Ultrastructural Studies of the Interaction Between Liposome-Activated Human Blood Monocytes and Allogeneic Tumor Cells in Vitro

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Human blood monocytes were activated to become tumoricidal by incubation with liposomes containing muramyl tripeptide-phosphatidylethanolamine, a lipophilic derivative of muramyl dipeptide. The interaction of both tumoricidal and control monocytes with target melanoma cells was analyzed by means of light microscopy and scanning and transmission electron microscopy. The authors found increased clustering around the melanoma cells by tumoricidal monocytes as compared with the control monocytes. The initial clustering of the tumoricidal monocytes around the tumor cells was followed by the establishment of numerous focal points of contact (binding), some of which actually exhibited areas of discontinuous membrane, a finding confirmed by stereophotography. By 24–48 hours of cocultivation, many of the target cells exhibited zones of vacuolation in the immediate vicinity of the tumoricidal monocytes, suggesting target cell damage. (This finding was confirmed by time-course cytotoxicity assays.) The authors conclude that tumor cell lysis mediated by activated human blood monocytes occurs as the final step in a process that includes the establishment of a direct cell–cell contact, damage to the target cell membrane, and the development of areas of vacuolation in the target cells. (Am J Pathol 1983, 112:101–111)

MONOCYTES or macrophages obtained from healthy, normal donors are usually not cytotoxic *in vitro* to tumorigenic target cells. Various agents such as lymphokines, whole bacteria, and/or bacterial products can activate cells of the monocyte–macrophage series to become tumoricidal.[1–7] Tumoricidal macrophages of many species can lyse target tumor cells without harming nontumorigenic cells, even under cocultivation conditions.[8–9]

There is some controversy over the exact mechanism(s) by which the lysing happens. Most,[1–11] but not all,[12,13] investigators have suggested that the first step in the process involves direct contact between the macrophage and the target cell. Subsequently, hydrolytic lysosomal enzymes may be transferred into the target cells,[10,11] or secretory products may be released from macrophages in the areas where they contact the target cells.[14–19] Most studies supporting these theories have used rodent models, and few comparable data are available on the interaction of human blood monocytes with susceptible tumorigenic target cells.

Recently, we reported that the endocytosis of liposomes (multilamellar vesicles) containing lymphokines[20] or muramyl tripeptide[21] by human blood monocytes rendered the cells tumoricidal *in vitro* against various allogeneic tumorigenic cells. We report here on ultrastructural studies of the interaction of activated monocytes with allogeneic melanoma cells and

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provide morphologic data that elucidate the mechanism(s) involved in the lysis of tumor cells by activated human blood monocytes.

Materials and Methods

Reagents

RPMI 1640, human AB serum, Hanks' balanced salt solution (HBSS), and fetal bovine serum (FBS) were purchased from M. A. Bioproducts, Walkersville, Maryland. N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide—MDP) and N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1′, 2′-dipalmitoyl) phosphatidylethanolamine (muramyl tripeptide-phosphatidylethanolamine—MTP-PE) were obtained from Ciba-Geigy Ltd., Basel, Switzerland. All reagents were free of endotoxins as determined by the *Limulus* amebocyte lysate assay (sensitivity limit, 0.125 ng/ml).

Target Cell Cultures

The human melanoma cell line A375 was adapted for growth in culture as described previously.[22] The cells were free of *Mycoplasma* and were maintained on plastic in Eagle's minimum essential medium supplemented with 5% heat-inactivated FBS, sodium pyruvate, and L-glutamine (M. A. Bioproducts) at 37 C in a humidified atmosphere containing 5% CO_2. Cytotoxicity assays were performed when the cultured target cells were in their exponential growth phase.

Phospholipids and Preparations of Liposomes

Chromatographically pure egg phosphatidylcholine (PC) and beef brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids, Birmingham, Alabama. The phospholipids dissolved in chloroform were admixed at a 7:3 mol ratio of PC to PS. MTP-PE was dissolved in methanol–chloroform (1:2) and added to the phospholipids. After evaporation of the solvents and appropriate drying procedures, HBSS was added and liposomes were produced by mechanical agitation.[23] The concentration of MTP-PE in the liposomes was 5 μg MTP-PE/μmol phospholipids. The incorporation of MTP-PE into the phospholipid bilayer membrane was confirmed by the ability of anti-MDP antibodies to specifically precipitate liposomes containing MTP-PE.[24] Practically all (>98%) of the MTP-PE was incorporated into the liposomes as determined from studies with radiolabeled MTP-PE.[24]

Isolation and Culture of Human Blood Monocytes

Human mononuclear leukocytes from the peripheral blood of normal donors were separated on lymphocyte separation medium (Litton Bionetics, Kensington, Md) and washed twice in HBSS. Peripheral blood monocytes were isolated from the mononuclear leukocytes by a further separation on a continuous preformed Percoll gradient as previously decribed.[25] Briefly, mononuclear blood leukocytes (40×10^6) were layered onto preformed Percoll gradients in 15-ml polycarbonate tubes and spun in swing-out buckets in a refrigerated centrifuge at 1000*g* for 20 minutes. When centrifuged, cell populations layered on the Percoll gradient were separated on the basis of their relative densities into two distinct bands. The upper band was enriched for monocytes (70–90%), as determined by nonspecific esterase staining and morphologic criteria. The cells from this band were harvested, washed twice in HBSS, resuspended in RPMI 1640 with 5% human AB serum, and adjusted to contain 1×10^6 monocytes/ml. For the *in vitro* cytotoxicity assays, 1×10^5 monocytes were added to each well of a 96-well, flat-bottom Microtest II plate (Falcon Plastics, Oxnard, Calif) that had been pretreated with FBS for 1 hour at 37 C (the serum was removed before the addition of monocytes). The monocytes were allowed to adhere for 1 hour at 37 C; the plating efficiency was >90%. After incubation, the nonadherent cells were removed, and the plates were washed three times with RPMI 1640. The purity of the monocytes at this point was >99%, as assessed by ingestion of carbon particles, morphologic features, and nonspecific esterase staining. Moreover, staining with a monoclonal antibody 61D3 directed against human monocytes revealed that all adherent cells were positive (Bethesda Research Laboratories, Bethesda, Md).

In Vitro Activation of Monocytes

Monocytes were incubated in RPMI alone, in RPMI containing 100 nmol/ml of liposomes containing MTP-PE, or in 100 nmol of control liposomes that contained HBSS and that were suspended in RPMI with free MDP at a concentration equivalent to that trapped within the liposome. Twenty-four hours later, the monocytes were washed thoroughly with RPMI; then radiolabeled target cells were added

for the cytotoxicity assay or morphologic examination.

Monocyte-Mediated Cytotoxicity

Cytotoxicity was assessed by a modification of a radioactive release assay previously described.[26] Target cells in exponential growth phase were incubated for 24 hours in the appropriate medium containing ^{125}I-iododeoxyuridine (0.3 μCi/ml; specific activity, 200 mCi/μmol) (New England Nuclear, Boston, Mass). The cells were then washed twice to remove unbound radiolabel and were harvested by a 1-minute trypsinization with 0.25% Difco trypsin and 0.02% EDTA. The labeled cells were resuspended in the medium, and 10^4 cells were plated into the culture well to obtain an initial target–effector-cell ratio of 1:10. Radiolabeled target cells were plated alone as an additional control group. After 24 hours, all cultures were aspirated to remove the nonplated cells, refed with fresh medium, and then cultured for up to an additional 2 days. At the indicated times the cultures were washed twice with HBSS, and the adherent viable cells were lysed with 0.1 ml of 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter.

Calculation of the Percentage of Monocyte-Mediated Cytotoxicity

The percentage of cytotoxicity was calculated according to the formula:

$$\text{Percent generated cytotoxicity} = \frac{\text{cpm in target cells cultured with control monocytes} - \text{cpm in target cells cultured with test monocytes}}{\text{cpm in target cells cultured with control monocytes}} \times 100$$

Experimental results were analyzed for their statistical significance by the Student two-tailed *t* test.

Morphologic Studies

All morphologic studies were carried out in parallel with the cytotoxicity assays. Purified human blood monocytes were plated on glass coverslips at a density identical to that in the Microtest II plates used in the cytotoxicity assay. Samples of monocytes were taken before and after the addition of liposomes and before and after the addition of target cells. To study the interaction of monocytes and tumor cells, samples were prepared 6, 12, 24, 48, and 72 hours after the addition of target cells. All samples were washed with serum-free medium and processed for examination by light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Preparation of Cells for Scanning Electron Microscopic Examination

Cells grown on coverslips were washed twice with HBSS and treated with a fixative containing 3% glutaraldehyde and 2% paraformaldehyde buffered with 0.1 M cacodylate buffer at pH 7.4. After 1 hour, the samples were washed twice with 0.1 M cacodylate buffer, fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes, and then washed three times with distilled water. The samples were dehydrated with a graded series of ethanol followed by a graded series of Freon 113, and they were critical-point-dried in Freon 13 in a Bomar critical-point dryer SPC-900/EX (Bomar Co., Tacoma, Wash). They were then coated with platinum-palladium alloy (100–150 Å of 80:20 alloy) by evaporation and examined in a Hitachi HFS-2 field emission scanning electron microscope.

Preparation of Cells for Light-Microscopic and Transmission Electron Microscopic Examination

Samples on coverslips were placed in a fixative containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 for 30 minutes at room temperature. The samples were then transferred to a freshly prepared solution of 1% tannic acid in 1% glutaraldehyde for 20 minutes. The samples were rinsed three times with cacodylate buffer, postfixed with 1% buffered osmium tetroxide for 1 hour, and rinsed with buffer and then with distilled water. Samples for light-microscopic examination were mounted on glass slides with Difco mounting medium. The samples were examined by phase and interference microscopy with the use of a Leitz Orthoplan microscope. For TEM, the samples were stained *en bloc* with 1% aqueous uranyl acetate for 20 minutes, rinsed briefly with distilled water, dehydrated with a graded series of ethanol, embedded in Emix resin (Ted Pella, Inc., Box 510, Tustin, Calif), and polymerized in a 60 C oven for 2 days. The coverslips were removed by transferring the block directly from the oven to liquid nitrogen and then to a 56 C water bath. The blocks were dried, placed in the 60 C oven for at least 1 hour, and cooled before sectioning. Thin sections were cut with a diamond

knife in an LKB Ultratome III. The sections were stained with Reynold's lead citrate for 3 minutes and examined in a Hitachi HU-12A transmission electron microscope at an accelerating voltage of 75 kv. Samples with cell–cell contact were examined in detail using the HK-6 specimen tilt accessory of the transmission electron microscope. Stereomicrographs were obtained to verify occurrence of membrane fusion.

Results

Generation of Tumoricidal Blood Monocytes by Liposome-Entrapped MTP-PE

Because we used an adherent cell assay in which cell–cell contact between effector and target cells is required to achieve killing, washing the cultures removed the error introduced by target cells that did not adhere but were not necessarily killed by monocytes. In this assay the initial plating efficiency of the target cells (4 hours) is >90% when plated alone or with either control or activated monocytes (Table 1). The results of time-course studies shown in Table 1 demonstrate that when target cells are cocultivated with monocytes activated by liposomes containing MTP-PE (but not control monocytes), loss of radioactivity begins after 24 hours and reaches a maximum after 48 hours. For this reason we routinely terminate the monocyte–tumor-cell interaction assay after 72 hours of cocultivation. In all of the assays the degree of monocyte-mediated cytotoxicity was assessed by the amount of radioactivity associated with the DNA of adherent viable target cells. Loss of radioactivity, however, could also be attributed to the detachment of viable target cells and not necessarily to lysis. Therefore, we examined the distribution of ^{125}I-IUdR from A375 cells after 3 days of interaction with activated monocytes. The supernatant fluids from these cultures were filtered through a 0.45-μ Millipore filter. In three independent experiments, >93% of radioactivity was associated with filterable material. Moreover, we were unable to precipitate more than 10% of the total radioactivity associated with the unfiltered supernatants after centrifugation for 10 minutes at 250*g*. These data validate the conclusion that the measurement of ^{125}I-IUdR in remaining adherent target cells is a reliable index of monocyte-induced target cell lysis.

The results of previous studies from our laboratory have demonstrated that blood monocytes obtained from normal donors are not cytolytic to the human A375 melanoma target cells.[20,21] The data shown in Tables 1 and 2 closely agree with those of these studies. Monocytes incubated in medium containing liposome-encapsulated HBSS were not cytotoxic to the target cells. In contrast, blood monocytes incubated with liposomes containing MTP-PE were highly cytotoxic to the melanoma cells (47%, $P < 0.001$). These results are highly reproducible and are representative of >30 independent experiments recently carried out in our laboratories. Nonetheless, to assure a correlation between the functional (Table 2) and morphologic studies, we carried both out simultaneously with identical monocytes and tumor targets.

Table 1—Monocyte-Mediated Cytotoxicity Against A375 Melanoma: Time-Course Studies

Time of cocultivation (hours)	Percentage cytotoxicity mediated by*	
	Normal monocytes	Activated monocytes
4	7%	4%
24	2%	2%
36	3%	21%†
48	13%	55%†
72	10%	60%†

* Blood monocytes (10^5) were incubated for 24 hours in medium with 100 nmol liposomes containing HBSS (normal) or in medium with 100 nmol liposomes containing 5 μg MTP-PE/μmol lipid (activated). The monocytes were thoroughly washed before the addition of 10^4 ^{125}I-iododeoxyuridine-labeled A375 melanoma cells. The percentage of cytotoxicity was calculated by comparisons with tumor cells alone (normal-monocyte-mediated cytotoxicity) or with cultures with normal monocytes (activated-monocyte-mediated cytotoxicity).

† $P < 0.001$.

Light- and Scanning Electron Microscopic Analyses

Twenty-four hours after the incubation of monocytes with liposomes, the cultures were thoroughly washed, and A375 melanoma cells were added to the wells. By 4 to 6 hours later, the monocytes were clustered around the tumor cells. The degree of clustering was more pronounced with the tumoricidal monocytes (Figure 1A) than with control monocytes (Figure 1B). At 6, 12, 24, and 48 hours of cocultivation, the number of monocytes in contact with target cells was determined by examination of 40–50 samples prepared for SEM. At both 12 and 24 hours the average number of activated monocytes (10/tumor cell) was significantly higher than that of control monocytes (3/tumor cell). The number of monocytes attached to tumor cells decreased significantly after 48 hours. After 24 hours of cocultivation, the tumor cells incubated with nonactivated monocytes retained their polygonal shape (Figure 1D), whereas tumor cells incubated with activated monocytes exhibited

Table 2—*In Vitro*-Mediated Cytotoxicity Against A375 Melanoma by Monocytes Treated With Liposomes Containing MTP-PE

Monocyte treatment*	Residual radioactivity in viable cells on Day 3†
No monocytes, tumor cells alone	2624 ± 120
Medium alone	2401 ± 84
Liposomes containing HBSS suspended in free MDP	2584 ± 145
Liposomes containing MTP-PE	1202 ± 168 (47%)

* Blood monocytes (10^5) were incubated for 24 hours in medium, in medium with 100 nmol liposomes containing 5 μg MTP-PE/μmol lipid, or in medium with 100 nmol liposomes containing HBSS. The monocytes were thoroughly washed before the addition of 10^4 ^{125}I-iododeoxyuridine-labeled A375 melanoma cells.

† Radioactivity in viable cells after 72 hours of cultivation. Mean counts per minute ± SD of triplicate cultures. Number in parentheses is the percentage of generated cytotoxicity calculated by comparison with cultures with monocytes treated with liposomes containing medium ($P < 0.001$).

marked elongation with long cytoplasmic projections (Figure 1C). This effect was even more pronounced in the samples examined after 48 hours of cocultivation. By 72 hours of cocultivation, increased tumor cell density was observed in the wells with nonactivated monocytes. No proliferation of tumor cells was evident in the presence of activated monocytes.

Transmission Electron Microscopic Analysis

Transmission electron microscopic examination of human monocytes before incubation with liposomes indicated that the cell preparation was homogeneous. Many cells exhibited either a central or peripheral nucleus with an irregular or kidney shape. For this reason, thin sections of monocytes contained one or more portions of the nucleus. Bundles of microfilaments were often observed in the perinuclear region. Well-developed Golgi bodies were found in the indentation of the nucleus, and mitochondria were prominent in the cytoplasm. The monocytes were highly phagocytic, as evidenced by the abundance of endocytosed liposomes (Figure 2A). The liposomes containing HBSS and the liposomes containing MTP-PE appeared to be phagocytosed to a similar extent.

Clustering of monocytes around tumor cells was observed shortly after the populations were mixed. We conclude that the ability of monocytes to cluster around target cells is influenced by their state of activation, because only a few nonactivated monocytes (treated with liposomes containing HBSS) were found to aggregate around the melanoma cells or to form contact sites (Figure 2B). In contrast, the activated monocytes clustered around the target cells, established many wide areas of contact with tumor cells, and formed cytoplasmic processes probing into the tumor cell (Figure 2C). The cell-cell contact sites varied in their area, as shown in Figure 3A. Although measurements of actual contact areas were not made in this study, in some thin sections the contact areas measured up to 2 μ. The sites of contact between activated monocytes and tumor cells were often characterized by regions where the membranes of the opposing cells could not be defined clearly (Figure 3A). The contact sites also exhibited osmiophilic aggregates, presumably cytoskeletal components, as shown in Figure 3B in the ventral portion of the monocyte adjacent to the contact site with the melanoma cell.

To investigate further membrane continuity at the sites of contact, we examined thin sections at various angles of specimen tilt. Stereoanalysis of numerous sites of cell-cell contact demonstrated distinct membrane discontinuities, an example of which is shown in Figure 4A. An example of tumor cell-monocyte interaction that resulted in the regional vacuolation of the tumor cell cytoplasm is shown in Figures 4B and 4C. The vacuolated areas were devoid of mitochondria. In contrast, the rest of the melanoma cell cytoplasm contained morphologically intact mitochondria and abundant endoplasmic reticulum (Figure 4B). In some target cells, pronounced surface projections, blebs, and microvilli were easily visualized (Figure 4C). Tumor cells with focal areas of vacuolation were detected by 24 hours of cocultivation. Extensive cytoplasmic vacuolation, however, was seen in samples prepared after 48 hours of culture with activated monocytes, corresponding to the kinetics of tumor cell lysis shown in Table 1.

Discussion

The results we report here on the ultrastructural analysis of human blood monocytes interacting with allogeneic A375 melanoma cells are similar to previous observations derived from rodent systems.[6,7,10,11,17-19,27,28] In our study, we observed that monocytes activated with liposomes containing MTP-PE clustered around tumor cells to a higher degree than did monocytes incubated with liposomes containing HBSS (nonactivated monocytes) (Figure 1). These findings agree with the data published by Bucana et al[11] and by Marino and Adams[18,19] that indicate that activated rodent macrophages bind more readily to target cells than nonactivated macrophages. *In vitro*, at least, tumoricidal macrophages

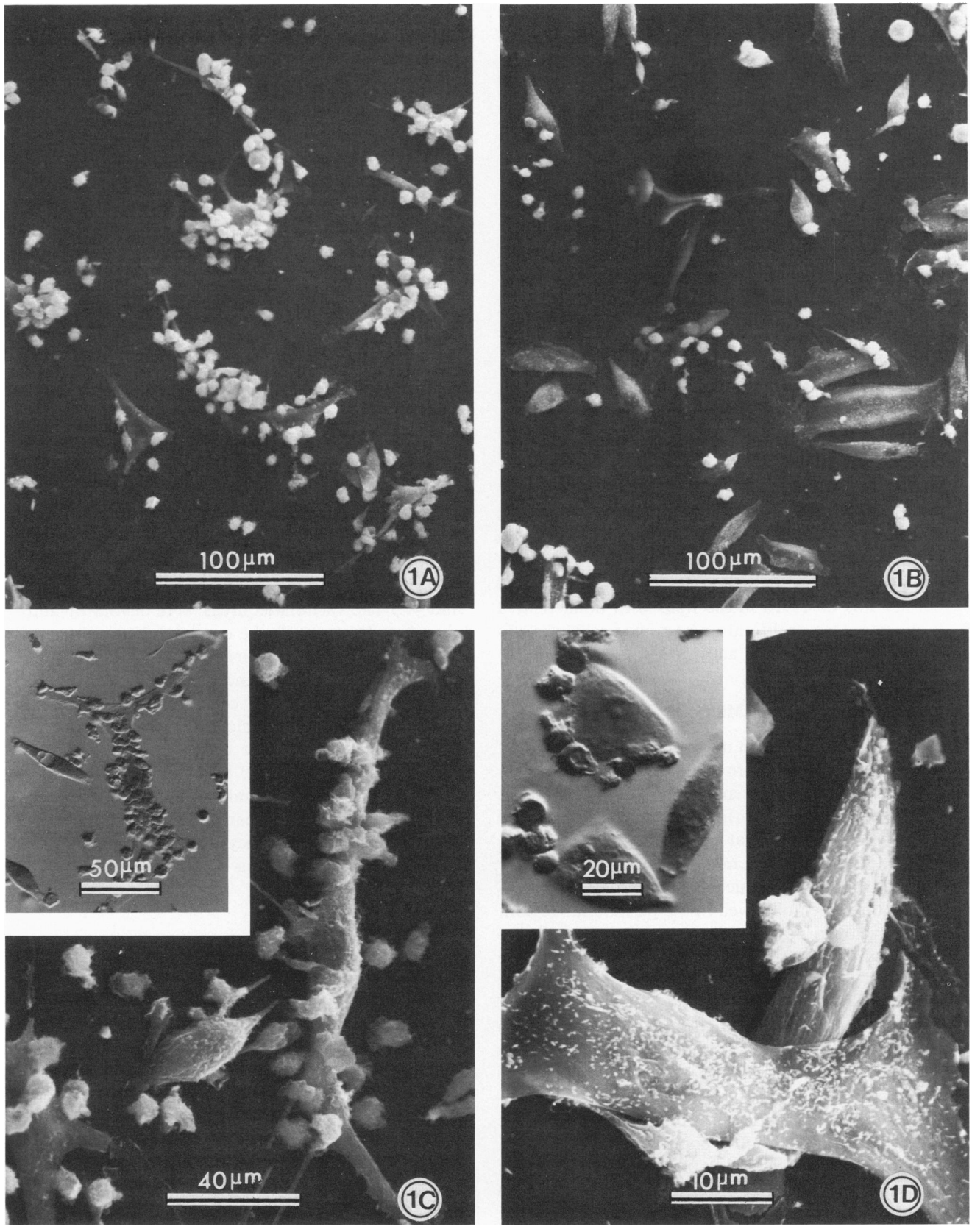

Figure 1 — Scanning electron micrograph of human blood monocytes cultured with allogeneic melanoma cells. **A** — Tumor cells and activated monocytes after 24 hour incubation. **B** — Tumor cells and nonactivated monocytes after 24-hour incubation. **C** — Tumor cells and activated monocytes after 48-hour incubation. Note the elongation and long processes of the tumor cells. **Inset** — Nomarski interference micrograph of a similar sample. **D** — Tumor cells and nonactivated monocytes after 48-hour incubation. **Inset** — Nomarski interference micrograph of a similar sample.

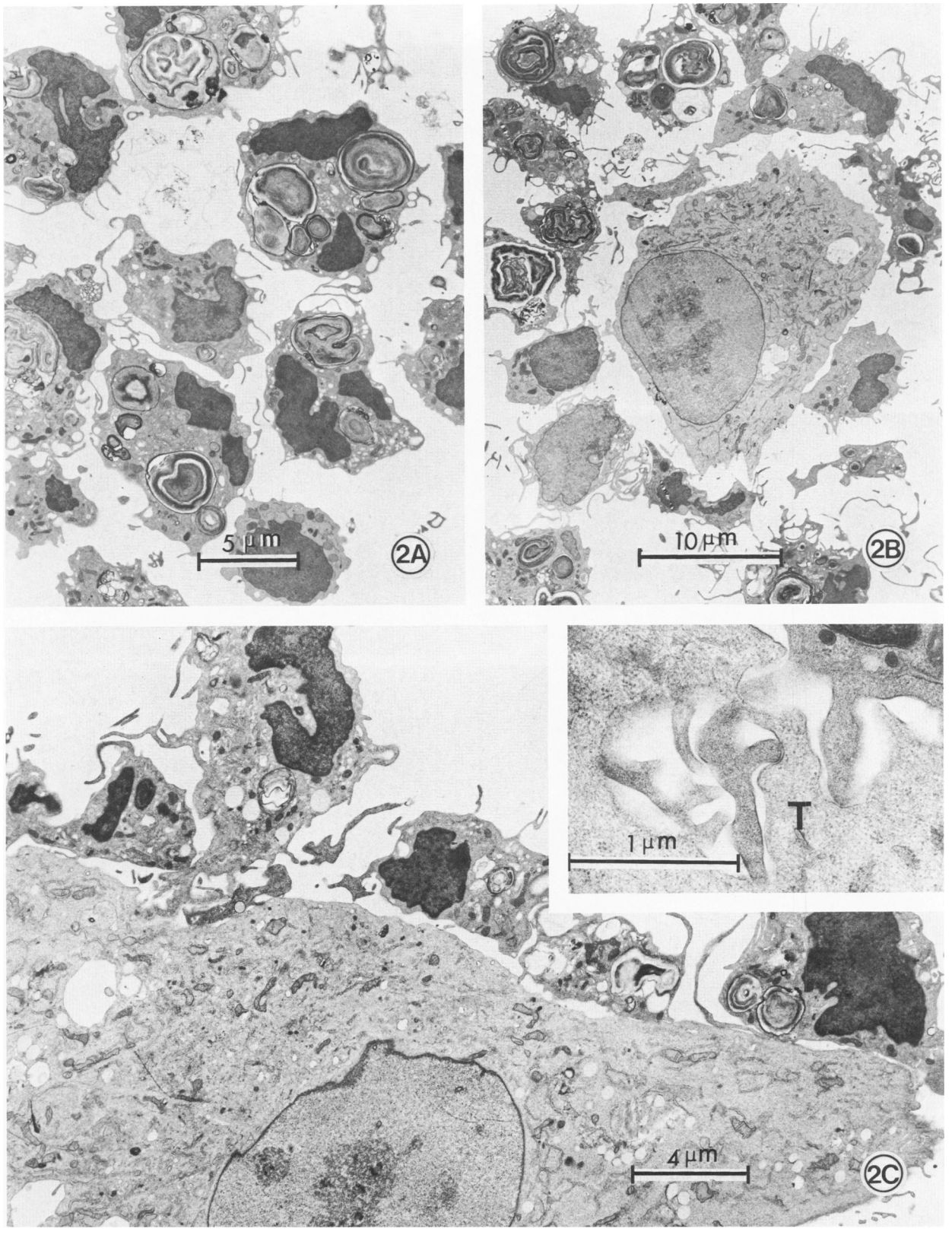

Figure 2 — Transmission electron micrograph of human blood monocytes interacting with allogeneic melanoma targets. **A** — Transmission electron micrograph of human blood monocytes subsequent to their incubation with liposomes. Note the presence of endocytosed liposomes within all the cells. **B** — Human blood monocytes treated with control liposomes surround a melanoma cell but do not exhibit cell-cell contact after 12 hours of cocultivation. **C** — Human blood monocytes (with endocytosed liposomes containing MTP-PE) attached to a tumor cell. This sample was taken at 12 hours of monocyte-tumor-cell cultivation. **Inset** — Serial section of cytoplasmic projection from a monocyte probing the surface of the tumor cell (*T*).

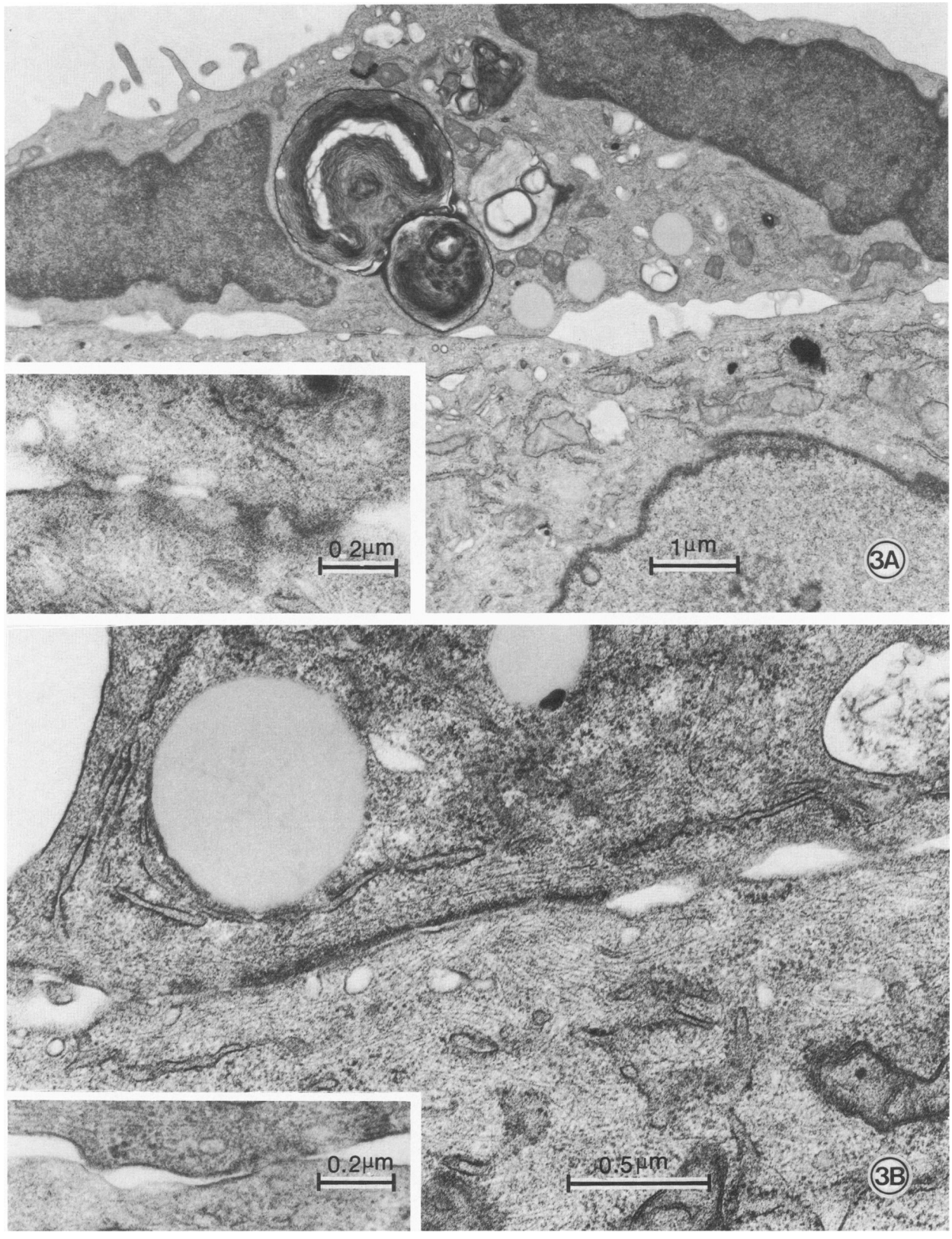

Figure 3A — Wide zones of close contacts between the activated blood monocytes (with endocytosed liposomes containing MTP-PE) and the melanoma cell can be readily seen. **Inset** — Sites of monocyte–tumor-cell contacts, with an appearance that suggests membrane discontinuities. **B** — Transmission electron micrograph of sites of contact between an activated monocyte and a tumor cell. Note the accumulation of osmiophilic material at the ventral side of the activated monocyte opposite the contact site with the tumor cell.

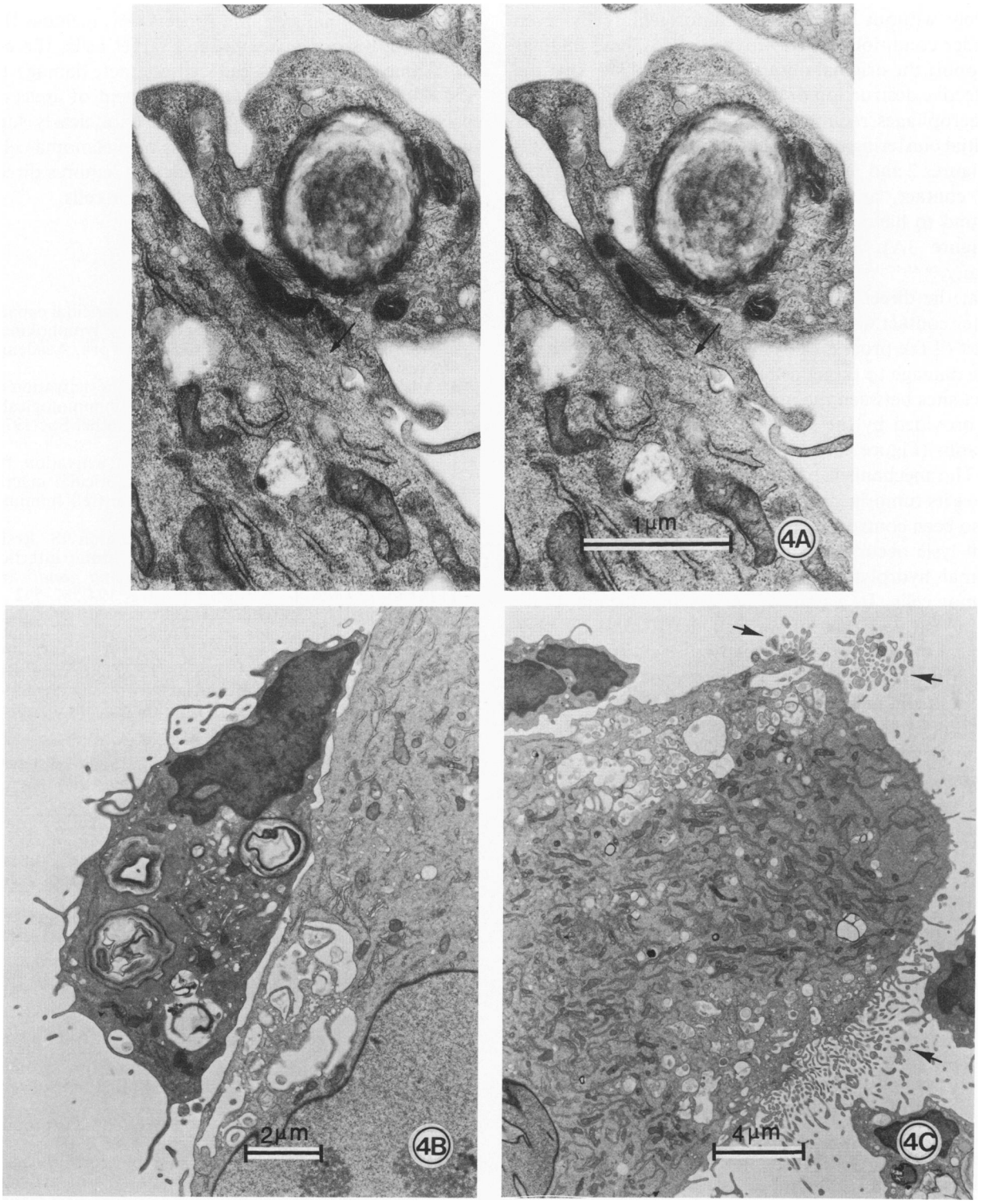

Figure 4A — Stereomicrographs showing a wide area of contact established between an activated monocyte and a tumor cell. The *arrow* indicates an area of membrane discontinuity that was verified by examining the specimen at various tilt angles. The area of membrane discontinuity was observed in a number of serial sections examined. **B** — Transmission electron micrograph of a sample taken at 48 hours of incubation showing regional vacuolation of the cytoplasm opposite the site of contact with an activated monocyte. In contrast to other areas in the cytoplasm, the vacuolated area is devoid of mitochondria. **C** — Transmission electron micrograph of a tumor cell with associated activated monocytes. Note focal areas of vacuolation in the tumor cell cytoplasm adjacent to sites of contact with monocytes. Pronounced cytoplasmic projections, blebs, and microvilli (*arrows*) are abundant at the cell surface immediately adjacent to the vacuolations in the cytoplasm.

acquire the ability to destroy tumorigenic cells selectively without harming nontumorigenic cells, even under conditions of cocultivation.[9,21] These findings support the original observation of Hibbs[8,10] that the selective destruction of tumorigenic cells by activated macrophages requires direct cell–cell contact. After initial clustering of monocytes around the tumor cells (Figures 2 and 3), we observed numerous focal points of contact, and some of these contact sites were found to have areas with discontinuous membranes (Figure 3A). These findings agree with those of many,[10,11,17–19,24,27–33] but not all,[13,34–36] investigators that the direct damage to the target cell membrane after contact with effector cells constitutes an integral part of the process of cytolysis. Further evidence for the damage to target cell membrane at intimate contact sites between the monocytes and melanoma cells is provided by the stereoanalysis of electron micrographs (Figure 4A).

The mechanism by which an activated monocyte lyses its tumorigenic target subsequent to binding has also been controversial. Hibbs[10] suggested that target cell lysis occurs as a result of the transfer of lysosomal hydrolytic enzymes from macrophages into tumor cells. In a guinea pig system, Bucana et al[11] demonstrated that tumor cell destruction by peritoneal macrophages activated by bacillus Calmette-Guerin was associated with direct exocytosis of lysosomes from macrophages and their subsequent endocytosis by the target cells. However, target cell lysis may not always require the translocation of lysosomes.[37] Rather, it may be caused by the release by macrophages of labile cytolytic substances such as neutral serine proteases,[17–19] hydrogen peroxide,[38,39] or the third component of complement (C3a).[40] If this is the case, the cytolytic products may be released at those areas where macrophages contact the target cells (Figures 3 and 4). Although our present studies are not definitive, the appearance of zones of vacuolation in target cells in the immediate vicinity of activated monocytes (Figures 4B and C) strongly suggests that a cytotoxic substance is transferred from the activated monocyte to the target cell at the intimate contact sites. The demonstration of membrane-bound proteolytic activity on the surface of mononuclear leukocytes also supports this mechanism.[41] In any event, the interaction of the activated monocytes with the tumorigenic target cells culminates in actual target lysis, as substantiated by data from the cytotoxicity assays. Control experiments ruled out that the detachment of target cells from the substratum can account for the loss of radiolabel.[20,21] Thus, we conclude that when human blood monocytes are rendered tumoricidal *in vitro* by incubation with liposomes containing MTP-PE, the lysis of tumor cells occurs as the final step in a process that includes the clustering of monocytes around target cells, the establishment of a direct cell–cell contact, damage to the cell membrane, and the development of areas of vacuolation in the target cells. The data clearly suggest that at least *in vitro*, the lysis of melanoma cells by activated human blood monocytes requires direct contact between monocytes and tumor cells.

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