Carbohydrate Polymers **23** (1994) 47–54

Immunological enhancement activity of muramyl dipeptide analogue/CM-curdlan conjugate

Yuichi Ohya,* Takehiro Nishimoto, Jun-ichi Murata & Tatsuro Ouchi

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

(Received 30 March 1993; revised version received 3 September 1993; accepted 6 September 1993)

In order to provide a novel synthetic biological response modifier exhibiting high antitumor activity, GADP (D-glucose analogue of muramyl dipeptide (MDP)) has been conjugated with carboxymethyl (CM)-curdlan (normal chain (1 → 3)-β-D-glucan) which has antitumor activity through stimulation of the host immunosystem. The immunological enhancement activities of the conjugate obtained were investigated against macrophage-like HL-60 (human promyelocytic leukemia) or U937 (human monoblast leukemia) cells to be enlarged by means of coupling of GADP with CM-curdlan. In this paper, in order to discuss the relationship between the immunological enhancement activities of the conjugate and its molecular structure, we synthesized GADP/CM-curdlan conjugates having various degrees of substitution of the carboxymethyl group (DCM) and degree of substitution of the GADP group (DGADP) per sugar unit. The GADP/CM-curdlan conjugate showed higher immunological enhancement activities than GADP derivative, CM-curdlan, or a mixture of GADP derivative and CM-curdlan in all the concentration ranges tested. The immunological enhancement activities of the GADP/CM-curdlan conjugate increased with increasing DGADP value. Although the immunological enhancement activities of CM-curdlan decreased with increasing in DCM value, those of GADP/CM-curdlan conjugate increased with increasing DCM value. We also synthesized the GADP/CM-dextran conjugate to investigate the effect of conjugation of GADP with immunologically active CM-curdlan. The immunological enhancement activities of GADP/CM-dextran conjugate was not higher than those of the mixture of GADP derivative and CM-dextran, or CM-dextran itself. These results suggested that the high immunological enhancement activities of GADP/CM-curdlan conjugates were due not only to giving polymeric character to GADP but also to the hybridization of GADP with immunologically active polysaccharide, curdlan. Moreover, from the results of the effect of DGADP and DCM values of the conjugate on activities and the results of the effect of the addition of calmodulin inhibitor to the macrophage-like cells activated by the conjugate on activities, the path of the activation of immunocompetent cells by the GADP/CM-curdlan conjugate was found to be different from that by CM-curdlan or free GADP derivative itself.

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 the 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 tumor growth (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, curdlan (normal chain (1 → 3)-β-D-glucan) from *Alcligenes faecalis* var. *myxogenes* is

*To whom correspondence should be addressed.

Carbohydrate Polymers 0144-8617/94/$07.00

known to have an antitumor activity (Sasaki *et al.*, 1979*a*; Takahashi *et al.*, 1988). The mechanism of its antitumor action is not completely clear, but curdlan cannot be shown to exert any direct action on tumor cells. Therefore, its antitumor action must be considered to be dependent on interaction with the immunocompetent cells of the host.

In order to provide a novel synthetic biological response modifier exhibiting high antitumor activity, we previously synthesized the hybrid type conjugate of carboxymethyl(CM)-curdlan and GADP (Ohya *et al.*, 1993) (Fig. 1). 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 (RES). So, by conjugation of water-soluble and low molecular weight GADP onto CM-curdlan through 6-amino hexanoic acid spacer, the prolongation of body circulation of GADP molecule is expected to be achieved. This conjugate can also be treated as a simple model of BCG cell wall having lipophilicity and polymeric character. Moreover, curdlan should express its immunological activity through the interaction with immunocompetent cells. Therefore, this conjugate can be expected to have the targetability to immunocompetent cells because of greater affinity to them. Furthermore, this conjugate can be expected to possess the synergistic effect of immunologically active curdlan and GADP.

Previously, we reported the synthesis of GADP/CM-curdlan and that the GADP/CM-curdlan conjugate showed higher immunological enhancement activity against macrophage-like cells *in vitro* than low molecular weight GADP derivative, CM-curdlan, or a mixture of GADP derivative and CM-curdlan (Ohya *et al.*, 1993). However, in the previous paper, the measurements of immunological enhancement activity were carried out by only one kind of conjugate and only one dose. The dose dependence and the relationship between the immunological enhancement activity of the conjugate and its molecular structure were not discussed.

In order to investigate the immunological enhancement activity of the GADP/CM-curdlan conjugate in detail, the present paper is concerned with the dose dependence of GADP/CM-curdlan conjugate on the immunological enhancement activity *in vitro*, glucose consumption, superoxide anion (O^{2-}) production, cytotoxic factor production and 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. Moreover, in order to discuss the relationship between the immunological enhancement activities of the conjugate and its molecular structure, we synthesized GADP/CM-curdlan conjugates having various degrees of substitution of the carboxymethyl group in mol % per sugar unit (DCM) and various degrees of substitution of GADP group in mol % per sugar unit (DGADP) in order to study the effect of DCM and DGADP on the immunological enhancement activities of the conjugate. We also synthesized GADP/CM-dextran conjugate by hybridization of GADP with dextran which has no immunological enhancement activity to investigate the effect of hybridization of GADP with immunologically active curdlan. Furthermore, we discussed the effect of the addition of calmodulin inhibitor to the macrophage-like cells activated by the conjugate on immunological enhancement activities to study the path of activation of the conjugate.

EXPERIMENTAL

Materials

Curdlan (a number-average degree of polymerization: approximately 500), lipopolysaccharide (LPS; from *E. Coli* 0111:B4), *p*-nitrophenyl-β-D-glucuronide, ferricytochrome c, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, calmodulin inhibitor) 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 purifications. Curdlan and dextran were carboxymethylated by the method described in the work of Sasaki *et al.* (1979*b*). GADP derivative and GADP/CM-curdlan conjugate (Fig. 1) were synthesized according to the previous paper (Ohya *et al.*, 1993). GADP/CM-dextran conjugate was synthesized by a similar method to that of GADP/CM-curdlan conjugate. By changing the reaction conditions, the ratio of GADP derivative to CM-curdlan in the reaction, and by using CM-curdlans having various values of DCM, we synthesized GADP/CM-curdlan conjugates having various values of DCM or DGADP. DCM and DGADP were determined by a colloidal 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 polycationic titrant, which was back-titrated with the polyanionic titrant (Tôei & Kohara, 1976). HL-60 cells, U937 cells, and K562 (human myeloid leukemia) 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 used in each test were cultured in 96-well flat-bottomed plates (Corning Laboratory Sciences Company) with 200 μl of culture medium.

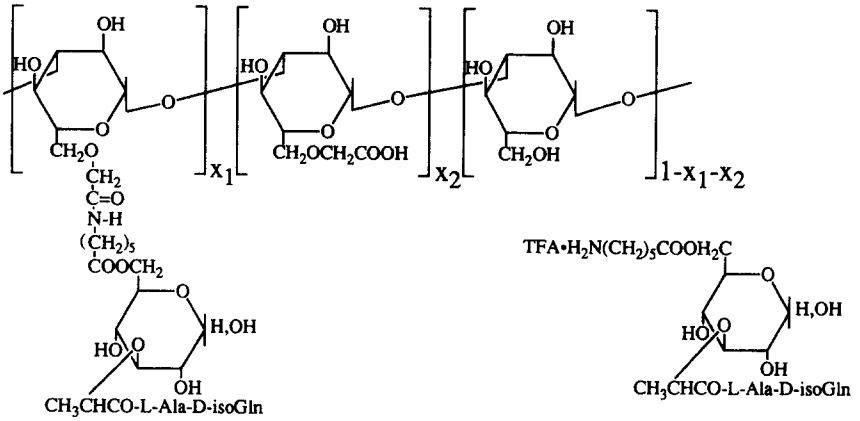

Fig. 1. Molecular structures of GADP/CM-curdlan conjugate and GADP derivative.

Measurement of immunological enhancement activities

The assay of glucose consumption activity 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 paper (Ohya *et al.*, 1993). The assay of cytotoxic factor production from PMA-differentiated U937 using K562 cells as target cells was also performed according to the same paper.

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 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 (1×10^6 cells/well) were cultured at 37°C for 5 days after treatment with 40 nM PMA (phorbol-12-myristate-13-acetate) to differentiate to macrophage-like cells (Ralph *et al.*, 1982; Harris & Ralph, 1985). After macrophage-like U937 cells were activated by GADP/CM-curdlan conjugate for 24 h, the macrophage-like U937 cells lysate prepared with 40 μl of 10% Triton X-100 were 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 the optical density at 405 nm using Corona MTP-120 microplate reader. The values of β-D-glucuronidase activity were calculated by comparing them with the values from the control experiment without treatment. In these measurements, free GADP derivative and CM-curdlan were used as references. The simple mixture of GADP derivative and CM-curdlan (weight ratio, GADP derivative: CM-curdlan = 27:73), which has the same composition as the GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit), was also used as a reference. This mixture was prepared by mixing 1·0 g of CM-curdlan (DCM = 34 mol %/sugar unit) and 370 mg of GADP derivative, and using it as solution in phosphate buffered saline.

The effects of the addition of calmodulin inhibitor, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Hidaka *et al.*, 1978*a*, *b*), on the immunological enhancement activities of the conjugate, GADP derivative, and MDP against macrophage-like cells were also investigated. The solution of W-7 in ethanol/water (1:1) (1×10^{-3} M) was added to the culture medium (30 μl/well) of PMA-differentiated HL-60 or U937 cells, at the same time as the addition of the conjugate, GADP derivative or MDP, and then the glucose consumption and β-D-glucuronidase activity were measured by the same methods as described above.

RESULTS AND DISCUSSION

Dose dependence of GADP/CM-curdlan conjugate on activities

The dose dependence of GADP/CM-curdlan conjugate on the glucose consumption, the superoxide anion production from PMA-differentiated HL-60 cells, the β-D-glucuronidase activity, and the cytotoxic factor production from PMA-differentiated U937 cells are shown in Figs 2–5, compared with those of GADP derivative, CM-curdlan, and the simple mixture of GADP derivative and CM-curdlan (weight ratio, GADP derivative: CM-curdlan = 27:73), which have the same composition as the GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit). The immunological enhancement activities of GADP/CM-curdlan conju-

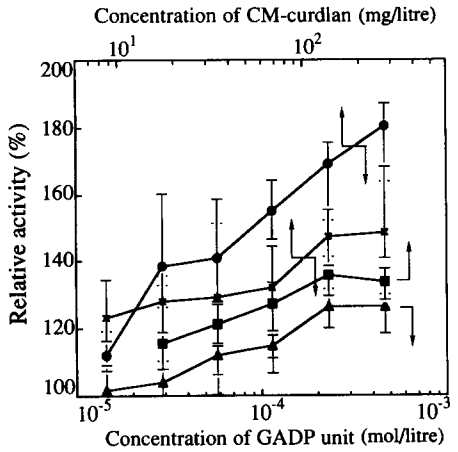

Fig. 2. Stimulating effect per unit mol of GADP unit for conjugate on glucose consumption from PMA-differentiated HL-60 cells. ●: GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit); ■: CM-curdlan; ▲: GADP derivative; ×: mixture of GADP derivative and CM-curdlan (weight ratio = 27:73).

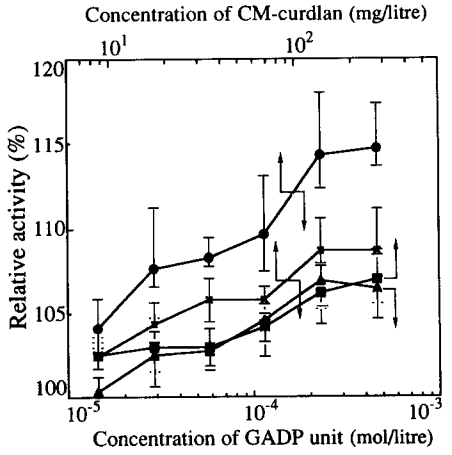

Fig. 3. Stimulating effect per unit mol of GADP unit for conjugate on the superoxide anion production from PMA-differentiated HL-60 cells. ●: GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit); ■: CM-curdlan; ▲: GADP derivative; ×: mixture of GADP derivative and CM-curdlan (weight ratio = 27:73).

gate were highest among them in the concentration range tested. It is clear that the high immunological enhancement activity resulted from the covalent fixation of GADP to CM-curdlan. Although the activation efficiencies of CM-curdlan and/or GADP derivative were saturated in a high concentration range of dose, the activation efficiency of GADP/CM-curdlan conjugate tended to increase linearly even in high dose ranges, especially in the assays of glucose consumption and β-D-glucuronidase activity. These results suggested that the activation mechanism of GADP/CM-curdlan conjugate was different from that of GADP derivative or CM-curdlan.

Comparison of activities of GADP/CM-curdlan conjugate with those of GADP/CM-dextran conjugate

Dextran is well known to be a polysaccharide having no immunogenecity and no immunological enhancement activity. In order to investigate the effect of hybridization of GADP with immunologically active curdlan and the effect of addition of polymeric character to GADP, we synthesized GADP/CM-dextran conjugate to compare immunological enhancement activities of itself with those of GADP/CM-curdlan conjugate. The immunological enhancement activities of the GADP/CM-dextran conjugate obtained were evaluated by test-

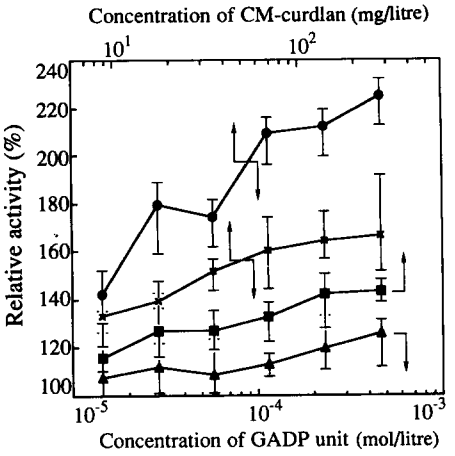

Fig. 4. Stimulating effect per unit mol of GADP unit for conjugate on β-D-glucuronidase activity from PMA-differentiated U937 cells. ●: GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit); ■: CM-curdlan; ▲: GADP derivative; ×: mixture of GADP derivative and CM-curdlan (weight ratio = 27:73).

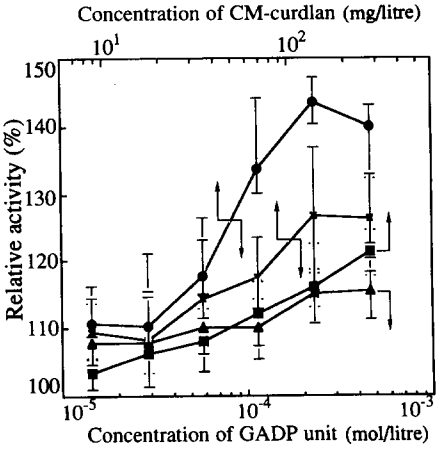

Fig. 5. Stimulating effect per unit mol of GADP unit for conjugate on cytotoxic factor production from PMA-differentiated U937 cells. ●: GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit); ■: CM-curdlan; ▲: GADP derivative; ×: mixture of GADP derivative and CM-curdlan (weight ratio = 27:73).

ing glucose consumption, superoxide anion production from PMA-differentiated HL-60 cells, β-D-glucuronidase activity, and cytotoxic factor production from PMA-differentiated U937 cells, compared with the activities of CM-dextran, the mixture of GADP derivative and CM-dextran, and GADP/CM-curdlan conjugate (Fig. 6). While the immunological enhancement activities of GADP/CM-curdlan conjugate were much higher than those of the mixture of GADP derivative and CM-curdlan, or CM-curdlan itself, the immunological enhancement activities of the GADP/CM-dextran conjugate were much lower than those of GADP/CM-curdlan conjugate containing equivalent amount of GADP unit, and were not higher than those of the mixture of GADP and CM-dextran, or CM-dextran itself. CM-dextran itself showed some immunological enhancement activity. Since some kinds of polyanion showed the activation of macrophage (Donaruma *et al.*, 1980), CM-dextran might act as a polyanion by introducting a carboxymethyl group and exhibiting some activation of macrophage-like cells. These results suggested that the high immunological enhancement activities of GADP/CM-curdlan conjugates were not only due to giving polymeric character to GADP but also to the hybridization of GADP with immunologically active curdlan.

Effect of DGADP and DCM on immunological enhancement activity

In order to investigate the relationship between the immunological enhancement activity of GADP/CM-curdlan conjugate and its molecular structure, we synthesized the conjugates having various values of DCM and DGADP (Table 1). In order to investigate the effect of the DCM of the conjugate on its activities, we used three kinds of conjugate (Nos 1, 2 and 3) having similar DGADP values (9–12 mol %/sugar unit)

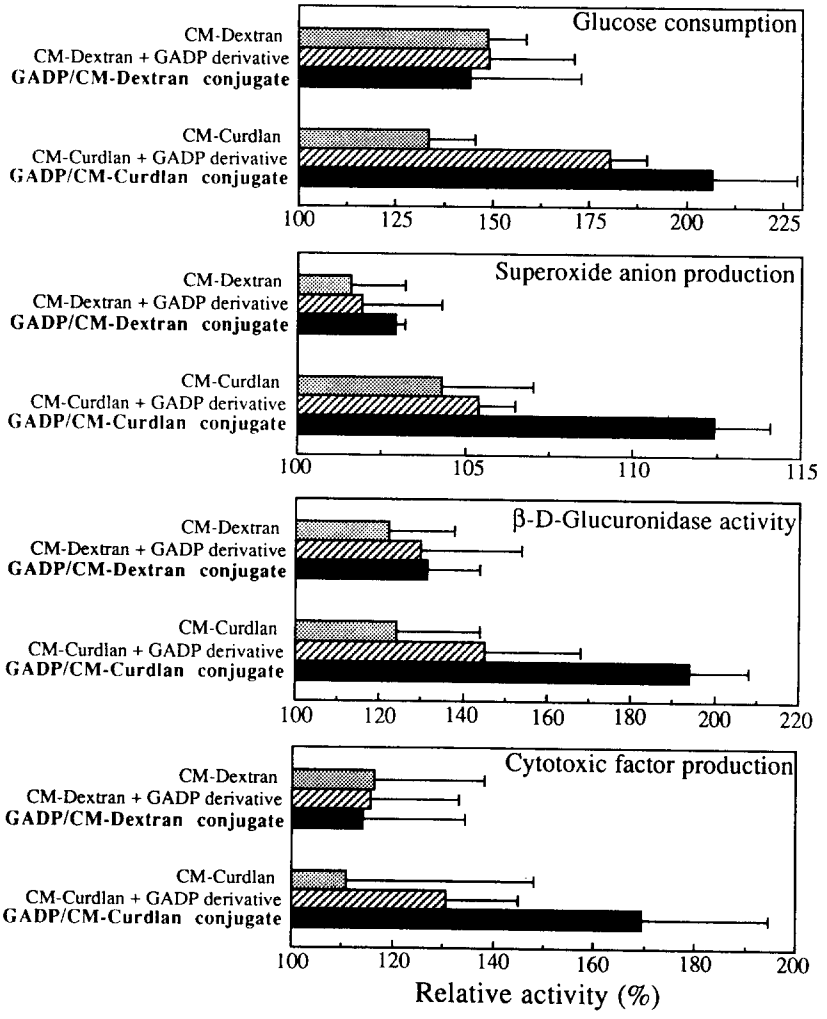

Fig. 6. Comparison of stimulating effect from PMA-differentiated HL-60 or U937 cells between GADP/CM-dextran conjugate and GADP/CM-curdlan conjugate. GADP/CM-dextran conjugate: DGADP = 16 mol %/sugar unit, DCM = 30 mol %/sugar unit; GADP/CM-curdlan conjugate: DGADP = 17 mol %/sugar unit, DCM = 25 mol %/sugar unit; CM-dextran: DCM = 46 mol %/sugar unit; CM-curdlan: DCM = 42 mol %/sugar unit.

Table 1. The values of DCM and DGADP of the GADP/CM-curdlan conjugates synthesized

No.	DCM (mol %/sugar unit)	DGADP (mol %/sugar unit)
1	12	11
2	22	12
3	33	9
4	28	6
5	25	17

and different DCM values, 12, 22 and 33 mol %/sugar unit, respectively. In order to examine the effect of DGADP of the conjugate on its activities, we used conjugate No. 2 and another two kinds of conjugate (Nos 4 and 5) having similar DCM values (22–28 mol % /sugar unit) and different DGADP values, 12, 6 and 17 mol %/sugar unit, respectively.

The results of the effect of DGADP on the immunological enhancement activities of the conjugate are shown in Fig. 7. The conjugates (1000 μg) containing various amounts of GADP unit or equivalent amounts of free GADP derivative for each GADP/CM-curdlan conjugate were added to the culture. The activities caused by GADP/CM-curdlan conjugate increased with an increase in the DGADP value of the conjugate. The free GADP derivative showed mild increase of activities depending on its dose. However, the increments with the DGADP value of the conjugate's activity were larger than that of free GADP derivative's activity.

The results of the effect of DCM on the immunological enhancement activities of the conjugate are shown in Fig. 8. Although the immunological enhancement activities of CM-curdlan decreased with increasing DCM value, those of GADP/CM-curdlan conjugate increased with increasing DCM value. As a cause of the decrease in activation seen in CM-curdlan, it was assumed that the triple helical conformation of curdlan was labilized by introduction of the carboxymethyl group. On the other hand, as the cause of the increase in activation seen in GADP/CM-curdlan conjugate, it was presumed that the polyanionic polymer, CM-curdlan, also acted as an effective immunocompetent cell activator, or that the high water-solubility due to the carboxymethyl group and/or the balance between the hydrophilicity and the hydrophobicity of the conjugate were preferable for the activation of macrophage-like cells.

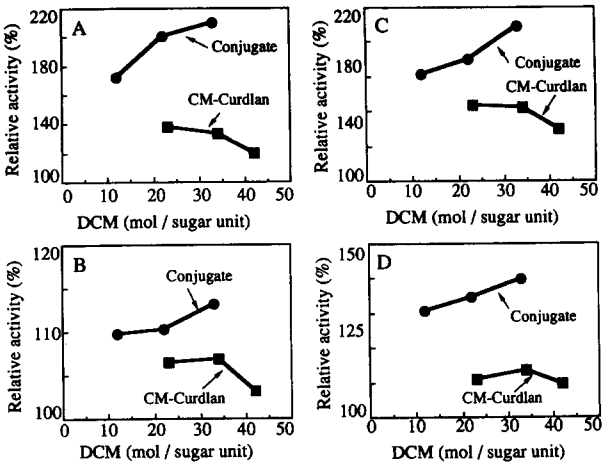

Fig. 8. Effect of DCM on stimulating effect of GADP/CM-curdlan conjugate and CM-curdlan. A: Glucose consumption, B: superoxide anion production, C: β-D-glucuronidase activity, D: cytotoxic factor production.

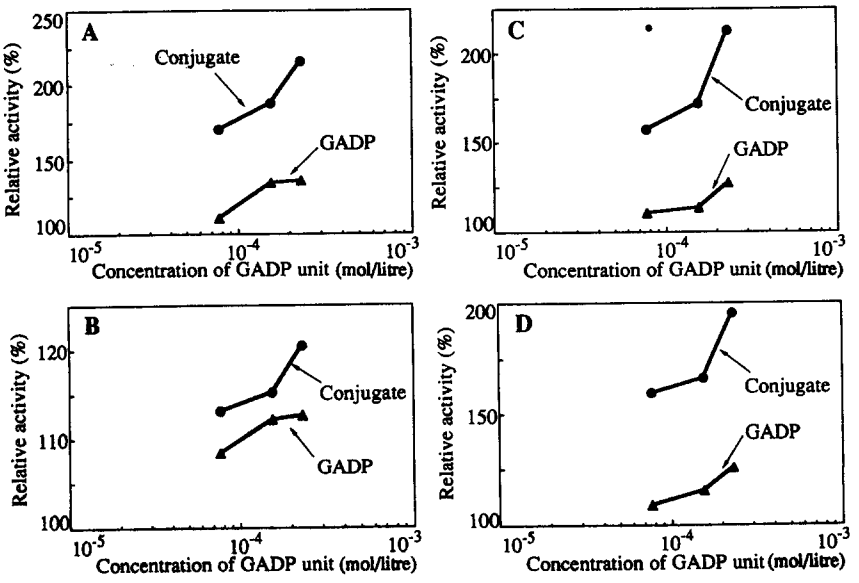

Fig. 7. Effect of DGADP on stimulating effect per unit mol of GADP derivative for conjugate from PMA-differentiated HL-60 or U937 cells. A: Glucose consumption, B: superoxide anion production, C: β-D-glucuronidase activity, D: cytotoxic factor production.

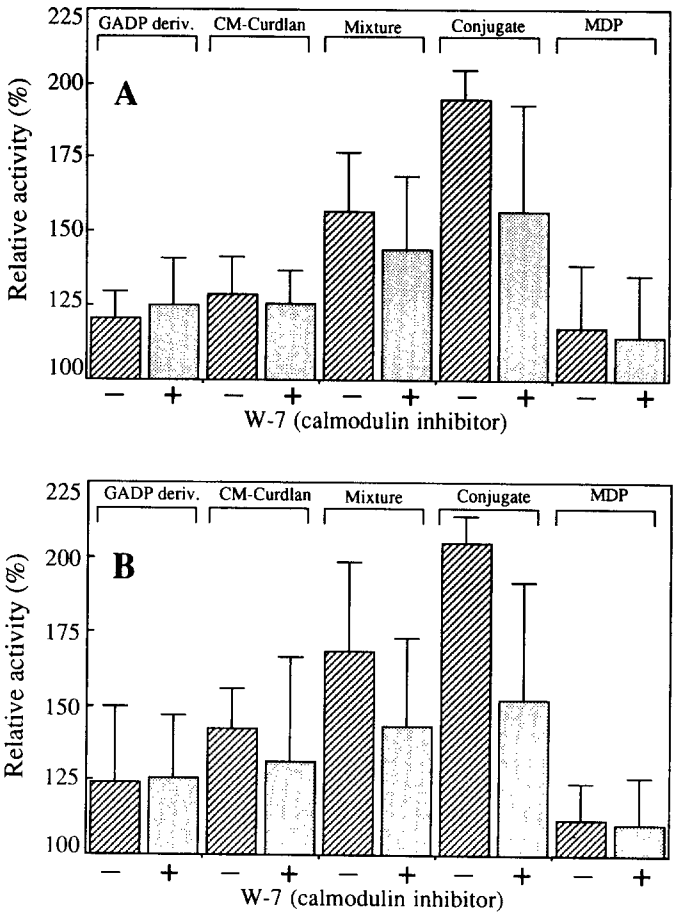

Fig. 9. The effect of the addition of W-7 on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) activated by GADP/CM-curdlan conjugate (DGADP = 12 mol %/sugar unit, DCM = 22 mol %/sugar unit), CM-curdlan (DCM = 23 mol %/sugar unit), GADP derivative, MDP and mixture of GADP derivative and CM-curdlan (weight ratio = 27:73).

These results suggested that the DGADP and DCM were very important to achieve the high immunological enhancement activity of the GADP/CM-curdlan conjugate. These results also suggested that the mechanism of the activation of immunocompetent cells by GADP/CM-curdlan conjugate was different from that by free GADP derivative and CM-curdlan.

Effect of the addition of the calmodulin inhibitor

Finally, in order to study the mechanism of immunological enhancement activity of GADP/CM-curdlan conjugate, the effect of the addition of calmodulin-specific inhibitor, W-7, (Hidaka *et al.*, 1978*a*, *b*), on macrophage-like cells was studied (Fig. 9). Calmodulin is well known to play an important role in the control of the cellular Ca^{2+} concentration. The activation of macrophage-like cells by the GADP/CM-curdlan conjugate was inhibited effectively by the addition of W-7. However, this inhibitor did not affect on the activation of macrophage-like cells by MDP and GADP derivative. Therefore, Ca^{2+} uptake into macrophage-like cells was clarified to be an important step in the immunological enhancement of macrophage-like cells by GADP/CM-curdlan conjugate but not in the enhancement by MDP and GADP derivative. Thus, Ca^{2+} uptake into macrophage-like cells was concluded to be one of the possible paths of activation of macrophage-like cells by GADP/CM-curdlan conjugate.

ACKNOWLEDGMENTS

The authors are grateful to Dr Hisanaga Igarashi and Mr Akihiko Sato of Shionogi Institute for Medical Science of Shionogi & Co. Ltd, for the supply of tumor cell line. This research was supported by the Science Research Promotion Fund of the Ministry of Education in Japan.

REFERENCES

Adachi, Y., Ohno, N., Ohsawa, M., Oikawa, S. & Yadomae, T. (1990). *Chem. Pharm. Bull.*, **38**, 477–81.

Alam, A., Capoor, A.K. & Rao, V. (1991). *Immunol. Lett.*, **27**, 53–7.

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**, 1885–7.

Donaruma, L.G., Ottenbrite, R.M. & Vogl, O. (1980). *Anionic Polymeric Drugs*. John Wiley & Sons, New York, USA.

Ellouz, F., Adam, A., Ciorbaru, R. & Lederer, E. (1974). *Biochem. Biophys. Res. Commun.*, **59**, 561–7.

Greenberger, J.S., Newburger, P.E., Karpas. A. & Moloney, W.C. (1978). *Cancer Res.*, **38**, 3340–8.

Harris, P. & Ralph, P. (1985). *J. Leukocyte Biol.*, **37**, 407–22.

Hidaka, H., Asano, M., Iwadare, S., Matsumoto, I., Totsuka, T. & Aoki, N. (1978*a*). *J. Pharmacol. and Experimental Therapeutics*, **207**, 8–15.

Hidaka, H., Yamaki, T., Totsuka, T. & Asano, M. (1978*b*). *Molecular Pharmacol.*, **15**, 49–59.

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**, 105–11.

Kusumoto, S., Tarumi, Y., Ikenaka, K. & Shiba, T. (1976). *Bull. Chem. Soc. Jpn*, **49**, 533–9.

Merser, C., Sinary, P. & Adam, A. (1975). *Biochem. Biophys. Res. Commun.*, **66**, 1316–22.

Ohya, Y., Nishimoto, T. & Ouchi, T. (1993). *Carbohydr. Polym.*, **20**, 43–9.

Phillips, N.C., Moras, M.L., Chedid, L., Lefrancier, P. & Bernard, J.M. (1985). *Cancer Res.*, **45**, 128–34.

Ralph, P., Williams, N., Moore, M.A.S. & Litcofsky, P.B. (1982). *Cellular Immunol.*, **71**, 215–23.

Sasaki, T., Abiko, N., Sugino, Y. & Nitta, K. (1979*a*). *Cancer Res.*, **38**, 379–83.

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

Takahashi, K., Yamazaki, M. & Abe, S. (1988). *J. Pharmacobio-Dyn.*, **11**, 472–8.

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