# IMMUNOMODULATING AND ANTITUMOR ACTIVITY OF POLYSACCHARIDES OF PLANT ORIGIN

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Among antitumor preparations those of greatest interest from the point of view of immunology are plant polysaccharides, because of the ability of some of them, in therapeutic doses, to stimulate the formation of cytolytic T lymphocytes (CTL) [1, 3-5].

In the investigation described below the effect of preliminary injection of polysaccharides (tagetan, palustran, and zymosan) into animals on CTL formation was studied in a one-way mixed culture, dose of the preparations and the schedule of their administration in order to maintain a high level of stimulation of these cells were determined, and the effect of immunostimulating therapy with these polysaccharides on growth of a Crocker's sarcoma also was investigated.

## EXPERIMENTAL METHOD

The polysaccharides palustran and tagetan were isolated from plants of the Rosaceae and Asteraceae families in the Laboratory of Chemistry of Natural Compounds, All-Union Oncologic Center, Academy of Medical Science of the USSR. The homogeneity of the polysaccharides was confirmed by gel-filtration on Sephadexes G-75 and G-200 and by electrophoresis on paper in borate buffer, pH 4.5. Zymosan was obtained from yeast cell membranes at the Tallian Pharmaceutical Chemical Factory.

Experiments to determine the effect of the polysaccharides on CTL formation were conducted on adult male BALB/c mice weighing 18-20 g, obtained from the "Stolbovaya" nursery, Academy of Medical Sciences of the USSR. The polysaccharides were dissolved in Hanks' solution immediately before injection into the animals and working concentrations of 10, 5, 2.5,1.125, and 0.06 mg/ml were prepared. To study the action of the polysaccharides in each concentration the experimental animals were divided into four groups. Mice of group 1 received a single intraperitoneal injection of the preparation 14 days before the mixed lymphocyte culture (MLC) was set up: mice of groups 2, 3, and 4 received the injection 7 and 3 days and 1 day respectively beforehand. Control animals were given an injection of Hanks' solution at the same times.

To determine the ability of the injected polysaccharides to induce an immunomodulating effect, the method of one-way MLC was used [2]. Splenic lymphocytes from experimental and control BALB/c H-$2^d$ (H-$2^d$) mice were used as reacting cells for the mixed culture, and spleen cells from C3H (H-$2^k$) mice irradiated in a dose of 1000 rads were used as stimulating cells. Reacting cells in a dose of $35 \times 10^6$ were mixed with $17.5 \times 10^6$ stimulating cells in 5 ml of medium DMEM, containing 10% inactivated embryonic calf serum (ECS), $5 \times 10^{-5}$ M 2-mercaptoethanol, $10^{-2}$ M HEPES, and $2 \times 10^{-3}$ M L-glutamine, and incubated in wells in 6-well plastic plates (No. 76-047-05, from Linbro, England) in a "Napco" humidified incubator with 5% $CO_2$.

On the 5th day of incubation $6 \times 10^6$ cells from each culture were centrifuged, the culture medium was decanted, and the residue resuspended in 0.6 ml of medium 199 with 10% ECS. Three aliquots, each consisting of 100 µl of the cell suspension, were added to a monolayer of $10^4$ $^{51}$Cr-labeled L-929 cells, seeded on the bottom of 96-well flat-bottomed plates (No. 3040, from Falcon, USA); the rest of the suspension (0.3 ml) was diluted twice with a fresh portion of medium, and again added in a volume of 100 µl to the next three wells with a monolayer of

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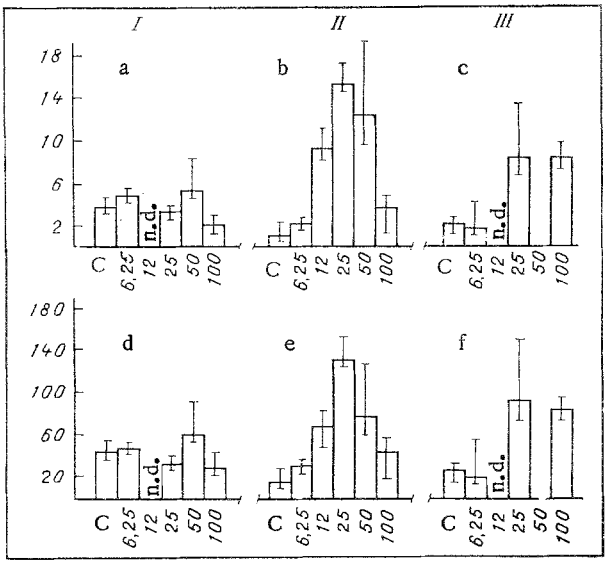


Fig. 1. Effect of different doses and schedules of tagetan administration on CTL formation in MLC. Abscissa, doses of tagetan (in mg/kg) injected into BALB/c mice 5 days (a, d), 7 days (b, e), and 13 days (c, f) being setting up MLC; ordinate: a-c) number of LU per $10^6$ lymphocytes, d, f) number of LU calculated per total number of lymphocytes in mixed cultures. I) $LU_{50}$, II) $LU_{40}$, III) $LU_{35}$. C) Control, n. d.) no data.

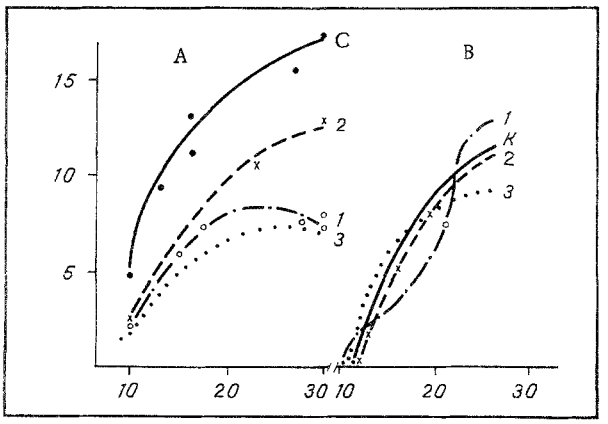


Fig. 2. Effect of tagetan (1), palustran (2), and zymosan (3) on growth of Crocker's sarcoma in BALB/c (A) and BALB/c nu/nu mice (B). Abscissa, time after transplantation (in days) ordinate, size of tumor (in $cm^3$). C) Control.

labeled L-cells. By means of these and subsequent dilutions of the cell suspension and addition of parts of it to the labeled L-cells we obtained lymphocyte—target cell ratio of 100:1, 50:1, 25:1, and 12.5:1 respectively. To determine the level of spontaneous lysis of the L-cells 100 µl of medium without lymphocytes was added to the monolayer. After addition of lymphocytes from each test culture to the labeled L-cells the plates were incubated for 3 h at 37°C. After the end of incubation the supernatant and cell residue, lysed with 10% Triton X-100, was aspirated into test tubes and the number of counts per minute was counted on a "Berthold" gamma-counter.

The percentage of cytotoxic activity of the CTL for each lymphocyte–target cell ratio was determined by the equation:

$$\text{cytotoxicity (in \%)} = \frac{A - B}{C},$$

where A, B, and C, denote mean numbers of counts per minute in supernatant of L-cells incubated with lymphocytes (A), of intact L-cells (B), and of L-cells lysed with Triton X-100 respectively. After determination of the values of cytotoxic activity and the standard deviation for all lymphocyte–target cell ratios in the control and experimental cultures, graphs were plotted to show how the cytotoxic activity depended on dose of effector cells, from which the value of one lytic unit (LU), corresponding to the number of lymphocytes causing lysis of the same standard number of labeled L-929, was determined. Depending on the magnitude of the cytotoxic effect in the series of cultures in one experiment, the number of lymphocytes inducing lysis of 35% ($LU_{35}$), 40% ($LU_{40}$), or 50% ($LU_{50}$) of labeled L-cells was taken as 1 LU.

The number of lytic units calculated per $10^6$ lymphocytes was defined as the relative cytotoxic activity, and the number of lytic units calculated per total number of lymphocytes in a given culture as the absolute cytotoxic activity. The ratio between values of absolute and relative cytotoxic activity in an experiment to the corresponding values in the control was defined as the indices of absolute and relative immunostimulating activity.

To determine correlation between the immunostimulating and antitumor activity of the polysaccharides, the preparations were injected intraperitoneally into male BALB/c and BALB/c nu/nu mice which in use have been found to give maximal indices of absolute and relative immunostimulating activity. These doses were 25 mg/kg for tagetan and 20 mg/kg for zymosan and palustran respectively.

To maintain a high level of stimulation of CTL throughout the course of immunotherapy, repeated injections of the polysaccharides were given at times when, in the experiments to choose the optimal dose, a decrease was observed in the values of relative and absolute immunostimulating activity. For tagetan these times corresponded to the interval between the 7th and 14th days, and for zymosan and palustran, that between the 3rd and 6th days. The first injection of tagetan was given 14 days, and the first of palustran and zymosan 6 days before subcutaneous transplantation of a Crocker's sarcoma into the animals, the injections were repeated at the moment of transplantation, and tagetan continued thereafter to be given every 14 days and zymosan and palustran every 7 days until death of the animals. In the course of observation on the animals the time of appearance of the tumor was recorded and every 3 days it was measured in three mutually perpendicular directions.

## EXPERIMENTAL RESULTS

The results of determination of the effect of various doses of tagetan on cytotoxic activity of the lymphocytes in a one-way mixed culture are shown in Fig. 1. Injection of tagetan into the animals in doses of between 6.25 and 100 mg/kg 5 days before the mixed culture was set up was not accompanied by statistically singificant increase in CTL formation, but on the 7th day after injection of the polysaccharide the cytotoxic activity of the lymphocytes rose sharply; at this time a marked dose– effect dependence was present: maximal activity was observed after injection of 25 mg/kg (average values of indices of relative and absolute immunostimulating activity were 12.3 and 7.8 respectively), and if the dose of the preparation was doubled or halved, activity fell only a little, whereas when doses of 100 and 6.25 mg/kg were given activity came close to the control level. On the 14th day after injection the stimulating effect of tagetan was preserved in doses of 25 and 100 mg/kg, but values of the indices of relative and absolute immunostimulating activity were considerably reduced.

Comparison of changes in the values of the indices of absolute and relative immunostimulating activity, depending on dose and time of injection, leads to the conclusion that the optimal doses of tagetan, stimulating CTL formation, were 100, 50, and 25 mg/kg, with a maximum of activity on the 7th day. The latent period, or time interval between injection of the polysaccharide and the time of setting up the MLC, during which stimulation of CTL formation was observed, was 5-6 days for tagetan, but a gradual decline of activity was observed in the interval between the 7th and 14th days. On the basis of these data it is possible to calculate the approximate number of injections of the preparation when giving immunotherapy. For tagetan the injection should be repeated in the interval between the 7th and 14h days, and in this case the latent period of the second injection of preparation will coincide in time with the

period of decline of activity after previous injection.

Similar experiments were carried out with the other two preparations -- zymosan and palustran. The optimal dose of zymosan was 25 mg/kg and the maximum of activity was observed on the 3rd day; the effect of stimulation was preserved in the interval between the 3rd and 6th days. Palustran gave an approximately equal level of stimulation in doses of 10 and 100 mg/kg, with a maximum of activity on the 5th day.

The results of immunotherapy of BALB/c and BALB/c nu/nu with tagetan (20 mg/kg every 14 days), palustran (10 mg/kg every 6 days), and zymosan (20 mg/kg every 6 days) are shown in Fig. 2. Tagetan and zymosan gave the strongest antitumor effect: when these polysaccharides were used the size of the tumor toward the end of the first month of immunotherapy was on average 2-2.5 times smaller in the experimental animals than in the control. Palustran had a weaker antitumor action. Inhibition of tumor growth by the use of palustran was approximately 30%. Injection of the same polysaccharides into BALB/c nu/nu mice, which are characterized by genetic absence of the T system of immunity, was not accompanied by inhibition of growth of Crocker's sarcoma compared with the control. These last data are direct evidence that inhibition of tumor growth under the influence of the three polysaccharides studied in this investigation is not connected with the direct effect of these preparations on tumor cells, but is mediated through stimulation of antitumor activity of the T cells.

## LITERATURE CITED


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