Antiviral Research, 11 (1989) 173–180
Elsevier

AVR 00359

Antiherpes activity of chemically synthesized lipid A-subunit analogue GLA-60 in immunosuppressed mice

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(Received 20 January 1989; accepted 24 March 1989)

Summary

Intraperitoneal administration of 10 μg GLA-60, a chemically synthesized lipid A analogue, to mice one day after treatment with 200 mg/kg of cyclophosphamide (CY) significantly increased the number of macrophages, lymphocytes and polymorphonuclear leukocytes (PMNs) in the peritoneal cavity. The intrinsic antiviral activity of macrophages against herpes simplex virus type 1 (HSV-1) as well as natural killer (NK) activity against YAC-1 target cells was stimulated by administration of GLA-60 to CY-immunosuppressed mice. When the mice were administered GLA-60 prior to HSV-1 infection, virus growth was inhibited and the mortality rate of infected mice was reduced. Thus, GLA-60 is a potent immunomodulator achieving its antiviral action through enhancement of nonspecific host defense mechanisms. Combined treatment of GLA-60 with the antiviral agent acyclovir (ACV) resulted in greater protection against HSV-1 in the CY-immunosuppressed mice than did single treatment with either GLA-60 or ACV.

Lipid A analogue; Herpes simplex virus; Immunocompromised host; Immunomodulator

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Introduction

Herpes simplex virus (HSV) can lead to severe infections in immunocompromised patients. In these patients, the lymphocyte, macrophage and NK cell counts decrease, resulting in a higher susceptibility to infectious diseases. According to Buhles and Shifrine (1977), administration of CY to mice leads to a marked reduction of various types of immunocompetent cells including PMNs, blood monocytes and subpopulations of lymphocytes. Therefore, CY is often used as an immunosuppressant to render mice susceptible to microbial infections (Collis, 1980; Dunger et al., 1981; Ikeda et al., 1988b).

GLA-60, a 4-*O*-phosphono-D-glucosamine derivative carrying 3-*O*-3-tetradecanoyloxytetradecanoyl [C_{14}-*O*-(C_{14})] and 2-*N*-3-hydroxytetradecanoyl (C_{14}-OH) groups is a synthetic compound that corresponds to part of lipid A known to be the active center of lipopolysaccharide (LPS) (Kiso et al., 1987). Among the various analogues we tested so far, GLA-60 had retained most of the activities of the parental lipid A, but not its pyrogenicity or toxicity (Ikeda et al., 1988a; Kumazawa et al., 1988). In a previous paper we showed that GLA-60 inhibited formation of pox tail lesions as well as growth of vaccinia virus at the lesion sites of infected mice (Ikeda et al., 1988a). We have now investigated whether administration of GLA-60 to CY-immunosuppressed mice can reinforce the host defense mechanisms and protect the mice against HSV-1 infection. Furthermore, we are investigating the chemotherapeutic effect of GLA-60 combined with acyclovir (ACV) on HSV-1 infection in CY-immunosuppressed mice.

Materials and Methods

Animals

Female ddY mice were obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan). The mice were bred for one week under a specific pathogen-free environment and used in the experiments when they were 5 weeks old.

Virus

HSV-1 (HF strain) was propagated in human embryonic lung cells and stored at −80°C until use.

Agents

GLA-60 was synthesized chemically according to a method described previously (Kiso et al., 1987). CY (Endoxan) was purchased from Shionogi Co. Ltd., Osaka, Japan. ACV was kindly provided by Japan Wellcome Co. Ltd., Osaka, Japan. GLA-60 was solubilized in pyrogen-free water by treatment with triethylamine as described in a previous paper (Matsuura et al., 1983). After complete removal of

trimethylamine by evaporation, the solution was diluted with sterile saline before use.

Preparation and analysis of peritoneal cells

Mice were given an intraperitoneal (i.p.) injection of 10 μg GLA-60 one day after treatment with CY (200 mg/kg). Peritoneal cells (PC) were obtained by lavage of the peritoneal cavity with Eagle's minimum essential medium (EMEM) according to the method described in a previous paper (Ikeda et al., 1985). The peritoneal cell number was determined in a hemocytometer and the ratio of macrophages, PMNs and lymphocytes to the total PC number was determined microscopically following Giemsa staining.

Intrinsic antiviral activity of macrophages

Peritoneal macrophages were obtained from normal or CY-treated immunosuppressed mice which had been given an i.p. injection of 10 μg GLA-60 3 days previously. Macrophages at a concentration of 5×10^5 cells/well were cultivated for 2 h at 37°C in 24-well culture trays and infected with HSV-1 at a multiplicity of infection (moi) of 0.1. After a 1 h virus adsorption period, unadsorbed virus was removed and the cells were washed twice with EMEM. Then RPMI-1640 medium containing 10% fetal calf serum and HEPES buffer (pH 7.4) was added to the cells. After 24 h cultivation at 37°C, the cells were harvested and disrupted twice by freeze-thawing. Virus titers in the cell homogenates were determined by plaque assay on 3T3 (Balb/c 3T3, clone A31) monolayer cells.

NK cell activity

NK cells were obtained from normal or CY-suppressed mice which were given an i.p. injection of 10 μg GLA-60 3 days before the NK assay. NK activity was assessed by determining the radioactivity released from ^{51}Cr-labeled YAC-1 target cells. The method was performed exactly as described previously (Ikeda et al., 1988a). For calculation of specific lysis of ^{51}Cr, the following formula was used: Specific lysis (%) = [(cpm of the test group - cpm of spontaneous release)/(cpm of complete release - cpm of spontaneous release)] × 100.

HSV-1 growth in the peritoneal cavity

Normal and immunosuppressed mice were given an i.p. injection of 10 μg GLA-60 3 days before i.p. challenge with 1×10^5 PFU of HSV-1. Twenty-four hours after challenge, virus in the peritoneal cavity was recovered by washing with 4 ml of EMEM. The washes were diluted in ten-fold steps with maintenance medium containing 1% newborn calf serum, 3 mM glutamine and 0.07% bicarbonate. One-tenth ml/well of the dilution was brought onto 3T3 cell monolayers of 24-well tissue culture plates and cultivated at 37°C in a CO_2 incubator. After an adsorption

period of 1 h, infected cells were overlaid with 0.5 ml EMEM containing 1% newborn calf serum and 0.8% agar noble, cultivated for 3 days at 37°C, and stained with 0.05% neutral red solution for visualizing virus plaques.

Protection of immunosuppressed mice against HSV-1 infection

Mice were given an i.p. injection of 200 mg/kg of CY 4 days before virus infection. GLA-60 was administered i.p. to CY-suppressed mice at various time intervals as indicated in the figures and tables. After i.p. inoculation of HSV-1 (1×10^4 PFU), the death of mice was recorded daily for 21 days and percentage survivals in the control and experimental groups were compared statistically by the χ^2 test.

Results

First, we determined the changes in total cell numbers and cell populations in the peritoneal cavity of normal and immunosuppressed mice. When mice were treated with CY at 200 mg/kg, 4 days before harvesting PC, the total numbers of PC decreased by 3-fold (Table 1). Administration of 10 μg GLA-60 to mice 1 day after treatment with CY resulted in a marked increase in the number of cells (i.e. macrophages, PMNs and lymphocytes) in the peritoneal cavity.

As shown in Table 2, HSV-1 replicated somewhat better in the macrophages of CY-suppressed mice than in macrophages of untreated mice. However, if the CY-immunosuppressed mice received 10 μg GLA-60 3 days before HSV-1 infection, virus growth in the macrophages of these mice was reduced as compared to virus growth in the macrophages from immunosuppressed mice not given GLA-60.

The NK activity of peritoneal cells obtained from normal and CY-suppressed mice given 10 μg GLA-60 3 days previously was assessed by determination of radioactivity released from target YAC-1 cells. Although NK activity of peritoneal

TABLE 1

Effect of GLA-60 on recovery of peritoneal cell numbers in immunosuppressed mice[a]

Treatment	Cell number ($\times 10^6$/mouse)			
	Total cells	Macrophages	PMNs	Lymphocytes
No treatment	3.82±0.33	1.58±0.14	0.14±0.01	2.10±0.18
CY(200 mg/kg, −4d)	1.07±0.15	0.53±0.07	0.06±0.01	0.48±0.07
CY(200 mg/kg, −4d)+GLA-60(10 μg, −3d)	2.86±0.38[b]	1.62±0.21[b]	0.08±0.01[c]	1.16±0.15[b]
GLA-60(10 μg, −3d)	7.13±0.59	3.39±0.28	0.51±0.04	3.22±0.27

[a]Peritoneal cells were harvested from mice treated i.p. with CY (200 mg/kg, at −4 days) and GLA-60 (10 μg, at −3 days). The number of peritoneal cells was counted by the hemocytometer and the cell population was determined microscopically following Giemsa staining.
[b]$P<0.001$, as compared with CY-suppressed mice (Student's t test).
[c]$P<0.05$.

TABLE 2

Effect of GLA-60 on the intrinsic antiviral activity of macrophages of immunosuppressed mice[a]

Macrophage source	Virus titer (PFU/ml)	Ratio
No treatment	$(3.37 \pm 0.21) \times 10^2$	1.00
CY (200 mg/kg, −4d)	$(4.30 \pm 0.46) \times 10^2$	1.28
CY (200 mg/kg, −4d) + GLA-60 (10 μg, −3d)	$(1.47 \pm 0.15) \times 10^{2b}$	0.47
GLA-60 (10 μg, −3d)	$(1.33 \pm 0.25) \times 10^2$	0.39

[a]The antiviral activity of peritoneal macrophages obtained from normal or CY-treated mice given 10 μg GLA-60 by the i.p. route was assessed by determination of virus growth in macrophages as described in Materials and Methods.

[b]$P<0.001$, as compared with CY-suppressed mice.

cells from CY-suppressed mice was diminished, the activity increased significantly if the mice were given GLA-60 1 day after CY-treatment (Fig. 1). These results indicate that administration of GLA-60 to immunosuppressed mice enhanced the peritoneal NK activity.

Interferon (IFN) has been recognized as an important factor in host defense mechanisms. The question arose as to whether GLA-60 would induce IFN in normal or CY-suppressed mice which had been infected with 1×10^5 PFU of HSV-1. However, no IFN was induced in peritoneal fluids of CY-suppressed mice (whether or not treated with GLA-60) following HSV-1 infection (data not shown).

Second, we investigated whether administration of GLA-60 could protect immunosuppressed mice against HSV-1 infection. When CY was administered i.p. to mice at a dose of 200 mg/kg 4 days before i.p. challenge with 10^4 PFU of HSV-1, mortality was markedly increased as compared to mortality of mice which had not been treated with CY. However, the survival rate of HSV-1-infected CY-suppressed mice was significantly increased if the mice had been treated with GLA-

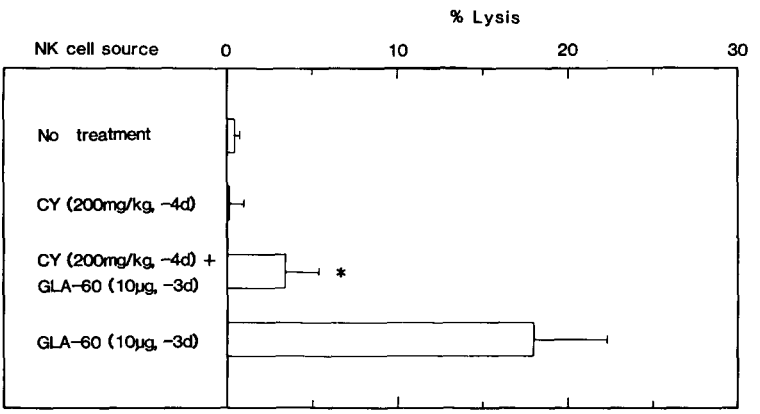

Fig. 1. Effect of GLA-60 on NK cell activity in immunosuppressed mice. The NK cell activity of peritoneal cells obtained from normal and CY-treated mice was assessed based on the radioactivity released from target YAC-1 cells. *$P<0.001$, as compared with CY-suppressed mice (Student's t-test).

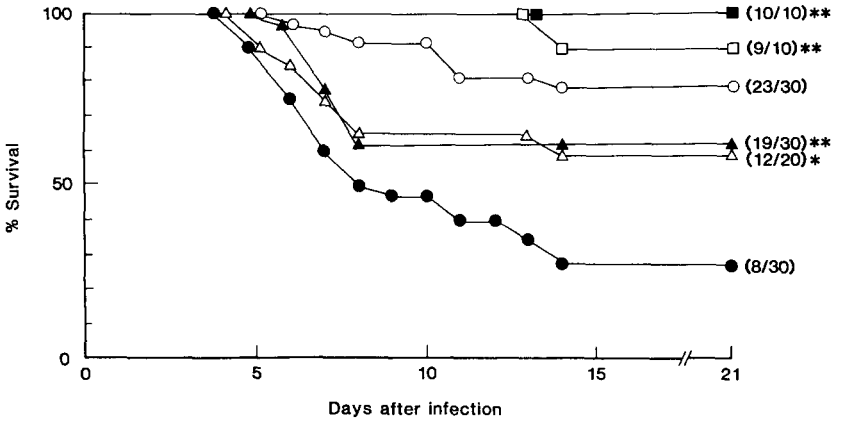

Fig. 2. Effect of GLA-60 on induction of viral resistance against HSV-1 in immunosuppressed mice. Mice were treated i.p. with 200 mg/kg of CY at 4 days before HSV-1 infection and treated i.p. with different doses of GLA-60 3 days before HSV-1 infection (i.p., 1×10^4 PFU). ○, not treated with CY; ●, CY-treated (immunosuppressed); △, CY-immunosuppressed and treated with GLA-60 at 1 μg; ▲, CY-immunosuppressed and treated with GLA-60 at 10 μg; □, CY-immunosuppressed and treated with GLA-60 at 30 μg; ■, CY-immunosuppressed and treated with GLA-60 at 100 μg. $^*P<0.01$, $^{**}P<0.005$, as compared with CY-immunosuppressed mice (χ^2 test).

60 between days 0 and 3 before virus challenge. As seen in Fig. 2, the protective activity of GLA-60 was dose-dependent and most pronounced when 100 μg GLA-60 was administered to CY-suppressed mice 3 days before HSV-1 infection. Even if treated with 10 μg GLA-60, susceptibility of the immunosuppressed mice to HSV-1 was significantly reduced, as shown in Table 3.

We then investigated combined immuno- and chemotherapy of GLA-60 plus ACV against HSV-1 infection. When CY-suppressed mice were given an i.p. injection of 10 μg GLA-60 3 days before virus infection and ACV (50 mg/kg/day) was administered i.p. for 3 days starting at 2 h after HSV-1 challenge, 18 out of 20 mice survived, as shown in Table 4.

TABLE 3

Effect of GLA-60 on HSV-1 growth in the peritoneal cavity of immunosuppressed mice[a]

Treatment	Virus titer (PFU/mouse)	Ratio
No treatment	$(4.28 \pm 0.60) \times 10^2$	1.00
CY (200 mg/kg, −4d)	$(8.28 \pm 0.32) \times 10^2$	2.00
CY (200 mg/kg, −4d) + GLA-60 (10 μg, −3d)	$(1.08 \pm 0.12) \times 10^{2}$[b]	0.25
GLA-60 (10 μg, −3d)	$(2.68 \pm 0.23) \times 10^1$	0.07

[a]Mice were treated i.p. with 200 mg/kg of CY at 4 days before HSV-1 infection. They were given 10 μg GLA-60 by the i.p. route 3 days before HSV-1 infection. Twenty-four hours after i.p. challenge with 1×10^5 PFU of HSV-1, peritoneal fluids were harvested and virus titers were determined based on a plaque assay.

[b]$P<0.001$, as compared with CY-suppressed mice.

TABLE 4

Combination of GLA-60 and ACV against HSV-1 infection in immunosuppressed mice[a]

Treatment	No. survivors	
	Total no.	Survival (%)
No treatment	17/20	85
CY(200 mg/kg, −4d)	16/40	40
CY + GLA-60(10 μg, −3d)	14/20	70[b]
CY + ACV(5 mg/kg, 0−2d)	12/20	60, ns
CY + GLA-60 + ACV(5 mg/kg)	15/20	75[c]
CY + ACV(50 mg/kg, 0−2d)	14/20	70[b]
CY + GLA-60 + ACV(50 mg/kg)	18/20	90[d]

[a]Mice were injected i.p. with 200 mg/kg of CY at 4 days before HSV-1 infection and were given 10 μg GLA-60 by the i.p. route 3 days before HSV-1 infection (i.p., 1×10^4 PFU). ACV was administered i.p. at 5 or 50 mg/kg/day for 3 days starting at 2 h after HSV-1 inoculation. Survivals were counted 21 days later and expressed as % survival.

[b]$P<0.05$.

[c]$P<0.01$.

[d]$P<0.005$.

ns, not significant, as compared with CY-treated mice (χ^2 test).

Discussion

The present study demonstrates that administration of GLA-60 to CY-immunosuppressed mice leads to an increase in the number of peritoneal cells and stimulates their functional maturation. GLA-60 inhibits the growth of HSV-1 in the peritoneal cavity of immunosuppressed mice, and the mice are protected against death due to HSV-1 infection. According to Kumazawa et al. (1988), administration of GLA-60 to mice leads to the appearance of colony-stimulating factor (CSF) in the blood. CSF may contribute to a rapid restoration of immunocompetent cells such as macrophages and granulocytes from the bone marrow and stimulate their functional maturation.

As shown in the present paper, replication of HSV-1 in immunosuppressed mice was inhibited by i.p. administration of 10 μg GLA-60 3 days before virus challenge. We also demonstrated that the intrinsic antiherpes activity of macrophages and NK cells was enhanced in immunosuppressed mice following administration of GLA-60. IFN is also an important factor in nonspecific host defense mechanism. Recently, we reported that GLA-60 induced interferon as well as tumor necrosis factor in *Propionibacterium acnes*-primed mice (Ikeda et al., 1988a; Kumazawa et al., 1988). However, IFN production was not detected in peritoneal fluid of CY-suppressed mice, whether or not GLA-60 was administered before HSV-1 infection. Therefore, IFN may not play a role in the nonspecific defense mechanisms of GLA-60 in CY-suppressed mice.

We also evaluated the protective activity of GLA-60 in combination with ACV. Hilfenhaus et al. (1987) demonstrated that the combination of acyclovir or bromovinyldeoxyuridine with passive immunization conferred better protective activ-

ity against HSV infection than did either chemo- or immunotherapy alone. However, this effect was less pronounced in immunocompromised mice than in immunocompetent mice. As shown in the present study, combined GLA-60 plus ACV therapy in CY-suppressed mice resulted in greater protection against HSV-1 than did single treatment with either GLA-60 or ACV.

Acknowledgement

This work was supported in part by a grant from The Kitasato Research Foundation (No. 3).

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