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Abstract

Scanning electron microscope (SEM) observations were conducted mainly on the in vitro interaction state between JTC-II cells derived from Ehrlich ascites tumor and regional lymph node cells obtained from the mice 10 days after transplantation of Ehrlich ascites tumor. Cells cultured on the cover glass were fixed with glutaraldehyde, dehydrated with graded acetone solution, and covered with carbon and gold, were observed by SEM. The results may be briefly summarized as follows. On the surface of JTC-II cells themselves are seen numerous fine microvilli projecting out regularly at right angle to the cell surface, which become attached to the glass, and there can be observed vacuoles in the cytoplasm. Such microvilli are lacking at the tip of the pseudopodial projection. The lymph node cells aggregated to JTC-II cell are lymphocytes of small or intermediate size, and the pattern of aggregation varies: some lymphocytes effect an intimate contact with the surface of target cell by their cellular projections; the contact is achieved by interdigitation of microvilli between lymphocyte and target cell; both cells form a bridge connection with a simple projection from each; or the two cells make a broad surface-to-surface contact. It is not possible to differentiate sensitized lymphocyte from nonsensitized one, their cell shape is spherical with rough surface and some cells show hole on the surface.

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SCANNING ELECTRON MICROSCOPY OF CONTACTUAL INTERACTION OF SENSITIZED LYMPHOCYTES WITH HOMOLOGOUS TARGET CELLS

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It is generally accepted that transplantation immunity and cancer immunity are mediated by lymphocytes. Sensitized lymphocytes, coming in direct contact with target cells *in vitro*, are known to adhere and aggregate onto the target cell, inhibit the growth and destroy the latter (1, 2, 3). Recently, it is considered that specific aggregation of sensitized lymphocytes represents the true nature of cellular immunity, and the destruction of target cells is a non-specific reaction occurring after aggregation (2, 3) so that a greater importance has to be given to elucidation of aggregation mechanism.

By means of nuclear counts and phase-contrast microscope observations or time lapse phase-contrast cinematography, HARA (4) and SATOH (5) of our laboratory studied antitumor activity of sensitized lymphocytes on target cells, and MANNAMI (6), by transmission electron microscopy (TEM), observed the direct attachment *in vitro* of regional lymph node cells from mice bearing Ehrlich ascites tumor with JTC-11 cells derived from Ehrlich ascites tumor cells. In the present paper a report is made on several findings about the interaction between JTC-11 cells and lymph node cells as observed by scanning electron microscopy, while using similar materials as MANNAMI did except for the observation methods.

MATERIALS AND METHODS

Animals: The animals used were Cb mice of about two months old weighing about 20g, bred in Okayama University Mouse Colony, and were fed on solid feed of Oriental Yeast Co.

Tumor cells: Ehrlich ascites tumor cells were maintained successively through the peritoneal cavity of Cb mice at Department of Pathology, Okayama University Cancer Institute.

Culture cells: The cells were derived from Ehrlich ascites tumor cells and

registered as JTC-11 cells at Japan Culture Association and maintained at Okayama University Cancer Institute. This strain of cells is capable of producing original tumors in mice.

Tissue culture medium: The culture medium was composed of the mixture of YLE solution and inactivated bovine serum (8 : 2, v/v) added with 100 r/ml keflin.

Sensitization: To Cb mice, 5×10^6 of Ehrlich ascites tumor cells were transplanted subcutaneously on the back between the scapulas.

Lymph node cell suspension: Ten days after transplantation mice were sacrificed and axillary and cervical lymph nodes were aseptically extirpated. These lymph nodes were cut into small pieces in cold Hanks solution and passed through 80-mesh filter. The filtrate was washed 3 times with Hanks solution by centrifugation at 1,500 rpm for 5 minutes, and then suspended in the culture medium. The lymph node cell suspension prepared in similar manner from normal mice served as controls.

Culture method: 1) Culture of JTC-11 cells alone: JTC-11 cells forming a monolayer were gently scraped off from the vessel wall with a rubber cleaner, and 2×10^4 cells/ml were put into a TD-15 bottle having a round cover glass 10 mm in diameter at its bottom and cultured alone at 37°C for 48 hr.

2) Mixed culture of lymph node cells and JTC-11 cells: 2×10^4 cells/ml of JTC-11 cells and 80×10^4 cells/ml of normal lymph node cells or sensitized lymph node cells were placed into TD-15 bottles similarly having a round cover glass at the bottom, and the mixed culture was carried out at 37°C for 48 hr.

Treatment of cells for scanning electron microscopy: After the completion of culture the cover glass with cells attached on it was taken out, and treated as follows: To remove the culture medium, the specimens were washed by immersing into physiological saline solution for about 5 min. Then they were put into a bath of the fixative fluid (2% glutaraldehyde adjusted to pH 7.4 with 0.1 M phosphate buffer) and fixed for 4 hr. The fixed specimens were further immersed in the saline solution for one hr and dehydrated with a series of graded acetone to be dried in air. Carbon and gold were evaporated in various perspectives in vacuum against these dry samples until the base of the block on which the samples were mounted took a slight golden hue.

Observation and photography were taken with a scanning electron microscope (JSM-U3 type, Japan Electron Optics Laboratory Co. Ltd., Tokyo), using an accelerating voltage of 5KV.

RESULTS

A. *Cultured JTC-11 cells*: It is said that a generation time of JTC-11 cells under the foregoing culture conditions is 12 hours (7), but there can be observed the cells at various growth phases with JTC-11 cells at the culture 48 hours. The majority of them have an irregular surface with

several pseudopodial projections by which they are attached to the glass surface. Inclusive of these projections these are seen on the entire cell surface fine microvilli which protrude rather regularly at right angle to the cell surface and attached firmly to the glass surface (Figs. 1-2).

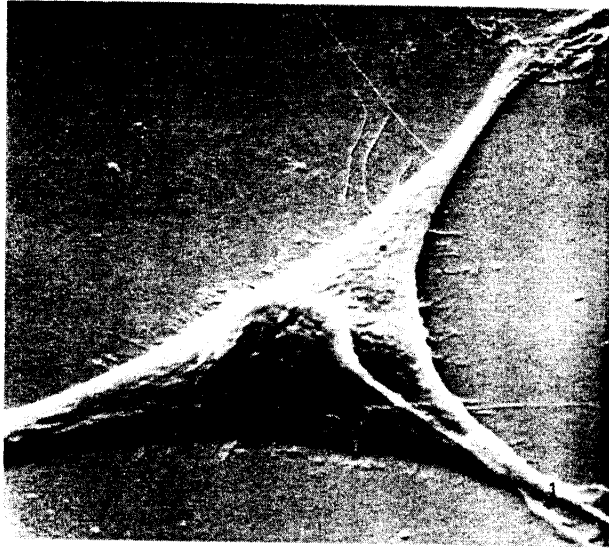


Fig. 1 JTC-11 cell showing bulge at the portion corresponding to the nucleus. $\times 2,400$



Fig. 2 JTC-11 cell $\times 4,000$

However, the peripheral end of pseudopodial projections is broadened and flat, and at this end there can be observed no fine microvilli. Within cytoplasm the portion that appears to be nucleus is bulging up markedly, and the cytoplasm reveals vacuoles here and there.

The cells considered to be at mitotic stage (Fig. 3) present a spherical shape, and the bulged portion, what appears to be the nucleus, becomes indistinct. The cell surface is covered with numerous short protrusions of varying size, and many long microvilli are projecting out regularly from the cell surface, which attach firmly to the glass surface.

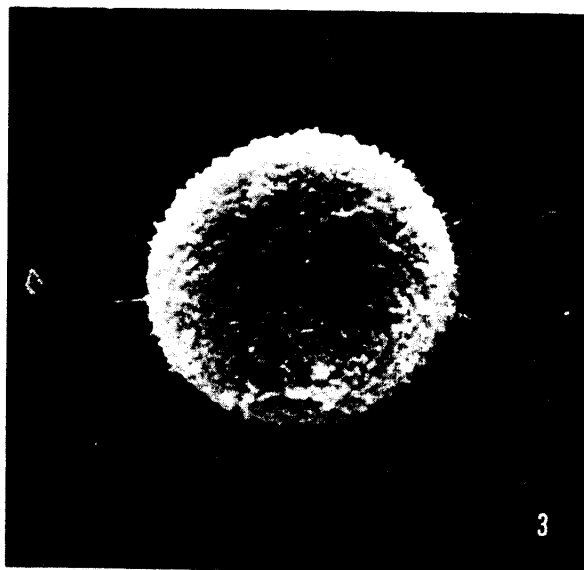


Fig. 3 JTC-11 cell at prophase $\times 4,500$

B. *Mixed culture of lymph node cells and JTC-11 cells*: Sensitized lymph node cells are seen aggregated onto JTC-11 cells (Fig. 4), but the manner of the aggregation varies. Some lymph node cells adhere to the target cell by protruding fine projections on both sides (Fig. 5), some attach to the target cell by projections on one side of lymph node cell (Fig. 6), and some are fused firmly by one relatively large projection to the surface of the target cell as in Figs. 7 and 8, while others adhere by a broad surface-to-surface contact (Fig. 9). The JTC-11 cells having sensitized lymph node cells aggregated on them show less microvilli on the cell surface and also poor growth of their pseudopodial projection, and some of them are undergoing cytolysis (Fig. 10). From the size of the lymph node cells aggregating on the target cell, most of them are lymphocytes of small or intermediate size.

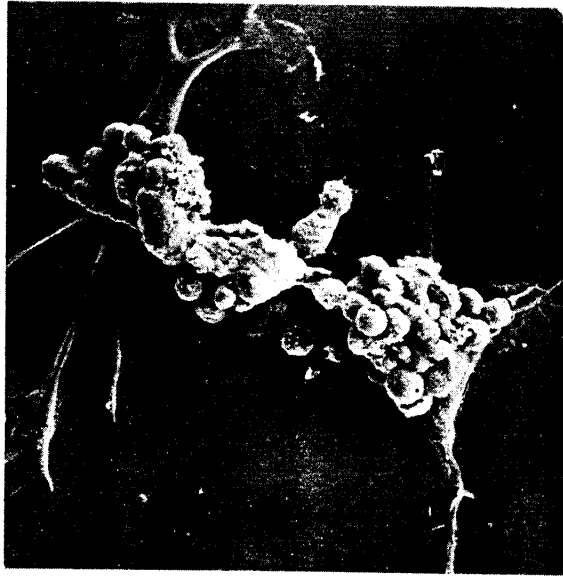


Fig. 4 Lymphocytes are seen aggregating onto JTC-11 cell in the mixed culture of sensitized lymph node cells and JTC-11 cells. Lymphocytes show small projections and some reveal hole. $\times 800$

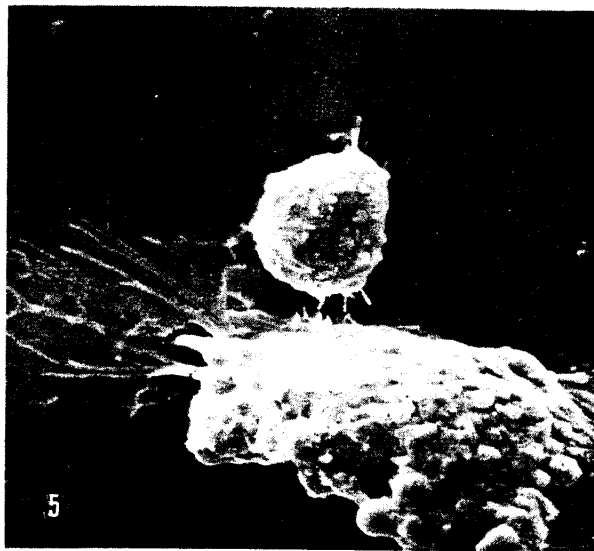


Fig. 5 Mixed culture of sensitized lymph node cells and JTC-11 cells. The union of lymphocyte and JTC-11 cell is achieved by short projections from one another. $\times 4,000$



Fig. 6 Mixed culture of sensitized lymph node cells and JTC-11 cells. Lymphocyte is attached to JTC-11 cell by its own projection. $\times 6,000$

Fig. 7 Mixed culture of sensitized lymph node cells and JTC-11 cells. Lymphocyte and JTC-11 cell are united by a relatively large projection forming a bridge between them, though it is not clear whose projection forms the bridge. $\times 10,000$





Fig. 8 A greater magnification of Fig. 7. $\times 30,000$

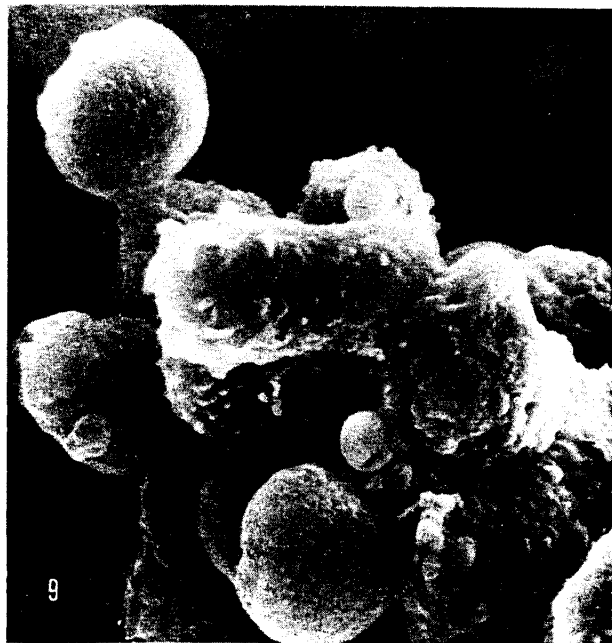


Fig. 9 Mixed culture of sensitized lymph node cells and JTC-11 cells. Lymphocyte and JTC-11 cell are seen fused by a broad surface-to-surface contact. $\times 7,000$

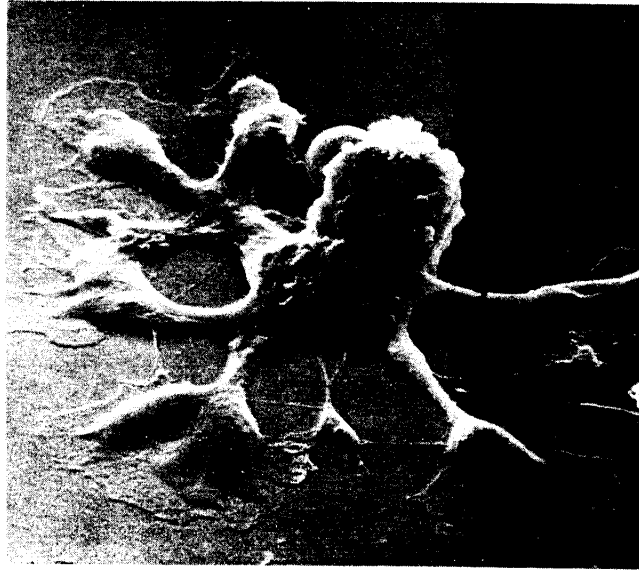


Fig. 10 Mixed culture of lymph node cells and JTC-11 cells. JTC-11 cell is seen undergoing cytolysis, showing cytoplasm flowing out of the tip of its pseudopodial projection. Bulging portion in the center appears to be the nucleus. $\times 3,000$

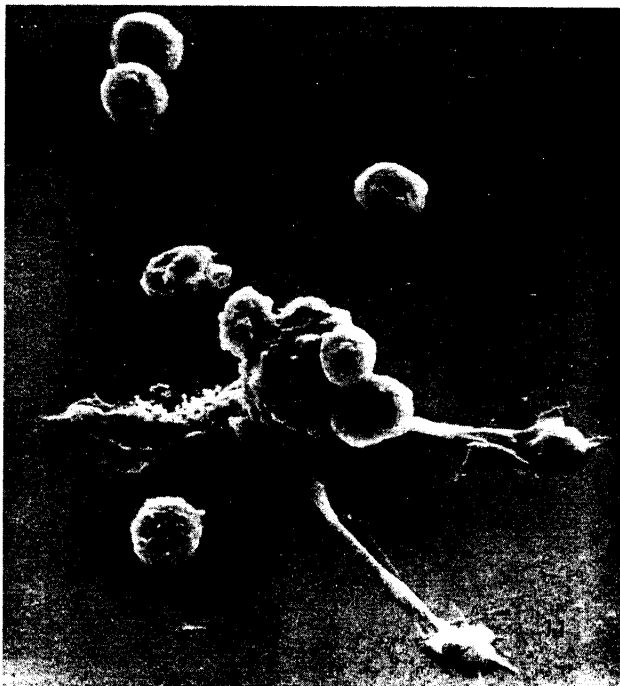


Fig. 11 Mixed culture of sensitized lymph node cells and JTC-11 cells. There can be observed some lymphocytes aggregating on JTC-11 cells, while some are free and scattered, but there is no distinct morphological difference between these two groups; some show small projections and others small hole. $\times 2,500$

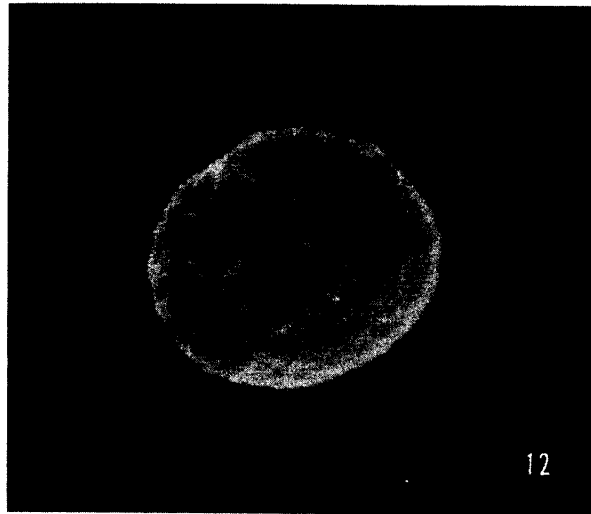


Fig. 12 Normal lymph node cell, showing a few small holes on its surface. $\times 12,000$

In the case of the mixed culture of JTC-11 cells with normal lymph node cells, there can be hardly seen lymph node cells fusing with target cell, and even if they do, the fusion is not so firm, showing only inter-digitation and no change at all in the shape of JTC-11 cell.

Looking at the lymphocytes aggregated and fused with the target cell and those lymphocytes that are free and scattered, there can be observed no special morphological difference between the two groups. Either of these lymphocytes is roughly spherical in shape, some with smooth surface, others with short protrusions, and still others show one to several holes on the cell surface (Figs. 4, 11, 12).

DISCUSSION

As the scanning electron microscopy (SEM) has been introduced to the study of cytology only recently, the reports on cultured cancer cells by this means are scarce. However, in the present investigation by SEM, it has been possible to demonstrate numerous fine microvilli projecting out at the right angle from the cell surface of JTC-11 cell, the presence of which had not been possible to detect by conventional methods of light microscopy, phase-contrast microscopy or transmission electron microscopy (TEM). Study on cultured lymphocytes by SEM is also extremely scanty (8), but small holes sometimes observable on the cell surface of lymphocytes can be said new findings.

It is well known that sensitized lymphocytes as the effector cells of cell-mediated immunity do specifically aggregate on target cell *in vitro*. By *in vitro* cultures of sensitized mouse lymph node cells obtained from mice previously transplanted with Ehrlich cancer together with JTC-11 cells (derived from Ehrlich cancer) HARA (4) and MANNAMI (6) and ORITA (9) of our laboratory have demonstrated that the sensitized lymphocytes do aggregate on JTC-11 cells. Tracing them by time lapse cinematography, sensitized lymphocytes begin to aggregate on JTC-11 cell about 6 to 8 hours of mixed culture, they gradually become fused and the number of aggregated cells also increases, and ultimately the growth of JTC-11 cells is inhibited, and some of them are destroyed in about 20-hour culture time. Observing them by cinematography, within a few to several seconds there can be seen a vigorous movement of cytoplasm, then cytolysis, and the shape appears as shown in Fig. 10. Looking at them by phase-contrast microscopy, the cytoplasm flowing out of the cell forms a round dark shadow.

Theoretically, antibody-like substance on the surface of lymphocyte would aggregate on antigens located on the cell surface of target cell. As the resolving power of SEM advances further, it would be possible to clarify more precisely antigens and antibody-like substances on the cell surface. Even by means of SEM it is not possible at present to differentiate clearly sensitized lymphocyte from normal lymphocyte.

The lymph node cells that aggregate on target cell are all composed of small or intermediate size-lymphocytes and no large lymphocytes corresponding pyroninophilic cell can be detected. This point agrees well with the finding of MANNAMI (6) who used TEM for his study. Practically under identical conditions as in the present study, MANNAMI conducted the mixed cultures of sensitized lymph node cells with JTC-11 cells, and with ultrathin sections of these cultured cells he conducted precise observations on the state of the contactual surface between the sensitized lymph node cells and JTC-11 cells, and reported several findings: namely, a) the contact between two membranes running approximately parallel; b) the contact being made between long, slender filamentous projections of JTC-11 cell and lymphocyte; c) the contact achieved by the interdigitation of both cell membranes; d) the contact being made by the projections of the lymph node cell penetrating into JTC-11 cells; and e) the membranes of the two cells showing discontinuity at the point of contact. Out of the SEM pictures in the present study, Fig. 5 seems to correspond to c), Figs. 6 to d), and Figs. 7 and 8 to e).

Up to date for ascertaining the actual state of contact whether lym-

phocyte is riding on target cell, or in actual contact, or fused with each other, mostly the phase-contrast microscopy had been used, but by SEM it has become possible to grasp readily the manner of aggregation in three dimensions. Therefore, SEM would be especially a useful method in elucidating the structures of the cell wall on which transplantation antigen and tumor specific transplantation antigen are located.

SUMMARY

Scanning electron microscope (SEM) observations were conducted mainly on the *in vitro* interaction state between JTC-11 cells derived from Ehrlich ascites tumor and regional lymph node cells obtained from the mice 10 days after transplantation of Ehrlich ascites tumor. Cells cultured on the cover glass were fixed with glutaraldehyde, dehydrated with graded acetone solution, and covered with carbon and gold, were observed by SEM. The results may be briefly summarized as follows.

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