Protective activity of the lipid A analogue GLA-60 against murine cytomegalovirus infection in immunodeficient mice

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The immunomodulating and murine cytomegalovirus (MCMV)-inhibiting effects of the synthetic lipid A subunit analogue GLA-60 were investigated in different strains of immunodeficient mice. Peritoneal natural killer (NK) cells obtained from nude (nu/nu) C57BL/6 mice or normal NMRI mice, which had been treated intraperitoneally with 10 µg of GLA-60 1 day earlier, exhibited a greater cytolytic activity than those from untreated mice. GLA-60 also stimulated NK cell activity in SCID (severe combined immune deficiency) mice (which are T cell-defective), but not in NK cell-defective beige (C57BL/6 bg/bg) mice. GLA-60 also enhanced the phagocytic activity of peritoneal macrophages in beige, nude and NMRI mice, but not in SCID mice. GLA-60, when administered as a single 150 µg dose 1 day before infection, completely protected beige mice against MCMV-associated mortality. It also caused a significant increase in the life-span of MCMV-infected nude and SCID mice.

Human cytomegalovirus (HCMV) causes serious infections in immunocompromised hosts including AIDS patients, transplant recipients and cancer patients under immunosuppressive therapy (Tyms et al., 1989; Rubin, 1990; Winston et al., 1990). In these patients, the lymphocyte, macrophage and natural killer (NK) cell counts decrease, resulting in a higher susceptibility to various infectious agents. HCMV also leads to severe clinical manifestations in the foetus or neonate (Stagno et al., 1982; Britt et al., 1991). Currently, only ganciclovir and foscarnet are used for the treatment of severe HCMV infections (Verheyden, 1988; Emanuel, 1990). However, both compounds may result in serious side-effects (i.e. neutropenia and kidney failure, respectively), and HCMV strains resistant to ganciclovir may emerge during therapy (Erice et al., 1989). It is therefore imperative to develop not only non-toxic antivirals but also compounds capable of stimulating or restoring the immune systems.

GLA-60, a 4-O-phosphono-D-glucosamine derivative carrying 3-O-3-tetradecanoyloxytetradecanoyl [C14-O-(C14)] and N-3-hydroxytetradecanoyl (C14-OH) groups, is a synthetic compound that corresponds to the non-reducing moiety of lipid A known to be the active centre of the lipopolysaccharide (Kiso et al., 1987). GLA-60 exhibits various immunopharmacological activities without pyrogenicity or Shwartzman reactivity (Ikeda et al., 1988b, 1990; Kumazawa et al., 1988; Saiki et al., 1990). Furthermore, GLA-60 is effective against vaccinia virus and Pseudomonas aeruginosa infections in animals, and also shows antitumour activity against Meth A fibrosarcoma and B16 melanoma in mice (Ikeda et al., 1988a, b; Nakatsuka et al., 1989, 1991; Saiki et al., 1989). GLA-60 inhibits the replication of herpes simplex virus type 1 (HSV-1) in the peritoneal cavity of cyclophosphamide (CY)-immunosuppressed mice by activation of macrophages and NK cells, thereby protecting the mice against a lethal HSV-1 infection (Ikeda et al., 1989).

NK cells are known to play an important role in the host's defence against murine CMV (MCMV) infection, particularly during the early stage of infection (Janeway, 1989; Staczek, 1990; Welsh et al., 1991). In addition to NK cells, macrophages also play an important role in the early defence mechanisms against virus infections (Morahan, 1984). Recently we reported that GLA-60 protects young Naval Medical Research Institute (NMRI) mice against lethal MCMV infection via activation of NK cells and macrophages (Ikeda et al., 1992). In the present study we investigated whether GLA-60 can activate NK cells and macrophages in different strains of immunodeficient mice, i.e. NK cell-defective beige (C57BL/6 bg/bg), T cell-defective nude (C57BL/6 nu/nu) and T and B cell-deficient SCID (BALB/c scid/scid) mice. We also monitored the protective activity of GLA-60 in these mice against a lethal MCMV infection.
Female NMRI, C57BL/6 bg/bg, C57BL/6 nu/nu and BALB/c scid/scid mice were bred in a specific pathogen-free environment at the Animal Center of the Rega Institute for Medical Research, and used at 5 to 7 weeks of age. Stocks of MCMV (ATCC VR194) were prepared from 10% homogenates of the salivary glands of NMRI mice that had been infected with a sublethal dose of MCMV. GLA-60 was synthesized chemically according to the method described previously (Kiso et al., 1987). GLA-60 was solubilized in pyrogen-free water as a triethylamine salt and complexed with BSA to evaluate its biological effects (Matsuura et al., 1983).

For the NK cell activity assays, peritoneal cells were obtained from different strains of mice which had been injected intraperitoneally (i.p.) with 10 µg of GLA-60 1 day before the assay. NK cell activity was assessed by determining the radioactivity released from 51Cr-labelled (sodium chromate, 3.7 MBq, Amersham) YAC-1 target cells (Ikeda et al., 1988a). Briefly, the peritoneal NK cells were incubated for 4 h at 37 °C with 51Cr-labelled target cells at an effector:target ratio of 30:1. The cell mixtures were prepared in quadruplicate, and the radioactivity released into the supernatant was counted in an autogamma scintillation spectrometer. The specific release of radioactivity was calculated according to the following formula: specific lysis (%) = [(c.p.m. of test groups - c.p.m. of the spontaneous release)/(c.p.m. of the complete release - c.p.m. of the spontaneous release)] × 100.

Phagocytic activity of macrophages was determined as described in a previous paper (Ikeda et al., 1988b). Briefly, peritoneal cells obtained from different strains of mice which had received 10 µg GLA-60 i.p. 1 day earlier were incubated for 2 h at a cell concentration of 6 × 105 cells/well in 24-well plates. After washing the wells twice with Eagle's MEM, non-adherent cells were removed and the resulting adherent cells were used as a macrophage source. 51Cr-labelled and antibody-sensitized sheep erythrocytes (51Cr-labelled EA) suspended in RPMI-1640 medium containing 10% fetal calf serum were added to each well (2 × 104 EA/0.5 ml/well). After incubation for 1 h at 37 °C, cells were treated with 0.85% ammonium chloride in Tris–HCl pH 7.6, to lyse 51Cr-labelled EA attached on the surface of macrophages. The radioactivity of 51Cr-labelled EA taken into macrophages was then determined.

Protective activity against MCMV was assessed by scoring the survival of MCMV-infected mice (Neyts et al., 1992). GLA-60 was administered i.p. at 150 µg/mouse. Mice were challenged 1 day later with 1 × 106 p.f.u. of MCMV per 0.2 ml MEM. The mice were observed for 21 days and the results were expressed as the percentage survival (seven to ten mice per group).

As shown in Fig. 1, GLA-60 when injected i.p. at 10 µg significantly stimulated the NK cell activity in nude, SCID and NMRI mice but not in beige mice. In particular, NK cells obtained from GLA-60-treated nude
respectively) as compared to that of untreated groups in infection, i.e. NK cells, macrophages, antibody-dependent cell-mediated cytotoxicity (ADCC), cytotoxic T cells cooperate in the host's defence against an acute MCMV in T and B cell-defective SCID mice, it may be surmised were challenged i.p. with MCMV at 1 x 10^5 p.f.u. As significantly increased (MDD: 16.6_+2.0 and 12.2_+3.2, the groups treated with t50 gg of GLA-60 was sig-
ificantly enhanced the phagocytic activity of macrophages in beige, nude and NMRI mice, but not in SCID mice (Fig. 2). Since GLA-60 stimulated macrophage activity in T cell-defective nude mice but not in T and B cell-defective SCID mice, it may be surmised that B cells participate in the activation of macrophages by GLA-60.

We then evaluated whether GLA-60 exhibits protective activity against lethal MCMV infection in the different strains of immunosuppressed mice. All mice were challenged i.p. with MCMV at 1 x 10^5 p.f.u. As shown in Fig. 3(a), 150 μg of GLA-60 injected i.p. 1 day before MCMV infection protected all beige mice against MCMV-associated mortality (P < 0.005). For SCID and nude mice, the life-span [mean day of death (MDD)] of the groups treated with 150 μg of GLA-60 was significantly increased (MDD: 16.6±2.0 and 12.2±3.2, respectively) as compared to that of untreated groups (MDD: 6.8±0.4 and 6.4±1.9, respectively) (P < 0.001).

It is probable that several cells and mechanisms cooperate in the host's defence against an acute MCMV infection, i.e. NK cells, macrophages, antibody-dependent cell-mediated cytotoxicity (ADCC), cytotoxic T cells and antibody (Staczk, 1990). In general, NK cells are considered to play a key role in the early defence mechanisms against acute MCMV infection, and cytotoxic T cells in the late defence mechanisms. It is well known that homozygous NK cell-defective beige mice are more susceptible to MCMV infection than their heterozygous littermates (Shellam et al., 1981, 1985), and congenitally athymic nude mice are much more sensitive to MCMV than are the heterozygous mice (Starr & Allison, 1977). SCID mice fail to develop functional T and B cell immunity (Bosma et al., 1983) and are therefore highly susceptible to infectious pathogens.

Recently we elaborated a model for MCMV infection in SCID mice. This model mimics CMV disease progression in the immunocompromised host more closely than the commonly used animals models (Neyts et al., 1992). According to our (preliminary) observations, SCID mice appeared to be the most susceptible to MCMV infection of the four mouse strains examined. In fact, all SCID mice eventually succumb after infection with MCMV, even with viral doses as low as 5 x 10^2 p.f.u. (data not shown).

GLA-60 was not able to induce NK cell activity as efficiently in beige mice as it did in NMRI mice in which NK cells had been depleted by in vivo treatment with anti-asialo GM1 (specific antibody for NK cells) (Ikeda et al., 1993). However in both strains of mice GLA-60 effectively stimulated macrophage activity and proved to be protective against lethal MCMV infection. Macrophages do not only clear MCMV particles and MCMV-infected cells but also present antigens to T cells or B cells. Since beige mice are NK cell-defective, but contain macrophages, T cells and B cells, it is surmised that in the beige mouse, macrophages activated by GLA-60 may (i) be able to clear MCMV particles and MCMV-infected cells during the early stage of infection, (ii) operate as antigen-presenting cells in order to establish specific immunity to MCMV and (iii) cooperate with cytotoxic T cells, ADCC and neutral antibody to block MCMV disease progression during the late stage of infection. Thus, the fact that GLA-60 completely protects the NK cell-defective beige mice against MCMV-induced mortality, whereas it increases the life-span of only nude and SCID mice but does not protect them from MCMV-induced lethality, indicates that macrophages, together with functional B and T cells, are essential for the protection against MCMV infections.

Kumazawa et al. (1988) reported that GLA-60 stimulates B cell activity and potentiates IgG antibody responses in mice immunized with BSA. The role of the humoral immune response during acute, chronic or latent MCMV infections is not well understood. However, the role of antibodies in the neutralization of MCMV infectivity (Arnaulo-Cruz et al., 1978; Selgrade et al., 1983; Shanley et al., 1981), and in antibody-
dependent cell immunity (Manischewitz & Quinnan, 1980; Quinnan & Manischewitz, 1979) is well recognized. GLA-60 is also able to induce the release of cytokines [i.e. interferon (IFN), colony-stimulating factor and tumour necrosis factor] and lymphokines (i.e. interleukin-1) (Ikeda et al., 1988b, 1990; Kumazawa et al., 1988; Saiki et al., 1990). IFN has been recognized as an important factor in host defence mechanisms. Also, it is well known that IFN plays a role in activating the effector cells. Therefore, all the factors induced by GLA-60 may act synergistically in macrophages and NK cells, and thus contribute to the defence of the immunodeficient hosts toward MCMV infection. Administration of GLA-60 to CY-immunosuppressed mice leads to an increase in the number of peritoneal cells (e.g. macrophages, polymorphonuclear cells and lymphocytes) and protects the mice against lethal HSV infection by activation of these cells, in particular macrophages and NK cells (Ikeda et al., 1989, 1990).

In conclusion, macrophages together with functional T and B cells are required to protect mice from lethal MCMV infections, and secondly, GLA-60 may be considered as an immunomodulator candidate drug to prevent or suppress herpesvirus infections in immunocompromised hosts.

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