

## INHIBITION OF TUMOR DEVELOPMENT BY CYTOPLASMIC MEMBRANES OF *PROTEUS MIRABILIS* STABLE L-FORM

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## ПОДАВЛЕНИЕ РОСТА ОПУХОЛИ ЦИТОПЛАЗМАТИЧЕСКИМИ МЕМБРАНАМИ СТАБИЛЬНОЙ L-ФОРМЫ *PROTEUS MIRABILIS*

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In the present work the effect of *Proteus mirabilis* VI stable L-form cytoplasmic membranes (CMPm) on the survival and tumor growth in hamsters with transplanted myeloid Graffi tumors was studied. Single doses of 0.25 mg or 0.5 mg CMPm were injected i.p. 1 week before tumor transplantation. Such treatment induced elongation of the latent time of tumor appearance, inhibition of tumor growth and enhancement of the mean survival time of experimental animals. The oncoprotective effect could be due to the preliminary established immunostimulating activity of the preparation.

**Key Words:** bacterial L-forms, transplanted tumor, cytoplasmic membranes, *Proteus mirabilis*.

В настоящей работе исследован эффект цитоплазматических мембран стабильной протопластной L-формы *Proteus mirabilis* VI (ЦМПм) на выживаемость и рост трансплантируемой миелоидной опухоли Graffi у хомячков. ЦМПм в дозах 0,5 мг и 0,25 мг вводили внутрибрюшинно за неделю до трансплантации опухолевых клеток. Установлено, что инокуляция ЦМПм удлиняет время до появления опухоли, вызывает ингибицию опухолевого роста и повышает выживаемость хомячков. Протекторное действие ЦМПм, вероятно, связано с иммуностимулирующей активностью препарата.

**Ключевые слова:** стабильные L-формы, иммуномодуляторы, перевиваемые опухоли.

The animal and human organisms fight against tumor progression by both humoral and cellular immune mechanisms, in particular, by antibody- and complement-dependent lysis of tumor cells, antibody- and complement-mediated opsonization, and antibody-mediated loss of adhesion ability of tumor cells. Cellular antitumor immune mechanisms represent: destruction of tumor cells by cytotoxic T-lymphocytes, antibody dependent cell mediated cytotoxicity, destruction by activated macrophages and by NK-cells [2].

Immunosuppression is the most important factor participating in the host antitumor immune response. Tumor cells produce humoral immunosuppressing factors. Some of them were shown to lead to induction of tumor-specific T-suppressor cells, which inhibit T- and B-cell proliferating activities, as well as NK cell activity in mice with progressing tumors [6]. Fujii et al [3] reported about suppressor activity of macrophages in tumor bearing mice resulting in inhibition of T-lymphocyte cytotoxic activity. Jesup et al [16] proved the exist-

tence of tumor tissue factor, suppressing the infiltration of tumors by macrophages. Recently it was established that apoptosis inhibitor exist in most human neoplasma, but not in normal differentiated tissues [1].

The cell wall deficient bacterial forms and their fractions possess potentials of modulators of immune response. The commercial preparation OK<sub>432</sub> (Picibanyl) based on streptococcal protoplasts is successfully used in the oncology. The preparation activates the NK-cell functions and interferon induction. Promising results in the treatment of stomach cancer, laryngeal carcinoma and thyroid cancer were obtained.

Earlier, we have studied the immunostimulating potential of some bacterial L-forms subfractions and have found out the immunoprotective effects of cytoplasmic membranes from stable protoplast type L-forms of *Escherichia coli* WF+ [12, 14, 23] and of *Lysteria monocytogenes* 4B [11] on the immune response of tumor Graffi bearing hamsters. Stimulating effect of cytoplasmic membranes of Streptococcal L-forms on the functions of macrophages and spleen lymphocytes in ICR mice was demonstrated as well [8].

Recently, we have shown that administration of cytoplasmic membranes from the stable protoplast type L-form of *Proteus mirabilis* VI (CMPm) to healthy ham-

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Abbreviations used: CMPm — cytoplasmic membranes of *Proteus mirabilis* VI stable L-form.

sters has stimulating effect on the number, migration and phagocytic activities of peritoneal macrophages and blood PMNs, as well as on the proliferating abilities of spleen lymphocytes in the presence of mytogens [13].

The aim of the present work is to study the effect of CMPm administration on the development of transplanted Graffi myeloid tumors in hamsters.

## MATERIALS AND METHODS

**Experimental animals.** In the experiments, "Golden siberian" 2 months old hamsters weighing 100 g obtained from the Bulgarian Veterinary Health Control Service (Sofia, Bulgaria) were used. The animals were grown up at standard conditions and separated in 3 groups (10 animals per group): group 1 — animals with transplanted tumors, treated i.p. by 0.5 mg CMPm/per animal; group 2 — animals with transplanted tumors, treated i.p. by 0.25 mg CMPm/per animal; group 3 — animals with transplanted tumors without treatment. All procedures were carried out according to the rules of Ethic Committee.

**Transplantation of tumors.** Graffi myeloid tumor induced by mouse Graffi virus adapted to hamsters [15] was maintained in hamsters by s.c. transplantation of  $1 \cdot 10^6$  viable tumor cells with 1 month intervals. In the present experiment  $1 \cdot 10^4$  viable tumor cells were injected s.c. in the inter-scapular field of the animals.

**Biometric estimations.** Transplantability of Graffi myeloid tumors was examined by daily palpation on the place of injection of tumor cells (in %). Tumor size was calculated as mean arithmetical value of two tumor diameters (in cm). The inhibition of tumor growth was calculated by the formula:  $ITG (\%) = (A-B)/A \cdot 100$ , where A is the tumor size values of untreated animals and B — the tumor size values of treated animals. This index was followed till day 30 after tumor transplantation. The mortality (%) was estimated till day 48<sup>th</sup> after tumor transplantation. Mean survival time (in days) for treated and untreated tumor bearing animals was followed.

**Microorganisms.** The stable protoplast type L-form of *Proteus mirabilis* VI was received by induction with subinhibitory concentrations of penicillin (400 U/ml) in Central Institute of Microbiology and Experimental Therapy (Jena, Germany) [23]. The culture was maintained in Tryptic soy broth (Difco, USA) at pH 7.8, supplemented with 1% Yeast extract (Difco, USA), 10% normal horse serum (Oxoid, England) and 3% NaCl. By electronic microscopy, it is shown that *Proteus mirabilis* VI L-form populations consisted of polymorphous, mainly round cells, surrounded by single cytoplasmic membranes, without any remnants of cell walls [19].

**Cytoplasmic membranes from *Proteus mirabilis* VI stable L-form.** Cytoplasmic membranes were prepared by [7]. Briefly, *Proteus mirabilis* VI stable L-form cells were collected by centrifugation, washed 3 times with 0.05 M Tris buffer (pH 7.8) and suspended in a lysing mixture containing 0.01 M  $MgCl_2$ , 50 ng ml<sup>-1</sup> DNA-se (Fluka, Germany) in 0.05 M Tris

buffer, pH 7.8. After 1 h incubation at 37 °C for realization of the enzyme reaction and 24 h freezing at -18 °C the lysed cells were centrifuged 3 times at 22 300 g at 0 °C. The purity of CM fraction was checked by phase contrast microscopy.

**Doses and way of application of CMPm.** CMPm were applied i.p. at doses 0.25 mg or 0.5 mg per animal 5 days before tumor transplantation.

**Statistical analysis.** Statistical analysis of the data was performed according to the Students *t*-test. The data are presented as mean values  $\pm$  SD, and  $p < 0.05$  was accepted to be significant.

## RESULTS AND DISCUSSION

Our experiments showed that the CMPm treatment elongates the latent time of tumor appearance after transplantation of Graffi myeloid tumor cells. In the group of animals treated by 0.5 mg of CMPm, tumors appeared at 13–17 days only in 25% of animals. At day 13<sup>th</sup> in the group of untreated animals 100% level of transplantation was observed. The protective effect of CMPm in dose 0.25 mg per animal was less pronounced (at day 9<sup>th</sup>, the level of tumor transplantation was 50%) (Figure, a).

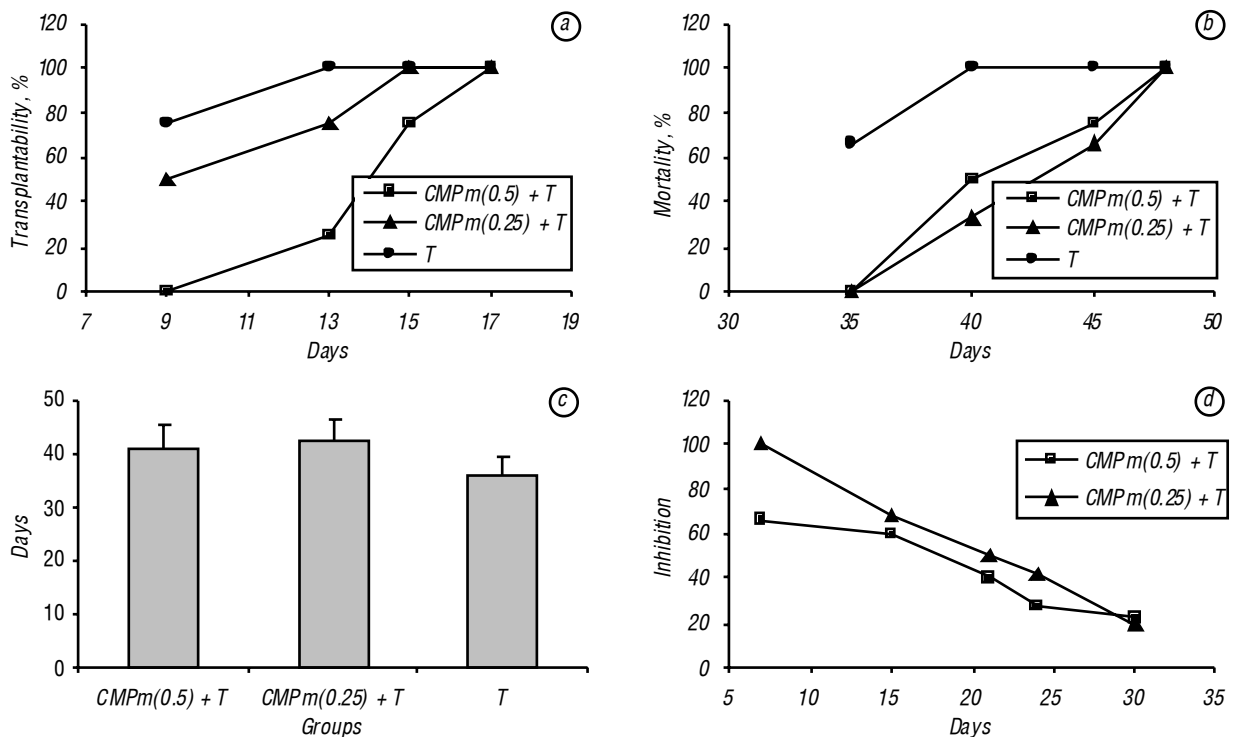
The CMPm treatment reduced the mortality of tumor bearing animals. In the 1 group of animals the mortality 0%, 50%, 75% and 100% was observed at days 35, 40, 45 and 48 respectively. Mortality in the 2 group was 33%, 66% and 100% at days 40, 45 and 48 respectively. Mortality in the control group of tumor bearing hamsters (TBH) was 66% at day 35 and 100% at day 40 (Figure, b).

The application of CMPm in doses 0.5 mg and 0.25 mg showed similar protective effect on the survival of TBH. The mean survival time (MST) was  $41 \pm 3.6$  days,  $42 \pm 2.5$  days, and  $36 \pm 1.7$  days for animals from groups 1, 2 and 3 respectively (Figure, c).

The inhibition of tumor growth (ITG) of the animals in both experimental groups under the action of CMPm was established. ITG in the 1<sup>st</sup> group of animals was 66%, 60%, 40%, 28.5% and 22%, determined at days 7<sup>th</sup>, 15<sup>th</sup>, 21<sup>st</sup>, 24<sup>th</sup> and 30<sup>th</sup>, respectively. ITG in the second experimental group was 100%, 68%, 50%, 42.8% and 20% observed at days 7<sup>th</sup>, 15<sup>th</sup>, 21<sup>st</sup>, 24<sup>th</sup> and 30<sup>th</sup> respectively (Figure, d).

The present investigation revealed an inhibiting effect of CMPm on the development of transplanted Graffi myeloid tumors in hamsters. This effect may be explained by the immunostimulating activity of CMPm, established previously [13]. Our results are in agreement with those of Karch and Nixdorff [17] showing that the cytoplasmic membranes from the stable protoplast type L-form of *Proteus mirabilis* VI as carriers of lipopolysaccharide (LPS) possess higher immunogenicity compared to the cell walls of the parental bacterial strain.

Our earlier investigations showed the beneficial effect of cytoplasmic membranes of some bacterial L-forms on anti-tumor immune response. Restoration of the suppressed functions of macrophages (number,



**Figure.** The level of tumor transplantation (%) (a), mortality rate (%) (b), mean survival time (days) (c) and inhibition of tumor growth (%) (d) in experimental and control groups of tumor bearing animals. Results are expressed as mean statistical values  $\pm$  SD

migration, phagocytic activity) [14], PMNs [10] and *in vitro* proliferation ability [24] of the spleen lymphocytes in Graffi tumor bearing hamsters after treatment with *E. coli* L-form cytoplasmic membranes (CMEc) has been proved. By scanning electron microscopy, a long termed stimulating effect of CMEc on the activation of the cell surface of peritoneal macrophages of TBH in the presence of tumor cells was established *in vitro* [25]. The same membranes stimulated *in vitro* the activation of peritoneal macrophages from Lewis lung carcinoma (LLC) bearing mice in the presence of LLC cells [9]. Cytoplasmic membranes from the stable protoplast L-form of *Listeria monocytogenes* 4B showed protective and immunorestitution effect on the macrophage, PMN and spleen lymphocyte functions in hamsters with progressing Graffi myeloid tumors [11]. The immunostimulating activity of the L-form cytoplasmic membranes is thought to be due to the presence of LPS. Previously, by mass-spectrometry and immunoelectronic microscopy we have shown the presence of LPS in the cytoplasmic membranes of *E. coli* WF+ [5, 18]. The study of Gmeiner and Martin [4] demonstrated the presence of LPS in *Proteus mirabilis* D<sub>52</sub> protoplast L-form cytoplasmic membranes identical by chemical composition with parent bacterium. The unusual location of LPS in cell wall deficient bacterial forms is due to the circumstance that the whole synthesis of the antigen in *Enterobacteriaceae* species is occurring inside cytoplasmic membranes [21, 22].

Another feature contributing to the immunostimulating activity of L-form cytoplasmic membranes is the high phospholipid content as well as increased level of unsaturated fatty acids [20]. Our investigations showed 2.5 fold increase in phospholipid content of the *Es-*

*cherichia coli* WF+ and B stable L-form cells and an increased quantity of unsaturated fatty acids compared with the respective parent cells [5].

In conclusion, administration of CMPm has the inhibiting effect on the development of transplanted Graffi myeloid tumor in hamsters and may be caused by the immunostimulating effect of this preparation [13], presence of LPS in *Proteus mirabilis* stable protoplast type L-forms [4], and/or by its enhanced phospholipid content [20].

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