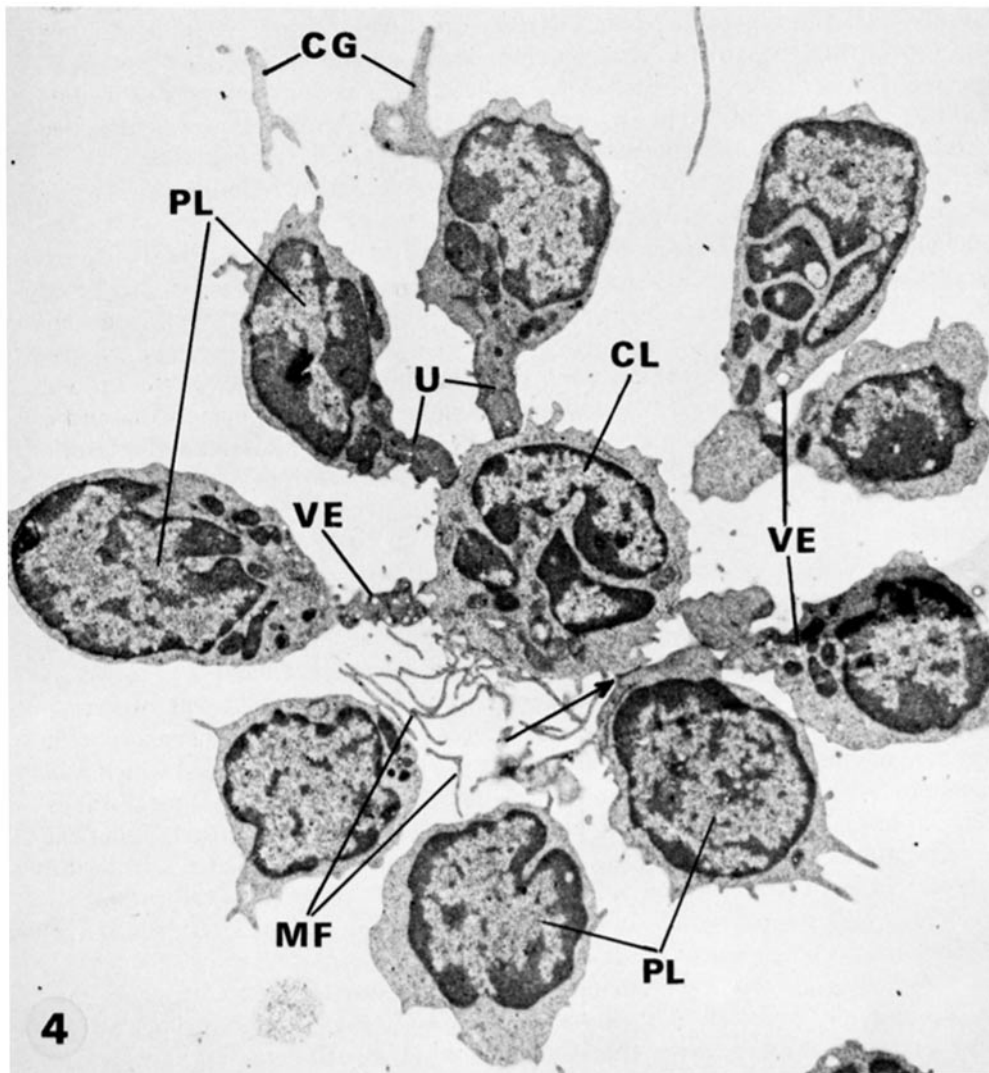


Fig. 4.   Peripheral lymphocytes (PL)—some with slender uropods (U) and one almost without a uropod (arrow)—are attached to the surface of the central lymphocyte (CL). Electron translucent vesicles (VE) are visible in the cell bodies, and in some of the uropods of the peripheral lymphocytes. (MF) denotes macrophage surface foldings, and (CG) cross-sectioned cristate pseudopodium on the peripheral lymphocyte. × 4,600.

The extensive area of surface contact between the central lymphocyte and the macrophage displayed many intercellular lagunae (Fig. 7), but it was shown by the complete penetration of ruthenium red that they were all continuous with the surrounding medium. When running in parallel the cell membranes of the macrophage and the central lymphocyte were 160 Å apart.
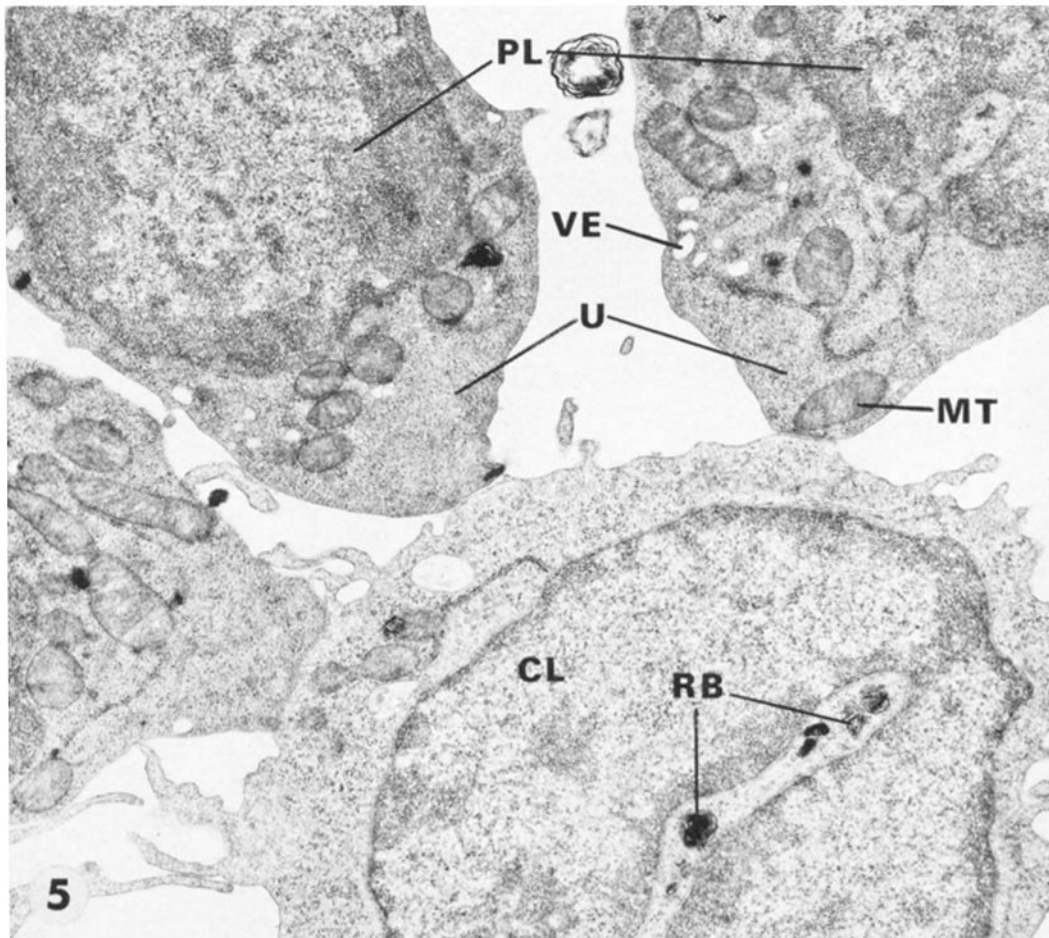


Fig. 5.  Peripheral lymphocytes (PL) with short cone-shaped uropods (U) are attached to the surface of a central lymphocyte (CL). They have a smooth surface and a rather electron-dense cytoplasm. The mitochondriae (MT) of the peripheral lymphocytes have a dense matrix. Vesicles (VE) with an electron-translucent content are usually located in the cytoplasm of the cell body, not in the uropod. The nuclear indentation of the central lymphocyte contains a few residual bodies (RB). × 19,200.

*Macrophage.* The shape and fine structure of the macrophage varied considerably from one cluster to another. In most clusters the macrophages had an ovoid shape. The macrophages usually displayed a vigorously folded surface membrane (MF, Figs. 1-4). The dominating cytoplasmic organelles were secondary lysosomal structures or residual bodies, often with abundant lipidlike
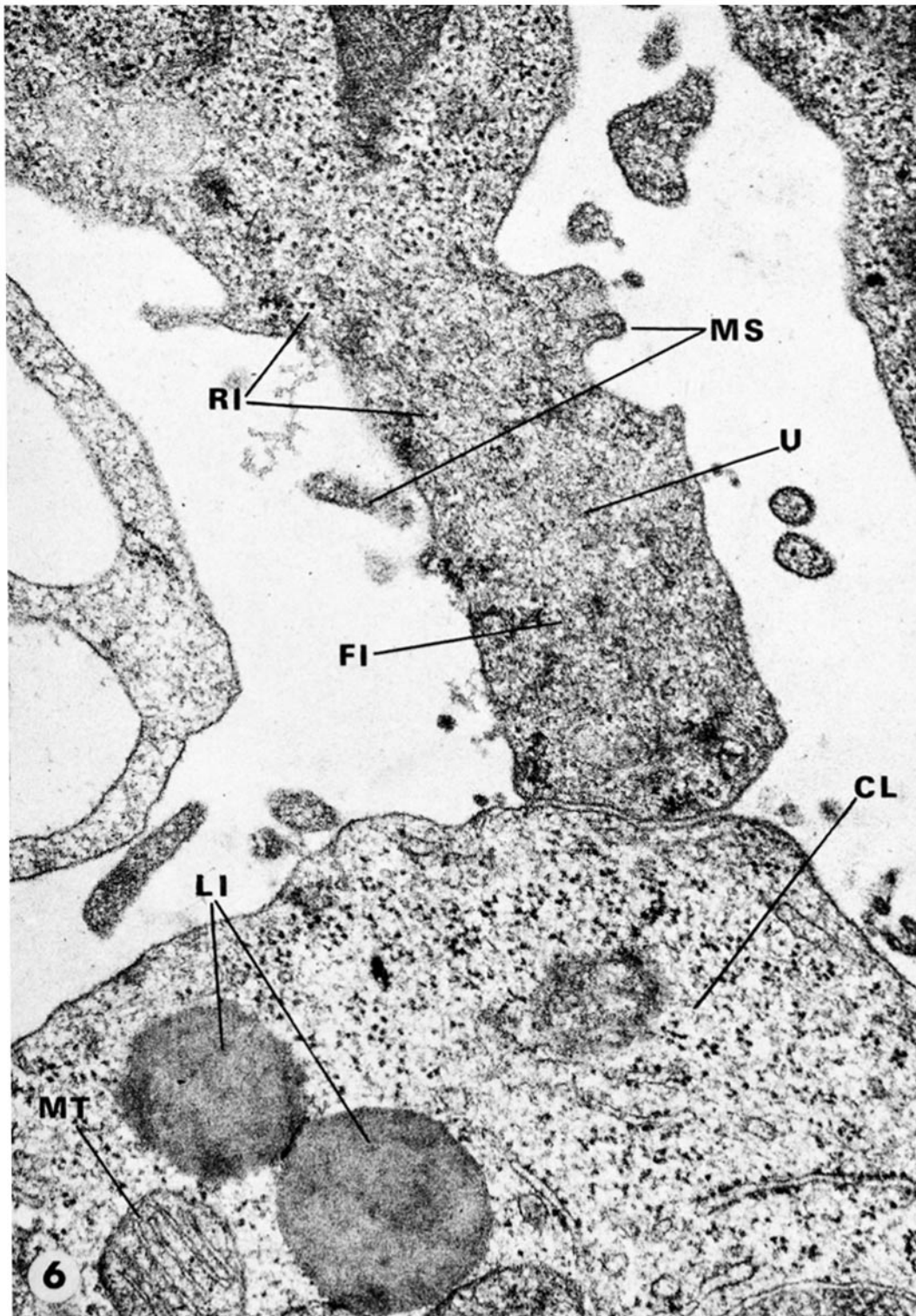
FIG. 6. Uropod (U) attached to the surface of a central lymphocyte (CL). Microvilli or spikes (MS) are seen on the proximal part of the uropod. The uropod contains few single ribosomes (RI) and many densely packed microfilaments (FI). Lipidlike dense inclusions (LI) and mitochondriae with a light matrix (MT) are visible in the central lymphocyte. × 62,000.

material. In some cells half of the cytoplasm was occupied by massive glycogen deposits, in others there was only a small amount (GL, Fig. 7). The macrophages contained several cisterns of granular endoplasmic reticulum (ER, Fig. 7), which were often layered in parallel. In many cells they were located near the surface and close to the area of contact with the central lymphocyte. Apart from this there was no regular orientation of the cytoplasmic organelles in the macrophage with respect to the central lymphocyte.

## Discussion

Morphological evidence for physical interaction between macrophages and lymphocytes in vitro has come mainly from light microscopic studies. Siegel (28) and Lipsky and Rosenthal (29) have investigated the antigen-independent binding of autologous thymocytes to macrophages. In the absence of antigen, nonimmune lymphocytes adhered to syngeneic macrophages after 1 h of culture. Each lymphocyte was closely attached to the macrophage with a broad area of contact, and no particular region of the lymphocyte surface was preferentially in contact with the macrophage (29). Others (17-19) have been concerned with the subject under study in the present report, namely antigen-dependent binding of autologous lymphocytes to macrophages. In the presence of PPD, immune cells formed clusters containing one macrophage and several lymphocytes. While light microscopic studies of these clusters indicated that the lymphocytes were all attached to the macrophage (1, 17-19), the present electron microscopic study revealed that only one of the cluster's lymphocytes is directly attached to the macrophage. The most simple cluster consists of one macrophage, one central lymphocyte attached to the macrophage with a broad area of contact, and several peripheral lymphocytes attached to the central one by their uropods. This structure is unique and has to our knowledge not been reported previously.

The peripheral lymphocytes are distinguished by the possession of a uropod. Studies of the lymphocyte uropod have mainly been conducted on cells stimulated by soluble phytohemagglutinin (PHA) in vitro (30-32) and on cells in mixed lymphocyte cultures (33-36). Observed by time-lapse cinematography (33-35) lymphocytes of such cultures exhibit increased cell motility and uropod formation, and have been seen to interact with lymphoblasts and macrophages in clusters, the point of contact being at the end of the uropod. The peripheral lymphocytes of the clusters described here have a fine structure, which only at few points differ from the fine structure of small lymphocytes studied in vitro during PHA stimulation (30-32) or in mixed lymphocyte cultures (35, 36). The major morphological differences were encountered in the fine structure of the uropods. The numerous microspikes and microvilli present on the tip of the uropod of PHA-stimulated lymphocytes (30-32) and on lymphocytes from mixed lymphocyte cultures, were not found by us, nor were the "vesicular bleps", the great number of mitochondriae, Golgi saccules, vesicles, and ribosomes (30, 32). On the other hand, the association of a single mitochondrion and one flat vesicle, which we have noted at the flattened tip of many uropods was not present in PHA-stimulated lymphocytes. These differences may reflect different functional
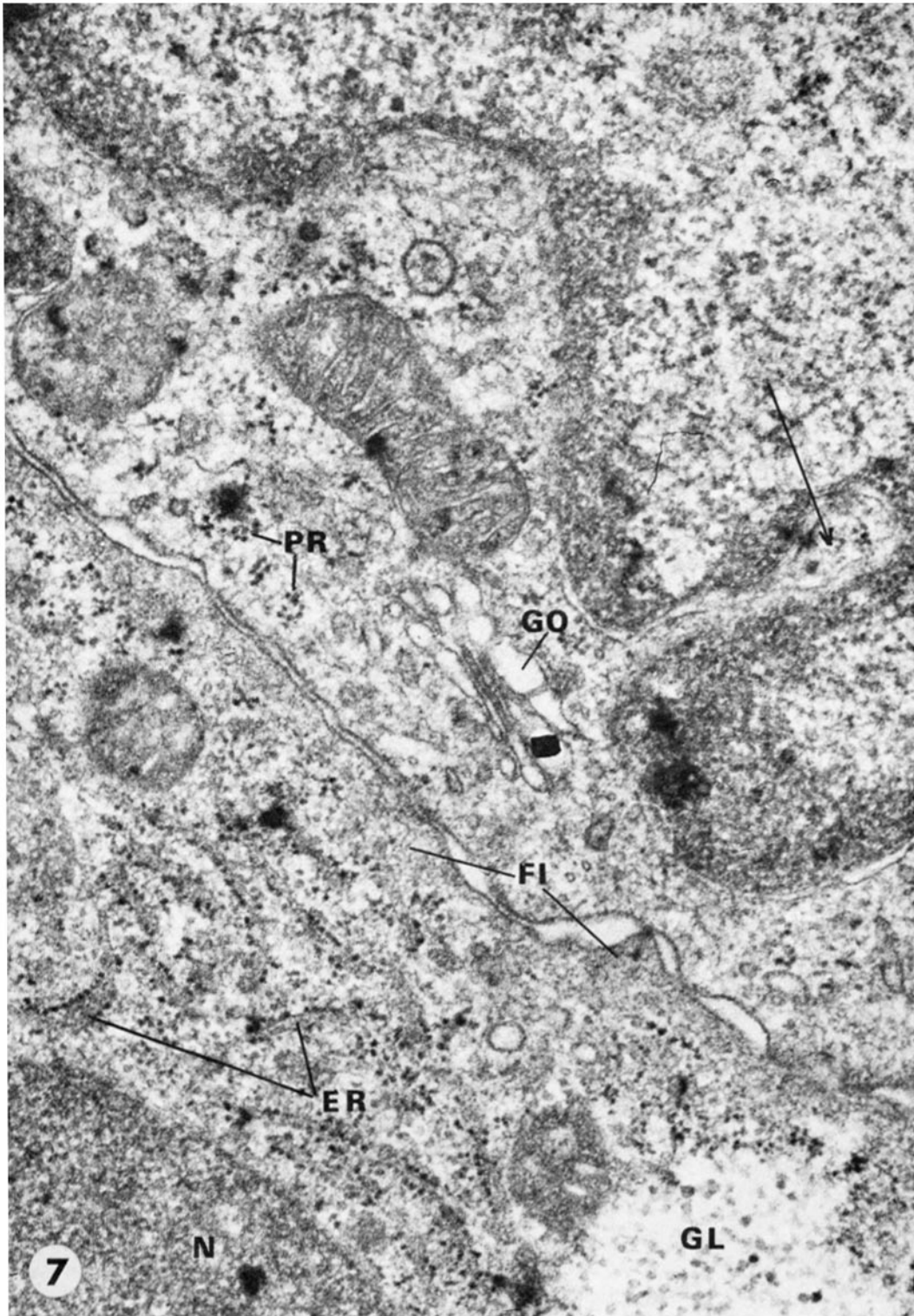
Fig. 7. Part of a central lymphocyte (upper right) and a macrophage (lower left). The indentations of the nuclear membrane (*arrow*) and the Golgi apparatus (GO) of the central lymphocyte face the macrophage. Polyribosomes are marked (PR). (N) denotes the nucleus, (ER) the granular endoplasmic reticulum, and (GL) glycogen granules of the macrophage cytoplasm. Microfilaments (FI) are visible in the macrophage cytoplasm only. × 67,500.

states of the lymphocytes. The marked accumulation of microfibrils possibly provides for the rigidity of the uropod, which has been observed in cinematographic studies (33). This could be of importance in maintaining the mutual position of the lymphocytes during interaction in the cluster. Previous publications have described bridge formation and cytoplasmic flow at the point of contact between lymphocytes (7), and between macrophages and lymphocytes (3, 7). In spite of thorough examination of the junction between peripheral and central lymphocyte, and between macrophage and central lymphocyte, we have consistently failed to demonstrate fusion of plasma membranes in the clusters.

The fine structure of the central, not uropod-bearing lymphocyte differed from the fine structure of the peripheral lymphocytes. The nucleus was larger than the nucleus of the peripheral lymphocytes, contained less heterochromatin, and had one or two enlarged nucleoli. The mitochondriae displayed an electron-translucent matrix, the Golgi apparatus was large, and polyribosomes replaced the single free ribosomes of the peripheral lymphocytes. These morphological characteristics indicate that the central lymphocyte is in an early stage of blast transformation. In fact, its structure is very similar to the structure of the large transformed lymphocytes of PHA-stimulated cultures (32). One might speculate that the constant orientation of the nucleus and cytoplasmic organelles with respect to the macrophage attachment indicates that the central lymphocyte attached initially to the macrophage through a uropod, which disappeared during the subsequent stages of cell interaction. Probably, the peripheral lymphocytes attached to the central one after its first contact had been established with the macrophage.

The central lymphocytes were easily identified in the scanning micrographs by their position in the clusters, and by their complex surface structure with multiple protrusions resembling microvilli covering the free surface. This contrasted to the peripheral lymphocytes which in general had a smooth surface with only a few ridgelike digitations. A recent scanning electron microscopic study by Polliack et al. (37) revealed that circulating human B lymphocytes have a villous surface, while circulating T lymphocytes in general have a smooth surface. Comparison between the scanning micrographs of typical B lymphocytes and those of the central lymphocytes described in the present paper have shown a striking resemblance. On the other hand, other workers, employing immunological markers for transmission electron microscopy, found no constant difference between the ultrastructure of nonstimulated murine T and B lymphocytes (38, 39). The exact nature of the central lymphocyte thus remains to be established, and is currently being investigated in our laboratory. However, the finding (1) that immune LNC enriched in T lymphocytes by column-purification produced almost twice the number of clusters per culture as did the same number of not column-purified LNC on monolayers of autologous macrophages with PPD present, suggests that the lymphocytes incorporated into clusters are T lymphocytes.

The peripheral lymphocytes are distinguished by the possession of a uropod. Uropod formation has mainly been observed in cell cultures stimulated by soluble PHA in which the responding cells are primarily T lymphocytes (40), and mixed lymphocyte cultures in which the responding cells are exclusively T lymphocytes

(41). A recent study directly indicates that in guinea pigs uropod-formation is exhibited exclusively by lymphocytes which lack easily detectable surface membrane immunoglobulin (42). Furthermore, uropod-bearing lymphocytes from mice all display T-lymphocyte surface antigens (43). These data strongly indicate that the peripheral lymphocytes of clusters are T lymphocytes, and support the suggestion based on experiments on cluster formation by column-purified cells (1) that both peripheral and central lymphocytes belong to the T-lymphocyte population.

As discussed in the companion paper (1) the role of macrophage-lymphocyte clusters in the immune response may be to provide a microenvironment suitable for cooperation between macrophages and lymphocytes through surface contact and/or soluble factors. It is tempting to speculate that the clusters may function as cooperation units in which lymphocytes receive the stimulatory signal that leads to blast transformation. The peculiar arrangement of the cluster's lymphocytes suggests that the microenvironment may also enable lymphocytes to cooperate with lymphocytes through surface contact. One can only speculate about the biological relevance of interaction between blast-transformed and non blast-transformed lymphocytes.

## Summary

Macrophage-lymphocyte clusters are formed when lymph node cells and autologous peritoneal exudate cells from guinea pigs immunized with tubercle bacilli are cultured in the presence of purified protein derivative of tuberculin (PPD) for 20 h. We have studied the ultrastructure of these clusters employing transmission and scanning electron microscopy. The most simple macrophage-lymphocyte cluster consisted of one macrophage, one large central lymphocyte with a blastoid appearance attached to the macrophage with a broad area of contact, and from a few to more than 20 small peripheral lymphocytes attached to the central lymphocyte by their uropods. Some clusters were of more complex type, containing two or three macrophages or one macrophage with more than one central lymphocyte attached to the surface, but even in these clusters each peripheral lymphocyte was attached only to one central lymphocyte. By morphological criteria the peripheral lymphocytes were T lymphocytes.

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