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Antitumor and Immunostimulant Activities of Polysaccharide Produced by a Marine Bacterium of the Genus *Vibrio*

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Antitumor activity of the polysaccharide preparation from marine *Vibrio* sp. and its effects on immune responses were studied by using mice.

Antitumor activities of the material were examined against P388 leukemia and Sarcoma-180 solid tumor cells. The saccharide showed a weak antitumor activity against both tumors. In the test of lymphocyte responsiveness to phytohemagglutinin (PHA), the test sample reduced the immunological response to PHA. The cellular immune responses were measured by the delayed hypersensitivity reaction to sheep red blood cells using footpad assay. The preparation suppressed the production of antibody against sheep erythrocytes in mice at high doses. The appearance of antibody forming cells after the injection of sheep red blood cell antigen was studied by the plaque technique. The number of plaque forming cells without treatment of this material is about 1×10^4 cells per spleen, which increased to about 5×10^4 by treatment of this material.

In the previous papers,[1-8] it was reported that the polysaccharide preparation obtained from a marine *Vibrio* sp. has a inhibitory activity against tumor cells.

Macromolecular substances such as the polysaccharide derived from various species of bacteria and plant have attracted attention from views of their antitumor activity.[4-10] It is generally thought that the antitumor action of these high molecular weight substances is mediated through the stimulation of immunological reactivity of the host in contrast to usual antitumor substances which directly inhibit tumor cell growth.

To investigate the mode of action of the antitumor activity of the bacterial polysaccharide, the immunostimulating effects of this substance were studied. The present paper deals with the effects of the polysaccharide preparation from marine *Vibrio* sp. on the growth of experimental tumors and on the immune response of animals.

Materials and Methods

The test sample used in the present experiments was an extracellular polysaccharide preparation.[1]

Animals and Tumors

Female mice of CDF_1, ICR-CR, BALB/c strains, and males of ddY strain were used. Mice of CDF_1, ICR-CR and BALB/c were purchased from Charles River Japan Inc., Kanagawa and mice of ddY were supplied from Institute for Experimental Animals, Tokushima. The tumors used were P388 lymphocytic leukemia and Sarcoma-180, Which were supplied by the Japanese Foundation for Cancer Research.

Assay for Antitumor Activity

Mice of CDF_1 strain weighing 20–21 g were inoculated with 10^6 cells of P388 lymphocytic leukemia on day 0. The preparation was injected intraperitoneally into mice daily with the specified dose levels through day 1 to 9 starting 24 h after tumor transplantation. Six mice were used at each dose. Antitumor activity was evaluated by comparing the survival time of the treated animals to that of the control animals and increase in life span was determined.

Sarcoma-180 cells (10^6 in 0.05 m*l*) obtained from ascites fluid were subcutaneously inoculated into the right flank of ICR-CR female mice of 20 g. The test sample (0.1 m*l*) was injected intratumorally for 14 consecutive days starting 24 h after tumor transplantation. Physiological saline was used as the control. The growth of solid tumor was charted weekly. The tumor size was measured by a caliper with the overlaying skin. Antitumor activity of test sample was evaluated by suppressive effect of tumor size during the observation of 42 days and also by tumor weight at the end of the experiment. All the survivors were sacrificed on the 42nd day of the experiment, and the antitumor

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activity was evaluated by comparing the mean tumor weight in treated animals to that in the control.

Proliferative Responsiveness of Splenic Lymphocytes to Mitogen

Lymphocytic cultures were performed in microphate. For tests of mitogen responsiveness, the spleen cells (10^6 cells/m*l*) of BALB/c mice were suspended in RPMI-1640 containing 10 μg/m*l* of phytohemagglutinin (PHA, from *Phaseolus vulgalis*, Seikagaku Kogyo Co., Tokyo) and 11, 33, or 100 μg/m*l* of the test sample as the final concentration, respectively. The mixture was incubated for 60 h at 37°C in a CO_2 incubator, and then the culture was labeled with 1 μCi of ^{3}H-thymidine. After 16 h the cells were harvested on a glassfiber filter. Radioactivity was counted with a liquid scintillation counter. The results were expressed in count per minutes (cpm). The degree of response was estimated by the stimulation index (SI) as follows:

$$\text{SI with PHA} = \frac{\text{cpm in the presence of the sample}}{\text{cpm in the absence of the sample}}$$

$$\text{SI without PHA} = \frac{\text{cpm in the presence of the sample}}{\text{cpm in the absence of the sample}}$$

Delayed Hypersensitivity Reaction

The delayed hypersensitivity reaction against sheep red blood cell (SRBC) was examined by measurement of the swelling of the mice footpad. In order to produce immunity to SRBC, the footpad of right hind leg of a ddY mouse was injected with 40 μl of SRBC (10^7 cells). After 4 and 14 days, the foot pad of the left hind leg was injected with the same volume of the SRBC (10^7 cells). Thickness of the footpad of left hind leg was measured with a dial thickness guage 24 h after the injection of the SRBC. The same volume of saline was used as a control. The test sample was injected intraperitoneally on every two days through day 0 to 14 after immunization with SRBC.

Hemolytic Plaque Forming Cells

Mice (BALB/c) were immunized by a single intravenous injection of 0.5 m*l* of a 20% suspension of SRBC (1×10^7 cells). Test sample was injected intraperitoneally into mice on day 1 to 4 after the immunization of SRBC. On five days after immunization the spleen was removed from mice. The appearance of antibody forming cells after the injection of SRBC antigen have been estimated by the plaque forming technique.[11-18]

Results and Discussion

Antitumor Activity

The antitumor activity of the polysaccharide preparation was assayed *in vivo* against P388 lymphocytic leukemia in CDF_1 mice. The average survival time of mice bearing P388 lymphocytic leukemia was prolonged by treatment with this material to 12.5±1.0 days (increase by 15% of control) at 50 mg/kg and to 14.5±0.5 days (increase by 34% of control) at 100 mg/kg. No significant acute toxicity was seen at any of the dose levels.

The results presented were revealed that the test sample exhibited a positive but weak antitumor activity against P388 lymphocytic leukemia by intraperitoneal injection.

As shown in Table 1, growth of the solid tumor of Sarcoma-180 was inhibited by treatment with the polysaccharide. The tumor weights were 13.2±6.3, 7.7±4.9 and 4.2±3.0 g at dose levels of 0, 25, and 100 mg/kg of the polysaccharide, respectively. This indicated that the daily injection of 25 mg/kg of this material showed 42% inhibition and 100 mg/kg showed 69% inhibition of tumor growth at 42 days after inoculation of tumor cells. The polysaccharide cured tumor

Table 1. Effect of the polysaccharide on subcutaneous growth of Sarcoma-180 tumor cells

Dose (mg/kg)	Tumor growth on day*1			Complete regression*2
	14	28	42	
Saline	11.3±2.7	19.2±4.8	30.6±6.3	0 / 6
25	12.0±2.5	13.8±8.6	21.6±13.4	1 / 6
100	9.5±1.9	9.3±5.8	14.7±11.9	2 / 6

Values are mean±SD.

*1 $\sqrt{\text{length}\times\text{width}}$ mm

*2 Number of complete regression/number of animals used.

completely in 33% of mice at 100 mg/kg.

Proliferative Responsiveness of Splenic Lymphocytes to Mitogen

The stimulation rate of lymphocyte responsiveness to PHA was 1.2 ± 0.2 and 0.95 ± 0.1 at dose levels of 11 and 33 μg/ml, respectively, which were regarded as no responsiveness. The magnitude of the proliferative response of splenic lymphocytes to PHA was reduced to 0.41 ± 0.1 in the presence of 100 μg/ml of the test sample. It was also noted that the stimulation rate of lymphocyte responsiveness to the test sample as mitogen was 3.3 ± 0.6 at dose level of 100 μg/ml.

In conclusion, the test sample reduced the immunological response to PHA to some extent, but it showed mitogen effect.

Delayed Hypersensitivity Reaction

Delayed hypersensitivity reaction was used as an index of the cellular immunity. As indicated in Table 2, treated mice showed lower reaction rates than nontreated group both on 4 and 14 days of eliciting. The polysaccharide seems to be rather immunosupressive to delayed hypersensitivity reaction immunized with SRBC in this experimental system. In general, various immunosensitive reactions including footpad test, plaque forming cell test and proliferative responsiveness of lymphocyte, have been used as indicator of the tumor resistance of hosts. However, the results of these tests are sometimes controversial.

Hemolytic Plaque Forming Cells

The numbers of plaque forming cell per spleen were $11{,}900\pm2{,}890$, $35{,}000\pm13{,}700$ and $54{,}100\pm14{,}500$ at dose levels of 0, 25 and 100 mg/kg of the polysaccharide, respectively. The polysaccharide showed 3 and 5 fold increase in number of plaques at doses of 25 and 100 mg/kg, respectively, compared with the control.

DRESSER *et al.*[18] studied the kinetics of the appearance at antibody forming cells after the injection of SRBC antigen to mice by the plaque technique, and it has been found that the kinetics of serum hemolytic antibody appearance are consistent with its production by plaque forming cells.

In the present experiment, the polysaccharide enhanced the production of plaque forming cells and the number of plaques formed varied with the concentration of the material. It seems possible that the intraperitoneal injection of this material can contribute to the improvement of the production of antibody to SRBC in mice.

Table 2. Effect of the polysaccharide on the footpad reaction immunized with SRBC

Days after immunization	Swelling of footpad* ($\times10^{-2}$ mm)		Percent of control
	Control	Treated	
4	84.0 ± 8.6	74.2 ± 5.7	88
14	81.3 ± 5.0	58.8 ± 8.9	72

* Mean$\pm$SD.

In conclusion, the polysaccharide preparation has antitumor activities against P388 lymphocytic leukemia and Sarcoma-180, acts as a mitogen, and enhances the production of hemolytic plaque forming cells. It seems that such an immunostimulant effect of this material plays some roles in this antitumor activity.

Further study would elucidate the mechanism of the action of this material.

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