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LIMITING EFFECT OF DIAZEPAM ON LEWIS LUNG CARCINOMA METASTASIS IN ANXIOUS MALE MICE

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Aim: **It has been shown previously that chronic social defeat stress produces development of strong anxiety and increases intensity of experimental metastasis in the losers in comparison with the winners and control mice. The question was: is it possible to decrease the number of metastases in the losers by chronic or acute diazepam treatment.** ***Materials and Methods:*** **Sensory contact model was used for generating male mice with repeated experience of social victories or defeats in daily agonistic interactions. Tumor cells of Lewis Lung Carcinoma (LLC) were injected into the tail vein of animals after 10 days of agonistic interactions. Then mice were treated acutely or chronically (7 days) with diazepam (1 mg/kg, i. p). Number of metastases in the lung was calculated in 16 days after tumor cell transplantation.** ***Results:*** **Diazepam decreased the number of LLC metastases in anxious losers, whereas in the winners and control mice, without anxiety state, diazepam was ineffective.** ***Conclusion:*** **Well-known anxiolytic diazepam may decrease intensity of metastasis in anxious mice.**
Key Words: **agonistic interactions, anxiety, Lewis lung carcinoma, metastasis, diazepam.**

It has been experimentally shown that chronic stress of different etiology is the factor provoking a rapid tumor growth and metastasis [1–7]. Our data obtained earlier indicated expressed and replicable effects of the social status of mice on experimental metastasis of two transplantable tumors Lewis lung carcinoma (LLC) and Hepatocarcinoma-29: in the losers with repeated experience of social defeats the number of metastases in the lungs was significantly higher than that in the winners with repeated social victories or control mice [8, 9]. Winners and losers are considered to be animals with positive or negative emotional states, respectively [10]. In has been shown the development of strong anxiety in the losers under social defeat stress [11–13]. Anxious behavior in the losers was sensitive to anxiolytic drugs: chronic [14] but not acute treatment [14, 15] by different anxiolytics had positive effects on losers' psychoemotional state. Question was: does the intensity of experimental metastasis depend on duration of agonistic interactions and could pharmacological correction of psychoemotional state decrease the number of metastases in anxious animals. It this experiment we studied animals after ten agonistic interactions because the losers, but not the winners, demonstrated high level of anxiety estimated in different behavioral tests (plus maze, partition tests etc) in comparison with controls [11]. Anxiolytic diazepam, which is shown to be active in this model [16] was used. The intensity of experimental metastasis was studied after chronic and acute injections of diazepam.

Experimental animals. Mice of C57BL/6J strain were bred and kept under the standard vivarium conditions at the Institute of Cytology and Genetics, Siberian Division, Russian Academy of Sciences. Mice were housed on a 12 h/12 h light/dark regimen and received the standard food (pellets) and water *ad libitum.* Weaned at the age of one month, the males were housed before the experiment in groups of 8–10 individuals in 36 x 23 x 12 cm cages. Mice used in experiments were 10–12 weeks old. All experimental procedures were in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Technique for generation of male mice with repeated experience of agonistic interactions. Winners and losers were generated using the sensory contact model [10]. Pairs of animals were placed in steel cages (28 x 14 x 10 cm) divided in two compartments by a perforated transparent partition allowing the animals to see, hear and smell their neighbor, but not to contact them physically. Test sessions commenced 2 days after adaptation of the animals to these new housing conditions (sensory contact). Every afternoon (between 2.00 p. m. and 5.00 p. m.) the steel cover of the cage was replaced by a transparent one, and 5 min later (the period needed for adaptation to the lighting condition) the partition was removed for 10 min to allow agonistic interactions. Superiority of one of the partners was evident within 2–3 daily test sessions. One partner attacked, bit, and chased the other, who displayed defensive behavior only (sideways, upright postures, withdrawal, lying on the back or freezing). Agonistic interactions were discontinued by lowering the partition if intensive attacks lasted more than 3 min. Every day after the test session, each defeated mouse was placed in another two compartment cage with a partition, in which another winner was present in the other compartment. The winners remained in their own compartments. The procedure yielded equal numbers of males with an opposite social experience of aggression, evidenced by victories (aggressors, winners) and defeats (defeated mice, losers) in agonistic interactions. In this experiment, the control group was represented by the males that lived together for a long time in groups with stable dominant-subordinate relationships. It was shown that, irrespective of the so-

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Abbreviations used: LLC – Lewis lung carcinoma; T10 winners, T10 losers – animals after 10 days of agonistic interactions; T20 winners, T20 losers – animals after 20 days of agonistic interactions.

cial status (dominant or subordinate), animals in such groups are not stressed [17].

Tumor model and animal's treatment. First we studied experimental metastasis of Lewis lung carcinoma (LLC) in male mice after 10 and 20 days of agonistic interactions (T10 and T20 losers and T10 and T20 winners). LLC was maintained by i.m. passages on C57BL/6J mice. To produce the tumor cell inocula, tumor transplants were removed, cut up with scissors; the suspension was filtered through a stainless steel mesh and spun two times at 500 g. The pellet was washed in physiological solution and resuspended to a final concentration of 5×10^5 cell/mL. LLC was transplanted to mice via i. v. injections (0.5 ml of cell suspension) into tail vein. In each experiment, the tumor cells were grafted simultaneously to mice of all groups: the winners, losers and controls. After tumor transplantation, the animals were kept in their compartments without agonistic interactions for 16 days until the end of the experiment. On day 17, the mice were decapitated; the lungs were fixed in 10% formalin and tested for the presence of metastases using a binocular magnifier (magnification 8 ×).

Chronic and acute injections of diazepam. After cell inoculation, each group of T10 winners and T10 losers as well as control mice were divided into two subgroups. Beginning the day after tumor transplantation, the mice of one of the subgroups received 7 daily i. p. injections of diazepam (Polfa Tarchomin S.A.) at dose of 1 mg/kg, and the animals of every other subgroup received saline (10 ml/kg body weight) in the same way. The experiment was terminated on 17 days after tumor challenge and the number of metastases in the lungs was calculated. Similar experimental design was used to study effect of acute injection of diazepam: beginning the day after tumor transplantation, the mice of one of the subgroups received acute injection of diazepam (1 mg/kg), and the animals of every other subgroup received acutely saline. Experiment was terminated on 17 days after tumor challenge and the number of metastases in the lungs was calculated.

Statistical analysis. Number of metastases was statistically processed using one-way ANOVA with the factor "social group" (winners, losers, control) for animals with T10 and T20 days of agonistic interactions. Two-way ANOVA was used for experiments with pharmacological treatment with the factor "social group" (winners, losers, control), factor "treatment" (saline, diazepam) and interaction of these factors. Then, paired comparisons of groups were performed using Student's *t*-test. Differences between experimental groups were considered to be significant if $p < 0.05$.

The data on the number of experimental lung metastases in the control group, winners and losers inoculated i.v. with tumor cells after 10 and 20 days of agonistic interactions are presented in Fig 1, *a*, *b*. One-way ANOVA showed that the number of metastases was reliably determined by the social group of animals with 10 days of agonistic interactions [$F(2,34) = 4.0$; $p < 0.027$] and 20 days of agonistic interactions [$F(2.68) = 6.85$; $p < 0.002$]. Comparisons of the data for mice after 10 days of social experience using Student's *t*-test did not show differences in the number of metastases between the control and T10 winners ($p > 0.05$). However, the T10 winners *vs* T10 losers, as well as the control mice *vs* T10 losers differed significantly by this parameter (for both comparisons $p < 0.01$). No differences were found in the number of metastases between the T20 winners and control mice ($p > 0.05$), whereas the T20 losers and T20 winners as well as the T20 losers and control mice differed significantly ($p \leqslant 0.01$ and $p \leqslant 0.001$, respectively). Thus, the data obtained are in agreement with our previous findings [8, 9]. Moreover the intensity of experimental metastasis in the losers depends on duration of agonistic interactions. The number of metastasis was about 2,6 times more in T20 losers and about 2.0 times more in T10 losers in comparison with the respective controls.

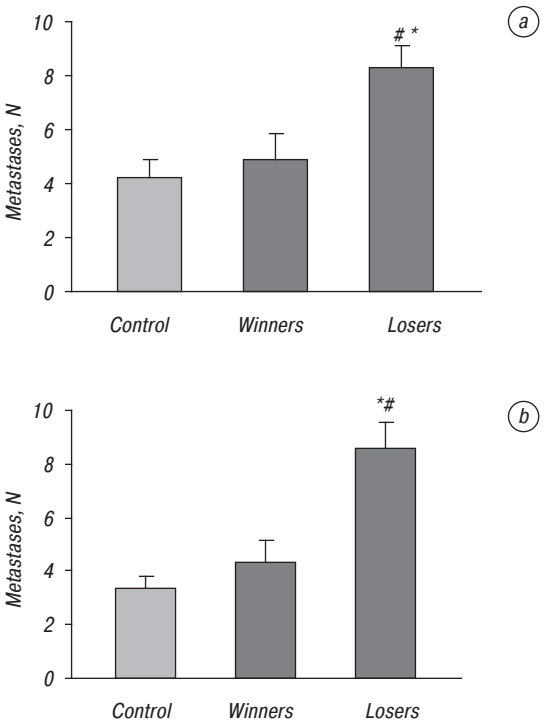

Fig. 1. Number of LLC metastases in lung of mice with repeated social defeats (losers), repeated social victories (winners) and the control after 10 (*a*) and 20 (*b*) days of agonistic interactions. $^*p < 0.01$; *vs* Control; $^\#p < 0.01$; *vs* Winners.

Chronic diazepam injections during 7 days had limiting effects on metastasis in T10 losers. Two-way ANOVA showed that the number of metastases was reliably determined by the social group of animals [$F(2,61) = 3.32$; $p < 0.05$], and interaction between effects of diazepam and groups of animals [$F(2.61) = 3.63$; $p < 0.05$] was found. In diazepam-treated losers, the number of metastases is decreased significantly in comparison with saline-treated losers ($p < 0.05$) and was similar with the metastasis levels in the winners and control animals. In the winners and control mice, however, diazepam neither potentiated nor inhibited lung metastasis ($p > 0.05$) (Fig. 2, *b*). Acute injection of diazepam was not too effective in decreasing the number of metastasis in the losers: there were no influence of interactions

between effects of diazepam and groups of animals [$F(2.61) = 0.54$; $p > 0.05$] (Fig. 2, *a*).

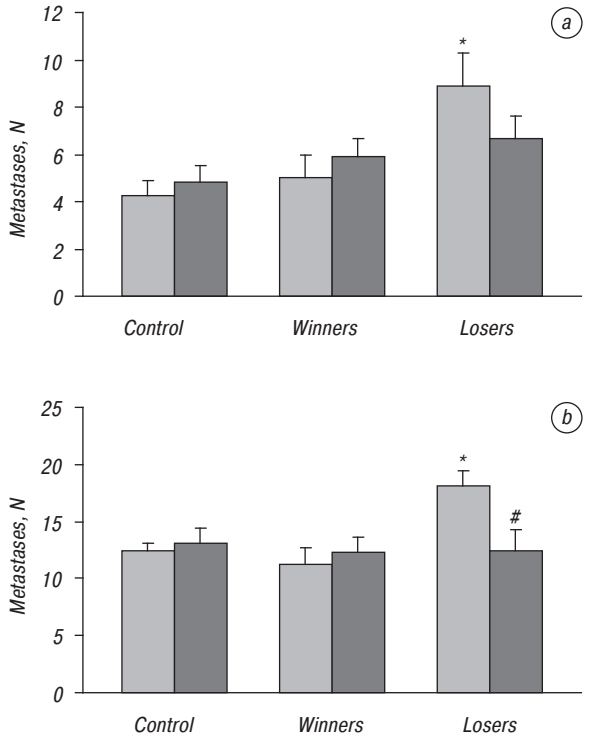

Fig. 2. Number of LLC metastases in lung of mice with repeated social defeats (losers), repeated social victories (winners) and the control after acute (*a*) and chronic (*b*) diazepam treatment. Light column — saline injections; dark columns — diazepam injections. *$p < 0.05$ *vs* Control and Winners; #$p < 0.05$ *vs* saline-treated Losers.

Noteworthy, T10 losers, but not T10 winners, had high level of anxiety in comparison with control [11] and diazepam specifically decreased the number of metastases only in anxious mice, defeated losers. It has been shown recently [18] that other drug — ethanol chronically injected during 7 days starting with day of tumor cells injections also decreased the number of lung metastases in T20 losers. Since ethanol has been demonstrated to have anxiolytic effects [13], these results are in agreement with effects of diazepam. Thus, it may be supposed that pharmacological correction of psychoemotional state with anxiolytics may decrease the intensity of metastasis in anxious individuals.

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