

Ion Exchange Separation of the Antitumor Component(s) of Yogurt Dialyzate¹

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ABSTRACT

The active antitumor component of yogurt obtained by dialysis was further fractionated by ion exchange chromatography into acidic, basic, and neutral fractions, which were analyzed for activity by *in vitro* agar diffusion, cell culture, and *in vivo* mouse assay. Neither dialysate nor fractions showed any antitumor activity when tested by agar diffusion or cell culture techniques. When tested by *in vivo* mouse assay, the dialyzate as well as the anionic fraction showed significant inhibitory activity. The cationic fraction showed no activity. There was no direct relationship between stage of purification and antitumor activity. Moreover, the response of the dialyzate or anionic fraction was not linear with concentration. The antitumor activity of the yogurt fraction(s) may be related to a host-mediated immunological reaction or "activation" of the "inactive" component(s).

INTRODUCTION

Reddy et al. (6) observed that yogurt made with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures, possessed antitumor activity. In subsequent studies we (1) reported that the antitumor component(s) of yogurt can be separated either by dialysis or by ether extraction. This communication describes additional purification of the dialyzate fraction by ion exchange chromatography and determination of antitumor activity by *in vitro* and *in vivo* methods.

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MATERIALS AND METHODS

Yogurt dialyzate powder (1) was separated by ion exchange chromatography into acidic, basic, and neutral fractions.

Ion Exchange Chromatography

Two and one-half grams of dialyzate powder were dissolved in 2.0 ml of distilled water and applied to a 150-ml bed volume of Dowex 50W × 8 cation exchange resin (Sigma Chemical Co.). The sample was washed through the column at a flow rate of 2.0 ml/min with distilled water until the effluent was negative to the Molisch test, indicating the absence of reducing sugars in the effluent. The column was eluted with approximately 6.5 bed volumes of 2N NH₄OH. The NH₄OH was removed from the eluate by repeated dilution and evaporation under vacuum. Both the effluent (anionic and neutral fractions) and eluate (cationic fraction) were concentrated by vacuum evaporation to approximately 5 ml. The pH of each fraction was adjusted to 6.0 with either NaOH or HCl, and the final volume was made to 10 ml with distilled water. Both samples were sterilized by millipore filtration (.45 μm pore size, Millipore Corp.) and stored at 4°C.

A five-milliliter aliquot of the effluent from the cation exchange column was applied to a 100-ml bed volume of Amberlite IRA-400 anion exchange resin (Mallinckrodt Chemical Works). The sample was washed through the column with distilled water at a flow rate of 2.0 ml/min until the effluent was again negative to the Molisch test. The anion exchange column was eluted with approximately 10 bed volumes of 2N acetic acid. Similarly, the acetic acid was removed by repeated dilution and evaporation under vacuum. Both the anion exchange column effluent (neutral fraction) and eluate (anionic fraction) were concentrated to approximately 5 ml. The samples were filter sterilized and used in subsequent trials to determine their antitumor activity.

Determination of Antitumor Activity

Two *in vitro* techniques (agar plate diffusion and cell culture) and one *in vivo* method (mouse assay) were used to determine the antitumor activity of yogurt dialyzate and the ion-exchange fractions.

In Vitro Determination of Activity. The agar plate diffusion technique measures the number of cells inhibited by an antitumor agent and is based upon the decrease of methylene blue reducing activity by the dead tumor cells from inactivation of cell dehydrogenase. Basal agar medium containing 1.0 g peptone, .5 g glucose, .5 g NaCl, .3 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 g agar in 100 ml distilled water and adjusted to pH 7.2 was used as the growth medium. To 6.0-ml portions of basal medium, 1.0 ml of fetal calf serum from Grand Island Biological Company (GIBCO) and 1.0 ml of 7 day-old Ehrlich ascites cells (2×10^7 cells/ml) were added. After thorough mixing, the contents were poured into sterile petri dishes and allowed to set. Paper discs (1.26 cm) saturated with test material (yogurt dialyzate at 1 mg/ml, Dowex effluent concentrate, Dowex eluent concentrate, Amberlite effluent concentrate, Amberlite eluate concentrate, mercuric chloride at 1 mg/ml as a standard or saline as a control) were placed on the agar plates. The plates were held in a refrigerator at 4°C for 10 h to permit the test materials to diffuse into the agar. The plates then were incubated at 37°C for 8 h, after which the paper discs were removed and surfaces of the plates washed with sterile distilled water. Each plate was flooded with 10 ml of .05% methylene blue solution. After the surface of the agar was uniformly dyed (ca. 5 s), the methylene blue was decanted, and the plates again were incubated at 37°C for 3 h. Diameters of circular blue zones on the agar then were measured with a metric vernier caliper to the nearest .1 mm.

The cell culture method for determination of the effect of streptolysin S against Ehrlich tumor cells described by Ginsburg and GROSSOWICZ (4) was adopted with modifications. Seven-day-old ascites cells from the peritoneal cavity of Swiss mice (Sasco Inc.) were trypsinized for 20 min with .25% trypsin (1:250, GIBCO), centrifuged at $610 \times g$, and washed three times with minimum essential medium (MEM) obtained from GIBCO. The washed cells

were resuspended to approximately 10^8 cells per milliliter in MEM containing 10% calf serum (GIBCO) and .1% antibiotic-antimycotic. One-tenth milliliter aliquots of cell suspension were pipetted into sterile test tubes, and .2 ml of the test material was added. Tubes were incubated at 37°C, and one tube of each treatment was removed after 0, 30, and 120 min to determine percent viable cells by .4% Trypan blue reduction.

In Vivo Determination of Antitumor Activity. Mice in each treatment were inoculated intravenously with 2.5 mg of the appropriate fraction in .1 ml saline, whereas control mice were inoculated with only .1 ml saline. Mice were sacrificed on the 8th day, and progression of ascites tumor growth was determined (1).

RESULTS AND DISCUSSION

In Vitro Determination of Antitumor Activity

Agar plate diffusion and cell culture techniques were used to assess activity of yogurt dialyzate and its ion exchange fractions against Ehrlich ascites tumor cells. Although a standard mercuric chloride solution produced a 15.7-mm zone of inhibition similar to that reported in (3), none of the yogurt samples or saline control exhibited measurable zones of inhibition. The anionic fraction, however, showed a faint, dispersed blue area of inhibition.

Similarly, when tested in cell culture, the yogurt dialyzate, ion exchange fractions, and saline increased the percent viable cells from 70 to 80% during the first 30 min, probably because of recovery of cells injured during the transfer process. During the next 90 min there was a general stabilization of viable cells, except for the anionic fraction where there was a slight but not significant decrease (82 to 78%) in viable cells. Mercuric chloride produced an immediate and significant reduction ($P < .01$) in the number of viable ascites cells as evidenced by a drop from 70 to 14% viable cells within the first 30 min and from 14 to 5% during the remaining 120 min incubation.

In Vivo Determination of Antitumor Activity

The absence of antitumor activity in yogurt dialyzate and its ion exchange fractions on Ehrlich ascites cells *in vitro* necessitated further investigation in a mouse system. As in Table 1,

TABLE 1. Effect of inoculating yogurt dialyzate fractions upon the proliferation of ascities tumor in mice.^a

Treatment	Total tumor cells (million/mouse)	Inhibition (%)	DNA ($\mu\text{g/ml}$ susp.)	Inhibition (%)
Saline (control)	46.7	521.9
Yogurt dialyzate	28.7 ^b	38.5	321.1 ^b	38.5
Dowex eluate (cationic)	43.2	7.5	482.82	7.5
Dowex effluent (anionic + neutral)	39.3	15.9	435.5	16.6
Amberlite eluate (anionic)	28.6 ^b	38.7	319.8 ^b	38.7
Amberlite effluent (neutral)	47.2	0	527.0	0

^aAverage of two trials (each with four replications).

^bDifferent ($P < .05$) from control.

inoculation of 2.5 mg yogurt dialyzate or the anionic fraction significantly inhibited (38.5%) cell proliferation. However, injection of 2.5 mg of the cationic, anionic plus neutral, or the neutral fraction did not result in significant inhibition of tumor proliferation.

If 1 U of activity is equivalent to 100% inhibition of ascites tumor proliferation in the mouse assay system, the following specific activities can be calculated: yogurt dialyzate .1539 U/mg, cationic fraction .0300 U/mg, anionic and neutral fraction .0634 U/mg, anionic fraction .154 U/mg, and neutral fraction .0. Because the yogurt dialyzate and anionic fraction possessed the same specific activity, the degree of purification with ion exchange cannot be established.

Several explanations are possible for the observation that yogurt dialyzate and its anionic fraction possess definite antitumor activity in vivo that was not demonstrable in vitro. First, these antitumor principles may be "inactive" when ingested or inoculated, but later "metabolized" or converted by the host into active components. Second, the "inactive" form might elicit the activation of immunological responses in the host against the tumor without participating directly. Third, technical problems such as poor diffusion of the sample in the agar or a lack of effect within 120 min in cell cultures might account for the apparent negative effect. The first two hypotheses are supported in part that there

was no direct relationship between stage of purification and in vivo activity. Bogdanov and his associates (2) also reported that the immunological mechanism of host played an important role in antitumor activity of blastolysin, a glycopeptide fragment from the cell wall of *L. bulgaricus*, after they failed to demonstrate activity in vitro. Host-mediated and immunological activities of sporamycin from *Streptosporangium pseudovulgare* also have been established by Komiyama et al. (5).

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