Lymphocyte–Target Cell Interaction In Vitro

Ultrastructural and Cinematographic Studies

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It is well-established that sensitized lymphoid cells may participate in the destruction of incompatible grafts and tumor cells possessing tumor-specific antigens, as well as in some autoimmune diseases. Several investigators [1,2] have described similar models *in vitro* for the study of sensitized lymphoid cell–target cell interaction, resulting in cell lysis. The reaction is specific,[3] but the mechanism of cell lysis is not clearly understood. Recently it has been suggested that sensitized lymphocytes elaborate a lymphotoxin or "lymphokines," which may be instrumental in cell lysis.[4,5]

The purpose of this experiment was to investigate the ultrastructural events leading to and associated with target cell destruction *in vitro* and to correlate the findings with time-lapse cinematographic studies of this reaction.

Materials and Methods

The sensitization of animals, isolation of lymphocytes, and preparation of tissue cultures have been described in detail previously.[1,6]

Briefly, BALB/c mice were inoculated intraperitoneally and intrasplenically with L cells of C3H mouse origin. Sensitized lymphoid cells were obtained from these animals, suspended in Medium 199, and adjusted to a concentration of 2 million cells/ml. Nonsensitized lymphoid cells were obtained from uninoculated animals and suspended at the same concentration in an identical medium.

For cinematography, the lymphoid cells were added to L cell monolayer cultures growing in Rose chambers. The experiments were conducted at a temperature of 37 C, an inverted microscope was used, and frames were taken at intervals of 20 sec.

For electron microscopy, the sensitized or nonsensitized lymphoid cells were added to previously established L cell monolayer cultures in plastic Petri dishes growing at 37 C in an air (95%)–CO_2 (5%) atmosphere and examined at intervals ranging up to 48 hr. The Petri dishes were covered with cold (4 C) 3% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate-HCl [7] for 1 hr, followed by 60 min postfixation in 1% osmium tetroxide in Veronal-acetate

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buffer (pH 7.4) with 4% sucrose added. The cells were then treated with 0.5–2.0% solutions of uranyl acetate in sodium maleate, pH 5.0, at 4 C [8] in the dark. Dehydration was accomplished in graded concentrations of ethanols in six 10-min steps. Araldite [9] was polymerized in a thin layer on the bottoms of the Petri dishes, and subsequently, with the cells on the deep surface, pulled from the dishes. Then the Araldite, cell layer down, was floated on toluidine blue at 56 C until the cells stained dark blue. The layers of Araldite were examined with a light microscope, and the lymphocyte–target cell junctions and other areas desired for electron microscopic examination were marked, cut out, and glued with epoxy resin onto Araldite blocks. Some cell monolayers were used for thick sections. Otherwise, silver and gray serial sections were made through each single cell layer. Sections for electron microscopy were double-stained with uranyl acetate and lead citrate, and electron micrographs were made with a Siemens Elmiskop I-A at 80 kV. Ultrathin sections of L cell–lymphocyte junctions were also viewed and photographed using a stereo drive allowing a 5° tilt of the preparation on either side of the horizontal plane.

Results

Time-Lapse Cinematographic Studies

The lymphocytes from sensitized mice collided at random with the large spindle-shaped L cells, resulting in an occasional attachment of the lymphocytes to the fibroblasts—no evidence of chemotaxis was noted Plate 1. Subsequently some of these lymphocytes became detached again. After attachment of one or several lymphocytes, the fibroblasts rounded up after varying intervals, swelled, and underwent lysis. At this time, swelling and death of the attached lymphocytes were also seen. Lysis was occasionally observed as early as 6 hr after addition of the lymphoid cells, but was most frequent at the 18–36-hr interval. In contrast, lymphocytes from nonsensitized animals rarely attached to target cells and lysis was not seen.

Electron Microscopic Studies

At OHr, Control and Sensitized. The ultrastructural appearance of the nonsensitized and sensitized spleen cells was similar prior to addition to the cultures. Primarily, the suspensions consisted of small lymphocytes (Fig 1) containing a few small, short strands of rough-surfaced endoplasmic reticulum. The Golgi apparatus was not prominent and polysomes were uncommon. Free ribosomes, a few mitochondria, and rarely vacuoles were found in the cytoplasm. Occasional large lymphoid cells were encountered in preparations from control and sensitized mice. The ratio of small to large cells was similar in both (97:3), counting 500 cells by electron microscopy. These large cells usually contained many free ribosomes but few polysomes.

At 18 Hr, Sensitized. Two sizes of lymphocytes were readily dis-

tinguishable: a majority of small cells indistinguishable from the small lymphocytes described above, and large lymphoid cells, which now contained numerous nonmembrane-associated polysomes (Fig 2) as well as smooth and coated vesicles. Some of the free-floating cells and most of the lymphocytes in close contact with L cell fibroblasts were of the large variety.

Large lymphoid cells near L cells sometimes displayed blunted extensions toward the L cells; such extensions were quite long in some instances. Lymphoid cells attached to L cells usually exhibited uniform, close, broad approximation to the external membrane of the target cell over a considerable area of the cell surface (Fig 3 and 4). Adjacent plasma membranes at these points of approximation remained nearly parallel (Fig 4). The contacts were either straight or undulating, and the interspace was not stained. A single large lymphoid cell sometimes revealed projections extending to more than one of the adjacent L cells. The morphology of the large lymphoid cells in contact with L cells was similar to that of those nearby with blunted extensions. Coated vesicles were especially prominent adjacent to areas of L cell contact (Fig 5). Fusion of the external membranes of lymphocytes and L cells was never observed in thin gray sections nor in multiple stereoscopic views. Centrioles were readily found in these large lymphoid cells, and multivesicular bodies were also noted occasionally (Fig 5). Within the L cell cytoplasm at points of close contact and directed toward contact areas were many microfilaments and small numbers of microtubules (Fig 5 and 6). Less frequently, filaments and microtubules were also seen within the large lymphoid cells, radiating toward contact areas.

At 18–48 Hr, Nonsensitized. Preparations studied at intervals from 18–48 hr were similar and hence are described together. The lymphoid cells retained their original size, rounded appearance, and general cytoplasmic and nuclear morphology (Fig 7). Close lymphocyte-L cell contacts extending over considerable areas of cell surface, as in sensitized cultures, were never observed.

At 26 Hr, Sensitized. Large lymphoid cells were in close contact with some L cell fibroblasts. In addition to the previously described contacts, spike-like interdigitating projections of large lymphoid cells and L cells were now more frequently noted.

At 28–40 Hr, Sensitized. Again, large lymphoid cells were seen in contact with L fibroblasts either by close and broad or spike-like contacts. It was now evident that the cytoplasmic organelles of large attached lymphoid cells with close contacts had become less dense, and

similar changes were observed in L cells. Whether this was the beginning of a specific lytic event or merely represented a nonspecific degenerative phenomenon within the aging culture was not determined. Numerous membrane-bound vacuoles occasionally developed within the cytoplasm of the L cells. Lymphoid cells were seen with disruption of the cell membrane and escape of the cytoplasmic components into the surrounding medium. Similarly, L cell changes consisting of edema, disruption of the plasma membrane, and escape of cytoplasmic components into the medium were observed. While lysis mediated by lymphoid cells could be easily recognized by time-lapse cinematography, delineation of specific lysis from nonspecific degenerative phenomena was more difficult to characterize by electron microscopy, since lymphocyte contacts might be present at sites other than the particular plane of sectioning. Occasionally the L cell cytoplasm adjacent to a close contact revealed loss of density—possibly an early event in target cell damage preceding lysis (Fig 8).

At 48 Hr, Sensitized. Both L cells and lymphoid cells were markedly decreased in number, so that lymphoid cell–target cell junctions were hard to find. When these were observed, however, the junctions were similar to those described previously.

On very rare occasions, a lymphocyte was found seemingly within an L cell but bound by cell membrane (Fig 9). By following such a configuration through serial sections, we noted that the lymphocyte in reality rested within an indentation of the L cell and was not surrounded by cytoplasm on all sides.

General Observations

Mitotic activity of the mouse lymphocytes was never observed. An occasional L cell undergoing mitosis was visible in preparations with either nonsensitized or sensitized lymphoid cells.

Discussion

Time-lapse cinematography from 0 to 48 hr after addition of sensitized lymphoid cells to target cells revealed several important features: (1) The movement of the lymphoid cells appeared to be random, and no chemotactic effect was observed. (2) After varying intervals, a minority of lymphoid cells from sensitized animals attached to target cells after an apparent chance collision. (3) Attachments of lymphoid cells to target cells were occasionally short-lived and not destructive; in many instances, however, attachment of lymphoid cells led to withdrawal of the cell processes of target cells, followed by rapid swelling and lysis.

(4) In contrast, lymphoid cells from nonsensitized animals rarely established contact with target cells, and lysis was not seen.

Ultrastructural studies added several important observations: When lymphocytes from sensitized animals were cultured with L cells, two populations of lymphoid cells became apparent—small lymphocytes, which contain free ribosomes, do not attach to target cells, and probably are not sensitized, and larger, apparently sensitized lymphoid cells, which may attach to target cells and produce cell lysis. These cells have many free polysomes and little endoplasmic reticulum. Since these latter cells were not observed at 0 time, it is likely that they developed *in vitro* after exposure to antigen (L cells). It is particularly noteworthy that among several hundred lymphoid cells adherent to target cells after varying periods of incubation, only two plasma cells (unattached to L cells) were seen by electron microscopy. This contrasts sharply with the observations of Weiss,[10] who noted numerous plasma cells adherent to target cells. In some of his preparations, as many as 50% of the adherent cells were plasma cells after 48 hr of incubation. However, our own findings indicate that plasma cells are not necessarily required for cytolysis in this system and that lysis may be effected by almost pure preparations of lymphocytes.

Contacts between lymphocytes from sensitized animals and target cells were of two distinct types: The most frequent and earlier-developing type was characterized by extensive, nearly parallel, close areas of contact between sensitized lymphoid cells and target cells. The close approximation would undoubtedly necessitate an alteration of the glycocalix. Fusion of the outer leaflets of adjacent plasma membranes was never observed by critical examination using ultrathin serial sections and stereo-electronmicroscopy. The other, later-developing type of contact was characterized by spike-like interdigitating junctions between lymphocytes and target cells. In areas of lymphoid cell attachment to target cells, filaments and microtubules were often seen in the adjacent cytoplasm of both cell types. These organelles radiated toward points of contact. The lymphoid cells also contained multivesicular bodies and coated vesicles, particularly near points of attachment.

In marked contrast, approximation of lymphocytes from nonsensitized animals to L cells was uncommon and it was never close. A space of at least several hundred Angstroms between the outer leaflets of the adjacent membranes was always maintained.

Time-lapse cinematographic studies suggest that the target cell membrane becomes altered subsequently to the attachment of lymphocytes because of the marked swelling of the target cells, progressing to lysis.

Granger *et al* [11] have isolated a lymphotoxin, a protein with a molecular weight of about 80,000, which appears to act on cell membranes. This substance was released by sensitized lymphoid cells upon contact with specific antigen or upon stimulation of lymphoid cells by phytohemagglutinin. One of us has also observed the release of a lymphotoxin by human lymphocytes treated with phytohemagglutinin.[12] This lymphotoxin is a protein susceptible to trypsin digestion. Granger noted that lymphotoxin may cause lysis of many different cell types. Thus the cytolytic effect of the sensitized lymphocytes upon L cells might be due to release of this lymphotoxin. In this connection, it is significant that the lymphocytes producing lysis have many free polysomes, suggesting active protein synthesis. Lymphotoxin may be one of the proteins synthesized.

Summary

The interaction of sensitized and nonsensitized mouse lymphoid cells with allogeneic mouse target cells was studied by time-lapse cinematography and electron microscopy.

Time-lapse cinematography revealed that the lymphoid cells moved about in a random fashion and became attached to target cells. After varying intervals, there was rounding up of the target cells, swelling, and lysis. Destruction of the attached lymphoid cells was also noted. In contrast, attachment of nonsensitized lymphoid cells was rare and no lysis was observed.

Seen by electron microscopy, lymphoid cells attaching to target cells were of the large variety, containing numerous polysomes, smooth and coated vesicles, and little endoplasmic reticulum. Sensitized lymphoid cells attached to target cells by two distinct types of contact: The most frequently occurring type was characterized by close contact of lymphocyte–target cell membranes over extensive areas; a later-developing type consisted of interdigitating spike-like projections. Swelling and vacuolization of target cells were observed. This swelling of target cells suggests cell membrane alterations, which may be related to the release of lymphotoxin by sensitized lymphocytes. In contrast, lymphoid cells from nonsensitized animals usually did not attach to target cells, and close contact of lymphoid cell–target cell surfaces did not occur.

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[*Illustrations follow*]

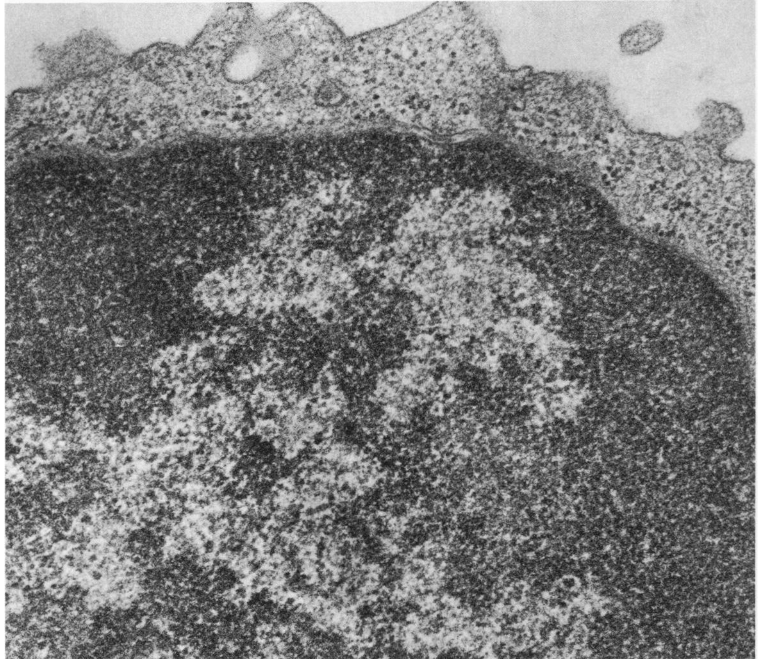


Fig 1. Nonsensitized control lymphocyte containing many free ribosomes. × 56,300.

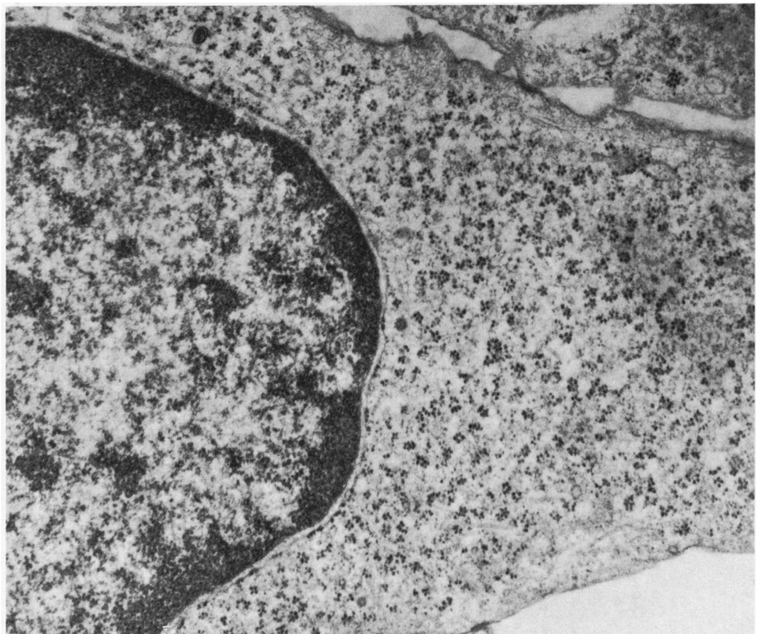
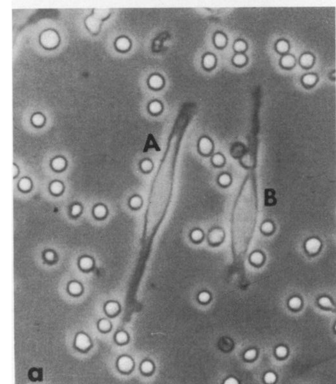


Fig 2. Portion of cytoplasm of lymphoid cell from sensitized preparation demonstrating prominent polysome clusters. × 22,200.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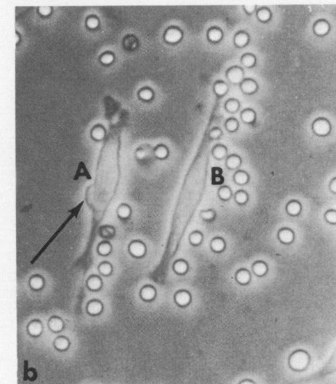
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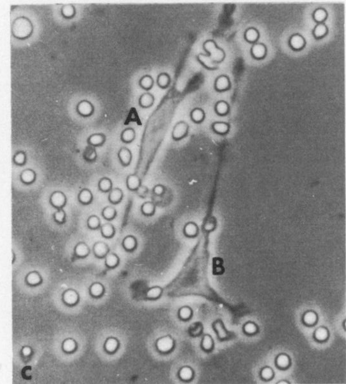
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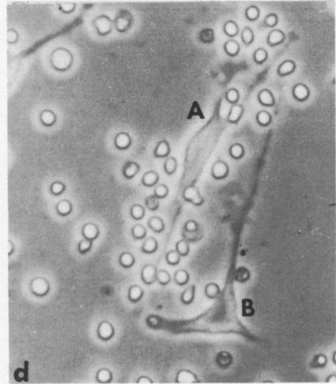
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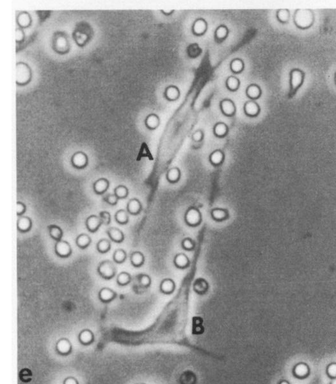
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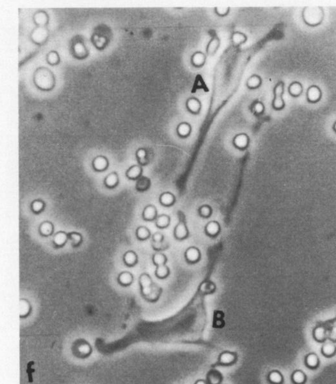
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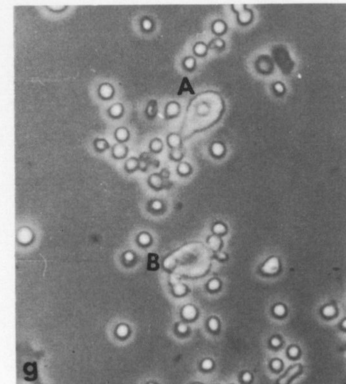
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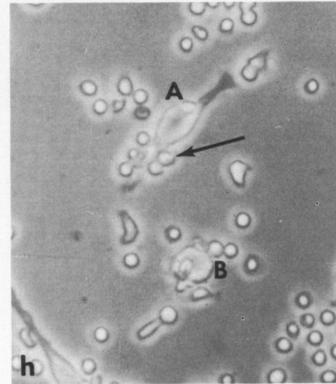
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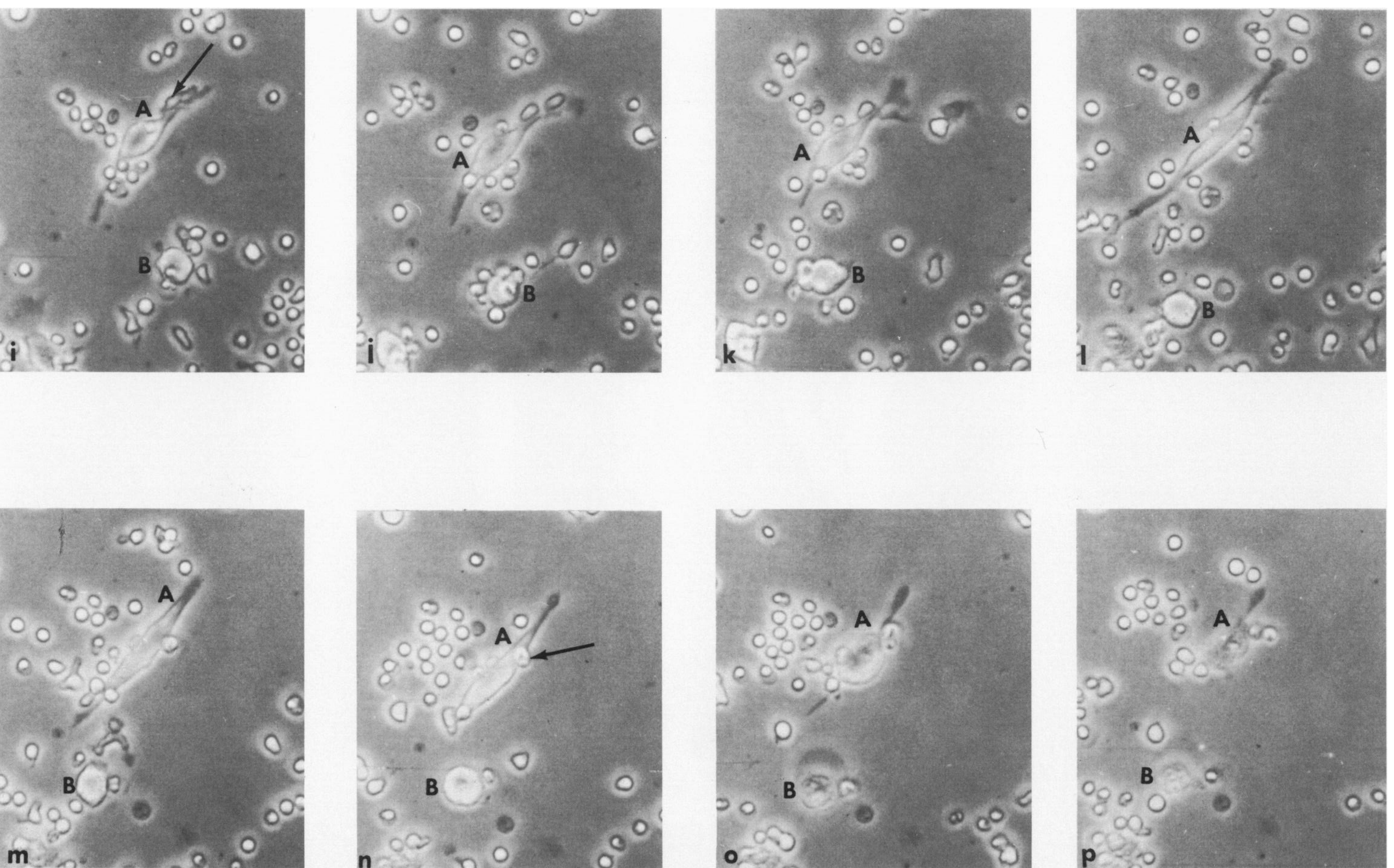

Plate 1. Time-lapse cinematography of interaction (*in vitro*) of L cell fibroblasts and lymphoid cells from sensitized BALB/c mice. Two fibroblasts, (*A*, *B*) are followed sequentially, demonstrating lymphoid cell attachment and finally cell lysis. Cell *A* rounds up (**Frame g**), recovers temporarily (**Frames h–n**), and then is destroyed (**Frame p**); such temporary recovery is an unusual event. These frames were taken at intervals of 10-30 min. Attached lymphocyte is indicated by arrow in some pictures.

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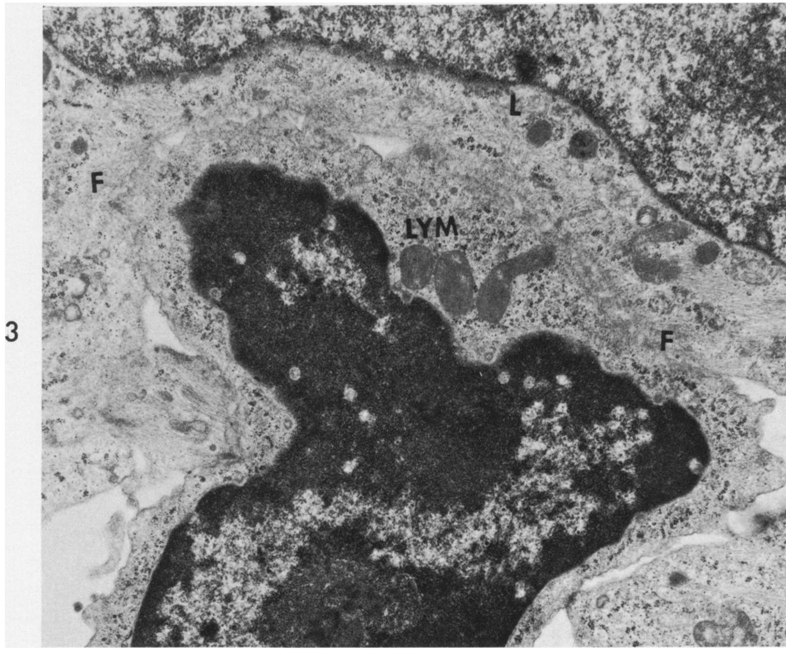

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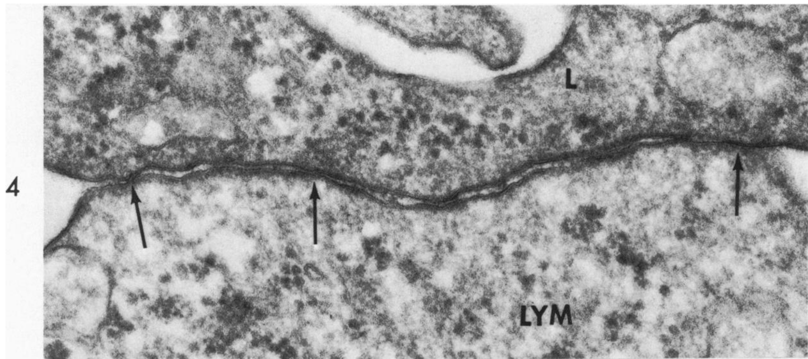

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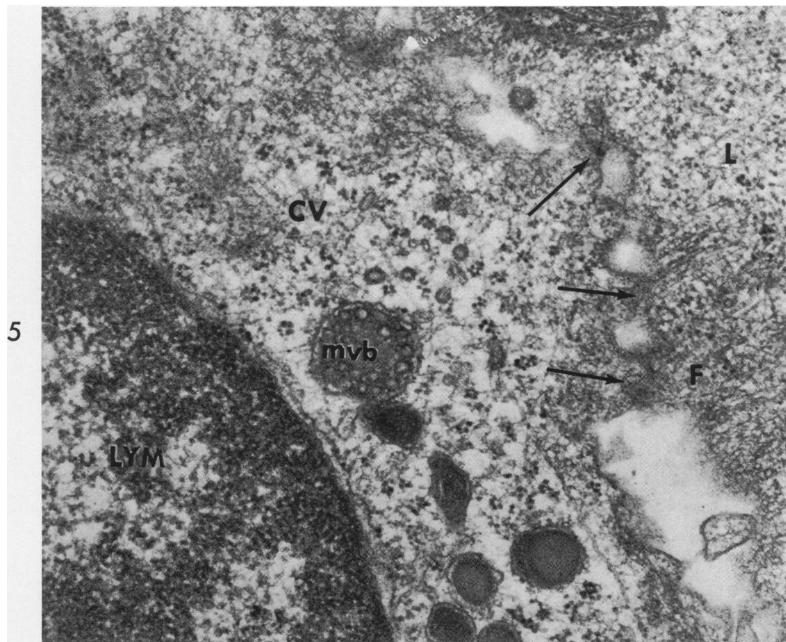

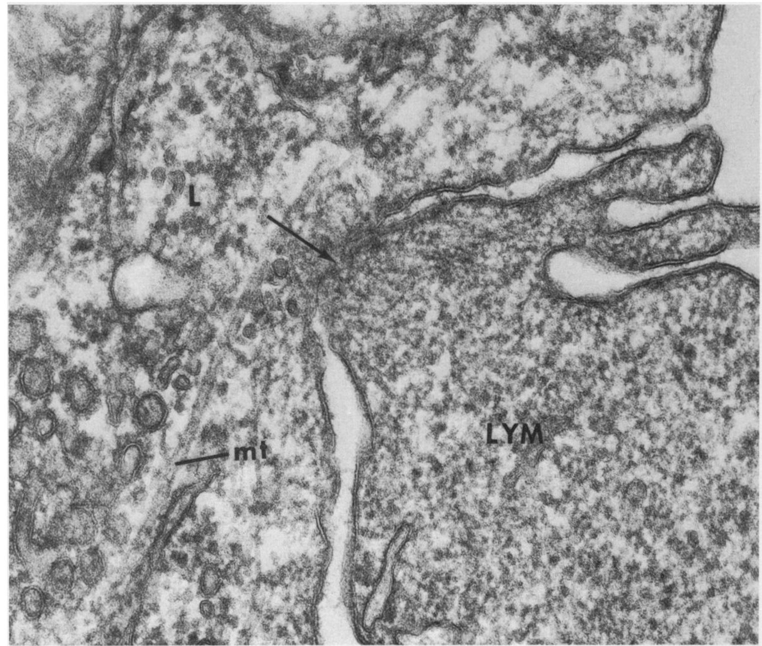

Fig 6. Contact site (*arrow*) between sensitized lymphoid cell (*LYM*) and L cell (*L*). Intercellular space is almost eliminated. Note microtubules (*mt*) in L cell cytoplasm. × 68,400.

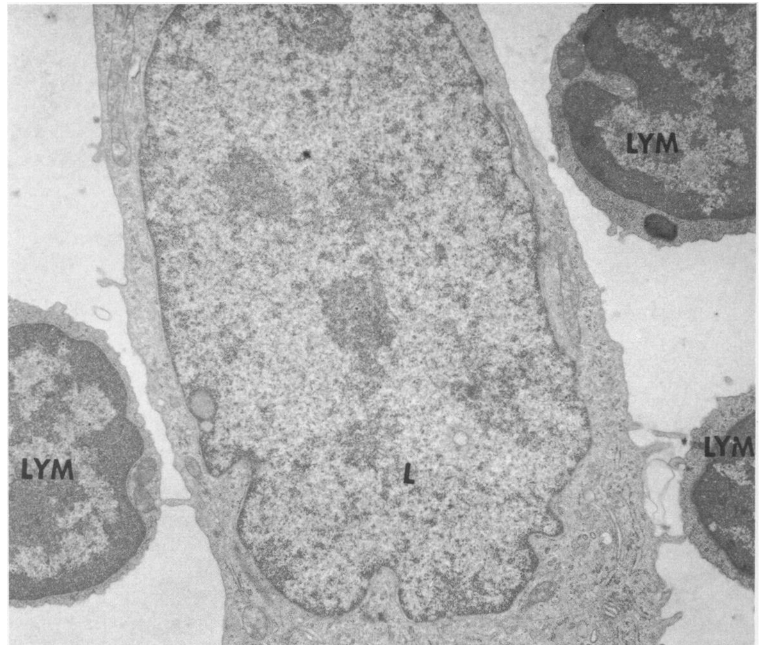

Fig 7. Control culture containing nonsensitized lymphocytes (*LYM*) and L cells. (*L*). Broad or spike attachments were never observed. × 7500.

Fig 3. Deep indentation in area of broad close contact between sensitized lymphoid cell (*LYM*) and L cell (*L*). Note numerous fibrils (*F*) in L cell cytoplasm along broad attachment site. × 10,900.

Fig 4. High-magnification of broad attachment, similar to that in Fig 4, between sensitized lymphoid cell (*LYM*) and L cell. Distance between outer leaflets of adjacent plasma membranes in some areas approximates 50 Å (*arrows*) and nearly eliminates intercellular space. Multiple serial sections, however, failed to reveal fusion of cell membrane components. × 67,700.

Fig 5. Spike-like attachments between sensitized lymphoid cell (*LYM*) and L cell (*L*). Indistinct appearance of cell membranes at these points (*arrows*) is due to tangential sectioning. Lymphoid cell cytoplasm contains multivesicular body (*mvb*) and many small coated vesicles (*CV*). *F* indicates fibrils. × 16,000.

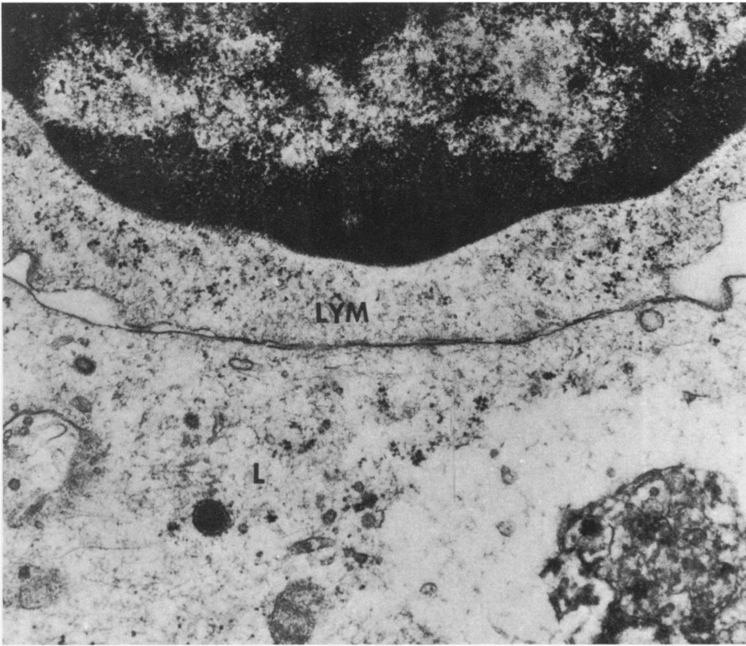

Fig 8. Area of broad close contact between sensitized lymphoid cell (*LYM*) and L cell (*L*). L cell cytoplasm exhibits loss of density and large phagosome. × 41,000.

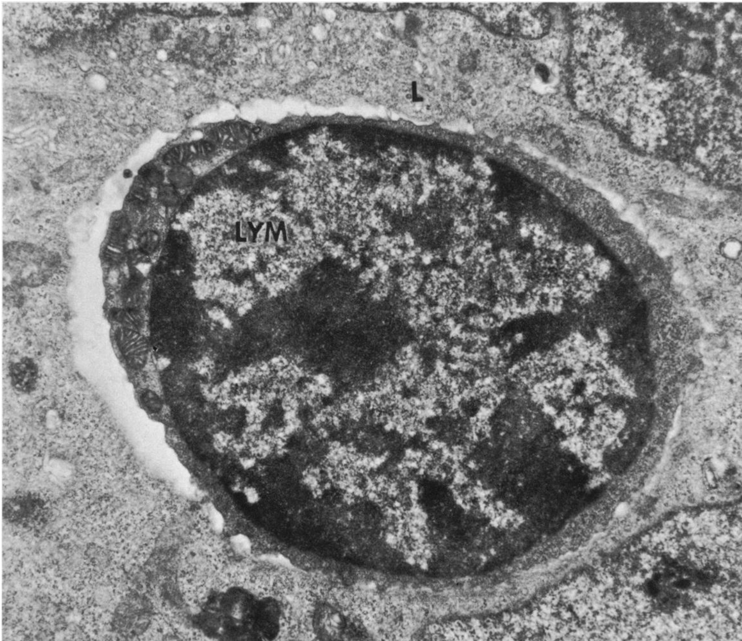

Fig 9. Deep invagination of L cell (*L*) cytoplasm by a lymphocyte (*LYM*). Lymphocyte appears to be within L cell cytoplasm; however, multiple serial sections always revealed this to be merely a deep invagination and all cell membranes remained intact. × 9950.