Time Course of the Cytotoxicity of Blood Mononuclears in Patients with Bladder Cancer during Endolymphatic Immunotherapy with Lymphokine-Activated Killers and Recombinant Interleukin-2

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A specific stimulation of mononuclear cells *in vivo* was observed after 3 or 4 endolymphatic injections of autologous lymphokine-activated killers and recombinant interleukin-2 in male patients with disseminated bladder cancer. This activation presented as an increase of the cytotoxicity of mononuclears towards target cells of bladder carcinoma. A statistically reliable increase of natural killer activity was observed, and in one patient a reliable increase of the cytolytic activity of mononuclear cells against Mel-1 target cells. After 1 or 2 injections the activity of natural killers increased to 80-90% for an initial level of 23-50%.

Key Words: *antitumor immunity; bladder cancer; immunotherapy; interleukin-2; lymphokine-activated killers*

Intravenous immunotherapy with interleukin-2 (IL-2) in a complex with autologous mononuclear cells (MNC) activated by it *in vitro* - lymphokine-activated killers (LAK) - is acknowledged to be more effective than therapy with lymphokine alone. It leads to remission in about 20% of patients with malignancies [7]. M. S. Pulley [6] developed, and later G. Pizza *et al.* [5] tried the method of endolymphatic injection of LAK in a complex with IL-2 to cancer patients. This method consists in a new mode of administering the agent and creates new conditions for the circulation of LAK and IL-2 in the organism. The authors succeeded in attaining complete or partial regression of metastases of various solid tumors after injections of native IL-2 (10,000 IU) and LAK (20×10^6 cells).

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Attempts at using lymphokines for the treatment of patients with bladder cancer started with intratumoral injection of IL-2, causing massive necrosis of the tumor and its complete (in 50% of patients) or partial regression. Injection of IL-2 in the involved regional lymph nodes confirmed the efficacy of this type of immunotherapy [4].

Data on the cytotoxic activity of immune system cells *in vivo* and *in vitro* during immunotherapy with lymphokines are very scarce, and there are no reports dealing with such treatment for bladder cancer. However, the antitumor cytotoxicity of MNC and LAK reflects the efficacy of their stimulation and may serve as a scientific approach to the validation of the methods and protocols of immunotherapy [1,8]. Hence, this study was aimed at investigating the cytotoxicity of different MNC populations and assessing the efficacy of their stimulation in patients with bladder cancer in the course of endolymphatic immunotherapy with LAK and recombinant IL-2 (rIL-2).

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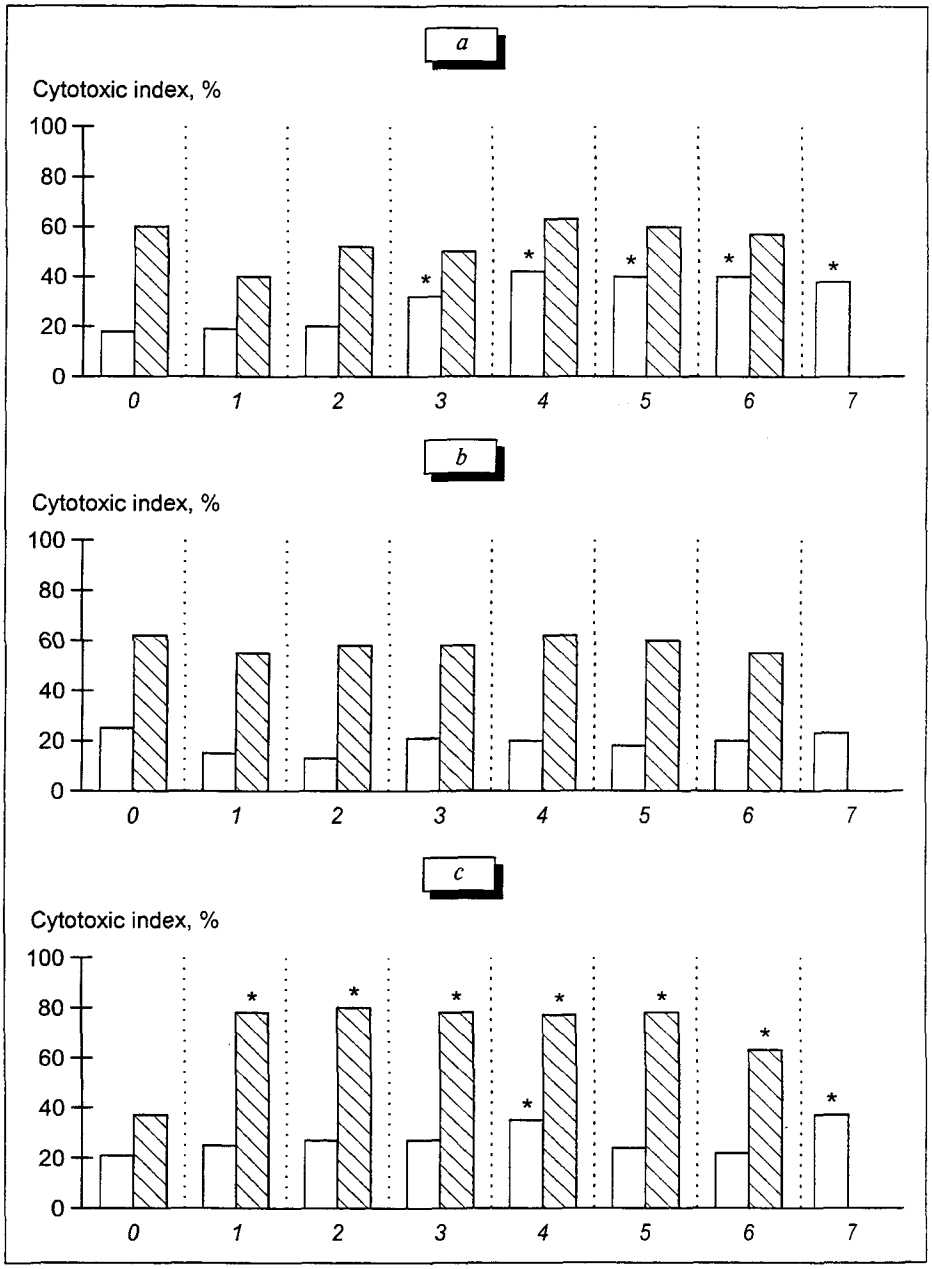

Fig. 1. Cytotoxic activity of mononuclear cells (white bars) and lymphokine-activated killers (LAK, cross-hatched bars) in patients with bladder cancer during adoptive therapy with low-dose LAK and rIL-2. *1-6*) LAK and rIL-2 injections; *7*) 3 months after injections. Asterisk shows a reliable increase of cytotoxicity ($p<0.05$) in comparison with the initial (*0*) level.

MATERIALS AND METHODS

Seven patients with bladder cancer, three of them with metastases untreatable by surgery, radiation, or chemotherapy, were examined. The diagnosis was morphologically confirmed in all patients, and all of them had normal parameters of hemopoiesis and of liver, kidney, and cardiovascular function (Table 1).

The cells were injected by catheterization of the lymph vessels on the feet. 0.4 ml of a 2.5% solution of vital stain was injected in 1-3 interdigital spaces. After numbing with 0.5% Novocaine solution, a lymph vessel was isolated on the posterior part of the foot and punctured with a Cordis needle. A syringe from a Schneider automatic injector was attached to the needle with an elastic catheter. At first LAK and rIL-2 were injected endovascularly, and then the diagnostic chemically inert x-ray-positive oily agent mayodyl. After injection the needle was removed from the lymph vessel and the wound sutured and covered with a sterile dressing. Then polypositional roentgenography was carried out, which was repeated after 24 h.

The course of treatment lasted for 2 to 6 weeks, with the drug injected once a week.

For assessment of the cytotoxicity of rIL-2 (Cetus), it was injected to the first three patients in increasing doses starting from 10,000 IU *pro dosi* (Table 1).

Side effects were evaluated in accordance with the scoring scale for assessing side effects of cytostatics, as recommended by WHO [3], and accord-

TABLE 1. Summary for Patients and Treatment Data

Patient	Age, years	Disease stage	No. of injections		Dose	
			rIL-2	LAK	rIL-2, $\times 10^3$	LAK, $\times 10^6$
A.	56	T1NOMO	6	6	10-100	12-77
F.	58	T4NOMO	6	6	10-100	16-100
Z.	39	T4NOMO	2	2	10-100	5-70
M.	60	T3N1MO	6	6	100	10-26
As.	55	T3bN2MO	6	6	100	35-57
S.	56	T3bNOMO	6	5	100	10-38
V.	60	T1NOMO	6	6	100	40-135

ing to a bulletin on drug therapy of tumors [2]. The efficacy of treatment was judged from the time course of tumor changes, which were measured and examined according to recognized standards: complete regression, partial regression (less than 50% reduction of tumor), and no effect. The patients were followed up for 3 months.

Preparation of MNC and LAK. MNC were isolated from the peripheral blood (100 ml) by centrifuging in Ficoll-Verograffin of 1.077 g/cm^3 density. One or two million MNC were cultured for 48 h in RPMI-1640 medium containing fetal calf serum, glutamine, gentamicin, and 1000 IU/ml rIL-2 (Cetus) at 37°C in an atmosphere with 5% CO_2 in Sany Glas glass culture flasks. The cells were then sedimented by centrifuging, washed with culture medium, and resuspended in 10 ml of normal saline.

Cytotoxic test. The cytotoxic activity of MNC and of LAK obtained from them after 48 hours was tested on allogeneic target cells of bladder cancer T-24 and human melanoma (Mel-1) (ratio of effector to target cells 50:1; the cells were provided by Dr. E. S. Revazova), and on K-562 cells sensitive to natural killers (effector to target cell ratio 25:1).

Half a million to one million target cells were incubated in a medium containing 50-100 μCi Na_2CrO_4 (Amersham) for 1 hour at 37°C, 5% CO_2, after which they were washed three times and resuspended in medium with 5% serum. Labeled target cells (1000 cells per sample) were mixed with effector cells (MNC or LAK) and incubated at 37°C, 5% CO_2 for 4 hours (K-562) or for 18 hours (T-24 and Mel-1). The test was repeated twice. The radioactivity of supernatants was measured on a gamma-counter. The cytotoxic index was calculated after the standard formula: $(Ce-Cs)/(Cc-Cs)\times 100\%$, where *Ce* is the radioactivity of a test sample, *Cs* the spontaneous release of the isotope from target cells not subjected to killers, and *Cc* the complete label per 1000 target cells.

Spontaneous release of the isotope in target cells of melanoma and bladder cancer was 15-20%, that for K-562 8-12%.

RESULTS

A reliable increase of the killer activity of MNC towards T-24 target cells was observed after 3-4 injections of rIL-2 and LAK, the cytotoxicity of immune cells increasing from the initial 18.7±2.6% to 32.8±2.5-42.3±4.0%. Subsequent endolymphatic infusions of rIL-2 and LAK did not lead to a further increase of MNC cytotoxicity (Fig. 1, *a*). A high level of MNC killer activity towards T-24 target cells persisted for 3 to 5 months after the end of immunotherapy.

Endolymphatic infusion of LAK and rIL-2 virtually did not affect the cytotoxicity of MNC for Mel-1 target cells (Fig. 1, *b*). In 5 out of 7 patients there was a tendency for MNC killer activity to increase after the end of treatment. A reliable increase of the activity of MNC from 9.0±2.1 to 20.6±5.5% was observed in only one patient after the second and fourth injections of LAK and rIL-2.

In tests with K-562 target cells the cytotoxicity of natural killers in the MNC population reliably increased from 21.0±1.8 to 30.8±1.0% in comparison with the baseline values only after the 4th injection of LAK and rIL-2, with an effector to target cell ratio of 25:1 (Fig. 1, *c*). Further injections led to a reduction of natural killer activity to a level close to the initial, but after the end of immunotherapy the cytolytic activity of natural killers again reliably increased. Their cytotoxic activity remained high for 2-3 months after the injections of LAK and rIL-2 were discontinued.

The activity of LAK towards T-24 and Mel-1 target cells did not change after the treatment in comparison with the initial levels (Fig. 1, *a, b*). However, it is noteworthy that in 5 patients with an initially high cytotoxicity (73.5-95.3%) the activity of LAK did not increase in the course of therapy, whereas in 2 patients with an initial activity of 23.2-48.2% the therapy did cause a reliable increase in activity (to 90.9±10.1% after 1 or 2 endolymphatic infusions of LAK and rIL-2). The cytotoxic activity of natural killers in the population of LAK reliably increased in 6 patients (from 30.5±4.5 to 75.9±6.7%, on average), as a rule after the

first infusion of LAK and rIL-2, and remained thus over the entire course of immunotherapy (Fig. 1, *c*). In one case, when the cytotoxicity of LAK before treatment was 73.5±7.8%, immunotherapy did not change it.

No appreciable side effects were observed in any of the patients in the course of therapy. In 5 patients blood biochemistry did not reliably change. In two cases there was a negligible increase of bilirubin and of urea and creatinine levels in the blood. Cytological analysis of the peripheral blood did not show the characteristic disorders associated with LAK and rIL-2 immunotherapy. Thrombocytosis was observed in three patients and thrombocytopenia in two. In two cases thrombocytosis was accompanied by lympho- and monocytosis.

Endolymphatic immunotherapy of patients with bladder cancer did not lead to tumor regression, but the clinical findings indicate that in most cases the progress of tumor growth was slowed.

Previously we demonstrated that MNC of melanoma patients, like patients with tumors of other localizations, are capable of generating LAK with a high cytotoxic activity, and immunotherapy of these patients with a combination of LAK and rIL-2 led in the majority of cases to an increase of MNC cytotoxicity *in vivo* [1]. The results of this study indicate that endolymphatic immunotherapy with LAK combined with low doses of rIL-2 leads to a specific stimulation of immunity against T-24 target cells in patients with bladder cancer, the maximal increase of cytotoxicity being observed after 3 or 4 injections of the agents.

These results demonstrating the stimulation of cytotoxic activity of immune cells *in vitro* and *in vivo* suggest that it is possible to attain a positive therapeutic effect if the doses of LAK and rIL-2 and the duration and number of courses of endolymphatic immunotherapy are properly selected.

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