The Alveolar Macrophage Delivery System

Kinetic Studies in Cultured Explants of Murine Lung

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The production of alveolar macrophages in a blood-free organ culture system has been studied to determine whether free macrophages undergo mitosis or whether their ongoing production is dependent upon continuing division of interstitial cells. Explants of murine lung were attached to cellophane or glass; after 6 days of culture, a population of cells identified morphologically and functionally as macrophages appeared around the central tissue. These cells did not divide, and they disappeared 4 days after removal of the central lung explant. ^{3}H-Thymidine labeling of these peripheral macrophages was observed only when the central tissue was present and when the thymidine pulse was 24 hours in duration. Actual cell division was observed only in the interstitial cells of the explant. It is concluded that the interstitial cell population provides a continuing pool of precursor cells that divide and migrate outwards, creating a "steady state" system in which macrophage loss at the periphery is balanced by cell production at the center. (Am J Pathol 83:123–134, 1976)

MONONUCLEAR PHAGOCYTES originate from precursor cells in the bone marrow, are transported in the blood as monocytes, and eventually become tissue macrophages.[1] Differences in morphologic, metabolic, and functional characteristics between the blood monocyte and the alveolar macrophage suggest a process of maturation before the macrophage reaches the air sac.[2] Based on a study using whole-body irradiation to suppress circulating monocytes, we proposed a pulmonary interstitial compartment intermediate between the blood monocyte and the functional free alveolar macrophage.[3] This conclusion was confirmed by the results of a later study in which the production of alveolar macrophages from proliferating interstitial cells was demonstrated in an *in vitro* system devoid of circulating monocytes.[4]

The demonstration that alveolar macrophages arise from a population of interstitial mononuclear cells does not answer the question of whether the free macrophage is capable of further division. The present study was designed to determine if the production of alveolar macrophages in organ

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cultures of lung is dependent upon continuing division of interstitial cells or upon proliferation of free cells.

Materials and Methods

Culture System

Male Wistar albino mice were sacrificed, the lungs removed aseptically, and 1 cu mm pieces were attached to sterile cover glasses (five per 25-mm-square glass) in plastic petri dishes measuring 35 × 10 mm. Two petri dishes were prepared from each animal sacrificed. Adhesion was achieved by placing the explants on the glass, adding T8 medium to cover most of the tissue, and placing the petri dish in an incubator at 37 C with a mixture of 95% oxygen and 5% carbon dioxide in constant flow.[4] After a few hours, the tissue became adherent and additional medium was added to cover it. The explants were cultured for varying periods up to 2 weeks, the medium being changed every 3 days.

After 6 days of culture, a halo of cells appeared around the explant. With removal of the central core of tissue, the cover glass preparations were suitable for light microscopy; for electron microscopy, the explants were cultured on squares of sterile cellophane.

Morphologic Studies

The cells on cover glasses were fixed in a 10% formalin in 50% ethyl alcohol solution and stained with hematoxylin and eosin for light microscopy. The cells on cellophane squares were fixed by buffered 2% osmic acid, a narrow strip of cellophane containing a halo of cells was cut out, processed, and embedded in Spurr for electron microscopy.

Phagocytic and bactericidal activity of the macrophagic monolayer was tested by adding 0.1 ml of a dilute broth culture of either *Bacillus subtilis* or *Staphylococcus albus* to several petri dishes. Acid phosphatase activity in the cell halo was demonstrated by incubating the monolayers on cellophane in Gomori's medium prior to electron microscopy.[5]

Kinetic Studies

Two petri dishes each containing five explants were used, and 5000 monolayer cells were examined at each time studied. Autoradiographs were prepared using Kodak NTB 2 emulsion on Feulgen-stained cover glass preparations. The following experiments were performed.

Experiment 1

Explants were cultured for intervals up to 14 days, with ^{3}H-thymidine (0.075 μCi/ml) being added to the medium for the final 24 hours.

Experiment 2

Explants were cultured for 6 days with colchicine (0.04 mg/ml) added to the medium for the final 4 hours to arrest dividing cells in metaphase. The lung explants were sectioned for microscopy, and the monolayers stained by hematoxylin and eosin.

Experiment 3

Two series of explants were cultured for 6 days. In one, the lung pieces were removed; in the other, they were left undisturbed. Each was then returned to the incubator. Subsequently, the cell number around each explant was determined at daily intervals over the next 5 days.

Experiment 4

Experiment 3 was repeated with ^{3}H-thymidine added to the medium for the final 24 hours of culture. In addition to the cover glass monolayer preparations, autoradiographs were made from sections of the lung explant.

Experiment 5

Lung explants were cultured for 6 days, pulse labeled with ^{3}H-thymidine for 24 hours and then cultured further in unlabeled medium for periods up to 10 days.

Results

Morphologic Studies

A few mononuclear cells were observed by light microscopy at the periphery of each explant after 3 days of culture; by 5 to 6 days a distinct halo of cells was visible to the naked eye. The size of the peripheral monolayer did not increase after the eighth day of culture. Figure 1 illustrates the monolayer following removal of the central core of tissue. The cells of the monolayer were 20 to 50 μ in diameter, with uniform round nuclei and few, if any, cytoplasmic inclusions (Figure 2). The cells adhered firmly to glass and cellophane (Figure 3) and were not removed by repeated washings. Numerous pseudopodia were observed closely apposed to the supporting cellophane (Figures 3 and 4). The cytoplasm contained numerous microtubules and many small lysosomes which were shown to contain acid phosphatase (Figures 5 and 6). Collectively, these features indicate the macrophagic nature of the peripheral monolayer. The function of these macrophages was tested by the addition of broth cultures of bacteria and it was found that 90% of the cells phagocytosed the bacteria (Figure 7).

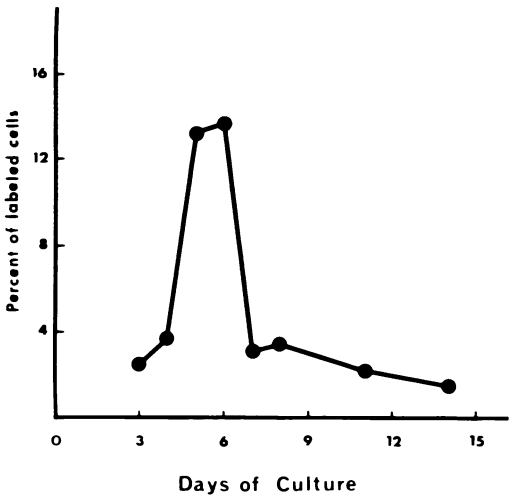
Kinetic Studies

Experiment 1

Explants were cultured up to 14 days and pulse labeled in the final 24 hours with ^{3}H-thymidine. The percentages of labeled macrophages on the cover glass are shown in Text-figure 1. Maximum DNA labeling was observed on Days 5 and 6 of culture; subsequently the value dropped and a basal level of about 3% was maintained for the remainder of the 14-day period.

Experiment 2

Explants were cultured for 6 days with colchicine added for the final 4 hours. Numerous mitoses were observed in the interstitial cells within the

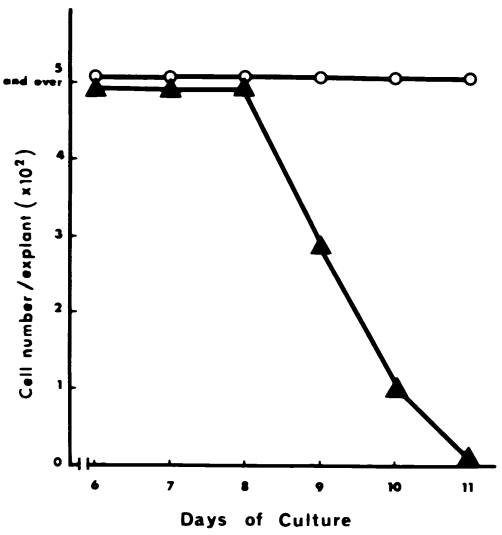


TEXT-FIGURE 1—Percentage of labeled cells on cover glass following a 24-hour pulse with ^{3}H-thymidine after varying intervals of culture (Experiment 1).

explant (Figure 8); none was seen in the peripheral outgrowth of macrophages.

Experiment 3

Text-figure 2 demonstrates the effect of removing the central core of lung tissue after 6 days of culture. In the presence of the lung explant, the number of macrophages is maintained, whereas after removal of the explant the number of cells in the monolayer declines rapidly over the subsequent few days.



TEXT-FIGURE 2—Number of cells per monolayer when central explant is removed (*triangles*) or left in place (*circles*) following 6 days of culture (Experiment 3).

Experiment 4

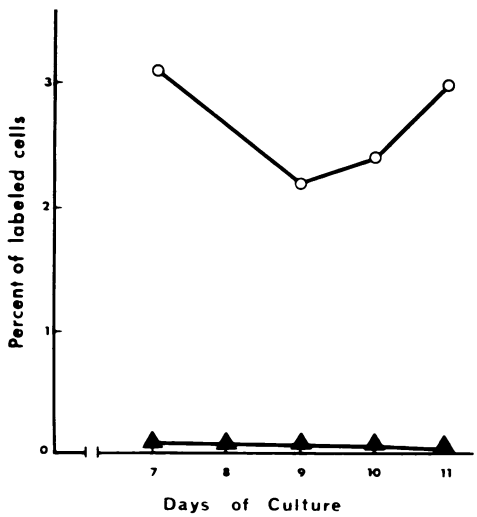
Thymidine incorporation into the macrophages under the conditions of Experiment 3 was negligible in cultures from which the explant had been removed. With the explant present, the basal level of 2 to 3% was maintained following a pulse label of 24 hours' duration (Text-figure 3). Autoradiographs prepared from the explant revealed that the interstitial cells of the lung and macrophages at the tissue edge were labeled with the tritiated thymidine (Figure 9). Morphologic evidence supporting outward migration of macrophages from the central explant is provided by Figure 10, in which a macrophage is shown apparently squeezing between two cells at the periphery of the explant.

Experiment 5

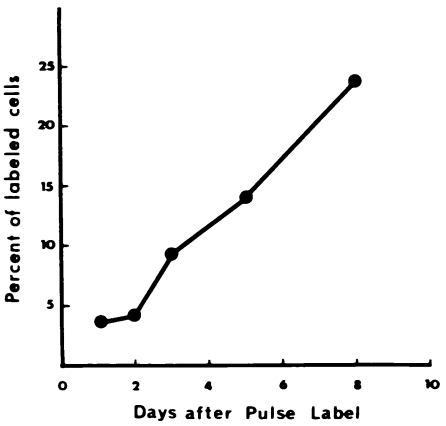
When lung explants were cultured for 6 days, pulsed with ^{3}H-thymidine for 24 hours, and then cultured further in unlabeled media, the percentage of labeled macrophages in the monolayer rose from 3 to 24% in the subsequent 8 days in the absence of free isotope (Text-figure 4).

Discussion

The kinship of the alveolar macrophage and the circulating monocyte is established,[1,2,6] and there is evidence suggesting an intermediate compartment of macrophage precursor cells in the interstitium of the lung.[3,4,7] In organ cultures of lung, a new population of large mononuclear cells has been observed at the periphery of the explanted tissue after 3 days.[4] In the present study, attachment of pulmonary explants to cover



TEXT-FIGURE 3—Percentage of labeled cells in monolayers after 24 hour ^{3}H thymidine pulse, when central explant is removed (*triangles*) or left in place (*circles*) following 6 days of culture (Experiment 4).



TEXT-FIGURE 4—Percentage of labeled cells in monolayer following culture for 6 days, 24-hour pulse with ^{3}H-thymidine, and further culture in unlabeled medium (Experiment 5).

glass or cellophane allowed the collection of these cells at the tissue periphery. The morphologic features of these outgrowing cells and their ability to phagocytose inert particles and bacteria characterize them as macrophages, and since the culture system is free of circulating monocytes, the production of a cellular halo around each explant confirms a local origin for these macrophages.

Although the size of the peripheral cellular halo remained constant after the initial 8 days of culture, daily pulsing with ^{3}H-thymidine gave a steady 3% labeling index from 8 to 14 days. This may be explained either as DNA synthesis without division or as macrophagic turnover, with the production of new cells balanced by the disappearance of older cells. DNA synthesis, as evidenced by ^{3}H-thymidine uptake, could not be demonstrated in the cells of the macrophagic monolayer subsequent to removal of the central explant. In addition, the progressive disappearance of derived macrophages from the cover glass following removal of the central explant suggests that continuing production of macrophages is dependent on the immediate presence of other cells. It is known that factors secreted by cells such as L fibroblasts will initiate mitotic activity in cultured alveolar macrophages.[8] In the present instance, no mitotic activity was observed in the macrophage monolayer either in the presence or absence of the explant. It is concluded, therefore, that the peripheral macrophages in this culture system do not divide.

Labeling of the peripheral macrophages with ^{3}H-thymidine was demonstrated, but only in the presence of the explant and after a pulse label of sufficient duration to allow for division of a macrophage precursor cell. Autoradiographic evidence of interstitial cell and macrophage labeling coupled with the demonstration of mitoses in the interstitial cells of the

explant suggest that the macrophages of the monolayer are the progeny of a dividing population of interstitial cells in the explant. This conclusion is supported by the results of Experiment 5, in which labeled explants with monolayers were transferred to unlabeled medium. Subsequently, a progressive increase in the percentage of labeled macrophages was observed; since these cells do not divide, the labeled cells could only have been derived from dividing labeled precursor cells in the explant. It is concluded that the macrophagic population around the explants arises by continuing division of interstitial cells and migration of daughter cells into the "airways" of the explant from which they further migrate to the cover glass or cellophane. This confirms our earlier findings of a temporal relationship between interstitial cell proliferation and macrophagic production.[3,4]

The adaptive capacity of the lung to increase the output of macrophages by a purely local mechanism is important to its handling of foreign material, particularly in an overload situation.[9] In some instances, such as in viral infections or granulomatous diseases, a phase of interstitial cell proliferation precedes the increase in macrophagic output;[10,11] in other cases, the local macrophagic delivery system may be supplemented by direct migration of blood monocytes.[12] It is also possible that, under certain conditions, free macrophages may divide *in situ*.[13,14] Such local proliferation of free alveolar macrophages could perhaps be operative in the inflammatory response to alveolar injury. The results of the present study support the hypothesis that the free alveolar macrophage usually exists in the inactive G_0 phase of the mitotic cycle and is an "end of the line" cell whose continuing production and delivery to the air sacs is dependent upon division and migration of cells from the pulmonary interstitial compartment.

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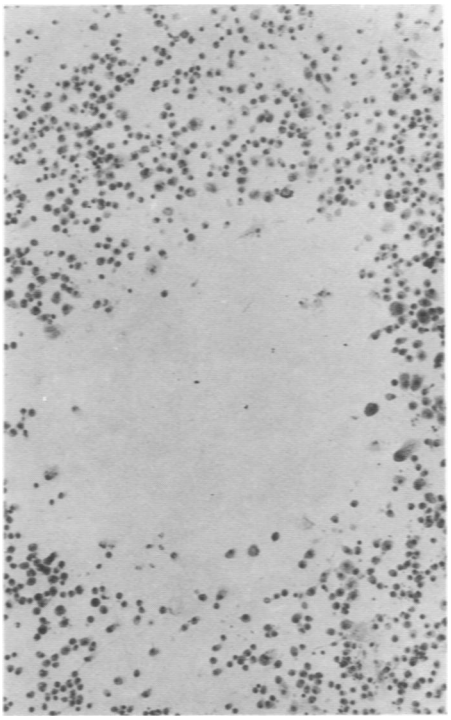

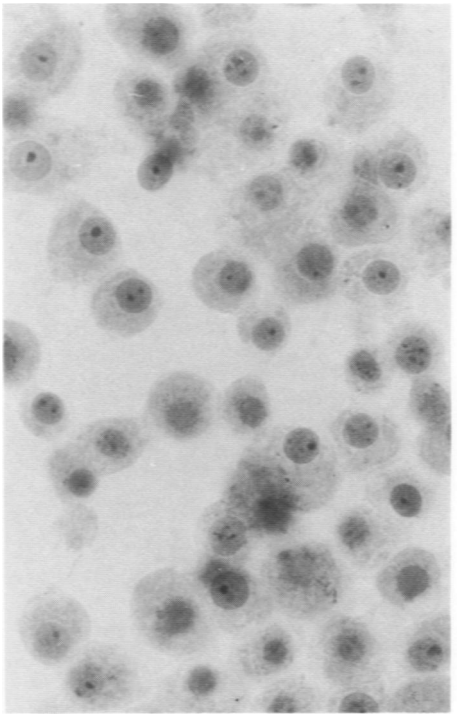

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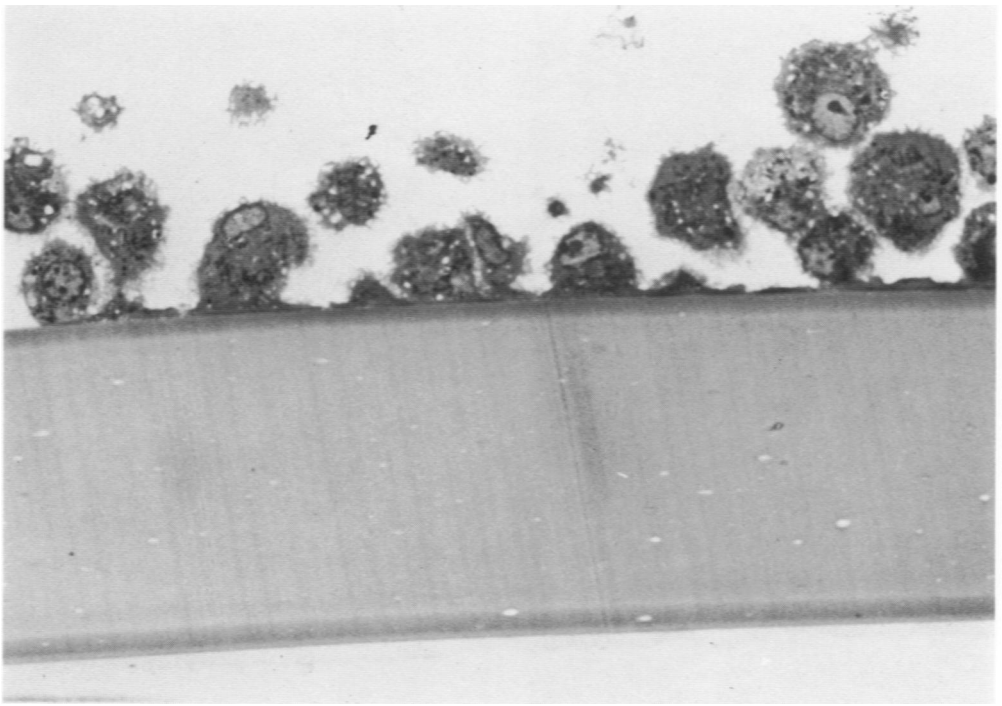

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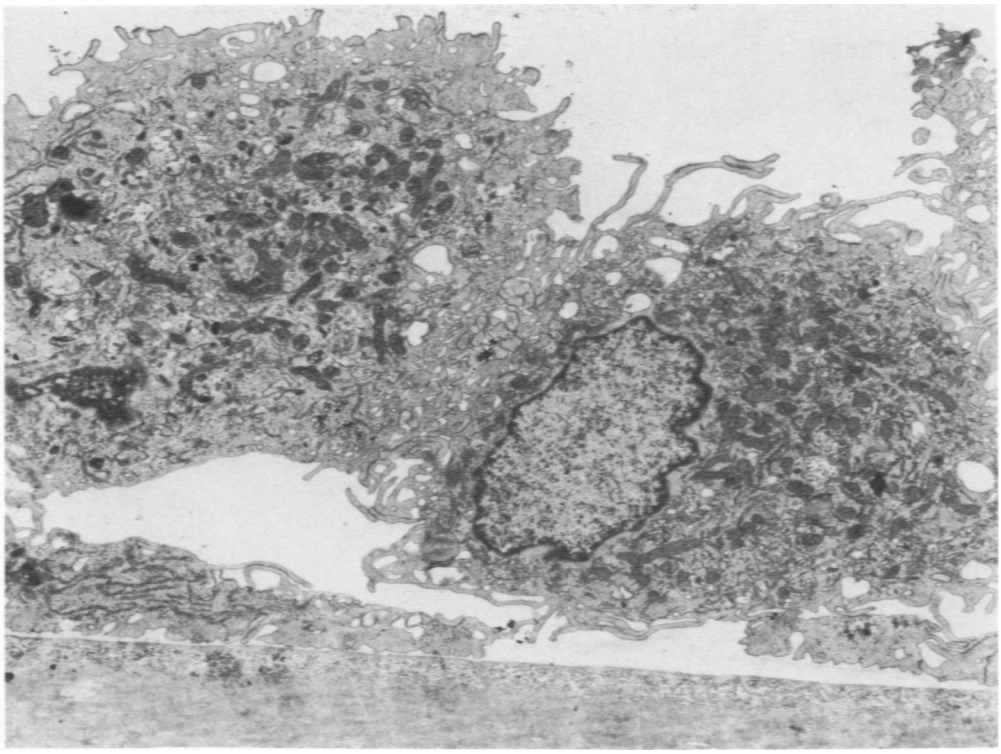
Figure 1—Peripheral halo of cells which develops when a pulmonary explant is cultured on cover glass; the central core of tissue has been removed (H&E, ×40). **Figure 2**—Higher magnification of Figure 1; the large mononuclear cells resemble macrophages (H&E, × 200). **Figure 3**—Section (0.5 μ) of peripheral monolayer showing pseudopodal attachment of cells to underlying cellophane (Toluidine blue, × 400).



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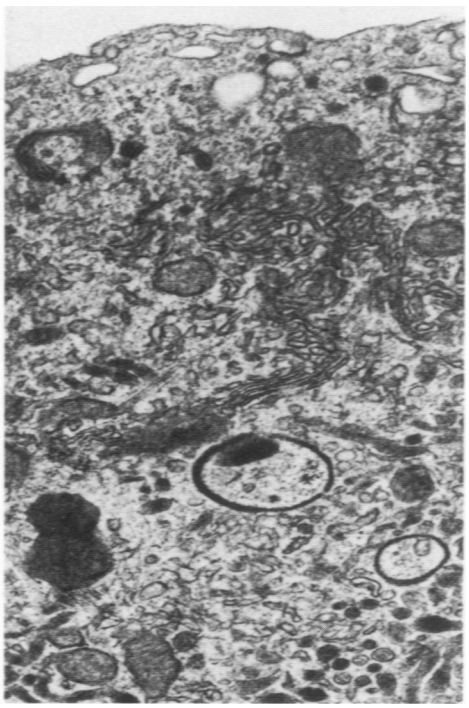

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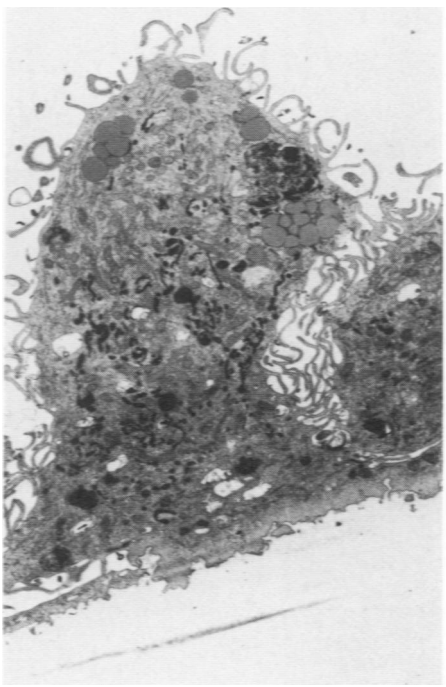

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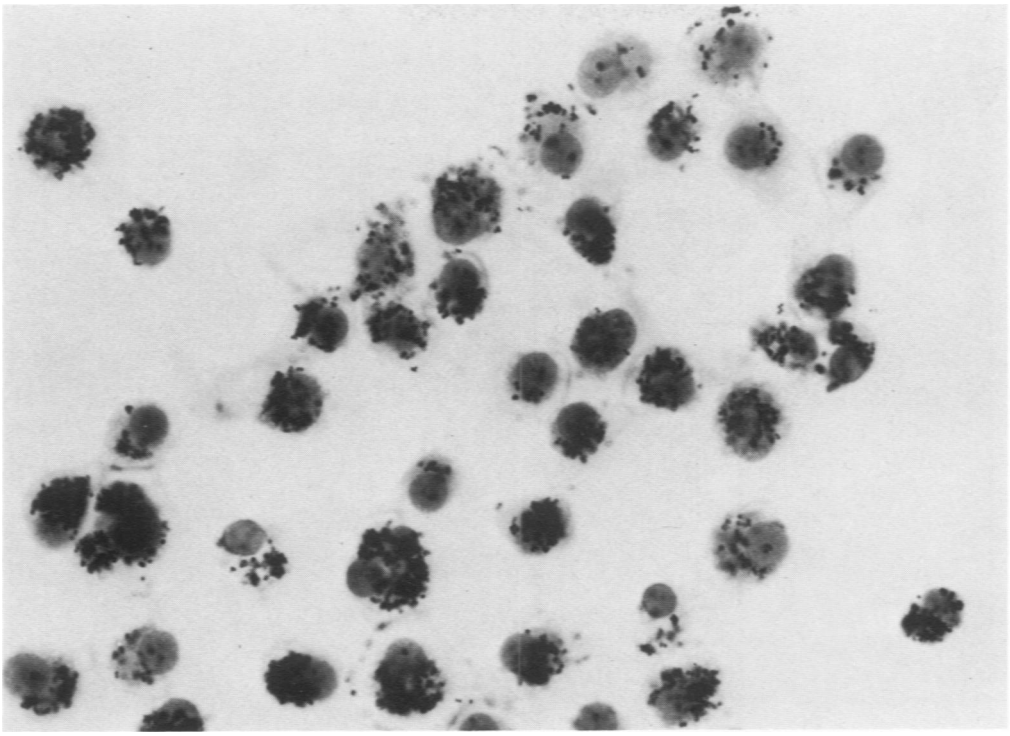
Figure 4—Cells of the peripheral monolayer cultured on cellophane; these cells have the ultrastructural appearance of macrophages (× 3800). **Figure 5**—Fine structure of cytoplasm showing microtubules and lysosomal bodies (× 20,000). **Figure 6**—Multiple dense deposits mark the lysosomal sites of acid phosphatase activity in the monolayer cells cultured on cellophane (×3000).



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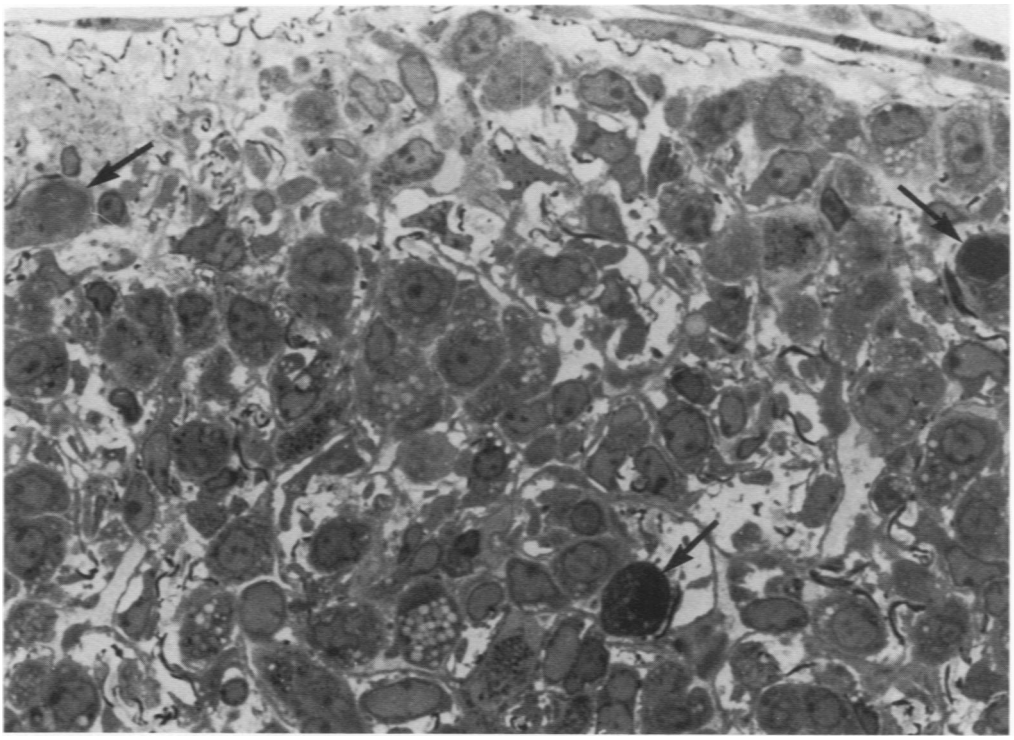

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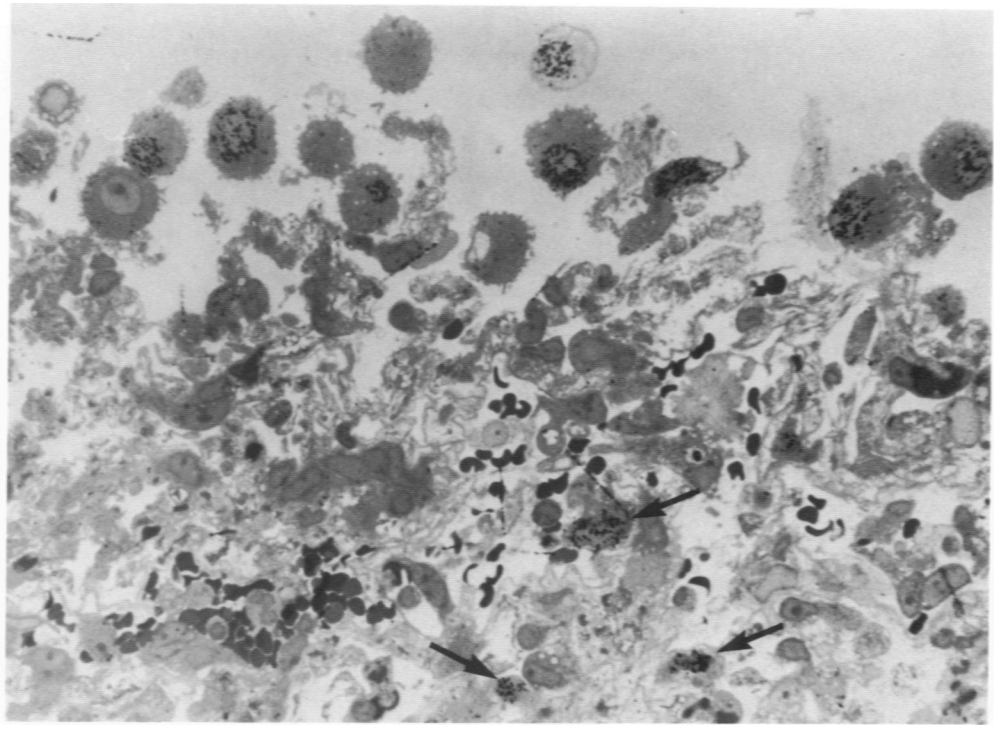
Figure 7—Phagocytosis of *Staphylococcus albus* by cells of the monolayer (H&E, × 350). **Figure 8**—Colchicine-arrested mitoses (*arrows*) in interstitial cells of cultured explant (0.5-μ section, toluidine blue, × 500)



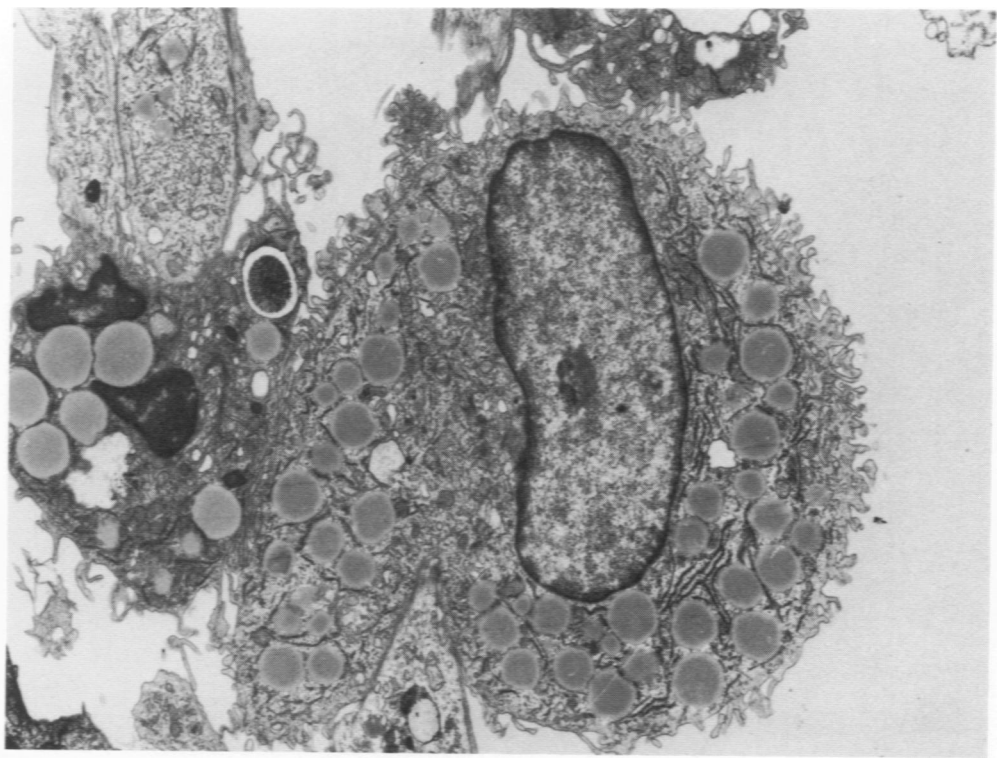


Figure 9—Autoradiograph of cultured explant after a 24-hour pulse of ^{3}H-thymidine (Experiment 4). Interstitial cells (*arrows*) and the macrophages near the edge of the explant carry the nuclear label. (0.5-μ section, toluidine blue, × 500) **Figure 10**—Electron micrograph of a cell apparently squeezing between two cells at the periphery of the explant (× 5500).