Ultrastructure of Lymphocyte Tumor Cell Interaction with Localization of Cell-bound Antibody by Ferritin Labeling

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INTRODUCTION

There is general agreement that the lymphocyte plays an important role in immunosurveillance (6, 8, 13, 16), but the precise mechanism by which the lymphocytes carry out this activity remains a matter for speculation (7, 10, 18). The cytotoxic effect of sensitized lymphocytes on target cells may represent at least 1 method by which such immunosurveillance can occur.

Many investigators (4, 19, 20, 21, 23, 24) have utilized the destruction of tumor cells in tissue culture to study lymphocyte cytotoxicity, and Heberman (10) has recently reviewed many of the methods developed to quantitate this cytotoxic effect on tumor cells. However, there has been scant attention paid to the morphological events that occur during this reaction, especially at the subcellular level, since the report of Deodhar et al. in 1972 (7). Therefore, this study was undertaken to describe in detail the sequence of cellular and subcellular morphological events that leads to tumor cell destruction by sensitized lymphocytes in a tissue culture system. Phase microscopy and scanning and transmission electron microscopy were utilized, and ferritin-labeled immunoglobulin techniques were performed to further define morphological observations noted during these experiments.

MATERIALS AND METHODS

Tumor-bearing Animals. Walker carcinoma (9) tumors were maintained in 80- to 150-g Sprague-Dawley rats by weekly s.c. implants of solid tumor fragments. Walker carcinoma grows locally at the site of implantation and tumors, 2 to 3 cm, are present 7 to 10 days after transplantation.

Preparation of Sensitized Lymphocytes. Lymphocytes were harvested from the spleens of tumor-bearing rats after 10 days of tumor growth, at which time the tumors had large necrotic centers. The rats were anesthetized with ether and the spleens were removed and sectioned 2 to 3 times. Three ml of TC 199 (Division of Becton Dickinson and Co.) were injected under the splenic capsule with a 25-gauge needle, and the lymphocytes were collected. This produced a uniform population of small lymphocytes, mixed with red cells and a few histiocytes. Nonsensitized (control) lymphocytes were harvested in a similar manner from the spleens of non-tumor-bearing animals.

Tissue Culture Techniques. Walker carcinoma 256 tissue culture was obtained from the American Type Culture Collection, Rockville, Md., and maintained in TC 199 and 5% horse serum (Microbiological Associates Inc., Bethesda, Md.). The tumor cells were grown in plastic tissue culture flasks at 37° without carbon dioxide. This line of cells characteristically forms small tumor balls when growth is heavy, and uniform monolayers are difficult to obtain. These tissue culture flasks were inoculated with the splenic lymphocytes from tumor-bearing rats (sensitized lymphocytes) and 5% non-tumor-bearing rats (control lymphocytes), after counting and adjusting to an approximate ratio of 50 to 100 lymphocytes/tumor cell. The lymphocyte-tumor cell preparations were observed at frequent intervals by phase contrast microscopy, and specimens were collected for electron microscopy at 0, 1, 2, 5, 10, 24, 36, and 48 hr after incubation with the lymphocytes.

Electron Microscopy. The lymphocytes and tumor cell cultures were fixed for transmission electron microscopy by the addition of a 2% phosphate-buffered glutaraldehyde. After 4 to 6 hr of fixation, the tissue culture contents were gently scraped off the plastic flasks and placed in cool agar for further processing. The hardened agar blocks were post-

SUMMARY

Splenic lymphocytes derived from Walker carcinoma-bearing rats were harvested and incubated with Walker carcinoma cells growing in tissue culture. The sequence of events leading to target cell death was studied by phase microscopy and scanning and transmission electron microscopy. The sensitized lymphocytes adhere to the tumor cells by multiple cytoplasmic appendages, but no ultrastructural changes are seen at this interface. After 1 hr these lymphocytes release cytoplasmic components consisting of membrane-lined vesicles, cell membranes, endoplasmic reticulum, and cytoplasmic material. This material adheres closely to the surface of the tumor cells and is subsequently seen within the cytoplasm of the tumor cell. The tumor cells then undergo degenerative changes and cell death occurs in 24 to 36 hr. The lymphocyte-derived material appears to contain immunoglobulin components as determined by specific ferritin labeling.

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fixed in osmium tetroxide and, after graded alcohol dehydration, were embedded in Epon 812. Transmission electron micrographs were taken on an RCA EMU-4.

Specimens to be examined by scanning electron microscopy were fixed in 2% phosphate-buffered glutaraldehyde and were allowed to remain attached to the bottom of the plastic flasks for 5 days. Samples were dehydrated in graded alcohol and dried in a Pelco critical drying point apparatus. The specimens were then coated with gold in a Varian evaporator and scanned with a Hitachi SEM-2.

**Ferritin-labeling Techniques.** Two sets of experiments were done using F-Anti-lgG. The antiserum was obtained from Cappel Laboratories Inc., Downingtown, Pa. The specificity of the antiserum was tested by protein immunoelectrophoresis, showing that the serum contained only rabbit γ-globulin and that it was conjugated to ferritin with no free ferritin present. The specificity of the antiserum for rat immunoglobulin was tested by absorption procedures.

**Experimental Design with Ferritin Label.** Utilizing the tissue culture techniques described above, the following set of test and control cultures was prepared. F-Anti-lgG was diluted 1:10 with tissue culture media (v/v) and added to flasks containing sensitized lymphocyte-tumor cell mixtures after 1, 2, 5, 10, 24, 36, and 48 hr. The F-Anti-lgG was allowed to incubate with the cells at 37° for 15 min, and the cultures were then washed twice with TC 199 to remove any excess ferritin-labeled antiserum and unbound lymphocytes. Immediately after washing, the cultures were flooded with 2% phosphate-buffered glutaraldehyde and prepared for transmission and scanning electron microscopy. The following cell cultures were treated in precisely the same fashion to form control systems: (a) tumor cells plus nonsensitized (control) splenic lymphocytes plus F-Anti-lgG; (b) tumor cells alone plus F-Anti-lgG; (c) tumor cells plus ferritin alone; (d) Lymphocytes plus ferritin alone; (e) Lysed tumor cells (debris) plus F-Anti-lgG; (f) Tumor cells plus sensitized (control) splenic lymphocytes plus F-Anti-lgG; (g) Lymphocytes, the tumor cells undergo vacuolation with loss of the tumor cell wall (Fig. 5). Transmission electron micrographs taken on an RCA EMU-4.

**Results**

**Phase Microscopy.** When viewed by inverted phase microscopy, the sensitized lymphocytes adhere to the tumor cells within 30 min after incubation. By 1 hr, there are 3 to 5 lymphocytes attached to individual tumor cells and a maximum number of lymphocytes is involved in this process by 5 hr. The lymphocytes remain fixed to the tumor surface despite vigorous agitation of the tissue culture media and washing of the lymphocyte-tumor cell mixture with fresh media. These lymphocytes remain firmly adherent to the tumor cells until 24 to 36 hr postincubation, at which time the tumor cells show cytoplasmic vacuolation and become detached from the plastic surface. They are small and rounded with granular cytoplasm at this point.

The control (nonsensitized) lymphocytes adhere poorly and in few numbers to the tumor cells. There is no phase microscopic evidence of tumor cell degeneration over a 5-day incubation period. Tumor cells cultured alone have a similar appearance.

**Scanning and Transmission Electron Microscopy.** The sensitized lymphocytes have a single, large round nucleus. The cytoplasm contains a few mitochondria, single ribosomes, and a few profiles of rough endoplasmic reticulum. Numerous cytoplasmic appendages are present (Fig. 1). The control lymphocytes are morphologically indistinguishable from sensitized cells. Scanning electron microscopy demonstrates a relatively uncomplicated surface with few cytoplasmic appendages (Figs. 2 and 3) but many small surface spikes. By 1 hr, there are numerous lymphocytes attached to a single elongate Walker carcinoma cell. At 5 hr, a maximum number of lymphocytes is attached to tumor cells (Fig. 2). Scanning electron microscopy reveals extensive plasma membrane contact between the lymphocytes and tumor cells (Figs. 2 and 3), and transmission electron micrographs through such junctions show numerous lymphocytic cytoplasmic appendages making close contact with the tumor cell membrane (Fig. 1). There are no specific fine structure changes seen at this interface. In this same time period, there are numerous cellular particles present on the surface of the tumor cells (Figs. 2 and 4).

Some of the lymphocytes show cytoplasmic budding (Fig. 2), and others are apparently undergoing lysis while fixed to the tumor cell wall (Fig. 5). Transmission electron microscopy reveals that these cytoplasmic particles are derived from the lymphocytic cytoplasm and consist of partially enclosed vesicles, cell membranes, endoplasmic reticulum, and ribosomes (Figs. 5 and 8). No mitochondria or lysosomes are seen in this material. At 10 hr, the lymphocytes are still firmly attached to the tumor cells. A few lymphocytes have undergone degeneration with cell death, but the majority remains intact.

At 24 to 36 hr after the incubation with the sensitized lymphocytes, the tumor cells undergo vacuolation with loss of adhesion to the plastic surface (Fig. 6), and most of the lymphocytes are no longer attached to the tumor cells. By 48 hr, only a few tumor cells remain attached to the plastic surface. Occasional lymphocytes have developed extensive endoplasmic reticulum, but most of them remain histologically unchanged, with the exception of a few cytoplasmic vacuoles.

The control lymphocytes obtained from spleens of nontumor-bearing rats adhere poorly to the Walker carcinoma cells and do not develop extensive cytoplasmic budding. The Walker carcinoma cells in the control flasks reveal no significant electron microscopic alterations.

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7 The abbreviation used is: F-Anti-lgG, ferritin-conjugated rabbit anti-rat lgG immunoglobulin.
vacuoles (Fig. 8). Finestructure studies of tumor cells at membrane of the tumor cells as well as within phagocytic particles, which are in the main closely adherent to the labeled with ferritin (Fig. 7), as are the free cytoplasmic particles, which are in the main closely adherent to the membrane of the tumor cells as well as within phagocytic vacuoles (Fig. 8). Fine structure studies of tumor cells at periods of time greater than 5 hr reveal decreased numbers of cytoplasmic particles along the cell surface. Control lymphocytes demonstrated some cell membrane labeling, but very little labeled or unlabeled cytoplasmic material was adjacent to tumor cells. In all control groups treated by ferritin labeling, little ferritin was seen, and it was not related to specific cell structures.

In the 2nd ferritin-labeling experiment, in which sensitized lymphocytes and tumor cells were incubated together for 2 and 5 hr before the addition of F-Anti-IgG and then harvested after 10, 24, and 48 hr of further incubation, many tumor cells contained accumulations of intracytoplasmic ferritin (Fig. 9). There were areas of cytoplasmic degeneration associated with this intracellular ferritin (Fig. 9). Control cultures consisting of tumor cells alone or tumor cells incubated with nonsensitized lymphocytes or with ferritin alone demonstrated only small amounts of ferritin within tumor cell cytoplasm, and no cytoplasmic damage was associated with its presence.

**DISCUSSION**

Our studies demonstrate an orderly sequence of morphological events after sensitized lymphocytes are introduced into the tumor cell culture. In the 1st step, the sensitized lymphocytes adhere to the cytoplasmic membrane of the tumor cell within 30 hr after their introduction. This intimate contact consists of multiple cytoplasmic appendages closely approximated to the tumor cell wall. Such contact does not appear to cause any direct cytotoxic effect to the tumor cells, as no morphological alterations are seen at this interface. This type of lymphocyte-target cell interaction has been described in other cell-mediated hypersensitivity reactions, such as Hashimoto’s thyroiditis (5), and during allograft rejection (22). It seems probable that this initial contact serves as a time of lymphocyte recognition of a specific antigen and perhaps initiates the release of surface material (14) from the sensitized lymphocytes.

The 2nd event occurs within 1 hr after the sensitized lymphocytes have fixed onto the tumor cell membrane, when sensitized lymphocytes shed surface membrane and in some cases undergo cellular lysis. This material, which appears amorphous when viewed by scanning electron microscopy, consists of membranes and cytoplasmic material, as revealed by transmission microscopy examination. It is found almost exclusively on the surface of tumor cells, suggesting that there is specificity to this process. This membrane-like material is morphologically similar to that identified by Kennel and Lerner (12) in the isolation of membrane-bound lymphocyte immunoglobulin.

The 3rd step in this sequence is the presence of lymphocyte-derived material within the tumor cell cytoplasm, as determined by ferritin labeling, and this is followed by tumor cell degeneration within 24 to 36 hr.

Amorphous material associated with stimulated lymphocytes has been reported by a number of investigators. Mamson and Roitt (15) have described an amorphous material surrounding blast cells in anti-allotype serum-stimulated cultures. Bjorklund et al. (3), in a study of lymphocyte-target cell interaction utilizing scanning electron microscopy, have described the presence of amorphous material on tumor cells and suggest that these particles might play a role in target cell destruction.

This study confirms and extends the above observations. We have shown that the amorphous material appears to be derived from the sensitized lymphocytes, that it is composed of membrane-bound cytoplasmic contents, and that it is specifically labeled by F-Anti-IgG. It adheres in a specific fashion to the tumor cell membrane, and ultimately the ferritin label appears within the tumor cell cytoplasm. This event is followed by cytoplasmic vacuolation and death of the cultured tumor cells.

The sequence of morphological events described here is consistent with the theory of delayed hypersensitivity proposed by Karush and Eisen (11), in which they suggest that the mechanism of this reaction was through the production by lymphocytes of a high-affinity antibody released near a surface antigen of the target cells. Marchalonis and Cone (14) have demonstrated that both T- and B-cell lymphocytes shed membrane-associated surface immunoglobulin into their environment, although the presence of surface immunoglobulin on T-cells remains controversial (20). The T-cell lymphocyte has been well-documented as the class of cell responsible for cell-mediated killing. The lymphocytes that attach to the tumor cells in these experiments have the morphological features of T-cells in that they contain few profiles of rough endoplasmic reticulum and, under scanning microscopy, demonstrate short, broad cytoplasmic spikes. However, we recognize that the morphological differentiation of T- and B-cells is unresolved (2, 17). These experiments do not prove that cell-bound immunoglobulin was responsible for the target cell death but rather that specific membrane-associated cellular fractions are shed from sensitized lymphocytes, which, upon contacting tumor membranes, result in tumor cell death. They strongly suggest that the material derived from the lymphocytes contains surface immunoglobulin that is shed with some membrane component. Such an interpretation is concordant with the work of Vitetta and Uhr (20) and may represent the morphological events that accompany the immunoglobulin shedding that they have measured by radioisotope techniques. Studies are in progress (1) using radioactive 131I-labeled surface immunoglobulin to characterize further the lymphocyte-tumor cell interaction described in this report.

**REFERENCES**

Lymphocyte-Tumor Cell Interaction


Fig. 1. Three small lymphocytes (L) are making contact with Walker carcinoma tumor cells (T) after 1 hr postincubation. × 6000.

Fig. 2. Numerous lymphocytes (L) are present on an intact tumor cell (T). Cytoplasmic material (cm) is present on the surface of the tumor cell. One of the lymphocytes (L₁) has undergone partial lysis. × 500.

Fig. 3. Some sensitized lymphocytes (L) extend large pseudopods toward the tumor (T) surface after establishing contact. Bar, 7 μm.

Fig. 4. Particles of cytoplasmic material (cm) derived from lysed or shedding sensitized lymphocytes are seen by scanning microscopy on the tumor cell (T) surface. × 2000.
Fig. 5. A sensitized lymphocyte (L) adjacent to a tumor cell shows cytoplasmic material (cm), consisting of vesicles, ribosomes, and endoplasmic reticulum, being released. x 15,000.

Fig. 6. By 36 hr most of the tumor cells incubated with sensitized lymphocytes have undergone degenerative changes. Tumor cytoplasm (cyto) can be identified in lysed cells. x 10,000.

Fig. 7. This transmission micrograph shows a sensitized lymphocyte (L), with F-Anti-IgG (F) surrounding cytoplasmic material (cm) and portions of its cell wall. x 20,000.
Fig. 8. Cytoplasmic material (cm) is seen adjacent to a tumor cell (T). Ferritin particles (F) can be seen phagocytosed into the tumor cytoplasm. \( \times 11,000 \).

The inset shows detail of ferritin-labeled material adjacent to cell wall. \( \times 55,000 \).

Fig. 9. Some tumor cells (T) show areas of vacuolization (V) adjacent to deposits of intracellular ferritin (F). \( \times 12,500 \).
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