Autoimmunity, 1991, Vol. 9, pp. 21–32
Reprints available directly from the publisher
Photocopying permitted by license only

EFFECTS OF PROLONGED ADMINISTRATION OF THE 19-NOR-TESTOSTERONE DERIVATIVES NORETHINDRONE AND NORGESTREL TO FEMALE NZB/W MICE: COMPARISON WITH MEDROXYPROGESTERONE AND ETHINYL ESTRADIOL

LYDIA W. KEISLER

Research Associate, Department of Medicine, University of Missouri-Columbia, Columbia, MO 65212

ANN B. KIER

Associate Professor, Department of Pathology, University of Cincinnati Medical School, Cincinnati, OH 45267-0529

SARA E. WALKER

Chief, Rheumatology Section, Harry S. Truman Memorial Veterans Hospital and Professor of Medicine, University of Missouri-Columbia, Columbia, MO 65201

(Received July 24, 1990; in final form November 1, 1990)

To evaluate effects of commonly used progestational estrogenic contraceptive steroids in a hormone-responsive model of lupus, we treated female NZB/W mice before clinical disease (6 wks of age) and after onset of lupus (24 wks of age) with doses of hormones titered to suppress reproduction. We report efficacy of norethindrone (NE) and norgestrel (NG), progestins derived from 19-nor-testosterone, in delaying expression of anti-DNA antibodies. Mice implanted with NG at 24 wks of age had prolonged lifespans. In contrast, the hydroxyprogesterone derivative, medroxyprogesterone acetate (MP), did not affect autoimmune disease. These observations suggest that prolonged administration of 19-nor-testosterone derivatives, in small doses adequate to suppress reproduction, may have ameliorative effects in systemic lupus erythematosus. Mice receiving ethinyl estradiol (EE) plus courses of tetracycline to suppress cystitis had active anti-DNA responses. In 60% of EE-treated mice, however, early deaths resulted from malignant lymphomas and complications of obstructive uropathy. Estrogen toxicity, rather than accelerated lupus, was the major cause of death in NZB/W mice treated with EE.

KEY WORDS: Autoimmunity, systemic lupus erythematosus, oral contraceptives, synthetic progestins, medroxyprogesterone, ethinyl estradiol.

INTRODUCTION

Systemic lupus erythematosus (SLE), a disease characterized by circulating autoantibodies and immune complex nephritis, occurs mostly commonly in women of reproductive age[1]. Active lupus is a relative contraindication to pregnancy[2], and it may be necessary for affected women to postpone childbearing. Oral contraceptive steroids are a dependable and reversible means of preventing conception. However, use of these drugs in lupus patients is controversial. Oral contraceptive therapy has been associated with lupus flares[3]. In a prospective study, 9 of 20 patients who received combination pills containing ethinyl estradiol (EE), 0.03–0.05 mg per pill, had exacerbations of lupus[4]. Because treatment with estrogenic hormones may be hazardous, progestational drugs have been suggested as alternative contraceptive agents[4,5]. As progestogens become available to increasing numbers of women with lupus, it is important to understand the influences of these drugs on the course of autoimmune disease.

F_1 hybrid NZB × NZW (NZB/W) mice spontaneously develop immune complex-mediated disease which closely resembles SLE, characterized by antibodies to DNA (anti-DNA) and proliferative glomerulonephritis. Unmanipulated female mice are expected to die with renal failure at the mean age of 10 mos, and mean longevity in males is 16 mos[6,7]. The

Address reprint requests to: Sara E. Walker, M.D., 111-F, Harry S. Truman Memorial Veterans Hospital, 800 Hospital Drive, Columbia, MO 65201. 314/443-2511, Ext. 6656

lupus-like disease of NZB/W mice is mediated by gonadal hormones[8,9].

The current studies were designed to examine effects of 3 commonly used progestational contraceptives – norethindrone (NE), norgestrel (NG), and medroxyprogesterone acetate (MP) – on expression of autoimmunity in the NZB/W model. For comparison, additional groups of mice received EE. Earlier investigations of effects of progestational hormones in NZB/W mice utilized implants containing undefined amounts of progesterone[9] or compared a range of doses given in the diet[10]. The experiments in the current study were planned to avoid using pharmacologic amounts of progestins. Preliminary experiments determined implanted hormone doses which suppressed reproductive function in NZB/W females[11]. Because it was important to avoid infections which might alter immune responses and longevity[12,13], hormone-treated and control animals were maintained in a pathogen-free environment. Earlier reports relied on assays of autoantibodies and limited tissue examinations to evaluate disease activity in hormone-treated NZB/W mice[8,9,14–16]. We felt it would be instructive to follow the animals until spontaneous death and perform necropsies to determine accurately why they died.

We report delayed expression of autoimmune disease in NZB/W mice treated with progestins derived from 19-nor-testosterone. In contrast, the hydroxyprogesterone derivative, MP, did not influence anti-DNA levels or longevity. In the course of these experiments, mice receiving long-term EE therapy had active anti-DNA responses and died prematurely. However, EE-treated mice did not develop premature lupus; the most common causes of death were malignant lymphomas and obstructive uropathy with necrotizing cystitis. These observations emphasize that long-term treatment with derivatives of 19-nor-testosterone are potentially beneficial in female NZB/W mice, but a synthetic estrogen may produce profound toxicity.

MATERIALS AND METHODS

Animals

Female NZB/W mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 4 wks of age and housed in barrier facilities in Micro-Isolator housing units (Lab Products, Inc., Maywood, NJ). Mice were caged in groups of 5 and exposed to controlled lighting (12 h light and 12 h dark).

Screening for Pathogens

At 3 intervals during the longevity study, groups of 2 to 9 untreated sentinel mice (NZB/W, DBA/1, DBA/2, PN) which were maintained in the same room under the barrier protocol were examined for agents which commonly infect colonies of experimental mice[12,13]. Complete health surveillance screening including necropsy examinations, appropriate cultures, and serologic studies were performed in the Research Animal Diagnostic and Investigative Laboratory of the University of Missouri College of Veterinary Medicine. Sera from an additional 19 NZB/W mice in the same colony were screened for mouse hepatitis virus by ELISA, and 5 of these animals were tested by ELISA for Sendai and pneumonia virus of mice. These tests showed no evidence of infection with naturally occurring mouse pathogens.

Hormones

Crystalline implants were made of 1-cm lengths of Silastic tubing (0.078 inch diameter × 0.125 inch outer diameter; Dow Corning, Midland, MI) packed with hormone powder. Ends of the tubing were sealed with wooden plugs and Silastic adhesive (Dow Corning) to form a capsule.

Earlier experiments[11] utilized serum concentrations of LH and FSH, uterine weight, endometrial proliferation, and formation of corpora lutea to determine the lowest dose of each contraceptive hormone which effectively suppressed reproductive function in female NZB/W mice. Based upon the dosing studies, suppressive doses chosen for the treatment groups were: (1.) NE (Parke-Davis Division of Warner-Lambert Co., Ann Arbor, MI), 5 mg, (2.) NG (Wyeth Laboratories, Philadelphia, PA), 7.5 mg, (3.) MP (UpJohn Company, Kalamazoo, MI), 10 mg, (4.) EE (Wyeth Laboratories) 0.5 mg. Implants were inserted into subcutaneous tissue through a small incision over the left scapula in mice anesthetized with Metofane (Pitman-Moore, Inc., Mundelein, IL), and the incision was closed with an Autoclip (Clay Adams, Parsippany, NJ). Each hormone was given in one implant except for MP, which was given in two capsules, each containing 5 mg. Age-matched control mice received empty implants.

When mice from the current study were necropsied, ovaries were sectioned and evaluated for luteal tissue. Absence of corpora lutea was used as an indicator that implanted steroids had contraceptive efficacy. Satisfactory sections were available from 20 to 40 mice in each group. Corpora lutea were identified in 30% of control animals, whereas only 6% of mice treated with contraceptive steroids had luteal tissue.

Treatment

Mice treated at 6 wks of age

Five groups of NZB/W females (30 mice per group)

received NE, NG, MP, EE, or empty capsules at the age of 6 wks, before clinical evidence of autoimmune disease appeared[6]. Because the contents of crystalline implants are absorbed gradually, every hormone-containing capsule was excised and inspected 12 wks after implantation. All capsules retained powder; nevertheless, they were replaced with identical implants. These mice were bled from the orbital plexus and urine was tested for albumin-uria when capsules were implanted, at 16, 24, and 32 wks after implantation, and at spontaneous death.

Mice treated at 24 wks of age

Additional groups of NZB/W females were implanted at the age of 24 wks, in the early stage of autoimmune disease[6]. Five groups, each containing 40 animals, were treated with NE, NG, MP, EE, or empty capsules. The mice were bled and urine was tested one wk before implantation. At that point, each group contained equivalent numbers of mice with active lupus. Active disease was defined as the presence of two of the following abnormalities: anti-DNA greater than 20% binding, BUN greater than 40 mg/dl, 4+ proteinuria. Bleeding and urine testing were repeated 8 wks after implantation and at spontaneous death.

Four wks after capsules were implanted, young EE-treated mice which had been treated from the age of 6 wks began to die with distended, deep red bladders which contained bloody or cloudy white urine. In 3 instances, the bladders were packed with stones. Analysis of two sets of stones showed that they contained Ca, Mg, PO_4, and NH_4, consistent with struvite. It was felt that the animals should be treated to prevent premature death from cystitis. All mice treated with EE, therefore, received continuous cycles of treatment (2 wks on, 2 wks off) with oxytetracycline HCl (Terramycin, Pfizer Agricultural Division, New York, NY), 18 g/liter, in sucrose-flavored drinking water and the acidifying agent methionine (Methigel, Evsco Pharmaceuticals, Immunogenetics, Inc., Buena, NJ), 8 mg, placed on the tongue daily. Ten young control mice and 10 old control mice were given identical preventive treatment.

Necropsy Protocol and Tissue Evaluation

Mice were examined daily for signs of disease. Moribund animals were bled, urine was tested for albuminuria, the animals were sacrificed, and complete necropsies were performed according to the protocol described in an earlier publication[17]. Because abnormal bladders were observed in the first EE-treated mice which died, each necropsied mouse was examined specifically for bladder distention, hydroureter, hydronephrosis, bladder stones, and bloody urine. Sections of superficial cervical, mediastinal, and lumbar lymph nodes, salivary glands, thymus, lung, heart, spleen, liver, kidney, ovaries, uterus, and bladder were examined by light microscopy.

Severity of glomerular lesions was assessed by counting numbers of specified abnormalities in 20 glomeruli on a cross section of each kidney. Lesions counted were mesangial hypercellularity, thickening of the mesangial stalk, focal glomerular hypercellularity, thickening of the basement membrane, diffuse glomerular hypercellularity, fibrin, and crescent formation. This grading system, which assigns a numerical score, has been used in this laboratory to effectively evaluate progressive glomerular lesions in autoimmune NZB/W mice[18].

Vascular lesions were identified when the arterial wall had loss of architecture and narrowing of the lumen. The majority of these abnormalities resembled the degenerative vascular lesions described in autoimmune mice by Berden, *et al.*[19].

Lymphoid tissue was examined carefully to avoid confusing hyperplastic and intermediate ("preneoplastic") changes with lymphoid malignancy. Lymphomas were diagnosed and classified as lymphoblastic or composite according to guidelines developed in this laboratory[20] based on the criteria of Collins *et al.*[21], Goldenberg *et al.*[22], and Della Porta *et al.*[23]. Lymphomas were considered widespread when two or more sites were involved. Localized lymphomas were restricted to one site, usually thymus or lymph nodes. For purposes of this classification, all lymph nodes were considered to be one site.

Bladders from 20 mice implanted with EE at 6 wks of age and from 29 mice implanted with EE at the age of 24 wks were evaluated for hyperplasia of smooth muscle, infiltrates of inflammatory cells, and abnormal epithelium. For comparison, bladders were dissected from formalin-preserved carcasses of 24 mice which had been implanted at the age of 6 wks with NE ($N = 6$), NG ($N = 6$), MP ($N = 6$), or empty capsules ($N = 6$). The mice, chosen at random from each respective treatment group, were 25–53 wks of age at death. Bladders were dissected free with attached urethra, bisected, embedded to orient cut edges against the microtome blade, and sectioned serially.

Assessment of Active Autoimmune Disease

Mice were bled from the orbital plexus into glass capillary tubes and serum was separated by centrifugation and stored at −20°C. Binding of heat-inactivated mouse serum to ^{14}C-labeled *Escherichia coli*-derived DNA (Amersham Corporation, Arlington Heights, IL) was measured in a modified Farr assay. Values greater than 20% binding indicated the presence of anti-DNA[24,25]. Blood urea nitrogen (BUN) values were measured using a colorimetric assay kit (American Monitor Corp., Indianapolis, IN) as

described in an earlier publication[26]. In this laboratory, the mean BUN level in stored frozen sera from normal female mice was found to be 30 mg/dl ± 4 SEM (95% confidence limits 23–39)[26]. Urine was tested with Albustix (Ames Co., Elkhart, IN). Results were graded 0–4+: 1+ = 30 mg/dl, 2+ = 100 mg/dl, 3+ = 300 mg/dl, 4+ > 2000 mg/dl.

Direct Immunofluorescence

Representative numbers of kidneys (N = 14–29) were examined from each control and treated group. Kidneys collected at necropsy were snap-frozen in liquid nitrogen, sectioned on a cryostat, and stained with previously determined working dilutions of fluorescein-conjugated goat antisera to mouse IgG, IgM, IgA, and C3 (Cappel, Cooper Biomedical, Inc., Malvern, PA)[27]. Glomerular fluorescence was graded 0–4+: 0 = no fluorescence, 1+ = fluorescence of 10–25% of glomerular areas, 2+ = fluoresence of 50% of glomerular areas, 3+ = fluorescence of 75% of glomerular areas, 4+ = fluorescence of 100% of glomerular areas. Kidney sections from 6-wk-old female DBA/1 mice (negative) and NZB/W female mice greater than 5 mos of age (positive) were included as controls for each conjugate in each run.

Statistical Analyses

For variables measured at specified time intervals, treatment groups were compared to age-matched controls. Ages at death, anti-DNA, BUN, and glomerular lesion scores were analyzed by analysis of variance, using SAS-GLM procedures (SAS; Cary, NC). Numbers of mice with glomerulonephritis and vasculitis, neoplasms, albuminuria, and glomerular immunofluorescence were analyzed by chi-square calculations[28].

RESULTS

Mortality

Figure 1 illustrates cumulative deaths in female NZB/W mice implanted with contraceptive hormones or empty capsules (controls) at the age of 6 wks, before the appearance of clinical lupus, and followed until spontaneous death. Mean longevity in controls was 31 wks ± 1 SEM. Lifespans were not affected by treatment with progestogens. Mice implanted with NE lived 33 wks ± 1, mice implanted with NG lived 35 wks ± 1, mice implanted with NG lived 35 wks ± 2, and mice implanted with MP lived 34 wks ± 1. Longevity was diminished significantly in EE-treated mice, whose mean lifespan was 23 wks ± 1 ($P < 0.0001$ vs. controls).

Mice implanted at 24 wks of age, at the onset of active SLE, had a different pattern of mortality (Figure 2). Mean longevity was 33 wks ± 1 in control mice, and lifespans were not influenced by therapy with NE or MP. In NG-treated mice, mean longevity was 36 wks ± 1 (compared to controls, $P = 0.03$),

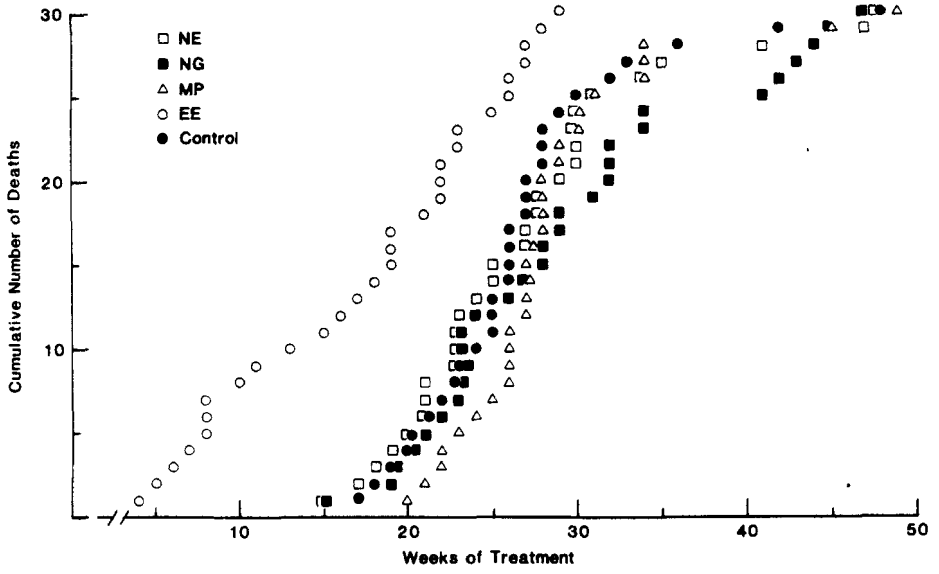

Figure 1 Cumulative deaths in female NZB/W mice implanted at 6 weeks of age with Silastic capsules containing contraceptive hormones in doses calibrated to suppress reproductive function[16]. Mice were examined daily and sacrificed and necropsied when they appeared moribund. Longevity was not affected by long-term treatment with the progestogens NG, NE, and MP. EE-treated mice died prematurely: the major factors contributing to death in EE-treated mice were lymphomas, glomerulonephritis, and obstructive uropathy with necrotizing cystitis.

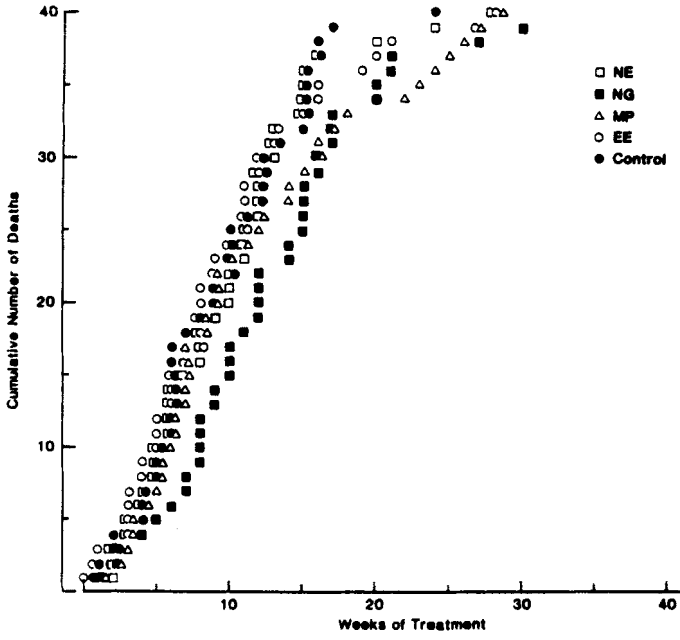

Figure 2 Cumulative deaths in female NZB/W mice implanted at 24 weeks of age, after the expected onset of lupus. One NG-treated mouse which died of iatrogenic causes was not included in mortality data. Mean lifespan was increased in NG-treated mice. Although EE-treated mice did not die prematurely, detailed necropsies showed that lymphomas and obstructive uropathy were the most common causes of death.

showing that NG had modest therapeutic effectiveness in mice with active autoimmune disease. Based on the work of others, it was anticipated that EE-treated mice would have accelerated onset of SLE and die prematurely[8,9,14,15]. In mice implanted with EE at the age of 24 wks, however, longevity was comparable to control mice.

Findings at Necropsy

Glomerulonephritis and vascular lesions, reflecting advanced autoimmune disease, were prominent findings in all control mice and in 98% of mice treated with the progestogens NE, NG, and MP (Table 1). Unexpectedly, deaths in mice implanted with EE were not explained solely on the basis of early onset of lupus. Histological evidence of advanced SLE was found in only 40% of mice implanted with EE at the age of 6 wks, compared to 100% occurrence of SLE lesions in controls ($P < 0.005$). Mice treated with EE from a more advanced age – 24 wks – did not die prematurely. Renal disease and vascular lesions were found in 40% of these animals, whereas all corresponding control mice had lesions reflecting severe autoimmune disease ($P < 0.005$). In most of the animals receiving EE, deaths were attributed to lymphomas, glomerulonephritis, and/or necrosis and hemorrhage of the bladder combined with obstructive uropathy. Sudden death was prevalent in the EE treatment groups; 21% of these animals died suddenly and unexpectedly, and tissue was lost by autolysis.

Neoplasms occurred in 9% of untreated control mice, and therapy with NE, NG, and MP did not predispose to development of malignancy (Table 1). In the EE treatment groups, numbers of mice with tumors found at necropsy were increased. Forty-four percent of mice treated with EE from the age of 6 wks had neoplasms (chi square = 6.6, $P < 0.005$ vs. controls), and neoplasms were found in 50% of older mice implanted with EE (chi square = 13.6, $P < 0.005$ vs. controls). Sixty-three neoplasms originated in lymphoid organs, and 2 mice had localized lung tumors. Malignant extension of lymphomas was influenced by the age at which treatment began. In mice implanted at the age of 6 wks, 96% of lymphomas involved more than one organ and were classified as widespread; in contrast, 62% of lymphomas were widespread in mice implanted at 24 wks of age. Fifty-eight percent of the lymphomas were classified as composite lymphoma (Figure 3); the remainder were lymphoblastic lymphoma (38%), benign thymoma (3%), and plasmacytoma (1%).

When EE-treated mice were necropsied, the urinary tract was strikingly different from other treatment and control groups. In 88% of NZB/W females implanted with EE at 6 wks of age, the bladder was distended markedly at necropsy. Forty-four percent of these

Table 1 Numbers of Abnormal Findings at Necropsy in Female NZB/W Mice Implanted with Contraceptive Hormones

Implant	*Complete Necropsies*	*Glomerulo-nephritis, Vasculitis*	*Lymphomas*		*Abnormal bladder*	*Hemor-rhage*	*Other**	*Autolysis, Iatrogenic death*
			Widespread	*Localized*				
Mice implanted at 6 wks of age								
NE	30	30	0	0	1	0	1	0
NG	29	28	4	0	0	0	0	1
MP	28	28	8	0	0	0	0	2
EE	25	10‡	10	1†	22	2	0	5
0 (control)	30	30	3	0	0	0	1	0
Mice implanted at 24 wks of age								
NE	38	36	4	1	0	0	2	2
NG	39	39	1	5	0	0	0	1
MP	39	38	5	3	0	0	1	1
EE	30	12‡	9	6‡	24	5	1	10
0 (control)	39	39	1	2	1	0	0	1

Subcutaneous implants of norethindrone (NE), norgestrel (NG), medroxyprogesterone (MP), and ethinyl estradiol (EE) were used in groups of 30 mice aged 6 wks and in groups of 40 mice aged 24 wks. Complete necropsies at spontaneous death identified abnormalities, often occurrifng in various combinations, which contributed to death. Numbers of mice with each abnormal finding are listed. Mice lost because of autolysis and one mouse, killed inadvertently, were excluded from analysis. Glomerulonephritis was defined as glomerular lesion score ≥ 30; glomerulonephritis and/or vascular lesions indicated that the animal died with established autoimmune disease. Malignant lymphomas involved 2 or more sites and localized lymphomas were restricted to one site. Statistical calculations compared all lymphomas (widespread + localized) within a group. Abnormal bladders were identified by gross examination as distended and/or containing stones or bloody urine. *Pneumonia (2 mice), pulmonary adenoma (1), pulmonary adenocarcinoma (1), necrosis of small bowel (1), necrosis of liver (1); †$p < 0.025$ vs. controls: ‡$p < 0.005$ vs. controls.

animals had associated hydroureter and hydronephrosis, and 24% of the mice had bladder stones (Figure 4A). In mice treated with EE from the age of 24 wks, the occurrence of bladder distention was 80%, hydroureter and hydronephrosis were present in 3% of mice, and 10% of mice had bloody urine.

Twenty bladders were available for detailed light microscopic examination from mice treated with EE from the age of 6 wks; 90% were abnormal. Bladders from these mice were characterized by massive thickening of smooth muscle in the bladder wall, often associated with fibrosis, massive infiltration with inflammatory cells, and necrosis and sloughing of bladder epithelium (Figure 4B); the lumens of two bladders contained masses of bacteria. In 9 instances, diseased bladders were felt to have contributed substantially to death of the animal. Similar abnormalities were found in mice implanted with EE at

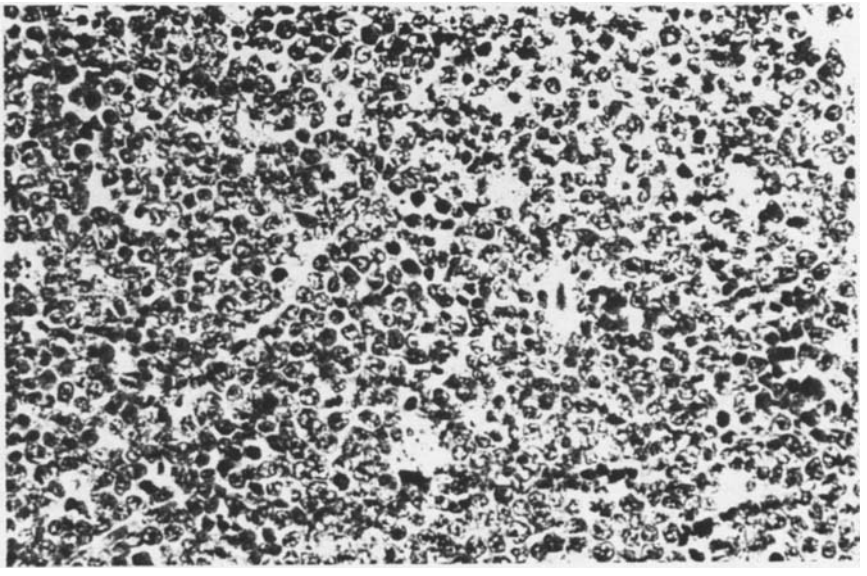

Figure 3 Composite lymphoma in a NZB/W mouse implanted at the age of 6 wks with EE, 0.5 mg. The animal died 23 wks later; malignant cells invaded thymus, spleen, and lymph nodes, and metastases were found in lung, liver, kidney, and ovary. A heterogeneous population of anaplastic lymphocytes is present, and cells contain nuclei with prominent nucleoli. There are numerous mitotic figures (mouse # 612, H & E, × 355).

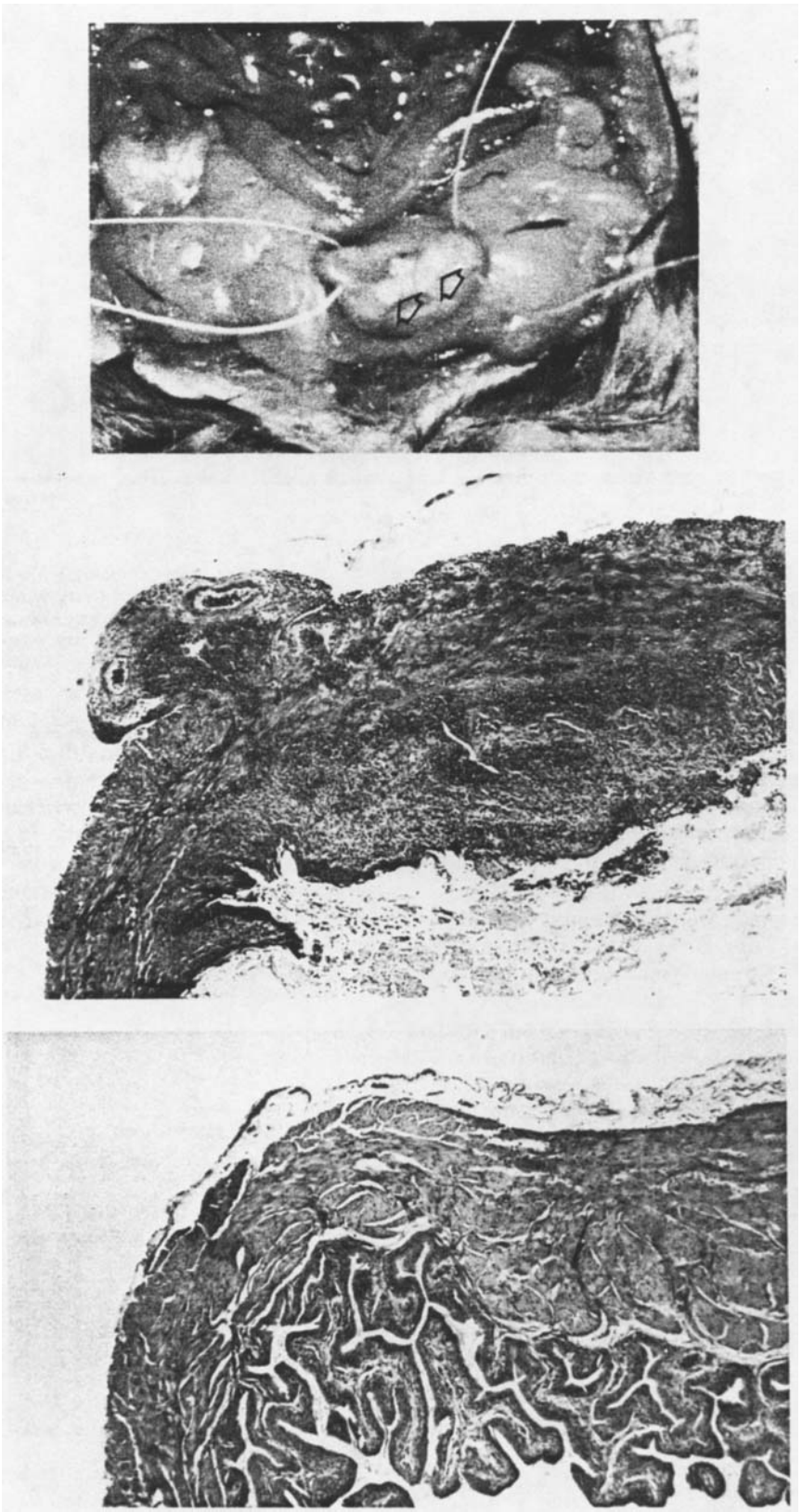

Figure 4 Abnormal bladders in EE-implanted mice. A. Struvite stones (arrows) packed in the thick-walled bladder of a NZB/W mouse (# 584) treated with EE from the age of 6 wks. The animal had 3 negative tests for anti-DNA (1–6% binding) and no glomerulonephritis. It died after 17 wks of treatment with BUN = 204 mg/dl. B. NZB/W mouse # 520, treated with EE beginning at 6 wks of age, died after 10 wks of treatment. There was no evidence of glomerular disease or vascular lesions at necropsy, and death was attributed to bladder stones and necrotizing cystitis. Inflammatory cells are present deep into the muscle layers of the bladder, and lining epithelium is denuded (H & E, × 47). C. A NZB/W control mouse (# 493) was implanted with a blank capsule at the age of 6 wks and died with renal failure 23 wks later. The normal bladder is contracted, causing folding of the epithelium (H & E, × 47).

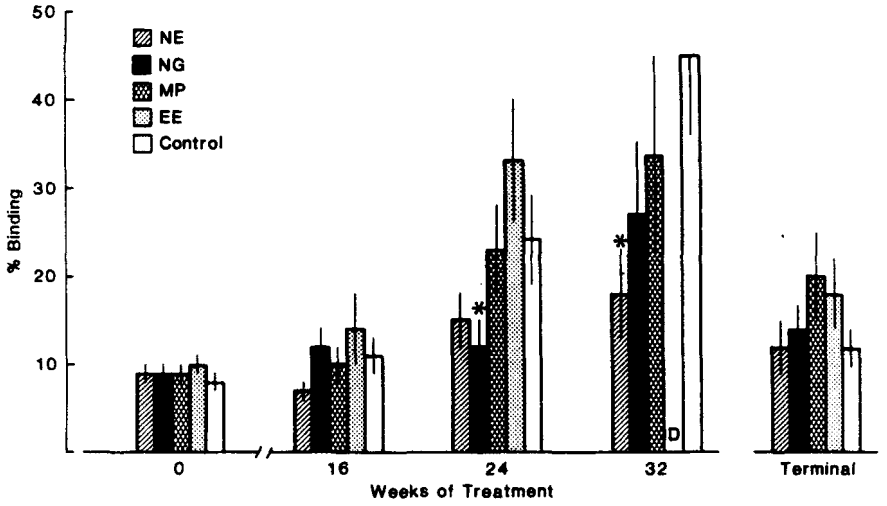

Figure 5 Serial anti-DNA antibody levels assayed by modified Farr technique. Values are mean % binding ± SEM. Groups of 30 NZB/W females were implanted at 6 weeks of age with contraceptive steroids or empty capsules (controls) and tested 0, 16, 24, and 32 weeks after implantation, and at spontaneous death. After 24 weeks of treatment, mice treated with NE and NG had blunted anti-DNA responses; anti-DNA was suppressed in NE-treated mice at 32 weeks. D, mice in this group were dead. *p = 0.05 vs. controls.

24 wks of age. Twenty-nine bladders were examined; 79% were abnormal. Severe necrotizing cystitis, hemorrhage from dilated blood vessels adjacent to bladder wall ulcerations, and infection with the presence of large clumps of bacteria in bladder lumens were judged to be major causes of death in 7 mice in this group.

Only two mice which were not treated with EE had abnormalities of the urinary tract observed at necropsy: one NE-treated mouse had a distended bladder, and one control mouse had bloody urine. Microscopic examination of bladders from controls revealed no bladder wall thickening (Figure 4C); two small, localized foci of lymphocytes were observed in sections of 6 bladders. In two instances, mice treated with NE, NG, and MP had thick bladder walls. Sections of 71% of bladders from these mice contained one or two small foci of inflammatory cells.

In both groups of EE-treated mice, necropsy as a rule revealed massive enlargement of the uterus with edema and thickening of uterine walls and uterine discharge associated with chronic ulceration of the vulva. In 3 instances, mice treated with EE had severe endometritis which may have contributed to mortality.

Anti-DNA

Figure 5 illustrates serial assays for anti-DNA in mice treated from the age of 6 wks. As control mice grew older, DNA binding increased from a mean pretreatment value of 8 ± 1 SEM to 45 ± 9, 32 wks after treatment began. Mean anti-DNA fell to 13 ± 3 in terminal control sera. These findings were in accord with earlier reports from this laboratory[24,29] which showed that anti-DNA binding in untreated NZB/W females increased with age, whereas anti-DNA values were usually suppressed in moribund mice with established renal failure. Twenty-four wks after capsules were implanted, anti-DNA responses were blunted in mice treated with NE and NG; in the NG group,

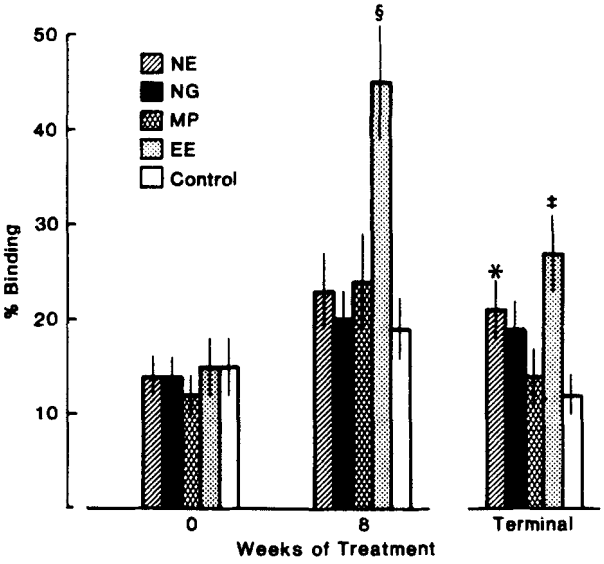

Figure 6 Anti-DNA antibody levels in groups of 40 female NZB/W mice, implanted at 24 weeks of age and tested at 0 and 8 weeks, and at spontaneous death. *p = 0.0002 vs. controls; ‡p = 0.04 vs. controls; §p = 0.002 vs. controls.

suppression of anti-DNA compared to controls was significant at the 0.05 level. After 32 wks of treatment, the anti-DNA level in NE-treated mice remained below the control level ($P = 0.05$) but mice implanted with NG were not suppressed significantly. Based upon earlier studies in which 17-β-estradiol therapy stimulated anti-DNA in NZB/W mice[8,9], it was expected that implanted EE would stimulate anti-DNA. In this treatment group, however, the apparent elevation of anti-DNA after 24 wks of treatment was not significant.

Mice implanted at 24 wks of age had responses which differed from mice implanted at the age of 6 wks (Figure 6). Animals treated with NE from 24 wks of age died with elevated anti-DNA ($P = 0.04$). Eight wks after EE capsules were implanted in mature animals, anti-DNA was increased significantly ($P = 0.0002$ vs. controls). EE-induced stimulation of anti-DNA in this treatment group was confirmed in terminal sera from moribund animals ($P = 0.002$ vs. controls).

Parameters of Renal Disease

Table 2 lists serial assays of BUN and percentages of mice with 3+–4+ albuminuria. In control mice treated from 6 wks of age, assays performed 16, 24, and 32 wks after implantation reflected age-dependent increases of BUN. Advanced renal disease, the major cause of death in controls, produced the terminal BUN value of 116 mg/dl ± 11. Similar patterns of progressive uremia were found in mice implanted with NE, NG, and MP. EE-treated mice did not have premature renal failure. BUN values in mice surviving to 24 wks of treatment did not exceed controls, and mean BUN in terminal sera from the EE treatment group was significantly lower than the corresponding value in controls ($P = 0.003$). Albuminuria in controls and in mice implanted with NE, NG, and MP at the younger age increased as the animals grew older, and heavy proteinuria was the rule in moribund animals. In contrast, albuminuria was suppressed in terminal tests of EE-treated mice (chi square = 29.7, $P < 0.01$ vs. controls). Mice implanted at the age of 24 wks had similar findings, suggesting that death with renal failure and proteinuria was expected. The exception was EE-treated mice, in which there was retarded development of renal insufficiency and proteinuria. At spontaneous death, terminal sera and urine from these animals had relatively low BUN compared to controls ($P < 0.001$) and decreased occurrence of 3+–4+ proteinuria (chi square = 36.7, $P < 0.01$ vs. controls).

Glomerular lesion scores, a useful means of evaluating severity of renal pathology in NZB/W mice[20], are described in Table 3. Control mice and animals implanted with progestogens died with severe glomerulonephritis; in mice treated with NG and MP from the age of 6 wks, mean glomerular scores were significantly greater than controls (in both groups, $P = 0.02$). Glomerular lesions were diminished in both groups of EE-implanted mice; in animals treated at 6 wks $P = 0.0003$ vs. controls, and at 24 wks of age $P = 0.001$ vs. controls.

The relatively mild renal inflammation in EE-treated mice at death was reflected in diminished severity of glomerular deposits in mice treated at 6 wks of age: IgG (chi square = 4.7, $P < 0.05$ vs. controls), IgA (chi square = 5.3, $P < 0.05$ vs. controls), and C3 (chi square = 6.1, $P < 0.05$ vs.

Table 2 Parameters of Renal Disease

Implant	*BUN (mg/dl)*					*Albuminuria (% 3+–4+)*				
	0	*16*	*24*	*32*	*T*	*0*	*16*	*24*	*32*	*T*
			Wks of Treatment					*Wks of Treatment*		
Mice implanted at 6 wks of age										
NE	20 ± 1	26 ± 2	41 ± 9	45 ± 9	144 ± 12	0	14	54	78	96
NG	20 ± 1	26 ± 2	31 ± 3	33 ± 4	89 ± 17	0	7	42	50	93
MP	19 ± 1	22 ± 1	30 ± 3	38 ± 5	92 ± 8	0	3	65	75	100
EE	18 ± 1	25 ± 2	32 ± 2	D	62† ± 12	0	0	33	D	28*
0 (control)	19 ± 1	23 ± 2	38 ± 5	44 ± 5	116 ± 11	0	3	58	33	100
Mice implanted at 24 wks of age										
	0		8		T	0		8		T
NE	23 ± 1		34 ± 5		111 ± 9	12		54		95
NG	26 ± 2		46 ± 9		112 ± 9	12		62		100
MP	26 ± 2		34 ± 5		110 ± 11	12		48		95
EE	24 ± 2		30 ± 7		51‡ ± 9	8		9		24*
0 (control)	25 ± 2		39 ± 6		134 ± 15	10		32		97

Groups of 30 mice treated from the age of 6 wks were tested after 0, 16, 24, and 32 wks of treatment and at spontaneous death (T). Groups of 40 mice aged 24 wks were implanted with contraceptive hormones and tested at 0 and 8 wks of treatment, and at spontaneous death. BUN (mean ± SEM) was quantitated using a colorimetric assay (28). Percentages of mice with 3+–4+ albuminuria, tested on Albustix, are shown. D, mice in this group were dead. *$p < 0.01$ vs. controls; †$p = 0.003$; ‡$p = 0.001$.

Table 3 Glomerular Lesions and Direct Immunofluorescence Studies

Implant	Glomerular score (lesions/20 glomeruli)	Kidneys with glomerular deposits (% 3+–4+) IgG	IgM	IgA	C3
Mice implanted at 6 wks of age					
NE	45 ± 1	61	63	53	47
NG	50† ± 2	71	73	67	67
MP	52† ± 1	67	80	67	93
EE	28‡ ± 2	27*	32	18*	26*
0 (control)	42 ± 2	67	47	58	61
Mice implanted at 24 wks of age					
NE	38 ± 1	50	53	42	60
NG	45 ± 2	58	70	57	80
MP	43 ± 2	76	88	81	82
EE	27§ ± 3	47	47	21	38*
0 (control)	42 ± 1	81	65	64	80

Glomerular lesions (mean ± SEM) were counted in 20 glomeruli in a hematoxylin and eosin stained section of each kidney. Frozen sections of kidney were stained by direct immunofluorescence (29). Glomerular deposits were graded 1+ to 4+ based upon extent of fluorescence in glomeruli. Percentages of mice with extensive deposits (3+–4+) are listed. *$p < 0.05$ vs. controls; †$p = 0.02$ vs. controls; ‡$P = 0.0003$ vs. controls; §$p = 0.0001$ vs. controls.

controls). In mice implanted at the age of 24 wks, contraceptive hormones did not consistently affect glomerular deposition of immunoglobulins; glomerular C3, however, was diminished significantly in EE-treated mice compared to controls (chi square = 3.8, $P < 0.05$).

DISCUSSION

This report describes results of prolonged exposure to low doses of exogenous contraceptive steroids in female NZB/W mice. The NZB/W murine model of SLE was chosen for this study because autoantibody levels and longevity are affected by gonadal steroids. It has been reported previously that implanting females with slow-release capsules containing 17-beta-estradiol was associated with elevation of anti-DNA at 4 and 5 mos of age and premature death[8]. In contrast, NZB/W females treated with testosterone had suppressed anti-DNA levels and prolonged survival[30]. There have been few investigations of the effects of progesterone in the NZB/W model of lupus. In an earlier study, castrated females implanted with an undefined amount of progesterone powder in Silastic capsules developed very high levels of anti-DNA and died prematurely[9,16].

The current study showed that intact female NZB/W mice implanted after puberty with the synthetic progestogens, NE and NG, had delayed appearance of anti-DNA. One treatment group receiving NG had modest, but significant prolongation of life. MP had no influence on disease in NZB/W females. We propose, therefore, that derivatives of 19-nor-testosterone have suppressive properties in female NZB/W mice whereas MP is not beneficial.

NE and its analog, NG, are synthetic derivatives of 19-nor-testosterone which lack the angular methyl group (C19) attached to C10. Nineteen-nor-progestogens, given alone or combined with mestranol, have been reported to interfere with T-cell dependent antibody responses[31], but these compounds were not consistently immunosuppressive[32–34]. Verheul and associates[10,35] verified the beneficial effects of 19-nor-testosterone and its progestational derivative, lynestrenol, in intact female NZB/W mice. Animals receiving daily doses of lynestrenol, 1 mg/day, had vaginal smears compatible with persisting diestrous, diminished anti-DNA levels at 39 wks of age, and increased longevity.

MP, a 17-alpha-hydroxyprogesterone derivative of progesterone[36], had no effect on lupus activity in the current study. Receptors for progesterone have been identified on rat thymus[37]. Although this finding suggested that progesterone and its derivatives had the potential to alter thymic function, both progesterone and MP failed to consistently affect immune responses mediated by T-cells in experimental animals[38,39]. It is of interest to note that MP has potent androgenic properties. MP binds competitively to androgen receptors[40] and stimulates androgenic activity in a beta-glucuronidase assay in mouse kidney[41]. Nevertheless, MP did not have beneficial effects on the androgen-responsive disease of the NZB/W mice.

In a number of studies[7,8,11,12,29], therapy with estrogen stimulated autoantibody levels in NZB/W mice. It was therefore assumed that estrogen induced premature nephritis, leading to early lupus-related death. These conclusions were supported by histopathological studies limited to kidneys from 9 NZB/W females, castrated and implanted with 6 to 7 mg of 17-beta-estradiol at the age of 2 wks. The animals were studied at 4, 5, 8, and 10 mos of age, and results were compared with 12 controls[9]. These important early therapeutic experiments[8,9] did not describe results of complete necropsies to exclude complications of estrogen therapy which may have contributed to death. The current study included 55 intact EE-treated NZB/W females and 69 corresponding controls. These mice were implanted at relatively advanced ages (6 and 24 wks) with the highly potent estrogen, EE. The 0.5 mg dose of EE was chosen because it predictably suppressed ovulation[11], but this dose was adequate to produce toxicity. Complete necropsies were performed when the mice were moribund. These differences in methodology may explain our finding early deaths with lymphomas and urinary tract abnormalities, results which were not in accord with the findings of Roubinian *et al.*[8,9] and others[14,16,19].

The carcinogenic effects of estrogenic hormones have been demonstrated in susceptible strains of rats and mice. Continuous administration of various estrogens has been reported to lead to lymphomas, mammary cancer, cancer of the cervix, adrenal tumors, and pituitary adenomas[42,43]. Estrogen stimulates production of high levels of prolactin, a hormone with potent mitogenic properties in nonreproductive target tissues[44]. Prolactin is a potent stimulator of growth in Nb rat lymphoma cells[45,46]. We have shown, in a separate study, that intact female NZB/W mice develop striking elevations of serum prolactin as early as 3 wks following implantation of Silastic capsules containing 0.5 mg EE (SE Walker, Unpublished observations). We would suggest that the genesis of lymphomas in EE-treated mice in the current study was mediated in part through the stimulatory properties of prolactin.

Obstructive uropathy has been recognized for at least 50 years as a complication of estrogen treatment in mice[47,48]. Earlier work in this laboratory documented deaths with distended bladders and hydronephrosis in NZB/W mice injected with mestranol or 17-beta-estradiol[18], and another group of investigators noted deaths with wasting and enlarged bladders in NZB/W mice of both sexes implanted with capsules containing 5 mg estradiol[49]. In a more recent study, Proteus mirabilis and *Escherichia coli* were cultured from urine aspirated from distended bladders of EE-treated NZB/W mice (SE Walker, Unpublished observations). It is postulated that obstruction with urinary stasis and chronic infection with a urea-splitting organism caused the struvite bladder stones found in these animals.

Unrecognized toxic effects of estrogens may have caused problematic observations in earlier studies of NZB/W mice implanted with gonadal hormones[9]. Estrogen toxicity could explain the ability of capsules containing estradiol, 3.5 mg, to cause early deaths and apparently abrogate protective effects of dihydrotestosterone in female NZB/W mice. Toxic effects might also have accounted for paradoxical early deaths in NZB/W males implanted with estradiol plus progesterone[9]. In a separate study, NZB × DBA/2 F_1 females were castrated and implanted with capsules containing 2 to 5 mg of estradiol. Although it might be expected that the hormone-treated mice would have lifespans resembling intact females, the treated mice died earlier than controls[15].

Results of this study suggest that synthetic progestogens do not cause flares of SLE. These results are in accord with reports of safe use of progestational contraceptives in women with lupus[4,5]. The possibility that 19-nor-testosterone derivatives have the potential of diminishing lupus activity awaits confirmation in appropriate clinical trials. The potential stimulation of anti-DNA levels in the lupus-prone model treated with EE suggests that a prudent course is to avoid using estrogenic contraceptives when there is risk of developing clinical complications of SLE.

Acknowledgements

Excellent technical assistance was provided by Ronald Owen, Mrs. Maxine Frangenberg, and Mrs. Karen Kanuckel. Kathryn L. Owen, D.V.M., M.S., supervised monitoring of the pathogen-free mice used in these studies. Periodic testing for mouse pathogens was performed through the Research Animal Diagnostic and Investigative Laboratory, College of Veterinary Medicine, University of Missouri-Columbia.

This work was supported by the National Institutes of Health-National Institute of Child Health and Human Development through contract HD 52909, and by funds from the Medical Research Service, Department of Veterans Affairs. Dr. Kier was the recipient of a National Institutes of Health Research Career Development Award DHHS 1RO1RR-OOO13. Dr. Walker was supported by a Clinical Investigator Award from the Department of Veterans Affairs.

References

1. Dubois EL. The clinical picture of systemic lupus erythematosus. In: Dubois EL, ed. *Lupus Erythematosus*. Los Angeles: University of California Press, 1974; 232–42
2. Zurier RB. Systemic lupus erythematosus and pregnancy. In: Lahita RG, ed. *Systemic Lupus Erythematosus*. New York: John Wiley and Sons, 1987; 541–54
3. Chapel TA, Burns RE. Oral contraceptives and exacerbation of lupus erythematosus. *Am J Obstet Gynecol* 1971; **110**: 366–9
4. Jungers P, Dougados M, Pelissier C, Kuttenn F, Tron F, Lesavre P, *et al.* Influence of oral contraceptive therapy on the activity of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 618–23
5. Mintz G, Gutierrez G, Deleze M, Rodriguez E. Contraception with progestagens in systemic lupus erythematosus. *Contraception* 1984; **30**: 29–38
6. Howie JB, Helyer BJ. The immunology and pathology of NZB mice. *Adv Immunol* 1968; **9**: 215–66
7. Steinberg AD, Pincus T, Talal N. DNA-binding assay for detection of anti-DNA antibodies in NZB/NZW F_1 mice. *J Immunol* 1969; **102**: 788–790
8. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F_1 mice. *J Exp Med* 1978; **147**: 1568–83
9. Roubinian J, Talal N, Siiteri PK, Sadakian, JA. Sex hormone modulation of autoimmunity in NZB/NZW mice. *Arthritis Rheum* 1979; **22**: 1162–9
10. Verheul HAM, Schot LPC, Deckers GHJ, Schuurs, AHWM. Effects of tibolone, lynestrenol, ethylestrenol, and desogestrel on autoimmune disorders in NZB/W mice. *Clin Immunol Immunopathol* 1986; **8**: 198–208
11. Keisler LW, Walker, SE. Suppression of reproductive function in autoimmune NZB/W mice: Effective doses of four contraceptive steroids. *Am J Reprod Immunol Microbiol* 1987; **14**: 115–21
12. Smith AL, Bottomly K, Winograd DF. Altered splenic T cell

function of BALB/cByJ mice infected with mouse hepatitis virus or Sendai virus. *J Immunol* 1987; **138**: 3426–30
13. Loew FM, Fox JG. Animal health surveillance and health delivery systems. In: Foster HL, Small JD, Fox JG, eds. *The Mouse in Biomedical Research*. Vol 3. New York: Academic Press, 1983; 69–82
14. Talal N, Roubinian JR, Dauphinee MJ, Jones LA, Siiteri PK. Effects of sex hormones on spontaneous autoimmune disease in NZB/NZW hybrid mice. In: Hadden J, Chedid L, Mullen P, Spreafico F, eds. *Advances in Immunopharmacology. Proceedings of the First International Conference on Immunopharmacology*. New York: Pergamon Press, 1981; 127–37
15. Steinberg AD, Melez KA, Raveche ES, Reeves JP, Boegel WA, Smathers PA, *et al*. Approach to the study of sex hormones in autoimmunity. *Arthritis Rheum* 1979; **22**: 1170–6
16. Siiteri PK, Jones LA, Roubinian J, Talal N. Sex steroids and the immune system – I. Sex difference in autoimmune disease in NZB/NZW hybrid mice. *J Steroid Biochem* 1980; **12**: 425–32
17. Walker SE, Bole GG. Augmented incidence of neoplasia in NZB/NZW mice treated with long-term cyclophosphamide. *J Lab Clin Med* 1973; **82**: 619–33
18. Walker SE, Bole GG. Influence of natural and synthetic estrogens on the course of autoimmune disease in the NZB/NZW mouse. *Arthritis Rheum* 1973; **16**: 231–9
19. Berden JHM, Hang L, McConahey PJ, Dixon FJ. Analysis of vascular lesions in murine SLE. *J Immunol* 1983; **130**: 1699–1705
20. Walker SE, Kier AB, Siegfried EC, Harris BG, Schultz JS. Accelerated autoimmune disease and lymphoreticular neoplasms in F_1 hybrid PN/NZB and NZB/PN mice. *Clin Immunol Immunopathol* 1986; **39**: 81–92
21. Collins RD, Cousar JB, Russell WG, Glick AD. Diagnosis of neoplasms of the immune system. In: Rose NR, Friedman H, eds. *Manual of Clinical Immunology*. Second edition. Washington, D.C.: Americal Society for Microbiology, 1980; 84–101
22. Goldenberg GJ, Paraskevas F, Israels LG. Lymphocyte and plasma cell neoplasms associated with autoimmune diseases. *Semin Arthritis Rheum* 1971; **1**: 174–93
23. Della Porta G, Chieco-Bianchi L, Pennelli N. Tumours of the haemopoietic system. In: Turusov VS, ed. *Pathology of Tumours in Laboratory Animals. Tumours of the Mouse*. Vol 2. Publication no. 23. Lyon: International Agency for Research on Cancer, 1979; 527–36
24. Walker SE, Solsky M, Schnitzer B. Prolonged lifespans in female NZB/NZW mice treated with the experimental immunoregulatory drug frentizole. *Arthritis Rheum* 1982; **25**: 1291–7
25. Walker SE, Bole GG. Selective suppression of autoantibody responses in NZB/NZW mice treated with long-term cyclophosphamide. *Arthritis Rheum* 1975; **18**: 265–72
26. Walker SE, Hewett JE. Responses to T-cell and B-cell mitogens in autoimmune Palmerston North and NZB/NZW mice. *Clin Immunol Immunopathol* 1984; **30**: 469–78
27. Walker SE, Gray RH, Fulton M, Wigley RD, Schnitzer B. Palmerston North Mice, a new animal model of systemic lupus erythematosus. *J Lab Clin Med* 1978; **92**: 932–45
28. Snedecor GW, Cochran WG. *Statistical Methods*. Sixth edition. Ames, IA: Iowa State University Press, 1967
29. Walker SE, Anver MR. Accelerated appearance of neoplasms in female NZB/NZW mice treated with high-dose cyclophosphamide. *Arthritis Rheum* 1979; **22**: 1338–43
30. Melez KA, Boegel WA, Steinberg AD. Therapeutic studies in New Zealand mice. VII. Successful androgen treatment of NZB/NZW F_1 females of different ages. *Arthritis Rheum* 1980; **23**: 41–7
31. Hulka JF, Lieberman MW. Effect of synthetic progestational agents on allograft rejection and circulating antibody production. *Endocrinology* 1965; **77**: 897–901
32. Vecchi A, Tagliabue A, Mantovani A, Anaclerio A, Barale C, Spreafico F. Steroid contraceptive agents and immunological reactivity in experimental animals. *Biomedicine (Paris)* 1976; **24**: 231–7
33. Rojo JM, Portoles MP, Portoles A. Immune responses and lymphocyte mitogenic activation in mice under oral contraceptive treatment. *J Reprod Immunol* 1980; **2**: 29–35
34. Rojo JM, Portoles MP. Lymphoid-organ variations and blastogenic responses of splenocytes in estrogen-progestogen treated virgin and multiparous mice. *Int J Clin Pharmacol Ther Toxocol* 1982; **20**: 139–41
35. Verheul HAM, Stimson WH, Den Hollander FC, Schuurs AHWM. The effects of nandrolone, testosterone and their decanoate esters on murine lupus. *Clin Exp Immunol* 1981; **44**: 11–7
36. Murad F, Haynes RC Jr. Estrogens and progestins. In: Gilman AG, Goodman LS, Gilman A, eds. *The pharmacological Basis of Therapeutics*. Sixth edition. New York: Macmillan Publishing Co, 1980; 736–44
37. Pearce PT, Khalid BAK, Funder JW. Progesterone receptors in rat thymus. *Endocrinology* 1983; **113**: 1287–91
38. Fabris N, Piantanelli L, Muzzioli M. Differential effect of pregnancy or gestagens on humoral and cell-mediated immunity. *Clin Exp Immunol* 1977; **28**: 306–14
39. Marotti T, Sirotkovic M, Pavelic J, Gabrilovac J, Pavelic K. *In vivo* effect of progesterone and estrogen on thymus mass and T-cell functions in female mice. *Horm Metab Res* 1984; **16**: 201–3
40. Gupta C, Bullock LP, Bardin CW. Further studies on the androgenic, anti-androgenic, and synandrogenic actions of progestins. *Endocrinology* 1978; **102**: 736–44
41. Bullock LP, Bardin CW, Sherman MR. Androgenic, antiandrogenic, and synandrogenic actions of progestins: Role of steric and allosteric interactions with androgen receptors. *Endocrinology* 1978; **103**: 1768–82
42. Gardner WU. Estrogens in carcinogenesis. *Arch Pathol* 1939; **27**: 138–70
43. Noble RL, Hochachka BC, King D. Spontaneous and estrogen-produced tumors in Nb rats and their behavior after transplantation. *Cancer Res* 1975; **35**: 766–80
44. Tougard C, Tixier-Vidal A. Lactotropes and Gonadotropes. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York: Raven Press, 1988; 1305–33
45. Buckley AR, Putnam CW, Russell DH. Prolactin as a mammalian mitogen and tumor promoter. *Adv Enzyme Regul* 1988; **37**: 371–91
46. Ly Y. Prolactin stimulates transcription of growth-related genes in Nb2 T lymphoma cells. *Mol Cell Endocrinol* 1990; **68**: 21–8
47. Selye H. On the toxicity of oestrogens with special reference to diethylstilboestrol. *Canada Med Assoc J* 1939; **41**: 48–9
48. Burrows H, Kennaway NM. On some effects produced by applying oestrin to the skin of mice. *Am J Cancer* 1934; **20**: 48–57
49. Melez KA, Reeves JP, Steinberg AD. Modification of murine lupus by sex hormones. *Ann Immunol (Paris)* 1978; **129C**: 707–14