Acta Medica Okayama

Volume 33, *Issue* 6 1979 *Article* 3

DECEMBER 1979

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Abstract

Peripheral blood lymphocytes and the various lymphocyte fractions from patients with cancer of the colon were cultivated with target cells (P-4788) derived from the colon cancer. Changes in the surface ultrastructure during tumor cell destruction were studied by scanning electron microscopy (SEM). P-4788 cells adhering to the coverslip showed various surface activity. The surfaces of some cells were relatively flat; others were smooth or had fine granules. Still other cells were villous, round or had marked blebs. When host lymphocytes were added to the target cells, adhesion of the two cell groups began by many fine projections. After incubation for 6 h, some lymphocytes had adhered to the target cells. Many lymphocytes had adhered to the target tumor cells by 24–48 h incubation. Ultimately the tumor cells became swollen and disrupted. Most lymphocytes adherent to the target cells had few microvilli. Lymphocytes after elimination of phagocytes by carbonyl iron treatment also adhered readily. Some target cells showed adhesion with lymphocytes passed through nylon-wool columns, although the number of lymphocytes adhering was fewer than in the case of lymphocytes not passed through nylon-wool columns. T cells were collected from lymphocytes that form rosettes with SRBC by isolation with NH4Cl. They had markedly elongated microvilli which in places were sparsely scattered and tended to be localized on the side, a finding which suggests loss of cell activity by the time of SEM. Only a few T cells adhered to target cells and they seemed to be T cells without activity. It was thought that there are cytotoxic cells among T cells and that the co-existence of T cells, non-T cells and monocytes caused target cell destruction.

KEYWORDS: canning electoron microscope, colon cancer-derived cell P-4788, target cell destruction, lymphocytes, lymphocyte fractions

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*PMID: 161468 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **33**, (6), 431—442 (1979)

SCANNING ELECTRON MICROSCOPY OF INTERACTION OF PERIPHERAL BLOOD LYMPHOCYTES FROM COLONIC CANCER PATIENTS WITH HUMAN COLONIC CANCER-DERIVED CELLS; P-4788

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Received June 6, 1979

Abstract. Peripheral blood lymphocytes and the various lymphocyte fractions from patients with cancer of the colon were cultivated with target cells (P-4788) derived from the colon cancer. Changes in the surface ultrastructure during tumor cell destruction were studied by scanning electron microscopy (SEM). P-4788 cells adhering to the coverslip showed various surface activity. The surfaces of some cells were relatively flat; others were smooth or had fine granules. Still other cells were villous, round or had marked blebs. When host lymphocytes were added to the target cells, adhesion of the two cell groups began by many fine projections. After incubation for 6 h, some lymphocytes had adhered to the target cells. Many lymphocytes had adhered to the target tumor cells by 24–48 h incubation. Ultimately the tumor cells became swollen and disrupted. Most lymphocytes adherent to the target cells had few microvilli. Lymphocytes after elimination of phagocytes by carbonyl iron treatment also adhered readily. Some target cells showed adhesion with lymphocytes passed through nylon-wool columns, although the number of lymphocytes adhering was fewer than in the case of lymphocytes not passed through nylon-wool columns. T cells were collected from lymphocytes that form rosettes with SRBC by isolation with NH_4Cl. They had markedly elongated microvilli which in places were sparsely scattered and tended to be localized on the side, a finding which suggests loss of cell activity by the time of SEM. Only a few T cells adhered to target cells and they seemed to be T cells without activity. It was thought that there are cytotoxic cells among T cells and that the co-existence of T cells, non-T cells and monocytes caused target cell destruction.

Key words: scanning electron microscope, colon cancer-derived cells P-4788, target cell destruction, lymphocytes, lymphocyte fractions

Many experimental tumors and human cancers have tumor specific antigens (TSA) and tumor associated antigens (TAA), thanks to which the cancer bearing host is able to resist the tumor by lymphocyte suppression of tumor proliferation. In the cancer bearing hosts with identical tissue systems, the existence of com-

mon antigens has been generally demonstrated. Common antigens have also been demonstrated in colon cancer (5, 6, 7).

The ultra-structure of target cell destruction in experimental tumor has been studied with SEM using JTC-cells derived from Ehrlich ascites tumor and regional lymph node cells (9). However, to date, there has been little morphological study by SEM using human cancer as both target cells destruction and common antigens. Therefore, with P-4788 cells derived from the colon cancer as the target cells, peripheral blood lymphocytes and various fractionated lymphocytes from patients with cancer of the colon were used as effector cells and their *in vitro* interaction was observed by SEM. The findings are presented here.

MATERIALS AND METHODS

Patients. Patients used as a source of lymphocytes all had colon or rectal cancer. They had been admitted to our hospital or to hospitals affiliated with Okayama University, and had positive cytotoxic reactions in tests using the Takasugi-Klein method (8). Histologically, all tumors were adenocarcinoma.

Target cells. P-4788 cells derived from the colon cancer were used. These cells were the 53rd generation of cells established from metastatic thoracic fluid of rectal cancer (adenocarcinoma) by Moore and Koike of Rosewell Park Memorial Institute (1). The cells, when transplanted to mice bearing ACTH-producing tumors, proliferated as a solid tumor. The histology was identical to the original tumor. These cells grew as monolayers and were subcultured twice weekly.

Medium. RPMI 1640 medium with 20% fetal calf serum and 200 ug/ml Kanendomycin was used.

Lymphocytes. From patients with colon cancer, 10 ml heparinized peripheral blood was collected and lymphocytes were isolated by the following procedures: 1. lymphocytes were purified by the Conray 400-Ficoll method (10) (these cell preparations contained about 90% lymphocytes mixed with phagocytes and monocytes). 2. The heparinized blood was mixed with 10% carbonyl iron (2) and incubated for one hour at 37°C. Lymphocytes were then purified by Conray 400-Ficoll gradient centrifugation (more than 98% of non-phagocytic cells had lymphocyte morphology). 3. After purification of heparinized blood from patients with colon cancer by the Conray 400-Ficoll method, lymphocytes were collected by passage through nylon-wool columns (3) (this gave about 85% T cells). 4. After purification of heparinized blood from a patient with colon cancer by the Conray 400-Ficoll method, lymphocytes were made to form rosettes with SRBC, then NH_4Cl was added to isolate genuine T cells (about 99% T cells mixed with fewer than 1% B cells) (4).

Culture method. 1. Culture of P-4788 cells alone: one day before mixed culture of lymphocytes and target cells, target cells were harvested by treating with 0.25% trypsin and gently pipetting. After washing and resuspension in fresh medium, the cells were counted and diluted to about 1×10^4 cells/ml. Of this, 0.2 ml of target cells was placed in each well of multiwell tissue culture plates

(Falcon No. 3040, Oxnard, Calif., U.S.A.). Each well had a coverslip with a surface area equal to the bottom of the well. The multiwell plates were incubated for 24 h at 37°C in a humidified incubator containing 5% carbon dioxide.

2. Mixed culture of lymphocytes and target cells: next day, 10 ml heparinized peripheral blood was taken from a patient with colon cancer. Lymphocytes were purified by the Conray 400-Ficoll method, fractionated, washed, and resuspended in medium to a final concentration of 1×10^6 cells/ml.

After living cancer cells had adhered to the coverslip on the floor of the well, the medium with free cells and debris was removed by aspiration. Then, 0.2 ml of the medium containing lymphocytes was layered on the target cells. Plates were incubated at 37°C in an atmosphere of 5% carbon dioxide.

Preparation of specimens for SEM. After completion of the culture, coverslips with attached cells were taken out at 6 h, 12 h, 24 h, 48 h after mixed culture of target cells and lymphocytes, and treated as follows: to remove culture medium, the specimens were washed in phosphate buffer solution (PBS) for about 5 min. Then they were fixed for about 24 h with glutaraldehyde in PBS, further immersed in PBS for one hour, dehydrated in a graded series of alcohol for 5 min at each stage, and suspended in amylacetate. Samples were dried by the critical point drying method using liquid CO_2, and coated with a thin layer of carbon and gold palladium in a vacuum evaporator. The specimens were examined with SEM (JSM-U3 type, Japan Electron Optics Laboratory Co., Ltd., Tokyo) at 25 KV.

RESULTS

1. Human colonic cancer-derived cells P-4788: P-4788 cells in monolayer culture had various shapes (such as spindle, ovoid, and triangular) when attached to the coverslips. With SEM, their surfaces varied from relatively flat or relatively smooth, to surfaces with fine granules. Other cells were villous, round, or had marked blebs (Fig. 1).

Cells of the same population showed marked variation in different phases of the cell cycle. Some P-4788 cells had pseudopodia or lamellipodia. Others had long filopodia which extended from the cell margin, but most had no ruffle. Most P-4788 cells were relatively flat (Fig. 2). Further study of this with nonmitotic cells showed that the central portion of the surface (probably the site of the nucleus) was covered with numerous small granular protuberances (Fig. 3).

Fig. 1. P-4788 cells show various surfaces: relatively flat, relatively smooth, with fine granules, villous, round, or having marked blebs. (×1,000)

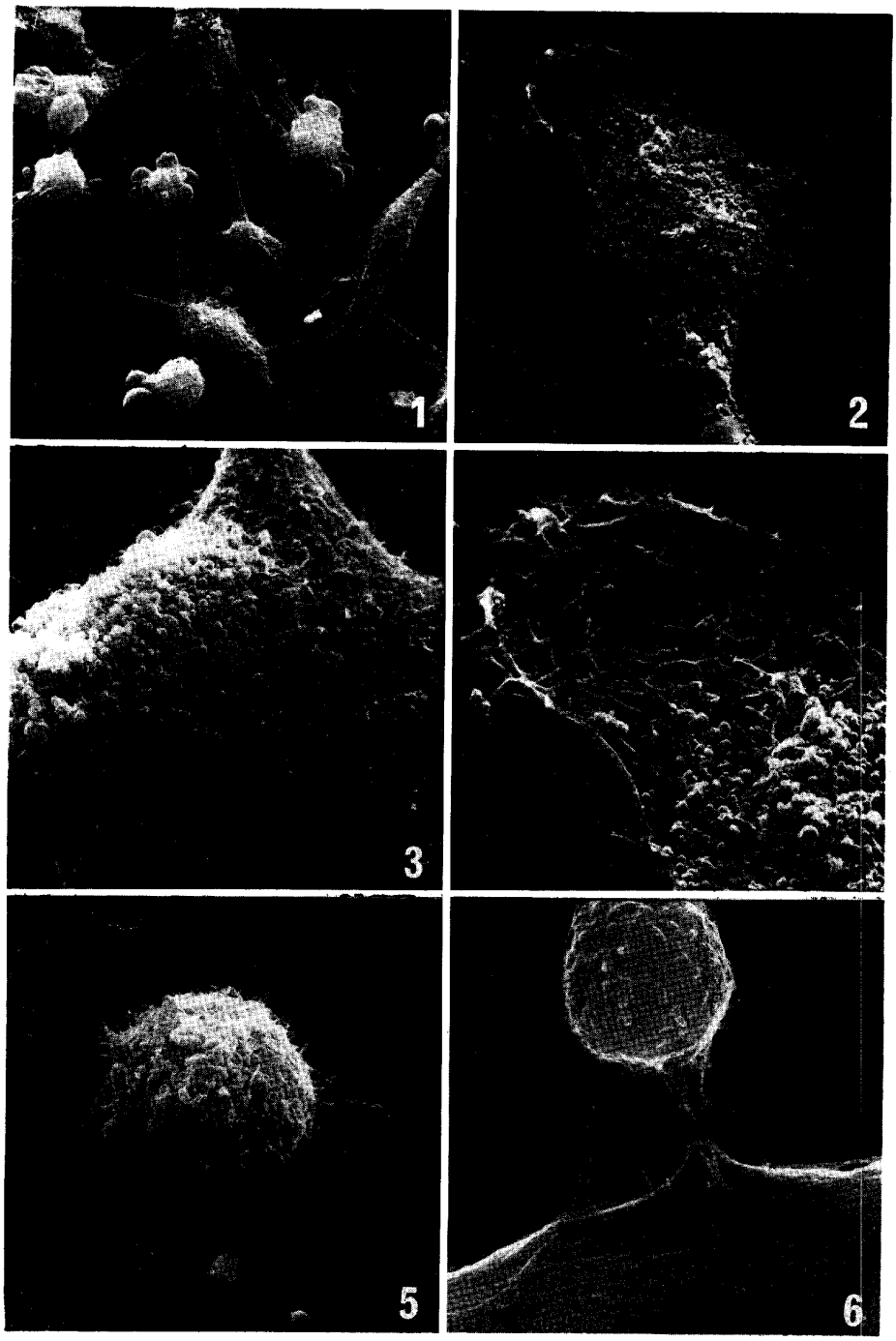
Fig. 2. Most P-4788 cells are relatively flat (×2,000).

Fig. 3. In observing P-4788 cells more precisely, there can be seen numerous short and fine granules in the center of the cell (×6,000).

Fig. 4. The periphery of P-4788 cells has slender microvilli (×6,000).

Fig. 5. This cells seems to be at a mitotic stage. It is round and covered with microvilli (×3,000).

Fig. 6. When the lymphocyte starts to make a contact, the target cell protrudes slender projections (×10,000).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The cell periphery (considered to be the cytoplasmic region) had numerous slender microvilli (Fig. 4).

The cell shown in Fig. 5 seems to be at a mitotic stage. It is round and covered with microvilli and there are long fine projections (filopodia) protruding from the cell surface and attached to the coverslip.

2. Mixed culture of lymphocytes and target cells:

(1) Lymphocytes purified by the Conray 400-Ficoll method (about 90% lymphocytes mixed with some phagocytes) adhered to, and destroyed, target cells. The adhesion began by the projection of microvilli from target cells (Figs. 6, 7) or from lymphocytes (Fig. 8). With lapse of time, the area of the adhesion increased giving the appearance of fusion of the two cells involved (Fig. 9). After 6 h of mixed culture, the lymphocytes appeared to be mounted on the target cells (Fig. 10). After incubation for 24–48 h, many target cells had numerous lymphocytes attached (Fig. 11). Granular shapes had developed on the surface of target cells (Fig. 12).

Ultimately target cells became swollen and disrupted (Fig. 13). The adherent lymphocytes had few microvilli and were intermediate or small in size (Fig. 14). Phagocytes were attached to the periphery of the target cells (Fig. 15). However, few target cells had phagocytes attached.

(2) When lympocytes treated by carbonyl iron (about 98% non-phagocytic

Fig. 7. At the contact of the lymphocyte, the target cell show protruding numerous fine projections (×10,000).

Fig. 8. The lymphocyte itself also protrudes slender microvilli to make a contact with the target cell (×10,000).

Fig. 9. As the time elapses, the contact of lymphocyte grows more firm with a wider surface (×10,000).

Fig. 10. After incubation for 6 h, lymphocytes begin their contact as if they are riding on the target cell (×2,000).

Fig. 11. After mixed culture for 24 h, many lymphocytes show adhered to the target cell (×2,500).

Fig. 12. Some target cells have granular substances on the cell surface. Such substances appear most markedly in the vicinity where many lymphocytes are attached (×2,500).

Fig. 13. Ultimately the target cell boeomes swollen and disrupted (×3,000).

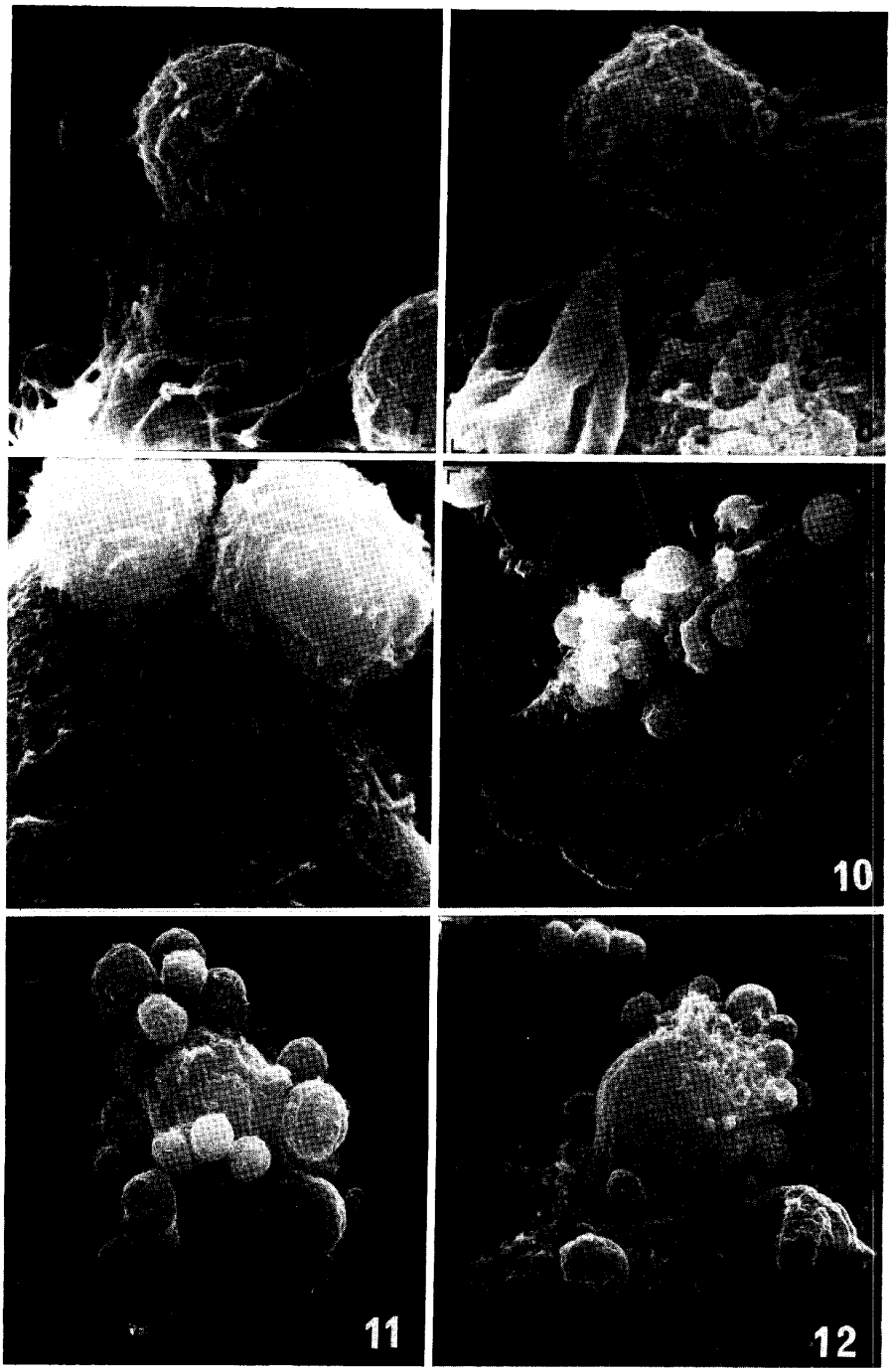
Fig. 14. Many lymphocytes show attached to the target cell. Those lymphocytes adhered have less microvilli and they are intermediate or small in size (×2,000).

Fig. 15. Here the phagocyte (monocyte) is seen adhering to the target cell (×3,000).

Fig. 16. Lymphocytes treated by carbonyl iron are attached in a considerable number to the target cell (×2,000).

Fig. 17. Lymphocytes passed through nylon wool columns show attached to some target cells (×3,000).

Fig. 18. T-cells isolated with the use of NH_4Cl from lymphocytes that form rosettes with SRBC have markedly elongated microvilli which in places are sparsely scattered and localized on the side. Most likely they might have lost the cell activity (×3,000).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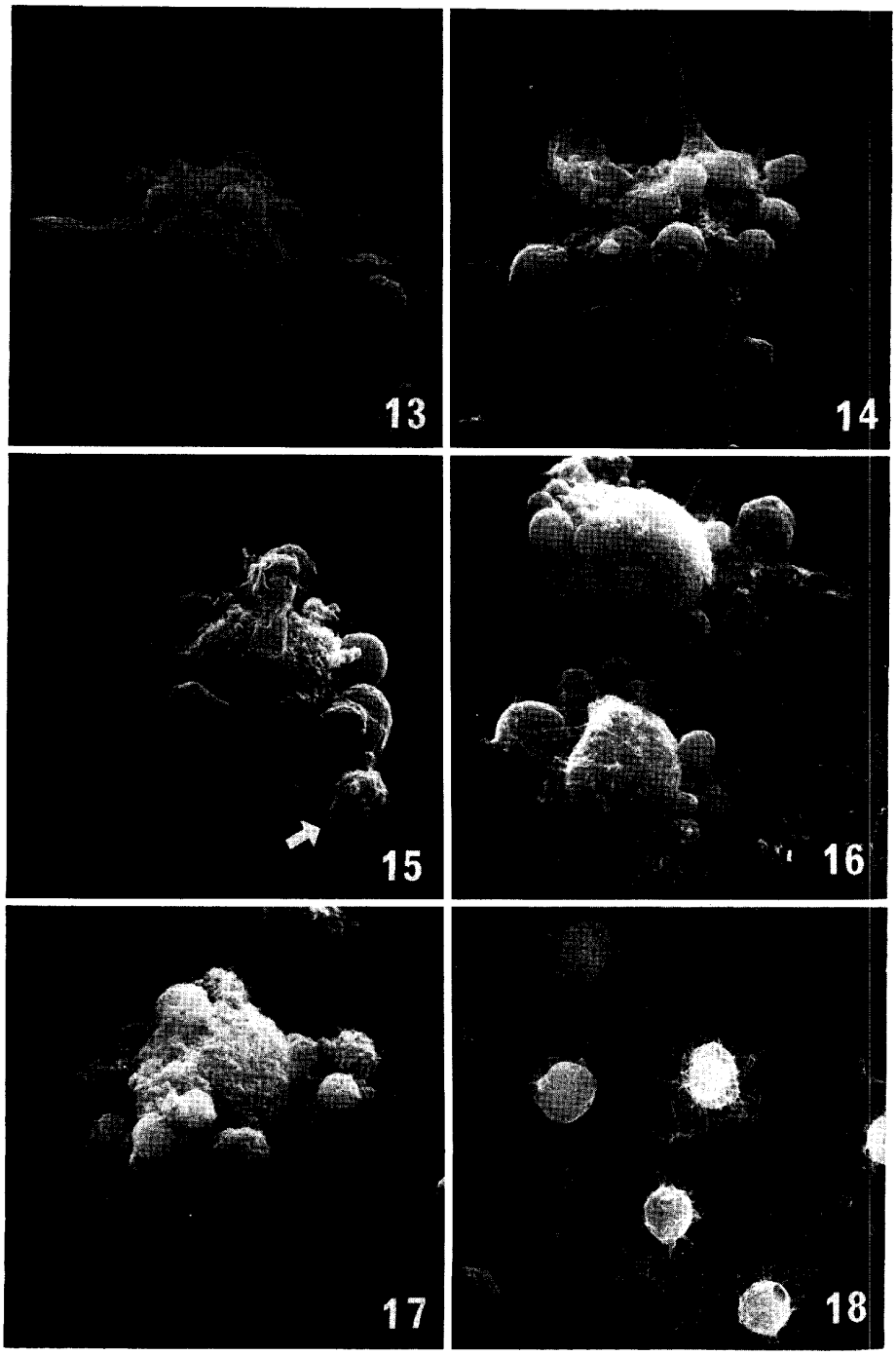
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cells with lymphocyte morphology) were added to target cells, intermediate or small lymphocytes preferentially adhered to target cells (Fig. 16). It is thought that if even a few phagocytes are present, adhesion to target cells can occur.

(3) Many of the lymphocytes passed through nylon-wool columns (about 85% T cells) became adherent to target cells (Fig. 17). However, the number of target cells with such lymphocytes attached was fewer than when passage through nylon-wool columns was omitted.

(4) T cells isolated with NH_4Cl from lymphocytes that form rosettes with SRBC (about 99% T cells) had markedly elongated microvilli. In places, these microvilli were sparsely scattered and localized on the side, suggesting that cell activity may have been lost by the time they were observed by SEM (Fig. 18). When such T cells were added to target cells, target cells with many T cells attached were few in number. Even if T cells were attached, they were apparently inactive T cells (Fig. 19).

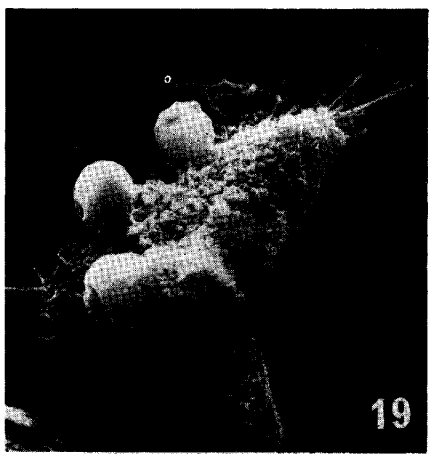

Fig. 19. T-cells isolated with NH_4Cl from lymphocytes that form resettes with SRBC show attached to some target cells, but such attached T-cells have most likely lost the cell activity (×4,000).

DISCUSSION

Since Govaerts 1960 (11), many investigators have demonstrated that sensitized lymphocytes destroy target cells *in vitro*. Techniques have included phase microscopy, phase microscopic cinematography, cytolytic plaque test, dye exclusion test, ^{51}Cr release cytotoxicity test, and colony inhibition test, although SEM as reported here is rare.

There have been many studies of cultured malignant cells *in vitro* (12, 13, 14). Cultured malignant cells are characterized by having more surface features

such as blebs, ruffles, and microvilli than their non-neoplastic counterparts (12, 13, 14). The P-4788 cells in this study had surface features similar to cultured malignant cells and characteristically had marked blebs. The behaviour and function of these blebs is poorly understood although it is known that most telophase cells display blebs, and that about half the small number of cells covered with blebs are in telophase (12, 15). All of these surface structures appear to be transient: that is, constantly forming and reforming (12).

A number of authors have described surface changes accompanying mitotic divisions of various cell lines (14, 15). Approximately 60% of fully rounded cells covered with microvilli are in metaphase or anaphase (15). Microvilli are generally regarded as a device for increasing the surface area and facilitating the transport of metabolites (12). Lang *et al.* suggested that the development of microvilli is a mechanism by which cell membrane area is conserved when a cell assumes a morphology characterized by decreased surface area (14). In the present study, microvilli were seen to become protrudings or probes at the time of lymphocyte attachment.

There are two steps in target cell destruction by cytotoxic effector cells. First, effector cells attach to target cells and cluster around them. Second, following the contact established in the first step, the target cells lyse. Lin *et al.* observed the interaction between bovine leukemic lymphocyte (NBC-6) and African green monkey fibroblasts (VERO) by SEM, and described the following: 1. contact between microvillous tips of the two cell types. 2. probing of VERO cell surfaces by NBC-6 microvilli, and 3. penetration of NBC-6 microvilli into VERO cells (16). Similar results were obtained in the present study.

Lang *et al.* observed the interaction between effector cells (lymphocytes and monocytes) collected from the peripheral blood of melanoma patients and human melanoma cells *in vitro* and described the following: when co-cultivated with effector cells, a number of the target cells developed surface microvilli, primarily in the area where effector cells were attached (14). In the present study, however, there was no apparent difference in the number of microvilli between the area where lymphocytes were attached and areas where lymphocytes were not attached.

Transmission electron microscopy (TEM) has shown that the contact of target cells and lymphocytes is accomplished by fusion of broken cell membranes of the two cell types (17, 18). Communication of cytoplasm between the two cells is probably established (19). With autoradiography of co-cultures of sensitized lymphoid cells labeled with ^{3}H-amino acid and target tumor cells, however, contact of lymphoid cells with target cells and destruction of the target cells occur within a few hours, but labeled grains in the attached lymphoid cells do not migrate into tumor cells at any stage (20). In the present study of the contact

surface between lymphocytes and target cells, SEM showed that lymphocytes adhered to the surface of the target cell without embedding as if they were riding on it. It was not clear whether there was any communication of cytoplasm.

One target cell destruction theory postulates that lymphocytes produce "cytotoxic substance" which destroys the target cell (21, 22, 24). In the present study, "granular substances" were observed at times in the vicinity of the contact area on the surface of the target cell. Such granular substances were located especially around the lymphocytes which had become "empty cell husks". Such granular substances may be produced by degeneration of the target cell due to lymphocytes, or by lymphocytes.

At present, lymphocytes and macrophages (monocytes) are thought to be the effector cells which directly impede the target cell (21). TEM observations suggest that macrophages engulf the surface protuberances of the target cells, and this may provide a stimulus for the synthesis of a "mediator" for cell destruction by macrophages, or may act directly to bring about cell destruction (23). Cells of the monocyte-macrophage series have been implicated as effector cells in tumor immunity (24). Lymphocytes treated by carbonyl iron consist of 98% non-phagocytic cells, but the author considers that if even a few phagocytes (monocytes) are present, target cell destruction may occur.

For a considerable time it has been assumed, on the basis of little evidence, that T cells are the major effector cell in cell-mediated immunity (24). Plata *et al.* reported that, with the ^{51}Cr release test, T cells were necessary for tumor cell immune lysis, and that there appeared to be no requirement for non-T cells (26). Lamon *et al.* demonstrated that the cytotoxic cells could be removed from cell suspensions by exposure to anti-immunoglobulin columns and concluded that the major cytotoxic cell was the B cell (24, 27). Grant *et al.* considered that the population of cytotoxic T cells is only one transient facet of the efferent limb; many other cell-dependent cytotoxic mechanisms being present at the same time. In other words, the cytotoxic T cell is only a minor component in a complex battery of effector cells, and only operates early in an immune response (24, 25). O'Toole *et al.* reported that after passage through anti-immunoglobulin, lymphocytes previously specifically cytotoxic for tumor cells lost all such activity. Non-T cells were necessary for the *in vitro* expresion of cell-mediated immunity (28).

In the present study, the numbers of target cells to which lymphocytes passed through nylon-wool columns were attached were fewer than in the case when such passage was omitted. Therefore, the author considers that passage through nylon-wool columns comparatively diminishes the cytotoxic activity. Few lymphocytes isolated by the use of NH_4Cl from lymphocytes which form rosettes with SRBC became adherent to target cells, indicating loss of their cell

activity.

Currie reported that cytotoxic T cells occur but that in most test systems where they have been found they act as effector cells only transiently. Cytotoxic cells are obtained from whole blood by methods designed to isolate lymphocytes and the lymphocyte has for a long time been incriminated. Morphological criteria, however, are far from adequate (24). Improvements in the isolation of living genuine T cells and elucidation of the definitive morphology of the effector cell are problems for future study.

Acknowledgment. The author wishes to express his profound thanks to Prof. Kunzo Orita for guidance and advice throughout this work and for critical reading of the manuscript, and to Dr. Eiji Konaga for his critical reading of the manuscript.

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