Polysaccharides in Fungi. XXXVII. 1) Immunomodulating Activities of Carboxymethylated Derivatives of Linear (1→3)-α-D-Glucans Extracted from the Fruiting Bodies of Agrocybe cylindracea and Amanita muscaria

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Immunomodulating activities of three carboxymethylated derivatives (AG-AL-CMS, AG-AL-CMI, and AM-APP-CM) of linear (1→3)-α-D-glucans from Agrocybe cylindracea and Amanita muscaria were evaluated with murine peritoneal macrophages playing an important role in tumor immunity. The ratio of macrophages in peritoneal exudate cells increased more than 50% after the administration of three carboxymethylated (1→3)-α-D-glucans. These carboxymethylated (1→3)-α-D-glucans exhibited higher potentiating activities for macrophages than carboxymethylated linear (1→3)-β-D-glucan (CMPS) in the potency of reduction of nitro blue tetrazolium, products of nitric oxide and the soluble cytotoxic factor, the amount of glucose consumption, and the activation of acid phosphatase. AG-AL-CMS, AG-AL-CMI, and AM-APP-CM were found to induce the tumor regressing factor in mouse serum, although the ability of the induction of this factor was weaker than that of CMPS. The reticuloendothelial system-potentiating activation of three carboxymethylated α-D-glucans similar to that of the carboxymethylated β-D-glucan, AG-AL-CMS and AG-AL-CMI, but not AM-APP-CM, were suggested to possess a higher-order structure, resulting from the formation of a fluorescent complex with aniline blue.

Key words polysaccharide; (1→3)-α-D-glucan; carboxymethylation; macrophage; tumor regressing activity; immunomodulator

Two β-(1→6)-branched (1→3)-β-D-glucans, lentilian and schizophran, have been used in the clinical therapy of cancers of the stomach and uterine cervix, respectively, in Japan. 2) Antitumor (1→3)-β-D-glucans show no direct cytotoxic activity against tumor cells, 3) but rather, their activity has been reported on conformation, such as triple helical or single helical structure. 4) The administration of (1→3)-β-D-glucans induces regulation of the system of immunological surveillance and raises the resistance of host against tumor cells. 5) Macrophages have been reported to be important cells in the immune system. When macrophages are activated by the administration of (1→3)-β-D-glucans to mice, the induction of cytokines such as interleukin-1 and tumor necrosis factor are reported. 6) However, such immunomodulating activities of chemical modified linear (1→3)-α-D-glucans have not been investigated.

We recently isolated linear (1→3)-α-D-glucans, AG-AL and AM-APP, from the fruiting bodies of Agrocybe cylindracea 9) and Amanita muscaria, 10) respectively, and reported their structures and antitumor activities of carboxymethylated derivatives of AG-AL and AM-APP, which were named AG-AL-CMS, AG-AL-CMI and AM-APP-CM, respectively. 9, 10) The degrees of substitution (DS) and the location of carboxymethyl groups in AG-AL-CMS, AG-AL-CMI and AM-APP-CM were determined by the method developed in our own laboratory. 11) The DS of each α-D-glucan derivative was estimated to be 0.80, 0.82, 12) and 0.95, 10) respectively. The carboxymethyl groups in AG-AL-CMS and AG-AL-CMI were mono-substituted at O-2, at O-4, and at O-6, and di-substituted at O-2 and O-4 and at O-2 and O-6 on each glucose unit. Water-soluble AG-AL-CMS had more substituents at O-2 and at O-6 than gelatinous AG-AL-CMI. 12) Also, the groups in AM-APP-CM were mono-substituted at O-2, at O-4, and at O-6, and di-substituted at O-2 and O-6 and at O-4 and O-6 on each glucose unit. AM-APP-CM had more substituents at O-2, at O-2 and O-6, and at O-4 and O-6 than AG-AL-CMS and AG-AL-CMI, compared with the amounts of substituents at O-2, at O-4 and unsubstituted glucose residue. 10) Water-soluble AG-AL-CMS, gelatinous AG-AL-CMI and water-soluble AM-APP-CM exhibited the most potent antitumor activity against sarcoma 180 solid tumor implanted in mice, although the original (1→3)-α-D-glucans had little antitumor activity. 9,10)

In this paper, the immunomodulating activities of three carboxymethylated (1→3)-α-D-glucans (AG-AL-CMS, AG-AL-CMI and AM-APP-CM) are studied regarding their potentiating activities for various functions of murine macrophages, the induction of tumor regressing activity and cytotoxicity against L929 fibroblasts, and their reticuloendothelial system-potentiating activity. These activities were compared with that of a carboxymethylated linear (1→3)-β-D-glucan (CMPS: DS of carboxymethyl groups, 0.68; the carboxymethyl groups were mono-substituted at O-4 and at O-6, and di-substituted at O-4 and O-6 on each glucose unit) 12) which has been reported to exhibit antitumor activity against sarcoma 180 transplanted in mice. 13)

MATERIALS AND METHODS

Materials and Animals Two carboxymethylated derivatives (AG-AL-CMS and AG-AL-CMI) of a linear (1→3)-α-D-glucan from Agrocybe cylindracea and a carboxymethylated derivative (AM-APP-CM) of a linear (1→3)-α-D-glucan from Amanita muscaria were prepared by the method previously reported. 9,10) A linear (1→3)-β-D-glucan (PS, degree of polymerization: 540) from

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Alcaligenes faecalis var. myxogenes IFO 13140, and the carboxymethylation product of PS\textsuperscript{14}\textsuperscript{14} (CMPS\textsuperscript{13}) were kindly supplied by Takeda Pharmaceutical Industry Co., Ltd., Osaka, Japan. Carboxymethylcellulose (CMC) was kindly provided by Daicel, Osaka, Japan. All reagents were of special reagent grade. Mice of the ddY and ICR strain were purchased from Japan SLC, Hamamatsu.

**Determination of Molecular Weight** Each polysaccharide (2.4 mg/0.5 ml) was applied onto a Toyopearl HW-65 column (1.5 cm \( \times \) 97.5 cm, Tosoh Industry) equilibrated with 1 m NaOH containing 0.1% NaBH\textsubscript{4} or with 0.1 m NaCl, and then eluted with the same solution at a flow rate of 7 ml/h. An aliquot of each fraction (4 ml) was analyzed by the phenol-sulfuric acid method.\textsuperscript{15}\textsuperscript{15} Molar weight (Mw) standards used were dextran T-110 (Mw: 105000), dextran T-150 (154000), dextran T-250 (253000), and dextran T-500 (495000) (Pharmacia Fine Chemicals).\textsuperscript{9}\textsuperscript{9}

**Formation of Complexes with Aniline Blue** Polysaccharides were dissolved in 10 mg/l of aniline blue solution in 0.1 m NaOH containing 0.5 m NaCl. Fluorescence intensity was measured at an excitation wave length of 395 nm and an emission wave length of 495 nm using a Hitachi 204-S fluorescence spectrophotometer.\textsuperscript{16}\textsuperscript{16}

**Assay for Induction of Tumor Regressing Factor** The assay was performed according to the method described by Kunimoto et al.\textsuperscript{17}\textsuperscript{17} Each polysaccharide dissolved in sterile saline was administered intraperitoneally (i.p.) to ICR female mice (6 weeks old) bearing 14d-old sarcoma 180 at a dose of 100 mg/kg. Blood was collected 14—16 h after the administration, kept on ice for 30 min, and the serum was prepared by centrifugation. All sera were stored at -40°C until use. Two hundred \( \mu l \) of the serum was injected intravenously (i.v.) via the tail vein to other ICR female mice (6 weeks old) bearing sarcoma 180 on day 14 of tumor growth. Three mice were used for each sample. The tumors were extripated 24 h later, minced, and incubated at 37°C for 2 h with stirring in an enzyme mixture containing 0.125% trypsin, 0.1% collagenase, and 0.02% deoxyribonuclease I in Eagle’s minimum essential medium (EMEM) using a volume of 20 times the tumor weight. In the resulting cell suspensions, the total number of tumor cells and other cells was detected by differential counting of fixed and stained preparations under a phase-contrast microscope, or by viable tumor cell counting under a phase-contrast microscope.

**Carbon Clearance Test** The test was examined in mice as described previously.\textsuperscript{18}\textsuperscript{18} Male ddY mice (5 weeks old) received an i.p. injection of polysaccharide samples and zymosan (Tokyo Kasei Kogyo Co.), as a positive control, dissolved in sterile saline at a dose of 50 mg/kg. Forty-eight h after administration, 0.3 ml of colloidal carbon solution consisting of 3 ml of Perikan drawing ink 17 black (Perikan AG, Germany), 4 ml of saline, and 4 ml of 3% gelatin solution was administered i.v. via the tail vein. A 50 \( \mu l \) aliquot of the blood was taken every 5 min from the orbital vein with a heparinized hematocrit tube after the injection of the carbon solution, then immediately mixed with 4 ml of 0.1% (w/v) Na\textsubscript{4}C\textsubscript{2}O\textsubscript{4}. The concentration of the colloidal carbon was estimated by the absorbance at 675 nm. The clearance rate of carbon is expressed as the half-life of carbon in the blood (\( t_{1/2} \), min), calculated by means of the following equation:

\[
K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1) \\
\]

\[
t_{1/2} = 0.693/K \\
\]

where OD\textsubscript{1} and OD\textsubscript{2} are the optical densities at times \( t_1 \) and \( t_2 \), respectively.

**Assay for Induction of Peritoneal Exudate Cells** Polysaccharide samples dissolved in sterile saline or sterile saline only for the control group were injected i.p. at a dose of 10 mg/kg into ddY male mice (6 weeks old). The mice were sacrificed on days 3, 6, and 10 after administration, and peritoneal exudate cells were prepared with Hanks’ balanced salt solution (HBSS). After the total number of cells was determined, cells were fixed on a glass plate and stained with Giemsa’s solution. The numbers of macrophages, neutrophils and lymphocytes were estimated by differential counting of stained preparations under a binocular microscope.\textsuperscript{19}\textsuperscript{19,20}\textsuperscript{20}

**Preparation of Macrophages from Peritoneal Exudate Cells** Peritoneal exudate cells were collected with HBSS from mice, and resident macrophages in peritoneal exudate cells were isolated by discontinuous density gradient centrifugation with 46% Percoll solution (Pharmacia Fine Chemicals). The fraction containing more than 98% of macrophages was used for the experiment. After two washings with HBSS, the isolated macrophages were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin G and 100 \( \mu \)g/ml of streptomycin.

**Assay for Glucose Consumption** Macrophages were plated into a 96-well culture plate at a density of 2 \( \times \) 10\textsuperscript{5} cells per 100 \( \mu l \) well per well. One hundred \( \mu l \) of the polysaccharide samples dissolved at a concentration of 200 \( \mu \)g/ml in RPMI 1640 containing 10% fetal bovine serum were added to the culture medium and the plate was incubated at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. After incubation for 24 and 72 h, 20 \( \mu l \) of the supernatant of the culture medium was withdrawn and the glucose concentration was measured using Glucose B-test Wako (Wako Pure Chemical Industries) based on the glucose oxidase method.\textsuperscript{21}\textsuperscript{21} Glucose consumption (%) was obtained by the equation of \( (1 - S/C) \times 100 \), where \( S \) is the glucose amount in the culture with polysaccharide, and \( C \) is the glucose amount without polysaccharide.

**Assay for Acid Phosphatase Activity** Male ddY mice (6 weeks old) were injected i.p. with test polysaccharides dissolved in saline at a dose of 10 mg/kg once a day for 3 d. Macrophages were prepared 24 h after the last injection and suspended at a density of 1 \( \times \) 10\textsuperscript{7} cells/ml into RPMI 1640 medium. A 100 \( \mu l \) aliquot of the suspension was withdrawn and centrifuged. After the collected macrophages were suspended in 0.1 ml of 0.1% Triton X-100, the acid phosphatase activity in the macrophages was measured by the use of ACP test BMY monostet (Boehringer Mannheim Co.) according to the manufacturer’s manuscript.

**Measurement of Nitric Oxide** The production of nitric oxide by macrophages was assayed according to the reported method.\textsuperscript{22}\textsuperscript{22} Male ddY mice (6 weeks old) were administered i.p. the polysaccharide samples at a dose of
of 10 mg/kg once a day for 3 d and 2 ml of protease peptone on day 4 from the first administration of the test polysaccharides. Forty-eight h after the injection of protease peptone, macrophages were prepared from peritoneal exudate cells and seeded into a 96-well plate at a density of $2 \times 10^5$ cells/well with RPMI 1640 containing 10% fetal bovine serum. To the culture medium, lipopolysaccharide (LPS, from E. coli 055: B5, Difco Laboratories) was added at a final concentration of 10 µg/ml and the plate was incubated at 37°C for 24 and 72 h in a CO₂ incubator. Nitric oxide in the supernatant of the culture medium was assayed using Griess' reagent with NaNO₂ as a standard. Fifty µl of the supernatant was withdrawn, mixed with an equal volume of the reagent, and allowed to stand at room temperature for 10 min. The absorbance of the mixture was measured at 570 nm with a microplate reader.

**Assays for Other Biological Activities of Macrophage**

Superoxide anion produced by macrophages was determined with nitro blue tetrazolium (NBT) as described in the previous paper. Male ddY mice (8 weeks old) received an i.p. injection of the test polysaccharides at a dose of 10 mg/kg once a day for 3 d and 2 ml of 10% protease peptone dissolved in saline on day 4 from the first injection. Forty-eight h after the last administration, macrophages were prepared and suspended at a density of $3.5 \times 10^6$ cells/ml into RPMI 1640 medium containing 10% fetal bovine serum, and seeded at a density of $3.5 \times 10^5$ cells/well onto a 96-well microplate. To the culture medium, NBT and phorbol 12-myristate 13-acetate (PMA) or NBT alone were added at final concentrations of 2 mg/ml and 0.7 µg/ml, respectively, and the plate was incubated at 37°C for 30 min under a humidified atmosphere with 5% CO₂. The supernatants of the culture medium were discarded and the plate was washed twice with HBSS and once with methanol. The resulting NBT formazan was dissolved in dimethylsulfoxide containing 1 M KOH, and the optical density of the solution was measured at 660 nm with a microplate reader.

The assay for tumor necrosis factor-like soluble monokine was carried out by the reported method involving a biological assay with L929 fibroblasts. L929 cells were commonly maintained in EMEM containing 10% fetal bovine serum, 100 unit/ml of penicillin G, and 100 µg/ml of streptomycin. DdY male mice (5 weeks old) received an i.p. injection of Corynebacterium parvum-PE (CP, RIBI Immunochem Research, Inc.) suspended in saline at a dose of 35 mg/kg. The polysaccharide samples dissolved in saline were administered i.p. at a dose of 10 mg/kg once a day for 5 d, and saline alone as the control was administered i.p. at a dose of 10 ml/kg once a day for 5 d, starting from next day of CP injection. Three days after the completion of administration with the test polysaccharides, 2 ml of 10% protease peptone was injected once and then, 2 d later, macrophages were prepared from peritoneal exudate cells. Macrophages were suspended at a density of $4 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum, 100 unit/ml of penicillin G, 100 µg/ml of streptomycin, and 10 µg/ml of LPS or without LPS, and they were seeded at a density of $4 \times 10^5$ cells/well onto a 96-well culture plate; the plate was incubated at 37°C for 24 h in a CO₂ incubator. The supernatant of the culture medium prepared by slow speed centrifugation was used for the cytotoxicity assay. L929 cells were plated at a density of $2.3 \times 10^4$ cells/well in 50 µl of EMEM containing 10% fetal bovine serum, 100 unit/ml of penicillin G, and 100 µg/ml of streptomycin onto a 96-well culture plate and the plate was incubated at 37°C for 3.5 h in a CO₂ incubator. To the culture medium, 50 µl of the supernatant and actinomycin D at a final concentration of 1 µg/ml were added and the plate was incubated at 37°C for another 41 h in a CO₂ incubator. The proliferation of L929 growth was assayed with 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1, Dojin Chemical Co., Kumamoto). To the culture medium, 10 µl of the reagent consisting of 5 nm WST-1, 0.2 nm 1-methoxy-5-methylphenazinium methylsulfate (Dojin Chemical Co., Kumamoto), and 20 nm HEPES (pH 7.5) was mixed and allowed to stand at 37°C for 3 h. The resulting water soluble WST-1 formazan was measured at 415 nm with a microplate reader. The cytotoxicity was calculated using the equation:

$$\text{cytotoxicity} \%(%) = \frac{A_s - A_i}{A_s} \times 100$$

where $A_s$ is the absorbance in the case of the test samples or saline alone (control) and $A_i$ is the absorbance in the case without macrophages (blank).

**Statistics**

The significance of differences between means was evaluated using Student's t test.

**RESULTS AND DISCUSSION**

Measurement of molecular weights of carboxymethylated (1→3)-α-D-glucans, AG-AL-CMS, AG-AL-CMI and AM-APP-CM, were performed by gel filtration on Toyopearl HW-65 in two different solutions, 0.1 M NaCl and 1 M NaOH. The average molecular weights of AG-AL-CMS, AG-AL-CMI and AM-APP-CM in 0.1 M NaCl as an elute solution were estimated to be about 390000, 730000, and 840000, respectively, while in 1 M NaOH as an elute solution, those of AG-AL-CMS, AG-AL-CMI and AM-APP-CM were about 155000, 320000, and 100000, respectively. The molecular weights of AG-AL-CMS and AG-AL-CMI in 0.1 M NaCl were more than double those determined in 1 M NaOH, but the molecular weight of AM-APP-CM showed a similar value in both solutions.

To study the higher-order structure of AG-AL-CMS, AG-AL-CMI and AM-APP-CM, the fluorescence intensity of the complex formed between the glucan and aniline blue was measured when the test compounds were dissolved in 10 mg/l of aniline blue solution in 0.1 M NaOH containing 0.5 M NaCl (Fig. 1). However, the data of AG-AL was not given because of its insolubility in the solution. The intensities of AG-AL-CMS, AG-AL-CMI, AM-APP-CM and AM-APP were weaker than that of CMPS (degree of polymerization: 540), but stronger than that of laminaran, CMC, amylose, dextran T-250, pullulan. The complex of higher molecular, carboxymethylated (1→3)-α-D-glucans, AG-AL-CMS or AG-AL-CMI, with aniline blue, showed higher fluorescence intensities than that of the lower molecular AM-APP-
CM. In the cases of higher molecular AG-AL-CMS and AG-AL-CMI, the enlargement of the molecular weight in a neutral solution compared with that in an alkaline solution and the high fluorescence intensity in the aniline blue test suggest the formation of a higher-order structure resulting from an aggregation of the AG-AL derivative molecules substituted by carboxymethyl groups. In contrast, no change in molecular weight between a neutral or an alkaline solution, and the low fluorescence intensity of lower molecular AM-APP-CM imply that the AM-APP-CM molecule may be insufficient in aggregation-ability for the formation of a higher-order structure. These facts may be ascribed to the differences in molecular size, DS, and the location of the O-carboxymethyl group in the molecule of each (1→3)-α-D-glucan derivative.

Kunimoto et al. reported\textsuperscript{17} that the tumor regressing factor which was induced in the serum by administrations of several polysaccharides, including (1→3)-β-D-glucans, to the mouse implanted with sarcoma 180, caused a decrease in the number of tumor cells and an increase in polymorphonuclear leukocytes (PMN) in the tumor lump. They also suggested that the antitumor activity of CMPS was exerted through the action of the tumor regressing factor induced in the serum. We examined the activity of the induction of the tumor regressing factor by the carboxymethylated derivatives of (1→3)-α-D-glucans. The cellular compositions in the tumor lumps carried by mice given i.v. injections of the carboxymethylated glucans are shown in Fig. 2. The numbers of sarcoma 180 cells in the groups injected the sera which were prepared by the administration of AG-AL-CMS, AG-AL-CMI, and AM-APP-CM decreased in comparison with that of the control group, which received an injection of the serum prepared by the administration of saline. The numbers in the AG-AL-CMS and AG-AL-CMI groups were less than half that in the control group. And also, the numbers of neutrophils in the AG-AL-CMS, AG-AL-CMI, and AM-APP-CM groups increased to twice that in the control group. These results showed that the induction of tumor regressing factor was caused by injection of the sera treated with AG-AL-CMS, AG-AL-CMI or AM-APP-CM, although the activities of these derivatives of α-D-glucans were lower than that of CMPS.

Next, the effect of the murine reticuloendothelial system on the phagocytic activity was examined by the carbon clearance test after the administration of AG-AL-CMS, AG-AL-CMI and AM-APP-CM at a dose of 50 mg/kg. The rate of carbon clearance by the administration of three α-D-glucan derivatives was shorter than that of the control group, longer than that of the zymosan group, and nearly equal to that of CMPS (Fig. 3). These results suggest that these derivatives of α-D-glucans possess potentiating activity for the reticuloendothelial system. It is known that schizophyllan
shows enhancing activity for the rate of carbon clearance, but lentil does not. The shortening of the clearance rate caused by the administration of schizophyllan has been suggested to be related to its antitumor activity.\(^4\,\text{26}\)

We examined the influence of the administration of carboxymethylated (1→3)-\(\alpha\)-D-glucan derivatives on the number of peritoneal exudate cells and the ratio of cell species. The number of murine peritoneal exudate cells by the administration of AG-AL-CMS, AG-AL-CMI and AM-APP-CM increased more than that of the control group (saline), and was equivalent to that of the CMPS group (Fig. 4). The ratio of macrophage occupied more than 50% of the peritoneal exudate cells.

Since macrophages have been suggested to play important roles in immunological surveillance,\(^27\) we examined immunomodulating activities such as glucose consumption, lysosomal enzyme activity, induction of nitric oxide, and reduction of NBT, focusing on macrophage activation. The activating effects of macrophages were first evaluated by the measurement of glucose consumption. After incubation of resident macrophages with the medium containing AG-AL-CMS, AG-AL-CMI, AM-APP-CM, and CMPS (100 mg/l) for 24 and 72 h, the glucose consumption of the macrophages significantly increased to 1.5 to 2 times that of the control group (Fig. 5). A large quantity of glucose is known to be consumed by macrophages for enhancing their functions, and glucose is utilized to produce the ATP and NADPH required for phagocytosis and also to produce active oxygen species.\(^28\)

These results suggested that resident macrophages are stimulated directly by AG-AL-CMS, AG-AL-CMI, and AM-APP-CM in vitro.

The effect of AG-AL-CMS, AG-AL-CMI and AM-APP-CM on macrophages in vitro was evaluated by measurement of the activity for the lysosomal acid phosphatase. The phosphatase activity by the administration of AG-AL-CMS, AG-AL-CMI and AM-APP-CM was positively compared with the control group, while the CMPS group did not affect this activity (Fig. 6).

Nitric oxide produced by the macrophages treated with AG-AL-CMS, AG-AL-CMI and AM-APP-CM was measured with Gries’ reagent (Fig. 7). In the presence of LPS, the production of nitric oxide from macrophages treated with AG-AL-CMS, AG-AL-CMI and AM-APP-CM increased significantly compared with the control group. In the absence of LPS, only the macrophages treated with AM-APP-CM among the compounds tested.

Fig. 4. Changes in the Numbers and Populations of Peritoneal Exudate Cells and in Mice Treated with Carboxymethylated Glucans

Each sample (10 mg/kg) was administered an i.p. injection for 3 consecutive days. The day after the final injection, peritoneal exudate cells were isolated with HBBSS. After the total cells were counted by the dye exclusion method with trypan blue, the cells were fixed and stained with Giemsa’s solution, and then the ratio of the cells was estimated. (A) The value expresses the arithmetic mean ± S.E. from four samples; □, macrophages; □, peritoneal exudate cell. Significant difference from the control, * \(p<0.05\), ** \(p<0.01\), *** \(p<0.005\), **** \(p<0.001\). (B) The cell population is expressed as the percent of whole peritoneal exudate cells. □, lymphocytes; □, macrophages; □, PMN.
produced nitric oxide significantly. The ability of the production of superoxide anion by the activated macrophages which were isolated from the peritoneal exudate cells after the administration of carboxymethylated glucan derivatives, was assayed by the NBT method. Macrophages prepared by the administration of AG-AL-CMS, AG-AL-CMI and AM-APP-CM reduced NBT to a similar extent as the CMS group in both the presence and absence of PMA (Fig. 8). AG-AL-CMS showed an ability to produce superoxide anions that was 1.5 times greater than the CMS group. These results suggest that macrophages primed by the i.p. injection of three carboxymethylated (1→3)-α-D-glucans possess the ability to induce nitric oxide and superoxide anion.

The functions of macrophages, such as glucose consumption, lysosomal acid phosphatase activity, and the induction of superoxide anion and nitric oxide, were
found to be enhanced by three carboxymethylated (1→3)-α-D-glucans. We examined the cytotoxic activity against L929 cells in the supernatant of the culture medium prepared by cultivating macrophages from mice which received in vivo injections of carboxymethylated (1→3)-α-D-glucans. As shown in Fig. 9, the cytotoxic activities of AG-AL-CMS and AG-AL-CMI groups increased 2 times compared with the CMPS group in the presence of LPS, while the activities did not appear in the absence of LPS. The activity of AM-APP-CM was similar to that of the control group regardless of whether it was with or without LPS. Since tumor necrosis factor has been reported to be a soluble cytotoxic factor which is produced by macrophages from mice administered antitumor (1→3)-β-D-glucans, the administration of AG-AL-CMS and AG-AL-CMI may cause the induction of tumor necrosis factor.

Although the mechanism of the antitumor action of AG-AL-CMS, AG-AL-CMI and AM-APP-CM had not yet been elucidated in detail, the present study reveals that three carboxymethylated (1→3)-α-D-glucans exhibit potentiating activities for macrophages. The antitumor activity of three carboxymethylated (1→3)-α-D-glucans may be related to the potentiation of macrophages participating in the augmentation of the host-mediated defense system against tumors, and may be exerted through the action of tumor regressin factor induced in the serum by the three carboxymethylated (1→3)-α-D-glucans.

The activities of the carboxymethylated (1→3)-α-D-glucans were higher than that of the carboxymethylated (1→3)-β-D-glucan in the consumption of glucose, lysosomal enzyme activity, reduction of NBT, induction of nitric oxide, and cytotoxicity against L929. The reticuloendothelial system-potentiating activation of the three carboxymethylated α-D-glucans was similar to that of the carboxymethylated β-D-glucan, while the activities of the carboxymethylated (1→3)-β-D-glucan were higher than that of the carboxymethylated (1→3)-α-D-glucans in the test of tumor regressing factor. This study also shows that the activation of macrophages by the carboxymethylated (1→3)-α-D-glucans is higher than the activation of macrophages by the carboxymethylated (1→3)-β-D-glucan. These effects are presumably related to the induction of antitumor activity by activation of the system of self-defense. On the other hand, differences in activation on macrophages were observed between the carboxymethylated derivatives from AG-AL and the carboxymethylated derivative from AM-APP. The functions of the derivatives from AG-AL were more effective than the functions of the derivative from AM-APP in the test of tumor regressing factor, glucose consumption, reduction of NBT, and cytotoxicity against L929. The other functions of the derivative from AM-APP were higher than those of derivatives from AG-AL in the aspects of activities on lysosomal enzyme and the induction of nitric oxide. AG-AL-CMS was higher in the test of glucose consumption and reduction of NBT than AG-AL-CMI.

The distinction of these immunomodulating activities between the carboxymethylated derivatives of AG-AL and that of AM-APP may be attributed to the difference in structural features arising from the molecular behavior, i.e. size and shape. These activities of the carboxymethylated α-D-glucans seemed to be generally similar to that of β-(1→6)-branched (1→3)-β-D-glucans, such as lentillin and schizophrenyllan. The higher-order structure of carboxymethylated AG-AL may be one of the factors which induces antitumor activity, but that of AM-APP-CM is not shown and the participation of the structure to the activity remains obscure.

REFERENCES AND NOTES