

Tamoxifen Decreases Renal Inflammation and Alleviates Disease Severity in Autoimmune NZB/W F1 Mice

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It has been documented that sex hormone may play a role in the pathogenesis of murine lupus. To determine the effect of tamoxifen (TAM) on NZB/W F1 female mice, a total dose of 800 µg (22 mg/kg body weight) of TAM was administered subcutaneously every 2 weeks. The control mice were injected with peanut oil only. After treatment with TAM for 5 months, the mice were killed and immunological parameters were evaluated. The results suggest that NZB/W F1 mice treated with TAM had less severe proteinuria and increased survival rate compared to controls. Flow cytometric analysis of splenocytes revealed a significantly lower percentage of B cells and CD5⁺ B cells in the TAM-treated group. There was a significantly lower serum level of soluble tumour necrosis factor (TNF) receptor I and II molecules in the TAM-treated mice. Immunohistological study showed that control mice had severe immune complex deposition in the kidney. In contrast, TAM-treated mice had much less pathological change. In summary, this study demonstrated that TAM treatment might be able to alleviate the symptoms of lupus nephritis, influence B-cell count, modulate the expression of cytokine receptors and thereby subsequently affect immune function. Further studies to determine the cellular mechanisms in lupus nephritis may increase our understanding of this complex disease and provide additional targets for therapeutic intervention.

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INTRODUCTION

The immunological predominance of females and their proneness to autoimmune disease is often attributed to estradiol [1]. Evidence obtained from studies involving animal models and human subjects supports the concept that female sex hormones, particularly oestrogen, contribute to the pathogenesis of systemic lupus erythematosus (SLE) [2,3]. Murine lupus models such as NZB×NZW F1 (NZB/W F1), NZB.H-2^{bm12}, NZB×SWR F1 (SNF1), MRL.*lpr/lpr* and BXSB mice have led to a better understanding of the pathogenic mechanisms of lupus [4,5]. All of these species of mice develop immunoglobulin G (IgG) anti-dsDNA antibody; a characteristic of lupus, and die of uremia in early life. Among these murine lupus models, the natural course of NZB/W F1 mice is closer to human lupus than MRL.*lpr/lpr* and BXSB mice.

Tamoxifen (TAM), a synthetic antiestrogen with high affinity

for oestrogen receptor [6,7], is known for its antitumor action *in vivo* [7]. However, it is well accepted that many of its effects are elicited via oestrogen receptor-independent routes. In addition to its antioestrogen activity, TAM has also been reported to inhibit protein kinase C [8,9], calmodulin [9], antioxidant activity [10], inhibition of the membrane translocation [11], down-regulation of insulin-like growth factor-I [12,13], induction of apoptosis [14], interaction with P-glycoproteins [15] and stimulation of transforming growth factor-β (TGF-β) secretion [6]. It is well established that sex hormones influence various immune system features, although their mechanisms of action have not yet been clearly established.

In the present study, we used a lupus prone mouse species, NZB/W F1, to investigate the effect of TAM on disease severity, the immune properties of cells, *in vitro* cytokine production, serum levels of autoantibody and cytokine receptors, and on renal pathological changes.

MATERIALS AND METHODS

Experimental animals. Six to 8-week-old female NZB/W F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the Animal Center of National Taiwan University, College of Medicine. Each group consisted of 20 mice. NZB/W F1 mice were followed throughout their life span and proteinuria levels were monitored weekly to confirm the pathological changes of the mice.

Drug treatment. Mice were subcutaneously injected every 2 weeks with 800 µg of TAM (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 50 µl peanut oil. Drug treatment was given regularly throughout the life span and serological changes were monitored fortnightly immediately prior to injection. Control mice were injected with peanut oil only. To study the cellular mechanism of action of TAM, the mice were treated for 5 months and killed at 8 months of age. This dose of 800 µg TAM was determined based on preliminary experiments demonstrating that NZB/W F1 mice treated with this dose had less severe proteinuria and reduced sera titer of anti-dsDNA autoantibodies and an increased survival rate.

Determination of serum anti-ss, dsDNA IgG antibodies. For the detection of anti-DNA antibodies in serum of NZB/W F1 mice, enzyme-linked immunosorbent assay (ELISA) was carried out as described previously [16]. Plates were initially coated with methylated bovine serum albumin (mBSA, Sigma). For the assessment of anti-ssDNA antibody, dsDNA was denatured by boiling for 15 min and then cooled on ice. The results of anti-DNA antibody are presented as ELISA units (EU/ml) using a standard monoclonal antibody (MoAb) 742H.7D (concentration: 74 ± 0.5 ng/ml) specific for dsDNA.

Flow cytometry. Six mice of each group were killed by cervical dislocation. Single-cell suspensions were prepared from the spleen and cells obtained from peritoneal exudates of 8-month-old female NZB/W F1 mice. The spleens were removed, and the red cells were lysed with ammonia chloride-Tris buffer to isolate single cells. Direct staining was performed by incubating viable cells ($2.5\text{--}5 \times 10^5$ cells) of the splenocytes and cells obtained from peritoneal exudates at 4 °C in the dark with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or CyChrome conjugated MoAbs against CD3, CD4, CD8, CD5, B220 (PharMingen, San Diego, CA, USA) or CD40 (Serotec, Washington DC, USA). The cells were washed and resuspended in 0.5 ml of phosphate-buffered saline (PBS) with 0.1% sodium azide and subjected to fluorescence-activated cell sorting (FACScan) analysis. For three-colour analysis, samples were quantitated on a FACScan and analyzed using Cell Quest Software (Becton Dickinson Immunocytometry Systems, Mansfield, MA, USA). A total of 10 000 cells were counted, the frequency of each cell-surface marker was determined using appropriate software (FACScan, Becton Dickinson, Mountain View, CA, USA).

Stimulation of cells obtained from peritoneal exudates. Cells obtained from peritoneal exudates with a concentration of 5×10^6 cells/ml were placed in 24 well plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, 10 mM HEPES pH 7.3, 50 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 mg/ml amphotericin. These cells were then activated in the absence or presence of 10 µg/ml lipopolysaccharide (LPS, Sigma). After 24 h incubation, the supernatant fraction was collected and assayed by sandwich ELISA to determine cytokine levels. Predetermined concentrations of anti-interleukin (IL)-6 and TNF-α antibodies (PharMingen, San Diego, CA, USA) were coated to the ELISA plate and incubated at 4 °C overnight. After washing with PBS, the supernatant was added to the plates and incubated at room temperature for 2 h. After washing with buffer, biotin-conjugated anti-IL-6 or TNF-α antibodies was added

followed by incubation at room temperature for 2 h. After washing with PBS, horseradish peroxidase-conjugated streptavidin was added followed by incubation at room temperature for 2 h. The detectable sensitivity of mouse IL-6 and TNF-α is 15.6 pg/ml. Absorbance was determined as described previously [16].

Cytokine production by spleen cells. Spleen cells with a concentration of 1×10^7 cells/ml were placed in 24 well plates in medium as previous described [16]. Splenocytes were stimulated in the presence of hamster anti-mice-CD3 antibody and anti-mice-CD28 antibody (1 µg/ml, PharMingen) cross-linked with anti-hamster IgG (0.5 µg/ml, Jackson Immuno-research Laboratories, West Grove, PA, USA) antibody. To determine the optimal levels of cytokine, supernatants were collected at different time points and the maximal response was noted at 48 h. The cytokine production pattern of spleen cells was measured using sandwich-ELISA. Predetermined concentrations of anti-IL-2, γ-IFN, IL-4 and IL-5 antibodies (PharMingen) were coated to ELISA plates and incubated at 4 °C overnight. After washing in PBS, supernatant was added to the plates followed by incubation at room temperature for 2 h. Biotin-conjugated anti-IL-2, γ-IFN, IL-4 and IL-5 antibodies and horseradish peroxidase-conjugated streptavidin were then added. Enzyme activity was evaluated using ABTS as the substrate. The minimum detectable sensitivity of mouse IL-2, γ-IFN, IL-4 and IL-5 is typically less than 15.6 pg/ml.

Determination of cytokine receptor levels. Serum levels of two TNF-soluble receptors (TNF-sR55 and TNF-sR75) were tested in 7-month-old NZB/W F1 mice. Amounts of total sera TNF-sR55 (TNF-soluble receptor 55) and TNF-sR75 (TNF-soluble receptor 75) were measured with commercial ELISA according to the manufacture's instructions (R & D Systems, Quantikine® M, Minneapolis, MN, USA). The minimum detectable sensitivity of mouse TNF-sR55 and TNF-sR75 are typically less than 5 pg/ml.

Assessment of renal pathological changes. Renal disease was evaluated by the development of albuminuria and histological changes in the kidney. Proteinuria was measured colorimetrically using commercially available sticks (tetrabromophenol paper, Eiken Chemical Co., Tokyo, Japan) and fresh urine samples. This colorimetric assay, which is relatively specific for albumin, was graded 1+ to 4+ and the approximate protein concentrations were as follows: 1+: 30 mg/dL, 2+: 100 mg/dL, 3+: 1000 mg/dL, and 4+: > 1000 mg/dL. High-grade proteinuria was defined as greater than 2+ (100 mg/dL). Kidney histology was performed on mice that were killed at 8 months of age. Immunohistochemistry was used for detection of either immune complex deposition or CD40/CD40 ligand expression in kidney samples that had been stored in neutral-buffered formalin before being processed by a standard paraffin-embedding technique. Kidney specimens were sliced in 5 µm sections and placed onto slides. The slides were then dewaxed in xylene and dehydrated through a graded series of alcohol concentrations. In order to optimize the immunohistochemistry technique, sections were then subjected to proteolytic enzyme digestion modified from a previous description [17]. Briefly, the sections were incubated with 10 µg/ml of proteinase K (Boehringer Mannheim, Indianapolis, IN, USA) in PBS at pH 7.4 for 40 min at room temperature. Following proteolytic digestion, the sections were washed in tap water. To efficiently block endogenous peroxidase, the sections were also incubated in methanol solution containing 0.3% (v/v) H₂O₂ for 30 min. The slides were then incubated with 10% FBS serum in PBS to block nonspecific avidin binding. They were first incubated overnight at 4 °C with either anti-IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA), antimouse CD40 (Serotec) or antimouse CD40 ligand (BioSource, Camarillo, CA, USA) antibodies, all at the working concentration of 10 µg/ml. Sections were then incubated for 1 h at room temperature with biotin-conjugated MoAb

against the target antigen. The sections were then washed in PBS and an alkaline phosphatase streptavidin conjugate was then added. The reaction product was developed using a solution of 0.1% 3,3' diaminodenzidine (Sigma) and 0.02% H₂O₂ in the PBS as substrate for 5 min and then washed in running tap water. Coverslips were then applied to the sections using a permanent mountant and the sections were then examined by light microscope.

Statistical analysis. Differences in serological parameters between the TAM-treated and control group were evaluated with Student's *t*-test. Data were expressed as mean \pm SEM for each group. The mortality and albuminuria were plotted using the Kaplan-Meier method, i.e. using nonparametric cumulative survival plots. Statistical comparison between the different curves was performed using the Mantel-Cox log-rank test, that provided the corresponding χ^2 values. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Proteinuria and life span

The results for severity of proteinuria and life span of NZB/W F1 mice treated with or without TAM are summarized in Fig. 1. The mice treated with TAM showed less severe albuminuria ($P = 0.013$, Fig. 1A) and a longer life span (Fig. 1B) compared to mice in the control group. In NZB/W F1 mice treated with peanut oil only, the onset of proteinuria occurred at the age of 5 months. Fifty percent of the animals had proteinuria at 6 months of age (220 days, Fig. 1A). The survival rate of TAM-treated mice was significantly higher than that of the control group. The mortality duration of mice in the control group reached 50% at 5.5 months. Longterm administration (more than 5 months) of 800 $\mu\text{g/kg}$ of TAM significantly delayed the onset of the disease. TAM-treated NZB/W F1 mice had notably prolonged survival ($P = 0.0012$), with 50% cumulative mortality not even reached at 11 months (340 days) of age, when the experiment was stopped.

Anti-DNA antibodies levels

The humoral autoimmune response in female NZB/W F1 mice was evaluated by measuring circulating autoantibody levels. The levels of IgG anti-ssDNA and anti-dsDNA antibodies were followed up regularly throughout the life span beginning at 4 months of age (Fig. 2). IgG anti-dsDNA antibodies increased gradually with age and peaked at 7 to 8-months-old mice (Fig. 2A). The results showed no difference of serum levels of IgG anti-ssDNA (Fig. 2B) and anti-dsDNA antibodies between these two groups.

Phenotypic analysis and CD5⁺ B cells expression in spleen and in peritoneal exudates

The results of phenotypic analysis are summarized in Table 1. One of the major B-cell abnormalities in NZB/W F1 mice is the increased frequency of CD5⁺ B cells in both the spleen and peritoneal cavity [20]. In this study, the percent of CD5⁺ B cells in mice treated with TAM was significantly lower than that of the

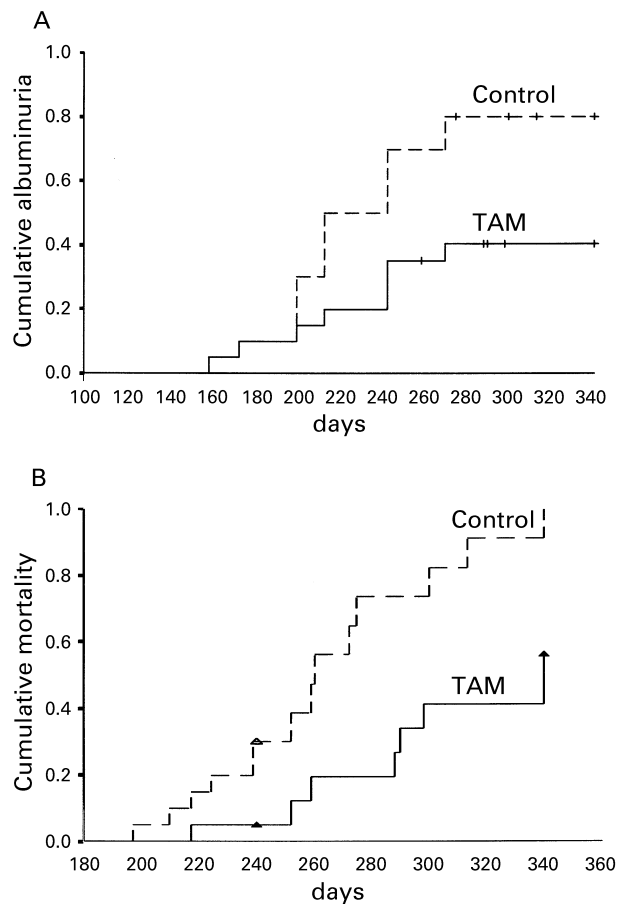


Fig. 1. Effects of TAM on NZB/W F1 mice showed less severe proteinuria (A) and a significantly increased survival rate compared to controls (B). Groups of 20 mice were injected subcutaneously every 2 weeks with TAM 800 $\mu\text{g/kg}$ (\blacktriangle) or oil only (\triangle). Albuminuria was measured weekly using dip sticks (Fig. 1A). Data show the percentage of mice with albumin concentration $> 100 \text{ mg/dL}$ albumin in the urine. Cumulative mortality was monitored throughout the 360-day duration of the experiment (Fig. 1B). Albuminuria and mortality rate were plotted using the Kaplan-Meier method and the logrank (Mantel-Cox) test was used for statistical comparison of the curves obtained. A significant difference was found in albuminuria and mortality between the TAM-treated group and the control group ($P < 0.05$).

control group ($P < 0.05$) both in spleen and in peritoneal exudates. The data also showed that TAM-treated mice had a significantly lower percentage of B cells in splenocytes and CD40⁺ B cells in peritoneal exudates. The numbers of B cells in peritoneal exudates were unchanged in these two groups of mice. No significant difference was observed in the number of major histocompatibility complex (MHC) class II-bearing cells, T cells or natural killer (NK) cells between these two groups.

Cytokine production by mitogen-stimulated splenocytes or cells in peritoneal exudates

Cytokines such as IL-2, γ -IFN, IL-4 and IL-5 produced by mitogen-stimulated splenocytes or IL-6, TNF- α secreted by cells

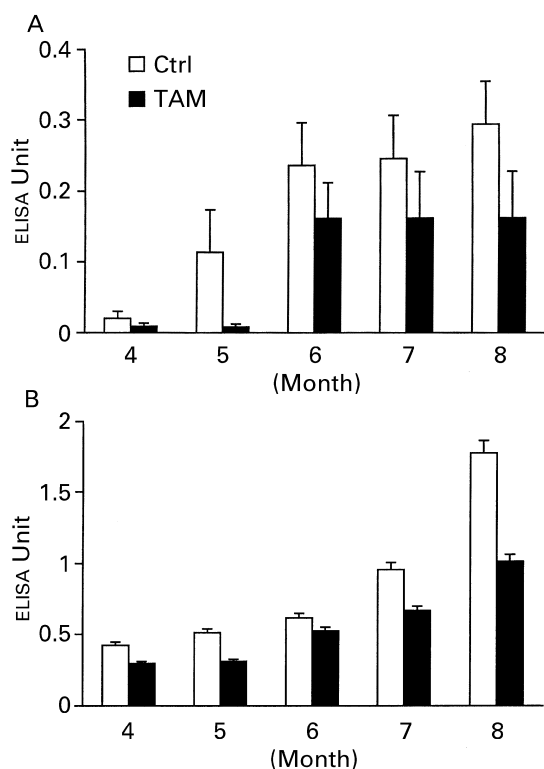


Fig. 2. The levels of serum IgG anti-ds DNA (A) and anti-ssDNA (B) autoantibodies in NZB/W F1 mice treated with or without TAM were quantitated by ELISA at different ages as described indicated in *Materials and Methods*. The mean and SEM values are shown for the two groups of mice. $n = 10$ mice of each group. Open bars represent the control group (Ctrl) and dark bars represent mice treated with TAM.

in peritoneal exudates of NZB/W F1 mice were determined and the results are summarized in Table 2. Analysis of the data revealed no significant difference in each cytokine production between these two groups. The ratio of γ -IFN/IL-4 tended to be higher in mice treated with TAM compared to that of the control group (6.7 ± 0.7 versus 5.1 ± 0.8 , $P = 0.1677$), although it was not statistically significant.

Serum levels of cytokine receptors are reduced by treatment with TAM

It is generally accepted that physiological modulators for TNF are present in a variety of body fluids including serum. Among these modulators are soluble TNF receptors (TNF-R) that are cleaved from the extra-cellular domain of the TNF-Rs. Two receptors of different structures are known to be expressed on monocytes, lymphocytes, granulocytes and other cells in the peripheral blood. The results show that TNF-sR55 and TNF-sR75 concentrations were significantly increased in the sera of control group mice (Fig. 3, $P < 0.005$). These results are consistent with previous findings in SLE showing elevated circulating levels of TNF-sR [18,19].

Table 1. Flow-cytometric analysis of immune cell populations in spleen cells and in cells from peritoneal exudates of NZB/W F1 female mice treated with TAM or control*

Cell types	Surface marker expression (%)	
	Control	TAM-treated
<i>Spleen cells</i>		
MHC II	47.8 ± 5.8	35.5 ± 12.0
T cells	31.8 ± 3.7	37.4 ± 3.8
B cells	51.1 ± 3.2	$38.1 \pm 2.4^\dagger$
CD4 ⁺ T cells	21.6 ± 1.8	22.4 ± 1.6
CD8 ⁺ T c cells	5.3 ± 0.9	7.1 ± 1.3
NK cells	2.9 ± 0.6	3.3 ± 0.3
CD5 ⁺ B cells	10.2 ± 0.6	$8.1 \pm 0.7^\dagger$
CD40 ⁺ B cells	20.9 ± 7.1	17.3 ± 3.3
<i>Peritoneal exudate cells</i>		
MHC II	87.6 ± 1.3	87.0 ± 1.9
T cells	2.5 ± 0.8	2.8 ± 1.2
B cells	90.3 ± 1.0	88.5 ± 1.2
CD4 ⁺ T cells	1.3 ± 0.2	1.3 ± 0.4
CD8 ⁺ T c cells	0.7 ± 0.4	0.5 ± 0.1
CD5 ⁺ B cells	72.0 ± 1.6	$61.9 \pm 2.8^\dagger$
CD40 ⁺ B cells	62.8 ± 3.5	$49.0 \pm 3.4^\dagger$

*Values shown are mean \pm SEM. Each group included six animals.

† The significance of difference among groups was analyzed statistically by Student *t*-test, differences were considered significant at $P < 0.05$.

Renal histology and glomerular antibody deposits in NZB/W F1 mice with or without TAM treatment

In agreement with the increased severity of proteinuria in sick mice, kidney histology as demonstrated by microscopic

Table 2. Summary of *in vivo* study of cytokine production in the spleen and cells from peritoneal exudates of NZB/W F1 female mice treated with TAM or control*

	Control	TAM-treated
<i>Spleen cells (pg/ml)†</i>		
IL-2	48.1 ± 21.2	64.1 ± 21.2
IFN- γ	863.4 ± 104.3	1392.2 ± 421.9
IL-4	186.5 ± 39.3	226.6 ± 84.4
IL-5	311.6 ± 86.4	545.2 ± 143.2
<i>Peritoneal exudates cells (ng/ml)‡</i>		
IL-6	4.2 ± 1.5	4.3 ± 1.8
TNF- α	1.3 ± 0.4	1.4 ± 0.5

*Values shown are mean \pm SEM. Each group included six animals.

† Splenocytes were stimulated in the presence of hamster antimice-CD3 antibody and antimice-CD28 antibody ($1 \mu\text{g/ml}$) cross-linked with antihamster IgG ($0.5 \mu\text{g/ml}$) antibody for 48 h. ‡ Peritoneal exudates cells were stimulated with LPS ($10 \mu\text{g/ml}$) for 2 days.

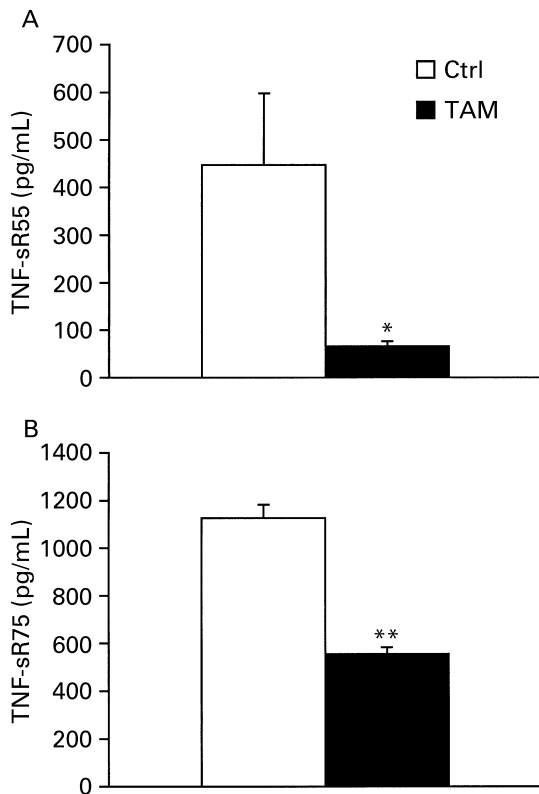


Fig. 3. Serum levels of soluble TNF receptors in NZB/W F1 mice treated with or without TAM. Sera collected at 7 months of age were evaluated for TNF-sR55 (A) and TNF-sR75 (B) using specific ELISA. Data are presented as mean SEM of soluble receptors in groups of six mice. TAM treatment lowered the sera levels of TNF-sR55 (*, $P = 0.044$) and TNF-sR75 (**, $P = 0.002$) in NZB/W F1 mice.

examination of hematoxylin-eosin stained sections from control mice (Fig. 4A) revealed accelerated endoproliferative and crescentic glomerulonephritis with enlarged glomeruli, and moderate to intense immune complex deposits. In contrast, mice treated with TAM (Fig. 4B) had intact glomeruli or only limited initial deposits along the capillaries. Glomerular injury was less severe in mice treated with TAM. Marked cellular proliferation, prominent fibrin and focal necrosis of glomeruli were noted in control mice. (Fig. 4A, HE stain. $\times 400$). In contrast, the TAM-treated group only showed mild mesangial expansion. Severe immune complex deposits were observed in the glomeruli of control mice but only mild deposits observed on the TAM-treated mice (Fig. 4C,D). Both glomerular and tubular CD40 and CD40 ligand expression were markedly expressed in 8-months-old NZB/W F1 mice of the control group (Fig. 4E,G). In contrast, TAM-treated kidney sections (Fig. 4F,H) had less CD40 and CD40 ligand expression, which was similar to that of young NZB/W F1 mice (data not shown). Our findings confirm the results of previous clinical study that proliferative lupus nephritis had markedly upregulated CD40 molecule expression [21,22]. The results of histochemistry staining demonstrated that many

CD40-positive cells and CD40-ligand-positive cells were infiltrated at the glomeruli of control group but much less in TAM-treated mice. It seems that the mechanism of action of TAM may occur through inhibition of lymphocyte infiltration or by changing the local cytokine secretion pattern.

DISCUSSION

In the present study, we demonstrated that treatment of lupus prone female NZB/W F1 mice with TAM at a dose of 800 $\mu\text{g/kg}$ every 2 weeks provided significant protection against nephritis, decreased the number of CD5⁺ B cells and prolonged the life span. The results are consistent with the findings of our previous study [16] in which we used another murine lupus model, MRL.*lpr/lpr*, and also found that TAM-treatment can alleviate disease severity. However, the beneficial effect of TAM seems to affect the different immune cell populations in these two strains as evidenced by the decreased double negative T-cell (CD4⁺CD8⁻, DN T cell) numbers in MRL.*lpr/lpr* mice but decreased CD5⁺ B cell numbers in NZB/W F1 mice.

Polyclonal B-cell activation and increased CD5⁺ B-cell number have been suggested to play an important role in the pathogenesis of autoimmunity in NZB/W F1 mice [23,24]. The most interesting finding of the present study is that the percentage of CD5⁺ B cells in TAM-treated NZB/W F1 mice was significantly lower than that of the control group. The clinical features of SLE are consistent with a prominent TH2 response [25]. Cytokines such as IL-5, which are produced by TH2 cells were closely related to the function and growth of auto-reactive B cells and responsible for autoantibody production [26–28]. It remains to be shown that oestrogen acts directly on B cells. It is plausible that because oestrogen affects the activity of CD5⁺ B cells [29] and enhances anti-dsDNA Ab expression in patients with SLE [30]. In contrast, TAM is a potent oestrogen antagonist, and therefore may play a biological role in immunoregulation. Further investigation of the underlying mechanisms of TAM, which contribute to the elimination of CD5⁺ B cells in NZB/W F1 mice, may suggest therapeutic strategies.

Recently, administration of the oestrogen antagonist TAM was studied by Stohoege *et al.* and Dayan *et al.* [31,32] in BALB/c male mice, prior to immunization with the 16/6 idiotype MoAb, and the beneficial effects of TAM were found to be associated with cytokine modulation. In contrast to the nonautoimmune BALB/c mice used in Dayan *et al.*'s [32] study, treatment of our lupus prone NZB/W F1 mice with TAM resulted in no change in serum autoantibody and cytokine production compared to the control mice. A previous study suggested that T-helper cells were dysregulated in autoimmune prone NZB/W F1 mice compared to nonautoimmune C57BL/6 mice [33]. This finding may explain why cytokine changes in the mice in this study was not as obvious as in the previous study.

Inflammation is a hallmark of disease progression in NZB/W F1 mice. The expression of both of the soluble TNF receptors was significantly lower in the serum of TAM-treated NZB/W F1 mice. TNF-soluble receptors (TNF-sR55 and TNF-sR75)

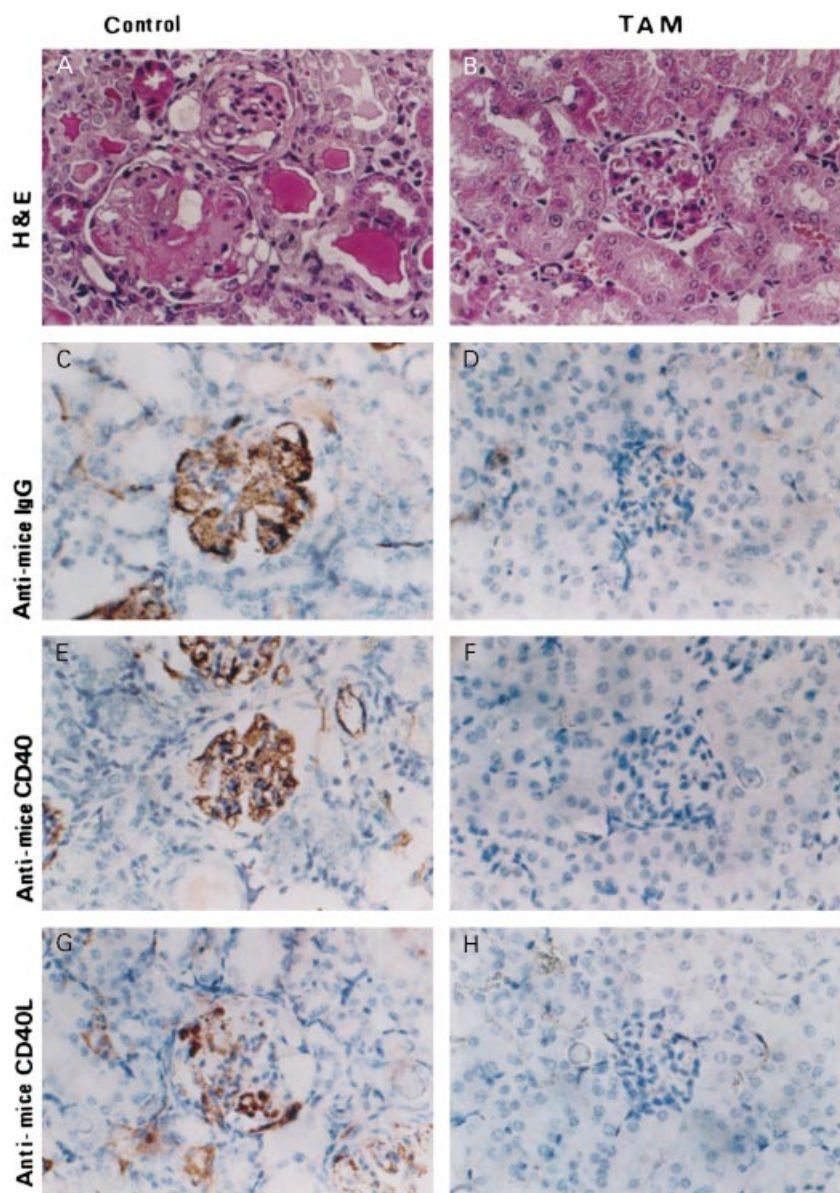


Fig. 4. Immunohistological examination of kidney sections from 8-month-old female NZB/W F1 mice treated with TAM and control. NZB/W F1 mice were s.c. injected 800 μ g/kg TAM or peanut oil, every two weeks. Five months after the start of treatment, mice were killed and their kidneys removed and analyzed for the presence of immune complex deposits as described in *Materials and Methods* (magnification $\times 400$). Hematoxylin -eosin staining of an untreated mouse (A) showed accelerated endoproliferative and crescentic glomerulonephritis with enlarged glomeruli, heavy deposits and fibrinoid necrosis; mice treated with TAM (B) had intact glomeruli or with initial deposits along the capillary walls and mild mesangial expression. However, control mice bearing immune complex deposit (C) had highly different histological characteristics from TAM-treated mice (D). IgG deposits were reduced in TAM-treated mice (D). Immunostaining for antimice CD40 or CD40 ligand antibodies was almost undetectable in TAM-treated mice (F,H), whereas the control mice (E,G) displayed an intense staining. In the control group, CD40 or CD40 ligand-positive cells were clearly present in the center of glomeruli.

represent stable and sensitive markers of an ongoing inflammatory reaction, and their levels are increased in the circulation of relapsing lupus patients [18,19] and in patients with rheumatic disease [34]. TAM may contribute to the lower circulating levels of TNF-sRs through inhibition of systemic inflammatory processes.

In this study, after 8 months of TAM treatment, the lymphoid cell infiltration and glomerular abnormalities were less severe than in the control mice. Furthermore, the TAM-treated mice showed much milder changes. Staining of the sections with anti-IgG antibodies, revealed severe immune complex deposition at the glomerulus of control mice but mild in TAM-treated mice. The control group mice of the glomeruli had marked cellular proliferation, prominent fibrin and focal necrosis. The TAM-treated group showed only mild mesangial expansion. Our present

study showed that glomerular CD40-CD40 ligand expression was markedly increased in the control group of NZB/W F1 mice that had a severe immune complex deposition in the kidney. In contrast, the TAM-treated mice had a downregulated expression of these surface molecules and less pathological change. The CD40 ligand is a membrane glycoprotein that is transiently expressed on the surface of activated CD4⁺-T cells [35]. Interaction of the CD40 ligand with its counterreceptor CD40 on macrophages is important for the effector function of macrophages and has been implicated in the pathogenesis of autoimmune glomerulonephritides [21,22,36]. Anti-CD40 ligand MoAb therapy markedly reduces the incidence and severity of glomerulonephritide in lupus-prone mice [37], even when administered after disease onset [38]. Some recent data suggested that a high expression of CD40 and CD40 ligand on cells of

inflammatory target organ was involved in certain immunological diseases [39,40]. Expression of CD40 molecule was noted on certain kidney cells such as parietal epithelial cells, mesangial cells, endothelial cells, and distal tubules [36]. It is of further interest that CD40–CD40 ligand interactions may play the key roles in the immunopathogenesis of murine lupus glomerulonephritide. Furthermore, Kuroiwa *et al.* [41] also suggested the possibility of the interaction of direct contact-dependent CD40–CD40 ligand mediated signals with infiltrated lymphocytes play a role in the immunopathogenesis in the kidney. Therefore, TAM may *alleviate* the cell-to-cell contact-mediated direct tissue injury in the kidney and may be a candidate for specific immunotherapy.

Our finding that treatment of NZB/W F1 mice with TAM delayed disease onset suggests that the administration of TAM might be beneficial for lupus patients suffering from nephritis. Therefore, it is even more urgent that the effects and molecular mechanisms of TAM on autoimmune disease severity be investigated to elucidate the possible therapeutic roles of TAM.

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