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Design of D-glucose analogue of MDP/CM-curdlan conjugate and its immunological enhancement activity

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It is well known that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is a minimum required structure of bacterial peptidoglycan responsible for immunoadjuvant activity. Although MDP itself has no antitumor activity, the MDP derivatives modified with lipophilic groups are reported to exhibit antitumor activities *in vivo*. On the other hand, curdlan (normal chain (1 → 3)-β-D-glucan) is known to exhibit an antitumor activity through stimulation of the host immuno-system. In order to provide the novel synthetic biological response modifier exhibiting high antitumor activity, we synthesized a hybrid type conjugate of curdlan immobilizing D-glucose analogue of MDP (GADP). The immunological enhancement activity of the conjugate obtained was evaluated by the test of glucose consumption of DMSO-differentiated HL-60 (*human promyelocytic leukemia*) cells, the test of superoxide anion (O^{2-}) production from PMA (phorbol-12-myristate-13-acetate)-differentiated HL-60 cells, and the test of cytotoxic factor production from PMA-differentiated U937 (*human monoblast leukemia*) cells. These results suggested that the immunological enhancement activity of GADP was increased by the hybridization with curdlan.

INTRODUCTION

Since *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is elucidated to be the minimum required structure responsible for the immunoadjuvant activity attributable to peptidoglycan of the bacterial cell wall, MDP has been of interest from the standpoint of tumor immunotherapy (Ellouz *et al.*, 1974; Kotani *et al.*, 1975; Merser *et al.*, 1975; Kusumoto *et al.*, 1976) (Fig. 1). However, MDP itself has no effect in suppressing the growth of a tumor (Azuma *et al.*, 1976). On the other hand, *Mycobacterium bovis* Bacille de Calmette-Guerin (BCG) cell wall is well known to be highly effective in tumor immunotherapy.

One of the remarkable differences between such a cell wall and MDP is seen in the lack of lipophilicity in the latter, since the BCG cell wall is covered with mycolic acid (a kind of fatty acid) on the surface (Azuma *et al.*, 1968). Actually it is clear by chemical modification of the primary hydroxy group of MDP that the antitumor effect of the cell wall might be attributed to the lipophilic character of the mycolic acid moiety.

Another marked difference of MDP from the cell wall might be recognized in the lack of polymeric character in MDP because the main chain of the cell wall consists of such polymeric materials as peptidoglycan (Azuma *et al.*, 1968). Moreover, the immunoadjuvant activity of the D-glucose analogue of MDP (GADP) was found to be higher than that of MDP itself (Kiso *et al.*, 1980).

On the other hand, curdlan (normal chain (1 → 3)-β-D-glucan) from *Alcaligenes faecalis* var. *myxogenes* has an antitumor activity and this activity is increased by carboxymethylation of curdlan (Sasaki *et al.*, 1979; Takahashi *et al.*, 1988). The mechanism of its antitumor action is not completely clear, but curdlan can not be shown to exert any direct action on tumor cells. Its

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antitumor action must therefore be considered to be dependent on the reaction of the host immuno-system. So we synthesized a hybrid type conjugate of curdlan immobilizing GADP (Fig. 1), evaluated its immunological enhancement activity *in vitro* by the test of glucose consumption of DMSO-differentiated HL-60 (*human promyelocytic leukemia*) cells, the test of superoxide anion (O^{2-}) production from PMA (phorbol-12-myristate-13-acetate)-differentiated HL-60 cells, and the test of cytotoxic factor production from PMA-differentiated U937 (*human monoblast leukemia*) cells. When carboxymethyl curdlan (CM-curdlan) fixes GADP through 6-amino hexanoic acid as hydrophobic spacer, GADP of water-soluble and low molecular weight can be suppressed to excrete from the body, this conjugate can be treated as a simple model of BCG cell wall, and expected to give the targetability to immunocompetent cells by enlargement of the affinity to them. Furthermore, this conjugate can be expected to possess the synergistic effect of GADP and curdlan. The present paper concerns the synthesis of the hybrid type conjugate of CM-curdlan immobilizing GADP, and its immunological enhancement activity.

EXPERIMENTAL

Materials

Acetone, methanol and tetrahydrofuran (THF) were purified by distillation. D-Glucose, sodium hydride (fine powder dispersed in mineral oil at 60% concentration), L-α-alanine, benzyl alcohol, D-glutamic acid, 6-amino hexanoic acid, dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (HOSu), *N,N'*-carbonyl-di-imidazole (CDI), triethylamine (TEA), trifluoroacetic acid (TFA), *O-t*-butyl *S*-(4,6-dimethyl-2-pyrimidinyl) thiocarbonate, dicyclohexylamine (DCHA), and palladium black were commercial grade reagent and used without further purification. Curdlan (number-average degree of polymerization: approximately 500), lipopolysaccharide (LPS; from *E. Coli* 0111:B4), ferricytochrome c and superoxide dismutase (SOD; from bovine erythrocyte) was purchased from Wako Pure Chemical Industry. Other reagents were commercially supplied and purified by the usual methods. A spray reagent of sugar coloration in TLC used the mixture of an equal volume with 20% sulfuric acid aqueous solution and 0·2% 1,3-dihydroxynaphthalene ethanol solution. Curdlan was carboxymethylated by the method described in Sasaki *et al.*, (1979). The degree of substitution (DCM, the average number of carboxymethyl group substituted per anhydroglucose unit) was 34 mol%/glucose unit. DCM was determined by a colloid titration method where a negative colloid solution (CM-curdlan) can be titrated with polycationic (methyl glycol chitosan) and polyanionic (potassium polyvinylsulfate) titrant to a conductometric end-point. With the conventional toluidine blue indicator method, a negative colloid solution was treated with excess of the polycationic titrant, which is back-titrated with the polyanionic titrant (Tôei *et al.*, 1976). HL-60, U937, and K562 cells (Shionogi & Co., Ltd) were maintained in RPMI-1640 medium (Nissui Seiyaku Co.) containing 10% heat-inactivated fetal calf serum (Hazeleton Biologics, Inc.), 2 mM of L-glutamine, 18 mM of sodium bicarbonate and 60 mg/liter of kanamycin at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cells using each test were cultured in 24-well or 96-well flat-bottomed plates (Corning Laboratory Sciences Company) in 2000 μl or 200 μl of culture medium, respectively.

Synthesis of GADP derivative

The synthesis of the GADP derivative unit was performed through five reaction steps as shown in Scheme 1.

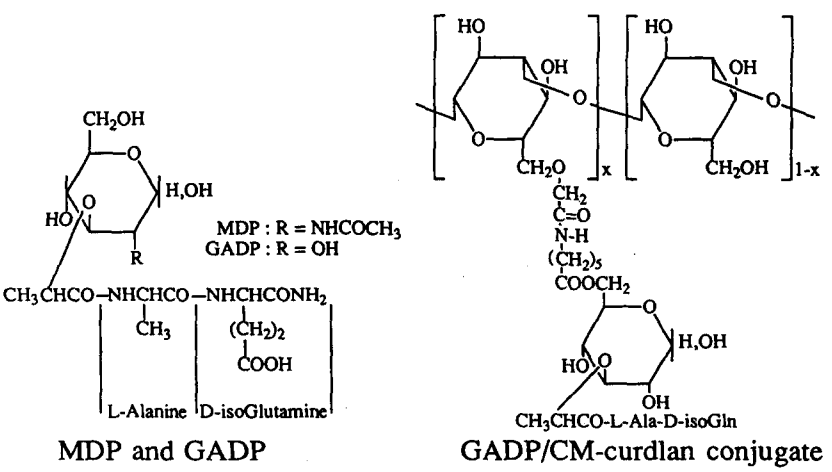
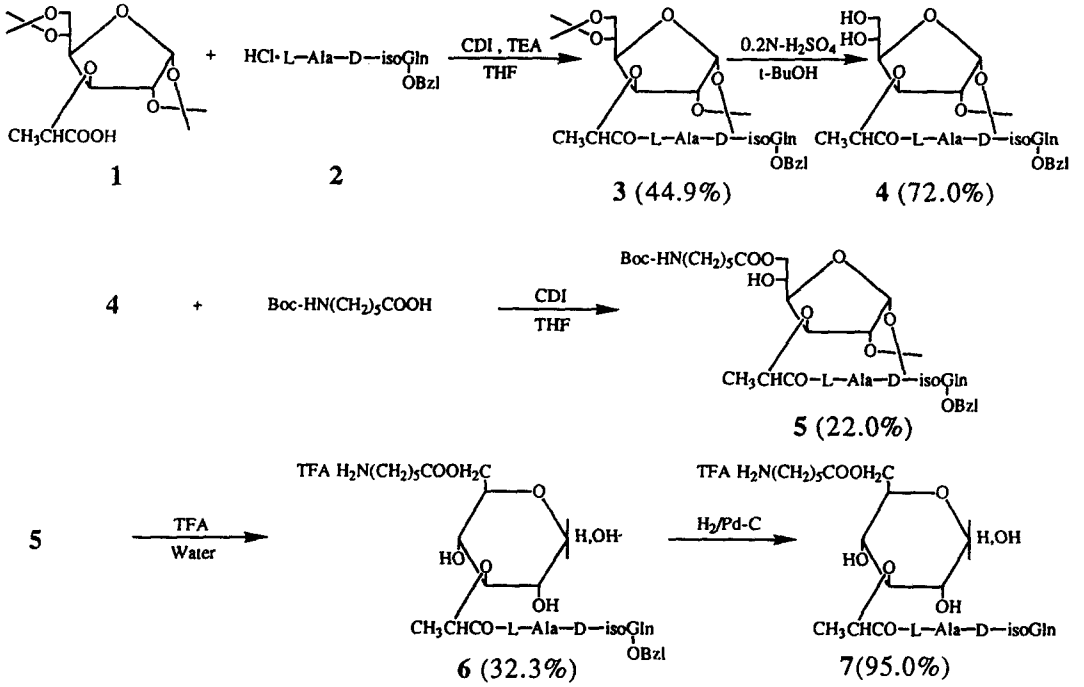


Fig. 1. Molecular structure of MDP, GADP and GADP/CM-curdlan conjugate.



Scheme. 1. Synthesis route of GADP derivative.

1,2:5,6-Di-*O*-[1-(*R*)-carboxyethyl]-α-D-glucofuranose (**1**) and L-Alanyl-D-isoglutamine γ-benzyl ester HCl salt (**2**). For the synthesis of these compounds refer to Ouchi *et al.* (1988).

1,2:5,6-Di-*O*-[(*R*)-(L-alanyl-D-isoglutamyl γ-benzyl ester) carbonyl ethyl]-α-glucofuranose (**3**). This compound was synthesized according to the method of Kiso *et al.* (1980).

N-[2-(1,2-*O*-Isopropylidene-α-D-glucofuranose-3-O-yl)-(*R*)-propinoyl]-L-alanyl-D-isoglutamine γ-benzyl ester (**4**). This compound was synthesized according to the method of Ouchi *et al.* (1988).

t-Butoxycarbonyl-6-amino hexanoic acid/ester/monoacetone glucose-CH_3CHO-L-Ala-D-isoGln-OBzl (**5**). CDI (5·2 g, 32·3 mmol) was added to an ice-cooled solution of *t*-butoxycarbonyl-6-amino hexanoic acid (5·0 g, 21·5 mmol) in THF (40 ml). The reaction mixture was stirred for 2·5 h, and then **4** (10·4 g, 17·9 mmol) in THF was added. This mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure. The residual syrup was extracted with ethyl acetate and then the ethyl acetate extract was washed thoroughly successively with 10% aqueous citric acid, saturated sodium chloride solution, 4% sodium hydrogen carbonate aqueous solution, and saturated sodium chloride solution, and dried over anhydrous sodium sulfate. After the drying agent was filtered off, the filtrate was evaporated under reduced pressure. Further purification of the residual syrup was carried out with silica gel column chromatography. Fractions containing only the main product (monitored with thin-layer chromatography (TLC)) were collected and evaporated under reduced pressure to afford a colorless syrup, 3·9 g, 4·91 mmol (22·0%).

IR (neat NaCl): absorptions at 3300 (NH), 1800 (ester), 1660 (amide), 1520 (NH_2), and 750 cm^{-1} (Ph).

^{1}H-NMR ($CDCl_3$): δ1·2–1·5 (m, 6CH_3, $(CH_2)_5$, 28H), 2·0–2·2 (m, 2CH, 2H), 2·4–2·6 (m, $(CH_2)_2$, 4H), 4·0–4·5 (m, H-2-6 of furan, 5H), 5·1 (s, CH_2Ph, 2H), 5·9 (d, H-1 of furan, 1H), 6·2,6·7 (s, 2NH, 2H), 7·3 (s, Ph, 5H), and 7·4 ppm (s, NH_2, 2H).

^{13}C-NMR ($CDCl_3$): δ17·4–18·2 (CH_3), 26·1–28·3 ($(CH_2)_5$), 30·5 (CH_2Ph), 48·9–52·4 ($(CH_2)_2$), 66·6–82·4 (furan), 105·3 ($(CH_3)_2C$), 128·2, 128·3, 128·6, 135·6 (Ph), 154·7 ($(CH_2)_5C$), and 172·1–173·5 ppm (C=O).

R_f for TLC monitored with UV_{254} and sugar coloration (developing solvent: chloroform/methanol of 10:1 V/V) was 0·32.

6-Amino hexanoic acid/ester/GADP-OBzl TFA salt (**6**). **5** (3·9 g, 4·91 mmol) in trifluoroacetic acid was stirred at room temperature for 5 h. The reaction mixture was evaporated under reduced pressure at ice-water cooling. The residual syrup which was dissolved in ethyl acetate was reprecipitated with ether. The isolation of object compound **6** obtained was carried out with silica gel column chromatography. Fractions containing only the main product (monitored with TLC) were collected and evaporated under reduced pressure to afford a colorless syrup, 1·29 g, 1·69 mmol (32·3%).

IR (neat NaCl): absorptions at 3430 (NH), 1780 (ester), 1680 (amide), 1200 (CF_3), and 700 cm^{-1} (Ph).

^{1}H-NMR (DMSO-d_6): δ1·0–1·5 (m, $(CH_2)_5$, 10H), 2·4 (m, $(CH_2)_2$, 4H), 4·0–4·5 (m, H-2-6 of pyran, 5H), 5·1 (s,

CH_2Ph, 2H), 7·1 (s, NH, 1H), 7·4 (s, Ph, 5H), and 8·0–8·1 ppm (m, NH_2, 2H).

^{13}C-NMR (DMSO-d_6): δ17·3–17·8 (CH_3), 27·0–30·0 ($(CH_2)_5$), 48·2–51·4 ($(CH_2)_2$), 65·4–83·0 (pyran), 127·8, 127·9, 128·4, 136·1 (Ph), 154·7 ($(CH_2)_5C$), and 171·7–172·8 ppm (C=O).

R_f for TLC monitored with UV_{254} and sugar coloration (developing solvent: chloroform/methanol of 5:1 V/V) was 0·33.

6-Amino hexanoic acid/ester/GADP TFA salt (GADP derivative; **7**). A solution of **6** (0·65 g, 0·85 mmol) in methanol (20 ml) was hydrogenated in the presence of palladium black (0·2 g) at room temperature for 24 h. After removal of the catalyst by filtration, the filtrate was evaporated under reduced pressure, 0·56 g, 0·83 mmol (95·0%).

IR and NMR of **7** was comparable to those of **6** except the peak (Ph) disappeared.

Synthesis of GADP/CM-curdlan conjugate

The synthesis of a CADP/CM-curdlan conjugate was performed through two reaction steps as shown in Scheme 2.

CM-Curdlan/amide/C_5/ester/GADP-OBzl (**8**). CM-Curdlan (0·47 g; obtained by treatment with sodium hydroxide and monochloroacetic acid (Sasaki *et al.*, 1979)) in THF (300 ml) was stirred at room temperature for 3 days. **6** (0·67 g, 0·88 mmol) and TEA (0·3 ml) were added to this solution and it was stirred with ice-water cooling for 1 h. CDI (0·22 g, 1·32 mmol) was further added to the reaction mixture and then was stirred with ice-water cooling for 2·5 h. Moreover, stirring was continued at room temperature for 24 h. After the solution was evaporated under reduced pressure, water was added to the residual syrup and it was reprecipitated with acetone. The precipitant was washed with methanol to give 327 mg of compound required. The degree of substitution (DGADP, the average number of GADP groups substituted per glucose unit) was estimated as 9 mol%/glucose unit by colloid titration.

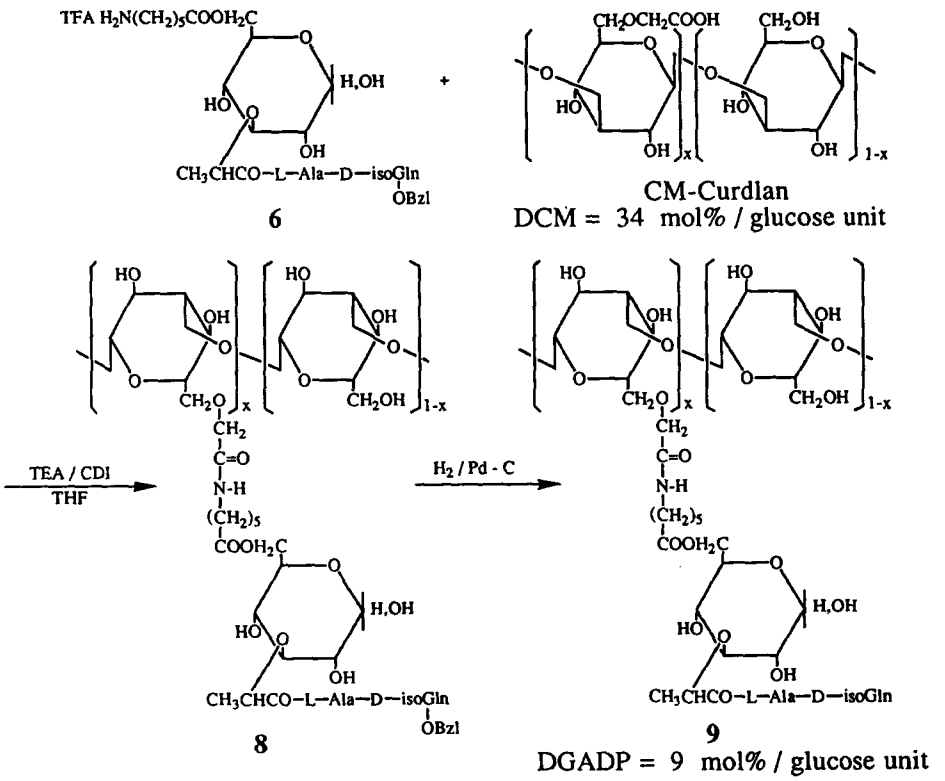
^{1}H-NMR (D_2O); δ1·0–1·5 (m, $(CH_2)_5$, 10H), 3·0–4·0 (m, H-2-6 of pyran, 5H), 5·1 (s, CH_2Ph, 2H), and 7·4 ppm (s, Ph, 5H).

CM-Curdlan/amide/C_5/ester/GADP (GADP/CM-curdlan conjugate; **9**). A solution of **8** (327 mg) in sodium hydrate solution (pH 8) was hydrogenated in the presence of palladium black (100 mg) at room temperature for 24 h. After removal of the catalyst by filtration, the filtrate was evaporated under reduced pressure and poured into a large amount of methanol to precipitate the compound **9** as a white powder.

^{1}H-NMR (D_2O): δ1·0–1·5 (m, $(CH_2)_2$, 4H), and 4·0–4·6 ppm (m, H-1-6 of pyran). The peak at δ7·4 ppm (s, Ph, 5H) disappeared.

Assay of glucose consumption activity

The HL-60 (*human promyelocytic leukemia*) cells were cultured in RPMI-1640 medium supplemented with



Scheme. 2. Synthesis route of GADP/CM-curdlan derivative.

10% fetal calf serum with kanamycin at 37°C in a 5% CO_2 atmosphere (Collins *et al.*, 1977). HL-60 cells (2×10^5 cells/well), neutrophilic differentiation (Huberman *et al.*, 1982; Harris & Ralph, 1985), were cultured for 6 days at 37°C after treatment with 0·2 M DMSO. After neutrophilic HL-60 cells activated by GADP/CM-curdlan conjugate (1000 μg/ml), LPS (1 μg/ml), CM-curdlan (760 μg/ml), and/or **7** (240 μg/ml) for 48 h at 37°C, glucose remaining in the culture supernatant was measured by the use of the Glucose B-test Wako (Adachi *et al.*, 1990; Suzuki *et al.*, 1991). The amounts of CM-curdlan and **7** were adjusted as for the contents of GADP/CM-curdlan conjugate. The supernatants (20 μl) obtained from the culture sampled for 72 h at 37°C were incubated with 3·0 ml of color reagent for 20 min at 37°C. The optical density at 505 nm of the solution was measured and the remaining glucose was determined from a calibration curve with standard glucose solution. The results were expressed as percentage glucose consumption, calculated from the following formula:

$$\text{Relative activity (\%)} = [(B - A)/(B - A_0)] \times 100$$

A_0, glucose content in culture medium cultured with DMSO-differentiated HL-60 cells; A, glucose content in culture medium cultured with DMSO-differentiated HL-60 cells and test samples; B, glucose content in culture medium without DMSO-differentiated HL-60 cells.

Assay of superoxide anion production activity

The measurement of the superoxide anion liberated from macrophage-like HL-60 cells was based on the reduction of ferricytochrome c as assayed by the increase in its absorbance at 550 nm (Newburger *et al.*, 1979; Pick & Mizel, 1981; Sato *et al.*, 1986). The HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum with kanaymycin at 37°C in a 5% CO_2 atmosphere (Collins *et al.*, 1977). HL-60 cells (1×10^6 cells/well), macrophage-like cell differentiation (Huberman *et al.*, 1982; Harris and Ralph, 1985), were cultured 2 days at 37°C after treatment with 40 nM PMA. After macrophage-like HL-60 cells were activated by GADP/CM-curdlan conjugate (1000 μg/ml), LPS (100 μg/ml), MDP (240 μg/ml), CM-curdlan (760 μg/ml) and/or **7** (240 μg/ml) for 2 h at 37°C, ferricytochrome c (6×10^{-5} M) was added to each well on a 96-well microtiter plate. After incubation for 2 min, the absorbance values of individual wells were read at 550 nm with a Corona MTP-120 microplate reader. The amount of CM-curdlan and **7** was adjusted as for the contents of GADP/CM-curdlan conjugate. In this test, SOD (100 μg/ml) was used as a negative control. Each value was calculated by comparing with the value of the control experiment without treatment.

Assay of cytotoxic factor production activity

The cytotoxic factor was measured by cytolysis assay against K562 (*human myeloid leukemia*) cells *in vitro* (Taniyama and Holden, 1979; Drysdale *et al.*, 1983; Nagano *et al.*, 1990). The U937 (*human monoblast leukemia*) cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum with kanamycin at 37°C in a 5% CO_2 atmosphere. U937 cells (1×10^6 cells/well), macrophage-like cell differentiation (Ralph *et al.*, 1982; Harris & Ralph, 1985), were cultured for 5 days at 37°C after treatment with 40 nM PMA. After macrophage-like U937 cells activated by GADP/CM-curdlan conjugate (1000 μg/ml), LPS (1 μg/ml) and MDP (240 μg/ml), CM-curdlan (760 μg/ml) and/or **7** (240 μg/ml) was adjusted as for GADP/CM-curdlan conjugate for 24 h at 37°C, each supernatant (100 μl) was added to K562 cells (4×10^4 cells/well) on a 96-well microtiter plate in the presence of actinomycin D (5 μg/ml). The amounts of CM-curdlan and **7** were adjusted as for the contents of GADP/CM-curdlan conjugate. After the K562 cells were incubated at 37°C for 48 h, cytotoxic activity was measured by the following MTT assay: MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. Stock MTT solution (10 μl per 100 μl medium) was added to all wells of an assay, and the plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0·04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Corona MTP-120 microplate reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm. The percentage cytotoxicity was defined as the relative absorbance (Abs) of sample versus control (culture medium only) wells:

$$\text{Cytotoxicity (\%)} = (1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

Values were calculated by comparing with the value of the control experiment without treatment.

Measurements

The proceeding reaction was monitored by thin-layer chromatography (TLC) with Merck F_{254} silica gel plates which were developed with chloroform/methanol (10:1 V/V) for compounds **5**, and with chloroform/methanol (5:1 V/V) for compounds **6–9**. The silica gel used for column chromatography was from the Wako Pure Chemical Industry. Optical rotations were determined with a Union Digital PM-101 polarimeter. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer. ^{1}H-NMR spectra and ^{13}C-NMR spectra were measured with a JEOL GSX-400 spectrometer at

400 MHz using TMS as the internal reference. UV spectra were measured on Shimadzu UV-240 and Corona MTP-120 microplate reader.

RESULTS AND DISCUSSION

Synthesis of GADP/CM-curdlan conjugate

GADP derivative **7** was synthesized by the five reaction steps shown in Scheme 1. The coupling reaction of **4** with *t*-butoxycarbonyl-6-amino hexanoic acid was conducted with CDI as a condensing agent. It was reported that MDP itself has no antitumor effect, but MDP which is modified by hydrophobic group does have the effect. So, 6-amino hexanoic acid was introduced to **4** as a hydrophobic spacer. Its structure was confirmed by IR, ^{1}H-NMR and ^{13}C-NMR spectra in which the signals assigned to 6-amino hexanoic acid and **4** were clearly observed. The acid removal of the 1,2-isopropylidene group and *t*-isopropylidene group and *t*-butoxycarbonyl group in **5** was carried out with trifluoroacetic acid at room temperature to afford **6** in good yield. The synthesis of CM-curdlan fixing **6** was carried out by the CDI method in THF as shown in Scheme 2. **8** was confirmed by ^{1}H-NMR spectra in which the signals assigned to the benzyl group and CM-curdlan were observed. Splitting off of the benzyl group in **9** was attempted by hydrogenation with palladium black as the catalyst in diluted alkaline solution (pH 8). It was confirmed in ^{13}C-NMR of **9** that the peak of δ7·4 ppm (Ph) disappeared.

The GADP/CM-curdlan conjugate was soluble in alkaline solution and RPMI-1640 medium, slightly soluble in water and THF. The following effects can be expected from this conjugate: the GADP unit of water-soluble and low molecular weight is suppressed to excrete from the body and this conjugate can be regarded as the simple model of BCG cell wall. The conjugate gives the targetability to immunocompetent cells by increasing the affinity to them. So, it can be expected that the antitumor activity of the conjugate is stronger than the BCG cell wall, for the conjugate increases each immunological enhancement activity and can produce a synergistic effect.

Immunological enhancement activity *in vitro*

The effect of the GADP/CM-curdlan conjugate on neutrophil or macrophage-like cell activation was evaluated by measuring the glucose consumption of DMSO-differentiated HL-60 cells, the superoxide anion production of PMA-differentiated HL-60 cells, and the cytotoxic factor production of PMA-differentiated U937 cells *in vitro*. As shown in Fig. 2, all DMSO-differentiated HL-60 cells stimulated with samples used in this assay consumed larger amounts of

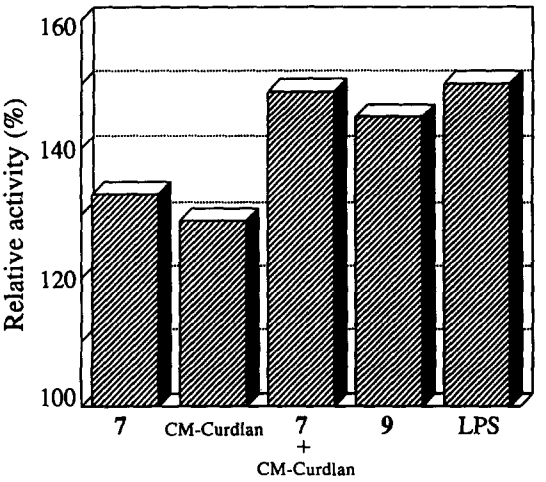


Fig. 2. Stimulating effect of GADP/CM-curdlan conjugate on glucose consumption from DMSO-differentiated HL-60 cells.

glucose than unstimulated DMSO-differentiated HL-60 cells. The GADP/CM-curdlan conjugate showed a larger increase of glucose consumption from DMSO-differentiation HL-60 cells than GADP derivative or CM-curdlan itself. The consumption of glucose by addition of GADP/CM-curdlan conjugate was comparable to a blend of GADP derivative and CM-curdlan. These results suggested that glucose consumption did not decrease with conjugation of them.

The effect of GADP/CM-curdlan conjugate on the superoxide anion production by ferricytochrome c reduction was evaluated (Fig. 3). The GADP/CM-curdlan conjugate showed a higher enhancement of the superoxide anion production from PMA-differentiated HL-60 cells than GADP derivative, CM-curdlan itself, and a blend of them. The activity of the superoxide anion production in the presence of superoxide dismutase was less than for the control. The activity of the cytotoxic factor production from PMA-

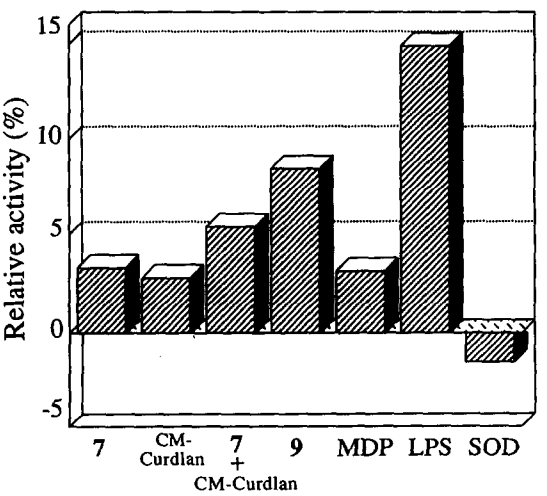


Fig. 3. Stimulating effect of GADP/CM-curdlan conjugate on the superoxide anion production from PMA-differentiated HL-60 cells.

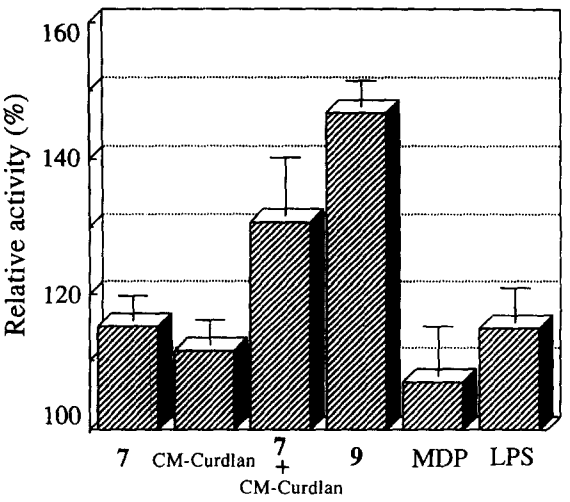


Fig. 4. Stimulating effect of GADP/CM-curdlan conjugate on cytotoxic factor production from PMA-differentiated U937 cells.

differentiated U937 cells is similar to the activity of superoxide anion production (Fig. 4). The highest activity was observed by stimulation with the GADP/CM-curdlan conjugate. It can be expected from these results that this conjugate has a high antitumor activity because the GADP unit is suppressed to excrete from the body and this conjugate gives targetability to immunocompetent cells by increase in the affinity to them *in vivo*.

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