ELSEVIER

PII:S0144-8617(96)00013-6

Carbohydrate Polymers 29 (1996) 111–118

0144-8617/96 $15.00+.00

Synthesis of muramyl dipeptide analogue–glucomannan conjugate and its stimulation activity against macrophage-like cells

Jun-ichi Murata*, Hiromu Nagae, Yuichi Ohya & Tatsuro Ouchi

Department of Applied Chemistry, Faculty of Engineering, Kansai University, Suita, Osaka 564, Japan

(Received 29 September 1995; revised version received 2 January 1996; accepted 5 January 1996)

Since the mannose receptors exist on the surface of macrophages, the branched mannose residues of glucomannan are expected to act as targeting moieties to macrophages. So, in order to achieve an efficient delivery of D-glucose analogue of muramyl dipeptide (GADP) via receptor-mediated endocytosis by mannose receptors on the surface of macrophages, the GADP/carboxymethyl(CM)–glucomannan conjugate was synthesized. Moreover, in order to study the relationship between the immunological enhancement activity of the conjugates and their mannose residues, we synthesized the GADP/CM–glucomannan conjugates having various degrees of substitution of carboxymethyl group in mol% per sugar unit (DCM) and GADP/CM–dextran conjugate through hybridization of GADP with dextran. The immunological enhancement activities of GADP/CM–glucomannan conjugates and GADP/CM–dextran conjugate were evaluated by measurements of the glucose consumption, the superoxide anion production and the β-D-glucuronidase activity from PMA (phorbol-12-myristate-13-acetate)-differentiated HL-60 (*human promyelocytic leukemia*) or U937 (*human monoblast leukemia*) cells as macrophage-like cells. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

It is well known that muramyl dipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine) is a minimum required structure of bacterial peptidoglycan responsible for the immunoadjuvant activity (Ellouz *et al.*, 1974; Kotani *et al.*, 1975; Merser *et al.*, 1975; Kusumoto *et al.*, 1976). Moreover, the immunoadjuvant activity of D-glucose analogue of MDP (GADP) was found to be higher than that of MDP itself (Kiso *et al.*, 1980). Although MDP itself has no effect in suppressing the growth of tumor (Azuma *et al.*, 1976), *Mycobacterium bovis* Bacille de Calmette-Guerin (BCG) cell wall is well known to be effective in tumor immunotherapy. The remarkable differences between such a cell wall and MDP are the lack of lipophilicity and polymeric character in the latter. Actually, it is reported that some MDP derivatives chemically modified with lipophilic groups showed antitumor activities (Phillips *et al.*, 1985; Azuma *et al.*, 1986; Alam *et al.*, 1991).

On the other hand, glucomannan purified from *Amorphophallus Konjac* is a copolymer of β-1,4-linked D-glucose and D-mannose, which has some mono-residual D-glucose or D-mannose branched at the 3-position of D-mannose units of the main chain. It is well known that macrophages, which are the target cells of the conjugates, express mannose receptors on their surfaces. Thus, an effective delivery of the conjugate to target cells can be expected to be achieved by use of cellular recognition.

In order to provide the novel synthetic biological response modifier exhibiting high antitumor activity, we previously synthesized the hybrid type conjugates of carboxymethyl(CM)–polysaccharides, such as CM–curdlan or CM–chitin, immobilizing GADP residues (Ohya *et al.*, 1993a, b, 1994). In comparison with a low molecular weight substance, a large molecule can diffuse slowly in body fluid and can be expected to prevent rapid clearance by the reticuloendothelial system. So, by conjugation of water-soluble and low molecular weight GADP onto CM–polysaccharides through 6-amino hexanoic acid spacer, the prolongation of body circulation of the GADP molecule is expected to be achieved. These conjugates can be also treated as a simple model of the BCG cell wall having lipophilicity and polymeric character. Moreover, curdlan and CM–chitin should

*Corresponding author.

express their immunological activities through the interaction with immunocompetent cells. Therefore, these conjugates can be expected to have the targetability to immunocompetent cells because of their enlargement of affinity to the cells. Furthermore, these conjugates can be expected to possess the synergistic effects of immunologically-active CM–polysaccharides and GADP.

To achieve an efficient delivery of GADP via receptor-mediated endocytosis by mannose receptors on the surface of macrophages, the present paper is concerned with the design of GADP/CM–glucomannan conjugates having mannose residues. Moreover, in order to study the relationship between the immunological enhancement activity of the conjugates and their mannose residues, we synthesized the GADP/CM–glucomannan conjugate having various degrees of substitution of carboxymethyl group in mol% per sugar unit (DCM) and GADP/CM–dextran conjugate through hybridization of GADP with dextran which have no immunological enhancement activity. The immunological enhancement activities of GADP/CM–glucomannan conjugate and GADP/CM–dextran conjugate were evaluated by measurements of the glucose consumption, the superoxide anion production and the β-D-glucuronidase activity from PMA (phorbol-12-myristate-13-acetate)-differentiated HL-60 (*human promyelocytic leukemia*) or U937 (*human monoblast leukemia*) cells as macrophage-like cells.

EXPERIMENTAL

Materials

Glucomannan obtained from *Amorphophallus Konjac* was purified according to the method described in the references (Nishida and Hashima, 1930; Sugiyama *et al.*, 1972). The content ratio of D-mannose to D-glucose for glucomannan was determined to be approximately 3/2 by gas-chromatography analysis after acidic hydrolysis of glucomannan, which is fairly well the content in previous reports (Kato and Matsuda, 1969; Maeda *et al.*, 1980). Lipopolysaccharide (LPS; from *E. coli* 0111:B4), *p*-nitrophenyl-β-D-glucuronide, ferricytochrome c, superoxide dismutase (SOD; from *Bovine erythrocyte*) and MDP were purchased from Wako Pure Chemical Industry. Organic solvents were purified by the usual distillation. Other materials were commercial grade and used without further purification. Glucomannan and dextran were carboxymethylated by the method described in Sasaki *et al.* (1979). GADP derivative and GADP/CM–glucomannan conjugates ($M_w = 1.4 \times 10^5$) in Fig. 1 were synthesized according to the previous papers (Ohya *et al.*, 1993a, b, 1994). GADP/CM–dextran conjugate ($M_w = 1.1 \times 10^5$) was synthesized by the similar method as that of GADP/CM–glucomannan conjugates. By changing the reaction condition, the feed ratio of GADP derivative to CM–glucomannan in the reaction, and by using CM–glucomannan having various values of DCM, we synthesized GADP/CM–glucomannan conjugates having various values of DCM or degree of substitution of GADP group in mol% per sugar unit (DGADP). DCM was determined by a colloidal titration method where a negative colloid solution (CM–glucomannan) can be titrated with polycationic (methyl glycol chitosan) and polyanionic (potassium polyvinyl sulfate) 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 was back-titrated with the polyanionic titrant (Tôei *et al.*, 1976). The DGADP value was determined by measurement of absorption of protected GADP derivative at 272 nm in water. HL-60 cells and U937 cells (Shionogi and 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/l of kanamycin at 37°C in a humidified atmosphere

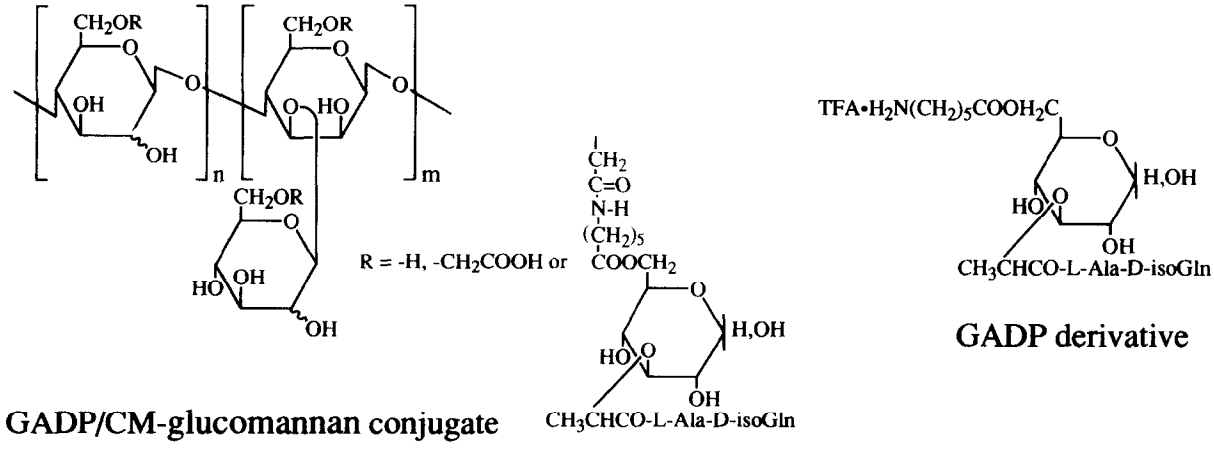

Fig. 1. Molecular structures of GADP/CM–glucomannan conjugate and GADP derivative.

containing 5% CO_2 in air. The cells used in each test were cultured in 96-well flat-bottomed plates (Corning Laboratory Sciences Company) with 200 μl of culture medium.

Measurement of immunological enhancement activity

The assay of the glucose consumption by the glucose oxidase method using glucose B-test Wako (Wako Pure Chemical Industry) and the assay of superoxide anion production activity by using ferricytochrome c against PMA-differentiated HL-60 cells were performed according to the previous papers (Ohya *et al.*,1993a, b, 1994).

The HL-60 (*human promyelocytic 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. HL-60 cells ($1x10^6$ cells/well), macrophage-like cell differentiation (Huberman *et al.*, 1982; Harris and Ralph, 1985), were cultured for 6 days at 37°C treatment with 40 nM phorbol-12-myristate-13-acetate (PMA). After macrophage-like HL-60 cells were activated by GADP/CM–glucomannan conjugate 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 supernatants (20 ml) obtained from the culture sampled for 72 h at 37°C were incubated with 300μl 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:

$$\text{Relative activity (\%)} = [(B-A)/(B \text{ to } A_0)]x100$$

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

The measurement of 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; Sato *et al.*, 1986; Pick and Mizel, 1981). The HL-60 (*human promyelocytic 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. HL-60 cells ($1x10^6$ cells/well), macrophage-like cell differentiation (Huberman *et al.*, 1982; Harris and Ralph, 1985), were cultured for 2 days at 37°C after treatment with 40 nM PMA. After macrophage-like HL-60 cells were activated by GADP/CM–glucomannan conjugate for 2 h at 37°C, ferricytochrome c ($6x10^{-5}$ M) was added to each well on 96-well microtiter plate. After incubation for 2 min, the absorbance value of individual wells was read at 550 nm with a Corona MTP-120 microplate reader. Each value was calculated by comparing with the value of the control experiment without treatment.

The β-D-glucuronidase activity in the cell lysate was measured by hydrolysis of *p*-nitrophenyl-β-D-glucuronide (Adachi *et al.*, 1990; Greenberger *et al.*, 1978). The U937 (*human monoblast leukemia*) cells were cultured in RPMI-1640 medium containing 10% fetal calf serum with kanamycin at 37°C in a 5% CO_2 atmosphere. U937 cells ($1x10^6$ cells/well) were cultured at 37°C for 5 days after treatment with 40 nM PMA to differentiate from macrophage-like cells (Ralph *et al.*, 1982; Harris and Ralph, 1985). After macrophage-like U937 cells activated by GADP/CM–glucomannan conjugate for 24 h, the macrophage-like U937 cells lysate prepared with 40 μl of 10% Triton X-100 was mixed with 100 μl of 6 M *p*-nitrophenyl-β-D-glucuronide in 0.1 M citrate buffer (pH 5.0). After incubation at 37°C for 2 h, the reaction was stopped with 100 μl of 0.2 M borate buffer (pH 9.8). The *p*-nitrophenol released by the enzyme-dependent hydrolysis of the substrate was quantified spectrophotometrically by measuring optical density at 405 nm using Corona MTP-120 microplate reader. The value of the β-D-glucuronidase activity was calculated by comparing with the value of the control experiment without treatment. In these measurements, free GADP derivative and CM–glucomannan were used as references. The simple mixture of GADP derivative and CM–glucomannan, which has the same composition as the GADP/CM–glucomannan conjugate, was also used as a reference.

RESULTS AND DISCUSSION

Conjugation effect of GADP with CM–glucomannan on immunological enhancement activity

The effect of the GADP/CM–glucomannan conjugate on activation of macrophage-like cells was evaluated by measuring the glucose consumption and the superoxide anion production from the PMA-differentiated HL-60 cells, and the β-D-glucuronidase activity from the PMA-differentiated U937 cells *in vitro*. It was known that the PMA-differentiation into macrophage-like cells made HL-60 cells increase the amount of glucose consumption and produce the superoxide anion, and U937 cells produce the β-D-glucuronidase (Huberman *et al.*, 1982; Harris and Ralph, 1985). It was observed that the macrophage-like cells unstimulated by the biological response modifier increased the amount of glucose consumption and produced the β-D-glucuronidase and the superoxide anion. Thus, the activation of stimulated macrophage-like cells was compared with that of unstimulated macrophage-like cells for the control. Moreover, the activation of stimulated macrophage-like cells

was made sure by different measurements of biological enhancement activity. The results of conjugation effect of GADP with CM-glucomannan on the glucose consumption from PMA-differentiated HL-60 cells and the β-D-glucuronidase activity from PMA-differentiated U937 cells are shown in Fig. 2, compared with those of free GADP derivative, CM-glucomannan, and the simple mixture of GADP derivative and CM-glucomannan, which has the same composition as the GADP/CM-glucomannan conjugate. The immunological enhancement activities of GADP/CM-glucomannan conjugate were highest among them. The revelation of such high activity for GADP/CM-glucomannan conjugate was suggested to be resulted from the covalent fixation of GADP to CM-glucomannan.

Dose dependence of GADP/CM-glucomannan conjugate on the activity

The results of dose dependence of GADP/CM-glucomannan conjugate on the glucose consumption from PMA-differentiated HL-60 cells, the β-D-glucuronidase activity from PMA-differentiated U937 cells are shown in Fig. 3, compared with those of GADP derivative, CM-glucomannan, and the simple mixture of GADP derivative and CM-glucomannan, which has the same composition as the GADP/CM-glucomannan conjugate. The stimulating effects of GADP/CM-glucomannan conjugate on the glucose consumption and the β-D-glucuronidase activity were highest among them in the concentration range tested. It was reconfirmed that the high immunological enhancement activity resulted from the covalent fixation of GADP to CM-glucomannan. Although the activation efficiencies of CM-glucomannan and/or GADP derivative were saturated in the high concentration range of dose, the activation efficiency of GADP/CM-glucomannan conjugate tended to increase linearly even in the high dose range. The dose-dependent increase of activity of GADP/CM-glucomannan conjugate tended to be larger than that of monomeric MDP and GADP derivative. Thus, these results suggested that the effect of multivalent-ligand formation might result from the conjugation of GADP derivative with the CM-glucomannan.

Effect of DCM on activity of GADP/CM-glucomannan conjugate

In order to investigate the relationship between the immunological enhancement activity of GADP/CM-glucomannan conjugate and its molecular structure, we synthesized the conjugates having different values of DCM (Conjugate I: DCM = 7 mol% / sugar unit, DGADP = 4 mol% / sugar unit, Conjugate II: DCM = 14 mol% / sugar unit, DGADP = 8 mol% / sugar unit, Conjugate III: DCM = 51 mol% / sugar unit, DGADP = 5 mol% / sugar unit).

The results of the effect of DCM on the stimulating activities of GADP/CM-glucomannan conjugate for the glucose consumption and the β-D-glucuronidase activity from macrophage-like cells are shown in Fig. 4. Although the immunological enhancement activities of CM-glucomannan and GADP/CM-glucomannan conjugate decreased with increasing DCM value, those of the mixture of GADP derivative and CM-glucomannan did not change even with increasing DCM value. As a reason of decrease of activities observed in CM-glucomannan and GADP/CM-glucomannan conjugates, the decrease of unmodified mannose residues of glucomannan by increase of introduction of carboxymethyl group can be pointed out. These results suggested that the balance of DGADP, DCM and degree of unmodified blanched mannose residues was very important to achieve high immunological enhancement activity of the GADP/CM-glucomannan conjugate.

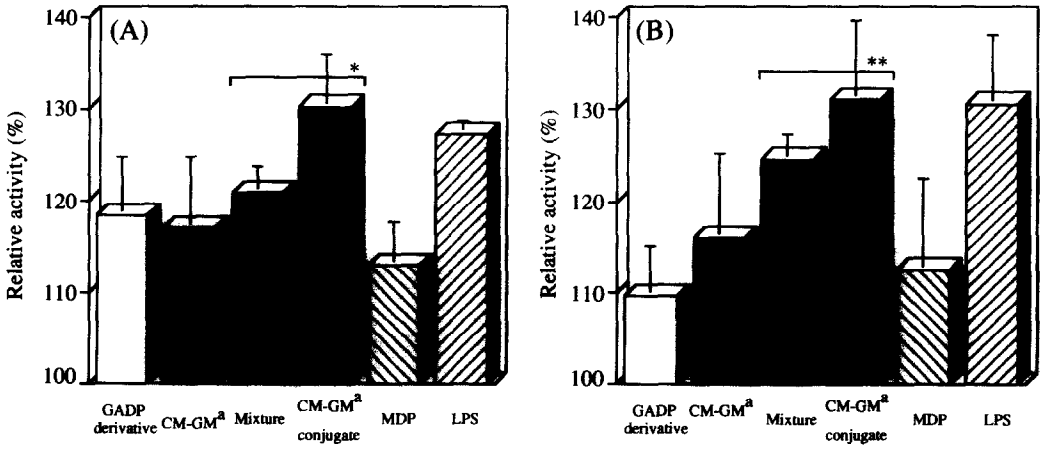

Fig. 2. Stimulating effects of GADP/CM-glucomannan conjugate on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) *in vitro*. GADP/CM-glucomannan conjugate: DGADP = 8 mol%/sugar unit, DCM = 14 mol%/sugar unit. [a] CM-glucomannan. *: $p < 0.05$, **: $p < 0.3$.

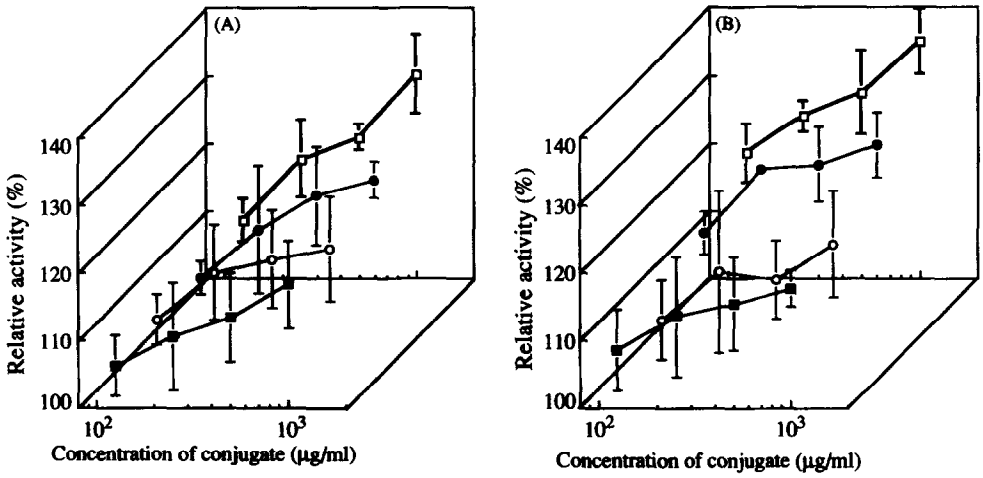

Fig. 3. Stimulating effects of GADP/CM–glucomannan conjugate on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) *in vitro*. ○: GADP derivative; □: GADP/CM–glucomannan conjugate (DGADP = 8 mol%/sugar unit, DCM = 14 mol%/sugar unit); •: mixture of GADP derivative and CM–glucomannan; ■: CM–glucomannan (DCM = 22 mol%/sugar unit). The amount of GADP derivative and CM–glucomannan was adjusted as for the content of the conjugate.

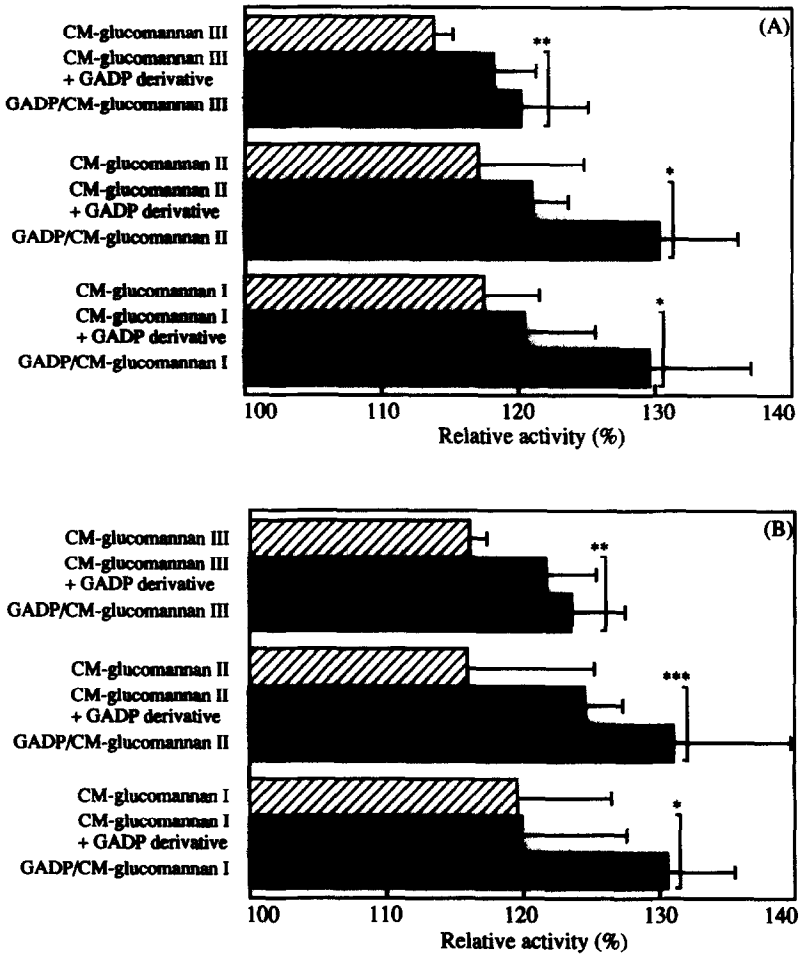

Fig. 4. Effects of DCM on the stimulating activity of GADP/CM–glucomannan conjugate, CM–glucomannan and mixture of GADP derivative and CM–glucomannan. (A): Glucose consumption from PMA-differentiated HL-60 cells, (B): β-D-glucuronidase activity from PMA-differentiated U937 cells. CM–glucomannan I: DCM = 11 mol%/sugar unit, GADP/CM–glucomannan conjugate I: DCM = 7 mol%/sugar unit, DGADP = 4 mol%/sugar unit, CM–glucomannan II: DCM = 22 mol%/sugar unit, GADP/CM–glucomannan conjugate II: DCM = 14 mol%/sugar unit, DGADP = 8 mol%/sugar unit, CM–glucomannan III: DCM = 56 mol%/sugar unit, GADP/CM–glucomannan conjugate III: DCM = 51 mol%/sugar unit, DGADP = 5 mol%/sugar unit. *: $p < 0.05$, **: $p < 0.35$, ***: $p < 0.3$.

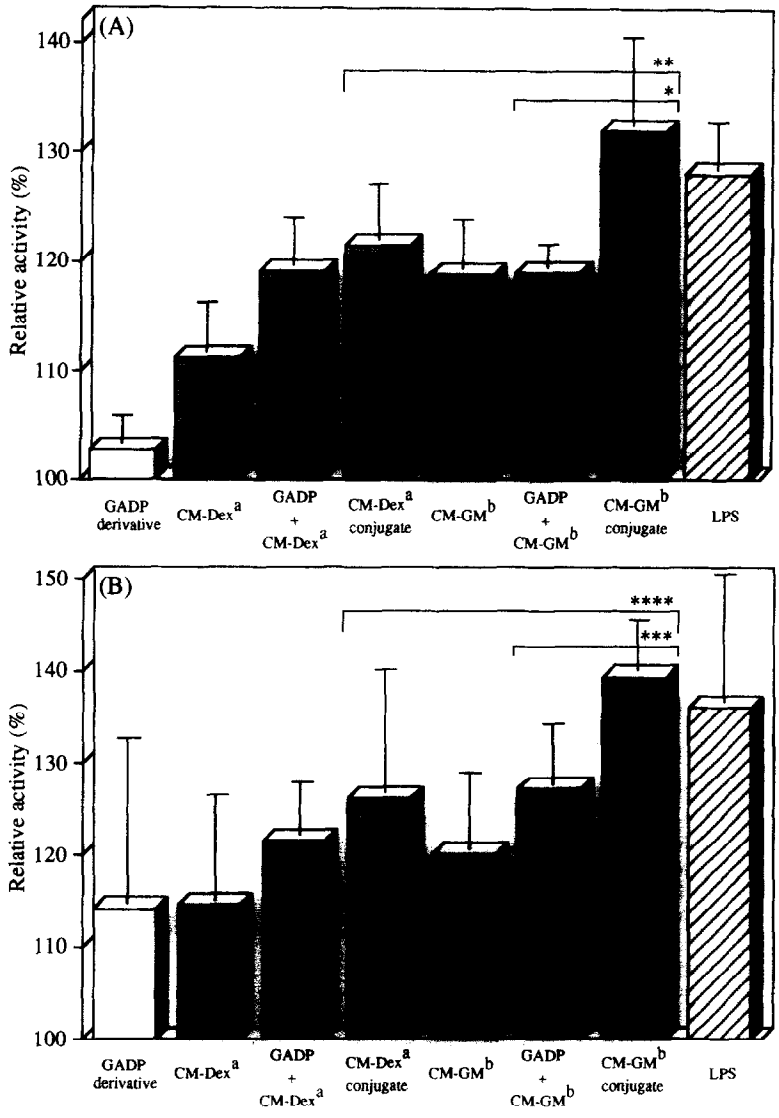

Fig. 5. Comparison of stimulating effects on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) for GADP/CM-dextran conjugate and GADP/CM-glucomannan conjugate. GADP/CM-dextran conjugate: DGADP = 6 mol%/sugar unit, DCM = 14 mol%/sugar unit GADP/CM-glucomannan conjugate: DGADP = 8 mol%/sugar unit, DCM = 14 mol%/sugar unit. [a]CM-dextran, [b]CM-glucomannan. *: $p < 0.005$, **: $p < 0.025$, ***: $p < 0.1$, ****: $p < 0.125$.

Comparison of activity of GADP/CM-glucomannan conjugate with that of GADP/CM-dextran conjugate

Dextran is well known to be a polysaccharide having no immunogenicity, no immunological enhancement activity and no mannose residues. In order to investigate the effect of hybridization of GADP with glucomannan having mannose residues and the effect of addition of polymeric character to GADP, we synthesized GADP/CM-dextran conjugate to compare immunological enhancement activity of itself with that of GADP/CM-glucomannan conjugate. The immunological enhancement activity of the GADP/CM-dextran conjugate obtained was evaluated by the glucose consumption from PMA-differentiated HL-60 cells and the β-D-glucuronidase activity from PMA-differentiated U937 cells, compared with the activities of CM-dextran, the mixture of GADP derivative and CM-dextran, CM-glucomannan, the mixture of GADP derivative and CM-glucomannan, and GADP/CM-glucomannan conjugate (Fig. 5). They were compared with LPS and MDP for positive control, which were known as the materials to make macrophage-like cells active. While the immunological enhancement activities of GADP/CM-glucomannan conjugate were much higher than those of the mixture of GADP derivative and CM-glucomannan, and CM-glucomannan itself, the immunological enhancement activities of the GADP/CM-dextran conjugate were much lower than those of GADP/CM-glucomannan conjugate containing an equivalent amount of GADP unit, as high as those of the mixture of

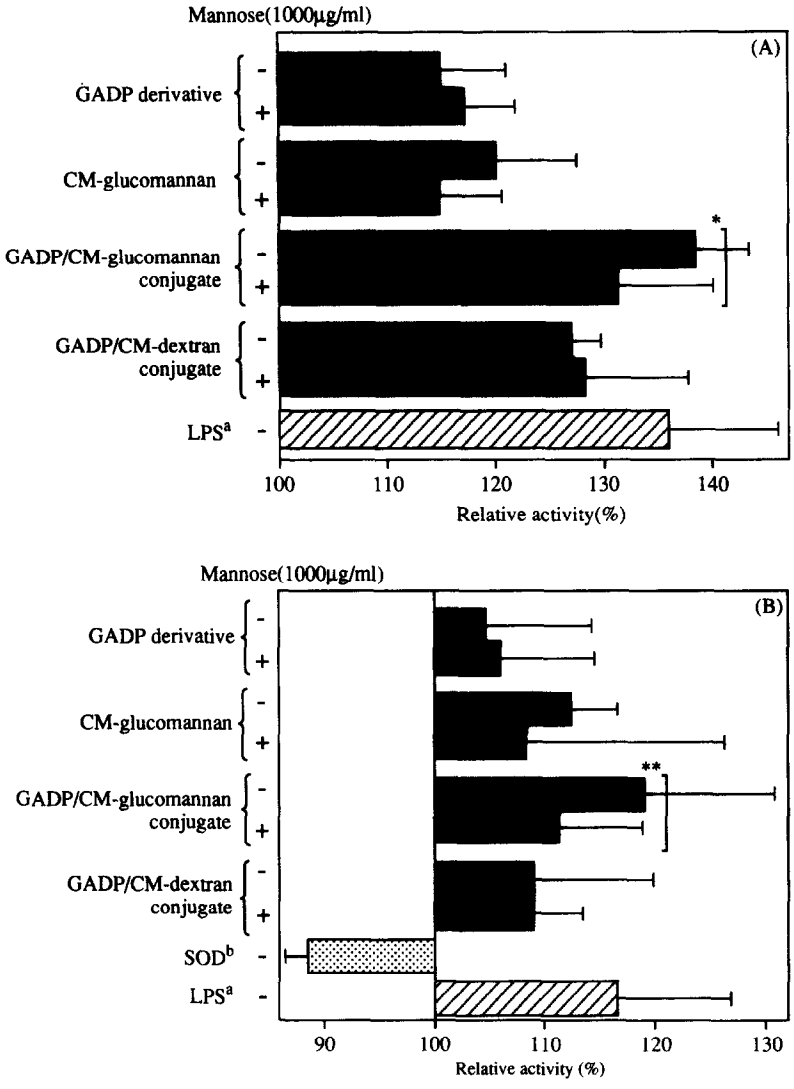

Fig. 6. Effects of mannose addition on the stimulating activity of GADP/CM–glucomannan conjugate and GADP/CM–dextran conjugate on β-D-glucuronidase activity from PMA-differentiated U937 cells (A) and superoxide anion production from PMA-differentiated HL-60 cells (B). +: addition of mannose (1000 μg/ml), –: absence of mannose, GADP/CM–dextran conjugate: DGADP = 6 mol%/sugar unit, DCM = 14 mol%/sugar unit, GADP/CM–glucomannan conjugate: DGADP = 8 mol%/sugar unit, DCM = 14 mol%/sugar unit. *: $p < 0.125$, **: $p < 0.15$. [a]: LPS was added as a positive control. [b]: In order to confirm the adequacy of assay of superoxide anion production, the addition test of SOD was carried out.

GADP and CM–dextran, the mixture of GADP and CM–glucomannan, and CM–glucomannan itself. These results suggested that the high immunological enhancement activity of GADP/CM–glucomannan conjugate was not only due to giving polymeric character to GADP, but also the hybridization of GADP with polysaccharide having branched mannose residues.

Effect of mannose addition on the stimulating activity

In order to confirm the targeting of GADP/CM–glucomannan to macrophage cells via receptor-mediated endocytosis, the inhibition effects of the stimulation activity of GADP/CM–glucomannan conjugate on the β-D-glucuronidase activity from PMA-differentiated U937 cells and superoxide anion production from PMA-differentiated HL-60 cells were investigated. The results of inhibition test by the addition of mannose are shown in Fig. 6. The adequacy of superoxide anion production could be confirmed by the decrease of its own activity with the addition of SOD (Fig. 6(B)). The stimulation activities of GADP/CM–glucomannan conjugate and free CM–glucomannan were decreased by the addition of mannose. On the other hand, those of GADP derivative and GADP/CM–dextran conjugate was little affected by the addition of mannose. These results suggested that the decreases of stimulation activity of GADP/CM–glucomannan conjugate and CM–glucomannan might be caused by the specific internalization via mannose receptor.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Hisanaga Igarashi and Mr Akihiko Sato of Shionogi Institute for Medical Science of Shionogi and Co. Ltd, for the supply of tumor cell line.

REFERENCES

Adachi, Y., Ohno, N., Ohsawa, M., Oikawa, S. & Yadomae, T. (1990). *Chem. Pharm. Bull.*, **38,** 477–481.

Alam, A., Capoor, A.K. & Rao, V. (1991). *Immunol. Lett.*, **27,** 53–57.

Azuma, I., Sugimura, K., Taniyama, T., Yamasaki, M., Yamamura, Y., Kusumoto, S., Okada, S. & Shiba, T. (1976). *Infect. Immun.*, **14,** 18–27.

Azuma, I., Yamamura, Y. & Fukushi, K. (1986). *J. Bacteriol.*, **96,** 1885–1887.

Ellouz, F., Adam, A., Ciorbaru, R. & Lederer, E. (1974). *Biochem Biophys. Res. Commun.*, **59,** 561–567.

Greenberger, J. S., Newburger, P. E., Karpas. A. and Moloney, W. C. (1978). *Cancer Res.*, **38,** 3340–3348.

Harris, P. & Ralph, P. (1985). *J. Leuk. Biol.*, **37,** 407–422.

Huberman, E., Braslawsky, G. R., Callaham, M. & Fugiki, H. (1982). *Carcinogenesis*, **3**(1), 111–114.

Kato, K. & Matsuda, K. (1969). *Agric. Biol. Chem.*, **42,** 1446–1453.

Kiso, M., Kaneda, Y., Okumura, J., Hasegawa, A., Azuma, I. & Yamamura, Y. (1980). *Carbohydr. Res.*, **79,** c17–c19.

Kotani, S., Watanabe, Y., Kinoshita, F., Shimona, T., Morisaki, I., Shiba, T., Kusumoto, S., Tarumi, Y. & Ikenaka, K. (1975). *Biken J.*, **18,** 105–111.

Kusumoto, S., Tarumi, Y., Ikenaka, K. & Shiba, T. (1976). *Bull. Chem. Soc. Jpn.*, **49,** 533–539.

Maeda, M., Shimahara, H. & Sugiyama, N. (1980). *Agric. Biol. Chem.*, **44,** 245–252.

Merser, C., Sinary, P. & Adam, A. (1975). *Biochem. Biophys. Res. Commun.*, **66,** 1316–1322.

Newburger, P.E., Chovaniec, M.E., Greenberger, J.S. & Cohen, H.J. (1979). *J. Cell Biology*, **46,** 315–322.

Nisida, K. & Hashima, H. (1930). *Nihon nougei kagaku kaishi*, **6,** 991–995.

Ohya, Y., Murata, J., Nishimoto, T. & Ouchi, T. (1993a). *J. Bioactive Compatible Polym.*, **8,** 351–364.

Ohya, Y., Nishimoto, T. & Ouchi, T. (1993b). *Carbohydr. Polym.*, **20,** 43–49.

Ohya, Y., Murata, J., Nishimoto, T. & Ouchi, T. (1994). *Carbohydr. Polym.*, **23,** 47–54.

Phillips, N.C., Moras, M.L., Chedid, L., Lefrancier, P. & Bernard, J.M. (1995). *Cancer Res.*, **45,** 128–134.

Pick, E. & Mizel, D. (1981). *J. Immunol. Methods*, **46,** 211–226.

Ralph, P., Williams, N., Moore M.A.S. & Litcofsky, P.B. (1982). *Cellular Immunol.*, **71,** 215–223.

Sasaki, T., Abiko, N., Nitta, K., Takasuka, N. & Sugino, Y. (1979). *Eur. J. Cancer*, **15,** 211–215.

Sato, T., Kojima, K., Ihda, T. & Sunamoto, J. (1986). *J. Bioact. Compat. Polym.*, **1,** 448–460.

Sugiyama, N., Shimahara, H. & Andoh, T. (1972). *Bull. Chem. Soc. Jpn.*, **45,** 561–563.

Suzuki, T., Ohno, N., Adachi, Y., Cirelli, A.F., Covian, J.A. & Yadomae, T. (1991). *Pharmacobio-Dyn.*, **14,** 256–266.

Tôei, K. & Kohara, T. (1976). *Anal. Chem. Acta*, **83,** 59–65.