FORMATION OF SPECIFIC ANTITUMOR CYTOTOXIC T LYMPHOCYTES IN MONOCULTURE

A. Yu. Lupatov and B. D. Brondz

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Analysis of the experimental and clinical data points to a significant influence of specific immunity on the course of some forms of cancer [11] In this connection some very important research is being carried out to create experimental models of generation of antitumor cytotoxic T lymphocytes (CTL). One model which has achieved widespread popularity is that in which the formation of CTL specific for a syngeneic tumor takes place after primary immunization in vivo as a result of tumor growth or inoculation of irradiated tumor cells, and of secondary immunization in vitro in a one-way mixed culture of lymphocytes and tumor cells. This model has been used to study the conditions of differentiation of memory cells into secondary CTL (CTL-2) [8], for adoptive immunotherapy of tumors [9], and to study activity of suppressor cells in suppressing the immune response to a tumor [3]. In all cases an essential condition for the formation of specific CTL is the presence in vitro of tumor cells as stimulators. Attempts to obtain CTL after a series of immunizations in vivo followed by maturation of the lymphocytes in vitro in the absence of stimulating tumor cells (in monoculture) leads to the formation of nonspecific CTL, which do not possess the Lyt2 (CD8) marker [10].

The development of a model by which specific CTL can be formed in monoculture may subsequently be used to develop methods of adoptive immunotherapy, and also to study factors involved in the appearance of immunoresistance to tumors.

The aim of this investigation was to create an experimental model enabling the generation of specific antitumor CTL during immunization in vivo and differentiation in vitro in a lymphocyte monoculture, and also to study some aspects of CTL generation in such a system.

EXPERIMENTAL METHOD

Male inbred C57BL/6 (B6) and C57BL/10 (B10) mice, identical with respect to the H-2 histocompatability locus were used. The animals were obtained from the nursery of the All-Union Oncologic Research Center, Academy of Medical Sciences of the USSR, and were used at the age of 5-10 months. A fibrosarcoma MCh-11 (induced in B10 mice) and an EL-4 T-cell lymphoma (induced in B6 mice) were maintained in ascites form by daily passage through syngeneic mice. To immunize the animals, the sarcoma MCh-11 and lymphoma EL-4 were irradiated in a dose of 30,000 rads and 5000 rads respectively. Next, $1 \cdot 10^7$ cells were injected into the hind footpads of a syngeneic recipient. Spleen cells (sc) from syngeneic animals, irradiated beforehand in a dose of 2000 rads, were used for the control immunization. To obtain a lymphocyte monoculture, regional (inguinal and popliteal) lymph nodes were removed from mice and a cell suspension was prepared from them, dead cells being removed by agglutination in 0.01 M Tris-HCl buffer, pH 7.4. The remaining cells were washed 3 times and placed in medium RPMI 1640 with the addition of 10% fetal calf serum, 2 mM L-glutamine, 4 mM HEPES, $5 \cdot 10^{-5}$ M 2-mercaptoethanol, and 50 μg/ml gentamicin. The cells were incubated in 24-well plates ("Linbro") with $5 \cdot 10^6$ cells per well, at 37°C in an atmosphere of 5% CO_2 for 3-4 days. The cells were then sedimented by centrifugation, counted, and their cytotoxic activity determined by measuring release of chromium-51 in a 6-h test by the standard method

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TABLE 1. Cytotoxic Response to Syngeneic Tumor Using Different Immunization Programs

Time after primary immunization (days)	Time after secondary immunization (days)	Time in monoculture (days)	Cytotoxic index (%)
Immunization by MCh-11			
4	—	4	7,5
6	—	4	9,2
6	4	4	0
8	4	4	9,0
10	—	4	4,9
10	4	—	5,4
10	4	4	43,1
15	4	4	42,0
Immunization by EL-4			
4	—	4	7,0
8	—	4	8,0
10	4	—	10,1
10	4	4	90,7

Legend. Tumor against which immunization was carried out was used as the target. The monoculture contained rIL-2 (100 U/ml).

[4]. To detect the CTL markers, the cells were treated with monoclonal antibodies (McAB) to antigens: Thy 1.2 (hybridoma G4 [2]); Lyt 2.2 (hybridoma TIB 150 [7]); L3T4 (hybridoma GK1.5 [5]) for 60 min at 4°C. After centrifugation the cells were incubated with nontoxic rabbit complement in a dilution of 1:20 for 40 min at 37°C.

EXPERIMENTAL RESULTS

The aim of the first series of experiments was to develop an immunization program by which active antitumor CTL could be obtained. For this purpose, B10 mice were immunized with irradiated sarcoma MCh-11. Irradiation in a high dose (30,000 rads) prevented viable tumor cells from entering the monoculture, for all the irradiated cells died in the course of 4 days. Since, unlike precursors of primary CTL, precursors of CTL-2 (pCTL-2) can be activated by killed cells [1], dead cells were removed from the monoculture.

Lymphocytes possessing cytotoxic activity against MCh-11 were not found in the regional lymph nodes of B10 mice, whether after immunization once or twice (Table 1). It also follows from the results in Table 1 that primary CTL are not formed even if the lymphocytes, after immunization, were incubated in monoculture with a large quantity of exogenous recombinant interleukin-2 (rIL-2, 100 U/ml). If secondary immunization in vivo was carried out 10 days after primary immunization, and this was followed 4 days later by incubation of the lymphocytes in monoculture containing rIL-2, CTL causing active lysis of MCh-11 were discovered in the cytotoxicity test. Similar results were obtained by immunization of B6 mice with thymoma EL-4. In this case also the CTL matured in monoculture only after secondary immunization in vivo (Table 1).

To study the conditions of CTL generation, different amounts of rIL-2 were added to the monoculture. Lymphocytes from donors immunized with syngeneic sc by the same program were used as the control. As the control of formation of lymphokine-activated killer cells (LAKC) a monoculture obtained from intact mice was used. Cytotoxicity was assessed relative to the two target tumors, identical as regards the H-2 locus. It will be clear from Fig. 1a that after immunization of the B10 mice with the MCh-11 tumor, maturation of specific CTL in monoculture took place only in the presence of rIL-2. In this case a high rIL-2 concentration (100 U/ml) led to LAKC formation. After immunization of B6 mice with the EL-4 tumor (Fig. 1b), even in the absence of exogenous IL-2, CTL specific for EL-4 were formed. In the final stage, the antigenic phenotype of the CTL was determined (Fig. 2). In both cases these were T lymphocytes bearing the phenotype Thy 1.2^+, Lyt 2^+, L3T4$^-$.

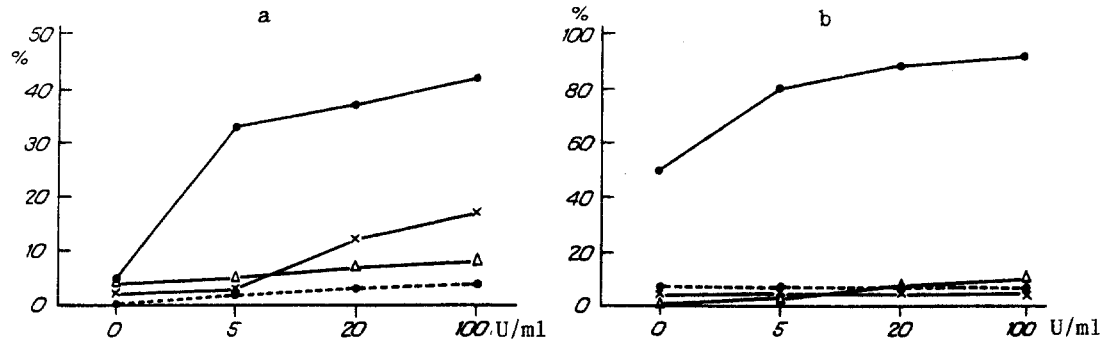

Fig. 1. Efficiency of CTL formation and their specificity depending on dose of rIL-2. Abscissa, dose of rIL-2 (U/ml); ordinate, cytotoxic index (CTI, %). a: B10 mice immunized twice with MCh-11 (●) or with syngeneic sc (△). Lymphocytes of immunized and intact (*) mice matured for 3 days in monoculture with different amounts of rIL-2, after which their cytotoxic activity was tested on MCh-11 (—), or EL-4 (– – – –) targets. Effector/target ratio 90:1, b: B6 mice immunized twice with EL-4 (●). Target was EL-4 (——), or MCh-11 (– – – –) respectively. Effector/target ratio 30:1.

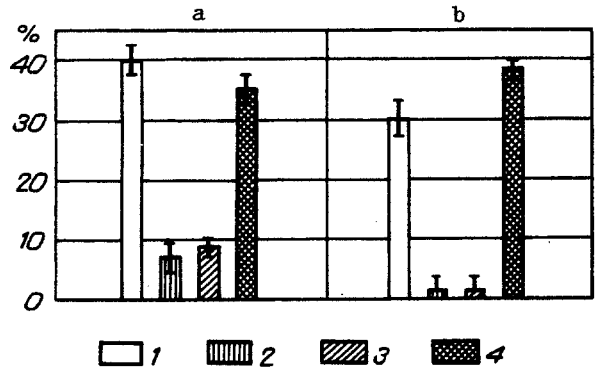

Fig. 2. Characteristics of phenotype of CTL specific for syngeneic tumor, Ordinate, CTI, %. CTL specific for MCh-11 (a) or EL-4 (b) were treated with medium (1), antiThy 1.2 UcAB (2), anti-Lyt 2.2 McAB (3), anti-L3T4 McAB (4), and complement followed by testing of cytotoxic activity. Effector/target ratio 90:1 (a); 30:1 (b).

As a result of this study we were unable to find primary CTL being formed either in vivo or in vitro The cytotoxicity discovered was evidently caused by CTL-2, which are analogous to those formed in secondary mixed culture of lymphocytes and tumor cells. For maturation of pCTL-2 to take place in mixed culture, there are two essential conditions: contact with antigen and the appearance of exogenous or endogenous IL-2 in the culture [1]. In the model described above these processes are separate, for immunization was carried out in vivo and maturation in vitro, in a culture not containing stimulating cells. The absence of mature CTL-2 in vivo may be connected with activation of suppressor cells, inhibiting IL-2-producing cells. This hypothesis is based on the fact that suppression does not interfere with the ability of pCTL-2 to recognize antigen; moreover, the formation of CTL-2 specific for MCh-11 takes place in monoculture only in the presence of exogenous IL-2. Generation of anti-EL-4 CTL-2 in the absence of exogenous IL-2 may perhaps be connected with the fact that EL-4, being a thymoma, may secrete IL-2 [6]. The appearance of IL-2 during immunization in vivo may indirectly promote maturation of CTL-2 in monoculture, for example, through activating secretion of endogenous IL-2 or other lymphokines by lymphocytes.

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SUPPRESSOR T CELLS OF TUMOR-BEARING MICE INHIBIT ANTITUMOR CYTOTOXIC T LYMPHOCYTE MATURATION IN MONOCULTURE

A. Yu. Lupatov and B. D. Brondz

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Limitation of the immune response by suppressor cells is one of the principal pathogenetic mechanisms formed during tumor growth. Suppression of T-cell type is observed in patients with various forms of cancer [1, 9, 11], suggesting that it may be one cause of the ineffectiveness of antitumor immunity. If transplantable tumors are used as the experimental model, the formation of two types of suppressor T cells (TS) may be discovered [7]. TS of the first type ($Lyt2^+$, $L3T4^-$) appear in the early stage of tumor growth and soon disappear. Their action is connected with inhibition of production of interleukin-2 (IL-2) by helper T cells [8]. In the later stages of tumor growth TS of the second type appear ($L3T4^+$, $Lyt2^-$), and are present until death of the tumor-bearing animal. They can suppress the manifestations of adoptive immunity [5] and inhibit the formation of cytotoxic T lymphocytes (CTL) in secondary mixed culture of lymphocytes and tumor cells [4]. The mechanism of action of suppressors of this type has so far received little study.

Previously the writers described a model whereby secondary antitumor CTL (CTL-2) can be generated during in vivo immunization and subsequent in vitro maturation, in the absence of stimulating cells (in monoculture) [3]. By using this model, new opportunities are presented for the study of the mechanism of suppression.

EXPERIMENTAL METHOD

Male inbred C57BL/6 (B6) and C57BL/10 (B10) mice were used. The animals were obtained from the nursery of the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, and used at the age of 5-10 months. Sarcoma MCh-11 and lymphoma EL-4 were maintained in ascites form by daily passage through syngeneic mice (B10 and

All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR. Moscow.

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