SCREENING OF HOST-MEDIATED ANTITUMOR POLYSACCHARIDES BY CROSSED IMMUNOELECTROPHORESIS USING FRESH HUMAN SERUM

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Abstract—On crossed immunoelectrophoresis, human serum C3 (the third component of complement) converted by antitumor polysaccharides (ATSO [antitumor polysaccharide oral], AB-P [Agaricus blazei polysaccharide], GU-P [Grifora umbellata polysaccharide], PS-K [polysaccharide Kureha] and zymosan) moved faster than native C3, appearing as the 3rd peak. The ratio of height of the 3rd peak to the α2-macroglobulin (α2-M) peak was linearly proportional to the dose of ATS. At the dose of 500 μg/ml antitumor polysaccharides, the ratios were higher than 0.78, and the ratios for the serum treated with polysaccharide of no antitumor activity (dextran and gum arabic) were less than about 0.52. This ratio readily determined in vivo can be used as a measure for the antitumor activity of polysaccharides.

We have previously described that ATS (1–5) and GU-P (2, 6, 7) inhibit the growth of Ehrlich solid carcinoma, pulmonary tumor 7423 and NF sarcoma. ATS, AB-P (8), GU-P, PS-K (9) and zymosan (10) have been known as host-mediated antitumor polysaccharides called immunopotentiators, the action of which has been explained as due to the potentiation of a host defense mechanism through cellular immunity.

So far, screening of host-mediated antitumor agents has exclusively depended on the test using implantable tumor in animals, but this procedure is very time-consuming. Here we propose a convenient and rapid method for the characterization of these agents by crossed immunoelectrophoresis in vitro.

Materials and Methods

Materials: AB-P (AHP-F [8]) was purified from Agaricus blazei Murrill according to the procedure described by Peat et al. (11). ATS (D-II [5]) (NSC-246149) was prepared from Coriolus versicolor Iwade, which is composed of a β-(1, 3)-linked D-glucose chain with a β-(1, 6)-branching one for every three glucose residues. GU-P (GU-2 [6]) was prepared from Grifora umbellata according to the method described previously, which is a β-(1, 3)-linked D-glucan with a small number of β-(1, 6)-linked branches. PS-K isolated from Coriolus versicolor CM 101 strain was supplied by the Sankyo Pharmaceutical Co., Tokyo, Japan. Zymosan (immunological reagent) was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Dextran (M.W. 200,000–300,000) and gum arabic were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All the other chemicals used were products of Nakarai Chemicals, Ltd., Kyoto, Japan.

Anti-human whole serum: Adult male rabbits (2–3 kg) were injected into their footpads with 0.25 ml of normal human serum obtained from healthy male volunteers, together with 0.25 ml of Freund’s complete...
adjuvant. The injection was repeated after 2 weeks. One week later, the animals were sacrificed. The sera were pooled, divided into small portions and preserved at -20°C until use. The anti-human whole serum was used for preparing an immunoplate.

**Assay for antitumor activity against Sarcoma 180 solid tumor:** Five-week-old male Swiss/NIH mice (25±2 g) obtained from our Institute of Laboratory Animals were housed in cages in an air-conditioned room and supplied with commercial diet (Oriental Yeast Co., Tokyo, Japan) and water ad libitum. Sarcoma 180 solid tumor was initially supplied by the National Cancer Research Institute of Japan and maintained in our laboratory. A fragment, about 3 mm in diameter, of the 14-day-old tumor was implanted s.c. into the right groin of mice by a trocar. Each test sample of an appropriate concentration in 0.15 M NaCl was injected at doses of 1, 10, 50 and 250 mg/kg/day i.p. once daily for 10 days, starting 24 hr after the implantation of Sarcoma 180. The antitumor activity was evaluated by the inhibition ratio, \((1 - \frac{T}{C}) \times 100\%\), where \(T\) is the mean tumor weight of the animal given polysaccharides and \(C\) is that of the control animals, and by the complete regression ratio (number of tumor-free mice/number of treated mice).

**Crossed immunoelectrophoretic assay:** The C3 conversion activity of human sera (5 ml) treated with polysaccharides was assayed by two-dimensional crossed immunoelectrophoresis according to the modified Laurel method (12). An adequate amount of each polysaccharide except for zymosan was dissolved in 0.15 M NaCl to final volume of 0.1 ml and added to 0.5 ml of human serum. The mixture was incubated at 37°C for 1 hr in the presence of 5 mM Mg-EGTA. For the control, 0.5 ml of human serum was incubated with 0.1 ml of 0.15 M NaCl. The first electrophoresis was performed on 1.0% agarose ME poured onto a 2.6×7.5 cm glass plate and electrophoresed in tricine veronal buffer (pH 8.6, \(\kappa=0.05\)) at 2 mA/cm for 100 min. The gel strip was separated, cut out and transferred to an immunoplate (7.5×7.5 cm) which was prepared with anti-human whole serum. Then, the second electrophoresis was carried out at 1.5 mA/cm for 5 hr under the same conditions as above.

**Assay for residual CH50:** An adequate amount of each polysaccharide was dissolved in 0.15 M NaCl to a final volume of 0.1 ml, and the solution was added to 0.5 ml of fresh normal human serum. After 1 hr incubation at 37°C, the residual CH50 was measured by the lysis of sensitized sheep erythrocytes according to the method of Mayer (13).

**Results**

**C3 conversion by ATSO in human serum:** Crossed immunoelectrophoretic patterns of human serum treated with ATSO, an antitumor polysaccharide, are shown in Fig. 1. The converted C3 migrated to a larger mobility region, appearing as a triple peak. We designate the anodal peak as the 3rd peak. The height of the 3rd peak increased with decreasing height of the native C3 peak in parallel to the dose of ATSO. The C3 conversion was observed even in the presence of 10 /µg/ml ATSO. The ratio of height of the 3rd peak to the \(a2-M\) peak was linearly proportional to the dose of ATSO up to 500 µg/ml, as shown in Fig. 2 and Table 1.

**Antitumor activity of polysaccharides and C3 converting activity:** As shown in Table 2, AB-P, ATSO, GU-P, PS-K and zymosan inhibited the growth of Sarcoma 180 solid tumor with considerable rates of complete tumor regression. On the other hand, dextran and gum arabic showed no significant inhibition. As shown in Table 1, the ratio of the height of the 3rd peak to the \(a2-M\) peak of human sera treated with ATSO up to 500 µg/ml was higher than 0.76, while that of the sera
treated with the same dose of dextran and gum arabic was nearly equal to the control value (0.40). The difference between the sera treated with the polysaccharide having antitumor activity and that treated with those with no antitumor activity is clearly shown in the ratio.

Discussion
The antitumor activity of so-called immunopotentiators has been explained as due to the potentiation of host defense mechanism through cellular immunity. It is well known that PS-K (9), zymosan (10), lentinan (14, 15), ATSO (1-5) and a water-soluble glucan from Grifola frondosa (2, 6, 7) exert suppressive effects against solid Sarcoma 180. Polysaccharides (100-200 µg/ml) such as ATSO, AB-P and GU-P have showed no direct cytocidal action against Sarcoma 180 ascites tumor cells, Hela S3 cells and chick-embryo fibroblasts in vitro (Data not shown).

In this study, we observed that intact mice and Sarcoma 180-bearing mice given i.p. with 250 mg/kg x10 of each polysaccharide showed no signs of toxicity. Suzuki et al. (16) reported that dextran also has no direct cytotoxic action against Sarcoma 180 ascites tumor cells in vitro. The mechanism of antitumor activity of AB-P, ATSO, GU-P, PS-K and zymosan appears to be an indirect effect.
Table 1. Ratio of height of the 3rd peak to 2-M peak of human sera treated with various polysaccharides

| Polysaccharides | dose (mg/ml) | 3rd peak* | 2-M peak* | 3rd peak/2-M peak |
|-----------------|-------------|----------|--------|------------------|
| Control         |             | 10.1±1.9 | 25.1±1.3| 0.40±0.03        |
| ATSO            | 10          | 12.2±0.7 | 23.2±0.9| 0.52±0.02*       |
|                 | 50          | 16.7±1.2 | 23.0±1.2| 0.76±0.06        |
|                 | 100         | 16.8±1.1 | 20.2±0.9| 0.83±0.04*       |
|                 | 500         | 22.9±1.0 | 22.2±0.9| 0.99±0.05        |
| AB              | 500         | 22.4±1.7 | 23.0±0.9| 0.97±0.06        |
| GU-P            | 500         | 20.7±1.4 | 22.7±0.9| 0.91±0.04*       |
| PS-K            | 500         | 18.5±1.2 | 23.4±0.8| 0.79±0.02*       |
| Zymosan         | 500         | 17.4±1.0 | 22.8±0.9| 0.76±0.05*       |
| Dextran         | 500         | 11.3±1.0 | 24.1±1.3| 0.48±0.03        |
| Gum arabic      | 500         | 9.9±0.9  | 22.6±1.0| 0.43±0.04        |

* The height of the peaks from the baseline in immunoelectropherograms is expressed in mm.

Table 2. Antitumor activity of polysaccharides against Sarcoma 180 solid tumor

| Polysaccharides | Dose (mg/kg/day for 10 days) | 1 | 10 | 50 | 250 |
|-----------------|------------------------------|---|----|----|-----|
| AB              | 20.4 (10/16)                 | 53 (28/32) |    |    |    |
| ATSO            | 72.4 (9/7)                   | 92.9 (16/20) |    |    |    |
| GU-P            | 76.9 (26/20)                 | 91.6 (13/4) |    |    |    |
| PS-K            | 76.9 (26/20)                 | 10.2 (0/10) | 98.4 (0/10) | 61.0 (2/10) |
| Zymosan         | 181.4 (8/17)                 | 71.2 (0/10) | 100 (9/10) | 6.5 (0/10) |
| Dextran         | -                            | 1.2 (0/1) | 0.0 (0/10) | - |
| Gum arabic      | -                            | -7.0 (0/7) | 2.4 (0/7) | 5.6 (0/7) |
| Avon            | 0.1 (0/3)                    |    |    |    |    |

An adequate amount of each polysaccharide was dissolved in 0.15 M NaCl to a final volume of 0.25 ml. The solution was administered intraperitoneally five times a day to MH mice bearing Sarcoma 180.

* Inhibition ratio (%), was determined at the end of 3 weeks after the tumor implantation.

** Complete regression ratio (number of tumor-free mice/number of treated mice) at the end of 5 weeks after the tumor implantation. - ratio in parenthesis.

Zymosan activates an alternative pathway of the complement system (17). C3 has been demonstrated to be associated with immune phagocytosis (18), anaphylatoxin generation (19, 20), immune adherence (21), immune hemolysis (21, 22), peptidase activity (23) and potentiation of viral neutralization (24). The antitumor polysaccharides activated C3 of human serum, but dextran and gum arabic that have no antitumor activity did not. These results suggest that the complement system might play a role in the antitumor mechanism of polysaccharides. When C3 was activated by an antitumor polysaccharide, the height of the 3rd peak increased with decreasing the height of the native C3 peak in the crossed immunoelectrophoretic patterns. In an in vivo experiment, C3 cleavage occurred by the activation of C3 after the i.p. administration of an antitumor polysaccharide, and the C3 cleavage product could bind to the C3 receptor of macrophages (data not shown). It is considered that the immune potentiating action of such polysaccharides is mediated through the activation of complement C3.
Okuda et al. (25) reported that all polysaccharides which have tumor inhibition activity of solid Sarcoma 180 had C3 inactivating activity in vitro. In other experiments carried out according to the method of Mayer (13), the residual CH50 of human serum used was 45.8±4.3, while that of sera treated with the doses of 10, 50 and 100 μg/ml ATSO were 33.8±3.0, 20.6±2.8 and 13.1±2.0, respectively. At the dose of 500 μg/ml ATSO, the residual hemolytic activity was diminished completely. These results indicate an inverse correlation between the ratio of the height of the 3rd peak to the α2-M peak and the residual CH50. It may be reasonable to estimate the antitumor activity through the C3 conversion by polysaccharides.

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