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PREPARATION OF SECONDARY T KILLERS

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The solution to the problem of antitumor and transplantation immunity is directly bound up with the study of differentiation and mechanisms of action of cytolytic T lymphocytes (CTL). Several model systems have been developed for obtaining CTL *in vivo* and *in vitro* [1, 7, 8, 13]. On immunization *in vivo* mainly small and medium lymphocytes, which can be obtained from the spleen, lymph nodes, or peritoneal exudate on the 11th-12th days after injection of a tumor allograft [8], possess cytolytic activity. Conversely, in mixed lymphocyte culture (MLC), the peak of cytolytic activity is found on the 4th-5th days and the target cells (TC) destroy large lymphocytes and lymphoblasts [4]. On immunization *in vitro*, CTL proliferate in response to a single antigenic stimulus, whereas *in vivo* the action of the antigenic stimulus continues until rejection of the allograft.

This paper decribes an attempt to reproduce *in vitro* the conditions corresponding to differentiation of CTL *in vivo*.

EXPERIMENTAL METHOD

Inbred BALB/c ($H\text{-}2^d$) and C3H ($H\text{-}2^k$) mice aged 8-12 weeks were used.

CTL were obtained in MLC by the method in [9]. Reacting spleen cells of BALB/c mice, in a concentration of $2\cdot10^6$ cells/ml, and stimulating spleen cells in concentration of $1\cdot10^6$ cells/ml, irradiated in a dose of 1000 R (10 Gy), from C3H mice were cultured in RPMI-1640 medium containing 15% embryonic calf serum(ECS), $3\cdot10^{-5}$ M mercaptoethanol, $2\cdot10^{-3}$ M L-glutamine, $5\cdot10^{-3}$ M HEPES, and penicillin and streptomycin each in a concentration of 100 units/ml medium, in Sani Glas flasks in an atmosphere of 5% CO_2 at 37°C for 5, 8, and 12 days. On the 5th day the cells were sedimented by centrifugation and transferred to freshly prepared medium without addition of mercaptoethanol.

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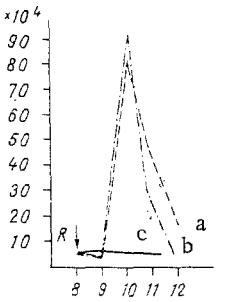

Fig. 1

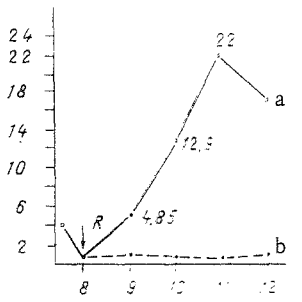

Fig. 2

Fig. 1. DNA synthesis in suspension of lymphocytes restimulated on 8th day of MLC. Abscissa, time of culture (in days); ordinate, number of cpm. a) Suspension of MLC cells from which adherent cells had been removed, b) suspension of unpurified MLC cells, c) unstimulated lymphocyte culture.

Fig. 2. Cytolytic activity of lymphocytes restimulated on 8th day of MLC. Abscissa, time of culture (in days); ordinate, number of lytic units (LU). a) Restimulated culture, b) culture not restimulated.

On the 8th day the lymphocytes were restimulated by addition of $4 \cdot 10^6$ spleen cells from C3H mice in a dose of 1 ml, irradiated in a dose of 1000 R (10 Gy), to $2 \cdot 10^6$/ml MLC cells in 1 ml. The restimulated cells in medium RPMI-1640 with all the additives were transferred to a monolayer of syngeneic macrophages.

In some experiments, before restimulation the population of cells adherent to nylon wadding was removed. For this purpose a 10-ml glass syringe was filled with 0.6 g nylon wadding (Leuko-Pak, Fenwall). Before incubation the column was washed with phosphate buffer solution. Cells in medium 199 with the addition of 10% ECS were incubated on the column for 60 min at 37°C with 5% CO_2 in the vertical position. Nonadherent cells were eluted with medium 199, heated to 37°C.

To determine the number of T cells the lymphocytes were treated with anti-Thy-1,2-serum (Cedarlen): $1 \cdot 10^{-5}$ to $2 \cdot 10^{-5}$ cells with antiserum in the corresponding dilution, in a total volume of 0.1 ml, were incubated in Cederlane Cytotoxicity Medium at room temperature. After 60 min the cells were sedimented by centrifugation at 1000 rpm for 7 min, the supernatant was harvested, a 10% solution of rabbit complement (Cederlane) was added, and the mixture was incubated at 37°C. After 40 min the cells were transferred to the cold (4°C) and stained with trypan blue and eosin; the number of living cells in 300 lymphocytes was counted.

To determine DNA synthesis, ^{3}H-thymidine (1 μCi/ml) was added to the cultures for 18 h. The cells were then treated in the Titertek Multiple Cell Harvester (Flow). The number of counts/min was determined in a Mark 2 Beta-spectrometer. The results were estimated as the arithmetic mean of four determinations of the number of counts per minute.

To determine cytolytic activity of the lymphocytes a culture of L fibroblasts ($H\text{-}2^k$) was used as the target cells. To obtain ^{51}Cr-labeled target cells, $5 \cdot 10^6$ to $6 \cdot 10^6$ L cells were incubated with 50-75 μCi $Na_2\,{}^{51}Cr_2O_7$ in 0.5 ml medium 199 with the addition of 5% ECS. 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 to 0.2 ml medium in flat-bottomed 96-well microplates (Falcon Plastics). To estimate cytolytic activity, at known times of culture lymphocytes were added to the target cells in the rates of 10:1, 5:1, 2.5:1, and 1.2:1 in medium 199 with 5% ECS in a total volume of 0.1 ml per well. After incubation for 3 h at 37°C with 5% CO_2, all the liquid was carefully collected from each well and radioactivity measured in a gamma-spectrometer (Nuclear Chicago). The percentage of specific yield of radioactive chromium was estimated by the equation:

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

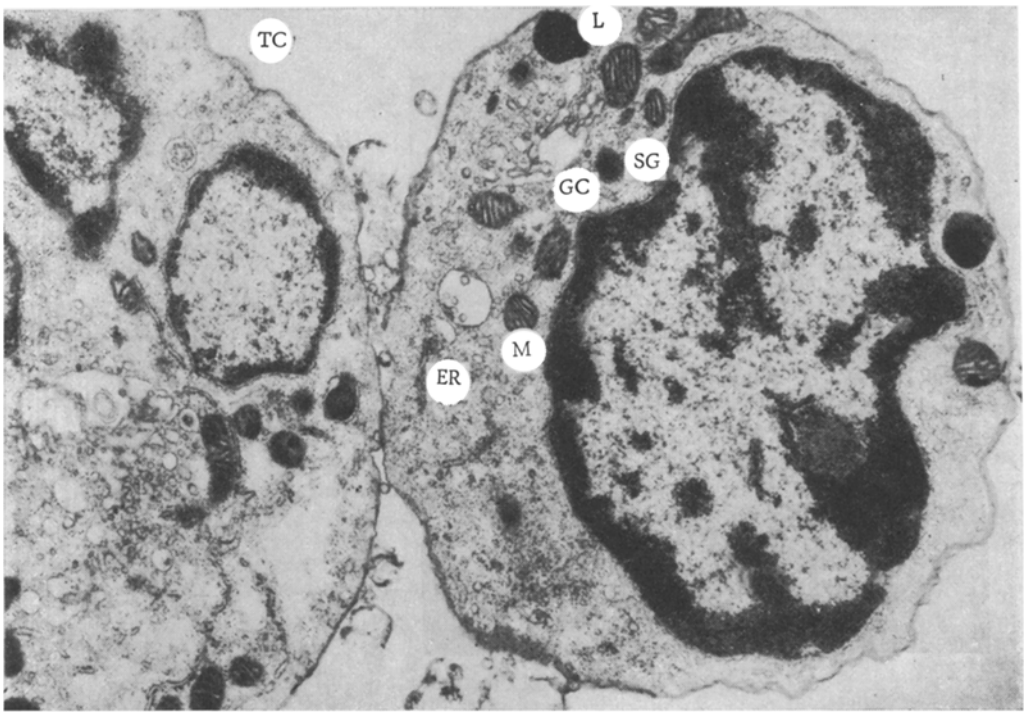

Fig. 3. CTL obtained on 4th day after restimulation and adsorbed on surface of target cell (30 min after beginning of interaction). Numerous mitochondria (M), lipid vacuoles (L), endoplasmic reticulum (ER), polymorphic secretory granules (SG), and Golgi complex (GC), facing zone of contact with target cell, where contents of secretory granules are found. TC) Target cells.

where experiment denotes the number of cpm after addition of CTL to target cells, and control denotes the number of cpm after incubation with target cells.

Cytolytic activity was expressed in lytic unit; (LU) per $1 \cdot 10^6$ cells; the LU was defined on the basis of the number of lymphocytes capable of producing lysis of 50% of target cells.

For the electron-microscopic investigation L cells were seeded overnight in flat-bottomed glass tubes measuring 20 × 10 mm, each containing $5 \cdot 10^4$ cells in 0.4 ml medium RPMI-1640 with 5% ECS. Lymphocytes were added in a ratio of 20:1, centrifuged at 200g for 2 min, and incubated for 30, 60, and 90 min at 37°C with 5% CO_2. After incubation the cells were washed and fixed with 1% glutaraldehyde, treated with 1% OsO_4 by the method in [10], and embedded in a mixture of Epon and Araldite. Ultrathin sections were examined under the YEM-100B electron microscope under magnifications of 3000, 30,000, 50,000, and 100,000.

EXPERIMENTAL RESULTS

To obtain secondary T killers the suspension of lymphocytes was restimulated on the 8th day of MLC and transferred to a monolayer of syngeneic macrophages. In some experiments before restimulation the population of adherent cells was removed on a column with nylon wadding.

The number of cells after incubation on the column with nylon wadding was 38.1-44.0% of that in the original suspension, whereas the proportion of T lymphocytes increased to 72.3-80% (as shown by the use of anti-Thy-1, 2 serum in dilutions of 1:8 and 1:16).

DNA synthesis increased very slightly during the first 24 h after restimulation, it rose sharply during the next 24 h, and thereafter fell to its initial level. Preliminary removal of the population of adherent cells shows that the peak of DNA synthesis 48 h after restimulation was due in fact to T lymphocytes (Fig. 1).

The cytolytic activity of the culture containing memory cells increased about tenfold during the 24 h after restimulation. As Fig. 2 shows, the lytic activity of the lymphocytes restimulated on the 8th day of MLC and cultured on a monolayer of syngeneic macrophages, in-

creased from 0.4 to 4.85 LU 24 h, to 12.4 LU 48 h, and to 22.0 LU 72 h after restimulation. Later the cytolytic activity of the restimulated lymphocytes fell gradually.

Large lymphocytes and lymphoblasts predominated in the culture 24-72 h after restimulation, but after 96-120 h (i. e., on the 11th-13th days of MLC) the culture contained chiefly small and medium lymphocytes, possessing high lytic activity.

After adsorption for 30-60 min on the surface of the target cells concentrations of ribosomes, cisterns of rough endoplasmic reticulum, mitochondria, lipids, and a particularly large number of cytolysomes of different sizes and different electron-optical density, multivesicular bodies, and secretory granules could be observed in the cytoplasm of these lymphocytes; the contents of the secretory granules were found in the intercellular space enclosed by membranes of the lymphocyte and target cell. The Golgi complex (GC) was hypertrophied and faced the zone of contact with the target cell (Fig. 3).

The fraction of lymphoblasts and large lymphocytes, isolaged by spontaneous sedimentation in an ESC gradient, exhibited 95% of the lytic activity of the whole culture [13]. The present writers previously found ultrastructures characteristic of secretory cells in the cytoplasm of T lymphoblasts adsorbed on the surface of target cells: a well-developed rough and smooth endoplasmic reticulum, empty vesicles, and a hypertrophied GC facing the zone of contact with the target cell [2, 3]. After incubation of 3-4 days the blast cells were converted without cell division into "secondary lymphocytes" and memory cells [13]. Memory cells do not synthesize DNA, they have low lytic potential, but they have not lost the power to be adsorbed on the surface of target cells. No signs of activation of the secretory apparatus could be seen in the cytoplasm of these lymphocytes. Restimulation of memory cells revealed a tenfold increase in lytic activity without any corresponding increase in the number of living cells or of DNA synthesis during the first 24 h of culture. It was accordingly postulated that during the first 24 h of re-exposure to the alloantigen activation of cytolytic activity is dissociated from proliferation [11]. Inhibitors of DNA synthesis, namely hydroxyurea and cytosine arabinoside C, had no effect on the cytolytic activity or viability of the lymphocytes during the first 24-48 h of culture in the presence of alloantigen [7, 12].

In the present experiments the lytic activity of the secondary T killers increased 10-15 fold and reached the level of activity observed on the 5th day of MLC during the first 24 h after restimulation. During the next 2 days the lytic activity doubled every 24 h.

Restoration of the lytic potential during the first days of culture may be evidence of reactivation of the secretory apparatus of the T killers, whereas the subsequent rise in lytic activity was accompanied by an increase in the number of T killers and by differentiation of their secretory apparatus. Restimulated lymphoblasts and large lymphocytes were converted on the 11th-12th day of MLC into medium and small lymphocytes (secondary killers), with high cytolytic activity. Signs of activation of the secretory apparatus could be seen in the cytoplasm of these lymphocytes adsorbed on the surface of target cells: hypertrophy and a change in orientation of GC, numerous cytolysomes, multivesicular bodies and secretory granules, the contents of which were found in the zone of contact between membranes of the lymphocyte and target cell.

A secondary antigenic stimulus thus induces differentiation of a clone of T killers, and this is accompanied by an increase in the lytic potential of these cells. It can be tentatively suggested that the secondary T killers obtained after restimulation *in vitro* correspond to the immune lymphocytes detectable after immunization *in vivo*.

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THE IMMUNODEPRESSIVE ACTION OF VACCINIA VIRUS

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KEY WORDS: living and inactivated smallpox vaccine; antibody formation; immunodepression.

Virus diseases (measles, influenza, rubella, mumps, chicken pox, etc.) are known to weaken immunity both to each other and to bacterial infections. However, the experimental study of this phenomenon only began comparatively recently. It was shown, for instance, that in influenza the number of T lymphocytes in the blood of affected humans and animals is reduced [5, 15], as also is the number of antibody-forming cells (AFC) in the spleen [3, 9], and macrophages and neutrophils are injured [7]. There is a certain amount of experimental evidence on immunoinhibitory properties of adeno-, arbo-, and cytomegaloviruses [2, 6, 10], measles virus [13], and rabies street virus [14].

The object of this investigation was to study the possibility of an immunodepressive action of vaccinia virus (living or inactivated).

EXPERIMENTAL METHOD

Noninbred albino mice weighing 12-14 g were used. Living vaccinia virus (batch 0342 produced by the Moscow Research Institute of Virus Preparations) in a titer of $10^{4.5}$ PFU was injected into the caudal vein in a volume of 0.2 ml. Inactivated smallpox vaccine (from the same producer, batch 8) was injected by the same route in a volume of 0.5 ml.

Sheep's red blood cells (SRBC) were injected into the caudal vein in a volume of 0.2 ml of 10% suspension. On the 4th day the number of AFC in the spleen was determined by Jerne's method [12].

The numerical results were subjected to statistical analysis by Student's t test and geometric means and confidence intervals were calculated [1].

EXPERIMENTAL RESULTS

Preliminary experiments showed that after injection of living or inactivated smallpox vaccine alone into the animals without SRBC the number of AFC per weight of organ on the 4th day after injection of the preparation was 42 and 12, respectively.

Three principal experiments were carried out on 60 animals. Twenty animals were used in each experiment (five in a group). The results were consistent (Table 1). The number of nucleated cells in the spleens of mice of all groups was almost identical. The number of AFC per $1 \cdot 10^6$ splenic karyocytes on the 4th day after injection of SRBC in group 2 was indistinguishable from the control (group 1). Different results were obtained in the animals of group 3: in mice receiving the living vaccine and SRBC 2 days later the number of AFC per $1 \cdot 10^6$ karyocytes was significantly less than in the control ($P < 0.01$). When inactivated smallpox vaccine was used (group 4) the number of AFC per $1 \cdot 10^6$ splenic karyocytes was a little higher than in the control.

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