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Effect of dimerization of the D-glucose analogue of muramyl dipeptide on stimulation of macrophage-like cells

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Abstract

N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is the minimum required structure responsible for the immunoadjuvant activity of the bacterial cell wall. The D-glucose analogue of MDP (GADP) was reported to show a higher immunoadjuvant activity than MDP itself. Although the mechanism of activation by MDP and the existence of receptor against MDP are not clear, the patch formation and cluster formation of receptors are important steps on the signal transduction by such bioactive molecules. It is expected that the cluster effect such as antennary oligosaccharides reported by Lee et al. increased the affinity of ligand against receptor and accelerated the patch formation and cluster formation of receptors. In order to discuss the effect of multivalent-ligand formation of GADP on the activation of immunocompetent cells in more detail, we have synthesized GADP dimers combined through various lengths of alkyl and poly(ethylene glycol) (PEG) spacer groups as the simple models of multivalent-ligand molecule of GADP and evaluated their immunological enhancement activities in vitro. The GADP dimers showed a higher level stimulatory activities against macrophage-like cells than free GADP and monomeric GADP derivatives. © 1997 Elsevier Science Ltd.

Keywords: MDP; Dimerization; Stimulation activity; Macrophage-like cells

1. Introduction

N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP, Fig. 1) is the minimum required structure responsible for the immunoadjuvant activity attributable to peptidoglycan of the bacterial cell wall [1–4]. Though MDP itself has no antitumor activities [5], *Mycobacterium bovis* Bacille de Calmette-Guerin (BCG) cell wall is effective in tumor immunotherapy. MDP is remarkably different from BCG cell wall in lacking lipophilicity and polymeric character. It is reported that some MDP derivatives chemically modified with lipophilic groups showed antitumor activities [6–8]. The D-glucose analogue of MDP (GADP, Fig. 1) was also reported to show a higher immunoadjuvant activity than MDP itself [9]. Previously, we synthesized hybrid type, of conjugates of immunoactive poly-

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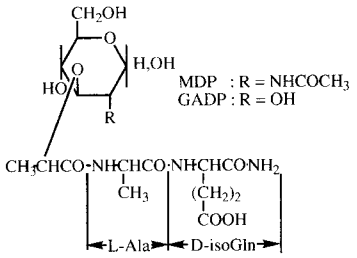


Fig. 1. Molecular structures of MDP and GADP.

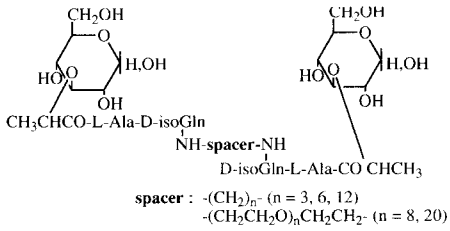


Fig. 2. Molecular structure of GADP dimers.

saccharides, such as curdlan and chitin, immobilizing GADP residues and evaluated their immunological enhancement activities in vitro [10–13]. The GADP/polysaccharide conjugates obtained showed higher stimulatory activities against macrophage-like differentiated cells than either free MDP, low molecular weight GADP derivative, each polysaccharide itself or the mixture of them. The revelation of a high level of stimulatory activity of GADP/polysaccharide conjugates was proposed as being due not only to the multivalent-ligand formation effect by immobilizing many GADP residues to the same polymer chain but also to the hybridization effect of GADP with immunoactive polysaccharides (Scheme 1). Generally, most hydrophilic bioactive molecules are known to be taken up into cells via binding to specific receptors on the cell surface. Patch formation and the cluster formation are important steps on the signal transduction by such bioactive molecules. Moreover, it is expected that the multivalent-ligand formation, such as an antennary oligosaccharides reported by Lee et al. [14–17] in the glycoprotein taken up by mammalian cells, will show cluster effects which will increase the affinity of ligand against receptor and accelerate the patch formation and cluster formation of receptors. The mechanism of enhancement of immunological activity by GADP is not yet clear, but may be similar to the activation mechanism described above. In order to discuss the effect of multivalent-ligand formation of GADP on the activation of immunocompetent cells in more detail, the GADP dimerization effect was investigated by using GADP dimer as the simplest model compound. The present paper deals with the synthesis of GADP dimers combined through various lengths of alkyl chain or poly(ethylene glycol) (PEG) spacer groups (as the simplest models of multivalent-ligand molecule of GADP; Fig. 2) and the evaluation of immunological activity of the GADP dimers against macrophage-like differentiated cells in vitro.

2. Results and discussion

Effect of dimerization on the stimulatory activity of GADP against macrophage-like cells.—In order to investigate the effect GADP dimerization on the immunostimulatory activities, two kinds of GADP dimer were compared with the corresponding monomeric GADP derivatives (GADP itself and the mixture of GADP and the corresponding spacer compounds) together with MDP and LPS as positive controls (Fig. 3). From the results shown in Fig. 3, **GD-12** was found to exhibit higher stimulating effects on the glucose consumption from PMA-differentiated HL-60

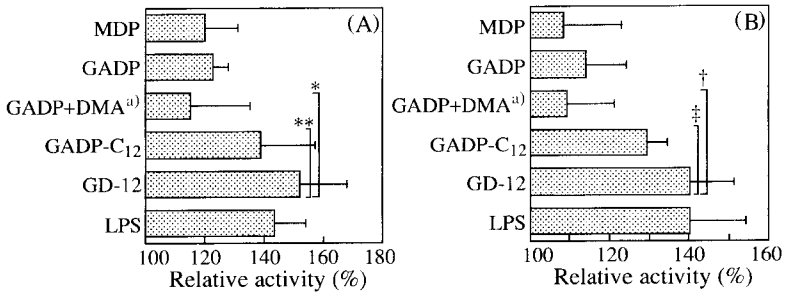


Fig. 3. Effect of dimerization of GADP through alkyl spacer group on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) in vitro. *: $p < 0.01$, **: $p < 0.15$, †: $p < 0.005$, ‡: $p < 0.05$. The dose per GADP unit mol (50 nM) of all samples was adjusted to be equal, except that of LPS (10 μg/ml). [a)] DMA: dodecamethyleneamine. Number of parallel experiments: 6, error bars: S.E.

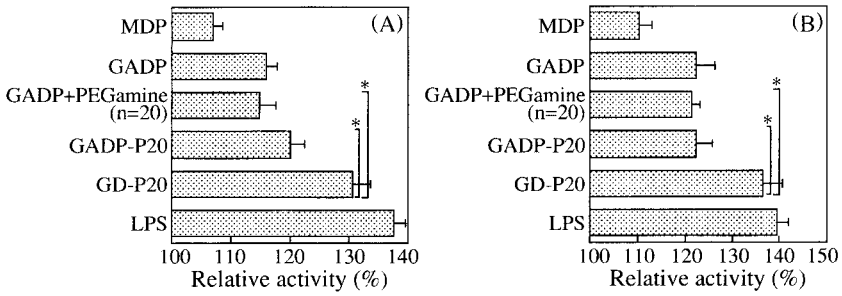


Fig. 4. Effect of dimerization of GADP through PEG spacer group on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) in vitro. *: $p < 0.0005$. The dose per GADP unit mol (50 nM) of all samples was adjusted to be equal except that of LPS (10 μg/ml). Number of parallel experiments: 6, error bars: S.E.

cells and the β-D-glucuronidase activity from PMA-differentiated U937 cells than monomeric GADP/dodecamethyleneamine derivative (**GADP-C_{12}**), GADP itself, and the simple mixture of GADP and dodecamethyleneamine, which had the same composition as **GADP-C_{12}**. Moreover, the GADP dimer combined through a PEG ($n = 20$) spacer group (**GD-P20**) exhibited higher stimulatory effects on the glucose consumption and the β-D-glucuronidase activity than monomeric GADP/PEG amine ($n = 20$) derivative (**GADP-P20**), GADP itself, and the simple mixture of GADP and PEG amine ($n = 20$), as shown in Fig. 4. These results suggested that the introduction of alkyl group into GADP was effective in increasing immunostimulatory activity, and that the dimerization of GADP caused an increase of immunostimulatory activity per GADP residue.

Effect of spacer length of the GADP dimer on the stimulatory activity against macrophage-like cells.— In order to study the GADP dimerization effect on the immunostimulatory activity in more detail, the effect of spacer length of GADP dimer was investigated by using the GADP dimers combined through alkyl spacer groups (**GD-3**, **GD-6**, **GD-12**) and the GADP dimers combined through PEG spacer groups (**GD-P8**, **GD-P20**). The effect on glucose consumption from PMA-differentiated HL-60 cells and the β-D-glucuronidase activity from PMA-differentiated U937 cells are shown in Figs. 5 and 6, respectively. The stimulatory activity of GADP dimer evaluated in both assays tended to increase with increasing spacer length, particularly for that of GADP dimer coupled with alkyl group. On the other hand, the stimulatory activity of **GD-P20** was not so different from that of **GD-P8** except in the low concentration range of GADP. In the high concentration range of GADP residue, both dimers showed higher stimulatory activities than expected from the increase of concentration of GADP residue. These results suggested that the remarkable increase of stimulation activity of GADP dimer would be controlled by the hydrophobicity of spacer, and that the dimerization effect on stimulation would be affected by the flexibility of spacer. Moreover, a phenomenon similar to the cluster effect [14–17], which would show higher stimulatory activities than expected from the increase of concentration

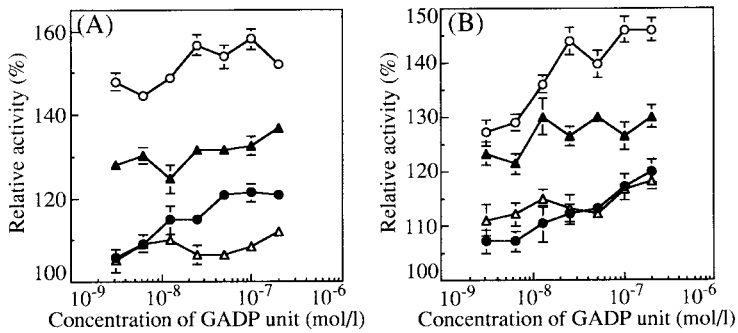


Fig. 5. Effect of spacer length of GADP dimers combined through alkyl spacer group on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) in vitro. ○: **GD-12**, ▲: **GD-6**, ●: **GD-3**, △: GADP. number of parallel experiments: 6, error bars: S.E.

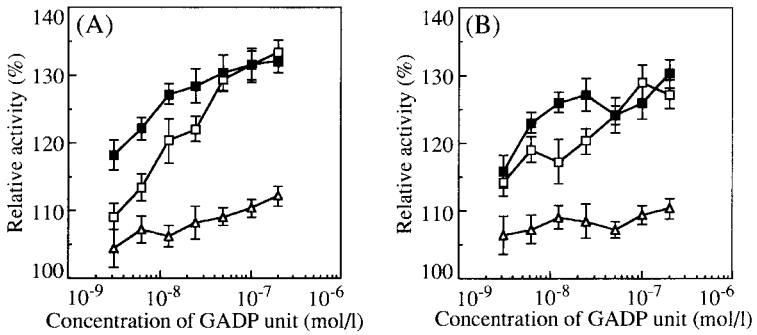


Fig. 6. Effect of spacer length of GADP dimers combined through PEG spacer group on glucose consumption from PMA-differentiated HL-60 cells (A) and β-D-glucuronidase activity from PMA-differentiated U937 cells (B) in vitro. ■: **GD-P20**, □: **GD-P8**, △: GADP. Number of parallel experiments: 6, error bars: S.E.

of GADP residue, was observed. This result meant that some receptor would be concerned with the mechanism of enhancement of immunological activity of GADP. Thus, multivalent-ligand formation could have an effect acceleration of patch formation and cluster formation on the cellular surface (Figs. 7 and 8) [13].

3. Experimental

Materials.—GADP was synthesized according to the method reported previously [9]. Lipopolysaccharide (LPS; from *E. Coli* 0111:B4), *p*-nitrophenyl-β-D-glucuronide, MDP and glucose B-test Wako were purchased from Wako Pure Chemical Industry. Organic solvents were purified by usual distillation. Other materials were commercial grade and used without further purification. HL-60 cells and U937 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/L 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.

Synthesis of GADP dimers combined through alkyl spacer group.—The synthesis of GADP dimers combined through alkyl spacer group (**GD-3**, **GD-6**, **GD-12**) was carried out by the coupling reaction of corresponding alkyldiamines with twice the equivalent of GADP (Scheme 1). The number of 3, 6, 12 corresponds to the number of methylene groups of spacer arm of each compound. As one example, the synthesis method of **GD-3** is described as follows: GADP (110 mg, 245 mmol) were reacted with CDI (*N,N'*-carbonyldiimidazole) (58.5 mg, 361 mmol) in THF (20 mL) at 0 °C for 2 h. 1,3-Propanediamine (10.0 mL, 119 mmol) and triethylamine (56.0 mL, 400 mmol) were added to the reaction mixture and stirred at 0 °C for 2.5 h and then at room temperature for 24 h. THF was evaporated from the reaction mixture under reduced pressure to afford the syrup. The purification of **GD-3** was carried out by silica gel column chromatography (gradient eluent: chloro-

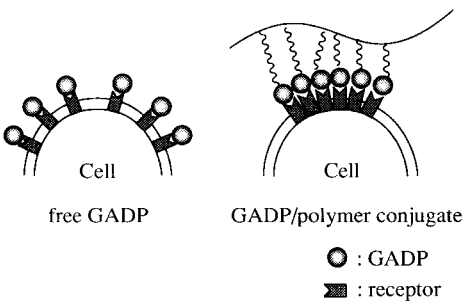


Fig. 7. The outline of interaction of free GADP and GADP/polymer conjugate with cellular surface.

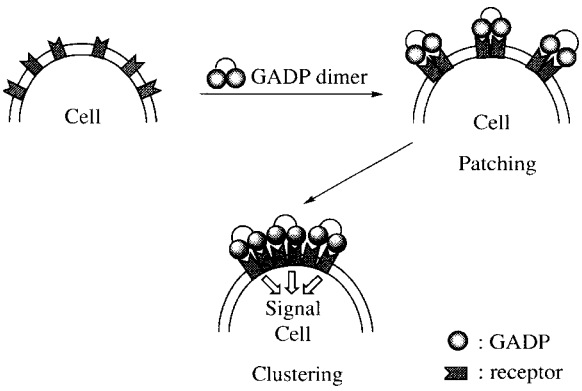
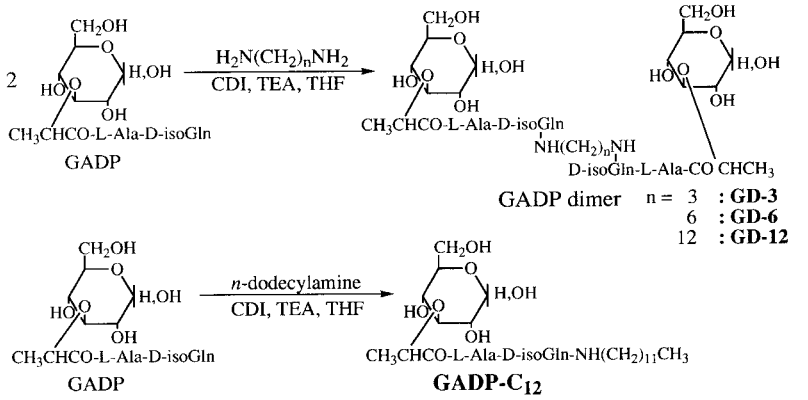


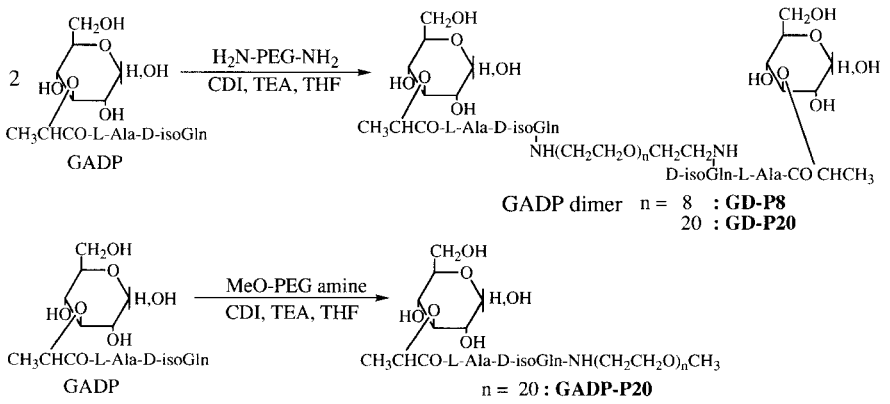
Fig. 8. The outline of the presumed cellular activation path by GADP dimer.

form/methanol = 5:1 → 2:1 v/v) (29.0 mg, 25.9%); ^{1}H NMR (D_2O): δ 1.02–1.06 (t, spacer -CH_2-, 2 H), 1.39–1.41 (d, prop, Ala $(CH_3)_2$-, 12 H), 1.93–2.18 (d, isoGln -$^{\beta}CH_2$-, 4 H), 2.22–2.26 (m, isoGln -$^{\gamma}CH_2$-, 4 H), 2.79 (s, spacer -$(CH_2)_2$-, 4 H), 3.03–3.84 (m, H2–6 of pyran, 12 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 4 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-, 2 H), 4.47–4.49 (d, $^{\beta}$H1), 5.19–5.20 (d, $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.33. The syntheses of the other GADP dimers (**GD-6** and **GD-12**) were also carried out by using 1,6-hexamethylenediamine and 1,12-dodecamethylenediamine instead of 1,3-propanediamine according to the procedure described above. As a control compound of **GD-12**, the synthesis of GADP derivative having dodecamethylene group (**GADP-C_{12}**) was synthesized the coupling reaction of GADP with *n*-dodecylamine. The purification of **GD-6**, **GD-12** and **GADP-C_{12}** was carried out by silica gel column chromatography (gradient eluent: chloroform/methanol = 10:1 → 5:1 v/v). **GD-6** (25.9 mg, 27.1%); ^{1}H NMR (D_2O): δ 1.28–1.43 (m, spacer -$(CH_2)_4$-, prop, Ala $(CH_3)_2$-, 20 H), 1.93–2.18 (d, isoGln -$^{\beta}CH_2$-, 4 H), 2.22–2.26 (m, isoGln -$^{\gamma}CH_2$-, 4 H), 2.55–2.59 (t, spacer -$(CH_2)_2$-, 4 H), 3.03–3.84 (m, H2–6 of pyran, 12 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 4 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-, 2 H), 4.47–4.49 (d, $^{\beta}$H1), 5.19–5.20 (d, $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.35. **GD-12** (17.2 mg, 32.3%); ^{1}H NMR (D_2O): δ 1.26–1.28 (d, spacer -$(CH_2)_8$-, 16 H), 1.39–1.44 (m, spacer -$(CH_2)_2$-, prop, Ala $(CH_3)_2$- 16 H), 1.93–2.18 (d, isoGln -$^{\beta}CH_2$-, 4 H), 2.22–2.26 (m, isoGln -$^{\gamma}CH_2$-, 4 H), 2.65–2.69 (t, spacer -$(CH_2)_2$-, 4 H), 3.03–3.84 (m, H2–6 of pyran, 12 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 4 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-, 2 H), 4.47–4.49 (d, $^{\beta}$H1), 5.19–5.20 (d, $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.50. **GADP-C_{12}** (37.3 mg, 50.8%); ^{1}H NMR (D_2O): δ 0.86–0.89 (t, spacer CH_3-, 3 H), 1.26 (m, spacer -$(CH_2)_9$-, 18 H), 1.39–1.44 (m, spacer -CH_2-, prop, Ala $(CH_3)_2$-, 8 H), 1.93–2.18 (d, isoGln -$^{\beta}CH_2$-, 2 H), 2.28–2.36 (m, isoGln -$^{\gamma}CH_2$-, 2 H), 2.65–2.65–2.69 (t, spacer -CH_2-, 2 H), 3.11–3.85 (m, H2–6 of pyran, 6 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 2 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-, 1 H), 4.48 (d $^{\beta}$H1), 5.15 (d $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.53.

Synthesis of GADP dimers combined through PEG spacer group.—The synthesis of GADP dimers combined through PEG spacer group (**GD-P8** and **GD-P20**) were carried out by using PEG-diamine (Mw = 400 (n = 8) and 1000 (n = 20)) instead of 1,3-propanediamine according to the procedure described above (Scheme 2). As a control compound of **GD-P20**, GADP derivative having methoxy(MeO)-PEG group (**GADP-P20**) was synthesized by the coupling reaction of GADP and MeO-PEG amine (Mw = 1000 (n = 20)). The purification of **GD-P8**, **GD-P20** and **GADP-P20** was carried out by gel filtration chromatography (LH-20, eluent: methanol, detect: 210 nm). **GD-P8** (8.70 mg, 6.84%); ^{1}H NMR (D_2O): δ 1.24–1.45 (m, prop, Ala $(CH_3)_2$-, 12 H), 1.97–2.20 (d, isoGln -$^{\beta}CH_2$-, 4 H), 2.35–2.38 (m, isoGln -$^{\gamma}CH_2$-, 4 H), 3.63–3.70 (m, PEG -CH_2CH_2O-) 3.03–3.84 (m, H2–6 of pyran, 12 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 4 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-,



Scheme 1. Synthesis of GADP dimer combined through alkyl spacer group and monomeric GADP derivative having alkyl group.



Scheme 2. Synthesis of GADP dimer combined through PEG spacer group and monomeric GADP derivative having PEG group.

2 H), 4.47–4.49 (d, $^{\beta}$H1), 5.19–5.20 (d, $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.38. **GD-P20** (53.4 mg, 15.1%); ^{1}H NMR (D_2O): δ 1.23–1.45 (m, prop, Ala $(CH_3)_2$-, 12 H), 1.97–2.20 (d, isoGln -$^{\beta}CH_2$-, 4 H), 2.35–2.38 (m, isoGln -$^{\gamma}CH_2$-, 4 H), 3.63–3.70 (m, PEG -CH_2CH_2O-) 3.03–3.84 (m, H2–6 of pyran, 12 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 4 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-, 2 H), 4.47–4.49 (d, $^{\beta}$H1), 5.19–5.20 (d, $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.21. **GADP-P20** (37.3 mg, 50.8%); ^{1}H NMR (D_2O): δ 1.23–1.45 (m, prop, Ala $(CH_3)_2$-, 6 H), 1.97–2.20 (d, isoGln -$^{\beta}CH_2$-, 2 H), 2.35–2.38 (m, isoGln -$^{\gamma}CH_2$-, 2 H), 3.35 (s, PEG CH_3O-, 3 H), 3.63–3.70 (m, PEG -CH_2CH_2O-) 3.03–3.84 (m, H2–6 of pyran, 6 H), 4.21–4.29 (m, prop, isoGln -$^{\alpha}$CH-, 2 H), 4.31–4.45 (m, Ala -$^{\alpha}$CH-, 1 H), 4.47–4.49 (d, $^{\beta}$H1), 5.19–5.20 (d $^{\alpha}$H1) ppm; R_f for TLC monitored with sugar coloration (developing solvent: *n*-butanol/acetic acid/water/pyridine = 4:1:2:1 v/v) was 0.20.

Measurement of immunostimulation activity against macrophage-like cells.—The stimulation activity of the obtained GADP dimers and the control monomeric GADP derivatives against cultured macrophages was evaluated by two kinds of assay of glucose consumption [10–12] and β-D-glucuronidase activity [18,19].

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 (1×10^6 cells/well) were cultured for 6 days at 37 °C after treatment with 40 nM phorbol-12-myristate-13-acetate (PMA) [20,21].

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 (1×10^6 cells/well) were cultured at 37 °C for 5 days after treatment with 40 nM PMA to differentiate to macrophage-like cells [20,22]. GADP dimer was added and after 24 h, cell lysates were prepared with 40 μL of 10% Triton X-100 was mixed with 100 mL 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) and the released *p*-nitrophenol quantified.

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