Immune mediated mechanisms of antimetastatic effect the carcinoma-specific transfer factor in conditions of growth of experimental tumours at mice C57BL/6

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Summary

The submitted experimental data testify to perspectivity of a method of prevention of metastasises by active formation of antineoplastic immune protection of an organism by the transfer factor (TF), specific to cells of a certain tumour. Adjuvant immunotherapy by carcinoma-specific TF prevents occurrence of lung metastasises Lewis lung carcinoma (LLC) at 50 % of mice C57BL/6 and suppresses growth of already developed metastasises in 75 % of cases (at adjuvant therapy nonspecific TF – 22 and 29 % accordingly). Use of carcinoma-specific TF at mice C57BL/6 after removal of melanoma B16 prevents of metastasises only at 11 % of animals, and at others – suppresses growth lung metastasises on 70 % (at adjuvant therapy nonspecific TF – 14 and 56 % accordingly), that testifies for the benefit of conformity carcinoma-specific TF to LLC antigenic structure. It is established, that tumor-specific TF is capable to transfer immune reactivity on antigens of this tumour to the recipient, to initiate for short time development of the productive immune answer of an organism on tumoral growth and to prevent or inhibit dissemination tumoral process that is a basis for the further development of this direction of biotherapy of cancer patients with the purpose of increase of efficiency of the basic methods of their treatment.

Key words: immunotherapy, carcinoma-specific transfer factor, Lewis lung carcinoma, melanoma B16, immune effects, antimetastatic effect.

Introduction

Despite of improvement of the basic methods of treatment of cancer patients, their results are unsatisfactory. Parameters of death rate from cancer till now remain at a high level practically worldwide. In Ukraine the 5-years survival rate of patients does not exceed 50 % [1]. The main problem in treatment of cancer patients remains

metastasises which serves as the reason of death more, than in 90 % of cases [2]. It is known, that there are metastasises already at the moment of an establishment of the diagnosis in 60-70 % of patients [3]. After surgical removal of a primary tumor at absence of attributes of its distribution at 15-33 % of patients further metastasises develop. Sometimes it occurs through a long time interval after radical surgical treatment [4].

In this connection search of methods of the therapy directed on prevention of metastasises is actual. Use adjuvant system cytostatic therapy in most cases promotes reduction of quantity of tumor cells which are destroyed by cells and factors of immune protection of an organism [5]. The role of immune system in antineoplastic protection proves to be true the increased risk of occurrence of a tumour on a background of immunosuppression, cases of spontaneous regression of a tumor at patients with the confirmed diagnosis and its regression on a background immunotherapy [6, 7]. So, immune reactions can prevent or at least inhibit growth of a tumor and it metastasises. In the same time, there are mechanisms which interfere with development of the antineoplastic immune answer of an organism, and the immune system can assist in some cases to progressing of disease. It connects with mechanisms of a chronic inflammation and hypoxia which realization breaks balance of an immune homeostasis and creates a basis for metastasises [8]. In such conditions purposeful correction and/or prevention of disorders in immune system by immunotherapy can become the important component of treatment.

In this aspect opportunities of immunotherapy methods, directed on formation of tumor-specific immune answer of an organism are studied [9]. Transfer of antineoplastic immunity by the transfer factor (TF) received from sensibilized to tumoral antigens lymphocytes, can create adequate conditions in an organism of the patient for realization of all potential of immune protection [10]. With this purpose TF preparations are applied, working which component are transfer factor polypeptides of the T-cellular origin with low molecular weight (3-12 kD) which are capable to transfer of cellular-mediated immune reaction to an antigen [11]. Transfer of antigen-specific immune reactivity by the TF is non-restricted by molecules of the

Major Histocompatibility Complex, such approach can be realized in allogeneic and xenogeneic systems [12].

In most cases in immunotherapy cancer patients apply TF preparations received from leucocytes of practically healthy people [11]. Such preparations are capable to raise some parameters of a cellular immunity, but they do not have sufficient tumor-specific activity [13]. On the contrary, certain specificity TF is capable to induce at the recipient formation of cellular-mediated immune reactions to tumor antigens, result of that are breaking metastasises and improvement of survival rate of patients [14].

In our opinion, active formation of antineoplastic immune protection of an organism by the tumor-specific TF for prevention metastasises is a perspective direction of biotherapy. The given work is devoted to definition antimetastatic effect carcinoma-specific TF in conditions of experimental model of Lewis lung carcinoma growth (LLC) and melanoma B16 at mice C57BL/6.

Object and methods of research

Experiments were carried out on 20 nonlinear rats in weight 100-120 g from National Cancer Institute vivarium and 110 mice C57BL/6 in weight 18-20 g, received from Bogomolets Institute of Physiology vivarium (National Science Academy of Ukraine). The maintenance of animals and work with them were carried out according to the standard international rules of carrying out of researches on experimental animals. Research has been approved by the Commission on questions of ethics of National Cancer Institute.

Xenogeneic carcinoma-specific TF has been received from a pool of spleen lymphocytes of 10 rats for 14th day after intraperitoneal immunization by alive cells of a mouse tumor – LLC as it is described [15]. Nonspecific TF received in the same way from a pool of spleen lymphocytes of 10 intact rats.

Antimetastatic effect of TF determined on model of LLC and melanoma B16 hypodermic growth at mice C57BL/6. For this purpose cells LLC or melanoma B16 ($5x10^5$) inoculated under the skin of a back of the mouse foot. As donor LLC or melanoma B16 used mice C57BL/6 for 14th day of growth of a hypodermic tumor. For 24th day after an inoculation at all animals under a narcosis (Thiopentalum-

natrium ("Київмедпрепарат", Ukraine) in a doze of 60 mkg, hypodermically) deleted a primary tumor by cutting off distal a piece of the struck finiteness with preliminary imposing of ligature. Further an animal of skilled groups carried out immunotherapy carcinoma-specific or nonspecific TF (intraperitoneally in a doze of 200 pg in 0,2 ml 0,9 % of NaCl solution, since 2nd day after operation and then each 7 day, sum total 3 injections); an animal of control group entered into the same terms of a physiological solution of 0,2 ml. Antimetastatic effect estimated on 44th and 49th day accordingly after inoculation LLC or melanoma B16 on such parameters:

- frequency metastasises (%);
- quantity of metastasises (pieces);
- quantity of metastasises in avascular growth phase (d≤0,5 mm);
- volume of metastasises (V, mm^3) under the formula:

$$V = a\frac{\pi(d_1)^3}{6} + b\frac{\pi(d_2)^3}{6} + c\frac{\pi(d_3)^3}{6},$$

where a, b, c – quantity of metastasises of the corresponding diameter; d_1, d_2, d_3 – diameter of metastasises (mm);

- index of suppression of metastasises (ISM, %) under the formula:

$$ISM = \frac{V_c - V_{exp}}{V_c} x\,100\,\%,$$

where V_c and V_{exp} – medium volume of metastasises at mice of control and experimental groups accordingly.

Research immune properties carcinoma-specific TF carried out on 1st, 3rd and 7th day after unitary intraperitoneal introductions in a doze 100 pg to mice C57BL/6 in test of suppression of adhesion of macrophages (SAM-test), proliferative and cytotoxic tests at the presence of LLC cells.

Formation of cellular-mediated immune answer determined by the spectrophotometric variant of the SAM-test [16], using macrophages of peritoneal exudate (MPE) and cells of LLC in the ratio 50:1. Results measured on spectrophotometer Uniscan-II ("Labsystems", Finland) and estimated on change of an index of adhesion (IA, %):

$$IA = \frac{OD}{ODc} x\,100\,\%,$$

where OD – optical density of experimental small cavity (adhesive MPE at presence of the test-antigen); OD_c – optical density of control small cavity (it is spontaneous adhesive MPE). Values of IA≤80 % were considered significant.

Proliferative answer of spleen lymphocytes in the mixed culture in vitro with LLC cells (in the ratio 20:1) estimated the with the help cytofluorimetry method as it is described [17]. Proliferative index (PI, %) expected under the formula:

$$PI = \frac{M2 + M3 + M4}{M1 + M2 + M3 + M4} x\,100\ \%,$$

where M1 – quantity of cells in a condition of dormancy (%); M2-M4 – quantity proliferate cells (%).

Cytotoxic activity of spleen lymphocytes against cells of LLC (in the ratio 50:1) determined by cytofluorimetry method as it is described [17]. The analysis of samples carried out on flowing cytofluorimetr FACScan ("Becton Dickinson", USA) with the help of the program "Cell Quest". Cytotoxic index (CI, %) expected under the formula:

$$CI = \frac{A - B}{C - B} x\,100\ \%,$$

where A – quantity of dead target cells (TC) in experience; B – quantity of dead TC in the control; C – total of the counted up TC.

In each group are 10 animals.

Statistical analysis was performed using the software package Excel (MS Office 2003, XP) and STATISTICA 6,0 (StatSoft Inc., USA). Results of research have been checked up on normal distributions by the Shapiro-Wilk test. For definition of significance level (p) of discrepancy between parameters in researched groups at normal distribution of values applied Student's t-test, to values which distribution differed from normal, used nonparametric Mann-Whitney U-test [18]. Results of research are submitted as M±m, where M – mean, m – its standard error. Statistical significance was defined as $p<0,05$.

Results and discussion

For definition of antimetastatic effect of carcinoma-specific TF in conditions of experimental model of growth LLC at mice C57BL/6 have been chosen adjuvant mode of its application (Figure 1).

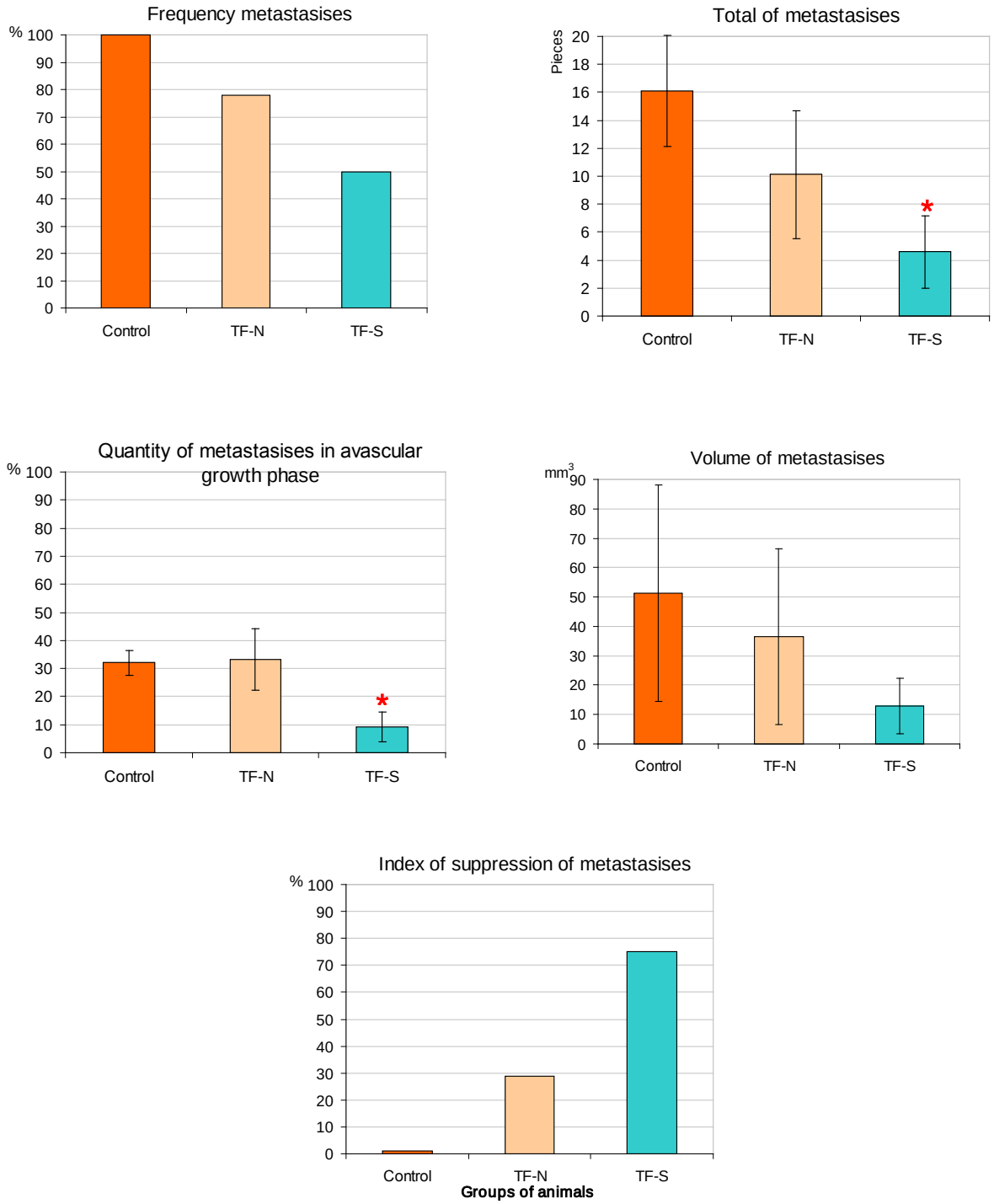


Figure 1. Influence of adjuvant immunotherapy by samples TF on metastasises of LLC at mice C57BL/6

Notes:
1. TF-N – nonspecific TF, TF-S – carcinoma-specific TF;
2. * – differences from control group are statistically significance ($p<0,05$).

Apparently from the data resulted in Figure 1, at animals with hypodermic LLC which have received only surgical treatment (control group), lung metastasises have in 100 % of cases: their quantity were (16,11±3,98) pieces; a share in avascular

growth phase – (32,06±4,50) %; volume – (51,23±36,74) mm^3. After adjuvant use nonspecific TF frequency of metastasises has decreased to 78 %, nevertheless the quantity and volume of lung metastasises in mice have remained at a level of values in control group (accordingly (10,11±4,55) pieces, (33,33±10,91) % and (36,54±29,83) mm^3, $p>0,05$). Thus ISM was 29 %.

On the contrary, adjuvant immunotherapy by carcinoma-specific TF essentially brakes metastasises of LLC: lung metastasises have only 50 % of mice, their total quantity and a share in avascular growth phase have considerably decreased (accordingly (4,59±2,58) pieces and (9,16±5,21) %) at comparison with parameters at animals of control group ($p<0,05$), and the volume of metastasises tended to reduction ((12,80±9,42) mm^3, $p>0,05$). Thus ISM at animals with lung metastasises of LLC was 75 %.

So, it is possible to approve, that use of carcinoma-specific TF in adjuvant regime is an effective means of prevention of development of metastasises of LLC at mice C57BL/6. Nevertheless there is obscure a selectivity antimetastatic effect of carcinoma-specific TF in conditions of growth another on histogenesis tumour at mice C57BL/6. Conformity of such effect of carcinoma-specific TF, received at immunization of rats by cells of LLC, to conditions of transfer immune reactivity on its antigens was a subject of check on experimental model of growth of melanoma B16 at mice C57BL/6.

Adjuvant use TF (dozes and schemes of introduction of samples similar to the previous experiment) at mice C57BL/6 with melanoma B16 has revealed some other answer to therapy (Figure 2).

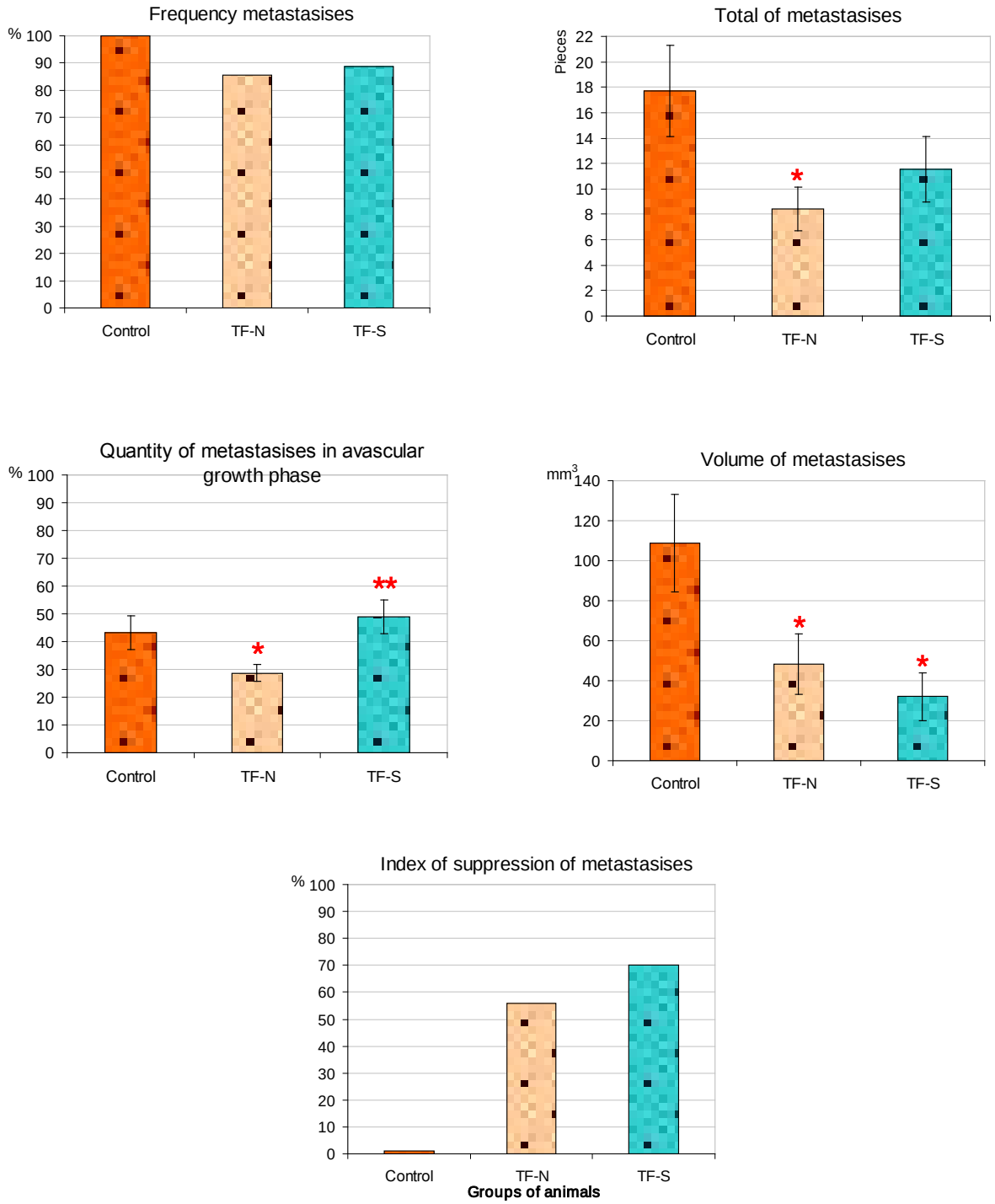


Figure 2. Influence of adjuvant immunotherapy by samples TF on metastasises of melanoma B16 at mice C57BL/6

Notes:
1. TF-N – nonspecific TF, TF-S – carcinoma-specific TF;
2. * – – differences from control group are statistically significance ($p<0,05$);
3. ** – difference from TF-N group is statistically significance ($p<0,05$).

Apparently, lung metastasises at animals of control group after removal of primary melanoma have been in 100 % of cases: their quantity was (17,71±3,61)

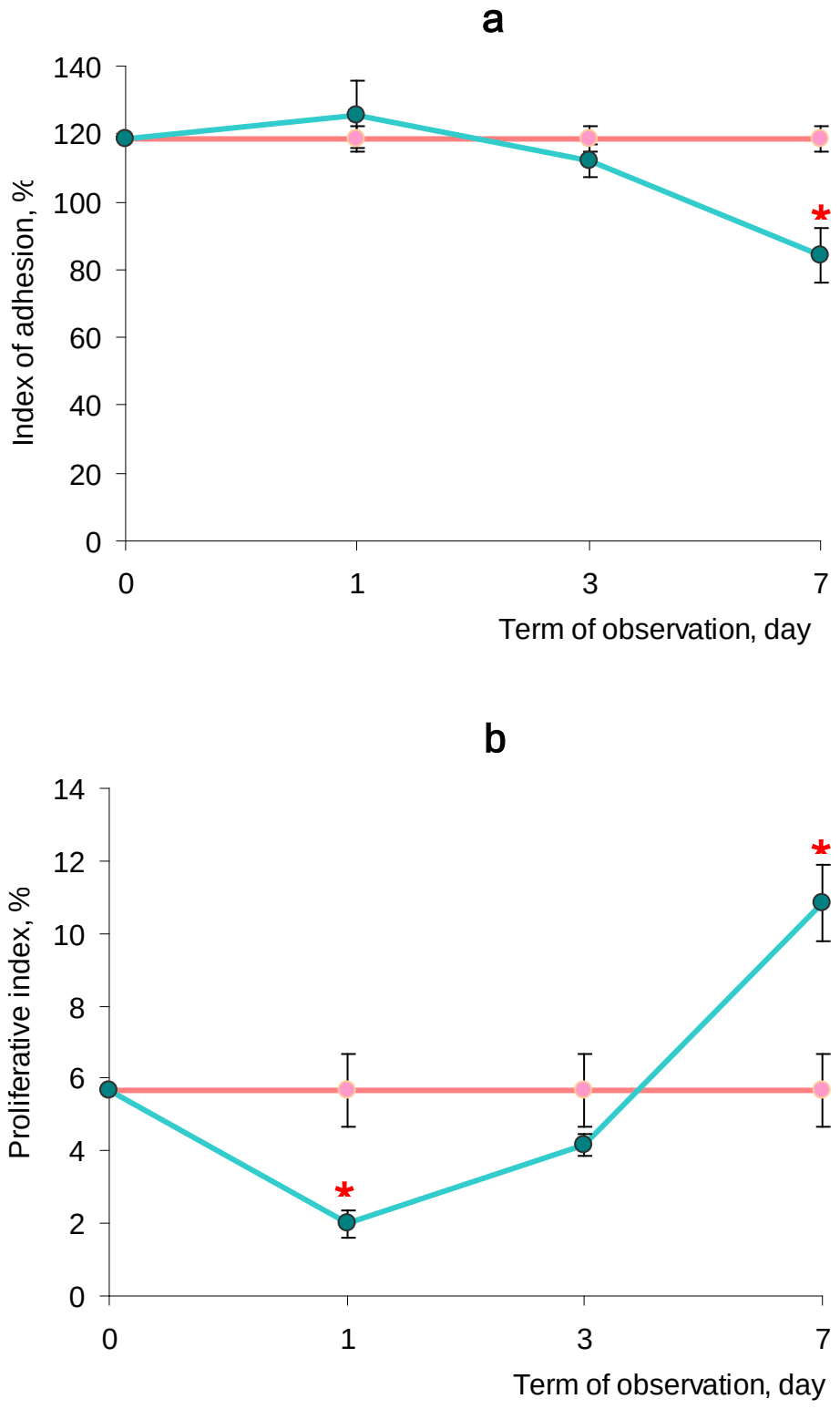
pieces; a share in avascular growth phase – (43,14±6,08) %; volume – (108,84±24,43) mm^3. After adjuvant use nonspecific TF frequency of metastasises was 85,7 %, the quantity of metastasises and their volume have considerably decreased (accordingly (8,43±1,73) pieces and (48,27±14,92) mm^3) from shares in avascular growth phase (28,67±3,12) % at comparable with similar parameters at mice of control group ($p<0,05$). ISM under such circumstances applications of nonspecific TF was 56 %.

Similar results are received after adjuvant immunotherapy carcinoma-specific TF: lung metastasises of melanoma B16 were in 88,9 % mice, the tendency ($p>0,05$) to decrease in quantity of metastasises to (11,56±2,57) pieces with statistically significance reduction of their volume to (32,13±11,91) mm^3 is registered at comparison with such at animals of control group. Nevertheless an essential difference at comparison with parameters at mice after use nonspecific TF it is not revealed, except for quantity of metastasises in avascular growth phase ((48,90±6,00) %), that statistically significance differed from such after use nonspecific TF, but conformed to control value. ISM has made 70 %.

So, essential difference in antimetastatic effect nonspecific and carcinoma-specific TF in conditions adjuvant use for mice C57BL/6 with melanoma B16 it is not revealed, that can testify for the benefit of the greater conformity carcinoma-specific TF to antigenic structure LLC.

Characteristic attribute TF is transfer of immune reactivity on an antigen from the immune donor to non-immune recipient that is determined in vitro with the help of the SAM-test which data well correlate with reproduction of delayed hypersensitivity in vivo [19]. It is known, that antigen-specific immune reactivity, induced TF, can be serially transferred from a small amount lymphocytes in vitro, involving thus in reaction of delayed hypersensitivity a significant population non-sensibilized cells [20]. It is obvious, that structure TF determines antigen-connecting properties and thus provides specific recognition [21]. TF initiates formation of immune reactivity during a short time interval, but reaction of delayed hypersensitivity which is transferred by the TF, is observed more year [19].

In our research ability to transfer of immune reactivity on antigens LLC to nonsensibilized recipient by carcinoma-specific TF in vivo has been confirmed with results of the SAM-test (Figure 3, a).



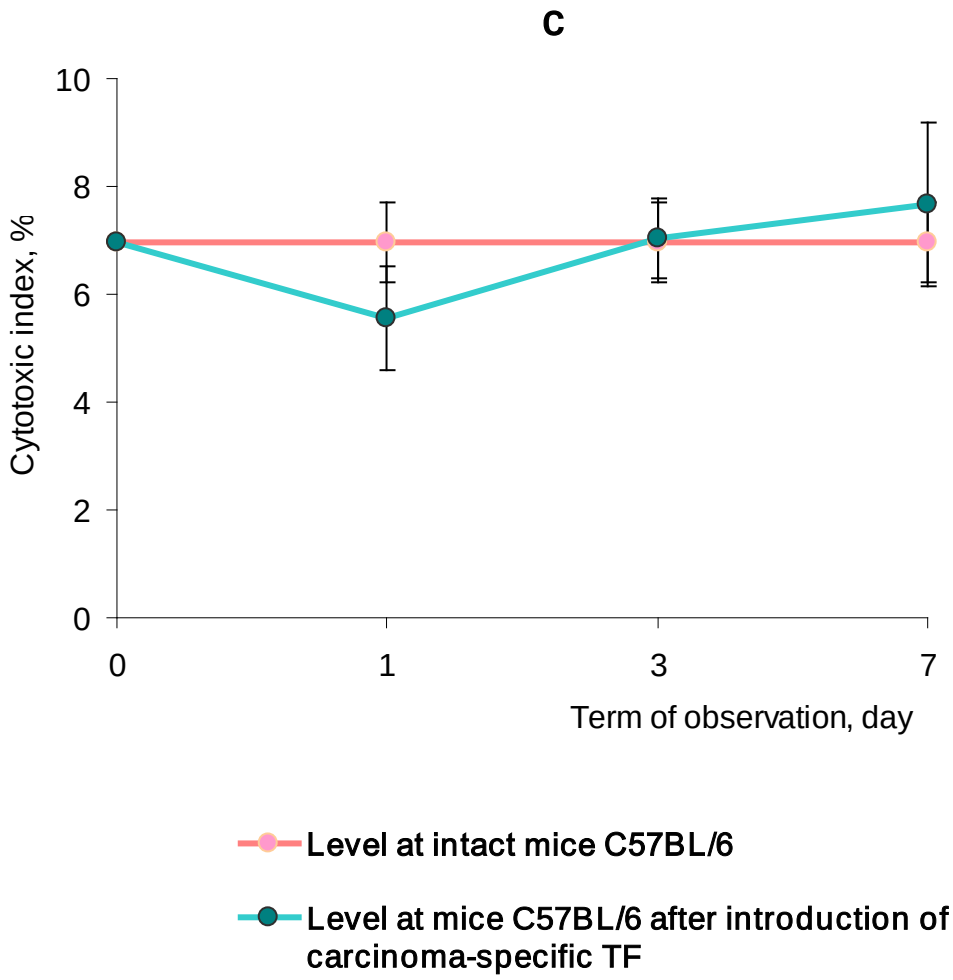


Figure 3. Formation of hypersensitivity of slowed down type on given to the SAM-test (a), proliferative (b) and cytotoxic (c) activity of spleen lymphocytes of mice C57BL/6 in answer on LLC cell in vitro after unitary intraperitoneal introduction of carcinoma-specific TF

Note. * – differences from intact animals are statistically significance ($p<0,05$).

Apparently from the data resulted on Figure 3 a, cellular-mediated immune answer to antigens LLC at mice C57BL/6 after introduction carcinoma-specific TF is formed gradually by 7th day to what testifies macrophage's IA at the presence of LLC cells in vitro, it size makes (84,4±8,0) against (118,5±3,8) % at intact animals, $p<0,05$. It's possible to explain such changes that TF is adsorbed first of all on cytoplasm membrane of Th1-cells [19] and operates, mainly, on effecter mechanisms of cellular-mediated immunity, inducing production Th1-cytokines (in particular,

IFN-γ, IL-1 and IL-2), that directs development of the immune answer on Th1-script, which plays the important role in antineoplastic protection of an organism [22].

Besides formation of cellular-mediated immunity to LLC antigens for 7th day after introduction carcinoma-specific TF to mice C57BL/6 proves to be true dynamics of lymphocyte's proliferation in the mixed culture with LLC cells in vitro (Figure 3, b). Apparently, for 1st day after introduction TF proliferate answer of lymphocytes on stimulating cells is reduced ((2,00±0,37) against (5,67±0,99) % at intact animals, $p<0,05$), on 3rd – is restored ((4,15±0,31) %), and on 7th – ability of lymphocytes to proliferation in vitro at the presence of LLC cells considerably raises ((10,83±1,05) %, $p<0,05$).

In the same time, at unitary intraperitoneal introduction of TF to intact mice C57BL/6, generation of tumor-specific effector cells in vivo was not observed, to what testifies cytotoxic activity of lymphocytes against LLC cells in vitro (Figure 3, c). So, cytotoxic activity of spleen lymphocytes against LLC cells does not change on 1st, 3rd and 7th day after introduction of TF: accordingly (5,56±0,97), (7,04±0,74) and (7,67±1,51) %, that does not differ from a parameter at intact animals ((6,97±0,73) %), $p>0,05$.

So, carcinoma-specific TF is capable to transfer immune reactivity on antigens of LLC to intact animals and to initiate development of the immune answer during 7 days after introduction in a low doze, but it is not capable to induce for this time generation in vivo of cytotoxic T-lymphocytes, specific to cells of LLC, without an available tumor in an organism.

Summing up the received data, it is possible to approve, that carcinoma-specific TF initiates in an organism of mice formation of immune reactions to LLC antigens that is shown in vitro active proliferation of lymphocytes and production of the factor which suppresses adhesion of macrophages at the presence of LLC cells. Besides as shown us earlier [23], adjuvant use of carcinoma-specific TF at mice with LLC results in increase of the contents of lymphocytes in peripheral blood and to restoration of weight and cellular contents of immunogenesis organs, that can be objective criterion of an estimation of efficiency of the combined treatment of mice with LLC. In turn, increase of functional activity of immune system correlates with

expressed antimetastatic effect which provides prevention of development of lung metastasises of LLC or breaking of their growth in case of occurrence which specifies system character of development of antineoplastic immune protection of an organism.

For creation of protective immunity we used of picogramm concentration of carcinoma-specific TF which are not toxic and does not stimulate growth of a primary tumor [23] that is important for development on this basis of more effective schemes of treatment. Use of immunotherapy in a combination with cytostatic and/or radiotherapy that promotes achievement of synergism their actions is pathogenetically proved [5, 24]. In particular, as we showed [25], adjuvant use of carcinoma-specific TF in a combination with cyclophosphamidum at mice C57BL/6 with LLC effectively prevents lung metastasises, that proves expediency of use in schemes of treatment of TF specific to cells of a concrete tumor.

Thus, tumor-specific TF is capable to transfer to the recipient immune reactivity on antigens of this tumor, to initiate for short time development of the productive immune answer of an organism on tumor growth and to prevent or brake of dissimination tumoral process that is a basis for development of a method of immunoprophylactics of cancer metastasises.

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