SCANNING ELECTRON-MICROSCOPIC STUDY OF THE SURFACE OF CYTOLYTIC T LYMPHOCYTES

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Elucidation of the mechanism of action of cytolytic T lymphocytes (CTL) on target cells (TC) is of great importance to solution of the problem of transplantation and antitumor immunity. The writers have put forward a secretory–receptor concept to explain the cytolytic effect of T killer cells [1-3]. It has been shown that ultrastructural features characteristic of secretory cells appear in the cytoplasm of CTL adsorbed on the surface of TC: hypertrophy and a change of orientation of the Golgi complex, outflow of secretory vacuoles in the zone of contact, bounded by membranes of the lymphocyte and TC. These phenomena are accompanied by an increase in extent and by shedding of the plasma membrane of the T killer cell [4].

In the investigation described below scanning electron microscopy (SEM) was used to study the time course of changes in CTL at different times after adsorption on the surface of TC.

EXPERIMENTAL METHOD

Inbred BALB/c (H-2^d) and C3H (H-2^k) mice aged 8-12 weeks were used. To obtain CTL a mixed lymphocyte culture (MLC) was obtained by the method in [6]. Splenocytes from BALB/c mice, mixed in the proportion of $2 \cdot 10^6$ to 10^6 (in 1 ml) of stimulating spleen cells, irradiated in a dose of 1000 R, from C3H mice, were used as reacting cells. The cells were cultured for 5 days in medium RPMI-1640 containing $3 \cdot 10^5$ M 2-mercaptoethanol, 10% embryonic calf serum (ECS), $2 \cdot 10^{-3}$ M L-glutamine, 5 mM HEPES, and 100 units each of penicillin and streptomycin in 1 ml in an atmosphere containing 5% CO_2 at 37°C in "Sany Glas" flasks.

The fraction of lymphoblasts and large lymphocytes was obtained by spontaneous sedimentation at 1g in an ECS gradient [9].

A monolayer culture of L-fibroblasts (H-2^k), incubated with 75 μCi $Na_2^{51}CrO_4$ in 1 ml of medium RPMI-1640 with 5% TEC was used as TC. After incubation for 45 min at 37°C the cells were washed 3 times and seeded overnight in a concentration of $4 \cdot 10^4$ cells in 0.2 ml of medium RPMI-1640 with 5% ECS and antibiotics in flat-bottomed 96-well microplates (Falcon Plastics, USA).

TABLE 1. Cytolytic Activity of T Killer Cells

Source of lymphocytes	Expt. No.	Number of lytic units (LU)
MLC	1	14,7
	2	10,3
	3	15,6
Fraction of lymphoblasts and large lymphocytes	1	38,5
	2	35,7
	3	34,0

Legend. Number of LU per 10^6 cells was determined from the number of lymphocytes capable of causing lysis of 50% of TC.

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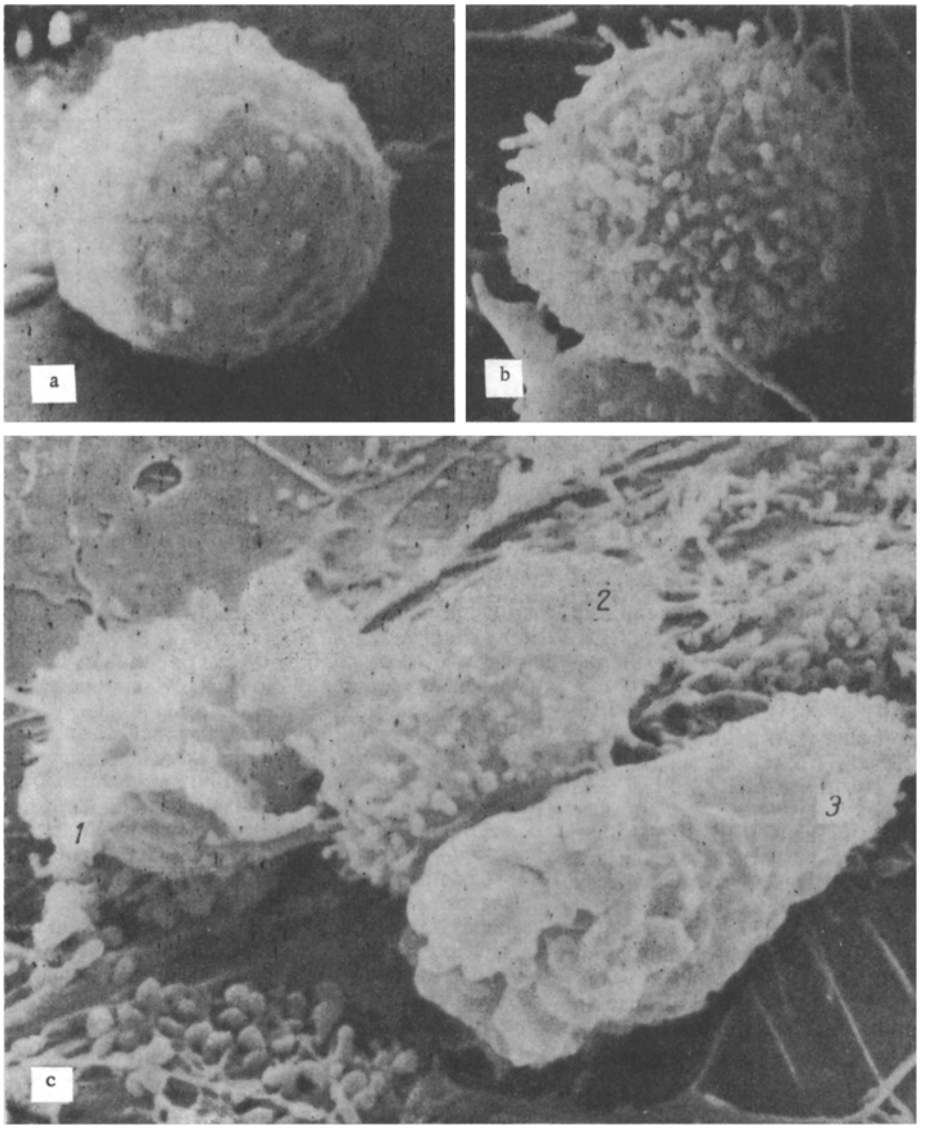

Fig. 1. Types of lymphocytes interacting with TC: a) type I lymphocyte: cell is spherical and has a smooth surface with single microvilli; b) type II lymphocyte: uniformly covered with microvilli; c) type III lymphocytes: irregularly shaped cells, with well-defined folded (1), villous (2), or nodular (3) surface. Magnification 5000.

To determine the cytolytic activity of the MLC, on the 5th day lymphocytes were added to a culture of labeled L-cells in the ratios of 10 : 1, 5 : 1, and 2.5 : 1 in medium RPMI-1640 with 5% ECS in a total volume of 0.2 ml per well. After incubation for 3 h at 37°C with 5% CO_2, 0.2 ml of liquid was carefully withdrawn from each well and radioactivity was measured in a Gamma-spectrometer (Nuclear Chicago, USA). Specific release of radioactive chromium was estimated, in percent, by the equation

$$\% \text{ lysis} = \frac{\text{experiment} - \text{control}}{\text{complete lysis} - \text{control}} \times 100,$$

where "experiment" denotes the number of counts per minute in the presence of CTL, "control" the number of counts per minute in the absence of CTL.

TC for electron-microscopic examination were seeded overnight in a concentration of 10^5 per well in 24-well microplates (Falcon Plastics, 3008), with coverslips arranged on the bottom of the wells. Lymphocytes from MLC were added to TC to the number of 10^6 and to the lymphoblast fraction to the number of $2 \cdot 10^5$ per well. After incubation for 8-10 min at 37°C with 5% CO_2, the unadsorbed lymphocytes were removed by washing twice with medium RPMI-1640 containing 5% ECS, heated to 37°C. The preparations were fixed with 1% glutar-

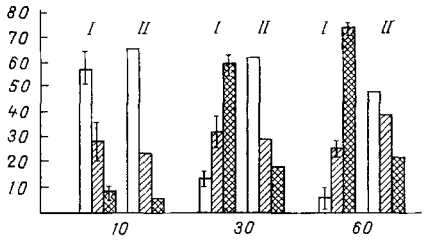

Fig. 2. Changes in surface of lymphocytes depending on duration of interaction with TM. Abscissa, time (in min); ordinate, percent of cells. I) Experiment: lymphocytes from MLC adsorbed on surface of TM for 10, 30, and 60 min; II) control: lymphocytes from MLC fixed with poly-L-lysine, and incubated for the same period of time. Unshaded columns – type I cells; obliquely shaded – type II, cross-hatched – type III cells.

aldehyde solution in 0.1M cacodylate buffer, pH 7.2, immediately after washing or after incubation for 30 and 60 min at 37°C and with 5% CO_2, and dehydrated with acetone. Drying was carried out at the critical point in an apparatus from "Balzers." The specimens were examined in a scanning electron microscope (Philips, The Netherlands).

EXPERIMENTAL RESULTS

Data on the cytolytic activity of the T killer cells are given in Table 1. Cells from MLC had an activity of 10.3-15.6 lytic units (LU), cells from the fraction of blasts and large lymphocytes an activity of 35.7-38.5 LU/10^6 cells.

Lymphocytes remaining unadsorbed 5-10 min after addition of CTL to the monolayer culture of L cells were removed and those remaining were fixed immediately or after incubation for 15, 30, and 60 min. In the control experiment, cells from MLC were attached to the coverslip with poly-L-lysine and were fixed simultaneously with the experimental specimens.

The study of CTL during interaction with TC revealed three types of cells: type I) spherical, almost smooth cells with single microvilli, type II) round and oval cells, uniformly covered with microvilli, type III) a heterogeneous population of lymphocytes, densely covered with microvilli, with a folded or nodular surface (Fig. 1).

With an increase in the time of interaction, the type of lymphocytes adsorbed to the surface of TC changed. Whereas in the first 5 min after the beginning of contact, cells of types I and II predominated (58-71 and 20-34% respectively), after 30-60 min the number of type III cells increased (to 72%). The same culture of lymphoid cells, attached to glass with poly-L-lysine, was used as the control. During the first minutes after adsorption, the proportions of cells of types I, II, and III corresponded to those of the experimental series, but with an increase in the duration of incubation the number of type III cells increased to only 20% (Fig. 2).

A rather different picture was found after addition of the fraction of lymphoblasts and large lymphocytes to the culture of TC. Immediately after adsorption the number of type III cells was already 46-60%, and after interaction for 60 min it increased to 76-90%.

Vos et al. [11], who studied the ultrastructure and surface of rabbit peripheral blood B lymphocytes, described three types of lymphoid cells: 1) small lymphocytes with a smooth surface, 2) medium-sized lymphocytes with an average number of microvilli, 3) activated large lymphocytes, covered with many microvilli. Polysomes, a well-developed endoplasmic reticulum, and a hypertrophied Golgi complex were seen in the cytoplasm of the type III cells. These workers consider that a villous surface of lymphocytes indicates their activation. A similar view also has been expressed in a study of human and mouse peripheral blood lymphocytes [7].

In the present experiments, with a longer duration of interaction the shape and character of contact between T killer cell and TC changed. Immediately after adsorption the lymphocytes were spherical in shape and made contact with TC by several microvilli. After interaction for 30-60 min the area of contact increased considerably, the lymphocytes became oval in shape and appeared to be spread over the surface of TC, and many cells became folded.

The appearance of microvilli and folding of the membrane has been observed after stimulation of secretory cells in vitro. Ten minutes after activation of the acinar cells of the rat pancreas by secretin their surface was covered with microvilli and folds appeared in the membrane [10]. Similar changes took place in the pancreatic β-cells secreting insulin [8], and in mast cells after release of histamine [5].

An increase in the number of microvilli and the appearance of folding can be regarded as the result of incorporation of the membrane of the secretory granules into the plasma membrane of the cell, i.e., as the result of exocytosis. The writers previously postulated that T killer cells are secretory cells with an interrupted type of secretion, for which contact with TC is the specific stimulus for activation of the secretory apparatus, hypertrophy, and a change in orientation of the Golgi complex and release of secretory vacuoles [1]. The types of T killer cells which we observed are thus evidently three stages of activation of lymphocytes interacting with TC.

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