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Enhancement of nonspecific resistance to viral infection by chemically synthesized lipid A-subunit analogs with different backbone structures and acyl groups

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Summary

Protection against vaccinia virus infection and induction of interferon (IFN) were investigated in *Propionibacterium acnes*-primed mice following treatment with chemically synthesized lipid A-subunit derivatives. The antiviral activity was based on the reduction of numbers of tail lesions in mice injected intravenously with the test compounds 1 day before virus infection. GLA-27, a 4-*O*-phosphono-D-glucosamine carrying 3-*O*-tetradecanoyl (C_{14}) and *N*-3-tetradecanoyloxytetradecanoyl [C_{14}-*O*-(C_{14})] groups, offered significant antiviral activity. Chemical modifications at the C_1 position of GLA-27, e.g. phosphorylation, replacement of OH by an SH, did not cause a significant change in antiviral activity. GLA-57 carrying an *N*-3-dodecanoyloxytetradecanoyl group showed stronger activity than GLA-27, but GLA-58 carrying an *N*-3-hexadecanoyloxytetradecanoyl group did not exhibit significant activity. GLA-59 carrying 3-*O*-3-hydroxytetradecanoyl and *N*-C_{14}-O-(C_{14}) groups was more active than GLA-27 and GLA-57. GLA-60 possessing the same fatty acid substituents as GLA-59 but in the reversed order was the most active of all compounds tested. This suggests that the nature and position of the acyl substituents are important for achieving the antiviral effects. The (R) isomers of GLA-59 and GLA-60 possessed stronger IFN-inducing activity than the (S) iso-

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mers, but no significant difference in antiviral activity was seen between the isomers.

Lipid A analog; Vaccinia virus; Interferon-inducing activity

Introduction

Lipid A, a lipid constituent of endotoxin or lipopolysaccharide (LPS), is the active moiety responsible for most of the various biological activities of LPS (Galanos et al., 1985; Homma et al., 1985; Kotani et al., 1985). Our investigations have been focused on the antiviral and toxic activities of chemically synthesized lipid A and its subunit analogs to clarify structure–activity relationships of lipid A (Matsuura et al., 1984, 1985, 1986; Kumazawa et al., 1985, 1986, 1987). GLA-27, a 4-*O*-phosphono-D-glucosamine (GlcN) derivative carrying *N*-linked 3-tetradecanoyloxytetradecanoyl [C_{14}-O-(C_{14})] and 3′-*O*-linked tetradecanoyl (C_{14}) groups, is a prototype compound fulfilling the structural requirement for manifesting beneficial activities without severe toxic activities (Matsuura et al., 1984, 1985; Kumazawa et al., 1985, 1986). To further increase desirable activities, new GLA-27 derivatives with different backbone structures and mono- and disaccharide-type compounds with different acyl and acyloxyacyl groups have been synthesized.

The aim of this study was to estimate the biological activity of 11 synthetic compounds in terms of enhancement of nonspecific resistance against vaccinia virus infection and interferon (IFN)-inducing activity in *Propionibacterium acnes*-primed mice.

Materials and Methods

Animals

Female 4-week-old ddY mice, obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan) were housed for one week under a specific pathogen-free environment and then used for determination of antiviral activity. Female ICR mice, 7 weeks of age, were used for testing IFN-inducing activity.

Cells and virus

The cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 3 mM glutamine, 0.07% bicarbonate and either 5% newborn calf serum for HEL cells or 10% fetal calf serum for L-929 cells. Vaccinia virus (Lister strain) and vesicular stomatitis virus (New Jersey strain) were grown in HEL cells and L-929 cells, respectively. The virus stocks were stored at −80°C until use.

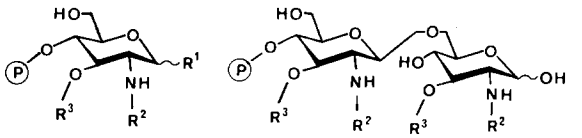
Synthetic compounds and control lipid A

The chemical structures of synthetic compounds used in this study are shown in

Fig. 1. The compounds were synthesized according to the method described earlier (Kiso et al., 1986, 1987a,b) and purified by high-performance liquid chromatography. The purity of synthetic compounds was more than 99%. Lipid A and LPS of *Escherichia coli* Re mutant (strain F515), kindly donated by Drs. O. Lüderitz and C. Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G., were used as controls. MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) was provided by the Research Institute of Daiichi Seiyaku Co., Ltd., Tokyo, Japan. The synthetic compounds and control lipid A were solubilized in pyrogen-free water by treatment with triethylamine and complexed with bovine serum albumin. The triethylamine was completely removed by evaporation (Matsuura et al., 1983). The solutions were diluted with sterile physiological saline before use.

Determination of IFN-inducing activity

IFN-inducing activity was assessed by measuring the IFN titer in pooled sera of five *P. acnes*-primed ICR mice per group as described previously (Ikeda et al.,



Monosaccharide-type Disaccharide-type

Synthetic compound	Substituent		
	R^3	R^2	R^1
Monosaccharide-type			
GLA-27	C_{14}	C_{14}-O-(C_{14})	OH
GLA-40	C_{14}	C_{14}-O-(C_{14})	H
GLA-66	C_{14}	C_{14}-O-(C_{14})	SH
GLA-70	C_{14}	C_{14}-O-(C_{14})	O-P
GLA-57	C_{14}	C_{14}-O-(C_{12})	OH
GLA-58	C_{14}	C_{14}-O-(C_{16})	OH
GLA-59	C_{14}-OH	C_{14}-O-(C_{14})	OH
GLA-60	C_{14}-O-(C_{14})	C_{14}-OH	OH
Disaccharide-type			
GLA-32	C_{14}	C_{14}-O-(C_{14})	
GLA-54	C_{14}-OH	C_{14}-O-(C_{14})	
GLA-55	C_{14}-O-(C_{14})	C_{14}-OH	

Fig. 1. Chemical structures of synthetic lipid A analogs. Ⓟ, $PO(OH)_2$; C_{14}, tetradecanoyl; C_{14}-OH, 3-hydroxytetradecanoyl; C_{14}-O-(C_{12}), 3-dodecanoyloxytetradecanoyl; C_{14}-O-(C_{14}), 3-tetradecanoyloxytetradecanoyl; C_{14}-O-(C_{16}), 3-hexadecanoyloxytetradecanoyl. Unless stated otherwise, the activities were determined using compounds with the (RS) configuration. Only GLA-59 and GLA-60 with three stereospecificities, (R), (RS) and (S), based on the asymmetric carbon at the C_3 position of C_{14}-OH and C_{14}-O-(C_{14}) groups were used.

1988). Briefly, mice were injected intravenously (i.v.) with 10-μg test samples 2 h before bleeding. Titers are expressed in international reference units. They correspond to the reciprocals of the highest dilution of test sera capable of reducing by 50% vesicular stomatitis virus-induced cytopathic effect in L-929 cells (Ikeda et al., 1985a).

Determination of antiviral activity against vaccinia virus

Antiviral activity was assessed by measuring the reduction of numbers of tail lesions in ten or twenty 5-week-old female ddY mice per group following i.v. infection of 10^4 plaque-forming units (PFU) of vaccinia virus (Ikeda et al., 1985b). Mice were injected i.v. with the indicated doses of the test compounds 1 day before the virus challenge, unless stated otherwise. Seven days after the challenge, the number of lesions was counted by staining with 1% fluorescein-0.5% methylene blue solution. Results are expressed as the percent inhibition ± SEM.

Statistical analysis

Statistical significance between the results of the test group and the control group was analyzed by the Student's *t*-test.

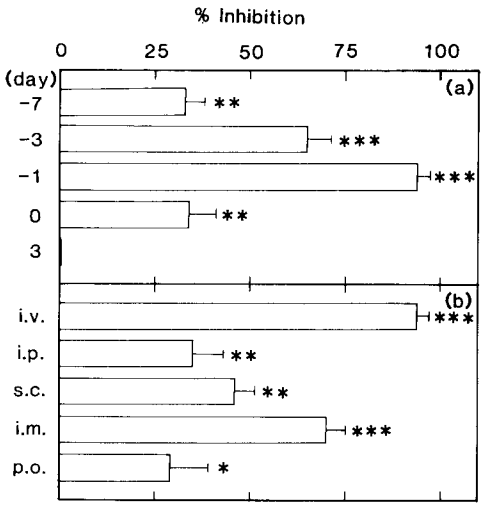


Fig. 2. Effects of time and route of treatment with LPS on lesion formation in mice infected with vaccinia virus. Female ddY mice were injected i.v. with 1 μg of LPS at the indicated days (a). With respect to the effect of route of LPS administration (b), 1 μg of LPS was administered by different routes [i.v. (intravenously), i.p. (intraperitoneally), s.c. (subcutaneously), i.m. (intramuscularly), p.o. (orally)]. Seven days after the challenge with 10^4 pfu of vaccinia virus, the lesions formed on the tail were counted. Results are expressed as the percent inhibition ± SEM of 10 mice per group. Statistically significant difference between the test group and the control group (*, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$) was determined by Student's *t*-test.

Results

Experimental conditions to estimate antiviral activity

Following i.v. inoculation of vaccinia virus, discrete dermal vesicles appeared on the tail of ddY mice on day 4. The maximum number, about 40 lesions, was reached on day 7. Therefore, the number of lesions was recorded at 7 days after infection. Virus titers of homogenates made from tails showing lesions, determined by a plaque assay on HeLa cell monolayers, closely correlated with the number of lesions (data not shown). To determine the optimum conditions for estimating antiviral activity of the test compounds, varying times and routes of LPS injection were examined. As shown in Fig. 2, the strongest antiviral activity of LPS was observed when mice were injected i.v. with LPS 1 day before infection. Therefore, all experiments were carried out under these conditions.

Protective effect of synthetic compounds against viral infection

Fig. 3 shows results of the antiviral activity of GLA-27 compared with that of *E. coli* LPS (Re mutant), its free lipid A and a synthetic immunomodulator MDP. Though the activity of GLA-27 was about 100-fold higher than that of MDP, lipid A as well as LPS exhibited much stronger antiviral activity than GLA-27.

The effect of chemical modification at the C_1 position of the backbone structure

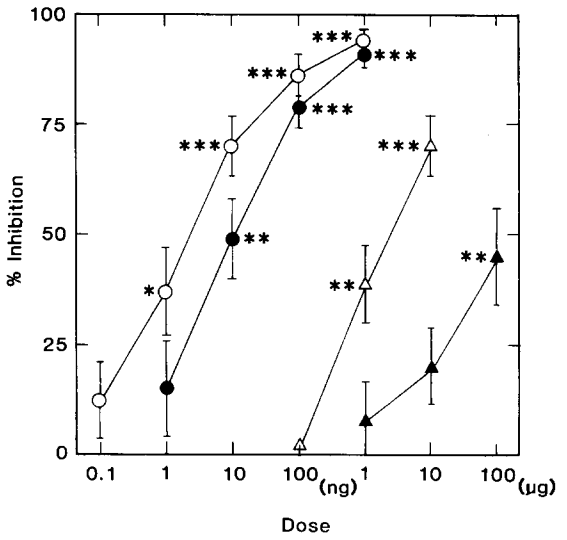


Fig. 3. Dose response curves for inhibitory effects of LPS, lipid A, GLA-27 and MDP on lesion formation induced by vaccinia virus infection. Female ddY mice were treated i.v. with the indicated dose of test samples, i.e., LPS (○), lipid A (●), GLA-27 (△) and MDP (▲), the day before i.v. challenge with 10^4 PFU of vaccinia virus. The lesions formed on the tail were counted on the 7th day after virus infection. Results are expressed as the percent inhibition ± SEM of 10 mice per group. Statistical differences between test and control groups: *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

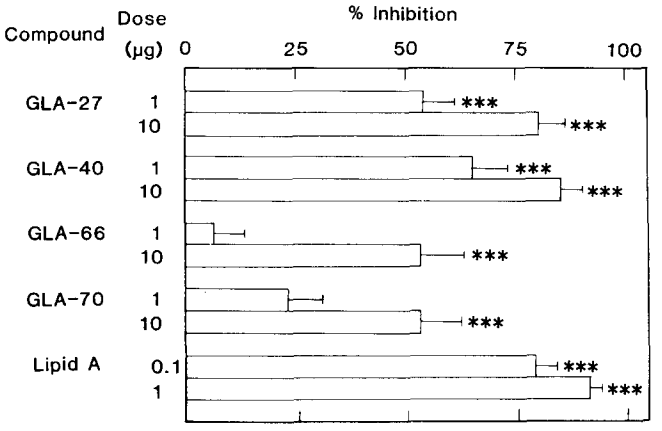


Fig. 4. Relationship between different backbone structures of compounds and their antiviral activity. See legend to Fig. 3.

of GLA-27 is shown in Fig. 4. GLA-40, a 1-deoxy derivative of GLA-27, achieved a similar antiviral activity as GLA-27. A 1,4-*O*,*O*-bisphosphorylated derivative with the same acyl groups as GLA-27 (GLA-70) and another derivative with a thiol group instead of an hydroxyl at the C_1 position of GLA-27 (GLA-66) exhibited weaker activity than the parent compound GLA-27.

The activity of the compounds composed of an *N*-(RS)-acyloxytetradecanoyl group with different carbon chain length, e.g. C_{14}-O-(C_{12}), C_{14}-O-(C_{14}) and C_{14}-

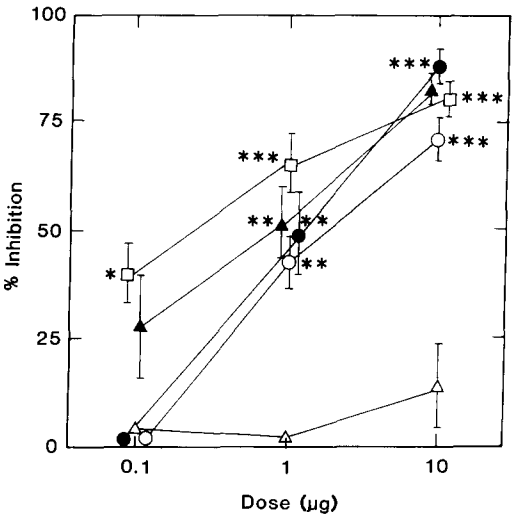


Fig. 5. Effects of different acyl groups at the 2-*N* and 3-*O* positions in glucosamine on the antiviral activity of the compounds. GLA-27 (○), GLA-57 (●), GLA-58 (△), GLA-59 (▲), GLA-60 (□). See legend to Fig. 3 for details.

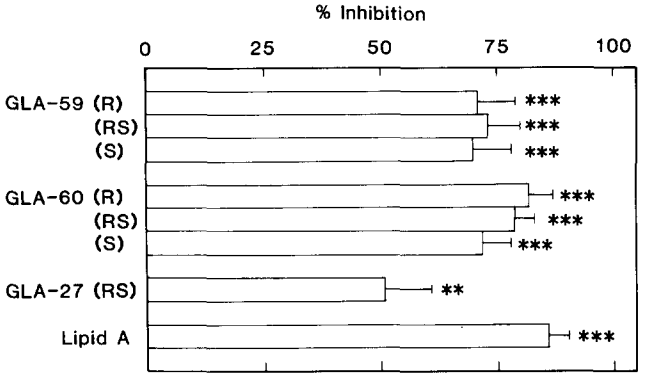


Fig. 6. Antiviral activity of stereoisomers of GLA-59 and GLA-60. Mice were injected i.v. with 1 μg of the test compounds one day before virus challenge. For other details, see legend to Fig. 3.

O-(C_{16}), termed GLA-57, GLA-27 and GLA-58, respectively, is shown in Fig. 5. GLA-57 with a C_{14}-O-(C_{12}) group exhibited stronger protective activity than GLA-27 with C_{14}-O-(C_{14}) group, while GLA-58 with a C_{14}-O-(C_{16}) group did not show detectable activity. GLA-59, a compound with *N*-C_{14}-O-(C_{14}) and 3-*O*-C_{14}OH groups, brought about greater resistance to the viral infection than did GLA-57. GLA-60, which contains the same fatty acid substituents as GLA-59 but in reversed order, emerged as the most active from all compounds tested.

Next, the antiviral activity of the (R) and (S) isomers of GLA-59 and GLA-60 (stereospecificity based on two asymmetric carbons at the C_3 position of C_{14}OH and C_{14}-O-(C_{14}) groups) was examined. As shown in Fig. 6, all (R) and (S) stereoisomers showed remarkable antiviral activity, and no significant difference in ac-

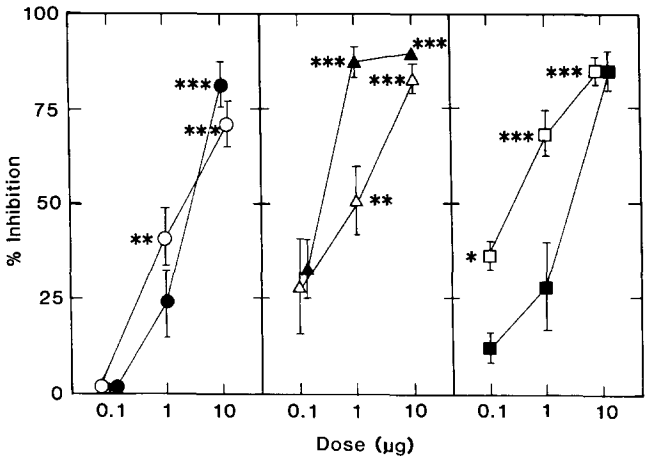


Fig. 7. Antiviral activity of disaccharide-type compounds. Open symbols represent monosaccharide-type compounds (○, GLA-27; △, GLA-59; □, GLA-60), and closed symbols indicate disaccharide-type compounds (●, GLA-32; ▲, GLA-54; ■, GLA-55). See legend to Fig. 3 for other details.

TABLE 1

IFN-inducing activity of synthetic lipid A analogs

Compound[a]	IFN titer (IU/0.1 ml)[b]	
	Exp. 1	Exp. 2
Mock control	< 10	< 10
Lipid A	1280	2560
Monosaccharide		
GLA-27 (RS)	320	440
GLA-66 (RS)	80	160
GLA-70 (RS)	< 10	40
GLA-57 (RS)	640	640
GLA-58 (RS)	< 10	20
GLA-59 (RS)	480	480
GLA-60 (RS)	640	640
Stereoisomer		
GLA-59 (R)	640	640
(RS)	320	320
(S)	160	160
GLA-60 (R)	640	640
(RS)	640	640
(S)	320	320
Disaccharide		
GLA-32 (RS)	40	40
GLA-54 (RS)	640	640
GLA-55 (RS)	20	40

[a] Ten μg of test samples were injected i.v. into *P. acnes*-primed mice 2 h before bleeding.

[b] IFN titer in sera of five mice was determined using L-929 cells and vesicular stomatitis virus. The experiments were carried out in triplicate. The titer represents the reciprocal of the serum dilution giving a 50% reduction of the virus-induced cytopathic effect.

tivity was observed among the stereoisomers of GLA-59 or GLA-60.

Since disaccharide-type compounds show stronger biological activities than monosaccharide-type compounds including GLA-27 and lipid X carrying different acyl substituents (Takahashi et al., 1987), we investigated whether 4′-*O*-monophosphorylated disaccharide-type compounds (GLA-32, GLA-54 and GLA-55) showed stronger antiviral activity than 4-*O*-phosphorylated monosaccharide-type compounds with the same acyl substituents. As shown in Fig. 7, the antiviral activity of GLA-54 was significantly stronger than that of monosaccharide-type compound GLA-59 at a dose of 1 μg/mouse, while the activity of disaccharide-type compound GLA-55 was weaker than that of monosaccharide-type compound GLA-60 at doses of 0.1 and 1 μg/mouse. No significant difference was observed between the disaccharide-type compound GLA-32 and the monosaccharide-type compound GLA-27.

IFN-inducing activity

IFN titers in the serum of *P. acnes*-primed mice injected i.v. with LPS and lipid

A were 10-fold higher than the IFN titers obtained in unprimed mice injected with LPS or lipid A (data not shown). The IFN-inducing activity of the synthetic compounds was thus determined in *P. acnes*-primed mice. The results are shown in Table 1. GLA-27 exhibited significant IFN-inducing activity in *P. acnes*-primed mice. It also did so in rabbits, as described previously (Matsuura et al., 1984). The IFN-inducing activity of GLA-66 with an SH group at the C_1 position was less than that of GLA-27 with an OH group. Bisphosphorylation of GLA-27 (compound GLA-70) diminished the IFN-inducing activity. Among the compounds containing (RS)-acyloxyacyl groups of different chain length, GLA-57 with an *N*-C_{14}-O-(C_{14}) group showed stronger activity than GLA-27 with an *N*-C_{14}-O-(C_{14}) group. On the other hand, the activity of GLA-58 with an *N*-C_{14}-O-(C_{16}) group was much lower. GLA-59 carrying *N*-C_{14}-O-(C_{14}) and 3-*O*-C_{14}OH groups produced higher IFN titers than GLA-27. GLA-60 with *N*-C_{14}OH and 3-*O*-C_{14}-O-(C_{14}) groups produced more IFN than did GLA-59. Among the (R) and (S) stereoisomers of GLA-59 and GLA-60, the (R) configuration was more effective in inducing IFN than the (S) configuration. A disaccharide-type compound, GLA-54, showed stronger activity than the corresponding monosaccharide-type compound GLA-59. However, GLA-32 and GLA-55 induced lower IFN titers than their monosaccharide-type compounds GLA-27 and GLA-60.

Discussion

Intravenous administration of some lipid A-subunit analogs, e.g. GLA-57, GLA-59 and GLA-60, significantly increased nonspecific resistance against vaccinia virus infection. These compounds possess significant immunostimulatory activities without detectable pyrogenicity and Shwartzman reaction (Kumazawa et al., 1988; Ikeda et al., 1988). Although LPS and lipid A are also active in inducing IFN and protect mice against vaccinia virus infection, pyrogenicity of natural free lipid A is apparent at much smaller doses than with GLA-59 and GLA-60. Thus, these synthetic analogs show a significant dissociation between antiviral activity and pyrogenicity.

There was a significant difference between GLA-27 and GLA-59 with respect to their antiviral and IFN-inducing activities. The only structural difference between GLA-27 and GLA-59 is the 3-*O*-linked fatty acid of the C_{14} and C_{14}OH groups. GLA-57 carrying an *N*-C_{14}-O-(C_{12}) group exhibited stronger activity than GLA-27, but GLA-58 carrying an *N*-C_{14}-O-(C_{16}) group did not show significant activity (Fig. 5 and Table 1). Other immunopharmacological activities are influenced in the same fashion (Kumazawa et al., 1988), suggesting that the nature of the acyl substituent in the lipid A-subunit analogs is very important for their antiviral and IFN-inducing activities. A significant difference was also observed in the antiviral and IFN-inducing activities of GLA-59 and GLA-60. GLA-60 was the most active of the two compounds. It was also more active in inducing nonspecific protection against *Pseudomonas aeruginosa* infection (Nakatsuka et al., unpublished data). The fatty acid substituents of GLA-59 are the same as those of GLA-60 but

their positions are reversed, suggesting that the positioning of the acyl groups is also important.

In the monosaccharide-type compounds, bisphosphorylation at the 1-*O* and 4-*O* positions diminished the antiviral and IFN-inducing activities, indicating that the presence of two phosphoryl groups in one glucosamine is less effective in entrusting antiviral and IFN-inducing activities than the presence of one phosphoryl group. Chemical modification at the C_1 position of the glucosamine backbone did not lead to an enhancement of the activities tested. Similar results were observed in terms of tumor necrosis factor induction and activation of macrophages (Kumazawa et al., in press).

Previous studies have shown that stereospecificity based on an asymmetric carbon of $C_{14}OH$ in GLA-27, GLA-40, GLA-59 and GLA-60 influences the biological activities (Matsuura et al., 1986; Kumazawa et al., 1987, 1988). As noted previously for the (R) and (S) stereoisomers of GLA-27 and GLA-40 (Kumazawa et al., 1987), the antiviral activity of the (R) and (S) isomers of GLA-59 and GLA-60 did not markedly differ (Fig. 6), although the IFN-inducing activity of the compounds with the (R) configuration was stronger than that of compounds with the (S) configuration.

Antiviral and IFN-inducing activities of GLA-27, GLA-59 and GLA-60 were compared with those of 4-*O*-monophosphorylated β (1→6)-linked D-glucosamine disaccharides carrying *N*, *N'*- and 3, 3'-*O*, *O'*-acyl groups, which have the same acyl substituents as GLA-27, GLA-59 and GLA-60, respectively. The activities of the disaccharide-type analogs were not always stronger than those of the monosaccharide-type analogs, suggesting that significant advantage is not always induced by the 4-*O*-monophosphorylated disaccharide structure.

The protective effect of GLA-59 and GLA-60 against vaccinia tail lesion formation may be primarily ascribed to the ability of the mononuclear phagocyte system to suppress virus infection, since GLA-59 and GLA-60 markedly stimulate peritoneal macrophages with respect to phagocytosis, level of cellular lysosomal enzymes and cytostasis-inducing activity (Kumazawa et al., 1988). Other mechanisms may also mediate protection against vaccinia virus infection, i.e. participation of natural killer cells as effector cells and IFN, since (a) i.p. administration of GLA-27 and GLA-60 cause marked enhancement of natural killer activity of peritoneal cells against YAC-1 target cells (Ikeda et al., 1988), (b) some analogs induce significant IFN production in *P. acnes*-primed mice, (c) lesion formation following vaccinia virus infection is partially suppressed by an adoptive transfer of LPS-injected sera with high IFN titer (unpublished data), and (d) IFN augments the activities of macrophages and natural killer cells (Ortaldo et al., 1981).

In conclusion, GLA-60 possessed the strongest antiviral activity among the compounds tested. We are conducting further investigations to decipher whether this analog can serve as a new type of antiviral agent for the treatment of infections due to other pathogens such as herpes simplex virus or Banzi flavivirus.

Acknowledgements

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