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Letter to the Editor

Antimacrophage Agents Decrease the Antitumor Effect of a Water-Soluble Carboxymethylated (1→3)-β-D-Glucan*

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PREVIOUSLY we reported the antitumor activities of a water-insoluble (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* (IFO 13140) and its water-soluble carboxymethylated derivatives against certain allogeneic tumors, particularly Sarcoma 180 in mice [1, 2]. Although the antitumor actions of polysaccharides are considered to be due to stimulation of the T cell-dependent humoral response [3], the mechanisms responsible for this antitumor activity are still unknown and possibly complex.

There is much current interest in tumor therapy through activation of macrophages. This prompted us to examine whether macrophages contribute to the antitumor activity of carboxymethylglucan (CM-glucan). This communication presents data showing that macrophages play an important role in the tumor growth-inhibitory activity of non-cytotoxic CM-glucan.

The 7-day-old Sarcoma 180 ascites tumor (6×10^6 cells in 0.05 ml) was transplanted s.c. into the right groin of female ICR-JCL mice weighing about 24 g. CM-Glucan prepared as reported previously [2], was administered at a dose of 10 mg/kg i.p. for 5 consecutive days from 24 hr after tumor inoculation. Silica particles, 5 μm in diameter (LiChrosorb SI60, Merck, Darmstadt), were suspended in Dulbecco phosphate buffered saline and injected i.v. at a dose of 125 mg/kg one day before tumor inoculation. Carrageenan (Type V, Sigma Chemical Co., St. Louis, Mo.) was suspended in saline by heating at 60°C for 10 min and injected i.p. into mice at a dose of 80 mg/kg one day before tumor inoculation. Poly-2-vinylpyridine *N*-oxide (PVNO, Polysciences, Inc., Warrington, Pa.) was injected s.c. at a dose of 200 mg/kg in saline 2 days before tumor inoculation. Mice were kept under observation for 5 weeks and then killed for final evaluation of the effect of treatment on tumor growth. Inhibition ratios were calculated by the following formula:

Inhibition ratio $(\%) = (A-B)/A \times 100$, where A is the average tumor weight of the control group and B is that of the treated group.

As shown in Table 1, treatment of ICR mice with CM-glucan clearly inhibited tumor growth (inhibition ratio, 100%). If macrophages play a role in its effect, the agents such as silica [4] and carrageenan [5], which decrease the macrophage number or function, should reduce the effect of CM-glucan. Suppression of macrophage function *in vivo* by injection of silica resulted in a significant decrease in the antitumor activity of CM-glucan: the tumor inhibition ratio was reduced to 56.6%, regression of tumors not being complete. The weight of tumors in mice treated with silica alone was 8.85 g, indicating promotion of tumor growth, as compared with that in controls (tumor weight, 6.95 g). Rios and Simmons [6] showed that PVNO, a macrophage-stabilizing agent, reverses the immunosuppressive effects of substances known to lyse macrophages. The tumor weight of mice treated with PVNO alone (6.68 g) was slightly,

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Table 1. *Inhibition of growth of Sarcoma* 180 *solid form in mice by CM-glucan and its reversal by antimacrophage agents*

Tumor treatment*		Average tumor weight (g)	Tumor inhibition ratio (%)	Complete regression†
Control		6.95±1.64‡	—	0/5
CM-Glucan	(10 mg/kg, i.p.)	0	100	4/4
CM-Glucan	(10 mg/kg, i.p.)			
Silica	(125 mg/kg, i.v.)	3.02±4.99	56.6	0/5
CM-Glucan	(10 mg/kg, i.p.)			
Silica	(125 mg/kg, i.v.)	0.10±0.10	98.6	4/6
PVNO	(160 mg/kg, s.c.)			
CM-Glucan	(10 mg/kg, i.p.)			
Carrageenan	(80 mg/kg, i.p.)	3.80±2.61	45.3	0/6
CM-Glucan	(10 mg/kg, i.p.)			
Carrageenan	(80 mg/kg, i.p.)	1.57±2.52	77.4	4/6
PVNO	(160 mg/kg, s.c.)			
PVNO	(160 mg/kg, s.c.)	6.68±1.92	3.9	0/5
Silica	(125 mg/kg, i.v.)	8.85±3.53	−27.3	0/4
Carrageenan	(160 mg/kg, i.p.)	8.47±4.74	−21.9	0/6

*On day 0, 6×10^6 Sarcoma 180 cells were injected s.c.; the ICR-JCL mice were killed and tumor weight were assessed on day 35.
†No. of tumor free mice/No. of mice tested.
‡Values are means±S.D.

but not significantly less than that of the control group (6.95 g). Combined treatment with PVNO, silica and CM-glucan resulted in a tumor inhibition ratio of almost 100% and a high rate of complete tumor regression. Thus the suppressive effect of silica on the antitumor activity of the glucan was completely reversed by the macrophage-stabilizing agent PVNO. Carrageenan also markedly decreased the antitumor activity of CM-glucan (inhibition ratio, 45.3%) and PVNO again effectively reversed this effect on the antitumor activity of CM-glucan (inhibition ratio, 77.4%) with complete tumor regression in 4 of 6 mice.

The present *in vivo* results demonstrate that the antimacrophage agents silica and carrageenan significantly decreased the antitumor activity of CM-glucan and that PVNO almost completely reversed these effects of the two agents on the activity of CM-glucan.

For assessment of macrophage cytotoxicity, mice were killed by cervical dislocation 15 days after tumor inoculation, since regression of Sarcoma 180 treated with CM-glucan occurred 10 days after the end of therapy and was not preceeded by retardation of tumor growth at the time of therapy [2]. Peritoneal cells were collected by i.p. injection of 5 ml of Hanks' balanced salt solution containing sodium heparin (2 U/ml). The cells were washed three times by RPMI-1640 medium and then allowed to adhere to plastic dishes for 40 min at 37°C. The dishes were washed thoroughly with RPMI-1640 medium to remove non-adherent cells, and the monolayers of macrophages were then harvested in RPMI-1640 medium containing 10% fetal bovine serum and Kanamycin (50 μg/ml). L5178Y Lymphoma cells which were syngeneic to DBA/2 mice, were used as target cells *in vitro* at a ratio of macrophages: target cells of 3:1. Monolayers of macrophages (3×10^5 cells) were incubated with L5178Y (1×10^5 cells) in a CO_2-gas incubator at 37°C for 48 hr and then viable L5178Y cells were counted 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 the viable L5178Y cells/ml of culture after incubation with macrophages from the control group and B is the number after incubation with macrophages from the treated group. Each experiment was performed twice, and similar results were obtained.

To exclude the possibility of direct cytotoxicity of CM-glucan on the target cells, we incubated L5178Y cells in the absence or presence of 50 or 100 μg CM-glucan per ml of RPMI-1640 medium for 48 hr. No significant

change in L5178Y cell viability was found in the presence of either 50 or 100 μg CM-glucan per ml.

Although macrophages from tumor-bearing control mice did not alter L5178Y proliferation significantly, macrophages harvested 10 days after the last injection of CM-glucan caused 60% inhibition of L5178Y cell proliferation. A typical experiment is shown in Table 2. The macrophage cytoxicity of mice treated with CM-glucan plus silica was greatly reduced to 2.7%. On the other hand, macrophages from mice treated with CM-glucan plus silica and PVNO showed the same cytotoxic activity as those from mice given CM-glucan alone.

Clearly the mechanism of suppression by polysaccharides of transplantable tumors in mice, requires further investigation. But both *in vivo* and *in vitro* studies with macrophages, indicate that the antitumor effect of CM-glucan is immunologically nonspecific and is mediated by macrophages.

Table 2. Effect of in vivo *treatments with CM-glucan, CM-glucan plus silica, and CM-glucan plus silica and PVNO on induction of macrophage-mediated cytotoxicity for L5178Y cells*

Macrophage source*	No. of L5178Y cells/ml of culture
Control	8.63×10^5†
CM-Glucan (10 mg/kg, i.p.)	3.45×10^5 (60.0%)‡
CM-Glucan (10 mg/kg, i.p.) Silica (125 mg/kg, i.v.)	8.40×10^5 (2.7%)
CM-Glucan (10 mg/kg, i.p.) Silica (125 mg/kg, i.v.) PVNO (160 mg/kg, s.c.)	3.30×10^5 (61.8%)

*Macrophages from 3 mice in each group were harvested and pooled 15 days after tumor inoculation (10 days after the last i.p. injection of CM-glucan) and allowed to react with L5178Y cells.

†Data represent the number of viable L5178Y cells after 48 hr incubation at 37°C; 1.04×10^5 L5178Y cells were initially seeded.

‡Figures in parentheses are percentage inhibitions compared with the control value (0%).

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