EFFECT OF SERUM FROM MICE TREATED WITH ANTITUMOR POLYSACCHARIDE ON EXPRESSION OF CYTOTOXICITY BY MOUSE PERITONEAL MACROPHAGES

TAKUMA SASAKI, MOTOHIRO TANAKA AND HIROYUKI UCHIDA

Chemotherapy Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo, 104 Japan

(Received July 22, 1982)

Treatment of ICR mice *i. p.* with the antitumor polysaccharide (1→3)- β -D-glucan (TAK-N) or its carboxymethyl derivative (CM-glucan) rendered macrophages cytotoxic to L5178Y lymphoma cells *in vitro*. The ability of macrophages to inhibit tumor cell proliferation appeared on days 4 and 9 after the injections of TAK-N and CM-glucan, respectively. Peritoneal macrophages from normal untreated mice were not cytotoxic. On *in vitro* incubation with serum from mice treated with the glucans, peritoneal macrophages from normal untreated mice became cytotoxic to tumor cells. This activating effect appeared transitorily in the serum on days 2—4 and 9—10 after administrations of TAK-N and CM-glucan, respectively, to mice. Neither the glucans alone, nor serum from untreated mice induced cytotoxicity of normal mouse peritoneal macrophages *in vitro*. On fractionation of mouse serum obtained on day 4 after injection of TAK-N at a dose of 80 mg/kg, two *in vitro* activators of normal macrophage were obtained: one was a peptide (mol. wt., 4,500 daltons), and the other was probably a peptidoglycan (mol. wt., 9000 daltons).

Keywords—Antitumor polysaccharide; (1→3)- β -D-glucan; carboxymethyl-glucan; macrophage; L5178Y lymphoma cell; mouse serum; macrophage activator

INTRODUCTION

Polysaccharides with antitumor activity against certain allogeneic tumors, particularly Sarcoma 180 in ICR mice, have been isolated from diverse sources including higher plants, fungi, lichen, bacteria, and yeast.¹⁾ These polysaccharides from plant sources have not been found to exert direct action on tumor cells. Their antitumor action must therefore be dependent on the reaction of the host; namely, their effect must be host-mediated.²⁾ But, although the antitumor actions of polysaccharides are considered to be due to stimulation of the T-cell dependent humoral response,³⁾ their exact mechanisms are still unknown.

We have reported that a well-defined (1→3)- β -D-glucan (TAK-N) produced by *Alcaligenes faecalis* var. *myxogenes* IFO 13140,⁴⁾ and its water-soluble carboxymethyl-derivative (CM-glucan),⁵⁾ strongly inhibit the growth of

several *s.c.* transplantable murine tumors, and that macrophages play a dominant role in this activity.⁶⁾ During these studies we found that sera from the mice treated with the glucans enhanced peritoneal macrophage-mediated cytotoxicity *in vitro*.

Thus, as described in this paper, we studied the relation between mouse peritoneal macrophage cytotoxicity and this serum from glucan-treated mice.

MATERIALS AND METHODS

Mice—Experiments were carried out on female ICR-JCL mice, weighing about 23 g, purchased from CLEA Japan, Inc.

Drugs—TAK-N (number-average degrees of polymerization, 540) obtained from the culture filtrate of *Alcaligenes faecalis* var. *myxogenes* IFO 13140, and CM-glucan (number-average degrees of carboxymethyl groups substituted per

anhydroglucose unit, 0.68) prepared as we reported previously⁵⁾ were used throughout. These glucans were supplied by Takeda Chemical Industries, Ltd., Osaka, Japan.

Macrophages — Mouse peritoneal macrophages were obtained either from normal untreated mice or from mice after *i.p.* administration of 80 mg/kg TAK-N or 100 mg/kg CM-glucan (both optimal doses for tumor regression). Peritoneal-exudate cells were collected by *i.p.* injection of 5 ml of Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing sodium heparin (2 units/ml). For preparation of the macrophage monolayer, the cells were washed 3 times with RPMI medium 1640 (Grand Island Biological Co.) and then allowed to adhere to plastic dishes (Falcon Plastics, Oxnard, C.A.) in a humidified atmosphere of 5% CO₂ in air in an incubator at 37°C for 40 min. The dishes were washed thoroughly with RPMI medium 1640 to remove nonadherent cells, and the monolayers of macrophages were then harvested in RPMI medium 1640 containing 10% heat-inactivated fetal calf serum (Grand Island Biological Co.) and Kanamycin (50 µg/ml). In experiments on *in vitro* activation, heat-inactivated sera from mice treated with the glucans or activators purified on a Sephadex column were added to the cells.

Preparation of Sera — ICR mice were given a single *i.p.* injection of 80 mg/kg TAK-N or 100 mg/kg CM-glucan, and then at intervals mice were bled from the heart. The blood was allowed to clot at 37°C for 30 min, and then sera were obtained by centrifugation at 3000 rpm for 10 min.

Purification of TAK-N-induced Serum — On day 4 after a single *i.p.* injection of 80 mg/kg TAK-N, blood was taken from the heart of 80 ICR mice. The serum was obtained by centrifugation at 3000 rpm for 10 min, and 20 ml of serum was fractionated with Diaflo ultrafiltration membranes (PM 30, PM 10, and UM 2; Amicon Co., Lexington, Mass.). The resulting fractions 1 (mol. wt. > 30000, 925 mg), 2 (10000 < mol. wt. < 30000, 5.8 mg), 3 (1000 < mol. wt. < 10000,

11.4 mg), and 4 (mol. wt. < 1000, 170 mg), were lyophilized. Fraction 3, which caused greatest *in vitro* activation of normal macrophages, was then purified by gel filtration on a Sephadex G-25 column (Pharmacia Fine Chemicals, Uppsala, Sweden) with water as solvent (Fig. 3). The UV-absorbance at 280 nm of the effluent was monitored, and the resulting fractions were lyophilized (1.67 µg) and assayed *in vitro* for ability to induce cytotoxicity of peritoneal macrophages from normal untreated mice.

Cytotoxicity Assay — L5178Y Lymphoma cells were used as target cells *in vitro* at a ratio of macrophages to target cells of 3: 1. Monolayers of macrophages (3×10^5 cells) in 1 ml of RPMI medium 1640 with heat-inactivated 10% fetal calf serum and Kanamycin (50 µg/ml) were incubated with L5178Y cells (1×10^5 cells) in an incubator under 5% CO₂ in air at 37°C for 48 h. Then the effect of the macrophages on target cell proliferation was estimated with a phase-contrast microscope. The percentage macrophage cytotoxicity was calculated by the following formula: Macrophage cytotoxicity (%) = $(A - B) / A \times 100$, where *A* is the number of viable L5178Y cells/ml of culture after incubation with control macrophages and *B* is the number after incubation with treated macrophages; in the case of *in vitro* activation, *A* is the number after incubation with normal macrophages plus normal serum and *B* is the number after incubation with normal macrophages plus either the glucan-induced serum or the isolated activators. Each experimental group consisted of 8 animals.

RESULTS AND DISCUSSION

As previously demonstrated,⁶⁾ macrophages are important for the antitumor activity of TAK-N and CM-glucan *in vivo*. Therefore, we firstly examined the kinetics of the macrophage-mediated cytotoxicity induced by the glucans.

Peritoneal macrophages were harvested at various times after *i.p.* administration of TAK-N or CM-glucan and tested *in vitro* for their ability to evoke cytotoxic activity of macrophages to inhibit L5178Y lymphoma cell proliferation. The

FIG. 1. Kinetics of the Appearance of Cytotoxic Activity of Stimulated Macrophages from Mice Given an i.p. Injection of Glucan

Peritoneal macrophages were harvested on days 1, 2, 4, 8, and 12 after administration of 80 mg/kg TAK-N (○), and on days 2, 4, 7, 9, 12, and 16 after administration of 100 mg/kg CM-glucan (●). Growth inhibition is shown as a percentage of the growth of the control.

FIG. 2. Time Course of Change in the Macrophage Activating Effect *in Vitro* of Sera from Glucan-treated Mice

Sera were obtained at the indicated times from mice treated with 80 mg/kg TAK-N (○), or 100 mg/kg CM-glucan (●). Peritoneal macrophages were harvested from normal untreated mice. Growth inhibition is shown as a percentage of control growth. Values are means $\pm$ S.D.

cytotoxic activity of the stimulated macrophages was measured on days 1, 2, 4, 8, and 12 after a single *i.p.* injection of 80 mg/kg TAK-N and on days 2, 4, 7, 9, 12, and 16 after *i.p.* injection of 100 mg/kg CM-glucan. The results in Fig. 1 indicate that the cytotoxic activity of peritoneal macrophages from the mice appeared sooner after treatment with TAK-N than after treatment with CM-glucan, the effects of TAK-N and CM-glucan being maximal on days 4 and 9, respectively. In contrast, macrophages from control untreated mice did not show any significant cytotoxicity at any time. These glucans that altered macrophage functions *in vivo* did not affect cell viability of tumor cells in the *in vitro* system. Moreover, incubation for up to 3 d with various concentrations of the glucans alone did not cause macrophage activation *in vitro* (data not shown).

Maeda *et al.* reported that with some exceptions,⁷⁾ the concentrations of three protein components (LA, LB, and LC) increase markedly in mouse serum soon after administration of lentinan, an antitumor polysaccharide from *Lentinus edodes*, and also that there is a close relation between increase in the levels of these protein components and the antitumor activity of the polysaccharide.⁸⁾ Accordingly, we studied the correlation between peritoneal macrophage-mediated cytotoxicity and mouse serum. Three protein components markedly increased in the serum of mice administered TAK-N or CM-glucan (data not shown); their amounts reached peaks on days 3 and 9 after injections of TAK-N and CM-glucan, respectively, and then gradually decreased, returning to similar levels to those in control mice on day 15. There was a close correlation between increase in their serum levels and the appearance of peritoneal macrophage-mediated cytotoxicity in mice treated with the glucans.

In view of these observations, we examined whether the serum of polysaccharide-treated mice could induce cytotoxicity of normal macrophages *in vitro*. Peritoneal macrophages from normal untreated mice were incubated with L5178Y cells in the presence of 50 μ l of heat-inactivated serum

TABLE I. Dose-dependent Effect of TAK-N-induced Serum in *in Vitro* Induction of Macrophage-mediated Cytotoxicity for L5178Y Lymphoma Cells

Serum sample ^{a)}	Dose (μ l/ml)	No. of L5178Y cells/ml of culture
Control	—	8.53×10^5 ^{b)}
Normal serum	25	8.77×10^5 (−2.8%) ^{c)}
Normal serum	50	8.46×10^5 (0.8%)
TAK-N serum	10	8.70×10^5 (−2.0%)
TAK-N serum	25	5.78×10^5 (32.2%)
TAK-N serum	50	3.05×10^5 (64.2%)

a) Sera were obtained from either normal untreated mice or mice on day 4 after a single *i.p.* injection of 80 mg/kg TAK-N.

b) Values are numbers of viable L5178Y cells after 48-h incubation at 37°C; 1.00×10^5 L5178Y cells were initially seeded.

c) Figures in parentheses are growth inhibitions as percentages of the growth of the control.

from mice treated with TAK-N (80 mg/kg) or CM-glucan (100 mg/kg). As shown in Fig. 2, in

FIG. 3. Elution Profile of Fraction 3 (5 mg) from Sephadex G-25

Fraction 3, the fraction with highest activity obtained by ultrafiltration of TAK-N-induced serum, was applied to the column, 1.1×30 cm, and fractions of 1.5 ml were collected. The absorbance at 280 nm (○) is plotted against the fraction number. Lyophilized material from each fraction (1.67μ g/ml of cell culture) was assayed for ability to evoke macrophage-mediated cytotoxicity (■) as described in the "MATERIALS AND METHODS." Values are inhibitions as percentages of the growth in the control cultures.

the presence of serum from mice treated with the glucans, normal macrophages showed significantly higher cytotoxicity than in the presence of serum from normal untreated mice. The activation of macrophage by sera from mice treated with TAK-N and CM-glucan reached peaks on days 3 and 9, respectively.

Next, we examined the dose dependency of the *in vitro* activation of macrophages by serum of TAK-N-treated mice. Serum was obtained from either normal untreated mice or mice on day 4 after a single *i.p.* injection of 80 mg/kg TAK-N. In the periods of exposure tested, the doses of sera used had no toxicity on either macrophages or tumor cells. At doses of 25 to 50 μ l per ml of culture, serum from normal untreated mice did not evoke any significant cytotoxicity of normal macrophages *in vitro*. In contrast, serum from TAK-N treated mice caused clear dose-dependent activation of normal macrophages. The results summarized in Table I indicate that even a dose of 25 μ l per ml of culture evoked cytotoxicity of normal macrophages.

These observations indicate that there is a macrophage-activator(s) in glucan-induced serum. Therefore, we next fractionated TAK-N-induced serum with Diaflo ultrafiltration membranes. Results showed that fraction 3 (1000 < mol. wt. < 10000) caused greatest *in vitro* activation of macrophages from normal untreated

mice. Purification of this fraction by gel filtration on a Sephadex G-25 column (Fig. 3) yielded two activators: a peptide (mol. wt., 4500 daltons), and another compound, which was probably a peptidoglycan (mol. wt., 9000 daltons). The pattern of serum from CM-glucan treated mice on Sephadex G-25 was close to that obtained from TAK-N treated mice. This marked similarity suggests that the macrophage-activators from CM-glucan-induced serum may be similar if not identical to that of TAK-N. Further structural studies on these activators will be described in a later publication.

Torikai *et al.* reported that LB, the serum protein with the lowest molecular weight, has a molecular weight of about 80000 daltons⁹⁾ and also it does not evoke cytotoxicity of normal macrophages *in vitro*.¹⁰⁾ Thus the *in vitro* activators of normal macrophages described here are chemically and biologically different from the serum proteins of Maeda *et al.*⁸⁾

The further studies that are in progress on these activators of normal macrophages should provide information on the nature of the antitumor action of polysaccharides.

Acknowledgement This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare and the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) R.L.Whistler, A.A.Bushway, P.P.Singh, W.Nakahara, and R.Tokuzen: Non-cytotoxic, antitumor polysaccharides, *Advan. Carbohydr. Chem. Biochem.*, **32**, 235–274 (1976).
- 2) W.Nakahara, F.Fukuoka, R.Tokuzen, and K.Aoki: A transplantable mouse sarcoma associated with the Friend virus, *Gann*, **56**, 309–311 (1965).
- 3) Y.Y.Maeda, K.Ishimura, N.Takasuka, T.Sasaki, and G.Chihara: "Antitumor polysaccharides and host defense against cancer," ed. by D.Mizuno, G.Chihara, F.Fukuoka, T.Yamamoto, and Y.Yamamura, Pro. 5th Internatl. Symp., Princess Takamatsu Cancer Research Fund, University of Tokyo Press, Tokyo, 1975, pp. 181–197.
- 4) T.Sasaki, N.Abiko, Y.Sugino, and K.Nitta: Dependence on chain length of antitumor activity of (1→3)- β -D-glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140, and its acid-degraded products, *Cancer Res.*, **38**, 379–383 (1978).
- 5) T.Sasaki, N.Abiko, K.Nitta, N.Takasuka, and Y.Sugino: Antitumor activity of carboxymethylglucans obtained by carboxymethylation of (1→3) β -D-glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140, *Eur. J. Cancer*, **15**, 211–215 (1979).
- 6) T.Sasaki and M.Tanaka: Antimacrophage agents decrease the antitumor effect of a water-soluble carboxymethylated (1→3)- β -D-glucan, *Eur. J. Cancer*, **16**, 1271–1273 (1980).
- 7) Y.Y.Maeda, S.T.Watanabe, M.Rokutanda, and G.Chihara: Genetic control of the antitumor action and unique increase of serum protein components induced by a potent immunostimulant, lentinan, *Int. J. Immunopharmacol.*, **4**, 263 (1982).
- 8) Y.Y.Maeda, G.Chihara, and K.Ishimura: Unique increase of serum proteins and action of antitumor polysaccharides, *Nature* (London), **252**, 250–252 (1974).
- 9) T.Torikai, O.Itoh, S.Toyoshima, and T.Osawa: Purification and biological activities of a mouse serum protein increased by administration of streptococcal preparation, OK-432, *Gann*, **69**, 657–665 (1978).
- 10) T.Torikai, O.Itoh, M.Satoh, and T.Osawa: Enhancement of macrophage tumoricidal activity by a serum glycoprotein that increased in OK-432-treated mice, *Gann*, **71**, 52–59 (1980).