American Journal of Pathology, Vol. 131, No. 3, June 1988


# *Sex Hormones Modulate the Response of Pulmonary Perivascular Inflammation to Cyclophosphamide Therapy in MRL/MpJ-lpr/lpr Mice*

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**Responses of pulmonary perivascular infiltrates to immunosuppressive therapy with cyclophosphamide (CY) were evaluated in the MRL/MpJ-lpr/lpr (MRL/l) mouse, a model for the study of systemic lupus erythematosus. Male and female mice were divided into the following groups: controls injected with saline; intact mice receiving CY; castrated CY-treated mice; castrated, hormone implanted, CY-treated mice. CY treatment began at 30 days of age and animals were killed at 60 days of age. Lungs were fixed–inflated to 26 cm $H_2O$ pressure with glutaraldehyde–formaldehyde fixative. The pulmonary perivascular response to immunosuppressive therapy was graded depending on the extent of infiltrates surrounding 15 pulmonary vessels per animal. Intact males treated with CY alone had almost complete clearing of perivascular infiltrates, whereas intact females did not respond to therapy. Castrated CY-treated males showed a decreased response to CY compared to intact CY-treated males. Castrated, estradiol-implanted males had no response to CY therapy. Estradiol interfered with the therapeutic response to CY in male MRL/l mice. (Am J Pathol 1988, 131:530–538)**

THE LUNG is an organ in which autoimmune disease is often expressed; this association has been demonstrated in the human prototype of immune complex disease, systemic lupus erythematosus (SLE). Histopathology of lung tissue in affected individuals is usually characterized by interstitial pneumonitis, local alveolar hemorrhage, and chronic pleural thickening with interstitial and perivascular infiltrates of lymphocytes and plasma cells.[1,2] In patients with SLE and symptomatic pulmonary disease, deposits of immunoglobulin and complement representing immune complexes have been detected in alveolar walls around capillaries and in small pulmonary vessels.[3–6] Because pulmonary involvement may contribute substantially to morbidity and lead to chronic restrictive lung disease in SLE,[7] it is necessary to seek therapeutic regimens that afford the possibility of arresting this complication.

The MRL/MpJ-lpr/lpr (MRL/l) mouse has been utilized in many laboratories as a model for the study of SLE. These animals exhibit a spontaneous autosomal recessive mutation (lpr) that produces generalized lymph node enlargement and premature death with autoimmune glomerulonephritis and vasculitis.[8] Pulmonary infiltrations with lymphocytes and plasma cells have been recognized in MRL/l mice as early as 2 months of age.[8–10] Because these lesions resemble the diffuse cellular infiltrates in pulmonary SLE, the MRL/l substrain is used as a model to determine if immunosuppressive therapy diminishes numbers of pulmonary lymphocytes and plasma cells.[10]

Other investigators have shown that therapy with the potent immunosuppressive drug cyclophospha-

Supported by the Research Service of the Veterans Administration.

Accepted for publication January 28, 1988.

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mide (CY) was associated with diminished lymphoproliferation, reduced autoantibody levels, and prolonged survival in MRL/l mice.[9] Because this therapy was successful in treating other manifestations of disease, CY was used to modify pulmonary inflammation in this model.[10] An unexpected result of this study was the discovery that CY was effective in males but not in females. Because sex was an important factor determining outcome, the studies were extended to examine influences of gonadal hormones on effectiveness of therapy with CY. The current report describes CY treatment in MRL/l mice of both sexes that were castrated or castrated and given implants of gonadal hormones. Our earlier finding that intact females were relatively resistant to therapy with CY was confirmed. Estradiol was found to be a critical factor regulating response of pulmonary inflammation to CY therapy.

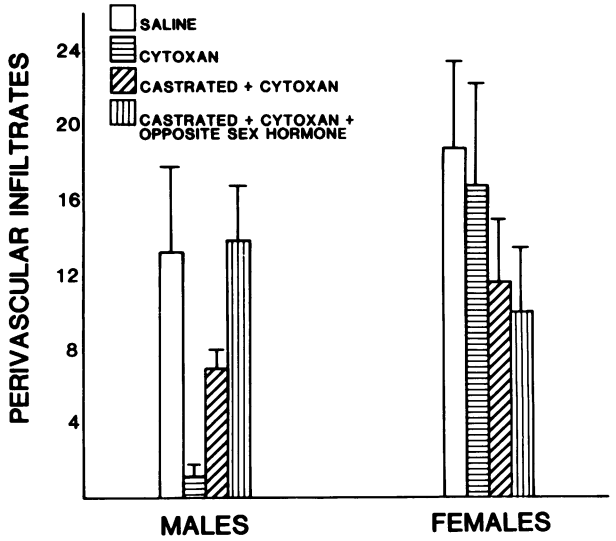



**Figure 1**—Mean pulmonary perivascular inflammation scores are represented by bars; lines indicate SEM. CY-treated males showed a highly significant reduction in pulmonary perivascular lung scores compared to saline-injected males ($P < 0.005$). Castration of males and implantation of estradiol completely abrogated this response to therapy; there was no difference between saline-treated controls and CY-treated castrates receiving estradiol. Comparisons of groups of treated females with corresponding controls revealed no significant differences in perivascular lung scores.

## Materials and Methods

### Animals

MRL/l mice were maintained in pathogen-free colonies in the Department of Laboratory Animal Medicine, University of Missouri Health Sciences Center (Columbia, Mo.). These animals descended from breeding pairs obtained from Jackson Laboratories (Bar Harbor, Me.).

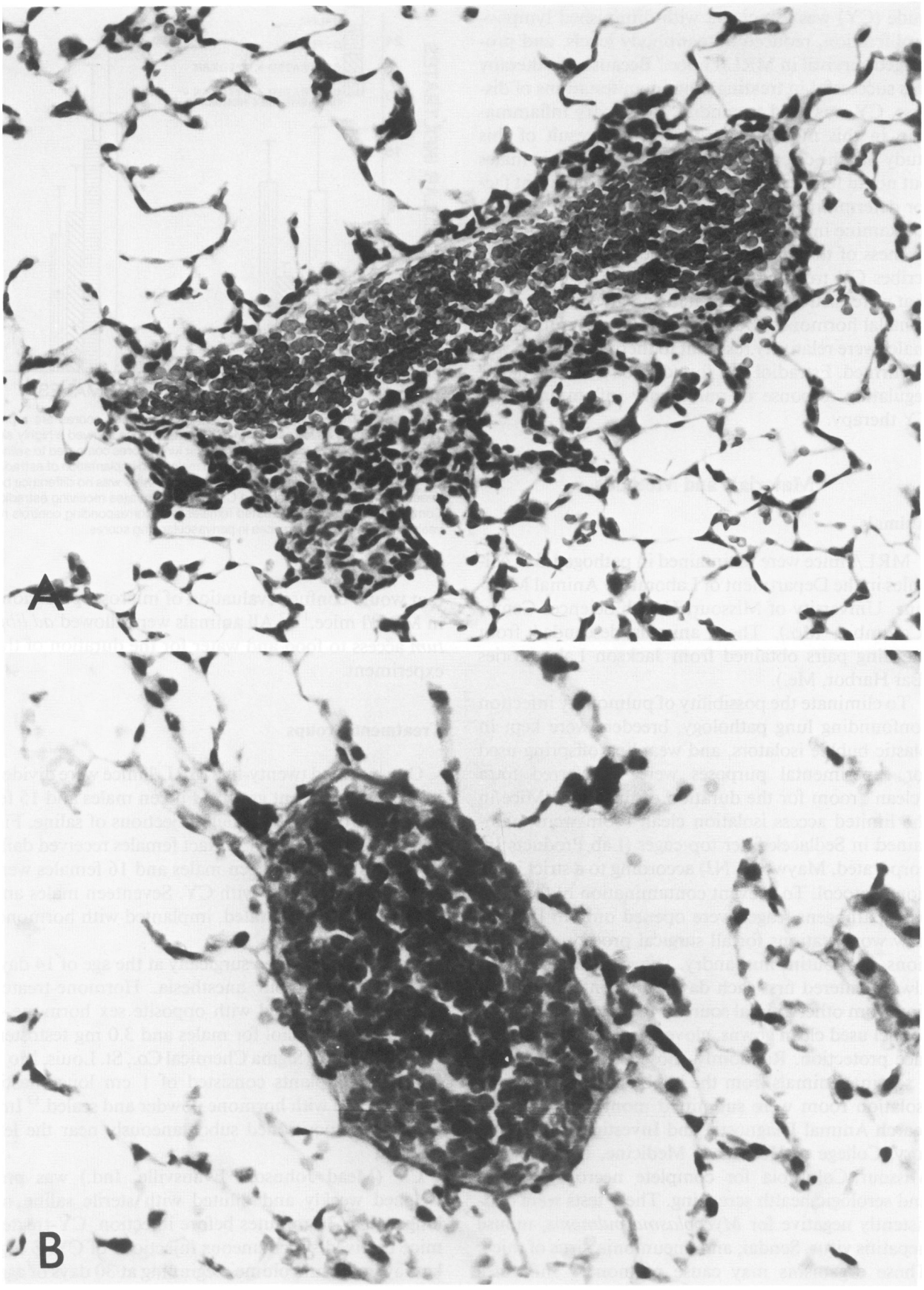
To eliminate the possibility of pulmonary infection confounding lung pathology, breeders were kept in plastic bubble isolators, and weanling offspring used for experimental purposes were transferred to a "clean" room for the duration of the study. Mice in the limited access isolation clean room were maintained in Sedlacek filter top cages (Lab Products Incorporated, Maywood, NJ) according to a strict isolation protocol. To prevent contamination of the mice with pathogens, cages were opened only in laminar flow work stations for all surgical procedures, injections, and routine husbandry. The isolation room was always entered first each day to prevent contamination from other animal sources. Prior to entry all personnel used clean gowns, gloves, and head, shoe, and face protection. Randomly chosen MRL/l and CD-1 sentinel animals from the bubble isolators and the isolation room were submitted monthly to the Research Animal Diagnostic and Investigative Laboratory, College of Veterinary Medicine, University of Missouri-Columbia for complete necropsy studies and serologic health screening. These tests were consistently negative for *Mycoplasma pulmonis,* mouse hepatitis virus, Sendai, and pneumonia virus of mice. Those organisms may cause pulmonary infiltrates that would confuse evaluation of microscopic lesions in MRL/l mice.[11,12] All animals were allowed *ad libitum* access to food and water for the duration of the experiment.

### Treatment Groups

One hundred twenty-five MRL/l mice were divided into eight treatment groups. Fifteen males and 15 female controls received daily injections of saline. Fifteen intact males and 15 intact females received daily injections of CY. Sixteen males and 16 females were castrated and treated with CY. Seventeen males and 16 females were castrated, implanted with hormone, and treated with CY.

Mice were castrated surgically at the age of 14 days by ketamine/rompin anesthesia. Hormone-treated mice were implanted with opposite sex hormone—2.5 mg 17$\beta$-estradiol for males and 3.0 mg testosterone for females (Sigma Chemical Co., St. Louis, Mo.). Hormone implants consisted of 1 cm long silastic tubes packed with hormone powder and sealed.[13] Implants were positioned subcutaneously near the left scapula.

CY (Mead Johnson, Evansville, Ind.) was preweighed weekly and diluted with sterile saline no longer than 30 minutes before injection. CY-treated mice received subcutaneous injections of CY, 8 mg/kg/day in 0.1 ml volume, beginning at 30 days of age.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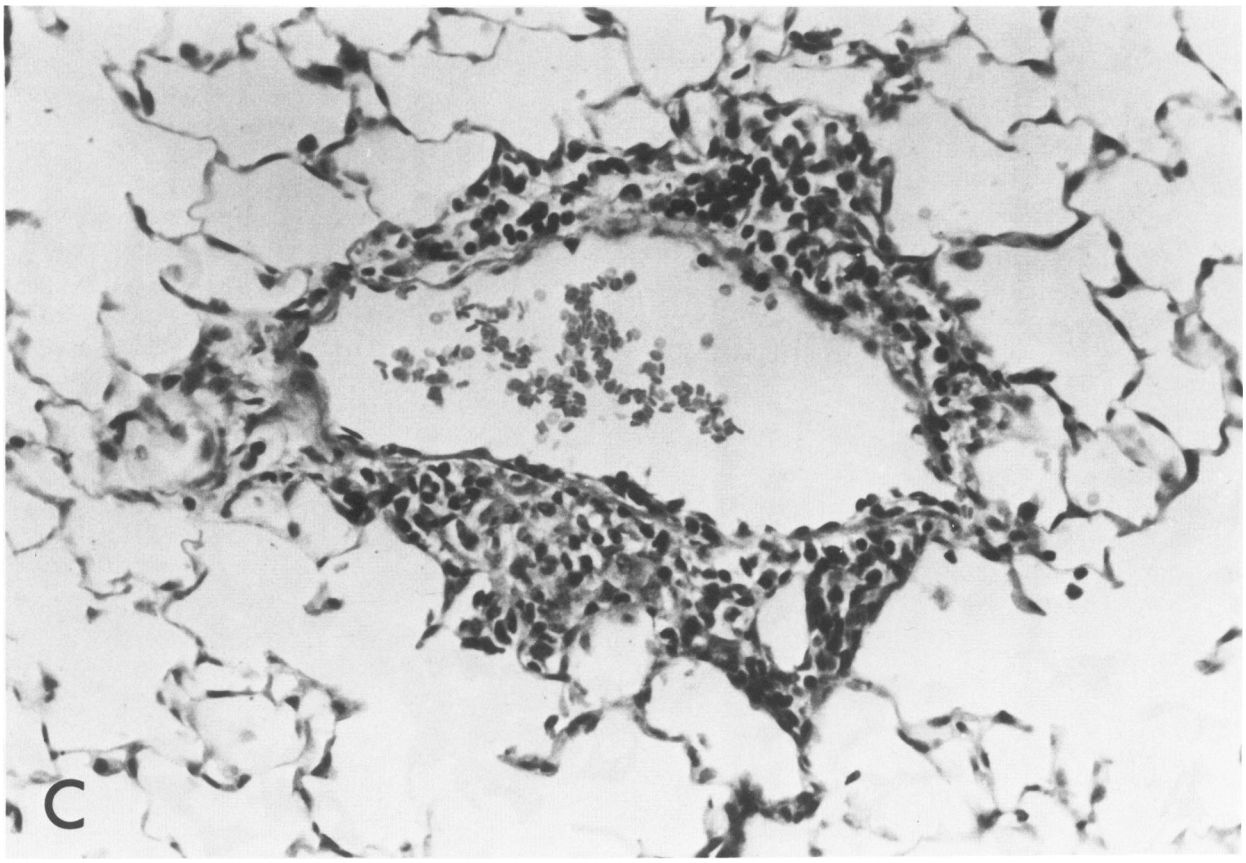



**Figure 2**—Effects of CY therapy on infiltrates around pulmonary vessels in 2-month-old MRL/l males. **A**—Lymphocytes and plasma cells surround a pulmonary vessel (grade = 3) in a control animal. **B**—No perivascular infiltrate is present in a vessel from a comparable intact CY-treated male. The vessel was graded 0. **C**—The perivascular infiltrate, graded 4, in a CY-treated male castrate implanted with estradiol resembles lesions in untreated controls (all photomicrographs; H&E, ×500).

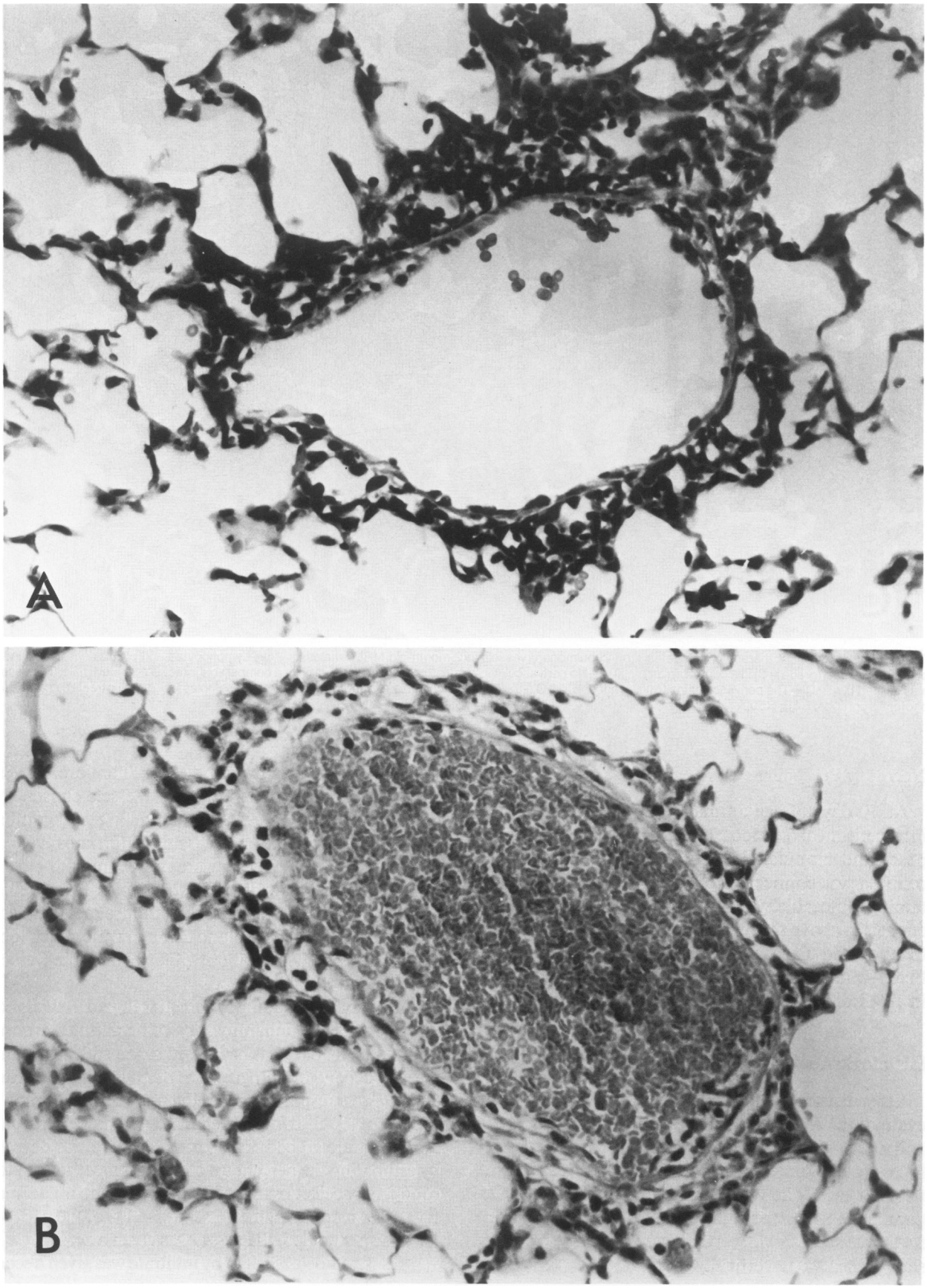
### Necropsy Procedure

At 60 days of age animals were anesthetized with intraperitoneal pentobarbital. The trachea was exposed and cannulated with a #16 gauge cannula. The cannula was connected to a 3-ml syringe barrel containing glutaraldehyde–formaldehyde fixative[14] at 26 cm height above the lung plane. Lungs were filled with fixative and fixed in inflation for 10–15 minutes. Fixed–inflated lobes were removed, sliced, and placed in 10% buffered formalin for light microscopy.

### Histologic Analysis

Lung tissues were randomized and coded before evaluation. A 6-$\mu$ section stained with hematoxylin and eosin was cut from each of the five pulmonary lobes in each animal. An approximate cross-section through each lobe was examined in a set pattern, beginning at the lower right corner of the section. The first three suitable vessels that were encountered as the slide was moved vertically were graded, whether or not a perivascular cuff was present. Therefore, 15 vessels were graded in each animal. Vessels chosen for grading were 50–100 $\mu$ in diameter. To avoid confusing peribronchial and perivascular infiltrates, vessels adjacent to larger airways were not evaluated. No attempt was made to distinguish arteries from veins. Approximately 25 vessels in each mouse met these criteria, depending on the plane through which each lobe was sectioned. Thus, about 60% of suitable vessels were graded in each animal.

The numerical grading system, developed in this laboratory to classify inflammatory cell infiltrates around pulmonary vessels, has been used successfully to evaluate age-dependent progression of pulmonary disease and to assess responses to CY therapy in MRL/l mice.[15] For each vessel, two characteristics of the perivascular infiltrate were graded. The perimeter score was derived by estimating the percentage of the vessel perimeter surrounded by cells. A score of 0 was given if no inflammatory cells surrounded the vessel. If 5–20% of the perimeter was surrounded, the vessel was given a score of 1. A vessel surrounded 25–45% by infiltrate was given a score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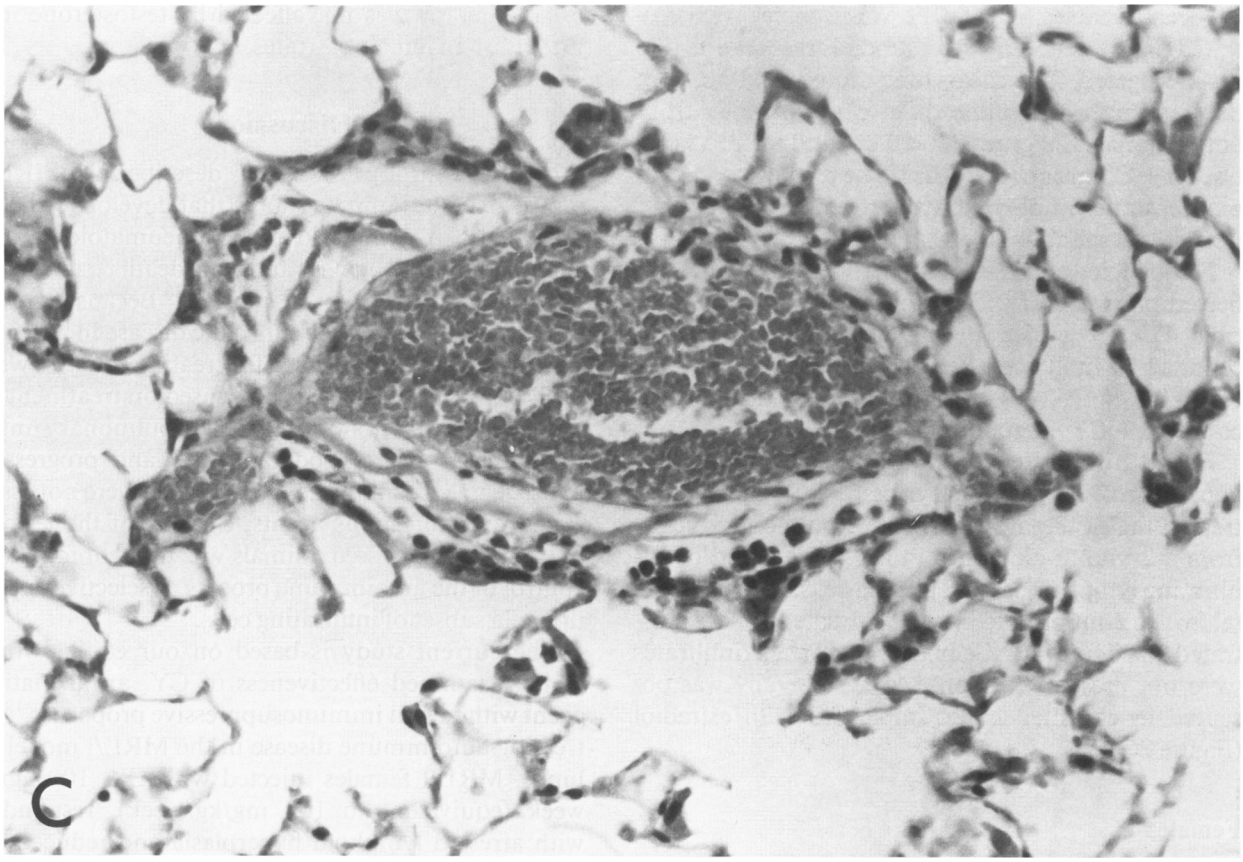


**Figure 3**—Pulmonary vessels from 2-month-old MRL/l females. **A**—A control female has extensive perivascular infiltration, graded 4, composed of lymphocytes and plasma cells. **B**—In this CY-treated female a similar perivascular infiltrate is graded 4. **C**—Castration and implantation of testosterone did not eliminate grade 3 infiltration around the vessel illustrated (all photomicrographs, H&E, ×500).

of 2. Three points were given if 50–70% of the vessel was surrounded, and 4 points were added to the score for a vessel surrounded 75–100%. The second measurement, the width score, was obtained using a 10 × 10 grid in one 10× eyepiece and a 40× objective (total magnification 400×) to score the width of each perivascular infiltrate; each measurement was made at a region that seemed to be the mean thickness for that vessel. Infiltrates extending into 50% or more of a grid were considered to occupy an entire grid. Based upon the number of small divisions in the 10 × 10 grid occupied by the width of the perivascular infiltrate, a numerical multiplier of 1, 2, or 3 was assigned to the vessel. This number was multiplied by the perimeter score for that vessel. The total number given to each slide was the sum of the scores for 15 pulmonary vessels.

The same examiner, reviewing coded slides repeatedly, gave 95% of vessels the same score. Three examiners, who evaluated coded slides independently, produced identical scores in 90% of involved vessels.

### Statistical Analyses

Pulmonary inflammation scores in treated and control groups were compared using analysis of variance,[16] and differences among means were determined by the least significant difference method of Fisher.[17]

## Results

### Males

Figure 1 contains mean perivascular infiltrate scores for saline-injected controls, and for treatment groups receiving CY alone, castration + CY, or castration + CY + implant of estradiol. In accord with our earlier results,[10,15] intact males treated with CY alone had almost complete clearing of perivascular infiltrates. The mean pulmonary perivascular infiltration score for intact CY-treated males was 1 ± 0.4 (mean ± SEM). This was a significant reduction compared to control males receiving saline, with a score of 13 ± 4 ($P < 0.005$). In castrated males CY treatment appeared to be partially effective. These mice did not differ significantly from saline-injected control males, but perivascular infiltrate scores in castrated + CY males were increased significantly compared to intact

CY-treated males ($P < 0.01$). When males were castrated and implanted with estradiol, response to CY was abrogated. The mean lung score was 14 ± 3 in these animals; this value differed significantly from both the male CY group ($P < 0.01$) and the male castration + CY group ($P < 0.05$). The response to CY in male castrates implanted with estradiol did not differ from intact saline controls.

Frequencies of vessels with perivascular cuffing reflected perivascular infiltration scores. In male controls 47% of graded pulmonary vessels were surrounded by infiltrates. Perivascular cuffing was present in 8% of CY, 45% of castrated + CY, and 58% of castrated + CY + estradiol males.

Figure 2 illustrates the contrasting histopathologic appearances of pulmonary tissue in control and treated males. Figure 2A shows a pulmonary vessel from a 2-month-old control male surrounded by infiltrating lymphocytes and plasma cells. A typical vessel from a 2-month-old intact CY-treated male is illustrated in Figure 2B. Cellular perivascular infiltrates were not present. Response to CY therapy was obscured in castrated males implanted with estradiol (Figure 2C).

### Females

Females in this therapeutic study were resistant to treatment with CY, and this resistance was not abolished by castration or implantation with testosterone (Figure 1). The mean perivascular infiltration score in saline-injected female controls was 19 ± 5, and the score in intact CY-treated females was 17 ± 5. In castrated females treated with CY the mean lung score was reduced to 12 ± 4. CY-treated castrated females implanted with testosterone had a mean lung score of 11 ± 4. Statistical comparisons of control females with each separate group of treated females, and comparisons among the three groups of treated females showed that perivascular infiltration was not altered by CY castration + CY, or castration + CY + testosterone.

In control females 55% of vessels that were graded had perivascular infiltrates. Frequencies of pulmonary vessels with cuffing were 46% in CY-treated females, 50% in castrated + CY females, and 44% in castrated + CY + testosterone females.

Figures 3A and 3B illustrate the perivascular infiltrates observed in control females and CY-treated females. Inflammatory mononuclear cells surrounded pulmonary vessels in both animals. Figure 3C illustrates the infiltrate surrounding a pulmonary vessel from a castrated female, implanted with testosterone and treated with CY. It was concluded that response to CY therapy was not affected by testosterone implantation in female castrates.

## Discussion

In 1978 Murphy and Roths[8] described the MRL/l mouse, an autoimmune model that develops striking hyperplasia of lymphoid tissue, rheumatoid factor, and antibodies to nuclear antigens; death results from glomerulonephritis and vasculitis.[8,18] Because immunosuppressive therapy of lupuslike disease in autoimmune models has relevance for treating humans with SLE, our recent studies have focused on treatment of the extensive lymphoproliferative pulmonary infiltrates that appeared spontaneously and progressed with age in MRL/l mice.[8] These lesions were not associated with infectious agents; clearing of the lesions in immunosuppressed animals was thought to reflect control of the autoimmune process or selective depletion of a subset of infiltrating cells.

The current study is based on our earlier work, which examined effectiveness of CY, an alkylating agent with potent immunosuppressive properties,[19] in treating autoimmune disease in the MRL/l model of lupus. MRL/l females injected with CY, 100 μg/g/week (equivalent to 100 mg/kg/week), responded with arrested lymphoid hyperplasia and reduced severity of renal disease.[9] The dose of CY employed in the current study (8 mg/kg/day) has been effective in preventing early death from glomerulonephritis and eliminating perivascular infiltrates of inflammatory cells in renal tissue in autoimmune mice.[20] It was therefore anticipated that our program of chronic immunosuppressive therapy with CY would suppress perivascular infiltrates in the lungs of MRL/l mice. This theory was supported in our initial study,[10] which produced evidence that CY eliminated perivascular, peribronchial, and interstitial infiltrates. However, an unanticipated result was the clear sex-determined difference in response to treatment. MRL/l males responded but females were resistant to a dose of 8 mg/kg/day.

The current study was designed to investigate hormonal influences on CY therapy by determining if castration and treatment with exogenous gonadal hormones affected pulmonary inflammation. As illustrated in Figure 1, the expected response to CY was abrogated in castrated MRL/l males implanted with estradiol. In females therapy with CY 8 mg/kg/day had no effect; this resistance to treatment was not suppressed by castration plus treatment with testosterone. It seemed likely that hormonal regulation of disease severity influenced outcome of CY therapy of pulmonary inflammation in MRL/l mice. In another

widely studied animal model of lupus, the NZB/W mouse, early onset of disease was triggered by sexual maturity in females,[21] which have accelerated disease and die at 10 months of age. In contrast, males live to a mean age of 16 months.[22] Female NZB/W mice with established autoimmune disease show improved survival when treated with androgens.[23] Prepubertal castration and therapy with 17$\beta$-estradiol led to early death in male NZB/W mice.[24] Therefore, it may be proposed that an analogous condition existed in intact MRL/l females and estradiol-treated males in the current study, and female hormones may have stimulated autoimmunity and pulmonary inflammation.

Exogenous estrogen clearly affects immune responsiveness in autoimmune mice. Implants of 17$\beta$-estradiol have been shown to stimulate elevated levels of antibodies directed against DNA and non-DNA nuclear antigens in MRL/l mice.[25] In other experiments short-term injections of estradiol depleted thymic populations of Lyt-2$^+$ T cells in C57Bl/6 mice bearing the lpr/lpr genome.[26] Hormone-mediated activation of B cells and removal of a population of cytotoxic/suppressor T-cells should theoretically have increased the inflammatory pulmonary disease in MRL/l mice.

Although female hormones stimulated the immune system in MRL/l mice,[25] opposing evidence exists that estradiol has little effect on the outcome of autoimmune disease in these animals. A modest sex-related difference in longevity has been described in this strain.[18] Studies in this laboratory[10] have established that there was no inherent difference in onset of pulmonary perivascular infiltrates in MRL/l males and females. This finding was confirmed in the current study, in which 2-month-old males had a mean lung score of 13 ± 5; this value did not differ significantly from comparable females (19 ± 5). Shear et al[27] found that castration and therapy with implanted 17$\beta$-estradiol did not alter autoantibodies, renal function, or longevity in MRL/l mice of both sexes. Because the MRL model of autoimmunity may not be responsive to exogenous estradiol, we considered the possibility that factors other than metabolic interference and immune stimulation accounted for resistance to CY therapy.

Based on recent studies of humans with SLE, it may be considered that degradation of estrogen in SLE-like mice differs from nonautoimmune mice. Lahita et al[28] found elevated levels of 16-$\alpha$-hydroxylated metabolites in both men and women with SLE and proposed that abnormally elevated estrogenic metabolites chronically stimulated the immune system. Although a recent study has shown that estrogen metabolism through the 2-hydroxylation pathway is accelerated in NZB/W mice,[29] 16-$\alpha$-hydroxylation may be abnormal. Delayed breakdown of estradiol in lupus mice and continued immunologic stimulation resulting from accumulated products of estrogen metabolism could potentially interfere with immunosuppressive therapy. This process could explain the resistance to CY treatment we observed in intact females in the initial study[10] and in the current study. Furthermore, accumulated estrogenic metabolites may have interfered with CY-induced immunosuppression in male castrates implanted with estradiol.

Explanations for the results observed in estradiol-treated MRL/l males should also take into account potential interactions between estradiol and CY as they undergo degradation in the liver. CY is inert, and *in vivo* activation is required to liberate active metabolites.[30] We are not aware that hepatic processing of estradiol interferes directly with CY metabolism, but related steroid hormones (prednisolone, testosterone) interfere with conversion of CY to its metabolites.[31] This inhibition, which occurs through the cytochrome P-450 system,[32] may have made the dose of CY used in this study inadequate to suppress autoimmunity.

In summary, this study confirmed our previous observation that intact MRL/l males responded to CY therapy and intact females did not.[10] Castrated CY-treated males showed a decreased response to CY compared to intact CY-treated males. Castrated, estradiol-implanted males responded to CY therapy much the same as intact females, and endogenous testosterone seemed to play a synergistic role in CY therapy in males.

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