

# Distinctive Serum Globulin Responses to Parenteral Treatment with 17- $\beta$ -Estradiol in Female NZB/NZW Mice

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**After 6 weeks of treatment with 17- $\beta$ -estradiol, two distinct groups of female NZB/NZW mice were identified. Group I females developed significant increases in serum  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -globulins and had few serologic abnormalities. Serum globulins did not increase in Group II females. This blunted response correlated with a pretreatment increase in the frequency of positive tests for ANA, LE phenomena and cryoproteins in 4-week-old Group II mice.**

Systemic lupus erythematosus (SLE) is an autoimmune disease that occurs predominantly in women (1), and symptoms of this disorder often appear during pregnancy or immediately postpartum (2). These clinical observations have stimulated repeated attempts to define the role of estrogenic hormones in the pathogenesis of SLE. Recent reports of exacerbations of SLE (3), joint pain and swelling (4), and the occurrence of positive tests for antinuclear antibodies or LE cells (4, 5) in wom-

en treated with oral contraceptives have also drawn attention to the possible interactions between female hormones and autoimmune disease. Clearly, adverse reactions to synthetic estrogen-progestogens are exceptional, since the majority of women using oral contraceptives do not demonstrate this response (6, 7). Precise identification of the hormonal, metabolic, or genetic processes responsible for these abnormalities would have important implications and help to define the specific host factors responsible for the evolution of autoimmune disease.

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This study was designed to evaluate the effects of daily administration of a naturally occurring female hormone, 17- $\beta$ -estradiol, on the course of autoimmune disease in the NZB/NZW mouse. First-generation offspring of matings between New Zealand Black and New Zealand White mice develop a disease resembling SLE characterized by positive tests for antinuclear antibodies (ANA), LE cells and the development of immune-complex nephritis (8). After 6 weeks of treatment with estradiol, two distinct patterns of serum globulin response were identified in NZB/NZW mice. Group I females developed high levels of  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -globulins. Group II females showed

insignificant increases of  $\alpha_2$ - and  $\gamma$ -globulins during treatment. After these groups of mice were identified on the basis of their serum protein responses to estradiol, it was noted that a significantly larger number of Group II mice had demonstrated serologic abnormalities (ANA, LE cells, and/or cryoproteins) before treatment started. Hypergammaglobulinemia was induced in Group I females, but the early course of disease in these mice did not differ from that found in the other animals.

## MATERIALS AND METHODS

**Animals.** Breeding pairs of New Zealand Black mice and New Zealand White mice were supplied by Mr. William D. Hall, The University of Otago, Dunedin, New Zealand. Each of our colonies is maintained by brother-sister matings. Swiss Webster mice were raised from breeding pairs purchased from the Spartan Company, Haslett, Michigan.

**Treatment Protocol.** Daily subcutaneous injections of 17- $\beta$ -estradiol, 2.5  $\mu$ g/kg body weight, were started when mice were 4 weeks old. Control mice received equivalent volumes of propylene glycol vehicle. Mice were bled from the orbital venous plexus before injections were started. Littermate male and female mice were randomly assigned to treatment or control groups. In the first experiment, 10-11 NZB/NZW and 6 Swiss Webster mice of each sex were bled after 6 weeks of injections and killed 2 weeks later at 12 weeks of age. After noting the serum  $\gamma$ -globulin response in treated NZB/NZW females, 12 additional females were studied before and after 6 weeks of treatment with 17- $\beta$ -estradiol. In a second experimental group, 12 four-week-old NZB/NZW females were bled before, after 2 and 4 weeks of treatment, and killed at 8 weeks of age.

**Serum protein determinations.** Electrophoresis of 2  $\mu$ liters of mouse serum on cellulose acetate strips was performed in a Shandon tank at constant current (2 mamp/strip) using Tris-barbital-sodium barbital buffer (pH 8.8). After staining, protein fractions were cut out and each was dissolved in a mixture of chloroform: absolute ethanol 9:1, v/v. The optical density (OD) of each fraction was determined using a Beckman DU-2 spectrophotom-

eter, and the protein concentrations were calculated as described by Scherr (9). To check the accuracy of protein recovery, duplicate strips of cellulose acetate were loaded with known amounts of protein and electrophoresis was performed. Comparison of OD of duplicate eluted strips showed that recovery was accomplished with an accuracy of  $\pm 2\%$ . Total serum protein was measured by a biuret method. Crystalline bovine serum albumin was used as standard.

Serum IgG levels were determined by radial immunodiffusion in 1% agar containing 1% goat antimouse IgG.\* This commercial antiserum was found by immunoelectrophoresis to contain a second precipitating antibody. Repeated cross absorptions with mouse red cell hemolysate removed this contaminating antibody from the antiserum to IgG. The IgG standard was separated from whole mouse serum by ion-exchange chromatography on a diethylaminoethyl (DEAE) cellulose column by the method of Fahey (10). Purity of the absorbed antiserum and the IgG standard was verified by immunoelectrophoresis.

**Serologic Studies.** Detection of ANA was accomplished by an indirect immunofluorescent procedure using conjugated goat antimouse 7S  $\gamma$ -globulin† and human leukocytes as substrate (11). Direct LE cell tests were performed using blood collected in capillary tubes and allowed to clot. The clot was disrupted with a fine steel wire and incubated for 2 hours at 37°C (12). The smeared buffy coat was stained with Wright's stain. LE tests were considered positive if at least one typical LE cell was identified on a slide. In this study, the presence of at least three pieces of typical extracellular nuclear material (ECM) noted on scanning the entire slide was also considered evidence of a positive LE reaction. Based on the work of Golden and McDuffie (13), ECM was identified as masses of round, homogeneous or mottled well-circumscribed purplish-staining nuclear material produced during *in vitro* incubation of the LE preparation. To test for the presence of cryoproteins, aliquots of serum were harvested at room temperature and stored in capillary tubes at 4°C and examined after 24 and 48 hours for the presence of a precipitate. The test was considered positive for cryoproteins if the precipitate disappeared on rearming to 37°C.

\*Kallestad Laboratories, Minneapolis, Minn.

†Immunology Inc, Glen Ellyn, Ill.

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**Histology.** Renal tissue fixed in Carnoy's solution was embedded in paraffin and 4- $\mu$  sections were prepared. The tissue sections were stained with hematoxylin and eosin, and with Schiff sulfite leucofuchsin (PAS) (14) and examined by light microscopy.

### RESULTS

#### Serum proteins

In Table 1, mean concentrations of the major serum protein fractions in treated and control NZB/NZW mice at 4 weeks of age are compared with values at 10 weeks of age. Serum levels of albumin,  $\alpha_1$ -globulins, and  $\beta$ -globulins were similar at 4 and 10 weeks of age. Age-dependent increases of mean  $\alpha_2$ -globulins and  $\gamma$ -globulins were noted in control females. Two distinctive patterns of serum protein response were found in 10-week-old female NZB/NZW mice after 6 weeks of treatment with 17- $\beta$ -estradiol. Based upon this response to estrogen, the mice were designated as belonging to Group I or Group II. In Group I females, there was an increase in levels of  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -globulins. In estrogen-treated Group II females, changes in  $\alpha_2$ - and  $\gamma$ -globulins were no greater than the age-dependent alterations in these serum protein fractions found in control females. Statistical analysis for groups with unequal numbers of individuals (15) demonstrated that post-treatment differences in Group I and Group II mice were significant. For  $\alpha_2$ -globulins  $t = 3.2$  with  $P < 0.05$ ; for  $\beta$ -globulins  $t = 2.5$  with  $P < 0.05$ ;<sup>\*</sup> and for  $\gamma$ -globulins  $t = 9.2$  with  $P < 0.001$ .

Individual NZB/NZW females could not be identified as Group I or Group II responders unless they received at least 6 weeks of treatment with estradiol. Treated

and control NZB/NZW females in a second experimental group were bled before and after 2 and 4 weeks of treatment. The mean serum  $\gamma$ -globulin value after 2 weeks of treatment was 4.5 mg/ml. At 4 weeks, the mean level of  $\gamma$ -globulins increased to 6.3 mg/ml. The mean responses of treated animals at 2 weeks and at 4 weeks were not statistically different from those of controls ( $P > 0.9$  and  $P > 0.9$ , respectively).

Serum proteins in male NZB/NZW mice are also listed in Table 1. Albumin levels were not affected by aging or by treatment with estradiol. An unexpected increase in  $\alpha_1$ -globulins was observed in 10-week-old male controls. Mean increases in levels of  $\alpha_2$ - and  $\gamma$ -globulins occurred in both control and treated males.  $\beta$ -globulins were essentially unchanged. The pattern of response of  $\alpha_2$ - and  $\gamma$ -globulins in treated males was similar to the response noted in estrogen-treated Group II females. Hypergammaglobulinemia was not found in treated males, and no NZB/NZW male demonstrated a Group I response to treatment with estradiol.

Swiss Webster mice were studied to evaluate the effects of estradiol on a second strain of mice that was not susceptible to the spontaneous development of autoimmune disease. The only significant difference between males and females was found in the mean  $\beta$ -globulin response after 6 weeks of treatment (males 13.7, females 7.7 mg/ml;  $P < 0.025$ ). Otherwise, mean serum protein values for equal numbers of mice of both sexes were similar. As observed in NZB/NZW mice, there were no changes in albumin and  $\alpha_1$ -globulin levels that could be ascribed to estrogen treatment. Treatment with estradiol did produce a mean increase in the level of  $\alpha_2$ -globulins from 6.3 to 12.8 mg/ml in male and female mice. The increase in serum  $\gamma$ -globulin levels was

<sup>\*</sup>For evaluation and interpretation of  $t$ , see Sec 4.9 (15). Given the similarities in  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -globulin values before treatment, these probabilities are based upon the chance of such divergence after 6 weeks of treatment.

similar in control (3.2 to 7.1 mg/ml) and in estradiol-treated mice (4.0 to 7.8 mg/ml). In contrast to female NZB/NZW mice, there was no evidence that the serum  $\gamma$ -globulin response in either male or female Swiss Webster mice was differentially affected by 6 weeks of treatment with 17- $\beta$ -estradiol.

To verify measurements of total serum  $\gamma$ -globulins by the Scherr procedure, IgG levels were determined by radial immunodiffusion using 35 specimens of sera from 29 female NZB/NZW mice. The mean IgG value in 15 pretreatment sera obtained from 4-week-old hybrid females was 1.2 mg/ml. This represented 63% of the total  $\gamma$ -globulin found in the serum of these animals by colorimetric measurement of the  $\gamma$ -globulin fraction separated by cellulose acetate electrophoresis. IgG levels were increased in older mice (2.9-6.6 mg/ml). In 14 estrogen-treated females the radial immunodiffusion measurements of IgG were

concordant with the total  $\gamma$ -globulin levels determined by the method of Scherr. In these animals, the mean serum IgG concentration accounted for 88 to 95% of the total  $\gamma$ -globulins.

#### Antinuclear antibodies, LE cell tests and cryoproteins

Positive tests for ANA, LE cells and/or cryoproteins in individual female NZB/NZW mice are recorded in Table 2. At the beginning of the study, 4-week-old female littermates were assigned to either control or estrogen treatment groups without knowledge of their pretreatment serologic status. After 6 weeks of estradiol therapy, Group I and Group II females were identified on the basis of their serum protein responses. At that time, analysis showed that 7 of the 13 females in Group II had at least one serologic abnormality before treatment. Only a single serologic

**Table 1. Serum Albumin,  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -Globulins in Control and 17- $\beta$ -Estradiol Treated NZB/NZW Mice**

	Age (wk)	No. of mice	Albumin	$\alpha_1$ Globulins	$\alpha_2$ Globulins	$\beta$ Globulins	$\gamma$ Globulins
			mg/ml				
<b>NZB/NZW Females</b>							
Controls	4	11	31.2 $\pm$ 1.4*	4.5 $\pm$ 0.4	7.0 $\pm$ 0.6	5.4 $\pm$ 0.3	3.2 $\pm$ 0.4
Controls	10	11	34.0 $\pm$ 0.9	5.4 $\pm$ 0.6	8.3 $\pm$ 0.6	5.2 $\pm$ 0.2	5.0 $\pm$ 0.3
<b>Treated</b>							
Before Treatment	4	23	29.1 $\pm$ 1.1	3.9 $\pm$ 0.2	6.1 $\pm$ 0.3	5.5 $\pm$ 0.3	3.3 $\pm$ 0.4
After Treatment†							
Group I	10	10	31.5 $\pm$ 2.4	5.6 $\pm$ 0.5	13.3 $\pm$ 1.2	9.1 $\pm$ 1.6	9.7 $\pm$ 0.5
Group II	10	13	29.2 $\pm$ 1.0	4.4 $\pm$ 0.3	8.2 $\pm$ 0.6	5.3 $\pm$ 0.3	4.9 $\pm$ 0.2
<b>NZB/NZW Males</b>							
Controls	4	9‡	27.7 $\pm$ 1.4	4.9 $\pm$ 0.5	6.0 $\pm$ 0.5	5.5 $\pm$ 0.3	3.4 $\pm$ 0.5
Controls	10	9	26.7 $\pm$ 1.1	9.0 $\pm$ 1.0	11.0 $\pm$ 1.1	5.6 $\pm$ 0.6	5.4 $\pm$ 0.3
<b>Treated</b>							
Before Treatment	4	8§	27.3 $\pm$ 0.6	4.0 $\pm$ 0.4	5.9 $\pm$ 0.6	4.9 $\pm$ 0.2	2.3 $\pm$ 0.3
After Treatment	10	8	28.7 $\pm$ 1.8	5.3 $\pm$ 0.8	9.1 $\pm$ 0.9	5.5 $\pm$ 0.5	5.4 $\pm$ 0.1

\* Mean  $\pm$  1 SE

† Treated with 17- $\beta$ -estradiol for 6 weeks (2.5  $\mu$ g/kg/day)

‡ One mouse died at time of initial bleeding

§ Two mice died at time of initial bleeding

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**Table 2. Antinuclear Antibodies, LE Cell Reactions, and Cryoproteins in Control and 17- $\beta$ -Estradiol Treated Female NZB/NZW Mice\***

Group	Age (wk)	No. of mice tested	Anti-nuclear antibodies	Positive LE cell tests	Cryo-proteins	Numbers of mice with serologic abnormalities
Controls	4	11	3	0	0	3
Controls	10	11	1	0	0	1
Before Treatment						
Group I	4	10	1	0	0	1
Group II	4	13	4		3	7
After Treatment†						
Group I	10	10	1	1	1	3
Group II	10	13	3	1	2	5

\* One 10-week-old control NZB/NZW male and one 10-week-old treated Swiss Webster female were ANA-positive. There were no other positive serologic tests in NZB/NZW males or Swiss Webster mice

† Treated with 17- $\beta$ -estradiol for 6 weeks (2.5  $\mu$ g/kg/day)

abnormality was found before treatment in the 10 Group I mice. The difference in the numbers of female mice with serologic abnormalities before treatment in Group I and Group II was significant ( $P < 0.05$ ). Therefore, clustering of pretreatment serologic abnormalities in Group II animals correlated with the absence of serum protein response to estradiol.

After 6 weeks of treatment with estrogen, the occurrence of serologic abnormalities in control, Group I and Group II mice was similar. One 10-week-old control NZB/NZW male and one 10-week-old treated Swiss Webster female were ANA positive. Detailed examination of the numbers of positive tests for ANA, LE cells and cryoproteins in individual mice before and after treatment failed to show that 17- $\beta$ -estradiol either stimulated or suppressed the appearance of early serologic abnormalities in these animals.

### Renal histology

Kidneys were removed at autopsy from 18 treated and control NZB/NZW females and tissue sections were examined by light

microscopy. These animals had received estradiol or propylene glycol for 4 weeks (11 mice) and 8 weeks (7 mice). They were 8 and 12 weeks of age, respectively, when they were killed. Two examiners, unaware of the treatment status of the mice, read the slides independently. Although overt renal disease is said to develop between 4 and 6 months of age in hybrid females (8), in this study definite histologic abnormalities were found in the kidneys of treated and control mice at 2 and 3 months of age. Mice in the younger age group demonstrated both hypercellularity and thickening of the mesangial stalk. In 3-month-old hybrid females there was swelling and diffuse hypercellularity of glomeruli, and focal areas of basement membrane thickening were found in PAS-stained sections. There was no conclusive evidence that these early renal abnormalities were influenced by treatment with low doses of 17- $\beta$ -estradiol for 8 weeks.

### DISCUSSION

A unique finding in this study was the division of female NZB/NZW mice into 2

groups based on their serum  $\alpha_2$ ,  $\beta$ - and  $\gamma$ -globulin responses to 6 weeks of parenteral treatment with very low doses of 17- $\beta$ -estradiol (2.5  $\mu\text{g}/\text{kg}/\text{day}$ ). These two groups were further distinguished by a clustering of pretreatment serologic abnormalities in the female mice that failed to demonstrate an increase in serum proteins after 6 weeks of treatment with estradiol.

Group I females responded to estrogen treatment with a threefold increase in serum  $\gamma$ -globulins. Significant increases in  $\alpha_2$ - and  $\beta$ -globulins were also observed. Based on the work of others, this response is expected in rodents. Varga found increased levels of  $\alpha$ -,  $\beta$ - and  $\gamma$ -globulins in rats treated with estrogen (16). Treatment of guinea pigs with diethylstilbestrol has been found to stimulate increased levels of serum  $\gamma$ -globulins (17). In our study, estradiol treatment stimulated increases of  $\alpha_2$ -globulins in Swiss Webster mice but  $\gamma$ -globulins were not increased in this strain. Group II NZB/NZW females—like NZB/NZW males—showed no increase in serum  $\gamma$ -globulin levels after treatment with 17- $\beta$ -estradiol. Work is currently in progress to define the effects of long-term treatment with estradiol in Group I and Group II female NZB/NZW mice.

In the current study, analysis of the frequency of positive tests for ANA, LE cells and cryoproteins showed that these serologic abnormalities were most common in 4-week-old female Group II mice before treatment with estradiol was started. This finding suggests that female hybrid mice that demonstrate early serologic markers of autoimmune disease are less responsive to the stimulating effects of estradiol on serum globulins. Studies by others (17–19) suggest that estrogen can either stimulate or suppress immunologic phenomena in experimental animals. This may be related to the species of animal, the maturity of the ani-

mal and the dose and duration of treatment. There was no evidence in this study that estradiol induced or suppressed ANA, LE tests or cryoproteins. Furthermore, there was no histologic evidence that 8 weeks of low-dose estrogen administered to young NZB/NZW females accelerated the initial appearance of renal disease in either Group I or Group II mice.

Although hypergammaglobulinemia is commonly considered one of the hallmarks of autoimmune disease, estrogen-induced hypergammaglobulinemia did not appear to accelerate the spontaneous development of disease in NZB/NZW mice. This is based upon failure to find increased numbers of serologic abnormalities or histologic evidence of accelerated renal disease in Group I animals. Thus, 17- $\beta$ -estradiol may produce a nonspecific stimulation of total serum  $\gamma$ -globulins without augmenting immunologic or biochemical reactions of critical importance in the evolution of NZB/NZW disease. Immune deficiency states have been shown to correlate with an increased incidence of autoimmune disorders and neoplasia (20, 21). The clustering of autoimmune abnormalities in Group II mice suggests that a blunted host response may in some instances be more important in the evolution of autoimmune disease than a hyperreactive state.

The current investigation demonstrates that exogenous estradiol treatment in a host uniformly predisposed to develop immunopathologic disease produces at least two patterns of response. The target organ effects of this hormone are conditioned by a number of metabolic factors (16, 17, 19), even in the offspring of two inbred strains of mice. Undoubtedly, the mechanism responsible for the apparent provocative effect of estrogen in drug-induced and spontaneous autoimmune disorders in humans is equally complex (1-7). Neverthe-

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less, the current findings strongly suggest that this hormone effects the immunologically susceptible female more selectively than the male. This differential response in the female NZB/NZW mouse offers an opportunity to examine in more detail the relevant extrinsic or immunogenetic determinants (8, 22, 23) important in pathogenesis of autoimmune disease in this experimental model. Chemical manipulation of the early phase of prototype disease in the NZB/NZW mouse should provide new insight into the host factors that control the evolution of the lesions that characterize human systemic lupus erythematosus.

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