Antiviral Research, 9 (1988) 37–46
Elsevier

AVR 00280

Antiviral and immunomodulating activities of chemically synthesized lipid A-subunit analogues GLA-27 and GLA-60

Satoru Ikeda[1], Chiaki Nishimura[1], Mitsunobu Nakatsuka[2], J. Yuzuru Homma[2], Makoto Kiso[3] and Akira Hasegawa[3]

[1]*School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan;* [2]*The Kitasato Institute, Minato-ku, Tokyo, Japan;* [3]*Faculty of Agriculture, University of Gifu, Gifu, Japan*

(Received 20 October 1987; accepted 29 December 1987)

Summary

Biological and antiviral activities of chemically synthesized lipid A-subunit analogues, GLA-27 and GLA-60, were investigated with respect to defense mechanisms such as macrophage and natural killer (NK) cell activation and interferon (IFN)-inducing activity. GLA-27, a 4-*O*-phosphono-D-glucosamine derivative carrying 3-*O*-tetradecanoyl (C_{14}) and 2-*N*-3-tetradecanoyloxytetradecanoyl (C_{14}-O-(C_{14})) group, and GLA-60, a similar analogue carrying 3-*O*-linked C_{14}-O-(C_{14}) and 2-*N*-linked 3-hydroxytetradecanoyl (C_{14}-OH) groups, strongly inhibited the formation of pox tail lesions and the growth of vaccinia virus at the tail lesion sites in infected mice. The antiviral activity of GLA-60 was about 1000-fold higher than that of muramyldipeptide (MDP), a representative immunomodulator. GLA-27 and GLA-60 had stronger immunomodulating activity than MDP in macrophage activation, NK cell activation and IFN-inducing activity, although it was weaker than natural lipid A. Toxic manifestations such as pyrogenicity, local Shwartzman reaction and lethality were far less pronounced for GLA-27 and GLA-60 than for natural lipid A.

Lipid A analogues; Vaccinia virus; Immunomodulator

Correspondence to: Satoru Ikeda, Department of Virology and Immunology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan.

Introduction

Lipid A is known as the active center of lipopolysaccharide (LPS): it exhibits a variety of biological functions such as Limunus amoebocyte lysate gelating activity, B cell activation, adjuvant activity and tumor necrosis factor (TNF)-inducing activity (Galanos et al., 1985; Homma et al., 1985; Kotani et al., 1985). In previous papers we reported that there is a close relationship between the chemical structures and biological functions of chemically synthesized lipid A analogues, corresponding to the nonreducing sugar moiety of parental lipid A (Matsuura et al., 1984, 1985; Kumazawa et al., 1985, 1986). Among the analogues investigated so far, GLA-27, a 4-*O*-phosphono-D-glucosamine derivative carrying 3-*O*-tetradecanoyl (C_{14}) and 2-*N*-3-tetradecanoyloxytetradecanoyl (C_{14}-O-C_{14}) groups, showed most of the activities of natural lipid A, but with very low pyrogenicity and toxicity.

With respect to antiviral activity of LPS, there are few papers (Gledhill, 1959; Borecky et al., 1970) which demonstrate that bacterial endotoxin has protective activity against ectromelia virus or encephalomyocarditis virus in mice. Masihi et al. (1986) showed that, although monophosphoryl lipid A alone did not protect mice against influenza virus, it reduced significantly the lung virus titers when combined with trehalose dimycolate, a known immunostimulator. On the other hand, there are several papers concerning IFN-inducing activity of LPS (De Clercq and Merigan, 1970; De Clercq, 1980). Studying the antiviral activity of various immunomodulators such as IFN inducers, MDP and its chemically synthesized analogues, against vaccinia virus (VV), herpes simplex virus and influenza virus (Ikeda et al., 1985a, b), we found that the VV tail lesion test is a sensitive system to assess the antiviral activity of immunomodulators. This paper addresses the antiviral activity as well as enhancing activity of host defense mechanisms by GLA-27 and the newly synthesized compound GLA-60.

Materials and Methods

Synthetic lipid A analogues

The structures of the synthetic analogues are shown in Fig. 1. GLA-27 and GLA-60 were synthesized according to a method described previously (Kiso et al., 1984, 1987) and provided by them. Synthetic muramyl dipeptide (MDP) was provided through the courtesy of the Research Institute of Daiichi Seiyaku Co., Ltd., Tokyo, Japan. Natural lipid A was prepared from *Escherichia coli* F515 strain (Re) by Drs. O. Lüderitz and C. Galanos, Max-Planck-Institute für Immunobiologie, Freiburg, F.R.G., and kindly donated for use in control experiments. The synthetic analogues of monosaccharide-type lipid A and control lipid A were solubilized in pyrogen-free water by adding triethylamine complexed with bovine serum albumine before use (Matsuura et al., 1983).

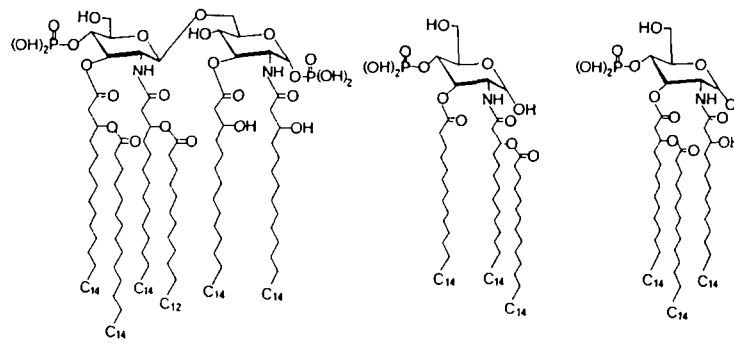

Fig. 1. Structures of chemically synthesized lipid A and its subunit analogues.

Determinations of pyrogenicity, local Shwartzman reaction and lethality

The toxicity tests were carried out according to the methods described in a previous paper (Homma et al., 1985). Fever response for each dose was recorded with three 2.5 kg Japanese white rabbits (Gokita Breeding Service, Tokyo) by intravenous injection. Local Shwartzman reaction was tested as follows; 18 h after preparatory intradermal injection with test samples in 0.1 ml water, a provocative injection of 20 μg/kg control lipid A was given intravenously, and intensity of the reaction was read 5 h after the provocative injection. The lethal toxicity was determined 24 h after intravenous administration of test samples to galactosamine-sensitized mice; that is, the test samples dissolved in 0.2 ml of saline were injected into male 10-week old C57BL/6 mice (Shizuoka Animal Center, Hamamatsu) immediately after intraperitoneal administration of 10 mg D-galactosamine in 0.5 ml phosphate-buffered saline (PBS).

Determination of macrophage activation

Phagocytic activity of macrophages was determined as described in a previous paper (Kumazawa et al., 1987). Briefly, peritoneal cells were obtained from 5-week old female ddY mice (Shizuoka Animal Center) which had been administered intraperitoneally 10 μg test samples 4 days previously. The cells were incubated for 2 h at a cell concentration of 5×10^5/well in 24-well plates. After washing three times with Eagle's minimum essential medium (EMEM), nonadherent cells were removed and resultant adherent cells were used as macrophage sources. Phagocytic activity was assessed by measuring the radioactivity of ^{51}Cr-labeled and antibody-sensitized sheep erythrocytes (^{51}Cr-labeled EA), which were phagocytosed by peritoneal macrophages. After incubation for 1 h at 37°C, cells were treated with 0.85% ammonium chloride Tris-HCl buffer (pH 7.6) to lyse ^{51}Cr-labeled EA attached on the surface of macrophages. The radioactivity of ^{51}Cr-labeled EA taken up into macrophages was determined in triplicate and results are expressed as mean phagocytic ratio ± SE of four experiments carried out separately. Phagocytic ra-

tios are expressed as a relative value of radioactivity recovered from macrophages originating from animals injected with the test samples compared to that of control macrophages.

Determination of natural killer (NK) cell activity

NK cell activity of peritoneal cells, obtained from 5-week old female ddY mice (Shizuoka Animal Center) which were administered, intraperitoneally, 10 μg test samples 1 days previously, was assessed by determining the radioactivity released from target YAC-1 cells (Engler et al., 1981). Peritoneal cells were incubated for 4 h at 37°C with ^{51}Cr-labeled YAC-1 cells at an effector:target ratio of 30:1. The cells were incubated in triplicate. To determine a spontaneous release of radioactivity, 100 μl aliquots of the culture medium were added to cultures without effector cells. A complete release of radioactivity was measured by lysing target cells alone with 100 μl aliquots of 1% Nonidet P40 (Iwai Kagaku Yakuhin, Co. Ltd., Tokyo). The radioactivity released into the supernatant was measured by an autogamma scintillator. The specific release of radioactivity was calculated according to the following formula: Specific lysis (%) = (cpm of tested groups − cpm of the spontaneous release) ÷ (cpm of the complete release − cpm of the spontaneous release) × 100.

Determination of IFN-inducing activity

IFN-inducing activity was assessed by measuring serum IFN titers, expressed as reciprocals of the highest dilution of sera that reduced vesicular stomatitis virus-induced cytopathic effect in L-929 cells by 50% as described previously (Ikeda et al., 1985a). Briefly, 6-week old female ddY mice were injected intravenously with 10 μg test samples. To detect serum IFN, mice were primed by intraperitoneal administration of 1 mg of formalin-killed *Propionibacterium acnes* 7 days previously (Okamura et al., 1982). Sera were separated from blood samples taken 2 h after the primer injection. IFN titers are expressed as international reference units, using standard IFN α/β (Lee Biomolecular, California).

Determination of antiviral activity against VV

Antiviral activity was assessed by measuring the reduction of lesion numbers formed on the tail of mice infected with VV, as described previously (Ikeda et al., 1985b). Briefly, 5-week old female ddY mice were injected intravenously with test samples one day before an intravenous challenge with 10^4 plaque-forming units (PFU) of VV (Lister strain). Seven days later, the number of lesions which appeared were counted upon staining with 1% fluorescein-0.5% methyleneblue solution. Antiviral activity was expressed by percent inhibition ± SE, using 10 mice per group.

Furthermore, the virus titers at the tail dermal lesions were determined by plaque assay on HeLa cell monolayers using 24-well plastic plates. The tail dermal lesions obtained from 4 mice of a group were homogenized at 4°C in 2 ml of EMEM containing 200 U/ml penicillin and 200 μg/ml streptomycin with a Microhomogenizer M-100 (Tokai Irika Co. Ltd., Tokyo). After making 10% homogenates in the me-

TABLE 1

Endotoxin activities of lipid A-subunit analogues.

Compound	Pyrogenicity M.E.D.(μg/kg)[a]	Shwartzman reaction M.P.D. (μg/mouse)[b]	Lethality LD_{50}(μg/mouse)[c]
MDP	16[d]	>100[e]	>100
GLA-27	>10	> 50	0.54
GLA-60	>10	> 50	0.75
Lipid A	0.001	1.25	0.0078

[a]Minimum effective dose (M.E.D.) of pyrogenicity was determined using 3 Japanese rabbits for each sample dose.

[b]Minimum preparatory dose (M.P.D.) in local Shwartzman reaction was determined by intensity of cutaneous reaction when rabbits were given a provocative intravenous injection of 20 μg/kg of natural lipid A 5 h previously.

[c]The 50% lethal dose (LD_{50}) was calculated by the method of Karber using galactosamine-sensitized mice.

[d]Kotani et al. (1976), Biken J. 19, 9–13.

[e]Nagao and Tanaka (1985), J. Exp. Med. 162, 401–412.

dium, the homogenates were centrifuged at low speed to remove debris, and supernatants were diluted appropriately with EMEM for virus plaque assay on HeLa cell monolayers.

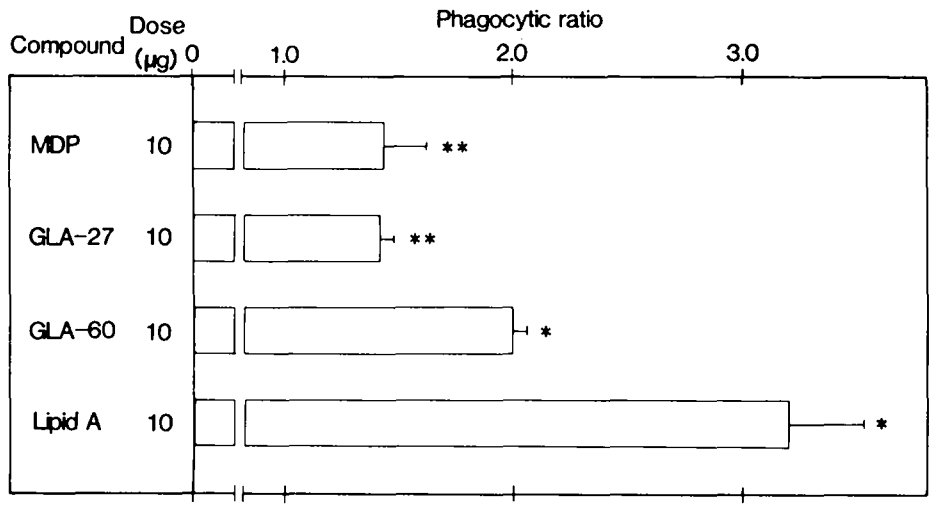

Fig. 2. Activation of macrophage phagocytic activity by lipid A-subunit analogues. Peritoneal macrophages were obtained from ddY mice injected intraperitoneally with 10 μg test samples 4 days previously and cultured for 1 h with ^{51}Cr-labeled EA. Phagocytic activity is expressed as a mean phagocytic ratio, which is the relative value of the radioactivity of macrophages originating from mice treated with the test samples to that of control macrophages, ± SE of four experiments carried out separately. *, $P<0.001$; **, $P<0.05$ (Student's t test).

Results

Toxicity and pathogenicity of GLA-27 and GLA-60

Control lipid A demonstrated remarkable pyrogenicity at a dose of 0.001 μg/kg in Japanese white rabbits (Table 1). However, GLA-27 and GLA-60 did not show pyrogenicity at a dose of 10 μg/kg. Although control lipid A induced local Shwartzman reaction at a dose of 1.25 μg, GLA-27 and GLA-60 were negative at a preparatory dose of 50 μg. GLA-27 and GLA-60 showed weak lethality which was 100-fold less than that of control lipid A in galactosamine-sensitized mice (Table 1).

Immunomodulating activity of GLA-27 and GLA-60

Fig. 2 demonstrates phagocytic activity of peritoneal macrophages recovered from mice injected intraperitoneally with 10 μg test sample 4 days previously. As seen in this Figure, phagocytic activity of macrophages obtained from mice administered control lipid A was quite remarkable. GLA-27 and GLA-60 significantly stimulated macrophage phagocytic activity in mice. The latter compound in particular exhibited stronger activity than GLA-27 and MDP. The enhancement of phagocytic activity by GLA-60, however, did not exceed that of control lipid A.

Results of NK cell activity are shown in Fig. 3. The enhancement of peritoneal NK cell activity was observed in mice which were injected intraperitoneally with

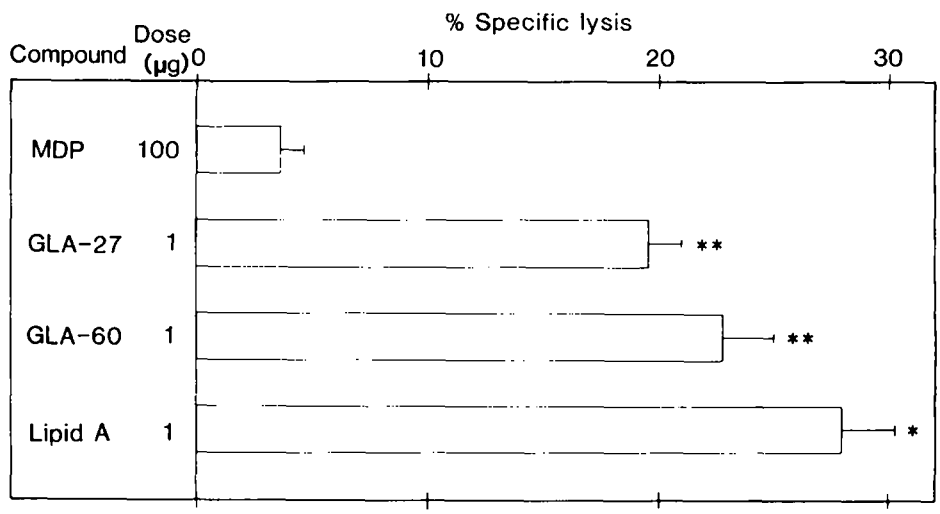

Fig. 3. Activation of NK cell activity by lipid A-subunit analogues. Peritoneal cells were obtained from ddY mice injected intraperitoneally with 1 μg test samples 1 day previously. Peritoneal cells (6×10^5/well in 96-well microplates) were cocultured for 4 h with target ^{51}Cr-labeled YAC-1 lymphoma cells in an effector/target cell ratio of 30:1. Lytic activity is assessed by the degree of release of ^{51}Cr from the target cells and expressed as mean ± SD of triplicate cultures. Specific lysis of untreated NK cells was 5.6 ± 1.9%. *, $P<0.001$; **, $P<0.01$ (Student's t test).

TABLE 2

IFN-inducing activity of lipid A-subunit analogues.

Compound[a]	IFN titer (IU/0.1ml)[b]	
	Exp. 1	Exp.2
MDP	<10	<10
GLA-27	640	320
GLA-60	1280	640
Lipid A	2560	1280

[a]Ten μg of test samples were injected intravenously into *P. acnes*-primed mice.
[b]Serum IFN titers were assayed in L-929 cells with vesicular stomatitis virus as the challenge virus. The IFN titer represents the reciprocal of the serum dilution giving a 50% reduction of the virus-induced cytopathogenicity.

control lipid A 24 h previously. This NK activity was completely reduced after treating the cells with an anti-asialo GM_1 antibody (data not shown). GLA-27 and GLA-60 showed remarkable stimulation of NK activity, and the activity of GLA-60 was stronger than that of GLA-27 but did not exceed that of control lipid A. The NK activity induced by MDP was very low.

Since serum IFN titers induced by intravenous injection of control lipid A in-

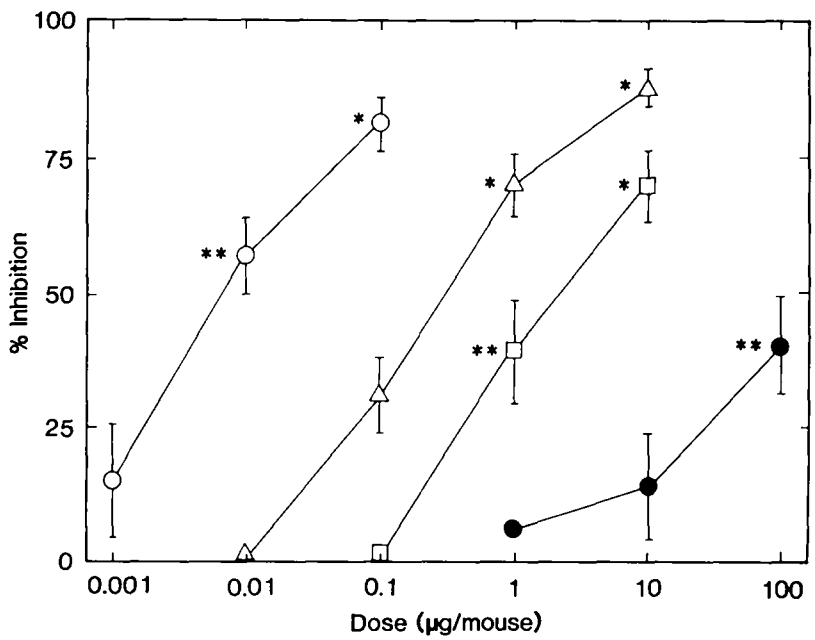

Fig. 4. Inhibitory effects of lipid A-subunit analogues on pox tail-lesion formation. Female ddY mice were administered intravenously with indicated doses of test samples the day before intravenous challenge with 10^4 PFU of VV per mouse. Seven days after challenge, the numbers of lesions formed on the tail were counted. Results are expressed as percent inhibition ± SE of 10 mice per group. Lipid A(○), GLA-27(□), GLA-60(△) and MDP(●). *, $P<0.001$; **, $P<0.01$ (Student's t test).

creased more than 10-fold when the mice were primed with *P. acnes* (data not shown), IFN-inducing activity of lipid A-subunit analogues were determined, using the same assay system. As shown in Table 2, IFN-inducing activity of GLA-60 was superior to that of GLA-27, though it was less than that of control lipid A.

Antiviral activity of GLA-27 and GLA-60 against vaccinia virus

Antiviral activity was determined based upon the tail lesion model in mice intravenously infected with VV. As shown in Fig. 4, GLA-27 and GLA-60 exhibited stronger antiviral activity than MDP, but the activity was weaker than that of control lipid A. The order was lipid A>GLA-60>GLA-27>MDP. The antiviral activity of GLA-60 was approximately 1000-fold higher than that of MDP. Table 3 demonstrates that the growth of VV at the sites of tail lesions was inhibited in parallel with the decrease in lesion numbers.

Discussion

The present study demonstrated that chemically synthesized lipid A analogues, GLA-27 and GLA-60, strongly inhibited the formation of pox tail lesions as well as viral growth at the lesion sites in VV-infected mice. The most active compound, GLA-60, showed antiviral activity about 10-fold higher than that of GLA-27 and about 1000-fold higher than that of MDP. Furthermore, GLA-27 and GLA-60 enhanced host-mediated defence mechanisms, e.g. macrophage and NK cell activation and IFN-inducing activity, and, unlike parental lipid A, were devoid of detectable pyrogenicity or local Shwartzman reaction.

Various efforts have been made by many investigators to remove the toxicity of natural lipid A, while trying to maintain its physiological activities, because lipid A is a vast treasure-trove of useful biological activities. Kotani et al. (1976) dis-

TABLE 3

Effects of lipid A and its subunit analogues on virus growth and lesion formation in mice infected with VV[a].

Compound	Dose (μg/mouse)	Virus titer in the lesions		Number of lesions	
		PFU/0.1 ml	Inhibition(%)	Mean±SE	Inhibition(%)
Control		$(6.9\pm0.4)\times10^{4}$	–	30.2±4.9	–
MDP	100	$(5.5\pm0.8)\times10^{4}$	(20)	20.9±3.7	(31)
GLA-27	1	$(9.4\pm0.2)\times10^{3}$[b]	(86)	10.2±2.4[c]	(66)
GLA-60	1	$(7.3\pm0.9)\times10^{3}$[b]	(89)	5.7±1.8[b]	(81)
Lipid A	1	$(3.6\pm0.7)\times10^{2}$[b]	(99)	2.6±0.7[b]	(91)

[a]Seven days after the virus challenge, the number of lesions formed on the tail were counted and the virus titers in the tail lesions were determined by plaque formation on HeLa cell monolayers. The virus titers are shown as plaque-forming unit (PFU) per 0.1 ml of 10% homogenates obtained from 4 mice.
[b]$P<0.001$.
[c]$P<0.01$ (Student's t test).

cussed in their paper that MDP has a variety of immunopharmacological activities but that pyrogenicity of MDP analogues is always associated with the expression of these activities. However, among the MDP analogues that have been developed, murabutide (butyl ester of MDP) and B30-MDP (6-*O*-acyl-MDP) have a high adjuvant activity and a low pyrogenicity, as reported by Parant and Chedid (1984) and Tsujimoto et al. (1986), respectively.

GLA-27 and GLA-60 are superior to MDP and its analogues from an antiviral and immunostimulatory viewpoint (Ikeda et al., 1985a, b, 1988). In the VV tail lesion model, a large number of lesions appeared on the tails of mice within 7 days after infection, but the development of these lesions was suppressed in the presence of GLA-27 and GLA-60. If mice had been treated previously with an anti-asialo GM_1 antibody, explosive virus growth occurred and lesion numbers on the tail increased (data not shown). This suggests that NK cells play an important role in protection from VV infection. Furthermore, MDP and its analogues activate macrophages so as to express an extrinsic antiviral activity against VV-infected cells (Ikeda et al., 1985b), suggesting that macrophages may participate in the protection.

GLA-27 and GLA-60 differ structurally in that the 3-*O*-acyl and 2-*N*-acyloxyacyl in GLA-27 is replaced by 3-*O*-acyloxyacyl and 2-*N*-hydroxyacyl in GLA-60. Such a reciprocal exchange of substituents at the 2- and 3-positions in the glucosamine ring and introduction of an hydroxy group in the acyl substituent resulted in a substantial increase of both the antiviral and immunomodulatory activities of GLA-60. This suggests that the conformation and hydrophilicity of lipid A analogues contributes to the biological functions in these compounds. We are now endeavouring at synthesizing compounds consisting of both MDP and lipid A subunit moieties covalently bound to each other.

References

Borecky, L., Lackovic, V. and Russ, G. (1970) Interferon production in leukocytes and the antiviral protection of mice treated with endotoxin. Ann. N.Y. Acad. Sci. 173, 320–330.

De Clercq, E. (1980) Interferon inducers. Antibiot. Chemohter. 27, 251–287.

De Clercq, E. and Merigan, T.C. (1970) Current concepts of interferon and interferon induction. Ann. Rev. Med. 21, 17–46.

Engler, H., Zawatzky, R., Goldbach, A., Schroder, C.H., Weyand, C., Hammerling, G.J. and Kirchner, H. (1981) Experimental infection of inbred mice with herpes simplex virus. II. Interferon production and activation of natural killer cells in the peritoneal exudate. J. Gen. Virol. 55, 25–30.

Galanos, C., Luderitz, O., Rietschel, E.T., Westphal, O., Brade, H., Brade, L., Freudenberg, M., Schade, U., Imoto, M., Yoshimura, H., Kusumoto, S. and Siba, T. (1985) Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. Eur. J. Biochem. 148, 1–5.

Gledhill, A.W. (1959) The effect of bacterial endotoxin on resistance of mice to ectromelia. Br. J. Exp. Path. 40, 195–202.

Homma, J.Y., Matsuura, M., Kanegasaki, S., Kawakubo, Y., Kojima, Y., Shibukawa, N., Kumazawa, Y., Yamamoto, A., Tanamoto, K., Yasuda, T., Imoto, M., Yoshimura, H., Kusumoto, S. and Shiba, T. (1985) Structural requirements of lipid A responsible for the functions: A study with chemically synthesized lipid A and its analogues. J. Biochem. 98, 395–406.

Ikeda, S., Matsuda, Y. and Nishimura, C. (1985a) Enhancement by muramyl-dipeptides of the activities of early-type inducers of interferon. Antiviral Res. 5, 345–353.

Ikeda, S., Negishi, T. and Nishimura, C. (1985b) Enhancement of non-specific resistance to viral infection by muramyldipeptide and its analogs. Antiviral Res. 5, 207–215.

Ikeda, S., Kumazawa, Y., Sai, K., Nishimura, C., Nakatsuka, M., Homma, J.Y., Yamamoto, A., Kiso, M. and Hasegawa, A. (1988) Immunopharmacological activities of chemically synthesized lipid A-subunit analogue GLA-27 combined with muramyl dipeptide via spacers of different carbon chain length. Int. J. Immunopharmacol. in press.

Kiso, M., Ishida, H. and Hasegawa, A. (1984) Synthesis of biologically active, novel monosaccharide analogs of lipid A. Agric. Biol. Chem. 48, 251–252.

Kiso, M., Tanaka, S., Fujita, M., Fujishima, Y., Ogawa, Y., Ishida, H. and Hasegawa, A. (1987) Synthesis of the optically active 4-*O*-phosphono-D-glutamine derivatives related to the nonreducing-sugar subunit of bacterial lipid A. Carbohydr. Res. 162, 127–140.

Kotani, S., Watanabe, Y., Shimono, T., Harada, K., Shiba, T., Kusumoto, S., Yokogawa, K. and Taniguchi, M. (1976) Correlation between the immunoadjuvant activities and pyrogenicities of synthetic *N*-acetyl-muramyl-peptide or -amino acids. Biken J. 19, 9–13.

Kotani, S., Takada, H., Tsujimoto, M., Ogawa, T., Takahashi, I., Ikeda, T., Otsuka, K., Shimauchi, H., Kasai, N., Mashimo, J., Nagao, S., Tanaka, A., Tanaka, S., Harada, K., Nagaki, K., Kitamura, H., Shiba, T., Kusumoto, S., Imoto, M. and Yoshimura, H. (1985) Synthetic lipid A with endotoxic and related biological activities comparable to those of a natural lipid A from an *Escherichia coli* Re-mutant. Infect. Immun. 49, 225–237.

Kumazawa, Y., Matsuura, M., Homma, J.Y., Nakatsuru, Y., Kiso, M. and Hasegawa, A. (1985) B cell activation and adjuvant activities of chemically synthesized analogues of the nonreducing sugar moiety of lipid A. Eur. J. Immunol. 15, 199–201.

Kumazawa, Y., Matsuura, M., Maruyama, T., Homma, J.Y., Kiso, M. and Hasegawa, A. (1986) Structural requirements for inducing in vitro B lymphocyte activation by chemically synthesized derivatives related to the nonreducing D-glucosamine subunit of lipid A. Eur. J. Immunol. 16, 1099–1103.

Kumazawa, Y., Ikeda, S., Takimoto, H., Nishimura, C., Nakatsuka, M., Homma, J.Y., Yamamoto, A., Kiso, M. and Hasegawa, A. (1987) Effect of stereospecificity of chemically synthesized lipid A-subunit analogues GLA-27 and GLA-40 on the expression of immunopharmacological activities. Eur. J. Immunol. 17, 663–667.

Masihi, K.N., Lange, W., Brehmer, W. and Ribi, E. (1986) Immunobiological activities of nontoxic lipid A: Enhancement of nonspecific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. Int. J. Immunopharmac. 8, 339–345.

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.

Matsuura, M., Kojima, Y., Homma, J.Y., Kubota, Y., Yamamoto, A., Kiso, M. and Hasegawa, A. (1984) Biological activities of chemically synthesized analogues of the nonreducing sugar moiety of lipid A. FEBS Lett. 167, 226–230.

Matsuura, M., Yamamoto, A., Kojima, Y., Homma, J.Y., Kiso, M. and Hasegawa, A. (1985) Biological activities of chemically synthesized partial structure analogues of lipid A. J. Biochem. 98, 1229–1237.

Nagao, S. and Tanaka, A. (1985) Necrotic inflammatory reaction induced by muramyl dipeptide in guinea pigs sensitized by tubercle bacilli. J. Exp. Med. 162, 401–412.

Okamura, H., Kawaguchi, K., Shoji, K. and Kawade, Y. (1982) High-level induction of gamma interferon with various mitogens in mice pretreated with *Propionibacterium acnes*. Inf. Immun. 38, 440–443.

Parant, M. and Chedid, L. (1984) Stimulation of non-specific resistance to infections by synthetic immunoregulatory agents. Infection 12, 230–234.

Tsujimoto, M., Kotani, S., Kinoshita, F., Kanoh, S., Shiba, T. and Kusumoto, S. (1986) Adjuvant activity of 6-*O*-acyl-muramyldipeptides to enhance primary cellular and humoral immune responses in guinea pigs: Adaptability to various vehicles and pyrogenicity. Inf. Immun. 53, 511–516.