

EXTENDED REPORT

Beneficial effects of the anti-oestrogen tamoxifen on systemic lupus erythematosus of (NZB×NZW)F1 female mice are associated with specific reduction of IgG3 autoantibodies

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Background: Sex hormones have been shown to influence the immune system and to modify the course of autoimmune disorders.

Objective: To examine the effects of the oestrogen antagonist tamoxifen on the course of systemic lupus erythematosus (SLE) in (NZB×NZW)F1 mice.

Methods: Groups of 8 week old (NZB×NZW)F1 female mice were treated with tamoxifen (800 µg/mouse; twice a week) or with double distilled water for four months. Mice were evaluated monthly for the presence of autoantibodies directed against DNA and nuclear extract (NE) by enzyme linked immunosorbent assay (ELISA). White blood cells and thrombocytes were quantified by a cell counter and proteinuria by combistix kit. At 6 months of age, all mice that did not die spontaneously were killed and evaluated for the presence of glomerular immune deposits by indirect immunofluorescence assay. IgG isotypes of autoantibodies in the mouse sera and glomeruli were determined by γ chain specific antibodies.

Results: Tamoxifen treatment significantly reduced autoantibody production directed against either NE or DNA. The latter reduction was mainly in autoantibodies of the IgG3 isotype. Furthermore, tamoxifen had significant beneficial effects on the course of SLE in (NZB×NZW)F1 mice. At 6 months of age, 40% of the untreated mice died spontaneously, whereas all the tamoxifen treated mice were still alive. All untreated mice showed severe thrombocytopenia and persistent proteinuria, with diffuse glomerular immune deposits of IgG2a and IgG3 isotypes in their kidneys. In contrast, the tamoxifen treated mice had a normal number of thrombocytes and only minimal proteinuria. Moreover, glomerular immune deposits were detected in <40% of the tamoxifen treated mice. The latter were mainly of the IgG2a but not of the IgG3 isotype.

Conclusion: The results clearly show the remarkable therapeutic effects of tamoxifen on SLE of (NZB×NZW)F1 female mice and suggest that these beneficial effects are related to the specific reduction of IgG3 autoantibodies.

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The immune system has been shown to be influenced by sex hormones.^{1–5} Moreover, certain immune disorders appear to be modified by sex steroids. Thus, in humans and in several animal models, female subjects have a higher incidence of certain autoimmune diseases such as systemic lupus erythematosus (SLE).^{6–10} Numerous studies have shown that oestrogens accelerate and androgens ameliorate the course of spontaneous SLE-like disease in (NZB×NZW)F1 and in MRL/lpr mice.^{11–12} In addition, it has been reported that the anti-oestrogen nafoxidine can ameliorate the lupus-like disease in (NZB×NZW)F1 mice.¹³ The mechanism(s) involved in disease immunomodulation by sex hormones are not elucidated yet. Comprehensive studies of androgen treatment of patients with SLE are lacking. The results of small and uncontrolled studies are inconclusive.^{14–15} Nevertheless, the potential role for androgens in the treatment of human SLE in young female subjects is limited owing to the adverse effects of these hormones.¹³ The use of dehydroepiandrosterone, an androgen without major adverse effects, in small numbers of female lupus patients resulted in clinical improvement.¹⁶ Thus, hormonal modulation of human SLE with oestrogen antagonists may be more beneficial.

We have demonstrated the induction of experimental SLE in naive mouse strains that are not prone to develop SLE spontaneously, by immunisation with a human anti-DNA monoclonal antibody which bears the common idiotype (Id)

designated 16/6 Id.^{17–18} We have shown that treatment of BALB/c female mice, afflicted with 16/6 Id induced experimental SLE, with the anti-oestrogen tamoxifen (a synthetic compound which binds specifically to the oestrogen receptor) led to a significant improvement of all SLE associated clinical manifestations (leucopenia, thrombocytopenia, and proteinuria).¹⁹ Moreover, the treatment with tamoxifen prevented the glomerular immune complex deposition which was seen in the untreated mice with experimental lupus.^{19–20} We have also shown that treatment with an anti-estradiol (anti-E2) monoclonal antibody led to similar beneficial effects.²⁰

This study aimed at investigating the effects of the oestrogen antagonist tamoxifen (a drug which is widely used in humans without significant adverse reactions²¹) on the development and the course of spontaneous SLE in (NZB×NZW)F1 female mice.

Abbreviations: DDW, double distilled water; ds, double stranded; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; Id, idiotype; IL, interleukin; NE, nuclear extract; SLE, systemic lupus erythematosus; ss, single stranded; TNF, tumour necrosis factor; WBC, white blood cells

MATERIALS AND METHODS

Mice

(NZB×NZW)F1 female mice were obtained from the Jackson Laboratory, Harbor, ME. All mice were used at the age of 8 weeks.

Tamoxifen treatment

The anti-oestrogen tamoxifen (Sigma Chemical Co, St Louis, MO) was diluted in double distilled water (DDW) and kept in dark glass tubes at 4°C. Tamoxifen was given as described previously^{19,20} at a dose of 800 µg in each injection/mouse twice a week, subcutaneously into the neck, in a volume of 100 µl. Tamoxifen treatment was started when the mice were 8 weeks old and was continued for four months to the age of 6 months. At that time, all mice which did not die spontaneously were killed and evaluated as below. The control (untreated) mice were injected concomitantly with 100 µl of DDW.

Assessment of SLE related disease manifestations

All mice were bled before the start of the treatment and thereafter at monthly intervals, until the end of the study. White blood cells (WBC) and thrombocytes were quantified by a cell counter (Ortho Diagnostic System, Westwood, MA,) as described previously.^{19,22} Proteinuria was measured, monthly, by a semiquantitative method using a combistix kit (Ames-Miles Inc, Stock Pages, Slough, UK).^{17–20}

Immunohistology of kidney sections

Kidneys obtained from tamoxifen treated and untreated mice were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections (5 µm) were air dried for at least two hours and fixed in acetone for 10 minutes. Immunoglobulin (IgG) deposits were detected with fluorescein isothiocyanate (FITC) labelled goat antimouse IgG (Jackson Immuno Research Laboratory, West Grove, PA), as described previously.^{17,18} All kidney sections were further evaluated for the specific IgG isotype deposition using FITC labelled goat directed against mouse IgG2a and IgG3 (γ_{2a} and γ_3 chain specific, respectively) antibodies.^{23,24} Immunofluorescent intensity, representing the amounts of the immune deposits (total IgG or IgG isotypes), was graded as negative (0), weakly positive (+), positive (++), and strongly positive (+++), as previously described.²⁴

Serological evaluation

The levels of autoantibodies directed against double stranded (ds) and single stranded (ss) DNA and nuclear extract (NE) were determined in duplicate in the mouse sera by an enzyme linked immunosorbent assay (ELISA). NE of HeLa cells and calf thymus DNA (Sigma Chemical Co) were used as antigens, as previously described.^{17–20} Briefly, HeLa NE was used for coating ELISA plates at a concentration of 5 µg/ml. For DNA assays, 96 well Maxisorb microtitre plates (Nunc) were coated with poly-L-lysine (Sigma). The plates were then washed and coated with either 10 µg/ml of denatured calf thymus DNA (Sigma) for ssDNA assay or λ -phage dsDNA (Boehringer Mannheim, 5 µg/ml) for dsDNA assay. After incubation with different dilutions of sera, goat antimouse IgG (γ chain specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch) was added to the plates, followed by the addition of the substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma). Results were read with an ELISA reader at 414 nm.²⁵ For the determination of the IgG isotypes of the specific autoantibodies, horseradish peroxidase labelled goat antimouse IgG1, IgG2a/2b and IgG3 (γ_1 , γ_{2a} , γ_{2b} and γ_3 chain specific, respectively) antibodies (Southern Biotechnology Associates, Inc, Birmingham, AL) were used. Results of assays to determine ssDNA and dsDNA were found to be similar. All DNA reactivity presented in our studies represents dsDNA reactivity.

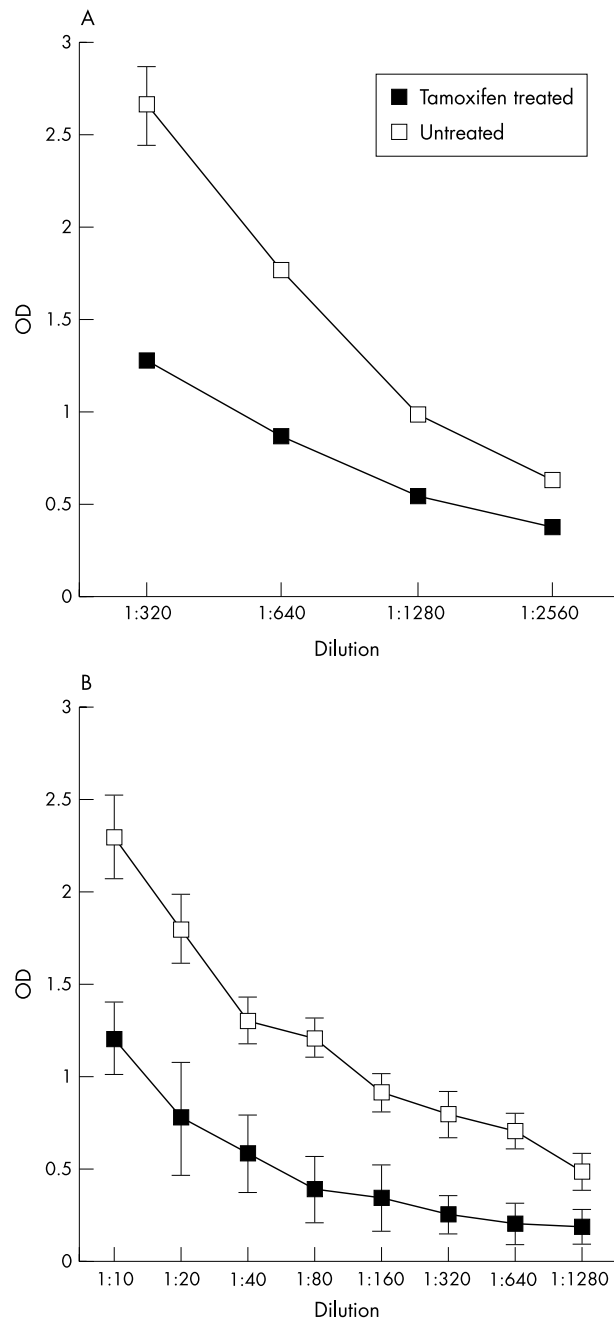


Figure 1 Autoantibodies of 6 month old (NZB×NZW)F1 female mice. Tamoxifen treated and untreated mice were bled, at 6 months of age, and the reactivity of their sera (total IgG) with dsDNA (A) and NE (B) was determined. Results represent mean (SEM) OD values (at 414 nm) of each individual mouse obtained from one representative of three consecutive experiments. Note the differences in sera dilution between the two assays.

RESULTS

Effect of tamoxifen treatment on autoantibody production

(NZB×NZW)F1 female mice, aged 8 weeks, were divided into several groups (5–13 mice in each group) of tamoxifen treated and untreated mice. In the treated groups, tamoxifen in DDW (800 µg/mouse subcutaneously into the neck, twice a week) was given for a period of four months, until the mice were 6 months old. The control, untreated mice, were injected with DDW alone. All mice were bled for serological evaluations before the start of the tamoxifen treatment and thereafter monthly until the end of the study. At that time, all mice

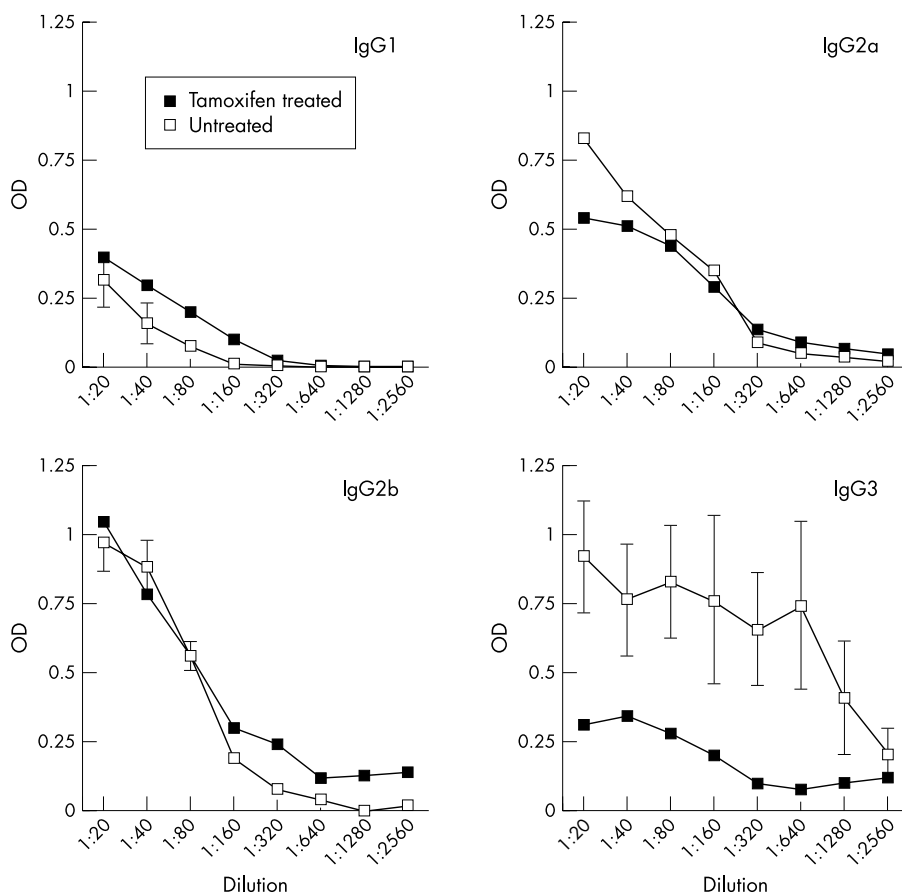


Figure 2 IgG isotypes of anti-dsDNA autoantibodies of 6 month old (NZB×NZW)F1 female mice. Tamoxifen treated and untreated mice were bled at 6 months of age and the isotypes of the anti-dsDNA autoantibodies in the mouse sera (mean (SEM) of each individual mouse) were determined by using γ_1 , γ_2a , γ_2b , and γ_3 chain specific goat antimouse antibodies. Results of one representative out of three consecutive experiments are presented.

which did not die spontaneously were killed and evaluated. Figure 1 shows that the untreated (NZB×NZW)F1 female mice produced significant amounts of anti-DNA autoantibodies and lower, though significant, levels of anti-NE autoantibodies. Tamoxifen treatment significantly decreased the levels of both anti-DNA (fig 1A) and anti-NE (fig 1B) autoantibodies. To investigate the effect of tamoxifen on the humoral response further, the IgG isotypes of the anti-DNA autoantibodies in the tamoxifen treated and untreated (NZB×NZW)F1 female mice were determined. Figure 2 shows that the main anti-DNA IgG isotype detected in the sera of the untreated (NZB×NZW)F1 female mice at 6 months of age was IgG3. The levels of the other IgG anti-DNA isotypes (IgG2a, IgG2b, and especially IgG1) were much lower. The tamoxifen induced reduction of anti-DNA autoantibody levels (fig 1) was mainly due to a specific reduction of the levels of IgG3 anti-DNA antibodies. Tamoxifen had no significant effect on the levels of the IgG1, IgG2a, or IgG2b anti-DNA autoantibodies (fig 2). Thus, the tamoxifen effect on the reduction of autoantibody production is due to its specific effect on the production of the IgG3 autoantibodies.

We further studied the kinetics of the autoantibody response in the tamoxifen treated and untreated (NZB×NZW)F1 female mice. Mice that were not treated with tamoxifen had significant amounts of anti-DNA autoantibodies at the age of 4 months (mean (SEM) OD 1.0 (0.02) units at 414 nm). The levels of the anti-DNA autoantibodies increased further with age, peaking at 5–6 months to about three times the levels found at 4 months. Before the start of the treatment, at the age of 2 months, all mice (tamoxifen treated and untreated) showed low though significant levels of anti-DNA

autoantibodies. Two months of tamoxifen treatment had no apparent effect on the levels of the anti-DNA autoantibodies because both mouse groups (treated and untreated) showed the same high levels of anti-DNA autoantibodies until 4 months of age. However, in contrast with the sharp increase in the levels of the anti-DNA autoantibodies seen in the untreated (NZB×NZW)F1 females at 5 and 6 months of age, the levels of the autoantibodies in the tamoxifen treated mice did not rise significantly at that time. The kinetics of the tamoxifen effect on anti-DNA levels completely correlated with the kinetics of the IgG3 anti-DNA autoantibody production in (NZB×NZW)F1 mice. IgG3 anti-DNA autoantibody titres increased significantly only at the age of 5 months (0.1 (0.02), 0.8 (0.04), 1.65 (0.06) mean (SEM) OD units for IgG3 dsDNA reactivity at 4, 5, and 6 months, respectively).

Effect of tamoxifen on mortality and clinical manifestations

The short term survival (up to 6 months) of tamoxifen treated (28 mice) and untreated (15 mice) (NZB×NZW)F1 female mice demonstrated significant effects of tamoxifen treatment. Thus, 40% of the untreated mice died spontaneously by the age of 6 months, whereas, all the tamoxifen treated mice were still alive at that time.

Table 1 shows that at the age of 6 months all untreated mice had significant thrombocytopenia and proteinuria, whereas the tamoxifen treated mice had a normal number of thrombocytes and only mild (near normal) proteinuria, similar to the control young (2 months old) (NZB×NZW)F1 female mice. The WBC counts were similar in all mouse groups ($6.2 (0.9) \times 10^9$, $6.6 (1.4) \times 10^9$, and $6.5 (1.4) \times 10^9$; WBC/l for young 2

Table 1 Clinical manifestations of tamoxifen treated and untreated (NZB×NZW)F1 female mice

Group	Thrombocytes (10 ⁹ /l) (SEM)	Proteinuria (mg/l) (SEM)	Glomerular Immune complex deposits (%)*
1. Tamoxifen	†815 (153)	†30 (29)	‡5/13 (38)
2. No tamoxifen	383 (65)	220 (109)	‡5/5 (100)
3. Young mice	794 (85)	<30	0/2 (0)

Thrombocytes, proteinuria, and glomerular immune complex deposits were determined at the age of 6 months (groups 1 and 2). At that time, the tamoxifen treated mice (group 1) had received tamoxifen for a period of four months. Young (2 months old) (NZB×NZW)F1 female mice (group 3) served as a control group. Results shown here were obtained from one representative experiment out of three consecutive experiments.

*Number of mice (%) with glomerular immune deposits (total IgG).

† $p < 0.0002$ between groups 1 or 3 and group 2. Not significant between groups 1 and 3.

‡In the tamoxifen treated mice (group 1), eight mice (62%) had no glomerular immune deposits (score 0), three mice (23%) were defined as weakly positive (+) and only two mice (15%) as positive (+/++). In contrast, in the untreated mice (group 2) one mouse had glomerular immune complex deposits defined as positive (++) and four mice (80%) as strongly positive (+++).

month old mice, tamoxifen treated, and untreated 6 month old (NZB×NZW)F1 mice, respectively; p not significant between all three groups).

Diffuse glomerular immune deposits (total IgG) were detected in kidney sections obtained from all untreated (NZB×NZW)F1 mice at the age of 6 months. In contrast, no such deposits were found in the kidneys of 62% of age matched tamoxifen treated mice (table 1). We further studied the IgG isotypes involved in the glomerular immune

depositions. Figure 3 shows that the kidney sections obtained from untreated (NZB×NZW)F1 female mice at the age of 6 months had diffuse glomerular immune deposits of both IgG2a and IgG3 isotypes (fig 3; 2B and 2C, respectively). On the other hand, most (>60%) kidney sections obtained from age matched tamoxifen treated (NZB×NZW)F1 mice did not show any glomerular immune deposits (fig 3; 4A, 4B, 4C) similar to the kidneys obtained from 2 month old (NZB×NZW)F1 mice (fig 3; 1A, 1B, 1C). The tamoxifen treated mice (<40%) that had glomerular immune deposits showed only mild IgG depositions, defined as weakly positive (+) or positive (+/++; fig 3; 3A) as compared with the strongly positive (+++) IgG deposits seen in the untreated mice (fig 3; 2A). Furthermore, the immune deposits in kidney sections of tamoxifen treated mice were mainly of the IgG2a (fig 3; 3B) with fewer deposits of IgG3 isotypes (fig 3; 3C). Thus, as was shown for the reduction in the levels of the pathogenic IgG3 anti-DNA autoantibodies in the mouse sera (fig 2), tamoxifen treatment concomitantly decreased the glomerular IgG3 depositions.

DISCUSSION

Our studies clearly demonstrate significant therapeutic effects of the oestrogen antagonist tamoxifen on the course of SLE in (NZB×NZW)F1 female mice. Tamoxifen treatment led to remarkable improvement of all lupus related clinical manifestations, including survival, thrombocytopenia, proteinuria, and glomerular immune depositions. These beneficial effects correlate with specific decrease in titres of the pathogenic IgG3 anti-DNA autoantibodies in the sera of the tamoxifen treated mice and with the reduction of IgG3 glomerular immune deposition.

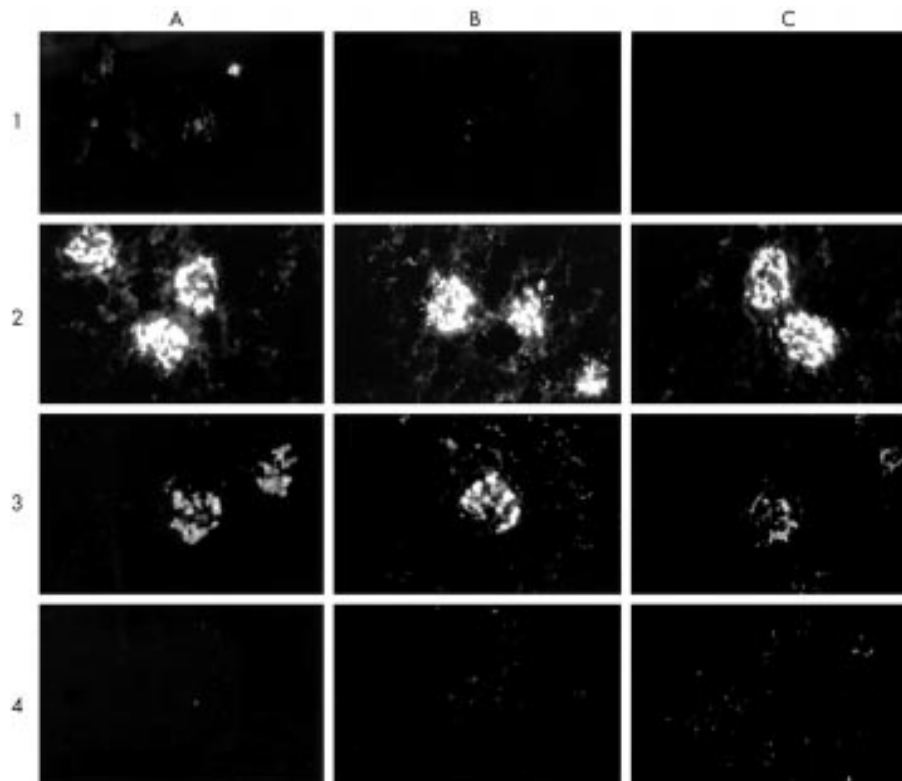


Figure 3 Immunohistology of kidney sections obtained from (NZB×NZW)F1 female mice. Kidney sections were obtained from 2 month old control mice (1A, 1B, 1C), 6 month old untreated mice (2A, 2B, 2C) and 6 month old tamoxifen treated (3A, 3B, 3C, 4A, 4B, 4C) (NZB×NZW)F1 mice. Mice were killed and their kidney sections (5 μ m) were stained with FITC labelled goat antimouse IgG (total IgG; panel A), IgG2a (panel B), or IgG3 (panel C) using γ , γ_{2a} , or γ_3 heavy chain specific antibodies, respectively. Representative figures ($\times 200$) are presented. Sections 1 and 4 were defined as negative (score 0), section 2 as strongly positive (+++) and section 3 as positive (+/++). Note the relatively low intensity of IgG3 deposits in kidney sections obtained from tamoxifen treated mice (3C).

Tamoxifen is a synthetic non-steroidal oestrogen antagonist which binds specifically to oestrogen receptor in the target organs.²⁶ It is widely used to treat patients with breast cancer. Long term treatment of patients with tamoxifen has not been associated with severe adverse effects.²¹ We used 800 µg of tamoxifen per mouse (twice a week) as the treatment protocol because such protocols have been shown to demonstrate anti-oestrogenic therapeutic effects in mouse mammary tumour models²⁷ and in murine experimental SLE models.^{19, 20}

Treatment of (NZB×NZW)F1 female mice for four months with tamoxifen led to significant therapeutic effects. Tamoxifen improved the short term (6 months) survival as well as all other lupus related clinical manifestations. The 6 month old tamoxifen treated mice had a normal thrombocyte count with minimal (near normal) proteinuria compared with the significant thrombocytopenia and massive proteinuria seen in all age matched, untreated mice (table 1). Furthermore, tamoxifen treatment completely prevented the glomerular immune deposition in 62% of the treated mice and significantly decreased the amounts of the immune depositions, especially IgG3, in the rest (38%) of the tamoxifen treated mice (table 1, fig 3).

Our observations correlate with previous reports which demonstrated amelioration by testosterone and exacerbations by oestrogen of the SLE in MRL/lpr and (NZB×NZW)F1 mouse strains.^{28–30} Recently, Kanda *et al* reported that oestrogen enhanced, in vitro, the production of dsDNA antibodies by peripheral blood lymphocytes obtained from patients with active SLE.³¹ In the study of Duvic *et al* only a modest beneficial effect of the anti-oestrogen nafoxidine on the course of SLE in (NZB×NZW)F1 mice was seen.¹⁵ The use of nafoxidine, which has a weak oestrogenic effect in addition to its anti-oestrogenic activity, may explain the difference between the two studies. The clinical beneficial effects of tamoxifen on SLE of (NZB×NZW)F1 mice (table 1, fig 3) are similar to our previous observations in BALB/c mice that had 16/6 Id induced experimental SLE.^{19, 20} More recently, Wu *et al* also reported that tamoxifen treatment (800 µg per mouse every two weeks) decreased proteinuria and glomerular immune deposits and increased survival of (NZB×NZW)F1 mice.³²

The mechanism(s) by which sex hormones (including anti-oestrogen agents such as tamoxifen) modulate the immune system and the course of SLE are not yet defined. Our previous studies of BALB/c female mice with 16/6 Id induced experimental SLE suggested that cytokine modulation is the basis for the tamoxifen (and anti-oestrogen antibodies) beneficial effects.^{19, 20} In those studies, tamoxifen treatment had no significant effect on the total IgG autoantibody levels (for example, anti DNA, NE antibodies) in the mouse sera¹⁹; however, a decrease in the IgG2a autoantibodies was seen in the tamoxifen treated mice.¹⁹ Wu *et al* did not find significant differences in the levels of anti-DNA autoantibodies between tamoxifen treated and untreated (NZB×NZW)F1 mice. However, they demonstrated a significant decrease in the levels of soluble tumour necrosis factor (TNF) receptors (TNFR55, TNFR75) in the sera of the tamoxifen treated mice.³² In our study we clearly demonstrate humoral suppression as a possible mechanism for the tamoxifen therapeutic effects, although other mechanisms, such as changes in cytokines, chemokines, or T cell subsets, cannot be excluded. The tamoxifen treated (NZB×NZW)F1 female mice had significantly lower levels of anti-DNA and anti-NE IgG autoantibodies than the age matched untreated mice (fig 1). Similar favourable effects on the humoral response of (NZB×NZW)F1 mice were found by Duvic *et al* using the oestrogen antagonist nafoxidine.¹⁵

Our previous studies in a different model of murine SLE (the 16/6 Id induced experimental lupus in BALB/c mice) demonstrated correlation between the beneficial effects of tamoxifen treatment and the reduction of IgG2a autoantibodies.¹⁹ Dang and Horbeck reported that IgG1 and IgG2a anti-DNA autoantibodies are the major IgG subclass

deposits in the glomeruli of untreated (NZB x NZW)F1 female mice.³³ Thus various IgG subclass autoantibodies have a role in the pathogenesis of murine lupus. Nevertheless, in the present studies of (NZB x NZW)F1 mice the beneficial effects of tamoxifen treatment correlated more with the reduction of IgG3 autoantibodies in the mouse sera (fig 2) and in the glomerular immune deposits (fig 3). This may explain the three months delay in the tamoxifen effect on the autoantibody levels. The tamoxifen suppressive effect on the humoral response was initially seen at 5 months of age when significant levels of IgG3 anti-DNA autoantibodies could be detected in the untreated mice. The pathogenic role of IgG3 in murine SLE was previously demonstrated in MRL/lpr and in (NZB×NZW)F1 mouse strains.^{34, 35} IgG3 anti-DNA monoclonal antibodies derived from MRL/lpr³⁶ and (NZB×NZW)F1³⁷ mice were shown to induce “wire-loop” lesions, characteristically described in human lupus nephritis. Moreover, the beneficial effect of early thymic irradiation of (NZB×NZW)F1 mice correlated with specific reduction of IgG3 autoantibodies.³⁸ The fact that the xid mutation, which causes a defect in IgG3 production, markedly reduced the development of lupus nephritis in lupus prone mice³⁹ also supports the pathogenic role of IgG3 autoantibodies. In humans, IgG3 has been found in glomerular immune deposits^{24, 25} of patients with lupus nephritis, further suggesting the pathogenic role of the latter.

Santiago *et al* were able to show that interleukin 4 (IL4) prevents the development of lupus-like disease in the (NZW×C57BL/6 Yaa) murine model of SLE.⁴⁰ The IL4 transgene introduced into the lupus prone mice prevented the development of lupus associated glomerulonephritis. Similar to the tamoxifen effects in our studies, the beneficial effects of IL4 correlated with the reduction of IgG3 autoantibodies.⁴⁰ The specific reduction of IgG3 autoantibodies may reflect down regulation of the Th1 immune response, which has been shown to accelerate murine lupus.^{41, 42}

To conclude, our studies clearly demonstrate significant therapeutic effects of short term tamoxifen treatment of (NZB×NZW)F1 female mice. These beneficial effects correlate with the specific reduction of IgG3 autoantibodies. Thus, tamoxifen might have a novel therapeutic role in patients with SLE.

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