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Short Communication

Effect of acyl substituents of synthetic lipid A-subunit analogues on their immunomodulating antiviral activity

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Summary

A chemically synthesized lipid A-subunit analogue, GLA-60, 2-deoxy-4-*O*-phosphono-2-[(3R)-3-hydroxytetradecanamido]-3-*O*-[(3*R*)-3-tetradecanoyloxytetradecanoyl]-D-glucose, has many of the activities of endotoxin but has little toxicity. Then, compounds with various lengths of acyl side chain of the acyloxyacyl group at the 3-*O* position of GLA-60 were synthesized and evaluated for interferon (IFN)-inducing activity, natural killer (NK) cell activation and antiviral activity. The compounds with acyl side chains between C_8 and C_{15} exhibited significant antiviral activity (inhibition of pox tail lesion formation in vaccinia virus-infected mice), serum IFN-inducing activity and NK cell activation. However, the compound carrying a C_2 or a C_{16} acyl side chain did not exhibit these activities. The compounds with a C_{13} or C_{14} acyl side chain showed strong protective against herpes simplex virus type 1 in cyclophosphamide-immunosuppressed mice.

Key words: Immunomodulator; Lipid A analogue; Interferon; Natural killer (NK) cell

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Introduction

GLA-60, a chemically synthesized lipid A-subunit analogue, exhibits a wide variety of immunopharmacological activities but not the toxicity of natural lipid A such as pyrogenicity and Shwartzman reactivity (Ikeda et al., 1988b; Kumazawa et al., 1988). Furthermore, GLA-60 is active against vaccinia virus, herpes simplex virus and *Pseudomonas aeruginosa*, and also shows antitumor activity against Meth A fibrosarcoma (Ikeda et al., 1988a,b, 1989; Nakatsuka et al., 1989). Structurally, GLA-60 is composed of a 4-*O*-phosphono-D-glucosamine backbone with (*R*)-3-hydroxytetradecanoyl (C_{14}-OH) and (*R*)-3-tetradecanoyloxytetradecanol [C_{14}-O-(C_{14})] substituents at the 2-*N*- and 3-*O*-positions, respectively. If in GLA-27, which has 3-*O*-C_{14} and 2-*N*-C_{14}-O-(C_{14}) groups on the same backbone as that of GLA-60, the acyl side chain at the 2-*N* acyloxyacyl group is replaced by C_{14}-O-(C_{16}), the antiviral and IFN-inducing activities are lost (Ikeda et al., 1988a).

In the present study we examined the effect of substitutions of the 3-*O*-acyloxyacyl group with acyl side chains of different length on the immunopharmacological and antiviral activities of the prototype compound GLA-60.

Materials and Methods

The chemical structures of 9 synthetic compounds are shown in Fig. 1. The compounds were synthesized as described previously (Kiso et al., 1987a,b). Natural lipid A prepared from *Escherichia coli* F515 strain (Re) was kindly supplied for use as a control by Drs O. Lüderitz and C. Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G. These compounds and control lipid A were solubilized in pyrogen-free water as triethylamine salts and complexed with bovine serum albumin before use (Matsuura et al., 1983).

For assay of NK cell activity, peritoneal cells were obtained from 5-week-old female ddY mice (Japan SLC, Hamamatsu), which had been administered 1 μg of test compound intraperitoneally (i.p.) 1 day previously. Then, NK cell activity was assessed by determining the release of radioactivity from target YAC-1 cells as described (Ikeda et al., 1988b). The specific release of radioactivity was calculated according to the following formula: Specific lysis (%) = [(cpm of tested groups – cpm of spontaneous release) ÷ (cpm of complete release – cpm of spontaneous release)] × 100.

For assay of IFN-inducing activity, 6-week-old female ddY mice were used after priming through the i.p. route by administration of 1 mg of formalin-killed *Propionibacterium acnes* 7 days previously. Such pretreatment of the mice enhanced serum IFN-production. Two hours after intravenous (i.v.) administration of 10 μg of test compounds to the primed mice, blood was collected and sera prepared for the IFN assay using L-929 cells and vesicular stomatitis virus (VSV; New Jersey strain) as described previously (Ikeda et al., 1988a). The IFN titers were expressed as international reference units calculated using standard IFN α/β (Lee Biomolecular, California).

Antiviral activity against vaccinia virus (VV; Lister strain) was assessed by measuring reductions in the numbers of lesions formed on the tails of VV-infected mice as described (Ikeda et al., 1988b). Seven days after inoculation of VV into the tail vein, the number of lesions formed was counted by staining with 1% fluorescein–0.5% methylene blue solution, and the antiviral activity was expressed by percent inhibition ± SE using 10 mice per group.

Antiviral activity against herpes simplex virus type 1 (HSV-1; HF strain) was based on the survival of mice infected with HSV-1 (Ikeda et al., 1989): the mice were given an i.p. injection of 200 mg/kg of cyclophosphamide (CY; Shionogi Co. Ltd., Osaka) 4 days before i.p. challenge with 10^5 PFU of HSV-1. Test compounds were administered i.p. to CY-immunosuppressed mice 3 days before HSV-1 infection. Surviving mice were counted 14 days later.

Results and Discussion

Results of NK cell activation and IFN-inducing activity of these compounds are shown in Table 1. The peritoneal NK cell activity was remarkably enhanced by administration of control lipid A (33.9 ± 1.5), and the enhanced activity disappeared completely after treatment of the cells with anti-asialo GM_1 monoclonal antibody (data not shown). This suggests that enhancement by lipid A is specific for NK cells. Compounds carrying C_8 to C_{14} acyl side chains in the 3-*O*-acyloxyacyl group showed significant stimulation of NK activity, although this stimulatory effect was less pronounced than that of natural lipid A. Among the compounds tested, GLA-90 and GLA-91 carrying C_{14}-O-(C_{11}) and C_{14}-O-(C_{13}), respectively, exhibited strong stimulation of NK cell activity (14.9 ± 1.3 and 15.5 ± 0.9), whereas GLA-87 carrying C_{14}-O-(C_2) with a short acyl chain and GLA-92 carrying C_{14}-O-(C_{15}) with a longer acyl chain did not activate NK cells at all.

With respect to IFN-inducing activity, substitutions of C_8 to C_{13} acyl side chains in acyloxyacyl group effected a high IFN-inducing activity, as shown in Table 1. IFN titers reached with GLA-89 (C_{10}), GLA-90 (C_{11}) and GLA-91 (C_{13}) were about 2000 IU/0.1 ml, which is comparable to that of natural lipid A. Compounds containing longer acyl side chains (more than 14) showed decreased IFN-inducing activity. GLA-87, carrying an extremely short acyl side chain (C_2), did not induce serum IFN at all.

The relationship between acyl chain length and antiviral activity was pursued by the pox tail lesion assay in VV-infected mice, GLA-87 (C_2) and GLA-64 (C_{16}) failed to inhibit the formation of pox tail lesions in mice. Compounds carrying acyl side chains between C_8 and C_{14} demonstrated significant antiviral activity as seen in Fig. 2. Substitutions of a longer acyl side chain in the acyloxyacyl group, i.e. C_{14}-O-(C_{15}) or C_{14}-O-(C_{16}), diminished the antiviral activity.

We also determined the protective activity of these compounds in HSV-1-infected CV-immunosuppressed mice. When CV was administered i.p. to mice at a dose of 200 mg/kg 4 days before i.p. challenge with 10^5 PFU of HSV-1, mortality was markedly increased as compared to that of mice not treated with CY. As shown

TABLE 1

Comparison of NK- and IFN-inducing activities by synthetic lipid A-subunit analogues

Compound	3-*O*-linked C_{14}-O-(C_n) n	NK activity[a] % Lysis, mean ± SE	IFN titer[b] (IU/0.1 ml)
No treatment		−3.1±0.2	< 10
GLA-87	(2)	−3.1±1.0[ns]	18
GLA-88	(8)	8.1±2.0[d]	1000
GLA-89	(10)	8.2±1.4[d]	2080
GLA-90	(11)	14.9±1.3[e]	2560
GLA-63	(12)	11.4±1.7[d]	1170
GLA-91	(13)	15.5±0.9[e]	2320
GLA-60	(14)	7.3±0.2[d]	640
GLA-92	(15)	1.5±1.4[ns]	540
GLA-64	(16)	3.3±1.4[c]	160
Lipid A		33.9±1.5[e]	2560

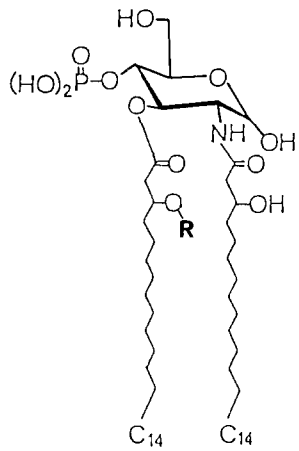
[a]Peritoneal cells were obtained from ddY mice which had been administered i.p. 1 μg of test samples 1 day previously. Peritoneal cells (6×10^5/well in 96-well microplates) were cocultured for 4 h with target ^{51}Cr-labelled YAC-1 lymphoma cells in an effector/target cell ratio of 30:1. Lytic activity was assessed by the degree of release of ^{51}Cr from the target cells and expressed as mean ± SE of three repeated experiments carried out independently.

[b]Ten μg of test samples were injected i.v. into *P. acnes* primed mice. IFN titer in sera was assayed using L-929 cells and vesicular stomatitis virus. The results were expressed as the average of two experiments.

[b]Ten μg of test samples were injected i.v. into *P. acnes* primed mice. IFN titer in sera was assayed

in Fig. 3, the survival rate of HSV-1-infected CV-suppressed mice significantly increased and approached that of the control mice following treatment with GLA-91 (C_{13}) or GLA-60 (C_{14}), but not any of the other compounds.

Among the synthetic lipid A-subunit analogues investigated so far, GLA-60 ex-



Compound	Substituent (R)	Number of carbons (n)
GLA-87	$COCH_3$	2
GLA-88	$CO(CH_2)_6CH_3$	8
GLA-89	$CO(CH_2)_8CH_3$	10
GLA-90	$CO(CH_2)_9CH_3$	11
GLA-63	$CO(CH_2)_{10}CH_3$	12
GLA-91	$CO(CH_2)_{11}CH_3$	13
GLA-60	$CO(CH_2)_{12}CH_3$	14
GLA-92	$CO(CH_2)_{13}CH_3$	15
GLA-64	$CO(CH_2)_{14}CH_3$	16

Fig. 1. Structures of chemically synthesized lipid A-subunit analogues.

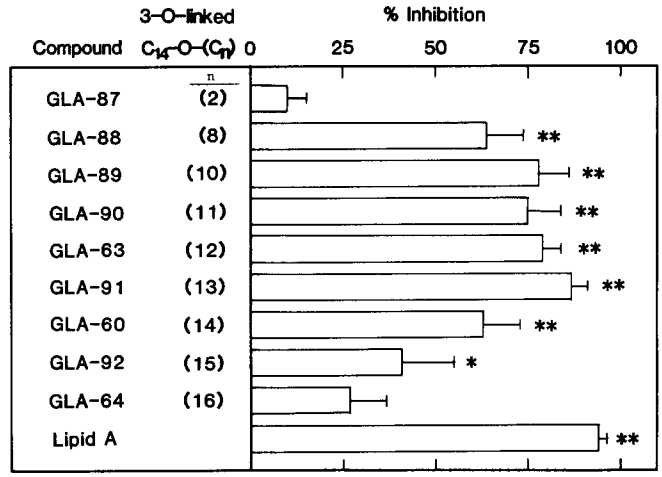


Fig. 2. Inhibitory effects of lipid A-subunit analogues on pox tail lesion formation. Female ddY mice were administered i.v. the indicated doses of test samples the day before i.v. challenge with 10^4 PFU of VV per mouse. Seven days after the virus challenge, the numbers of lesions formed on the tail were counted. Results are expressed as percent inhibition ± SE of 10 mice per group. *, $P < 0.05$; **, $P < 0.001$ (Student's t-test).

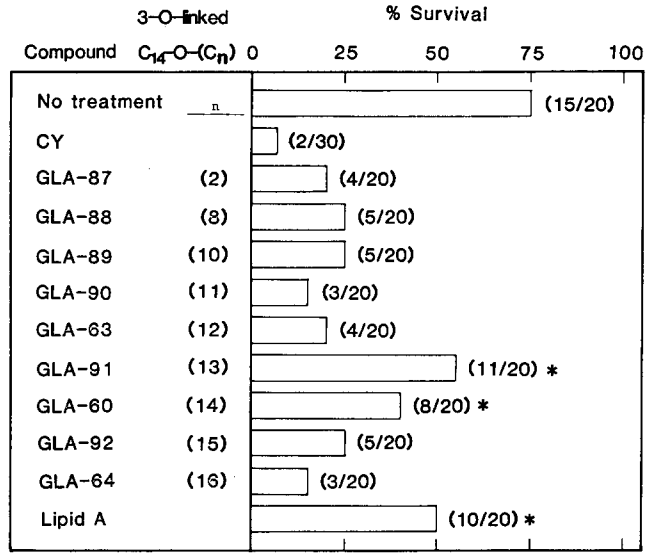


Fig. 3. Effect of lipid A-subunit analogues on induction of resistance against HSV-1 infection in immunosuppressed mice. Female ddY mice were treated i.p. with 200 mg/kg of CY at 4 days before HSV-1 infection and administered 30 μg test samples 3 days before i.p. challenge with 1×10^5 PFU of HSV-1. Surviving mice were counted 14 days later and the results are expressed as % survival. *, $P < 0.005$ as compared with CY-immunosuppressed mice (χ^2 test).

hibited the best immunopharmacologic activities and greatest protection against microbial infections (Ikeda et al., 1988a; Kumazawa et al., 1988; Nakatsuka et al., 1989). In further investigations assessing the role of the acyl side chain length of the 3-*O*-acyloxyacyl group of GLA-60, we found that compounds GLA-89, GLA-90, GLA-63 and GLA-91, containing C_{10} to C_{13} acyl side chains also exhibit marked immunopharmacological activities.

With respect to toxicity, natural lipid A is pyrogenic at a dose of 0.001 μg/kg in Japanese white rabbits, whereas none of the GLA-60 derivatives is pyrogenic even at a dose of 100 μg/kg (data not shown). These results indicate that the toxic activity of the parent molecule (lipid A) can be dissociated from its beneficial biological activities.

According to a recent paper by Lei and Morrison (1988), there is a specific LPS-binding protein (MW = 80 kDa) on the cytoplasmic membrane of murine splenocytes, and this protein may represent the receptor sites responsible for LPS-mediated cellular immunostimulation. Lipid A-subunit analogues should also bind to these receptor sites to achieve their biological effects. Appropriate length of the acyl side chains in the 3-*O* acyloxyacyl group may satisfy the conditions for binding to LPS-specific receptors on the surface of effector and target cells.

In this paper, we have described new compounds that exhibit stronger antiviral activity than those previously reported, and that approach the activity of natural lipid A. Further investigations are under way to decipher whether these compounds can serve as immunomodulating antiviral agents for treatment of viral infections in the normal and immunodeficient host.

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References

Ikeda, S., Kumazawa, Y., Nishimura, C., Nakatsuka, M., Homma, J.Y., Kiso, M. and Hasegawa, A. (1988a) Enhancement of nonspecific resistance to viral infection by chemically synthesized lipid A-subunit analogs with different backbone structures and acyl groups. Antiviral Res. 10, 167–178.

Ikeda, S., Nishimura, C., Nakatsuka, M., Homma, J.Y., Kiso, M. and Hasegawa, A. (1988b) Antiviral and immunomodulating activities of chemically synthesized lipid A-subunit analogues GLA-27 and GLA-60. Antiviral Res. 9, 37–46.

Ikeda, S., Tominaga, T., Nishimura, C., Homma, J.Y., Kiso, M. and Hasegawa, A. (1989) Antiherpes activity of chemically synthesized lipid A-subunit analogue GLA-60 in immunosuppressed mice. Antiviral Res. 11, 173–180.

Kiso, M., Tanaka, S., Fujita, M., Fujishima, Y., Ogawa, Y. and Hasegawa, A. (1987a) Synthesis of nonreducing-sugar subunit analogs of bacterial lipid A carrying an amide-bound (3*R*)-3-acyloxytetradecanoyl group. Carbohydr. Res. 162, 247–256.

Kiso, M., Tanaka, S., Fujita, M., Fujishima, Y., Ogawa, Y., Ishida, H. and Hasegawa, A. (1987b)

Synthesis of the optically active 4-*O*-phosphono-D-glucosamine derivatives related to the nonreducing-sugar subunit of bacterial lipid A. Carbohydr. Res. 162, 127–140.

Kumazawa, Y., Nakatsuka, M., Takimoto, H., Furuya, T., Nagumo, T., Yamamoto, A., Homma, J.Y., Inada, K., Yoshida, M., Kiso, M. and Hasegawa, A. (1988) Importance of fatty acid substituents of chemically synthesized lipid A-subunit analogs in the expression of immunopharmacological activity. Infect. Immun. 56, 149–155.

Lei, M. and Morrison, D.C. (1988) Specific endotoxin lipopolysaccharide-binding proteins on murine splenocytes. II. Membrane localization and binding characteristics. J. Immunol. 141, 1006–1011.

Matsuura, M., Kojima, Y., Homma, J.Y., Kubota, Y., Shibukawa, N., Shibata, M., Inage, M., Kusumoto, S. and Shiba, T. (1983) Interferon-inducing, pyrogenic and proclotting enzyme of horseshoe crab activation activities of chemically synthesized lipid A analogues. Eur. J. Biochem. 137, 639–642.

Nakatsuka, M., Kumazawa, Y., Matsuura, M., Homma, J.Y., Kiso, M. and Hasegawa, A. (1989) Enhancement of nonspecific protective activity against *Pseudomonas aeruginosa* infection and tumor regression by synthetic lipid A-subunit analogs, 4-*O*-phosphono-D-glucosamine derivatives carrying *N*- and 3-*O*-linked acyl groups. Int. J. Immunopharmacol. 11, 349–358.