

Effect of Methotrexate Treatment on the Onset of Autoimmune Kidney Disease in Lupus Mice

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In the present study, we examined the effects of methotrexate (MTX) on the development of autoimmune kidney disease in three kinds of autoimmune prone mice, NZB/NZW F1 (BWF1) mice, MRL/Mp-lpr/lpr (MRL/lpr) mice and NZW/BXSB F1 (WBF1) mice. The results showed that MTX delayed the appearance of proteinuria and prolonged survival of both BWF1 and MRL/lpr mice and inhibited the elevation of blood urea nitrogen (BUN) levels which accompanies the development of lupus nephritis. However, MTX treatment did not affect these in WBF1 mice. Furthermore, MTX could not suppress immunoglobulin G (IgG) class anti-deoxyribonucleic acid (DNA) and anti-trinitrophenol (TNP) antibody production in any variety of mice. These suggest that the therapeutic effect of MTX on BWF1 and MRL/lpr mice does not result in the suppression of IgG autoantibody production.

Keywords methotrexate; autoimmune kidney disease; autoantibody; lupus mouse

Introduction

Methotrexate (MTX) has been used successfully in the treatment of neoplastic, and nonneoplastic disease such as psoriasis, psoriatic arthritis, polymyositis, sarcoidosis and Reiter's syndrome.¹⁻⁶ In addition, recent clinical studies have shown that the administration of MTX to the patient with rheumatoid arthritis (RA),⁷ systemic lupus erythematosus (SLE)⁸ and steroid-resistant-asthma⁹ causes clinical improvement.

MTX interferes with folate metabolism *via* the inhibition of dihydrofolate reductase and blocks deoxyribonucleic acid (DNA) synthesis in rapidly proliferative cells.¹⁰ These actions induce immunosuppression. In fact, MTX inhibits immune responses such as the delayed-type hypersensitivity (DTH) response^{11,12} and antibody production.^{13,14}

In this study, to define the mechanism of action of MTX on the suppressive effect on autoimmune disease, we examined the effect of MTX on the development of autoimmune kidney disease using three kinds of autoimmune prone mice, NZB/NZW F1 (BWF1) mice, MRL/Mp-lpr/lpr (MRL/lpr) mice and NZW/BXSB F1 (WBF1) mice, which spontaneously develop autoimmune disease like SLE, RA and idiopathic thrombocytopenic purpura (ITP), and are widely used for drug evaluation. The results showed that MTX inhibits the development of autoimmune kidney disease in BWF1 and MRL/lpr mice, but not in WBF1 mice, without the suppression of autoantibody production.

Materials and Methods

Animals Female NZB×NZW F1 (BWF1) mice, MRL/Mp-lpr/lpr (MRL/lpr) mice and NZW×BXSB F1 (WBF1) mice were bred in our laboratories. The animals were specific pathogen free and were kept in cages in a room maintained at 24±2°C, with 50–60% relative humidity.

Experimental Protocol Methotrexate (Sigma Chemical Co., St. Louis, MO) was suspended in a 0.1% sodium carboxymethylcellulose solution and administered orally three times a week until the end of experiment.

Proteinuria Proteinuria was measured semi-quantitatively by means of Combisticks® paper (Sankyo Co., Ltd., Tokyo). Protein concentration in the urine was expressed by 5 categories from 0 to 4 grades: 0–30, 30–100, 100–300, 300–1000, >1000 mg/dl. Proteinuria over grade 3 was assessed as positive.

Blood Urea Nitrogen (BUN) The BUN level was measured by means of Unikit-BUN-s (Chugai Pharmaceutical Co., Ltd., Tokyo) using a Rapid-Blood Analyzer (Chugai Pharmaceutical Co., Ltd., Tokyo).

Anti-DNA and Anti-trinitrophenol (TNP) Antibody Serum anti-DNA and anti-TNP antibody levels were assessed by enzyme-linked immuno-

sorbent assay (ELISA) according to the method described previously.¹⁵

Serum Immunoglobulin G₁, G₂ and G₃ (IgG₁, IgG₂ and IgG₃) Levels Serum IgG₁, IgG₂ and IgG₃ levels were measured by single radial immunodiffusion, described previously.^{15,16}

Statistical Analysis Statistical significance of the differences was analyzed by the Student's *t*-test, except for the proteinuria excretion and survival rates. Proteinuria excretion and survival time were analyzed by a generalized Wilcoxon test.

Results

BWF1 Mice Mice were given *p.o.* 1.0 mg/kg of MTX three times a week from 24 to 50 weeks of age. MTX significantly delayed the appearance of proteinuria ($p < 0.01$) and showed a tendency to prolong the survival of this strain of mice (Fig. 1). Moreover, the elevation of BUN levels accompanied by the development of lupus nephritis was not observed in the MTX-treated group until

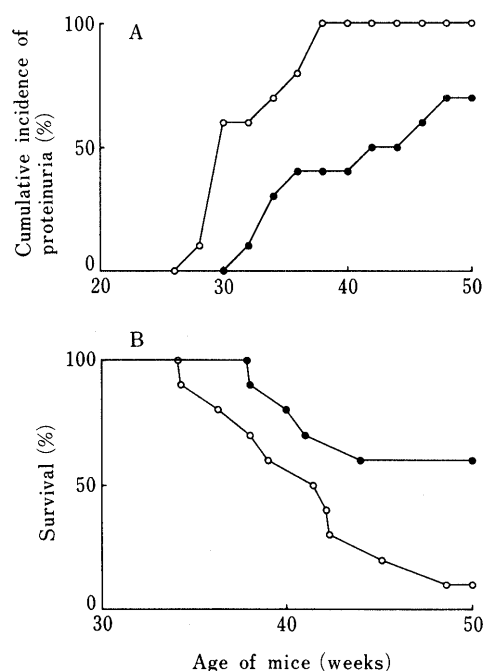


Fig. 1. Cumulative Incidence of Proteinuria (A) and Survival Time (B) in Female BWF1 Mice Treated with MTX

Female BWF1 mice were given *p.o.* 1.0 mg/kg of MTX three times a week from 24 weeks old to 50 weeks old. Proteinuria over 300 mg/dl was assessed as positive. Each group included 10 mice. —○—, control; —●—, MTX 1.0 mg/kg.

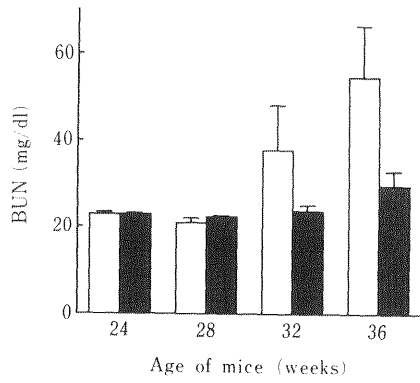


Fig. 2. BUN Levels in MTX-Treated Female BWF1 Mice
Data are expressed as the mean \pm S.E. of surviving mice at each age. The same batch of mice as in Fig. 1 was utilized. \square , control; \blacksquare , MTX 1.0 mg/kg.

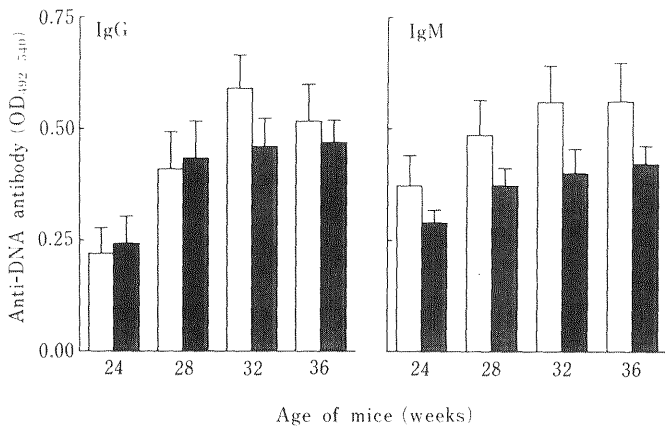


Fig. 3. Serum Anti-DNA Antibody Levels in MTX-Treated BWF1 Mice
Sera were diluted 1/200 and anti-DNA antibody levels were measured by ELISA. Data are expressed as the mean \pm S.E. of surviving mice at each age. The same batch of mice as in Fig. 1 was utilized. \square , control; \blacksquare , MTX 1.0 mg/kg.

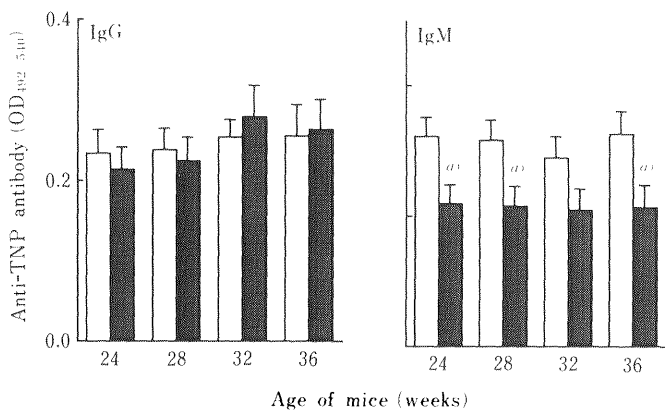


Fig. 4. Serum Anti-TNP Antibody Levels in MTX-Treated BWF1 Mice
Sera were diluted 1/200 and anti-TNP antibody levels were measured by ELISA. See legend for Fig. 3. \square , control; \blacksquare , MTX 1.0 mg/kg. *a*) $p < 0.05$.

the mice were 36 weeks of age (Fig. 2). MTX significantly suppressed IgM class anti-TNP antibody production and showed a tendency to suppress IgM anti-DNA antibody production, but did not suppress either IgG class anti-DNA or anti-TNP antibody production (Figs. 3 and 4). Serum levels of IgG subclasses at 32 weeks of age also were not changed (Table I).

MRL/lpr Mice Female mice were given *p.o.* MTX

TABLE I. Serum IgG Levels in 32-Week-Old NZB/W F1 Mice Treated with MTX and Their Controls

Treatment	n	IgG subclass (mg/dl)		
		IgG ₁	IgG ₂	IgG ₃
Control	10	139.5 \pm 16.9	349.5 \pm 54.5	109.0 \pm 14.1
MTX 1 mg/kg	10	157.2 \pm 16.4	346.6 \pm 47.9	115.7 \pm 7.6

Each value indicates mean \pm S.E. of 10 mice.

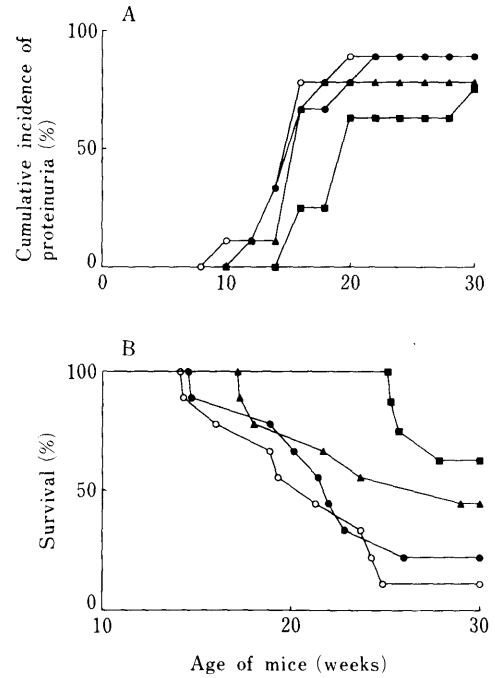


Fig. 5. Cumulative Incidence of Proteinuria (A) and Survival Time (B) in Female MRL/lpr Mice Treated with MTX

Female MRL/lpr mice were given *p.o.* 0.4–2.5 mg/kg of MTX three times a week from 8 weeks old to 30 weeks old. Proteinuria over 300 mg/dl was assessed as positive. Each group included 9 mice (8 mice for the 2.5 mg/kg MTX-treated group). \circ — \circ , control; \bullet — \bullet , MTX 0.4 mg/kg; \blacktriangle — \blacktriangle , MTX 1.0 mg/kg; \blacksquare — \blacksquare , MTX 2.5 mg/kg.

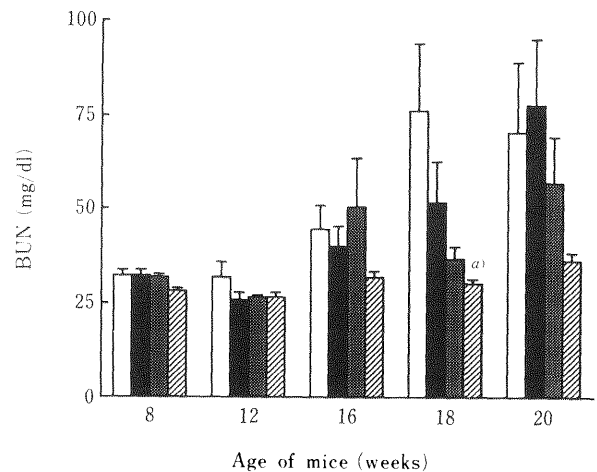


Fig. 6. BUN Levels in MTX-Treated Female MRL/lpr Mice

Data are expressed as the mean \pm S.E. of surviving mice at each age. The same batch of mice as in Fig. 5 was utilized. \square , control; \blacksquare , MTX 0.4 mg/kg; \blacksquare , MTX 1.0 mg/kg; \hatched , MTX 2.5 mg/kg. *a*) $p < 0.05$.

three times a week from 8 to 30 weeks of age. The appearance of proteinuria was significantly delayed only in the 2.5 mg/kg treated-group ($p < 0.05$) (Fig. 5A). Survival

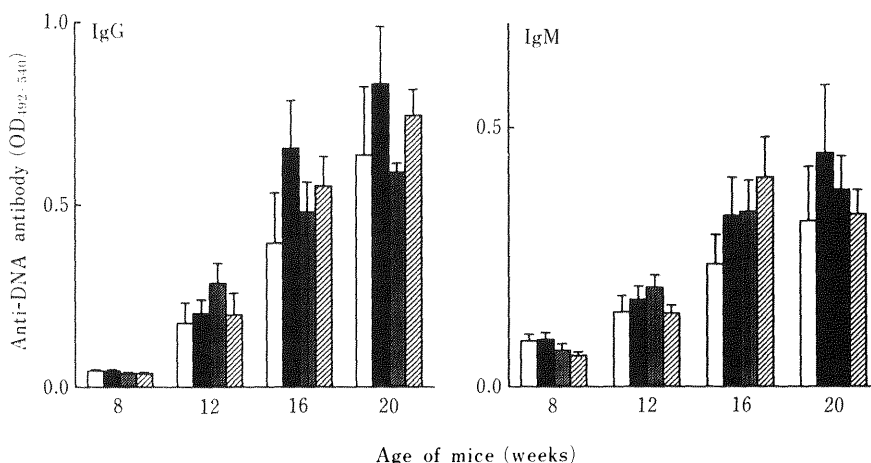


Fig. 7. Serum Anti-DNA Antibody Levels in MTX-Treated MRL/lpr Mice

Sera were diluted 1/500 for IgM class and 1/1000 for IgG class and anti-DNA antibody levels were measured by ELISA. Data are expressed as the mean \pm S.E. of surviving mice at each age. The same batch of mice as in Fig. 5 was utilized. \square , control; \blacksquare , MTX 0.4 mg/kg; \boxtimes , MTX 1.0 mg/kg; \boxplus , MTX 2.5 mg/kg.

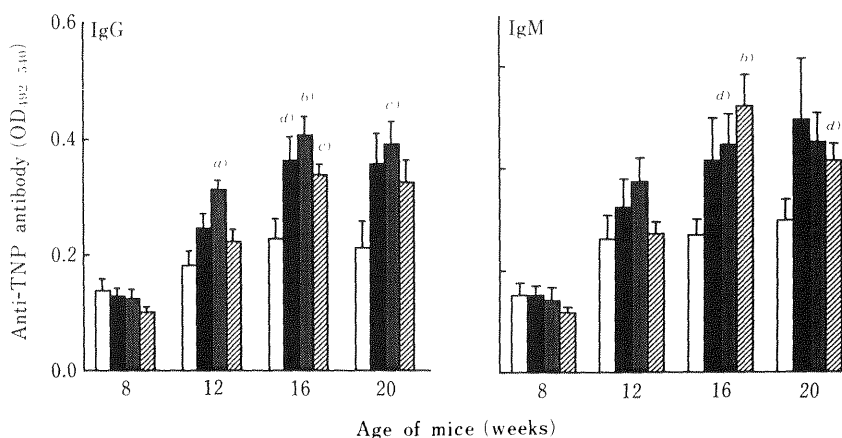


Fig. 8. Serum Anti-TNP Antibody Levels in MTX-Treated MRL/lpr Mice

Sera were diluted 1/500 for IgM class and 1/1000 for IgG class and anti-TNP antibody levels were measured by ELISA. See legend for Fig. 7. \square , control; \blacksquare , MTX 0.4 mg/kg; \boxtimes , MTX 1.0 mg/kg; \boxplus , MTX 2.5 mg/kg. a) $p < 0.001$, b) $p < 0.01$, c) $p < 0.02$, d) $p < 0.05$.

TABLE II. Serum IgG Levels in 16-Week-Old MRL/lpr Mice Treated with MTX and Their Controls

Treatment	n	IgG subclass (mg/dl)		
		IgG ₁	IgG ₂	IgG ₃
Control	7	797.7 \pm 203.1	795.3 \pm 73.7	374.1 \pm 40.0
MTX 2.5 mg/kg	8	992.5 \pm 164.4	671.8 \pm 49.2	511.6 \pm 47.0 ^{a)}

Each value indicates mean \pm S.E. a) $p < 0.05$.

was prolonged in a dose-dependent manner by the administration of more than 1.0 mg/kg, and the 2.5 mg/kg treated group was statistically significant ($p < 0.01$) (Fig. 5B). The elevation of BUN levels was also inhibited dose-dependently (Fig. 6). Serum anti-DNA antibody levels were not altered, in fact, serum anti-TNP antibody levels actually increased (Figs. 7 and 8). Serum IgG levels were measured at 16 weeks of age. As shown in Table II, serum IgG₃ levels were significantly increased, but IgG₁ and IgG₂ levels were not affected by 2.5 mg/kg of MTX.

Moreover, when male MRL/lpr mice were given *p.o.* 1.0 mg/kg of MTX three times a week from 8 to 23 weeks of age, the weights of mesenteric lymph node and spleen

were 664 \pm 152 (mean \pm S.E.) mg and 1182 \pm 176 mg in the controls and 702 \pm 75 mg and 1341 \pm 225 mg in MTX-treated group, respectively.

WBF1 Mice Male mice were given *p.o.* 1.0 mg/kg of MTX three times a week from 8 to 34 weeks of age. In this strain of mice, male mice have a Yaa (Y chromosome-linked autoimmune acceleration) gene derived from male BXSB mice and show an early onset of autoimmune disease, whereas female mice show a late onset. MTX did not affect the appearance of proteinuria, the survival rate, BUN levels, serum anti-DNA and anti-TNP antibody levels (Figs. 9–12).

Discussion

We examined the preventive effect of MTX on the development of autoimmune kidney disease in WBF1 mice, MRL/lpr mice and WBF1 mice and then obtained the results as follows. (1) MTX (1.0 mg/kg) delayed the appearance of proteinuria and prolonged the survival of female WBF1 mice. (2) In MRL/lpr mice, the appearance of proteinuria was delayed by 2.5 mg/kg of MTX. Survival was prolonged in a dose response manner by the administration of more than 1.0 mg/kg. (3) MTX, at a lower dosage (1.0 mg/kg), did not affect the appearance of

proteinuria and the survival of male WBF1 mice. At a higher dosage (5.0 mg/kg) MTX remarkably decreased body weights due to its toxicity 4 weeks after initiation of administration (data not shown). This suggests that MTX is ineffective on this strain of mice.

In all kinds of lupus mice, MTX did not suppress IgG class anti-DNA and anti-TNP antibody production. Besides the anti-DNA antibody, anti-gp70 antibody also seems closely related to the course of renal disease of lupus mice.¹⁷⁾ However, the possibility that MTX selectively suppresses the production of other pathogenic antibodies, such as anti-gp70 antibody, is unlikely because serum levels of IgG subclasses also remained unchanged by

treatment with MTX. These results suggest that the preventive effect on autoimmune kidney disease is not due to the suppression of antibody production, because IgG class antibodies are essential to the onset of autoimmune disease.¹⁸⁾ Furthermore, MTX did not suppress the lymphadenopathy in MRL/lpr mice. These lines of evidence suggest that MTX might not affect lymphocyte proliferation and antibody production under this experimental condition.

MTX, which interferes with folate metabolism *via* the inhibition of dihydrofolate reductase and blocks DNA synthesis, is an immunosuppressive drug. It is well-known that immunosuppressive agents such as cyclophosphamide, azathiopurine and steroids¹⁹⁻²¹⁾ show beneficial effects in autoimmune disease therapy and suppress autoantibody production. These results are inconsistent with our results that MTX did not influence autoantibody production in SLE prone mice.

Recently, Ueda *et al.*²²⁾ reported that MTX induces prostaglandin (PG) E₂ production from human peripheral blood mononuclear cells *in vitro* and that MTX injection

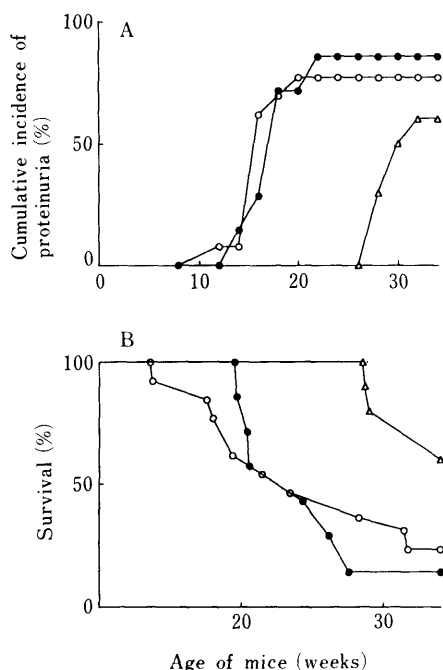


Fig. 9. Cumulative Incidence of Proteinuria (A) and Survival Time (B) in Male WBF1 Mice Treated with MTX

Male WBF1 mice were given *p.o.* 1.0 mg/kg of MTX three times a week from 8 weeks old to 34 weeks old. Proteinuria over 300 mg/dl was assessed as positive. Male (control group), MTX-treated group and female group included 13, 7 and 10 mice, respectively. —○—, male (control); —●—, MTX 1.0 mg/kg; —△—, female.

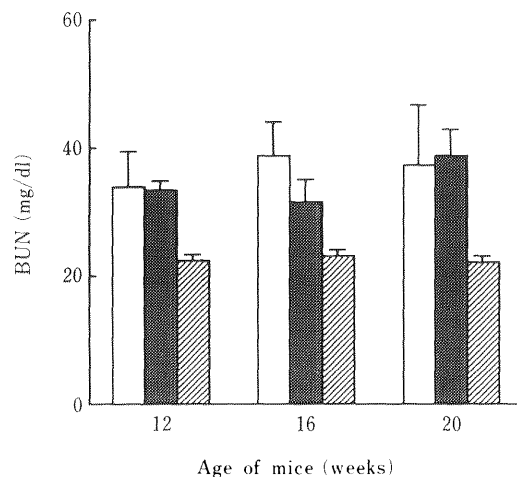


Fig. 10. BUN Levels in MTX-Treated Male WBF1 Mice

Data are expressed as the mean ± S.E. of surviving mice at each age. The same batch of mice as in Fig. 9 was utilized. □, male (control); ■, MTX 1.0 mg/kg; ▨, female. *a*) $p < 0.02$.

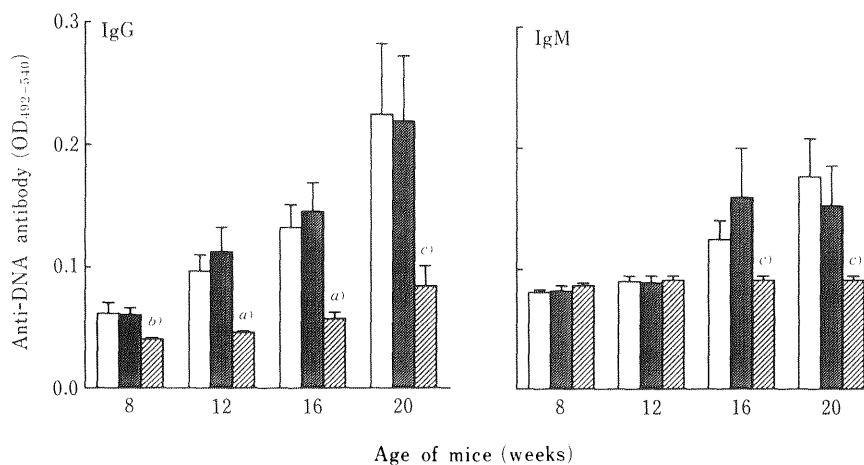


Fig. 11. Serum Anti-DNA Antibody Levels in MTX-Treated WBF1 Mice

Sera were diluted 1/300 and anti-DNA antibody levels were measured by ELISA. Data are expressed as the mean ± S.E. of surviving mice at each age. The same batch of mice as in Fig. 9 was utilized. Statistical significance of the difference from the control were analyzed. □, male (control); ■, MTX 1.0 mg/kg; ▨, female. *a*) $p < 0.01$, *b*) $p < 0.02$, *c*) $p < 0.05$.

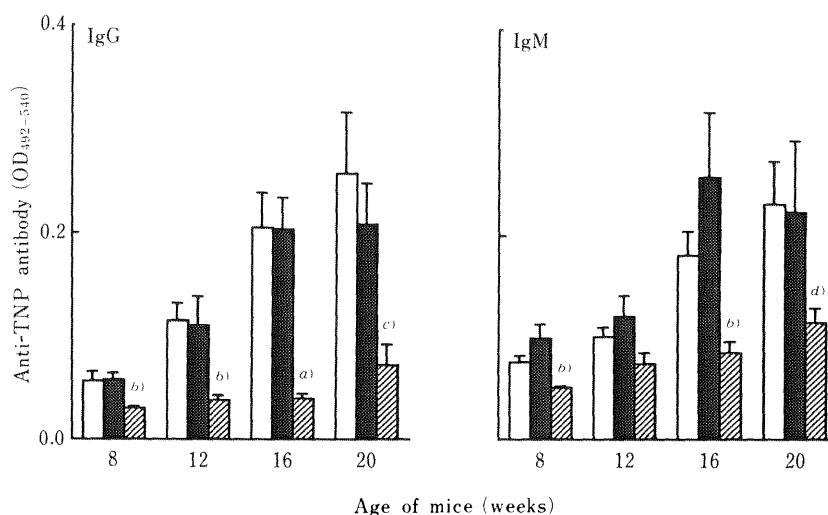


Fig. 12. Serum Anti-TNP Antibody Levels in MTX-Treated WBF1 Mice

Sera were diluted 1/300 and anti-TNP antibody levels were measured by ELISA. See legend for Fig. 11. □, male (control); ■, MTX 1.0 mg/kg; ▨, female. a) $p < 0.001$, b) $p < 0.01$, c) $p < 0.02$, d) $p < 0.05$.

also increases the PGE₂ level in the peritoneal exudate of mice. PGE₂ inhibits autoimmune kidney disease in BWF1 and MRL/lpr mice without suppressing anti-DNA antibody production, but it does not inhibit the development of autoimmune nephritis in BXS mice.²³⁻²⁵ These facts closely resemble our results. Taken together with these lines of evidence, it is suggested that PGE₂ induced by MTX participates in the inhibition of the development of autoimmune kidney disease in BWF1 and MRL/lpr mice. Izui *et al.*²⁵ also reported that BXS mice treated with PGE remained unchanged in respect to serum gp70 immune complex levels and development of renal disease. This may suggest the reason why MTX did not improve the development of renal disease in WBF1 mice. Further studies should be conducted for the confirmation of this point.

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