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Effect of chemical modification at C1 of the glucosamine backbone of lipid A-subunit analog GLA-27 on manifestation of immunopharmacological activity

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The effect of chemical modification at the C1 position of GLA-27, 4-*O*-phosphono-D-glucosamine carrying *N*-3-tetradecanoyloxytetradecanoyl [C_{14}-O-(C_{14})] and 3-*O*-tetradecanoyl (C_{14}) groups, was investigated. Replacement by SH or S-acetyl groups of the OH group resulted in the enhancement of mitogenicity but gave rise to a reduction, in macrophage-stimulating ability such as induction of tumor necrosis factor and enhancement of phagocytic and cellular acid phosphatase activities. Bisphosphorylation at C1 and C4 resulted in a slight decrease in mitogenicity or almost complete loss of the macrophage-stimulating ability.

Lipid A-subunit analog; GLA-27; Mitogenic activity; Tumor necrosis factor

1. INTRODUCTION

Various immunopharmacological activities of lipid A, the active principle of endotoxin, are manifested by chemically synthesized lipid A-subunit analogs, 4-*O*-phosphono-D-glucosamine derivatives carrying *N*- and 3-*O*-acyl substituents [1]. GLA-27, a glucosamine derivative with 4-*O*-phosphoryl, 3-*O*-C_{14} and *N*-C_{14}-O-(C_{14}) groups, is a prototype compound fulfilling the structural requirements for the expression of immunopharmacological activity without pyrogenicity and Shwartzman reaction [2–8].

The present investigation was undertaken in order to determine whether chemical modification at C1 of the glucosamine backbone of GLA-27 exerts an influence on the expression of immunopharmacological activity.

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2. MATERIALS AND METHODS

2.1. *Synthetic and natural compounds*

Lipid A-subunit analogs were synthesized chemically as in [9]. The chemical structures of the synthetic compounds are depicted in fig.1. Natural lipid A prepared from *Escherichia coli* F-515 (Re) was kindly donated by Drs O. Lüderitz and C. Galanos (Max-Planck-Institut für Immunobiologie, Freiburg, FRG) and used as a positive control. Samples were solubilized in pyrogen-free water by treatment with triethylamine and complexing with bovine serum albumin as described in [2].

2.2. *Mitogenic activity*

Mitogenic activity was assessed by determining tritiated thymidine ([^{3}H]TdR) uptake into C3H/He spleen cells (4×10^5 cells/well of 96-well microplate) which were incubated in triplicate at 37°C for 48 h with or without test samples [10].

2.3. *Tumor-necrosis factor (TNF)-inducing activity*

TNF-inducing activity was assessed by determining TNF titer in sera obtained from *Propionibacterium acnes*-primed mice which had been administered intravenously 10-μg test samples 1.5 h earlier, according to [4]. TNF titer was determined by measuring the percent inhibition of [^{3}H]TdR uptake into L-929 cells.

2.4. *Macrophage-activating activity*

The ability of peritoneal macrophages, obtained from

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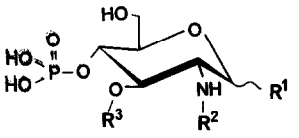

Fig.1. Chemical structures of lipid A-subunit analogs.

Compound	R^1 (1-)	R^2 (*N*-)	R^3 (3-*O*-)
GLA-27	-OH	-C_{14}-O-(C_{14})	-C_{14}
GLA-65	-S-$COCH_3$	-C_{14}-O-(C_{14})	-C_{14}
GLA-66	-SH	-C_{14}-O-(C_{14})	-C_{14}
GLA-70	-O-$PO(OH)_2$	-C_{14}-O-(C_{14})	-C_{14}

(BALB/c × DBA/2)F_1 (CDF_1) mice which had been administered i.p. 20 μg of test samples 4 days earlier, was investigated as in [7]. Phagocytic activity was assessed by evaluating the radioactivity of ^{51}Cr-labeled and antibody-sensitized sheep erythrocytes which were phagocytosed by peritoneal macrophages (5 × 10^5 cells/well, 24-well culture tray). Cellular lysosomal enzyme (acid phosphatase) activity of peritoneal macrophages (1 × 10^5 cells/well of 96-well microplate) was assayed as follows: macrophage lysates were mixed with 0.2 M acetate buffer, 24 mM *p*-nitrophenyl phosphate and incubated at 37°C for 30 min; 0.2 M sodium carbonate was then added to the reaction mixture and the absorbance at 405 nm estimated. Enzyme activity was calculated from the standard curve using acid phosphatase from potato.

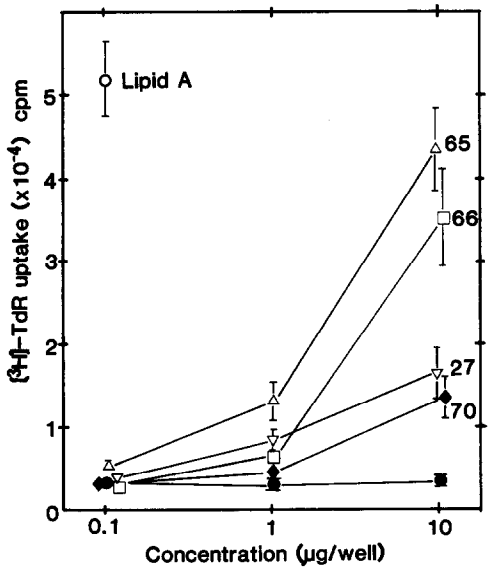

Fig.2. Mitogenic activity of lipid A-subunit analogs. C3H/He spleen cells were stimulated for 48 h with the indicated dose of natural lipid A (○), GLA-27 (▽), GLA-65 (△), GLA-66 (□) and GLA-70 (◆). Mock control (●). Mitogenic activity is expressed as cpm (means ± SD) of triplicate cultures. [Lipid A] 80366 ± 7284 (1 μg/well) and 82277 ± 2322 (10 μg/well).

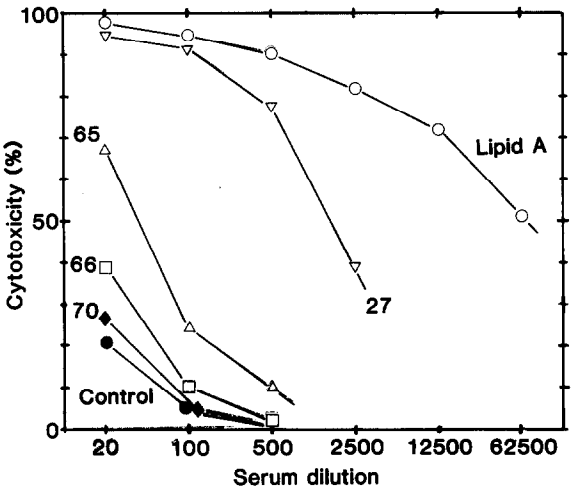

Fig.3. TNF-inducing activity of lipid A-subunit analogs. Sera were obtained from mice administered i.v. natural lipid A (○), GLA-27 (▽), GLA-65 (△), GLA-66 (□) or GLA-70 (◆) at a dose of 10 μg per mouse. Mock control (●). TNF activity in sera is expressed as percent inhibition of [^{3}H]TdR uptake into L-929 cells.

3. RESULTS

Our results on mitogenic activity are shown in fig.2. An increase in mitogenic activity at a dose of 10 μg/well was found to result from the replacement by either an SH or S-Ac group of the OH group at C1 of GLA-27. Mitogenic activity of compound (GLA-70) bisphosphorylated at C1 and C4 was somewhat weaker than that of GLA-27.

As reported in [2,4,5,8], GLA-27 exhibited significant TNF-inducing activity; however, the ac-

Table 1

Phagocytic activity of peritoneal macrophages stimulated in vivo with lipid A-subunit analogs

Compounds	Phagocytosis (%)[a]		
	Expt 1	Expt 2	Mean
Control	1.2 ± 0.1	0.9 ± 0.1	1.1
Lipid A	5.0 ± 0.2[c]	5.1 ± 0.2[c]	5.1
GLA-27	3.1 ± 0.3[c]	3.5 ± 0.2[c]	3.3
GLA-65	2.0 ± 0.3[b]	2.6 ± 0.1[c]	2.3
GLA-66	1.8 ± 0.2[b]	2.1 ± 0.1[c]	2.0
GLA-70	1.3 ± 0.1[ns]	1.1 ± 0.1[ns]	1.2

[a] Results are expressed as arithmetic mean ± SD of triplicate cultures; [b] $P < 0.01$ and [c] $P < 0.001$ (by Student's *t*-test); [ns], not significant

Table 2

Cellular lysosomal enzyme activity of peritoneal macrophages stimulated in vivo with lipid A-subunit analogs

Compounds	Acid phosphatase activity (mU/10^5 cells)[a]		
	Expt 1	Expt 2	Mean
Control	593 ± 73	613 ± 50	606
Lipid A	1093 ± 150[d]	1124 ± 65[d]	1109
GLA-27	802 ± 65[c]	837 ± 71[d]	820
GLA-65	708 ± 20[b]	693 ± 30[d]	701
GLA-66	670 ± 12[ns]	635 ± 30[ns]	653
GLA-70	613 ± 20[ns]	629 ± 13[ns]	621

[a] Results are expressed as arithmetic mean ± SD of triplicate cultures; [b] $P < 0.05$, [c] $P < 0.01$ and [d] $P < 0.001$ (by Student's t-test); [ns] not significant

tivity was weaker than that of natural lipid A (fig.3). Decreased TNF-inducing activity was observed in GLA-65 with an S-Ac group at C1. The activity of GLA-66 with an SH group was very weak. GLA-70 with 1,4-bisphosphates showed no detectable activity.

With respect to macrophage activation, the results concerning the phagocytic activity are listed in table 1. Natural lipid A induced a marked enhancement of phagocytosis of peritoneal macrophages in both experiments. Peritoneal macrophages from mice administered GLA-27 showed significant phagocytic activity. The activities of GLA-65 and GLA-66 were weaker than that of GLA-27. Administration of GLA-70 did not enhance the activity. As shown in table 2, the largest increase in level of cellular acid phosphatase activity was observed in peritoneal macrophages recovered from mice which had been administered lipid A. Significant acid phosphatase activity was observed in GLA-27 and GLA-65 but not in GLA-66 and GLA-70.

4. DISCUSSION

Previous studies showed that biological activities of 1-deoxy compound of GLA-27, GLA-40, were somewhat stronger than GLA-27 in expression of mitogenicity and polyclonal B cell activation but almost equal to those of GLA-27 in terms of macrophage activation, TNF-inducing activity and enhancement of antiviral activities against vaccinia virus infection [5,6]. The present study shows that chemical modification at the C1 position of the glucosamine backbone has varied effects on the activities of GLA-27. Replacement by an SH or S-Ac group of the OH group at C1 of GLA-27 induced an increase in mitogenicity but caused a reduction in macrophage-stimulating activity. The activities of GLA-65 with an S-Ac group were greater than those of GLA-66 which contains an SH group. This distinction indicates that different structural requirements for activation of lymphocytes and macrophages exist in the lipid A-subunit analog. Ikeda and colleages [11] reported that GLA-66 with an SH group was less effective compared to GLA-27 in enhancing the effect of nonspecific resistance in mice against vaccinia virus infection, in which nonspecific phagocytes participate as effector cells for viral clearance [11]. The above suggests that the introduction of an SH group at the C1 position can reduce the ability of the analog to induce nonspecific phagocytes which participate as effector cells for eliminating invaded pathogens.

A previous study indicated that phosphorylation at C1 of the glucosamine backbone is more effective for expression of lymphocyte activation than is phosphorylation at C4 [6]. Among the synthetic lipid A analogs which are glucosamine disaccharide compounds, compound 506, which is bisphosphorylated at C1 and C4′, demonstrated stronger biological and toxic activities compared to compounds 504 and 505, which are monophosphorylated at C1 or C4′ [12]. However, bisphosphorylation at C1 and C4 in the glucosamine monosaccharide resulted in a slight decrease in mitogenic activity or almost complete loss of TNF-inducing activity and macrophage activation, suggesting that 1,4-bisphosphorylation is the excess of negative charge in the monosaccharide compound for manifesting the activities.

A number of lipid A-subunit analogs retain particular biological activities of endotoxin without a toxic effect being exerted, such as pyrogenicity and local Shwartzman reaction. Further experimentation is required in order to clarify the structural requirements for the expression of a limited beneficial activity in host defense.

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