any effect on the magnitude of the immune response if injected two days after immunization. Injection of NSC on the third day after immunization considrably potentiated the immune response. Cells treated with diucifon at this same time reduced the immune response compared with that after injection of NSC.

Spleen cells treated with diucifon thus had the strongest action on the initial stages of the immune response. NSC have their maximal action if injected in the final stages of the immune response, i.e., they have an effect that is similar to the action of a stimulator of antibody producers [4]. Treatment of the cells with diucifon abolishes the immunostimulating action of NSC when injected in the final stages of the immune response. The impression is created that diucifon, which facilitates release of TSGF — a factor intensifying proliferation — prevents release of factors facilitating differentiation of cells into antibody producers. It is possible that simultaneous injection of cells treated with diucifon and NSC will have the strongest action on the magnitude of the immune response. The results may be further justification for the use of immunostimulating therapy by injection of autologous cells treated with diucifon or with other preparations, and also of cells incubated under suitable conditions, making use of the principles of plasmaphaeresis.

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EXOCYTOSIS OF CYTOTOXIC T LYMPHOCYTES STUDIED BY FREEZE-FRACTURING AND STEREOPHOTOGRAMMETRY

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KEY WORDS: cytolytic T lymphocyte; ring-shaped structures; secretory vacuoles; stereophotogrammetry; exocytosis.

Rejection of a graft of normal or tumor tissue takes place mainly through the activity of cytolytic T lymphocytes (CTL), whose mechanism of action has not yet been explained. To study this problem model systems have been developed *in vivo* and *in vitro*, and with them it has become possible to investigate interaction between CTL and target cells (TC). On the basis of the evidence so far obtained several hypotheses, explaining the cytolytic effect of T killer cells, have been put forward [1, 2]. The present writers have suggested a secretory mechanism of action of CTL [2-6]. Electron-microscopic investigations have revealed activation of the secretory apparatus of CTL during interaction with TC. In the zone of contact between the lymphocyte and TC an amorphous substance, corresponding in electron-optical density to the contents of secretory vacuoles demonstrable in the cytoplasm of CTL, has been found. However, discharge of secretory vacuoles was not observed.

The aim of this investigation was to detect exocytosis and to describe secretory vacuoles in the zone of contact between the lymphocyte and TC, using techniques of freeze-fracturing and stereophotogrammetry [7, 8].

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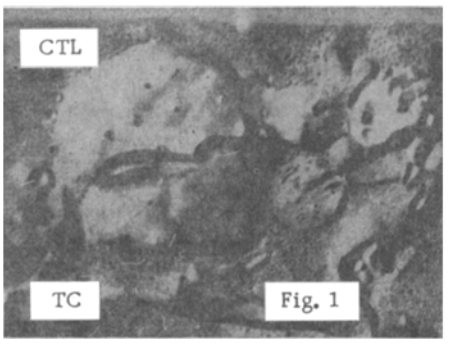

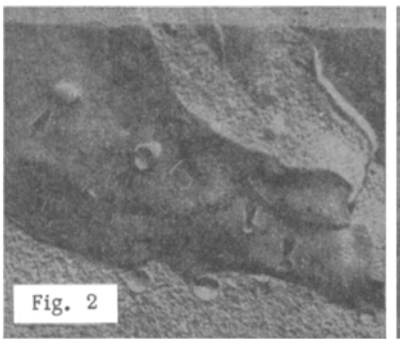

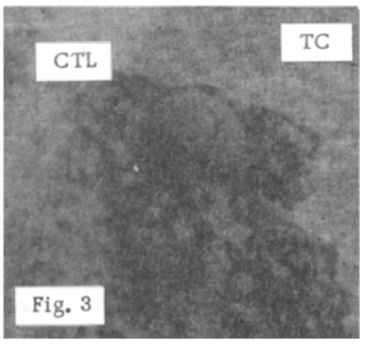

Fig. 1. Stereograph of model of CTL forming conjugate with TC. Arrows indicate zone of contact, vacuoles, and sites of release of secretory vacuoles. 15,000 ×.

Fig. 2. Stereograph of fragment of outer surface of CTL membrane. Arrows indicate sites of release of secretory vacuoles: circular raised areas of membrane with no intramembranous granules, and ring-like structures. 36,000 ×.

Fig. 3. Stereograph of vacuoles in zone of contact between lymphocyte and TC. 210,000 ×.

EXPERIMENTAL METHOD

Inbred mice of lines C3H ($H\text{-}2^k$) and DBA/2 ($H\text{-}2^d$), aged 8-12 weeks, were used. Cells of ascites leukemia L-1210 were transplanted into DBA/2 mice by intraperitoneal injection of 20×10^6 cells. C3H mice were immunized by a single intraperitoneal injection of 20×10^6 L-1210 cells, suspended in 2.5 ml of medium 199. On the 11th day after immunization peritoneal exudate cells were taken from the mice and purified on a column with nylon wadding [9]. L-1210 leukemia cells were used as targets.

To obtain conjugates, lymphocytes and TC were mixed in the ratio of 10:1 and centrifuged for 10 min at 250g; the residues were carefully resuspended and incubated at 37°C.

Cultures were fixed immediately or after 30 and 60 min with 2.5% glutaraldehyde in cacodylate buffer; a 25% aqueous solution of glycerin was used as the cryprotector. The cells were frozen in freon-22 at −150°C.

Shearing and spraying were done on the BAF-301 apparatus (Balzers). After removal of organic residues with 10% hypochloride solution the replicas were examined in the EM-400 transmission electron microscope under an accelerating voltage of 80 kV.

Stereoscopic photographs were obtained in the EM-400T electron microscope by tilting the goniometric specimen holder through 6° or more depending on the magnification and method of information processing.

Stereoscopic negatives were examined on a stereocomparator (Carl Zeiss). The stereograph negatives were oriented by the minimal cross parallax method.

EXPERIMENTAL RESULTS

A three-dimensional reconstruction of the shear fracture, obtained by the method of double projection of stereonegatives, is a three-dimensional model of a CTL forming a conjugate with TC. As Fig. 1 shows, the lymphocyte interacts with TC with a considerable area of its surface. The model clearly demonstrates intracellular organoids, membranes of interacting cells in the zone of contact, vacuoles, and sites of release of secretory vacuoles.

On the outer surface of the plasma membrane of CTL, round, raised areas of the membrane, with no intramembranous granules (IMG), from 50 to 80 nm in diameter and from 70 to 100 nm high are found. At the same time there are ring-like structures from 50 to 80 nm in diameter and from 70 to 100 nm high, the inner zone of which also is marked by absence of IMG (Fig. 2).

In shear fractures passing through the zone of contact of CTL and TC, vacuoles located in the slit-like space between membranes of lymphocyte and TC can be observed. The diameter of the vacuoles varies from 70 to 140 nm (Fig. 3). Some vacuoles are fixed to the surface or lie in the immediate vicinity of the plasma membrane of CTL (Fig. 2).

Secretory cells are divided into two types: cells with a continuous and an intermittent type of secretion. The latter are characterized by maturation and deposition of secretion in the vacuoles, which discharge only in response to the specific stimulus, resulting in activation of the secretory apparatus: hypertrophy of the Golgi complex (GC) and release of secretory vacuoles [14]. As the writers showed previously, contact of the corresponding TC with the plasma membrane is such a stimulus for CTL [4-6].

The technique of freeze-fracturing is widely used to study exocytosis in secretory cells. Exocytosis is characterized by fusion of the membrane of the secretory vacuole with the plasma membrane of the cell. The dynamics and topography of membrane fusion have been studied in several secretory cells, including cells of the anterior lobe of the pituitary [10], thyroid gland [12], parathyroid gland [15], and glandular epithelium of the prostate [11].

As a result of approximation of the secretory vacuole to the inner surface of the plasma membrane of the cell, a protuberance, not containing IMG, is formed on its outer surface.

In the next stage a ring-like structure or "pore," through which the secretion flows out, is formed in this place. Some workers have found an accumulation of IMG after release of the secretion into the hollow of the "pore." It is suggested that regions with densely packed IMG are zones of pinocytosis, i.e., they represent reutilization of surplus membrane [10, 12]. According to other workers, however, no zones with increased density of IMG are formed during release of secretion from cells of the pancreas [13] or glandular epithelium of the prostate [11].

Raised areas of the membrane not containing IMG, and ring-like structures whose internal zone does not contain IMG, also are found on the surface of the plasma membrane of a lymphocyte interacting with TC. Previously the writers described shedding of the plasma membrane of the lymphocyte in the late stages of contact with TC, which they regarded as removal of the excess of membrane, which is observed after release of multiple secretory vacuoles [3, 6]. Absence of IMG in the inner zone of the ring-like structures of the lymphocyte plasma membrane is thus evidence that the excess of membrane is removed by shedding and not by pinocytosis. When replicas with fracture surfaces having a well-marked relief are photographed in the electron microscope considerable distortion of the image can be obtained, and this may lead to incorrect interpretation of the results. Accordingly, we used the technique of stereophotogrammetry, as is used in geodesy and cartography, with certain modifications. By this method distortions arising during photography can be avoided and an analytical model of the object can be obtained by collection of values of the change of relief of the replica with a certain level of discreteness, followed by graphic or mechanical reconstruction of the three-dimensional model. By contrast with existing methods of three-dimensional reconstruction using a diffractometer or serial sections, which give information only on the configuration of the object studied, the technique of stereophotogrammetry enables a solid model to be obtained, including all the information present in the stereographs, and it allows undistorted dimensions of the test object to be obtained.

In the present experiments the use of stereophotogrammetry and three-dimensional reconstruction of the conjugate formed by CTL and TC enabled reliable dimensions to be obtained of the secretory granules and membranous structures formed in the course of exocytosis, and their relationship to the membrane surface to be correctly interpreted.

The discovery of exocytosis, and also of vacuoles in the zone of contact between lymphocyte and TC confirms the hypothesis that the cytolytic effect of T killer cells rests on a secretory mechanism.

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