Autoimmune Kidney Disease in MRL/lpr Mice Inhibited by OK-432; II. Effect of Indomethacin

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We have reported that OK-432 (a streptococcal preparation) prevents the development of autoimmune kidney disease in MRL/Mp-lpr/lpr (MRL/lpr) mice and prolongs their survival time. In the present study, to clarify the mechanism of this action of OK-432, we examined whether the cyclooxygenase inhibitor indomethacin (IND) affects this inhibition by OK-432. It was reconfirmed that OK-432 prevented the development of autoimmune kidney disease and prolonged the survival time. This OK-432 effect was counteracted when IND was coadministered. Furthermore, OK-432 produced tumor necrosis factor (TNF)- α and prostaglandin (PG) E₂ in the peritoneal fluids in this strain of mice. The coadministration of IND suppressed the PGE₂ but not the TNF- α production. These results suggest the possibility that the inhibition of autoimmune kidney disease by OK-432 might be due to the induction of cyclooxygenase metabolites of arachidonic acid.

Keywords — MRL/lpr mice; autoimmune kidney disease; OK-432; prostaglandin E

Introduction

OK-432, a heat- and penicillin-treated lyophilized Su strain of *Streptococcus pyogenes*, is a potent biological response modifier, which has been widely used for cancer immunotherapy in Japan.¹⁾ It is thought that the anti-cancer activity of OK-432 is effected through the stimulation of the immune system by producing several cytokines such as interleukin (IL)-1, IL-2,²⁾ IL-6³⁾ and tumor necrosis factor (TNF)- α .⁴⁾

In our previous papers, we have shown that the long-term administration of OK-432 delays the onset of autoimmune kidney disease and prolongs the survival time in both NZB/NZW F1 hybrid mice and MRL/lpr mice, and that this preventive effect in NZB/NZW F1 mice may result from the stimulation of the endogenous TNF- α production.⁵⁾ Based on the fact that TNF- α produces prostaglandin (PG) E₂^{6,7)} which prevents the development of autoimmune kidney disease,^{8,9)} it is suggested that PGE participates in the inhibitory effect of OK-432 in the autoimmune kidney disease. To clarify this point we examined whether indomethacin (IND), which inhibits PG production, could counteract the preventive effect of OK-432 on autoimmune kidney disease in MRL/lpr mice.

Materials and Methods

Animals — Male MRL/lpr mice were obtained from a colony maintained at the Laboratory Animal Center of our Research Laboratories by propagation from breeding pairs supplied by Dr. E. D. Murphy of the Jackson Laboratory (Bar Harbor, ME). Thirty-eight mice were used to determine survival time. In a separate experiment, 20 more mice were used to determine TNF- α and PGE₂ production. The animals were specific-pathogen free and were kept in cages in a room maintained at 24 ± 2 °C and 50—60% relative humidity.

Drugs — OK-432 is produced by incubating cultures of the avirulent Su strain of group A *Streptococcus pyogenes* of human origin, treated with penicillin G potassium, followed by lyophilization of the incubation mixture (Chugai Pharmaceutical Co. Ltd., Tokyo). One KE of OK-432 corresponds to 0.1 mg of the dried streptococci. The lyophilized OK-432 was resuspended in saline before administration and 2 KE was injected i.p. once every week from 8 weeks of age to the end of experiment. Indomethacin (IND: Sigma chemical, St. Louis, MO) was suspended in 0.1% sodium carboxymethylcellulose solution and administered *p.o.* at a dose of 3 mg/kg 30 min before each OK-432 injection.

Proteinuria — Proteinuria was measured semi-quantitatively with Combisticks® paper (Sankyo Co. Ltd., Tokyo) impregnated with tetrabromophenol blue. Protein concentration in the urine was expressed by 5 categories from 0—4 grades: 0—30, 30—100, 100—300, 300—1000, >1000 mg/dl. Proteinuria over grade 2 was assessed as positive.

Anti-DNA Antibody and Total IgM Levels — Anti-DNA antibody level in the serum was assessed by enzyme-linked immunosorbent assay and serum IgM levels were assessed by a single radial immunodiffusion test according to the methods previously described.¹⁰⁾

Serum Immunecomplex (IC) Level — Serum IC was measured as previously described.¹⁰⁾

TNF- α and PGE2 Production in Peritoneal Fluids — MRL/lpr mice were given i.p. 2 KE of OK-432 three times at 1 week intervals from 8 to 10 weeks of age. IND was orally administered 30 min before each OK-432 injection. Two hours after the last injection of OK-432, the mice were sacrificed and 2 ml of PBS was injected into the peritoneal cavity. Peritoneal fluids (PF) were subsequently obtained and centrifuged to remove peritoneal exudate cells. Cell-free PF was passed through a 0.45 μ m membrane filter to remove the OK-432. TNF- α activity in PF was evaluated by the cytotoxicity against L-929 cells

according to the methods previously described.^{4,5)} Rabbit anti-mouse TNF- α antiserum was obtained from Genzyme (Boston, Mass.).

PGE2 Assay — PGE2 concentrations in PF were measured by competitive radio immunoassay (RIA), according to the manufacturers instructions (New England Nuclear, Wilmington, DE). All determinations were performed in duplicate, and the mean was used to calculate PGE2 levels by interpolation from a standard curve.

Statistical Analysis — Statistical differences were analyzed by non-parametric Student's *t* test except for survival time and the incidence of proteinuria. Differences in the latter two were analyzed by the X^2 test with the Yates correction.

Results

Development of Autoimmune Kidney Disease

Administration of OK-432 (2 KE/week) starting from 8 weeks of age significantly prevented the appearance of proteinuria ($p < 0.02$) and prolonged survival time ($p < 0.02$) of MRL/lpr mice. This preventive effect was completely abolished by the combined treatment with 3 mg/kg of IND (Figs. 1 and 2). Treatment with IND alone had no effect on the development of autoimmune disease.

Anti-DNA Antibody and Total IgM Level

Treatment with OK-432, IND, or OK-

Fig. 1. Cumulative Incidence of Male MRL/lpr Mice with Positive Proteinuria

●, control ($n = 10$); ○, OK-432 2 KE ($n = 8$); △, OK-432 2 KE + indomethacin 3 mg/kg ($n = 9$); □, indomethacin 3 mg/kg ($n = 10$).

Fig. 2. Survival Curve of Male MRL/lpr Mice Treated with OK-432 and Indomethacin

The same batch of mice and symbols as in Fig. 1.

Fig. 3. The Development of Serum IgM Anti-DNA Antibody with Age

Error bars are the S.E. of mice surviving at each week.

■, control; ▨, OK-432; ▩, OK-432 + indomethacin; ▤, indomethacin. *a)* $p < 0.02$.

432/IND combination did not significantly affect the serum levels of IgG anti-DNA antibody (Data not shown). In contrast, OK-432 significantly increased IgM anti-DNA antibody ($p < 0.02$; Fig. 3) and serum IgM levels ($p < 0.01$; Fig. 4). And these increased IgM anti-DNA antibody and serum IgM levels were not influenced by the administration of IND.

Immune Complex Level

The administration of OK-432 increased the serum IC levels and IND did not affect these augmented levels (Fig. 5).

TNF- α and PGE2 Production in PF

TNF- α activity in the PF was measured to determine whether OK-432 stimulated TNF- α production in MRL/lpr mice. As shown in Table I, there was much higher TNF- α activity in the PF of OK-432-treated mice than in the control mice ($p < 0.001$). OK-432-induced TNF- α production was rather augmented by the coadministration of IND. This cytotoxic activity was completely neutralized by adding anti-TNF- α antiserum. On the other hand, PGE2 levels in the PFs were significantly higher in OK-432-treated

Fig. 4. Serum IgM Levels at 24 Weeks of Age

Error bars are the S.E. of mice surviving at 24 weeks.

a) $p < 0.01$, *b)* $p < 0.05$.

Fig. 5. Serum Immune Complex Levels at 24 Weeks of Age

Error bars are the S.E. of mice surviving a 24 weeks.

a) $p < 0.02$.

TABLE I. PGE2 Levels and TNF- α Activity in the Peritoneal Fluids from OK-432- and IND-Treated MRL/lpr Mice

Treatment	<i>n</i>	PGE2 (ng/ml) ^{a)}		TNF- α activity (%) ^{b)}	
Control	5	0.74 $\pm$ 0.13	<i>p</i> < 0.01	3.4 $\pm$ 1.3	<i>p</i> < 0.001
OK-432	5	2.65 $\pm$ 0.78		77.6 $\pm$ 8.9	
OK-432 + IND	5	< 0.25		93.4 $\pm$ 0.8	
IND	5	< 0.25		7.0 $\pm$ 2.6	

a) PGE2 levels in PFs were measured by competitive RIA. b) Diluted ($\times 1000$) peritoneal fluids and L-929 cells were cultured for 24 h at 37 °C. After culture, the cytotoxicity was measured by MTT assay. IND, indometacin.

mice than in control mice (*p* < 0.01). However, PGE2 did not appear in either OK-432/IND-treated group or the group treated with IND only.

Discussion

We have previously reported that the long term administration of OK-432 delays the development of autoimmune kidney disease in NZB/W F1 mice and MRL/lpr mice.^{5,11)} And it was suggested that, in the NZB/W F1 mice, TNF- α may participate in its inhibitory effect. In the present study, we found that this preventive effect of OK-432 was decreased by the combined administration of the cyclooxygenase inhibitor IND without suppressing TNF- α production. IND rather augmented TNF- α production. This is consistent with the fact that a decreased production of PGE induces an increase in TNF- α production.¹²⁾ These results strongly suggest that TNF- α alone does not directly participate in the suppressive effect of OK-432.

In contrast, our present data showed that IND treatment completely inhibited PGE2 production (Table I). It has been reported that PGE delays the development of autoimmune kidney disease and prolongs the survival time of both NZB/NZW F1 mice and MRL/lpr mice.^{8,9)} Together with the evidence indicating that the injection of TNF- α increases plasma PGE2 level,¹³⁾ it appears that OK-432 enhances the TNF- α production resulting in the increased synthesis of PGE. Both TNF- α ^{14,15)} and PGE⁸⁾ delay the development of autoimmune kidney disease in SLE mice without affecting anti-DNA antibody production, and these similarities also support our hypothesis that the effect of OK-432

may be attributed to the synthesis of PGE induced by TNF- α . In addition, OK-432 did not suppress the onset of autoimmune kidney disease in male NZW/BXSB F1 mice (unpublished data). It is very interesting that PGE does not suppress disease development in male BXSB mice.¹⁶⁾ However, the possibility can not be excluded that OK-432 directly induces PGE2 production. Furthermore, it is also possible that other arachidonic acid metabolites such as prostacyclin and thromboxanes are involved in the suppression of the disease development.

Since OK-432 did not reduce the serum levels of anti-DNA antibody, it is unlikely that the preventive effect of OK-432 is due to the inhibition of polyclonal B cell activation, as it is in immunosuppressants.^{17–20)} Furthermore, these immunosuppressive agents suppress lymphadenopathy, which is a characteristic of MRL/lpr mice. As previously reported, OK-432 does not suppress lymphadenopathy. In contrast, Kelley *et al.* reported that the injection of PGE suppresses lymphadenopathy in MRL/lpr mice.⁹⁾ If PGE plays an essential role in the preventive effect of OK-432, there seems to be a discrepancy between these two results. They administered large amounts of PGE (200 μ g twice daily 5 d of the week and once a day on Saturday and Sunday) to suppress completely the disease development. In contrast, we injected OK-432 only once a week resulting in the lesser degree of the suppression. These facts suggest that the PGE level induced by OK-432 was not high enough to affect lymphadenopathy.

Our previous report indicated that OK-432 increases the serum levels of IgM class antibodies.¹¹⁾ This is possibly related to the decreased IC deposition in the renal glomeruli resulting in the prevention of the development of autoim-

mune disease. However, our present study excluded this possibility, since IND abolished the effectiveness of OK-432 in preventing autoimmune disease without affecting the increased level of IgM antibody (Figs. 3, 4).

OK-432 is a heat- and penicillin-treated *Streptococcus pyogenes*. It would be very interesting to clarify which component of OK-432 is essential for its activity. In spite of many efforts to determine the active components of OK-432, we have only limited evidence.^{21,22} The search for the active components in the antinephritic activity of OK-432 is a subject of great interest which we will undertake in the future.

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