Interleukin-2 Gene Therapy Potentiates the Antitumor Effect of Cytostatic Agent in Mice with Advanced Transplantable Tumors

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1. Introduction

The attempts to augment the immune response in patients with solid tumors have been undertaken since a long time ago. Nowadays, vaccines and cytokines are the most actively investigated approaches in cancer immunotherapy. The initial application of cytokines involved systemic administration of pharmacologic doses of recombinant protein. The major cytokine that has been used that way in cancer patients is interleukin 2 (IL-2) (1,2). The systemic IL-2 administration yields the results of clinical response rate of about 20% in patients with renal cancer and melanoma, but in other cancers the response was rather modest (3). To maintain high levels of IL-2 in circulation, high doses of the cytokine had to be used by multiple bolus injections or by continuous infusion. It appeared, however, that although the concentration of lymphokine in vasculature was at so high levels that it often caused severe toxic side-effects, the amount of the cytokine at the site of tumor was frequently suboptimal to elicit effective antitumor response (4).

In the past five years, the combined application of cancer vaccines and cytokines resulted in new immunotherapy strategies aiming at the expression of a specific cytokine locally at the tumor site. Numerous reports have analyzed various biological effects of injections of tumor cells engineered to secrete different cytokines (5,6). The most important feature emphasized by all animal model studies is that the inflammatory responses induced by high local secretion of cytokines often resulted in the ultimate destruction of the transduced tumor cells without significant evidence of systemic toxicity. In some experimental models, systemic protection against parental non-transfected cells was observed after rejection of the cytokine-producing cells.

Since immunotherapy alone is rather insufficient in advanced cancer disease, it should be considered in clinical trials within a broader context of other treatment modalities i.e. chemotherapy, radiotherapy or surgery that could reduce the tumor load before immunological interventions have been initiated (7). Thus, more studies in model systems are required to assess potential synergies of gene therapy with other conventional treatments.

Here we present our studies in which non-tumorigenic murine plasmocytoma cells engineered to secrete mIL-2 were used for induction of antitumor response in mice bearing either parental tumor or non-related other murine tumors (fibrosarcomas or colon carcinoma). Using numbers of cytokine-secreting cells and different the time intervals between chemotherapeutic agent administration and adjuvant immunotherapy we aimed to achieve a more efficient response in mice with advanced tumors.

2. Material and methods

2.1. Cell lines

Mouse plasmocytoma X63Ag8-653 cells ("wild" line, **X63/0**) and murine IL-2 cDNA transfected subline (**X63-mIL-2**) cells were kindly provided by dr J. Bubenik from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague. Their properties were described elsewhere (8). **BFS-1 fibrosarcoma** (MCA-induced in BALB/c mice) was obtained from the Tumor Bank of the Institute of Pathology, Deutches Krebsforschungszentrum, Heidelberg. **F-69-3 fibrosarcoma** (MCA-induced in BALB/c nu/nu) was obtained from the Panum Institute of the University of Copenhagen. All cells were propagated under previously described conditions (10). **Colon 38 adenocarcinoma** was received from the tumor bank at the Radiobiological Institute TNO, Rijswijk, The Netherlands. It is maintained *in vivo* by s.c. transfer of tumor tissue fragments into C57BL/6 mice.

2.2. IL-2 bioassay

The mIL-2-bioactivity present in supernatants from X63-mIL-2 cell culture was determined using CTLL-2 cell line (IL 2-dependent mouse cytotoxic T lymphocyte line). The assay was conducted according to Mossman (9) with minor modifications (10). The optical density at 570 nm was measured on microplate ELISA reader. One laboratory unit (1 LU) of IL-2 activity was defined as the concentration of supernatant inducing 50% of maximal cell growth. The levels of IL-2 varied from approximately 1.5×10^3 to 5×10^3 LU/ml in different supernatants.

2.3. Therapeutics

1. Cyclophosphamide, CY was purchased from Asta Medica AG, Germany (Endoxan). The drug was dissolved in a sterile water to obtain concentration of 20 mg/ml.

2. Compound CBM-4A (racemic chlorobromofosfamide). The agent is a bromine-substituted derivative of fosfamide, which was synthesized in the Molecular and Macromolecular Studies Center, P.A.S., Łódź (11). It has been previously shown to possess significant antitumor activity in several murine tumor models. Its lethality was assessed in healthy animals (12). The dose of 150 mg/kg, used more frequently in these studies, is roughly an equivalent of 200 mg/kg of cyclophosphamide.

Both agents were injected intraperitoneally (i.p.) in the amount of 0.01 ml/g of body weight.

3. Recombinant human IL-2 (rhIL-2), Proleukin, Cetus Corp., Emeryville, stock solution of 10^6U/ml was diluted with saline.

2.4. Mice

Eight to ten-week-old male or female inbred BALB/c mice and F1 hybrids of BALB/c x DBA/2 (CD2F1) or C57BL/6 x DBA/2 (B6D2F1) mice were obtained from the Inbred Animals Breeding Center at our Institute. All experiments were conducted under conventional hygienic conditions (M.D. standard) of an experimental animal house. Free access to granulated chow and filtered tap water *ad libitum* was provided.

2.5. *In vivo* experiments

Tumors were inoculated subcutaneously into the right flank. The incidence and tumor growth were recorded 2-3 times a week. The tumor mass was calculated according to the formula 0.5 (ab^2), where a and b are the perpendicular diameters of tumor nodule (a — the longer and b — the shorter) measured with calipers. Experiments were usually terminated when the average tumor mass reached 4-6 grams (plasmocytoma) or 6-8 grams (fibrosarcomas), or when one half of animals in the control group died. Tumor weight inhibition in relation to the control was calculated according to the formula: [1-T/C] x 100%, where T — is the average tumor mass in treated group and C — in control group.

2.6. Statistical analysis

Differences in tumor incidence were evaluated with the Chi-square test and differences in average tumor mass were evaluated by Student's t test using CSS Statistica, StatSoft™ Inc.

3. Results

3.1. Characteristics of X63-mIL-2 cells

Interleukin-2-producing X63-mIL-2 cells have been previously shown to be non-tumorigenic when inoculated by different routes (i.p., s.c. or i.v.) either to intact syngeneic BALB/c, semisyngeneic CD2F1, athymic nu/nu NCr mice or to CD2F1 mice immunosuppressed 3 or 7 days earlier with cyclophosphamide (200 mg/kg, i.p.). The cells appeared to be immunogenic as the decrease of parental (X63/0) tumor take number and growth retardation of accepted tumors was observed in mice pretreated with the single s.c. inoculation of transfected cells (10^7/mouse). These effects depended on the time interval between vaccination and the subsequent tumor challenge. In all pretreated groups the number of non-accepted tumors was greater than in the control group, but the statistically meaning full difference was observed in the group of mice pretreated on day 7 ($p = 0.025$). Also the life-span of mice in this group was significantly higher than in the control ($p = 0.003$) or other groups. Mice that did not accept primary tumors were protected against the second challenge 3 months later.

Administration of a single peritumoral (p.t.) injection of mIL-2-secreting cells to BALB/c or CDF1 mice shortly after the subcutaneous inoculation of the parental plasmocytoma cells resulted in tumor rejection in up to 50% of the animals and in significant tumor growth retardation. With the delay of the treatment the number of cured animals decreased. However, two injections of transfected cells applied on days 6 and 12 were curative for 100% of animals (10).

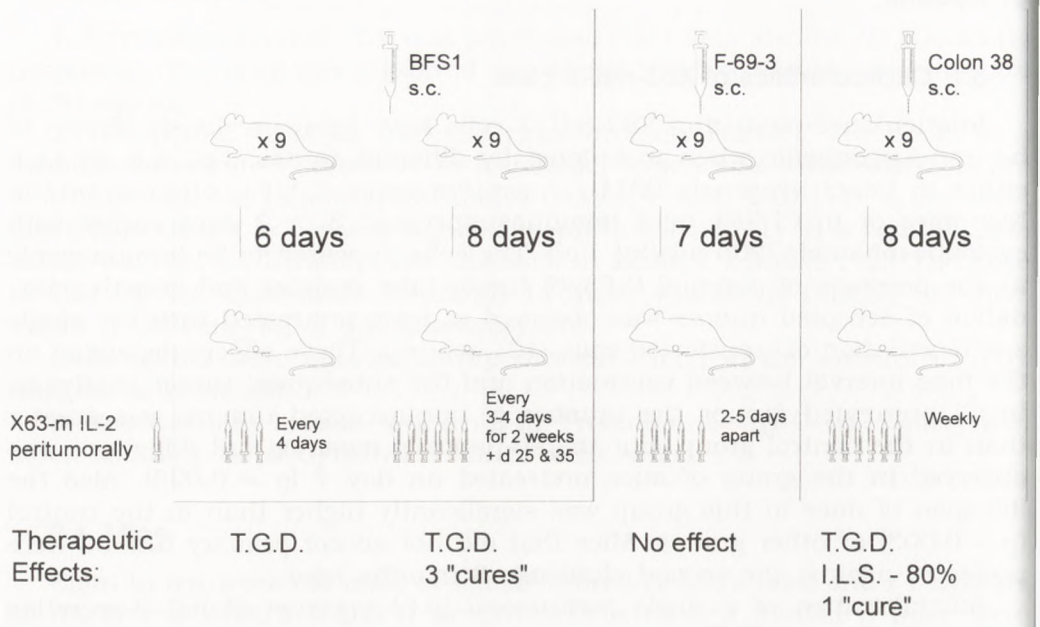
3.2. Immunotherapy in mice with non-advanced not-related tumors

A similar treatment (on days 6 and 13), applied in mice bearing BFS1 fibrosarcoma appeared to be ineffective. Increasing the total cell dosage (over 2×10^7/mouse) administered in three injections on days 6, 11 and 15 (experimental schemes are depicted diagramatically in Fig. 1) caused tumor growth retardation, although rejections were not observed.

In another regim, the treatment was initiated on day 8 after tumor challenge when small tumors became palpable. It was sustained for almost 4 weeks. During this time mice received six intermittent injections of alternately high and low doses of mIL-2-secreting cells. This treatment resulted in a significant tumor growth retardation. Moreover, 3 out of 8 treated mice remained tumor-free for more than 3 months.

Similar therapy applied in mice bearing F-69-3 fibrosarcoma tumors was ineffective, although no toxicity of this treatment was observed. In both experiments, mice receiving rhIL-2 injections at the same time as X63-mIL-2 cells, did not respond to the treatment.

Transfected plasmocytoma cells exerted antitumor effect in B6D2F1 mice bearing small palpable (25 to 150 mg) subcutaneous nodules of colon 38



T.G.D. - Tumor Growth Delay
T.G.I. - Tumor Growth Inhibition
"Cure" - Tumor-free for at least 3 months
I.L.S. - Increase in Life-Span over Untreated Control

Fig. 1. Immunotherapy with X63-m IL-2 Cells. Non-advanced tumor systems.

adenocarcinoma. Six peritumoral injections of X63-mIL-2 cells (one per week), initiated on day 8 after tumor transfer, resulted not only in significant tumor growth delay ($p = 0.0013$, as compared to the untreated control, measured on day 36) but also in substantial prolonging of the treated mice life span (I.L.S. = 80%). In addition, 1 out of 8 mice survived tumor-free for more than 5 months.

3.3. Combined chemoimmunotherapy in mice with advanced tumors

Immunotherapy becomes less effective with tumor progression; it is rational, therefore, to use it in combination with other modalities that could reduce the tumor load, thereby providing a chance for the immune system to eradicate the residual disease. Since the "wild type" plasmocytoma was found earlier to be extremely sensitive to a single optimal dose of cyclophosphamide (10), we considered the autologous system non-suitable for chemoimmunotherapy modelling.

In the initial experiment carried out on mice with advanced non-related BFS1 fibrosarcoma we found that the combination of the single dose of compound CBM-4A (150 mg/kg, i.p.) with two subsequent injections of X63-

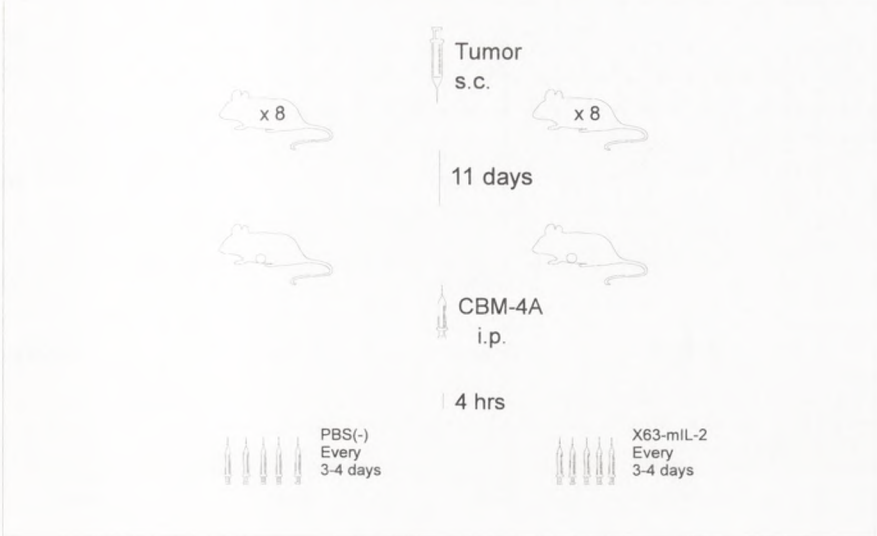


Fig. 2. Adjuvant immunotherapy with X63-mIL-2 Cells after Single-Dose Chemotherapy. F-69-3 fibrosarcoma.

mIL-2 cells (7 days apart, starting 4 hrs after cytostatic) was more toxic than the cytostatic alone. Although in all mice receiving combination therapy the augmentation of tumor growth inhibition was observed (insignificant difference, $p = 0.1$), ca 30% of mice died before control, and complete responses were not observed. On the oter hand, the treatment with the cytostatic alone proved more effective — 40% of mice responded with complete tumor regression (10).

Toxic effects were also observed in mice with F-69-3 fibrosarcoma which were treated with similarly scheduled chemoimmunotherapy (as depicted in Fig. 2). In mice that received 5 injections of transfected plasmocytoma cells (every 3-4 days, a 2 week therapy) tumor growth inhibition was exactly the same as in the group receiving only cytostatic, but after the last dose of mIL-2-secreting cells the treatment appeared lethal for 50% of animals.

Since we previously found that three subcutaneous injections of X63-mIL-2 cells administered on days 5, 8 and 13 after single-dose cytostatic treatment (compound CBM-4A 150 mg/kg, i.p.) were not toxic to healthy animals we concluded that, in order not to be toxic, the intervention with transfected cells, should be applied not earlier than 3 days after chemotherapy (10).

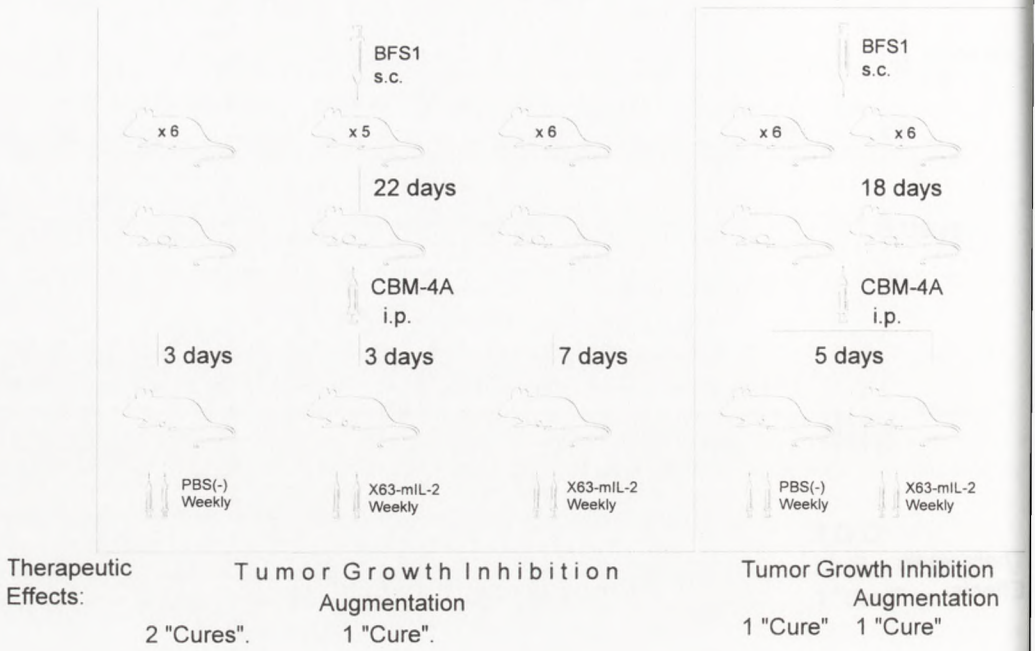


Fig. 3. Adjuvant immunotherapy with X63-mIL-2 Cells after Single-Dose Chemotherapy. Advanced BFS1 fibrosarcoma.

In the next series of experiments, carried out on mice with advanced BFS1 fibrosarcoma (as depicted in Fig. 3), we tried to optimize the immunotherapy timing following single-dose cytostatic. The treatment was initiated about 3 weeks after tumor challenge when average tumor mass (A.T.M.) reached ca 250 mg (in range of 70 to 500 mg). Two injections of 10^7 transfected cells were given with one week interval, beginning 3, 5 or 7 days after chemotherapy. The results of those experiments indicate that the most efficient augmentation of the tumor growth inhibition exerted by the compound CBM-4A was achieved when interleukin-2-secreting cells were administered 3 or 5 days after cytostatic agent. Upon delaying of immunotherapy until day 7 after the cytostatic, the augmentation of the tumor growth inhibition became weaker and complete tumor regressions were not observed.

In the last series of experiments, the antitumor effectiveness of each modality separately, as well as their combination, was compared in mice bearing colon 38 adenocarcinoma (Fig. 4). Mice with advanced tumors, in a range of 150 — 350 mg, (260 mg on average), were sensitive to the treatment with single-dose cytostatic. They responded to compound CBM-4A (day 16, 300 mg/kg, p.o.) with considerable tumor growth inhibition (the dif-

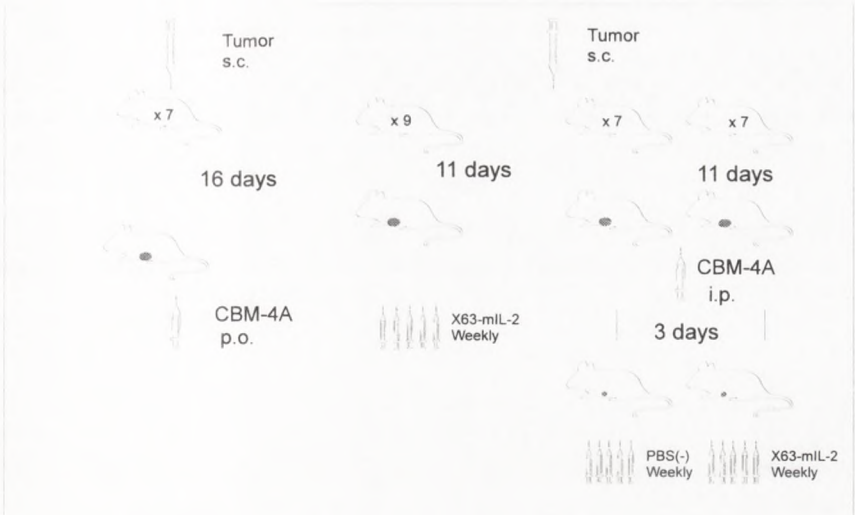


Fig. 4. Adjuvant immunotherapy with X63-mIL-2 Cells after Single-Dose Chemotherapy. Advanced Colon 38 adenocarcinoma.

ference in A.T.M between treated and untreated control was statistically significant, $p \leq 0{,}0001$, as measured on the day 42). The increase in life-span of treated mice over the control was 39% ($p = 0.028$). Treatment with of X63-mIL-2 cells alone, initiated in mice with small tumors (day 11), caused a marked tumor growth retardation and an increase in life-span by 15%. In mice with more advanced tumors the addition of locally injected mIL-2-transfected cells to the single-dose cytostatic therapy augmented antitumor response. This effect was expressed by much more pronounced tumor growth retardation as compared to each of the treatments applied alone. Tumors in mice treated with chemoimmunotherapy were the smallest at the time of evaluation (6 weeks after challenge), however, the variation in the tumor mass from the start of the experiment, as well as non-uniform response of mice to immunotherapy and chemotherapy, resulted in statistically insignificant differences between treatment groups (Tab. 1).

TABLE 1

COLON 38 TUMOR GROWTH INHIBITION AS THE EFFECT OF TREATMENT WITH COMPOUND CBM-4A, mIL-2-TRANSFECTED PLASMOCYTOMA CELLS, OR COMBINATION OF BOTH

Gr.	Treatment	A.T.M. (range) (g)	T.W.I. (%)	P		
				vs Gr. I	vs Gr. II	vs Gr. III
I	PBS(-) only	3.32 (1.6-5.5)				
II	X63-mIL-2 only	1.90 (0.2-4.7)	43	0.036		
III	CBM-4A only	1.40 (0.1-3.4)	58	0.004		
IV	CBM-4A + X63-mIL-2	0.64 (0.3-1.3)	81	< 0.0001	0.08	0.15

The tumors were measured on day 42 after tumor inocualtion (the day of the first death record). T.W.I. — tumor weight inhibition. P — Student's t-test of treated groups vs PBS(-) treated control.

4. Discussion

Murine plasmocytoma cells transfected with mIL-2 gene (X63-mIL-2) were used as the cytokine slow-release system for immunotherapy (transgenic immunotherapy) (13) or combination chemoimmunotherapy in mice inoculated subcutaneously with unrelated tumors. Immunotherapy alone was applied in mice with non-advanced stages of tumor growth. It was usually initiated on days 6-8 after tumor transfer, when small palpable tumors had appeared. Mice bearing BFS1 fibrosarcoma (the tumor induced by MCA in immunocompetent host) responded to the tretament in a manner dependent on the number of injections of transfected cells and the therapy duration. When the treatment period was short (3 injections during about 2 weeks), the antitumor effect was weak, while multiple injections of transfected cells for a prolonged time (4-week therapy) yielded significant tumor growth inhibition and complete tumor regression in ca 37% of animals. Mice inoculated with cells of another MCA-induced tumor, F-69-3 fibrosarcoma derived from athymic, nonimmunocompetent mice, were apparently non-responsive to a very similar therapy.

A distinctive antitumor effect of immunotherapy with transfected cells was observed in allogeneic mice inoculated with unrelated colon adenocarcinoma. All mice responded with significant tumor growth inhibition and with 1 out of 9 complete tumor regression. The therapy also resulted in the considerable survival time prolongation (80% over untreated control).

When combining the cytostatic agent with successive administration of cytokine, used either as a recombinant protein or secreted by genetically manipulated cells, adequate timing of both agents appears to be of great importance. We have already noted in another model system (BFS1 fibrosarcoma), that immunotherapy, initiated as early as 4 h after single dose of CBM-4A (150 mg/kg, i.p.) cells, augmented tumor inhibition elicited by the cytostatic agent, but lethal effects observed in a fraction of mice dis-

paraged this treatment approach (10). Comparably toxic response was also observed in the current study when a similar therapy was applied to mice with F-69-3 fibrosarcoma. However, no augmentation of tumor growth inhibitory effect of chemotherapy was observed on this system.

In other experiments, conducted in mice with BFS1 fibrosarcoma, cellular transgenic immunotherapy applied after cytostatic agent was found to augment tumor growth inhibition. The most significant potentiation was observed when the 3-day interval separated both ways of treatment. The combined therapy did not increase the number of "cured" mice, as compared to the cytostatic alone.

Similar results were observed in mice with colon carcinoma: transgenic immunotherapy was found to significantly augment tumor growth inhibition exerted by compound CBM-4A. In this experiment, however, both treatments, the cytostatic alone and its combination with immunotherapy, did not considerably improve the survival time of mice.

The results of the experiments aimed at programming of chemoimmunotherapy with local IL-2, as released by genetically manipulated non-tumorigenic plasmocytoma cells, show that the standardization of the chemoimmunotherapy protocols in our animal tumor systems is not complete, as yet. First of all, the non-uniform response to immunotherapy, or to chemotherapy, that was observed in all tumor systems, makes proper evaluation of the therapy outcome impossible. It has been frequently reported by others that mice with subcutaneously transplanted tumors, subjected to chemotherapy, radiotherapy or immunotherapy, responded with high variability (14-16).

Secondly, the optimal timing for the interleukin 2 therapy after cytostatic agent has not been established. It varied in different models — from the same day as chemotherapy (17) up to 7 days after it (17-19). In our experiments, administration of transfected cells 4 h after compound CBM-4A appeared to be toxic — a fraction of mice died from apparent cachexia. We have no explanation for this observation as yet, however it could be a result of high levels of tumor necrosis factor (TNF-α) produced at that time in mice in response to the combined treatment, in addition to other factors released from the decaying tumor. TNF has been reported to be more toxic in tumor-bearing than in healthy or sham-operated mice (16). Similarly, high doses of rhIL-2 were better tolerated by healthy than by tumor-bearing mice (14). It has also been reported that treatment with IL-2 could not only lead to the induction of TNF but also of upregulated soluble TNF receptors, thus modulating its biological activity (20). We have also observed that relatively low doses of rhIL-2 given to mice after CBM-4A were more toxic than the cytostatic alone (10). In the presented here experiments, administration of transfected cells 3 to 5 days after cytostatic agents was apparently the most favourable in antitumor effect augmentation.

The duration of the immunotherapy is yet another problem: how many injections should be given and for how long. These treatment parameters have to be elaborated for each tumor system separately, as they primarily depend on the tumor growth rate.

The best cytokine for tumor cell-targeted cytokine gene therapy has yet to be established. There are few promising candidates for vaccine development that can effectively increase tumor immunogenicity, leading to enhanced response against the parental tumor. Their effectiveness in therapy however, has, yet to be determined (21).

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Summary

The cells of non-tumorigenic X63-Ag8.653 mouse plasmocytoma line transfected with murine interleukin 2 cDNA (X63-mIL-2) were used as the slow-release system of IL-2 for immunotherapy and chemoimmunotherapy in mice challenged with subcutaneous injections of different non-related tumors (MCA induced fibrosarcomas BFS1 and F-69-3, colon carcinoma C38). The combination of one dose of the cytostatic agent (bromoanalog of ifosfamide) administration with subsequent peritumoral injections of the cytokine-producing cells was observed to be more efficient in the tumor growth inhibition as compared with the cytostatic alone.

Key words:
interleukin-2, immunotherapy, chemoimmunotherapy, fibrosarcome, colon carcinoma.

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