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APPLICATION OF DNA FROM MUSHROOM *PLEUROTUS OSTREATUS* FOR CANCER BIOTHERAPY: A PILOT STUDY

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Aim: **In present work, the attempt to study immunomodulatory activity and biotherapeutical potential of DNA isolated from the fruit body of *P. ostreatus* was made.** ***Methods:*** **The efficacy of biotherapeutic application of mushroom DNA was evaluated on the BALB/c mice with subcutaneously transplanted Ehrlich carcinoma. The effect of *Pleurotus ostreatus* DNA on NK activity was studied *in vitro* using nonspecific cytotoxicity assay.** ***Results:*** **Application of mushroom DNA resulted in augmentation of NK cytotoxic activity *in vitro* and significant increase of the life span of mice with solid Ehrlich carcinoma.** ***Conclusion:*** **The observed effects of *P. ostreatus* DNA administration can be probably explained by the presence of immunostimulatory unmethylated CpG motifs in DNA.**

Key Words: **cancer biotherapy, CpG DNA, medicinal mushrooms, murine Ehrlich carcinoma, NK cells, *Pleurotus ostreatus*.**

Numerous studies worldwide are aimed on the development of immunotherapy to target and remove tumor cells as well as substances such as immunopotentiators, immunoinitiators and biological response modificators that act to prevent carcinogenesis and induce carcinostasis [1]. The use of medicinal mushrooms in the fight against cancer is known for a very long time in Korea, China, Japan, Russia, USA and Canada. Higher basidiomycetes belong to the group of immunoceuticals by their mode of action. More than 650 mushrooms species have yielded potential immunoceuticals that exhibit anticancer activity *in vitro* or in animal models [2–4]. Medicinal mushrooms represent an unlimited source of potent new pharmaceutical products. Many, if not all, medicinal mushrooms contain biologically active substances in fruit bodies, cultured mycelium, culture broth that include hemicellulose, polysaccharides, lipopolysaccharides, peptides, proteins, glycoproteins, nucleosides, triterpenoids, complex starches, lectins, lipid derivatives and other metabolites which have been classified as molecules with potent immunomodulatory, anticancer, anti-proliferative, anti-inflammatory, antiviral, hypotensive and antithrombotic activities [5–8]. Unique biotherapeutic medicines such as Lentinan, Krestin, Schizophyllan, Sonifilan, Ganoderan, Grifolan, Pleuran and others were prepared and studied in experiment investigations and clinical trials [1, 6, 7].

Recent studies have demonstrated that unmethylated CpG motifs that are present in bacterial DNA or synthetic deoxyoligonucleotides exhibit potent immunostimulatory effects [9]. The ability of CpG DNA to activate vigorously the cells of innate immune system and indirectly to augment adaptive immunity advantage the successful use of CpG DNA as a potent immunoadjuvant in immunotherapy of infectious diseases, allergy and cancer [9–12].

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Abbreviations used: CpG – cytosine-phosphodiester-bond-guanine or cytosine-phosphorothioate-bond-guanine; ILS,% – increase in life span (%); MNC – mononuclear cells; MST – median survival time; NK – natural killer cells; PMA – phorbol myristate acetate; TC – tumor cells.

Although in large part studied bioactive substances isolated from medicinal mushrooms are polysaccharides or their derivates, we aim to study mushroom DNA containing unmethylated CpG motifs with probably immunomodulatory and antitumor activities. The aim of present pilot study was to isolate DNA from fruit body of edible mushroom *Pleurotus ostreatus* and to research immunostimulatory activity and antitumor potential of mushroom DNA.

MATERIALS AND METHODS

Isolation and analysis of mushroom DNA. *P. ostreatus* which belongs to higher *Basidiomycetes* was used. Genomic DNA was isolated from fresh fruit bodies of *P. ostreatus* using a standardized DNA extraction protocol [13]. Concentration of isolated DNA was estimated by spectrophotometry. Pulsed-field agarose gel (PFAG) electrophoresis [14] was used for identification of DNA in the samples. To detect the presence of unmethylated CG-dinucleotides, restriction analysis with endonuclease Hpa II (Sigma, USA) [15] according to the instructions of the manufacturer with the next PFAG electrophoresis followed by ethidium bromide staining was performed [16]. For DNase experiments, DNA prerarations were digested with 50 μg/ml DNase I (Sigma, USA) for 2 h in 10 mM $MgCl_2$, 50 mM Tris (pH 7.5). Isolated DNA was dissolved in 0.15 M endotoxin free phosphate buffer saline (PBS) at the concentration of 1 mg/ml. DNA samples were stored at –20 °C. Repeated freezing of DNA samples was avoided.

Animals and tumor model. In the study, age and sex-matched BALB/c mice (9–12 weeks old) obtained from vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine (Kyiv, Ukraine) were used. The animals were housed in standard facilities, with free access to water and food. All animals were maintained under strict ethical conditions according to International recommendations.

Murine Ehrlich carcinoma transplanted intraperitoneally to BALB/c mice has been used as a tumor model. Tumor cell suspensions in physiological saline were ordinarily prepared from tumor cells of ascitic

transplantable Ehrlich carcinoma to the final concentration of 1.25 x 10^6 cells/ml; and then inoculated subcutaneously into right flank with 2.5 x 10^5 viable tumor cells/mouse in 0.2 ml (on day 0).

***In vitro* NK activity assay.** Splenic mononuclear cells (MNC) were obtained as previously described [11]. MNC were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma, USA), penicillin (40 U/ml) (Kyivmedpreparat, Ukraine) and streptomycin (40 μg/ml) (Kyivmedpreparat, Ukraine). The final concentration of MNC was 7.5 x 10^6 cells/ml. MNC were preincubated with 0.05, 0.5 and 5.0 μg/ml of DNA for 2 h at 37 °C in humidified air with 5% CO_2, washed by centrifugation and used for cytotoxicity assay. Phorbol myristate acetate (PMA; Sigma, USA) at the concentration of 250 ng/ml was used for MNC stimulation. The viability of the effector cells after preincubation with mushroom DNA was determined by the conventional trypan blue dye exclusion test and was not less than 99%. Ascitic transplantable Ehrlich carcinoma cells isolated from peritoneal cavity of BALB/c 8–9 weeks old mice at 11th day were used as NK-resistant target cells for nonspecific cytotoxicity assay. Tumor cells were suspended in culture medium so that the concentration of cells was about 2.5 x 10^6 cells/ml. Cell number and viability (about 98%) were determined in microscopical supravital test with trypan blue. Nonspecific cytotoxicity test was performed according to [11]. Cytotoxic activity of NK cells was expressed as cytotoxicity index (CI, %).

***In vivo* studies.** At the 2nd day after tumor cells inoculation mice were treated by intraperitoneal injections of 0.1 ml the mushroom CpG DNA (100 μg/ml in PBS) at the dosage of 10 μg/mouse; such injections were repeated triply with 3 day intervals. At preventive therapy, mice were treated by the similar schedule. At the 8th day after the preventive therapy was completed, tumor cells of ascitic Ehrlich carcinoma (2.5 x 10^5 cells/mouse) were inoculated subcutaneously to treated mice. The control group composed of 9 mice (survival data of two control subgroups (for therapeutic and prophylactic) were summarized because difference was considered as insignificant) was treated with PBS.

The antitumor efficacy of therapy and preventive treatment by *P. ostreatus* DNA was evaluated using increase in host`s life span. Percentage increase in life span (% ILS) was calculated as:

$$\% \text{ ILS} = \frac{MST_T - MST_C}{MST_C} \times 100\%,$$

where MST_T — median survival time of treated mice, MST_C — median survival time in control group (untreated mice).

MST was calculated on the basis of mortality data.

Statistical analysis. All *in vitro* data were from at least three independent experiments. All *in vivo* experiments were performed twice. Survival curves were constructed by using the Kaplan-Meyer method. For statistical analysis, two-tailed Student's *t*-test was applied. p values < 0.05 were considered as significant. Statistical analyses were performed using one-way ANOVA.

RESULTS AND DISCUSSION

Mushroom DNA from fresh fruit bodies of *P. ostreatus* was routinely isolated (the 260/280 coefficient was 1.8–1.85). Native isolated mushroom DNA was visualized using PAAG electrophoresis followed by ethidium bromide staining (at the left on Fig. 1). No protein contamination was registered.

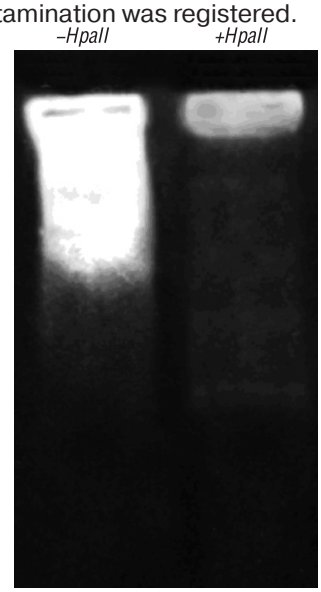
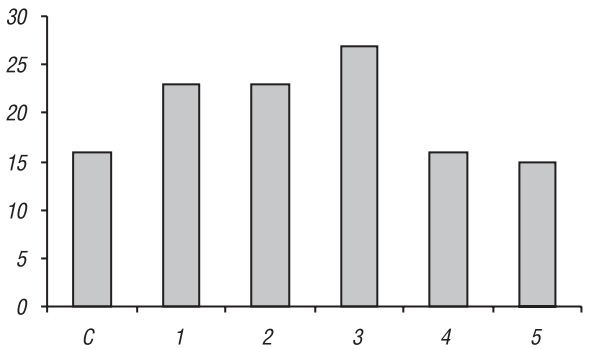


Fig. 1. Restriction analysis of DNA isolated from fruit body of mushroom *P. ostreatus* using Hpa II endonuclease

Early immunostimulatory effects of bacterial DNA were shown to be mainly due to a specific sequence of CpG motifs presented in the unmethylated state and occur at a higher frequency than in mammalian DNA [9]. Therefore, the content of these immunostimulatory DNA sequences and level of methylation of cytosine residues in such CpG motifs can be considered as screening factors of DNA-containing substances as potential candidates for immunotherapy of cancer and other diseases. To determine the level of unmethylated CG-dinucleotides in isolated mushroom DNA, restriction analysis with endonuclease Hpa II was applied [15]. As shown in Fig. 1, *P. ostreatus* DNA demonstrated moderate sensitivity to Hpa II. Earlier it was demonstrated that isolated DNA-containing fraction from culture medium after the 8th day of cultivation of *Bacillus subtilis* strain 7025 and *B. subtilis* strain GP1-807-03 (on the 9th day), showed high sensitivity to HpaII, that proves the fact of relatively high content of unmethylated CG-dinucleotides in isolated DNA sequences [17]. Furthermore, the sensitivity of *B. subtilis* DNA to HpaII correlated with its immunostimulatory and anticancer activity [11]. At the same time, DNA from chicken eryhrocytes was relatively resistant to Hpa II [17] and demonstrated low immunostimulatory activity [18].

Activation of NK cytotoxicity was the first effect of CpG DNA that was described [19]. Other studies also have demonstrated augmentation of NK cell activity by DNA containing unmethylated CpG-dinucleotides *in vit-*

ro or *in vivo* [11, 20]. We have studied the concentration-dependent effect *in vitro* of mushroom DNA on cytotoxic activity of NK cells (Fig. 2). Increase of NK cytotoxicity was observed when murine splenic MNC were incubated with mushroom DNA at the concentration of 0.05 and 0.5 μg/ml while at the concentration of 5.0 μg/ml *P. ostreatus* DNA showed none immunostimulatory effect. It is important to note that stimulatory effect of mushroom DNA on NK cell cytotoxicity was comparable with that when standard stimulant of lymphoid-macrophage lines, PMA, was used. To demonstrate that stimulation resulted from mushroom DNA rather than contaminants such as polysaccharides, we performed experiments using DNA preparations which had been digested by DNase I. As shown in Fig. 2, pretreatment of the mushroom DNA by DNase I completely abrogates the activation of NK cell cytotoxicity.



C – Control (TC + NK); 1 – TC + (NK + PMA);
2 – TC + (NK + DNA 0.05 μg/ml); 3 – TC + (NK + DNA 0.5 μg/ml);
4 – TC + (NK + DNA 5.0 μg/ml); 5 – TC + [NK + (DNA 0.5 μg/ml + DNase I)]

Fig. 2. Augmentation of NK cell activity after preincubation of murine MNC with different concentrations of *P. ostreatus* DNA (TC — tumors celles)

The results of application of mushroom DNA in biotherapy of mice with solid Ehrlich carcinoma are shown in the Table. All control animals developed tumors and died within 52 days (MST 33.8 ± 4.4 days). Mice treated with mushroom DNA in prophylactic regimen of application survived till 80 days (MST was 46.3 ± 8.4 days, and ILS — 37%). Immunization of tumor-bearing mice by *P. ostreatus* DNA in therapeutic regimen resulted in a dramatic increase of animal survival rate: MST was 71.0 ± 8.6 days, ILS — 110%. It is necessary to note that one animal survived till 110th day of experiment.

Table. Results of biotherapeutic application of *P. ostreatus* DNA in mice with solid Ehrlich carcinoma

Group/treatment	Number of animals in group	MST, days	ILS, %
Control	9	33.8 ± 4.4	–
Prophylactic regimen	9	46.3 ± 8.4	37
Therapeutic regimen	10	71.0 ± 8.6	110

The spectrum of detected pharmacological activities of various substances isolated from *P. ostreatus* is very broad. However, polysaccharides are the best known and the most potent mushroom-derived substances with antitumor and immunomodulating properties [6]. It was reported that *P. ostreatus* mushrooms possess potent antitumor activity against Ehrlich ascites carcinoma [7]. Oncoprotective and immunomodulatory effects of substances from *P. ostreatus* were demonstrated [21–23]. Besides, *P. ostreatus* diminishes the toxicity of cyclophosphamide in mice [24]. Antimutagenic effects were found for methanolic extracts of *P. ostreatus* [25]. A dried mushroom *P. ostreatus* diet reduced pathological changes in dimethylhydrazine-induced colon cancer in rats but did not influence significantly the incidence of tumors. This effect is explained by the antioxidant properties of this mushroom and by its fiber content [26]. Also, a pronounced hypocholesteremic effect of *P. ostreatus*, combined with inhibition of lipid peroxydation, was shown *in vivo*. Diet including 10% dried fruiting bodies of *P. ostreatus* significantly reduced the incidence and size of atherosclerotic plaques in rabbits [27].

In the present study biologically active mushroom product was isolated and characterized. Obtained data suggest that *P. ostreatus* DNA also has potent immunomodulatory activity similar to bacterial DNA or DNA from some viruses, yeast, nematodes, mollusks and insects and in contrast to various vertebrates (mammals, fish and frogs) and plants (e. g., corn) DNA which do not possess immunogenic activity [11, 20, 28].

In conclusion, DNA isolated from fruit bodies of higher Basidiomycetes mushroom *P. ostreatus* possesses immunomodulatory activity and biotherapeutic potential that probably can be explained by the presence of unmethylated CpG motifs. Based on the reported results, an application of *P. ostreatus* DNA for cancer biotherapy is a promising strategy.

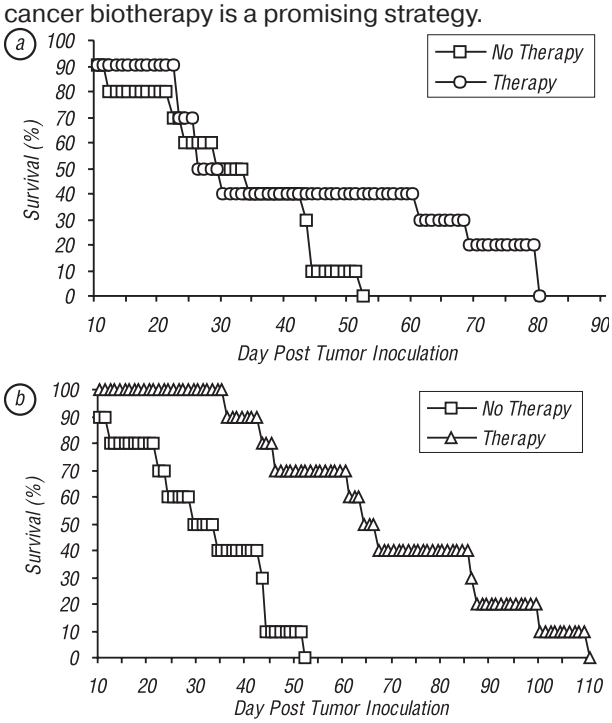


Fig. 3. Survival dynamics of tumor-bearing mice upon application of mushroom DNA (*a* — prophylactic regimen, *b* — therapeutic regimen)

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ПРИМЕНЕНИЕ ДНК ИЗ ГРИБА *PLEUROTUS OSTREATUS* ДЛЯ БИОТЕРАПИИ РАКА: ПОИСКОВОЕ ИССЛЕДОВАНИЕ

Цель: **в данной работе сделана попытка исследовать иммуномодулирующее влияние ДНК из плодового тела гриба *Pleurotus ostreatus* на активность NK-клеток, а также возможность ее применения в экспериментальной биотерапии опухолей.** ***Методы:*** **эффективность терапевтического применения грибной ДНК изучали у мышей линии BALB/c с подкожно трансплантированной карциномой Эрлиха. Влияние ДНК *P. ostreatus* на активность NK-клеток исследовали *in vitro* с помощью теста неспецифической цитотоксичности.** ***Результаты:*** **установлено, что интраперитонеальное введение ДНК повышает цитотоксическую активность NK-клеток *in vitro* и значительно повышает выживаемость мышей с солидной карциномой Эрлиха.** ***Выводы:*** **наблюдаемые эффекты, вероятно, можно объяснить наличием в составе ДНК из *P. ostreatus* иммуностимулирующих неметилированных CpG-мотивов.**

Ключевые слова: **биотерапия рака, CpG ДНК, карцинома Эрлиха, лекарственные грибы, NK-клетки, *Pleurotus ostreatus*.**