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Ultrastructural Aspects of the Adherence to Target Cells of *in Vitro* Differentiated Lymphocytes (38901)

M. KALINA AND H. GINSBURG
(Introduced by M. Wolman)

Department of Cell Biology and Histology, Sackler School of Medicine, Tel-Aviv University, and Department of Immunology, Technion, Israel Institute of Technology, School of Medicine, Haifa, Israel

Much attention has been given recently to cytolysis mediated by lymphocytes (CML) since it is thought that CML operates in certain autoimmune diseases, in graft rejection, and in some cases of tumor destruction (1). It is well accepted that there is contact between sensitized lymphocytes and target cells in all cases in which CML occurs (2–5). The nature of this contact as well as the mechanism by which the lytic process is brought about are far from being understood.

It has been shown that the specificity of this process is confined to the recognition phase as manifest by the specific adherence of hypersensitive lymphocytes to target cells (4). The lytic phase which follows may not display specificity. In our *in vitro* system, blast cells obtained by stimulation with various mitogens and antigens are plated on embryo fibroblast monolayers in the absence of any of the stimulating mitogens or antigens. Under these conditions the blast cells transform within 2 or 3 days into a distinctive type of lymphocytes termed "secondary lymphocytes" (4, 6). Secondary lymphocytes obtained after stimulation with pokeweed mitogen (PWM lymphocytes) are of particular interest since they show definite specificity in their ability to undergo reactivation (4). Thus, when PWM lymphocytes are exposed to target cells in the presence of PWM, more than 90% adhere within ½ to 6 hr. The adherence is followed by a rapid transformation to blast cells which resume motility and lyse the target fibroblasts. This specific and massive adherence, as well as the target cell lysis which follows, stimulated us to study the manifestations under the electron microscope.

Materials and Methods. Lymphocytes and embryos for preparation of fibroblast monolayers were obtained from rats of the Lewis strain. Fibroblast monolayers were prepared and maintained as previously described (7). Lymphocytes were prepared from Lewis rat lymph nodes as already described (8). The cells were centrifuged and resuspended in Dulbecco's modified Eagle's medium containing 15% horse serum and 5 μg PWM/ml medium. Lymphocytes were then cultured in 60-mm Falcon petri dishes; 30×10^6 cells per petri dish. Incubation continued for 4 days at 37° in a humidified incubator with 7% CO_2 in air. For preparation of secondary lymphocytes the blast cells were washed free of PWM and plated onto Lewis fibroblast monolayer in 100-mm petri dishes; 3×10^6 cells in 10 ml medium per dish, containing 15% horse serum. To obtain a suspension of secondary lymphocytes, these cultures were further incubated for 3–4 days.

In most experiments 3×10^6 PWM blasts or secondary lymphocytes were plated onto a Lewis fibroblast monolayer in a 60-mm petri dish; pokeweed mitogen was added at a concentration of 20 μg/ml. Condition of media, sera, and culturing were as previously described (4). Monolayers with adherent lymphocytes were taken at intervals ranging from ½–24 hr after plating the lymphocytes, and vigorously washed to eliminate nonadherent cells. Monolayers with adherent lymphocytes were fixed for 2 hr in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, postfixed in 1% osmium tetroxide, stained in the block with 0.5% uranyl acetate, dehydrated, and embedded in Epon 812. For scanning electron microscopy the glutaraldehyde-fixed monolayers were frozen in liquid nitrogen and prepared according to the freeze-drying technique (9).

Results and Discussion. Figure 1 illustrates a typical attachment of PWM secondary

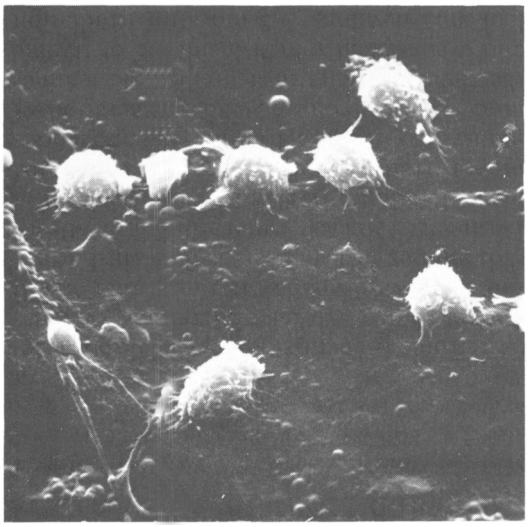

FIG. 1. Scanning electron micrograph of several PWM-secondary lymphocytes attached to a fibroblast monolayer by many pseudopods which appear on the monolayer side. Preparation was made after 2-hr adhesion. ×1300.

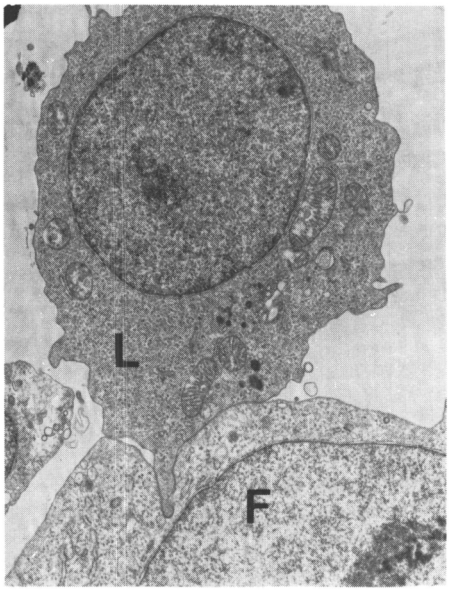

FIG. 2. A PWM-secondary lymphocyte (L) attached to a fibroblast (F), a single pseudopod can be seen penetrating the target cell. Prepared after 2-hr adhesion. ×6000.

lymphocytes to fibroblasts after a short adherence time. Several lymphocytes are seen attached to the monolayer by pseudopods which are present only on the monolayer

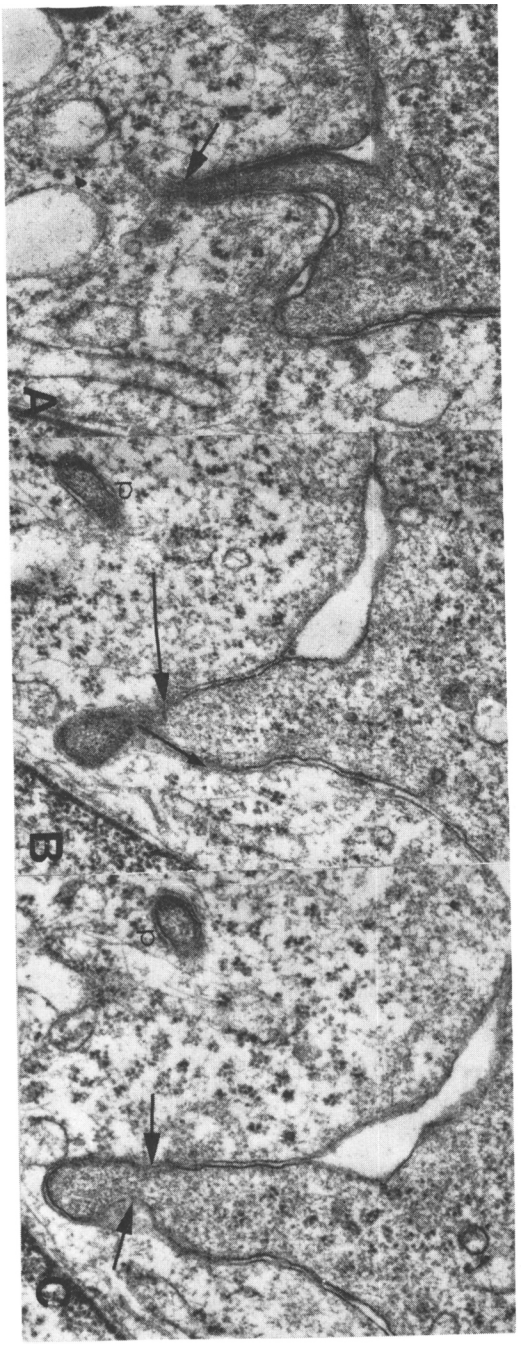

FIG. 3. Serial sections (a, b, c) through the pseudopod presented in Fig. 2. In (a) two pseudopods can be seen. In (b) and (c) cross sections of one of the pseudopods can be seen (P). In all sections there are areas in which the two distinct membranes (of the lymphocyte pseudopod and fibroblast) either fuse or disappear (arrows). Note the absence of cellular organelles except microfilament in the pseudopod. ×25,000.

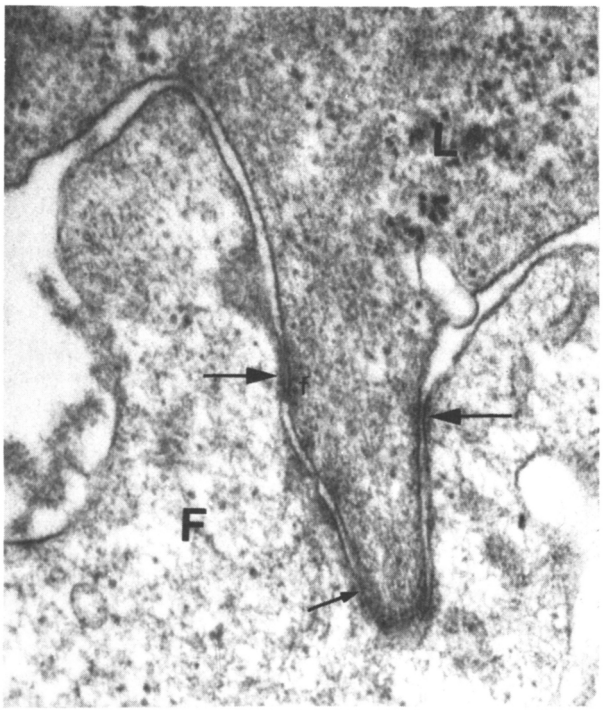

FIG. 4. A PWM-secondary lymphocyte (L) pseudopod penetrating a target fibroblast (F). A few contact areas can be observed between the two cells: (a) a close contact in which no intercellular space can be observed (small arrow); (b) more open contacts with a gap between the two membranes (large arrows). A few thick filaments can be seen in these contact regions (F); microfilaments are the only cellular component within the pseudopod. Preparation was made after 4-hr adhesion. ×50,000.

side. These pseudopods resemble the microvilli and micropikes described by McFarland *et al.* (10). After longer periods of adherence (1–15 hr), uropods could be observed but attachment occurred through pseudopods projecting from the uropods. Often the pseudopods were found to penetrate into the fibroblasts (Fig. 2), and sometimes more than one could be seen penetrating a single target cell.

In control experiments in which PWM was omitted, the few nonspecifically adhered lymphocytes (10% adhesion) rarely showed such penetrating pseudopods. Serial sections of a lymphocyte attached to a target cell showed that the pseudopods penetrate at different angles and can be curved inside the target cell (Fig. 3). In most cultures the most intimate contact between the lymphocyte and target cells appeared to be in the region of the penetrating pseudopod. Sometimes the plasma membrane of the cells in contact disappeared due probably to an oblique sectioning and not to a real fusion. Nevertheless, certain areas were in close contact, tempting to suggest a possible similarity to the known gap or tight junction (Figs. 3, 4). However, no definite statement can be given in the present communication.

The notion of transient junctions between lymphocytes and target cells had already been suggested. Sura *et al.* (11) claimed the occurrence of "bridges" between lymphocytes and target cells, and Koren *et al.* (12) observed discontinuities in the plasma membrane of the target cells. Recently Ferluga and Allison (13) observed an increased ion flux in the membranes of target cells after interaction with sensitized lymphocytes. These authors suggested as an hypothesis that a gap junction is established between the effector and target cells. This can be the form of the close contact observed in our study.

A characteristic observation made in the

present study was the lack of cellular elements with the notable exception of microfilaments within and in the vicinity of the lymphocyte pseudopods at the contact region (Figs. 2, 3, 4). Most of the lymphocyte organelles such as Golgi complex, endoplasmic reticulum and lysosomes, were found adjacent to the nucleus or in the uropod (14), but never close to the contact region. Golgi elements as well as vacuoles or vesicles which are characteristic of secretory process, were also absent from that region. The same observation was made at different adherence times up to 24 hr, at which time lysis of the fibroblast target cells was in an advanced stage (approximately 40% lysis). This absence of cellular organelles in the contact region may have some bearing on the lytic mechanism. It has been proposed that such a mechanism involves transport of cytotoxic agents from the lymphocyte to the target cell (15, 16). Assuming that such a mechanism exists, one would expect to find some morphological signs of secretion in the contact region. However, no secretory elements such as vacuoles or vesicles were observed. Nevertheless, the production and release of soluble factors by effector cells in these contact areas cannot be excluded.

Summary. The specific adherence to target fibroblasts of lymphocytes differentiated *in vitro* from blast cells after stimulation with pokeweed mitogen was studied under the electron microscope. Lymphocyte pseudopods were seen to penetrate into the target cells forming a close contact between the membranes of the two interacting cells. Microfilaments were the only cytoplasmic components that could be detected within or in the vicinity of the penetrating pseudopods. No morphological signs of secretion could be observed in the contact region. Morphological aspects of the contact region are discussed as related to the lytic mechanism.

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