CYTOTOXICITY OF LYMPHOCYTES FROM MELANOMA PATIENTS AGAINST AUTOLOGOUS TUMOR CELLS AND ITS POTENTIATION *IN VITRO*

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One of the most important trends in modern immunology is the development of methods of adoptive immunotherapy. Research on laboratory animals has led to definite advances in this field. After adoptive transfer of T cells, activated *in vitro*, regression of transplantable tumors has been obtained in mice and rats [3, 4]. It has been shown that to obtain rejection of tumors in mice, cells with the Lyt-2^+ phenotype are needed [9], and that their action can be potentiated by repeated injections of interleukin 2 (IL2) [5]. One result of adoptive transfer of immune cells is that the tumors of these animals contain highly active cytolytic T lymphocytes [6].

Approaches have now been developed to the adoptive immunotherapy of human tumors. For this purpose the study of specific antitumor immunity is patients and the development of methods of potentiating it *in vitro* are needed. In the investigation described below specific and natural cytotoxicity of peripheral blood lymphocytes from patients with melanoma was compared and stimulation with autologous tumor cells or a pool of allogeneic lymphocytes from five healthy blood donors also was used to potentiate the specific antitumor activity of the patients' lymphocytes.

EXPERIMENTAL METHOD

The group of patients with metastases of melanoma included six men and six women aged from 35 to 62 years.

Mononuclear cells were isolated from the patients' peripheral blood by the method in [2]. Some cells were incubated on a column with nylon wadding in medium RPMI-1640 containing 5% human serum, at 37°C and with 5% CO_2. Nonadherent cells were eluted 45-60 min later with the same medium, previously warmed to 37°C.

The tumor was minced mechanically to pieces with a volume of 2-8 mm^3 and subjected to trypsinization in a glass flask on a magnetic mixer at 37°C for 40-60 min. A 0.25% solution of trypsin (4 mg, from Serva, West Germany) was used for this purpose. At the end of incubation the trypsin was inactivated by the addition of 20% bovine serum to the suspension. The cells were washed twice with medium 199, transferred to plastic dishes, and allowed to stand in order to restore the surface antigens in medium RPMI-1640 containing 5% human serum for 16-18 h at 37°C. Suspensions containing no fewer than 80% of living tumor cells were used for the work. The cells were frozen by the standard program and kept in a container with liquid nitrogen.

To assess the cytolytic activity of the lymphocytes cells of an autologous tumor, cells of the K-562 line, autologous peripheral blood lymphocytes (PBL), and blast cells obtained from PBL after stimulation by phytohemagglutinin (PHA) were used as the target cells (TC). The TC ($0.5 \cdot 10^6$) were incubated in 0.5 ml of medium containing 25-50 μCi of $Na_2^{51}CrO_4$, in a water bath at 37°C. After 45-60 min the cells were washed 3 times and added, in a concentration of 10^4 per well, to 96-well plates with a conical bottom (Falcon, USA). The total volume of the contents of the well was 0.2 ml. Before incubation the plates were spun at 1000 rpm for 1 min. After incubation for 4 or 18 h at 37°C in the presence of 5% CO_2 the cells were

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TABLE 1. Cytotoxicity of Peripheral Blood Lymphocytes of Melanoma Patients against Autologous Tumor Cells and K-562 Cells (% of cytolysis of TC).

Patient No.	Autologous tumor			K-562 cells		
	ratio of lymphocytes to TC					
	50:1	25:1	12:1	50:1	25:1	12:1
1	10,8	8,8	5,8	85,6	77,8	69,6
2	0	0	1,9	63,6	28,6	23,4
3	13,4	12,5	4,3	—	—	—
4	15,0	14,9	7,1	30,2	21,6	14,2
5	—	0	—	—	8,9	—
6	18,7	15,9	—	54,2	48,0	33,0
7	23,6	25,9	20,9	26,9	16,1	9,1
8	10,5	7,4	6,6	19,0	14,0	9,5
9	—	19,3	—	—	72,8	—
10	0	0	—	29,9	13,8	15,8
11	6,0	4,4	2,9	—	38,2	23,2
12	—	0	—	14,2	10,2	7,2

sedimented by centrifugation of the plates at 1000 rpm for 10 min, and samples of supernatant were taken to determine radioactivity. The specific release of chromium was estimated by the equation:

$$\text{Cytolysis} = \frac{\text{experimental - spontaneous release}}{\text{total lysis - spontaneous release}} \times 100\%,$$

where the experimental release denotes release of the isotope from TC on the addition of lymphocytes; spontaneous release denotes release of the isotope by intact TC; total lysis denotes the total amount of isotope incorporated into TC.

To obtain a mixed culture of lymphocytes and autologous tumor cells, the patients' lymphocytes were mixed with tumor cells irradiated in a dose of 4500 R, in the ratio of 1:1 or 2:1. The cells were cultured in medium RPMI-1640 containing 5% human serum, $2 \cdot 10^{-3}$ M L-glutamine, $5 \cdot 10^{-3}$ M HEPES, $3 \cdot 10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) for 6 days in plastic flasks (Falcon). The concentration of lymphocytes with 10^6/ml. For allogeneic stimulation of the patient's PBL were mixed with PBL from healthy blood donors, irradiated with a dose of 3000 R, in the ratio of 2:1. The concentration of reacting lymphocytes was $2 \cdot 10^6$/ml. The cells were cultured in Saniglas glass flasks for 7 days.

EXPERIMENTAL RESULTS

The cytolytic activity of the peripheral blood monocytes of 12 patients with melanoma metastases against autologous tumor cells, obtained on the day of the operation, was studied. A cytolytic effect was observed in eight patients. The patient's monocytes were shown to produce lysis only of the tumor cells themselves, and not of autologous PBL or PHA-stimulated lymphoblasts, lysis of which in the present experiment did not exceed 1.5-2.5%, when effector cells and TC were in the ratio of 25:1.

Natural cytotoxicity was observed in all patients. The level of activity of natural killer cells (NKC), which were tested on NKC-sensitive K-562 target cells, was 2-10 times higher than the effect of the patients' lymphocytes against autologous tumor cells (Table 1).

These data are in agreement with results obtained by other workers. Depending on the location and the histological area of the tumor, autoreactivity was found in 9-45% on the patients; all patients, moreover, showed high NKC activity. The period of survival and the duration of remission were longer in patients with autoreactivity against the tumor cells themselves [10, 11].

The method of lectin-dependent cytotoxicity, whereby activity of potential killer cells, circulating in the patient's peripheral blood [1], can be detected, also was used. After treatment of the TC with concanavalin A (con A) the cytolytic effect of the patients' lymphocytes was increased five-sevenfold, and under these circumstances activity was higher if a population enriched with T lymphocytes was used (Fig. 1). These data suggest that specific antitumor immunity exists, but the absence of antigenic determinants or their masking on the surface of a certain proportion of the tumor cells prevents their contact with killer T cells.

The effect of killer T cells against autologous tumor cells can be considerably potentiated *in vitro* by stimulation of the patient's lymphocytes by irradiated autologous tumor

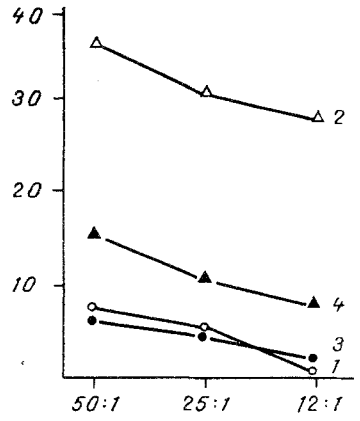

Fig. 1

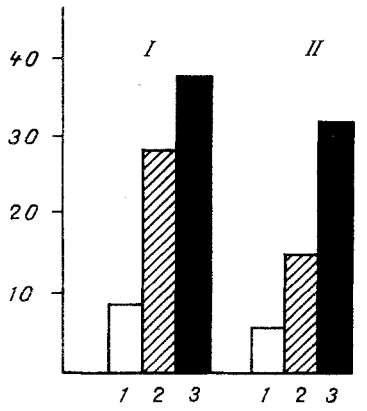

Fig. 2

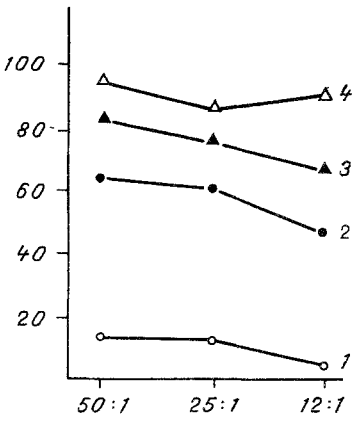

Fig. 3

Fig. 1. Cytolytic activity of T lymphocytes (1, 2) and monocytes (3, 4) from peripheral blood on autologous tumor cells untreated (1, 3) and treated (2, 4) with con A. Abscissa, Ratio of lymphocytes to TC; ordinate, cytolysis (in %).

Fig. 2. Cytolytic activity of peripheral blood lymphocytes against autologous tumor cells before (1) and after stimulation of unfractionated (2) and T cell enriched (3) populations in mixed culture of lymphocytes and tumor cells. Abscissa: I) patient Sh, II) patient V; ordinate, cytolysis of TC with ratio of lymphocytes to TC of 25:1 (in %).

Fig. 3. Cytolytic activity of lymphocytes, unstimulated (1, 3) and stimulated by alloantigens (2, 4) against autologous tumor (1, 2) and K-562 (3, 4). Abscissa, Ratio of lymphocytes to TC; ordinate, cytolysis (in %).

cells. Activity of effector T lymphocytes, which they generate *in vitro*, was higher if a cell population enriched with T-lymphocytes was used for stimulation (Fig. 2). Preliminary removal of adherent cells may perhaps abolish the effect of macrophages or of suppressor T cells.

The optimal effect was obtained by the use of a mixture of lymphocytes from allogeneic donors as the stimulating cells (Fig. 3). Activated T lymphocytes produce lysis of autologous tumor cells and NKC-sensitive K-562 target cells, but not of autologous peripheral blood lymphocytes or of lymphoblasts stimulated by PHA.

According to data in the literature such lymphocytes, when activated by alloantigens, PHA, or IL2, carry markers of OKT-3, OKT-4, and OKT-8 T lymphocytes, although their precursor cells have no marker characteristics whatsoever [7, 8]. Since the patient's tumor cells are not always accessible for use as specific stimulators, killer T cell generation *in vitro* can evidently be obtained by individual selection of certain nonspecific methods of stimulation.

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