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EXPERIMENTAL ARTICLES

Antitumor Activity of the Lipid Fraction of the Spores of an Anaerobic Bacterium *Clostridium butyricum*

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Abstract—Antitumor activity of the preparation of the lipid fraction of *Clostridium butyricum* spore extract was demonstrated in vivo on a transplantable mouse model of breast cancer. At a specific scheme of application, inhibition of tumor growth and improved survival dynamics compared to the control group were observed. Thin-layer chromatography (TLC) of the lipid fraction of the spore extract revealed, apart from a saturated hydrocarbon, cholesterol ester, cetyl palmitate, triacyl glycerol, and palmitic acid, also a phenolic lipid bound in a complex with a peptide component. Acetone extraction of the lipid pool revealed a phenolic lipid. According to the TLC and ^{1}H-NMR spectrum of the acetone extract of the lipid fraction of *C. butyricum* spores, the structure of the phenolic lipid was proposed, *n*-butyl benzoate substituted in the *para* position. The phenolic lipid is suggested to be responsible for the biological activity of the spore's lipid fraction.

Key words: resorcinolic lipids, lipid fraction of bacterial spores, antitumor activity.

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Metabolism of bacterial vegetative cells is known to change in the course of formation of the dormant forms. Bacterial cysts were found to contain twice the lipids of the vegetative cells [1]. This is sufficient to gain interest in the lipids of the anabiotic forms. The content of phenolic lipids was reported to increase sharply in the dormant forms. For example, in the course of encystment, 5-alkylresorcinols and 6-alkylpyrones substitute the phospholipids in the cytoplasmic membrane of *Azotobacter vinelandii* [2].

Amphiphilic lipids containing a benzene ring in their structure (phenolic lipids and resorcinolic lipids) exhibit diverse biological activity. The universality of their characteristics results from their effect on the rate of lipid peroxidation, as well as on the composition and features of the cell membrane [3, 4].

Literature data suggest that phenolic lipids determine the inhibition of metabolic processes in bacterial cells and promote the formation of anabiotic forms [5–7]. Inhibition of the metabolic processes is one of the ways to increase the overall resistance of an organism affected by various pathologies, including carcinogenesis.

Investigation on the antitumor effect of phenolic lipids isolated from various natural sources revealed high activity of some of them. For example, bilobol (5-*n*-pentadec-8-enylresorcinol), a phenolic lipid isolated from *Ginkgo biloba*, completely suppressed growth of the S180 mouse sarcoma cells during 4 days of application [8]. Similar activity was observed for alkylresorcinols (gravilol and 5-*n*-tridecylresorcinic acid) from the medicinal plant *Lysimachia japonica* against the cells of leukemia P-388, melanoma B-16, Carcinoma Hep-2, etc. [3]. Resorcinolic lipids attract attention due to their low toxicity to higher animals. For example, the 5 g/kg peroral dose as absolutely nontoxic to rats [3].

Apart from their antitumor activity, resorcinols of bacterial and plant origin exhibit bacteriostatic activity. Phenolic lipids with an alkyl substitute at the fourth position in the benzene ring have pronounced antibacterial characteristics, and bacteriostatic activity of 4-hexylresorcinol against seven species of phytopathogenic bacteria was demonstrated [3].

The lipid fraction of *Clostridium butyricum* spore extracts were shown to have a radiotherapeutic effect on laboratory animals subjected to lethal doses of irradiation; survival of rats was 37% higher than in the control [9]. The spore extracts were found to have a bacteriostatic effect on the vegetative cells of the producer culture. The original spore extract's preparation inhibited cell wall autolysis of *C. butyricum* vegetative cells by 30%, while the lipid fraction of the preparation, by 87% [10]. We believe that the inhibition of the cell's autolysate activity may be mediated by the membrane-acting effect of a phenolic lipid. Phenolic lipids affecting the activity of membrane-bound enzymes (e.g., proteases), which regulate cell division, may suppress tumor growth.

The goal of the present work was investigation of the antitumor effect of the lipid fraction of *C. butyricum*

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spores. We have previously demonstrated the presence of a phenolic lipid in this fraction [10].

MATERIALS AND METHODS

The culture *C. butyricum* 35/11 was obtained from the Department of Physiology of Spore-Forming Bacteria, Institute of Microbiology, Russian Academy of Sciences. *C. butyricum* spores were obtained as described in [11]. The method of obtaining spore extracts was described earlier [12]. The lipids were removed from the extract with a chloroform–methanol mixture according to the standard procedure [13, 14] and vacuum-dried. To isolate the phenolic lipid from the lipid fraction, acetone extraction was used in order to avoid extraction of other lipids [15]. Acetone (2–3 ml) was added to 10–15 mg of the dry fraction of spore lipids, and the mixture was incubated for 10–12 h on a shaker at room temperature. The mixture was then centrifuged (600 *g*, 20 min) and the supernatant was separated from the pellet and vacuum dried. Dry precipitate was dissolved in the chloroform–methanol mixture (2 : 1) and used for subsequent investigation. Thin-layer chromatography (TLC) was carried out on Kieselgel 60 F_{254} plates (Merck); phosphomolybdic acid, ninhydrin, and the Fast Blue B Salt reagent (Serva) were used for detection.

A Bruker Avance-600 spectrometer (United States) was used to record the ^{1}H NMR spectra at 22°C in the chloroform-*d1*–methanol-*d4* mixture (2 : 1) at the operating frequency of 600 MHz with a 5-mm sensor, and the signals of the residual protons in the methanol methyl group (3.30 ppm) were used as a standard. A small amount of the sample required signal accumulation with a number of scans about 1000. The spectra were analyzed with the XWinNmr 3.1 software package. Two-dimensional correlation spectra (*COSY*) were registered as absolute values. The spectral window was from –0.57 to 8.89 ppm (the spectra with smaller window sizes were also registered to improve the signal resolution). The data matrix was 16384×480 along the F2 and F1 dimensions, respectively. Duration of registration was 1.44 s with the relaxation delay of 2.6 s and the number of scans per fid about 64. During conversion, the data were multiplied by a weighted function of the unshifted sinebell type.

The preparation of the lipid fraction of *C. butyricum* spores was prepared as described previously [10]. The antitumor activity of the preparation was investigated on the CBRB-Rb(8.17)1Iem transplantable mouse model of breast cancer (further CBRB) [16]. Three groups of 4.5-month old CBRB male mice were used with ten animals in each group. The recipients were inoculated with 10^6 tumor cells obtained from the slowly growing spontaneous breast's adenocarcinoma from a syngeneic female as described in [17].

The preparation (2 mg/ml of the physiological saline) was filter-sterilized (0.2 μm) and introduced intraperitoneally (0.2 ml per mouse per injection). The control animals (group 3) were injected with the same volume of the physiological saline. The mice were divided into three groups according to the scheme of application of the preparation. The first group was injected both 5 days and 1 day prior to the tumor transplantation; the second group was additionally injected 1 h, 2 days, and 5 days after the transplantation.

The antitumor activity of the preparation was assayed as retardation of tumor growth and improved survival dynamics of the recipients as compared to the control for 16 weeks. Tumor growth was assayed weekly by measuring the average tumor size calculated from three orthogonally related measurements, as described earlier [17]. The first stages of tumor growth, prior to its visual manifestation (during the latent period, up to 5 mm in diameter) were determined by palpation; the significance of the differences was determined using the Wilcoxon–Mann-Witney parametric criterion [16].

RESULTS AND DISCUSSION

To test the antitumor effect of the lipid fraction of the spores on a model of breast cancer in CBRB mice, the animals were divided into groups with different schemes of the application of the preparation relative to the transplantation of tumor cells. The dynamics of tumor growth for different experimental groups is presented on Fig. 1.

In the first group (receiving the preparation only twice befor the transplantation), reliable stimulation of tumor growth was observed, compared to the control (from the 4th to the 8th week, $p < 0.01$). In this group, the latent palpable period of tumor growth decreased, while the tumor growth rate increased, compared to the control. Survival dynamics for the animals of this group was reliably worse than in the control group (Fig. 2).

In the second group of animals (injections of the preparation continued after the tumor transplantation), tumor growth was suppressed (from the 5th to the 6th week, $p < 0.05$). Interestingly, while the last treatment was carried out several days after the tumor cells transplantation, inhibition of tumor growth was observed up to the 8th week; the differences in tumor growth dynamics in group 2 compared to the control (group 3) were significant from the 4th to the 8th week ($p < 0.05$). Survival dynamics of the recipient mice in the second group improved significantly (Fig. 2), while no significant differences in life duration (compared to the control) were found.

Thus, the antitumor activity of the preparation of the lipid fraction of *C. butyricum* spores was revealed at quintuple application: twice before and three times after the transplantation of the tumor. Since two-times injection of the preparation prior to tumor transplantation (group 1) stimulated tumor growth and decreased survival, selection of the optimal mode of application for the preparation may increase the antitumor effect.

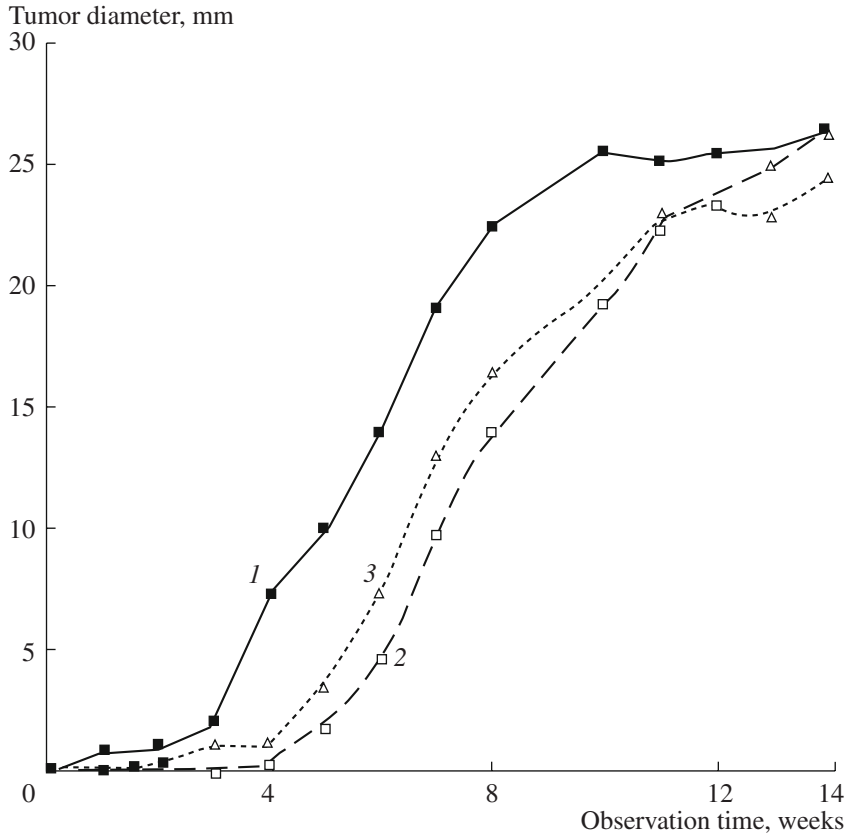


Fig. 1. Dynamics of tumor growth (mm) in mice of different experimental groups: injection of the preparation 5 and 1 days before tumor transplantation (*1*); injection of the preparation 5 and 1 days before and 1 h, 2 days, and 5 days after tumor transplantation (*2*); control (*3*).

Amphiphilic lipids of an aromatic nature (phenolic and resorcinolic lipids) exhibit a membrane-acting effect resulting in the modulation of activity of the membrane-bound enzymes, including proteases. Proteases are known to participate in division of bacterial cells and proliferation of tumor cells.

Clostridium is one of the big genera of anaerobic spore-forming bacteria, comprising obligate spore-forming anaerobes, mainly nonpathogenic, living in soil as saprophytes. Their strict anaerobiosis and ability to form spores make clostridia an original vector system for tumor therapy, and intravenously injected spores germinate in the region of hypoxia and necrosis of a solid tumor. Lysis of a rat's carcinoma tumor resulting from clostridial growth and having no negative effect on healthy tissues was first reported in 1927 [18]. Under normal physiological conditions, hypoxia does not develop in healthy tissues, which are therefore protected from germination of clostridial spores. Clinical investigation revealed that degradation of the tumor tissue was caused by the proteases of the spores germinating in necrotizing tissues under anaerobic conditions (see review [19]). The method of tumor therapy involving bacterial spores does not affect the peripheral parts of the tumor; it is therefore applicable only in combination with antibiotics, radiotherapy, etc. Experiments have demonstrated that tissue degradation caused poisoning of the organism, and the integral criterion (survival) did not improve [19].

In this work, for the first time antitumor activity of the lipid fraction of *C. butyricum* spores was demonstrated in vivo. Our results suggest biphasic effect of the spore's lipid fraction depending on the application time relative to the tumor transplantation. It may either promote tumor growth (before transplantation) or suppress it (after transplantation). A number of immunomodulatory drugs have the same biphasic effect (see, for example, [20]).

The TLC results in various systems demonstrated that the total lipids obtained from the *C. butyricum* spore extract contained a polar fraction. This fraction was found to consist of two components, an amino acid (or peptide) and a phenolic lipid [10]. The results of ^{1}H-NMR spectroscopy suggest the presence of α-aminotridecanoic acid and *n*-butyl benzoate substituted in the *para* position.

Separation of the acetone extract of the spore's lipid fraction by TLC in the chloroform–ethyl acetate system (7 : 3) and staining with phosphomolybdic acid revealed a spot with R_f 0.25; it was stained yellowish-pink with the Fast Blue Salt reagent, indicating the phenolic nature of this compound [15]. The spot also exhibited positive reac-

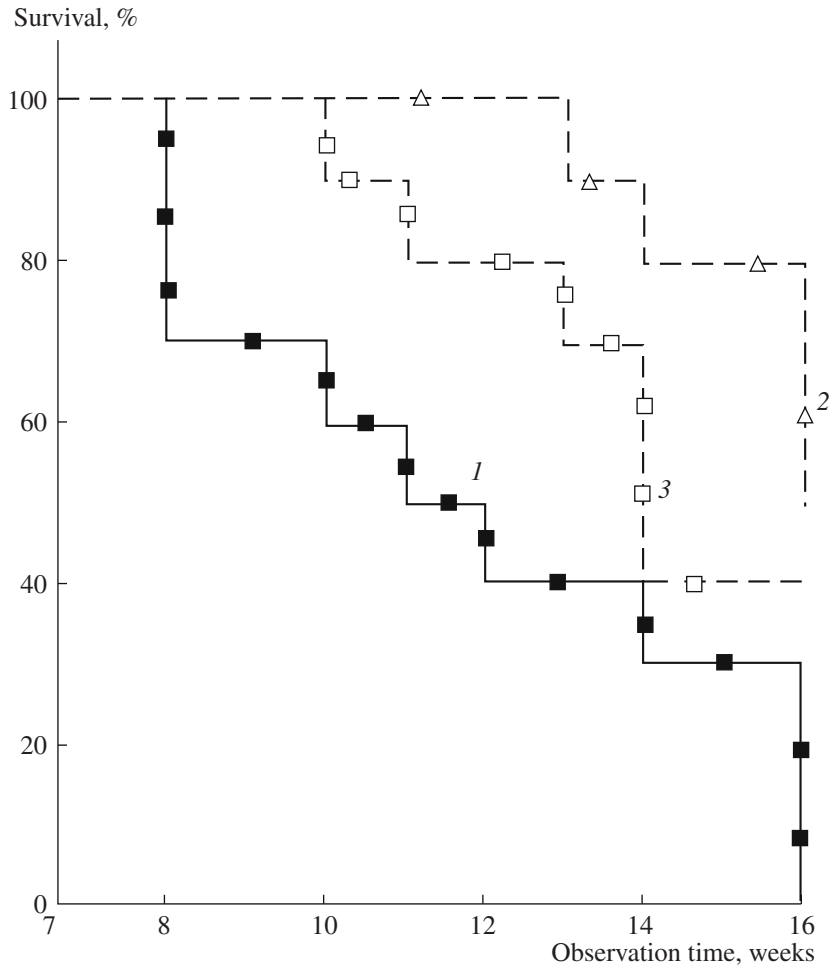


Fig. 2. Survival of CBRB mice in different experimental groups. The designations are the same as on Fig. 1.

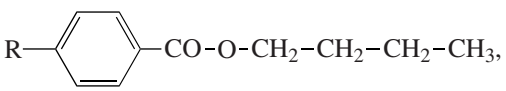
tion to the peptide group. The spot, therefore was a lipid (or a mixture) containing both phenolic and amino groups.

The results of ^{1}H-NMR spectroscopy suggest the presence in the acetone extract of an alkyl compound with the following conjectural structure:

$$CH_3–(CH_2)_{10}–CHX–COOH,$$

where X is an unidentified group which does not contain protons at the carbon atom. This is most probably an amino group, which was detected on TLC treated with ninhydrin. In the spectrum, this compound is represented by the signals with chemical shifts of 0.85 (t), 1.26–1.28(ush), 1.58 (m), and 2.25 (t), with the intensity ratio of 3 : 18 : 2 : 1. The presence of the COOH-group can be expected due to the position of the proton signal at the vicinal carbon atom (2.25 ppm). The relative position of the structural elements of the chain is confirmed by the interactions in the 2D spectrum of the *COSY*-type's homonuclear spin correlation with the cross peaks (0.85; 1.20), (1.26; 1.28), (1.28; 1.58), and (1.58; 2.25).

A compound with an aromatic structure was also revealed in the acetone extract, which exhibited a pair of multiplet signals with shifts of 7.55 and 7.68 ppm and the ratio of integral intensities of ~1 : 1, approximately symmetrical. The signals are bound by the spin–spin interaction; no other correlations were observed for them. They therefore, probably belong to a double-substituted benzene ring with the substitutions in the *para* position. The signals at 4.27 (t), 1.70 (m), 1.40 (m), and 0.95 (t) ppm bound by the spin–spin interactions in the order cited may also belong to this compound; no other interactions were observed for these signals. It may be suggested due to the close values of signal intensity at 7.69, 7.55, 4.27, and 1.70 ppm, which relate approximately as 1 : 1 : 1 : 1 (considering the errors of baseline correction and integration). For the signals 1.40 and 0.95, the intensity interval was not determined due to strong overlapping. The conjectural structure therefore looks as follows:



where the ester group is positioned according to the value of the chemical shift for the terminal CH_2 group.

The R substituent is probably an oxygen-containing group without the protons bound to carbon.

Thus, our results enable us to conclude that the antitumor effect of *C. butyricum* spores may be caused not only by the presence of proteolytic enzymes, but also by specific pore lipids. The most probable factor of antitumor activity in the lipid fraction of the spores is possibly the above-described phenolic lipid. Phenolic lipids are present in a number of organisms. Their biological activity, physiological role, and involvement in metabolic regulation were revealed, although are poorly studied [4, 8, 21]. Phenolic lipids possibly play a significant role in the general regulatory mechanisms in both pro- and eukaryotic cells, including the process of carcinogenesis.

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