Influence of Natural and Synthetic Estrogens on the Course of Autoimmune Disease in the NZB/NZW Mouse

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Antinuclear antibodies were found in 64% of serum samples obtained from male NZB/NZW mice after 6 weeks of treatment with mestranol. Both female and male mice responded to this synthetic female hormone with marked elevations of serum α_2- and β-globulins; γ-globulins were not increased. There was no evidence that mestranol accelerated the development of autoimmune renal disease in these animals. Treatment of hybrid New Zealand mice with mestranol, 17-β-estradiol and oophorectomy exerts divergent effects on serum proteins and the development of serologic abnormalities.

Systemic lupus erythematosus (SLE) is known to have a striking predilection for women (1). This observation has stimulated repeated attempts to define the relationship between female hormones and autoimmune disease. We have previously reported the results of treating hybrid New Zealand Black/New Zealand White (NZB/NZW) mice (animal models of SLE) with 17-β-estradiol (2). Daily administration of this naturally-occurring female hormone influenced serum globulin concentrations but had no effect on the early course of disease in NZB/NZW mice. Nevertheless, recent evidence suggests that exogenous administration of compounds containing synthetic estrogens may influence autoimmune phenomena in women. Systemic lupus erythematosus may exacerbate during treatment with oral contraceptives (3). Kay, Bole and Ledger (4) reported the appearance of antinuclear antibodies (ANA) or rheumatoid factor in the serum of women taking synthetic estrogen-progestogen compounds. This report describes studies undertaken to evaluate the influence of a synthetic estrogen, mestranol, on the evolution of autoimmune disease in NZB/NZW mice.

MATERIALS AND METHODS

Animals. Breeding pairs of NZB and NZW mice were initially supplied by Dr. William D. Hall, The University of Otago, Dunedin, New Zealand. Each strain is maintained by brother-sister matings.

Mestranol and Estradiol Studies. Mestranol (the 3-methyl ether of ethinyl estradiol)* was obtained through the courtesy of G.S. DuBoff, DSC; 17-β-estradiol was purchased from Mann Research Laboratories. †

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Littermate female and male mice were assigned at random to treatment or control groups. In the mestranol study, 29 NZB/NZW mice (18 females and 11 males) received daily subcutaneous injections of the steroid in a dose of 2.0 μg/kg body weight. This dose is equivalent, on a weight basis, to the amount of mestranol ingested daily by a 50-kg woman taking an oral contraceptive containing 100 μg of the estrogen per tablet. In the estradiol study, 31 NZB/NZW mice (23 females and 8 males) received daily injections of 17-β-estradiol, 2.5 μg/kg body weight, as previously described (2). Twenty-seven control mice were injected daily with equivalent volumes of proplyeneglycol vehicle. Injections were started when all mice were 4 to 6 weeks old. Animals were bled from the orbital plexus before injections started and at 4, 6, 8 and 12 weeks of treatment.* After 8 weeks, subgroups of control and treated mice were bled at 4-week intervals. At 12 weeks of treatment, sera were available, as noted in Table 3.

Oophorectomy Study. Ovaries were removed from 2 groups of NZB/NZW females: a) Immature: 12 mice, aged 4 to 6 weeks, were bled before and 6 weeks after surgery. Renal tissue was obtained from 1 female that died unexpectedly at 29 weeks of age and from 5 females that were sacrificed at 31 to 33 weeks of age. The evolution of autoimmune disease in these young animals was compared to the course of disease in immature control, mestranol- and estradiol-treated mice. b) Mature: 12 mice, aged 8 to 12 weeks. Nine control females 8 to 15 weeks of age had sham oophorectomies. To assess the long-term effects of gonad removal in these older NZB/NZW females, they were bled 6 and 12 weeks after surgery. Kidneys were examined from 3 mature oophorectomized females that died of renal insufficiency at 32 to 33 weeks of age and from 5 females that were sacrificed at the age of 43 to 46 weeks. Renal tissue was obtained from 4 sham-operated control females. One control died at the age of 28 weeks, and 3 controls were killed at 41 to 48 weeks of age. Tissue was not obtained from the other 5 animals in this group due to unanticipated deaths and autolysis.

Antinuclear Antibodies. Serum was stored in capillary tubes without preservatives at −20° C. Throughout this study, positive control sera from 12-month-old NZB/NZW females with positive lupus erythematosus cell preparations and negative control sera from immature Swiss Webster mice were used. When human leukocyte substrate was used, great care was exercised to prevent mechanical damage and drying of the cells, which may distort nuclei and interfere with the interpretation of positive tests (6). All glassware, except microslides, was siliconized. Heparinized blood was obtained from a single donor. Erythrocytes were sedimented with 5% glucose and 6% dextran in 0.15 M NaCl (7). After centrifugation, drops of the leukocyte-rich plasma supernate were transferred gently to gelatin-coated slides. The slides were placed in a moist chamber for 30 minutes to allow the white blood cells to settle. These cells were washed gently with phosphate-buffered saline (pH 7.0) and fixed in 95% ethanol for 30 minutes. Sera obtained from mestranol-treated mice after 4, 6 and 8 weeks of treatment were also tested on human thyroid tissue obtained at necropsy from a 5-day-old infant. Sections of thyroid tissue, 4 μ thick, were prepared on a cryostat and fixed in acetone. Samples of mouse serum were applied to white blood cell or thyroid substrate, and testing for ANA was performed using the indirect immunofluorescent procedure described by Friou (8). Fluorescein-conjugated goat antimouse 7S γ-globulin* diluted 1:20 was employed. Slides were examined without knowledge of the treatment status of mice.

Mestranol, dissolved in propyleneglycol, was diluted with ANA-negative serum from Swiss Webster mice, in concentrations ranging from 5.0×10^{-5} to 2.5×10^{-10} mg/ml. The mestranol-serum mixtures and propyleneglycol were applied separately to both leukocyte and thyroid substrates, and routine tests for ANA were performed.

Serum Protein Determinations. Electrophoresis of mouse serum on cellulose acetate and measurement of total serum proteins were preformed as described elsewhere (2). Measurement of total γ-globulins was compared with the determination of IgG levels in some animals by radial immunodiffusion (2).

Histology. Renal and liver tissue fixed in Carnoy's solution was embedded in paraffin and 4-μ sections were prepared. The tissue sections were stained with hematoxylin and eosin and examined by light microscopy. Pirani,

*In the thirteenth week of mestranol therapy, treated females and males began to die. At autopsy, bladder distention with hydronephrosis and hydroureters was found. Rapid autolysis after death prevented adequate microscopic examination of tissues removed at autopsy in most of these animals. The surviving 5 females and 3 males in the group treated with mestranol were bled and killed in 2 groups at 22 and 26 weeks of treatment, and renal and hepatic tissue was obtained for histologic study. After 12 weeks of treatment with estradiol, male mice began to die. These animals also had evidence of lower urinary tract obstruction. A high incidence of lower urinary tract obstruction and sudden death in mice injected with estradiol has been noted by other investigators (5). Renal tissue was not recovered from these mice. Kidneys and liver from 5 estradiol females which were killed after 16 to 20 weeks of treatment were available for examination.

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Table 1. Antinuclear Antibodies (Leukocyte Substrate) in Control and Treated NZB/NZW Mice

Mice	Treatment (wk) No. positive tests/total mice tested		
	0	6	12
Controls			
Females	4/18	3/14	2/9
Males	0/9	1/9	1/5
Mestranol			
Females	2/18	9/18	3/9
Males	3/11	7/11	6/10
17-β-estradiol			
Females	5/22	4/23	0/4
Males	0/8	0/8	0/6
Oophorectomy			
Immature females	3/12	3/10	ND
Sham-operated controls	ND	0/9	7/9
Mature females	ND	0/12	9/12

Pollak and Schwartz (9) described a reproducible semiquantitative method of analyzing abnormal renal morphology which can be used to assess histologic changes in the kidneys of NZB/NZW mice (10). A modification of this method was used to examine kidneys from mice in this study. An examiner who was not aware of the treatment status of the mice counted the number of histologic abnormalities in 20 glomeruli in a section from each kidney studied. Lesions counted were: a) thickening or hypercellularity of the mesangial stalk, b) focal glomerular hypercellularity, c) thickening of the basement membrane, d) diffuse glomerular hypercellularity, e) fibrinoid change, f) crescent formation and g) sclerotic glomeruli. For comparison, a number of kidneys from untreated female NZB/NZW mice, in our colony, have also been graded using this system. As untreated mice increase in age, there is essentially a linear increase in numbers of renal abnormalities.

Statistical Analysis. Data for the ANA were analyzed employing a χ^2 formula (11). Serum levels of α_2-, β- and γ-globulins were analyzed statistically using Cochran's approximation of the Behrens-Fisher test described by Snedecor (12). A probability value of < 0.05 was considered significant.

RESULTS

Antinuclear Antibodies

In Table 1, positive tests detected on leukocyte substrate in control mice are compared with results in NZB/NZW mice treated with mestranol, 17-β-estradiol and oophorectomy. The development of ANA was accelerated in young NZB/NZW males treated with mestranol. After 6 weeks of treatment, the appearance of ANA in 64% of these males compared to ANA in 11% of male controls was found to be significant by χ^2 analysis ($P < 0.025$). The apparent increase in numbers of positive tests in mestranol-treated females at 6 weeks and in males and females at 12 weeks compared to control mice was not significant.

The occurrence of serologic abnormalities in NZB/NZW mice before and after 6 weeks of treatment with estradiol has been described in detail elsewhere (2). Antinuclear antibodies in estradiol mice are listed in Table 1 to permit comparison between mestranol and estradiol groups after 6 and 12 weeks of treatment. Positive tests for ANA in mestranol females were significantly increased at 6 weeks when these mice are compared to estradiol females ($P < 0.050$). Comparison of positive tests in treated males shows that a significant number of mestranol males, compared to estradiol males, were positive for ANA at 6 weeks

Table 2. Patterns of Nuclear Fluorescence (Leukocyte Substrate) in NZB/NZW Mice Treated with Mestranol

Mice	No. of sera tested	No. of positive tests	Peripheral*	Homogeneous*	Mixed*
Untreated females	43	13	7	6	0
Mestranol-treated females	47	21	15	5	1
Mestranol-treated males	40	28	16	8	4

*Number of tests

($P < 0.005$) and 12 weeks ($P < 0.025$).

As NZB/NZW mice grow older, the incidence of ANA increases (13). Therefore, in Table 1 tests performed in immature castrated females are compared with results in young female controls (4 to 6 weeks). Older oophorectomy females (8 to 12 weeks) are compared with mature sham-operated controls (8 to 15 weeks). Oophorectomy failed to influence the formation of ANA in either group of mice.

Eighty-seven samples of sera from mestranol-treated female and male mice obtained at 4, 6, 8 and 12 weeks of treatment were tested for ANA on human leukocyte substrate. Forty-three serum samples from untreated NZB/NZW females, aged 4 to 16 weeks, were also tested. Each positive test demonstrated a homogeneous, peripheral or mixed pattern of nuclear immunofluorescent staining. Homogeneous staining is illustrated in Figure 1A. Peripheral staining (Figure 1B) was characterized by pale nuclear fluorescence with intense staining around the periphery of the nucleus (6). Other positive sera produced a mixed pattern of peripheral or homogeneous staining identified in individual nuclei on a single slide. The occurrence of each pattern is listed in Table 2. A peripheral pattern was most common in all 3 groups of animals. The occurrence of a peripheral pattern in 16 of 40 sera tested from mestranol males is significant when this group is compared to control females ($P < 0.025$).

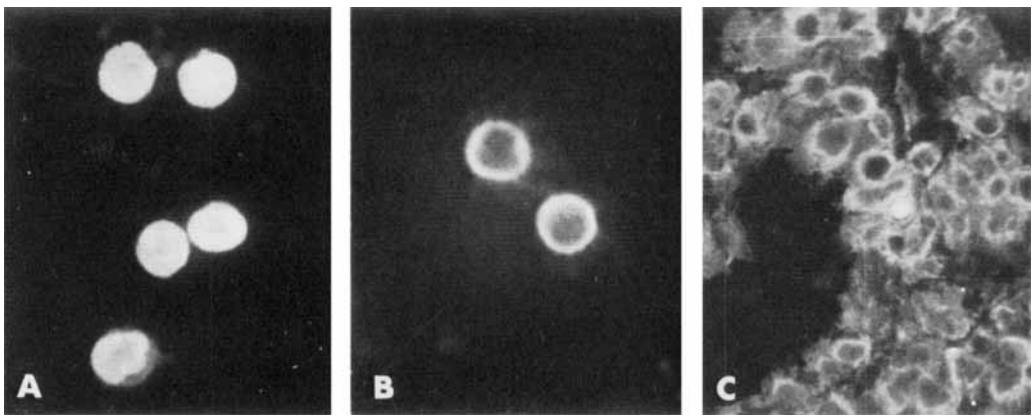

Fig 1. Photomicrographs of patterns of nuclear immunofluorescence in positive tests for ANA. **A.** Homogeneous pattern on human leukocytes (×500). **B.** Peripheral pattern on human leukocytes (×500). **C.** Peripheral pattern on human thyroid (×312).

After an unexpectedly large number of positive tests for ANA were found in male mestranol-treated mice initially tested on white cells, antinuclear activity was reassessed by repeat testing using both human leukocytes and human thyroid as substrate. Undiluted sera obtained from 21 mestranol females and males bled at 4, 6 and 8 weeks of treatment were divided into duplicate samples and applied to white cells and thyroid slices. As early as 4 weeks after mestranol therapy began, 8 of the 11 males in this treatment group had positive tests for ANA on leukocytes. Concomitant testing on thyroid showed that 6 of the 11 samples of sera were positive for ANA. The increased frequency of positive tests for ANA initially found in mestranol-treated males after 6 weeks of treatment (Table 1) was also confirmed by testing on thyroid tissue. Fifty-seven percent of the sera tested on thyroid at 6 weeks was positive. After receiving mestranol injections for 8 weeks, 7 of the 8 males tested were positive for ANA on white blood cells and 6 of these animals were positive on thyroid slices. In 15 instances, samples of sera found to be positive on white blood cells were negative when tested on thyroid. The most consistent correlation between the 2 substrates was found in sera from the 8 males that were positive for ANA (12 to 14 weeks of age) treated with mestranol for 8 weeks. All positive tests on thyroid slices showed peripheral staining (Figure 1C), and no homogeneous or mixed patterns of immunofluorescence were found.

To investigate the stability of patterns in diluted sera, serial dilutions of known positive sera from 6 mestranol-treated mice, aged 20 weeks, were tested on human leukocytes. Sera were positive at titers of 1:64 to 1:128. In 5 instances, the patterns were unchanged in diluted sera (4 homogeneous and 1 peripheral). One pattern changed from homogeneous in undiluted serum to peripheral in serum diluted serially from 1:2 to 1:64.

Both mestranol and propylene glycol were tested for ability to cause fluorescence of

Table 3. Mean Serum α_2-, β- and γ-Globulins in Control and Treated NZB/NZW Mice

Mice	Treatment (wk)									No. of mice bled at 12 weeks
	0*	6*	12	0	6	12	0	6	12	
	α_2-Globulins (mg/ml)			β-Globulins (mg/ml)			γ-Globulins (mg/ml)			
Controls										
Females	6.8	8.0	7.6	5.3	5.4	6.5	3.3	4.4	4.6	9
Males	6.0	11.0	7.6	5.5	5.6	5.1	3.4	5.4	6.4	5
Mestranol										
Females	5.0	13.6	11.3	5.3	9.4	9.0	2.7	3.6	5.1	10
Males	5.5	14.4	11.8	5.1	10.4	8.4	2.7	3.3	4.7	10
17-β-estradiol										
Females										
Group 1	6.7	13.3	10.4	5.5	9.1	7.2	3.0	9.7	11.6	1
Group 2	5.6	8.2	10.7	5.5	5.3	6.4	3.6	4.9	9.9	3
Males	5.9	9.1	8.0	4.9	5.5	5.4	2.3	5.4	8.0	6
Oophorectomy										
Immature females	7.3	7.9	ND	5.7	5.1	ND	4.0	5.4	ND	
Sham-operated controls	ND	7.2	8.9	ND	5.2	7.2	ND	5.8	7.4	9
Mature females	ND	8.2	10.2	ND	5.4	7.8	ND	5.5	6.8	12

*All animals in each treatment group were tested at 0 and 6 weeks.

mammalian tissue substrate in the immunofluorescent test for ANA. Direct application of these substances to white cells and thyroid followed by incubation, washing and addition of fluorescein-conjugated antimouse γ-globulin did not produce positive tests for ANA.

Serum Proteins

The effects of mestranol, estradiol and oophorectomy on serum protein concentrations were also studied in these animals. Serum albumin and α_1-globulin levels were not changed after hormone administration or ovarian ablation. Mean values for serum α_2-, β- and γ-globulins are listed in Table 3. After 6 weeks of mestranol therapy, α_2-globulins increased significantly in females ($P < 0.001$) and in males ($P < 0.025$) over values in female and male controls. Increases of α_2-globulins in mestranol-treated mice were sustained at significant levels after 12 weeks (females: $P < 0.010$; males: $P < 0.001$). Beta-globulin levels in mice of both sexes increased during mestranol treatment. These elevations represented highly significant increases over β-globulin levels in controls at 6 weeks (females: $P < 0.001$; males: $P < 0.001$) and at 12 weeks (females: $P < 0.010$; males: $P < 0.001$). After 6 weeks of treatment with mestranol, both female and male mice demonstrated an increase in serum γ-globulin levels compared to pretreatment values. However, this blunted response was significantly different from the age-dependent elevation of γ-globulins found in controls after 6 weeks (females: $P < 0.005$; males: $P < 0.001$). Gamma-globulins in 16- to 18-week-old mestranol-treated males at 12 weeks of treatment remained significantly lower than controls ($P < 0.001$).

The response to mestranol differed from that observed during treatment with 17-β-estradiol. After 6 weeks of treatment with 17-β-estradiol, female mice showed two distinct patterns of serum globulin response and were grouped according to this response (2). In 10 of 23 females (*Group 1*), the administration of estradiol for 6 weeks resulted in significant increases of serum globulins over control values. Serum globulin levels were not increased in the remaining 13 (*Group 2*) females or in treated males. After 6 weeks of therapy, differences in α_2-, β- and γ-globulins between Group 1 and Group 2 females were significant; α_2-, ($P < 0.050$), β-, ($P < 0.050$) and γ-globulins, ($P < 0.001$). The significance of this unique pattern of response has been discussed elsewhere (2). The small number of sera (four) from treated females available after 12 weeks of estradiol therapy precluded evaluation of long-term response of Group 1 or Group 2 females to estradiol therapy.

Mean α_2-, β- and γ-globulin levels in immature female mice treated with oophorectomy at 4 to 6 weeks of age are also listed in Table 3. These values resemble globulin levels in young female controls. Serum globulins in older oophorectomized females showed no significant deviations from levels measured in the sham-operated control group 6 to 12 weeks after surgery.

Histology

Mean numbers of glomerular lesions counted in 27 treated and 22 control NZB/NZW mice are listed in Table 4. The administration of mestranol or 17-β-estradiol failed to influence the number of glomerular lesions in 10 females between 4 and 30 weeks of age. There was no evidence in this study that the administration of female hormones or oophorectomy affected the spontaneous evolution of renal disease in female NZB/NZW hybrid mice. Fewer glomerular lesions were found in kidneys from the 3 mestranol-treated males than were identified in control animals.

No evidence of hepatic damage was found on microscopic examination of livers from 6 mestranol, 4 estradiol and 5 control mice killed at 24 weeks of age. Small periportal collections of lymphocytes were noted in the liver from 1 untreated female mouse. Infiltration of lympho-

Table 4. Glomerular Lesions in Control and Treated NZB/NZW Mice

Mice	No. of kidneys examined	Age at examination (wk)	Mean number of lesions in 20 glomeruli
Controls			
Females	13	20–34	24
Males	5	20–38	24
Mestranol			
Females	5	27–30	20
Males	3	26–29	9
17-β-estradiol			
Females	5	20–24	28
Males*			
Oophorectomy			
Immature females	6	29–33	33
Sham-operated controls	4	28–48	35
Mature females	8	32–46	42

*All males treated with 17-β-estradiol died before the age of 20 weeks.

cytes in the liver was not found in any of the animals treated with mestranol or estradiol.

DISCUSSION

This study is an extension of our investigation into the influence of estrogenic hormones on the evolution of autoimmune disease in hybrid New Zealand mice, evaluated by studies of ANA, serum proteins and renal histology. Our data suggest that low daily doses of mestranol accelerate the appearance of ANA in this animal model of autoimmune disease. Male NZB/NZW mice are expected to develop ANA at 4 to 6 months of age, about 2 months later than females (13). McGiven and Ghose (14) surveyed 59 samples of serum from NZB/NZW males, aged 1 to 6 months, for ANA and these sera were negative. In their experience, ANA were first detected in hybrid males at 7 months of age. Therefore, the early appearance of ANA in mestranol-treated NZB/NZW males was unexpected. Unfortunately, the high death rate in mestranol-treated mice prevented a long-term study of the course of disease in these animals after 20 weeks of age. The mechanism of stimulation of autoantibody production and the predilection of treated males to develop ANA remains unexplained. Studies of differences in hormone metabolism between female and male NZB/NZW mice may clarify the apparent sex-determined responses of these animals to individual female hormones.

The apparent stimulation of ANA production by mestranol in NZB/NZW mice has an analogue in human experience. Synthetic estrogen-progestogen compounds have been reported to produce serologic abnormalities in certain patients with incipient rheumatic disease (15). Kay *et al* (4) reported four normal women who developed ANA while taking oral contraceptives containing at least 80 μg of mestranol per tablet. Estrogenic hormones may also alter immune reactions in rodents. Nicol and his co-workers (16) have shown that diethylstilbestrol stimulates activity of the reticuloendothelial system and prolongs survival time after bacterial infection.

Comparison of the synthetic hormone mestranol with 17-β-estradiol and oophorectomy in NZB/NZW mice has shown important differences in effects on ANA and serum proteins. Divergent biologic responses may be produced by steroid hormones which are similar in struc-

ture. Mestranol exerts a weak estrogenic action on vaginal epithelium in comparison with natural estrogens (17) such as 17-β-estradiol. This study suggests that these two hormones may also differ in their effects on immunocompetent cells.

Other workers (14) have described homogeneous, "membranous" (peripheral) and speckled patterns produced on rat liver, human thyroid and dried human blood films when using sera from untreated NZB/NZW mice. However, the incidence of each type of immunofluorescent staining in these animals was not reported. In the present study, sera from mestranol-treated males produced significantly more peripheral patterns than sera from control females. Although the significance of patterns of nuclear immunofluorescence has not been established (18), some investigators have suggested that a "shaggy" (peripheral) pattern (6) is produced by antibodies to DNA. In our study, examination of kidneys from treated and control animals showed no evidence that mestranol therapy and subsequent induction of ANA formation accelerated the development of autoimmune nephritis in these NZB/NZW mice. Based upon current theories of the immune pathogenesis of NZB/NZW nephritis (19), it would be anticipated that the ANA induced by mestranol are not of anti-DNA type. This question is currently under investigation. Unlike other examples of drug-induced production of autoantibodies to nuclear constituents (20), mestranol-induced ANA may fail to participate in immune complex formation. For example, ANA of the IgM class do not fix complement; and these autoantibodies may be innocuous (21). Male mice treated with a synthetic estrogenic hormone develop low levels of serum complement (22). Pathogenic antigen-antibody-complement complex formation may be retarded in such animals. On the other hand, ANA were first detected in the sera of treated male mice 5 to 22 weeks before death. If long-term mestranol treatment were possible, persistence of autoantibodies in the sera of treated mice might have caused severe renal damage. The failure of estradiol administration or ovarian ablation to alter the progress of disease in NZB/NZW mice suggests that a simple excess or deficiency of natural estrogens does not play a determinate role in the evolution of autoimmune nephritis in this animal genetically predisposed to form pathogenic immune complexes.

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