PROLIFERATION AND CYTOTOXICITY OF THYMUS LYMPHOCYTES in vitro

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The proliferative and cytolytic activity of lymphocytes from the spleen and intact thymus was compared after alloimmunization. The number of living cells and of DNA-synthesizing cells in a monoculture of thymocytes was 90-97% less, and in a mixed culture of thymus cells about 80% less than the corresponding number of spleen cells. The index of stimulation of immune thymocytes was several times greater than that of immune spleen cells. The peak of cytotoxicity was observed on the fourth to fifth day of stimulation, when the cytolytic activity of the immune thymocytes was close to the activity of immune spleen cells. The low DNA synthesis and the considerable cytotoxic activity of the immune thymocytes mean that stimulation of thymus cells in vitro can be used to obtain a cell population with a high content of cytolytic T lymphocytes.

KEY WORDS: *thymus; spleen; proliferation; cytolytic T lymphocytes.*

Antitumor and transplantation immunity are nowadays regarded as manifestations of cellular immunity. To study this phenomenon a number of model systems have been developed in vitro [7], in which proliferation of lymphocytes has been demonstrated in response to antigenic stimuli, and later a cytotoxic action has been found on target cells carrying these surface antigens. It has also been shown that among the spleen cells not only immune lymphocytes of thymus origin, but also K cells, macrophages, and B lymphocytes, carrying the receptor for the Fe fragment of immunoglobulin G — an antibody bound with antigens of the target cell — possess cytotoxic activity [4, 8].

To study the cytology and mechanisms of action of T lymphocytes under conditions causing least trauma to the cell and enabling a population rich in T lymphocytes to be obtained, an attempt was made in the investigation described below to obtain alloimmunization of thymus lymphocytes in vitro.

EXPERIMENTAL METHOD

To obtain cytotoxic lymphocytes the spleen and thymus of BALB/c and C3H mice aged 8-16 weeks were removed under aseptic conditions, carefully crushed in a glass Potter's homogenizer, filtered through four layers of sterile gauze, after which the number of living cells was counted after staining with eosin and trypan blue.

Stimulation in vitro was carried out by the method of Nabholz et al. [6] with certain modifications. Lymphocytes from the thymus and spleen of BALB/c mice ($H\text{-}2^d$), in concentrations of $6 \cdot 10^6$ and $2 \cdot 10^6$ cells/ml, were used as reacting cells, and spleen cells from C3H mice ($H\text{-}2^k$), irradiated immediately before incubation in a dose of 1000 R, in concentrations of $1 \cdot 10^6$ and $2 \cdot 10^6$ cells/ml, respectively, were used as stimulating cells.

The cell suspensions were mixed in a ratio of 2:1 to obtain cytotoxic splenic lymphocytes and in a ratio of 3:1 to obtain cytotoxic thymus lymphocytes in a total volume of 20 ml, they were then incubated for 4-6 days in RPMI 1629 medium with the addition of 20% embryonic calf serum (not frozen during storage), and heated for 30 min at 65°C, with $2 \cdot 10^{-3}$

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TABLE 1. Proliferation and Cytotoxicity of Thymus Cells in vitro

Expt. No.	Culture	Culture time								
		4th day			5th day			6th day		
		DNA synthesis	proliferation index	CT, %	DNA synthesis	proliferation index	CT, %	DNA synthesis	proliferation index	CT, %
1	Thym-CTL	700	10	79	1420	9	40	1420	3,2	35
	Thymus cells	70		1	280		0	440		0
2	Thym-CTL	690		82	400		82	200		17
	Thymus cells	100	6,9	1	190	2	7	300	0,5	0
3	Thym-CTL	2110		76	1200		78	640		38
	Thymus cells	300	7	3,6	400	3	2	330	2	0

Legend. Thym-CTL — cytolytic thymus lymphocytes; CT — cytoxicity of thymus lymphocytes.

TABLE 2. Proliferation and Cytotoxicity of Splenic Cells in vitro

Expt. No.	Culture	Culture time								
		4th day			5th day			6th day		
		DNA synthesis	proliferation index	CT, %	DNA synthesis	proliferation index	CT, %	DNA synthesis	proliferation index	CT, %
1	SPL-CTL	4500		80	2800		71	2260		78
	Spleen cells	1800	2,7	6	1170	2,4	9	850	2,6	0
2	SPL-CTL	640		85	800		97	1000		70
	Spleen cells	400	1,6	4	530	1,5	7	1000	1	0
3	SPL-CTL	6060		100	1510		98	1180		76
	Spleen cells	1690	3,5	3,6	1500	1	4	1180	1	9

Legend. SPL-CTL — cytolytic splenic lymphocytes; CT — cytoxicity of splenic lymphocytes.

M L-glutamine, 5 mM HEPES, $3 \cdot 10^{-5}$ M mercaptoethanol, and 100 units each of penicillin and streptomycin to 1 ml medium in 50-ml and 100-ml glass flasks (Sany Glass). The incubation mixture was treated with 5% CO_2, after which the flasks were closed with rubber stoppers and incubated at 37°C. A monoculture of BALB/c lymphocytes or of C3H lymphocytes irradiated in a dose of 1000 R, incubated under the same conditions, served as the control.

On the day of the experiment the number of living cells was counted after staining with eosin and trypan blue, and the proportion of lymphocytes, conventionally divided into small (5.5-7.0 μ), medium (7.0-8.5 μ), and large lymphocytes and blast cells (greater than 8.5 μ), the maximal diameter of which did not exceed 13.2 μ, was determined with an ocular micrometer under phase contrast.

Under sterile conditions samples of $2 \cdot 10^5$ living lymphocytes were taken from the flasks, centrifuged for 7 min at 1000 rpm, and incubated in fresh RPMI 1629 medium (with 5% embryonic calf serum) with the addition of 1 μCi/ml [^{3}H]thymidine for 3 h at 37°C. After incubation the cells were washed 3 times with medium No. 199 and then treated by the method of Nabholz et al. [6]. The number of counts per minute was determined with a Mark-2 B-spectrometer.

To determine the proportion of DNA-synthesizing cells on the day of the experiment $5 \cdot 10^6$ lymphocytes from each experimental group were incubated as described above with 5 μCi [^{3}H]thymidine and washed 3 times, after which films were made, fixed with Carnoy's fluid, coated with type M (NIIKhimfoto) emulsion, and developed after 7 days. The films were stained with methyl green—pyronine and the proportion of labeled cells determined by counting 500 lymphocytes.

L cells (H-2^k), incubated the day before the experiment in medium No. 199 with 20% bovine serum and 100 μCi ^{51}Cr in the proportion of $8 \cdot 10^6$ cells to 1 ml medium for 1 h, washed 3 times with medium No. 199, and inoculated in a dose of $5 \cdot 10^4$ cells in 0.5 ml medium No. 199 with 10% bovine serum in 12 × 120 tubes with an oval bottom (Pyrex glass), set in sloping stands, were used as targets.

On the day of the experiment the L cells were rinsed and treated with stimulated lymphocytes and cells of a monoculture of the spleen and thymus of BALB/c mice in the proportion of 10 lymphocytes to 1 L cell in medium RPMI 1629 with 5% embryonic calf serum and antibiotics for 18 h in a total volume of 0.5 ml. To determine spontaneous lysis the same medium was added without lymphocytes. The supernatant (0.4 ml) was transferred to plastic tubes (5 tubes for each group of the experiments), which were examined in a Nuclear Chicago gamma spectrometer. The percentage of specific cytolysis was determined by the formula:

$$\text{Cytotoxicity } (\%) = \frac{\text{experiment} - \text{spontaneous lysis}}{\text{maximum} - \text{spontaneous lysis}} \times 100,$$

where experiment denotes the mean liberation of isotope into the medium after the addition of cytotoxic (or normal) lymphocytes, spontaneous lysis the liberation of isotope by intact cells (in these experiments 15-20%), and maximum the liberation of isotopes after treatment of the cells with a 2% solution of sodium dodecyl sulfate in 0.05 M borate buffer solution, pH 9.0, by the method described in [1].

EXPERIMENTAL RESULTS

As a result of stimulation of thymus and spleen cells of BALB/c mice in vitro the following data were obtained: The number of living lymphocytes was 11-13% of the initial number of thymus cells in the suspension and 50-60% of the number of spleen cells in the suspension. The proportion of blast cells and large lymphocytes determined by phase-contrast microscopy was 23-35% for stimulated thymus cells and 33-48% for spleen cells. The proportion of medium lymphocytes was 29-39 and 23-31% of cells respectively. The number of lymphocytes labeled with [^{3}H]thymidine determined autoradiographically was 15-26% for immune thymus lymphocytes and 32-41% for immune splenic lymphocytes. The number of living cells in the monoculture of thymus lymphocytes was 3.3% of the initial value and 0.05% of the cells were labeled, whereas in the monoculture of splenic lymphocytes the number of living cells was 30-40% and from 9 to 21% of the cells were labeled; the proportion of labeled lymphocytes in the monoculture increased with an increase in the culture time.

The results of three experiments in which cytotoxicity and DNA synthesis were demonstrated on the fourth to sixth day of stimulation of thymus and spleen cells respectively are given in Tables 1 and 2.

On alloimmunization of the animals in vivo only 1-2% of specifically immune lymphocytes can be found at the peak of the immune response [9]. The numerous "extraneous cells" make work with them difficult. Alloimmunization of spleen cells in vitro yields a much richer population of effector lymphocytes [5], and stimulation of thymus cells can yield a population of T lymphocytes virtually free from B cells.

The mitotic activity and cytotoxicity of cortisone-resistant thymus lymphocytes stimulated by alloantigens in a Marbrook chamber has been studied in detail by Wagner et al. [10-12]. These workers showed that for killer activity to be generated in vitro cells carrying θ antigen are necessary, and that mitotic activity of medullary (cortisone-resistant) thymocytes, immunized in vitro, is strictly specific and their cytotoxicity is 3 times greater than the activity of spleen cells. However, as a result of corticosteroid administration 75-85% of the cells are lost, whereas our own experiments were carried out on intact animals.

Only 5-6% of mature cells are found in the thymus [3], and this is evidently responsible for the high vulnerability of the thymocytes and the small proportion of them undergoing mitosis in vitro. A monoculture of thymocytes is a population of nondividing cells; for that reason the proliferation index for thymus cytotoxic lymphocytes (Thym-CTL) is significantly higher than that of splenic lymphocytes (SPL-CTL). However, DNA synthesis by itself in stimulated spleen cells was 3-5 times higher on the fourth day and twice as high on the fifth to sixth day of culture as in Thym-CTL. This could evidently be attributed to proliferation of B lymphocytes and of noncytolytic T lymphocytes.

By phase-contrast microscopy of living stimulated lymphocytes the approximate proportion of killer cells in the population can be determined even before the addition of target cells to the culture. This is in agreement with the observations of Andersson and Blomgren [3], who showed both in vivo and in vitro that large lymphocytes and blast cells are killers at the peak of the immune response.

The peak of proliferation of thymus and splenic lymphocytes is thus observed on the third to fourth day of immunization. The cytotoxicity of thymus lymphocytes is still present on the fourth to fifth day but falls by the sixth day of incubation, whereas SPL-CTL are active on the fourth to sixth day.

Both DNA synthesis and the proportion of blast cells among stimulated splenic lymphocytes were considerably higher than those of stimulated thymus lymphocytes, whereas the cytotoxicity of these cells was similar. The Thym-CTL are thus a cell population with a high content of cytolytic T lymphocytes which can be used with advantage for the study of the intimate mechanisms of transplantation and antitumor immunity.

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EFFECT OF SOLUBLE H-2 ANTIGENS ON THE CYTOTOXIC EFFECT AND ADSORPTION OF IMMUNE LYMPHOCYTES

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Serologically active preparations of soluble H-2 antigens were obtained by extraction with 3 M KCl from ascites cells of leukemia L1210 ($H\text{-}2^d$) and sarcoma MCh-11 ($H\text{-}2^b$). These preparations had no specific effect on the cytotoxic action of immune lymphocytes on target cells in vitro and did not inhibit adsorption of lymphocytes on a monolayer of the corresponding target cells.

KEY WORDS: *soluble H-2 antigens; cytotoxic effect; T cells.*

Conjecturally the receptors of T cells are molecules of special (T cell) immunoglobulins [10, 13], products of the Ir gene [8], or a complex of the V region of immunoglobulin with the H-2 molecule [5, 12]. To study the nature of receptors of cytotoxic lymphocytes, direct destruction of their membrane [18] or discovery of the conditions or interaction between living T cells and preparations of soluble H-2 antigens [19] have been used.

In the investigation described below the effect of soluble H-2 antigens, extracted with 3 M KCl, on the cytotoxic effect and adsorption of immune lymphocytes was studied.

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