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CHARACTERISTICS OF THE SECRETORY APPARATUS OF MEMORY T CELLS

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Much research has been devoted to the study of the mechanism of action of cytolytic T lymphocytes (CTL) on allogeneic or syngeneic target cells with modified H-2 haplotype [6, 9, 11].

The writers showed previously that a rough and smooth endoplasmic reticulum, tubular structures and a complex of tubular structures, immature secretory granules, empty vesicles, and a hypertophied Golgi complex (GC), facing the zone of contact with the target cell, are present in the cytoplasm of T lymphoblasts adsorbed on the surface of target cells and possessing high cytolytic activity, evidence of active synthesis and liberation of secreted material [1-3].

Depression of cytolytic activity in a mixed lymphocyte culture (MLC) is accompanied by disappearance of lymphoblasts and large lymphocytes and by the appearance of small and medium-sized lymphocytes, i.e., memory cells [4].

The object of the present investigation was to study the functions and ultrastructure of the secretory apparatus of memory T cells in MLC, which have the property of being specifically adsorbed on the surface of target cells, but do not cause their lysis in a 3-hour cytotoxic test.

EXPERIMENTAL METHOD

Inbred BALB/c ($H\text{-}2^d$) and C3H ($H\text{-}2^k$) mice aged 8-12 days were used. To obtain CTL, an MLC was used by the method in [7]. Reacting spleen cells from BALB/c mice were mixed in the number of $2\cdot10^6$/ml with $1\cdot10^6$ stimulating spleen cells in 1 ml, irradiated with a dose of 1000 R, from C3H mice in medium RPMI-1640 containing $3\cdot10^{-5}$ M 2-mercaptoethanol, 15% embryonic calf serum (ECS), $2\cdot10^{-3}$ M L-glutamine, 5 mM Hepes, and 100 units each of penicillin and streptomycin to 1 ml medium. The cells were cultured in "Sani-Glass" flasks in an atmosphere with 5% CO_2 at 37°C for 4, 5, and 8 days.

On the day of the experiment the number of living cells was counted after staining with eosin and trypan blue. The dimensions of the cells were determined under phase contrast, using an ocular micrometer. The cells were divided into small (5.5-7.0 μ), medium-sized (7-8 μ), and large lymphocytes and lymphoblasts (>8.5 μ); the maximal diameter of cells of this last category did not exceed 13.2 μ. The lymphocytes were fractionated by spontaneous sedimentation at 1 g in ECS by the method in [12]. To determine DNA synthesis the cultures were treated with ^3H-thymidine (spontaneous activity 14 Ci/mmole, from the Radiochemical Center, Amersham, England) for a period of 18 h. Later the cells were treated by the method described previously [13]. Radioactivity was measured with a Mark-2 β-spectometer. The result was expressed as the arithmetic mean of three determinations of the number of counts per minute.

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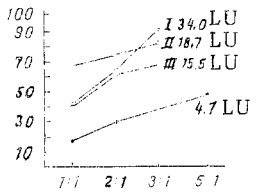

Fig. 1. Cytolytic activity of fraction of lymphoblasts and small lymphocytes isolated by spontaneous sedimentation in an ECS gradient. Abscissa, ratio of lymphocytes to target cells; ordinate, % of cytolysis of target cells. Empty cells denote lymphoblast fraction: experiment I — 34.0 LU, experiment II — 18.7 LU, experiment III — 15.5 LU; filled circles denote unfractioned lymphocytes (4.7 LU). Mean results of three experiments shown.

In some experiments DNA synthesis was determined autoradiographically. After incubation for 18-24 h with ^{3}H-thymidine the cells were washed 3 times and films made, which were then fixed by Carnoy's method and coated with type M emulsion. After exposure for 4-7 days at 4°C the films were developed and stained with methyl green and pyronine. The proportion of labeled cells was determined by counting 500 lymphocytes. The culture of L-fibroblasts (H-2^k) was used as target cells. To obtain ^{51}Cr-labeled target cells, $5.6 \cdot 10^6$ L-cells were incubated with 75 μCi Na_2CrO_4 in 1 ml medium RPMI-1640 with the appropriate additions. After incubation for 45-60 min at 37°C the cells were washed 3 times and seeded overnight at the rate of $4 \cdot 10^4$ cells in 0.2 ml medium RPMI-1640 with 5% ECS and antibiotics in flat-bottomed 96-well microplates (Falcon Plastics).

To determine cytolytic activity on the 5th, 7th, and 8th days of MLC the 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 0.3 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 its radioactivity measured in a Gamma-spectrometer (Nuclear Chicago). The specific release of radioactive chromium was estimated by the equation:

$$\% \text{ lysis} = \frac{\text{experiment} - \text{control}}{\text{total lysis} \cdot 0.75} \cdot 100,$$

where experiment denotes radioactivity in cpm in the presence of CTL, control denotes radioactivity in cpm without CTL.

The number of lytic units per 10^6 cells was determined from the number of lymphocytes capable of inducing lysis of 50% of the target cells.

For electron-microscopic investigation cells were seeded overnight in glass flat-bottomed tubes measuring 20 × 10 mm at the rate of 5×10^4 cells in 0.4 ml medium RPMI-1640 with 5% ECS. Next day CTL were added to the L cells in the ratio of 20:1 and, in order to sediment all the lymphocytes simultaneously, the tubes were centrifuged for 2 min at 200g and cultured for 10, 30, and 90 min at 37°C with 5% CO_2. After incubation, cells with the lymphocytes adsorbed on them were washed and fixed with 1% glutaraldehyde, treated with 1% OsO_4 by the method in [8], and embedded in a mixture of Epon and Araldite. Ultrathin sections were examined under the YEM-100B electronmicroscope with magnification of 3000, 30,000, 50,000 and 100,000.

EXPERIMENTAL RESULTS

After fractionation of cells obtained on the 4th-5th days of MLC by spontaneous sedimentation in an ECS gradient, mainly the fraction of lymphoblasts and large lymphocytes possessed cytolytic activity (Fig. 1). The mean number of lytic units (LU) per 10^6 cells in this fraction was 22.7 compared with 4.7 per 10^6 cells in the unfractionated MLC population. In the present experiments the fraction of lymphoblasts and large lymphocytes contained on average 84% of large lymphocytes and lymphoblasts, 2.5% of medium-sized lymphocytes, and 13.5% of small lymphocytes. The number of cells labeled with thymidine-^{3}H was 63.7%.

TABLE 1. Time Course of Cytolytic Activity and DNA Synthesis from 5th through 8th Days of Lymphocyte Culture on Monolayer of Syngeneic Macrophages

Days	Expt. No.	% cytolysis of target cells 10:1	5:1	2,5:1	1,25:1	Number of LU	DNA synthesis
5th	1	83,5	73,3	52,5	29,1	10,4	60 988
	2	66,2	40,9	18,6	13,5	3,72	66 703
	3	48	42	28	21	5,6	32 920
8th	1	6,5	6,1	4,2	0,8	0,84	3 375
	2	18,6	9,7	3	6,5	0,6	15 350
	3	12,7	5,4	7,5	1,75	0,54	9 351

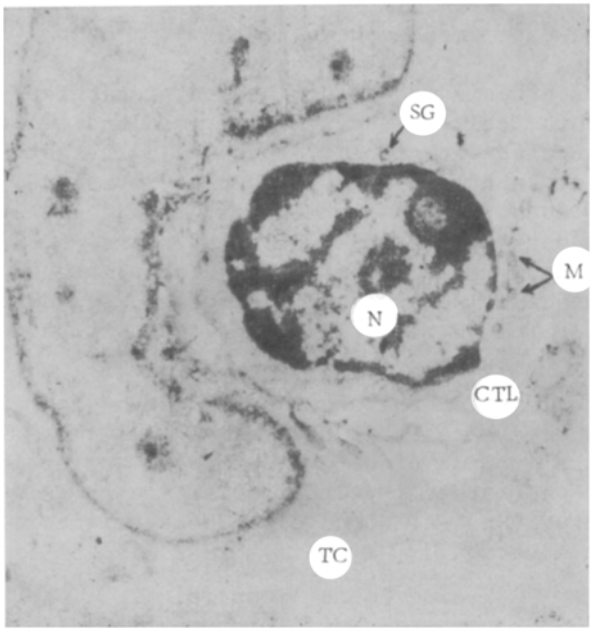

Fig 2. CTL (8th day after stimulation *in vitro*); 30 min after adsorption on surface of target cell (TC). N) Nucleus; M) mitochondrion; SG) secretory granules. Magnification 12,000 ×.

After culture of the cells of this fraction for 2-3 days on a monolayer of syngeneic macrophages the mean proportion of lymphoblasts and large lymphocytes was under 1.8%, that of medium-sized lymphocytes 39%, and small lymphocytes 59.2%. The content of thymidine-^{3}H-labeled cells determined autoradiographically did not exceed 1-2%. The cytolytic activity of these lymphocytes was reduced to 1.5-3 LU/10^6 cells but they preserved their ability to be adsorbed on the surface of target cells.

Lymphocytes obtained on the 4th-5th days of MLC and cultured for 2-3 days on a monolayer of syngeneic macrophages were thus converted into medium-sized and small lymphocytes, which were adsorbed on the surface of the corresponding target cells, but possessed low cytolytic activity.

The study of the ultrastructure of these cells revealed cisterns of smooth endoplasmic reticulum, a few mitochondria, lysosomes, and mature secretory granules in their cytoplasm. Hypertrophy of GC and other features of activation of the secretory apparatus were not found (Fig. 2).

According to data obtained by different workers cytolytic activity in MLC reaches a peak between the 5th and 11th days of culture [5, 7]. Memory T cells are usually studied on the 14th day of MLC, which corresponds to the time of appearance of memory T cells in the spleen

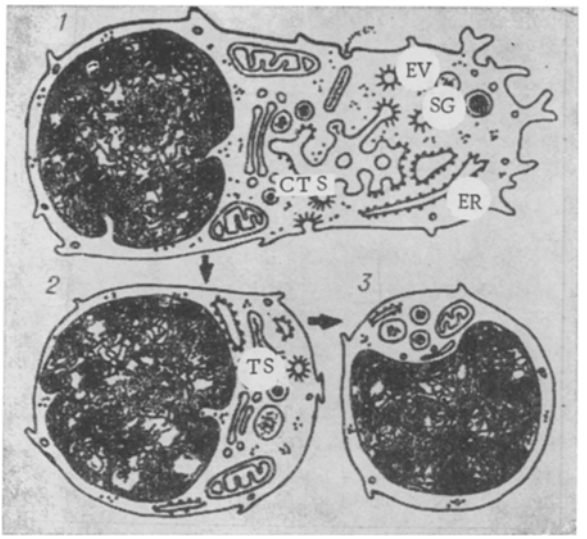

Fig. 3. Scheme of differentiation of T lymphoblast to medium-sized and small lymphocyte. 1, 2, 3) Lymphoblasts, medium-sized and small lymphocyte respectively. ER) Endoplasmic reticulum; TS) tubular structure; CTS) complex of tubular structures; EV) empty vesicle; SG) secretory granule.

of animals immunized with allogeneic cells. Restimulation of these cells with the corresponding alloantigen leads to the more rapid formation of T lymphoblasts, possessing cytolytic activity more than 10 times greater than the activity of precursor cells [10].

In the present experiments the peak of cytolytic activity was observed on the 4th-5th days but on the 6th-8th days of culture this activity was lower. On the 4th-5th days of MLC the fraction of lymphoblasts and large lymphocytes possessed cytolytic activity, but these cells were converted into small and medium-sized lymphocytes after 2-3 days of culture on a monolayer of syngeneic macrophages. The absence of signs of activation of the secretory apparatus corresponded to the low cytolytic activity of these cells. A suggested scheme of differentiation of the secretory apparatus in the cytoplasm of the lymphocyte from the lymphoblast stage to the small lymphocyte (memory cell) is shown in Fig. 3. Secretion synthesized in the rough endoplasmic reticulum is later found in the smooth reticulum, where it fills the tubular structures and complex of tubular structures. In the course of conversion of lymphoblasts into medium-sized and small lymphocytes the secretion is condensed in immature and mature secretory granules.

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