Pulmonary Inflammation in Autoimmune MRL/Mp-lpr/lpr Mice

Histopathology and Bronchoalveolar Lavage Evaluation

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Early detection of lupus pneumonitis is difficult because it requires lung biopsy. The authors describe here in detail 1) the age-related histologic changes in pulmonary inflammation, 2) the age-related changes in bronchoalveolar lavage (BAL), and 3) the effect of cyclophosphamide (8 mg/kg) on pulmonary inflammation and bronchoalveolar lavage in MRL/Mp-lpr/lpr mouse, an animal model of systemic lupus erythematosus. To assess the evolution of pulmonary inflammation and response to cyclophosphamide therapy, they compared the age-related progression of pulmonary inflammation with sequential changes in BAL cell populations in this autoimmune mouse model. A striking similarity was noted between age-related changes in pulmonary inflammation and lymphocyte counts in BAL. A trend to reduction in histologic evidence of inflammation was reflected by lymphocites in BAL in cyclophosphamide-treated (8 mg/kg/day) males but not in females. There was a striking sex-related difference in that the histologic evidence of pulmonary inflammation and bronchoalveolar lavage lymphocyte count in cyclophosphamide-treated males was significantly lower than cyclophosphamide-treated females of the same age. (Am J Pathol 1986, 124:353–362)

PULMONARY involvement may be an important cause of morbidity and mortality in systemic lupus erythematosus (SLE) and mixed connective tissue disease,[1-5] but the natural history of this pulmonary inflammation has not been defined. Lung disease secondary to SLE includes interstitial pneumonitis with mononuclear cellular infiltration, interstitial fibrosis, edema, and pulmonary vasculitis.[4] Even though in autopsy studies a high incidence of interstitial pneumonitis (98%) and bronchopneumonia (57%) are reported,[5] further studies have shown that when other causes such as infection are clearly excluded the incidence is reduced to 13%.[4] Despite adequate therapy and clinial improvement in acute disease, many of these patients experience recurrent episodes and frequently develop restrictive lung disease of varying severity.[1] The diagnosis of lupus pneumonitis is one of exclusion, and lung biopsy may be required for definitive diagnosis. Therefore, early detection is difficult, and there is a need for dependable, less invasive markers of lupus lung disease.

MRL/Mp-lpr/lpr (MRL/l) mice have been used as a murine model of SLE.[6-8] The autoimmune disease in these mice is characterized by generalized lymphadenopathy, immune complex glomerulonephritis, acute polyarteritis, hypergammaglobulinemia, anti-Sm antibodies, and high levels of circulating immune complexes. The usual cause of death in MRL/l mice is glomerulonephritis.[8] We have recently shown that this autoimmune mouse model spontaneously develops progressive pulmonary perivascular infiltrates.[9]

Bronchoalveolar lavage (BAL) has been used as a tool in the evaluation of interstitial lung disease, including pulmonary fibrosis associated with collagen vascular disease,[10-12] but sequential changes in BAL have not been reported in SLE. This study describes the age-related progression of pulmonary inflammation and the changes in BAL in MRL/l mice. In addition, we report the effect of cyclophosphamide therapy on pulmonary

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inflammation and BAL cell populations. The implications of this animal model toward understanding pulmonary inflammation in SLE and the role of BAL in the early diagnosis of this pulmonary inflammation are discussed.

Materials and Methods

Animals

Eighty-two male and 74 female MRL/Mp-lpr/lpr mice were obtained at the age of 21 days from our bubble-isolated breeding colony maintained by the Laboratory Animal Medicine Department at the University of Missouri. These mice were obtained from brother/sister mating of breeding pairs purchased from the Jackson Laboratory (Bar Harbor, Maine).

Disease Screening

To eliminate pulmonary infection from confounding our lung pathology, we transferred newborn animals from the breeding isolator to a maintenance isolator until they were weaned. Subsequently, weaned animals were transferred to a "clean" room until they were sacrificed. This "clean" room had limited access and was always entered first each day for prevention of contamination from other animal sources. Prior to entry, all personnel put on clean gowns, gloves, head, shoe, and face protection.

Randomly chosen animals from both isolators and the "clean" room were subjected to complete health screening on a monthly basis. Health screening was negative for mouse hepatitis virus and pneumonia virus of mice (PVM).

Lung Tissue

A total of 28 male and 25 female mice were sacrificed at 2, 4, and 6 months of age. The animals were anesthetized with 0.1 ml of intraperitoneal pentobarbital (50 mg/ml). The trachea was exposed and cannulated with a 16-gauge plastic catheter. The catheter was connected to a 3-ml syringe barrel containing glutaraldehyde-formaldehyde fixative[13] at 26 cm height above the lung plane. The lungs were filled with fixative and fixed in inflation for 10–15 minutes. Inflated lobes were removed, divided, and place in 10% buffered formalin for light microscopy. All tissues prepared for electron microscopy were postfixed with 1% osmium tetroxide for 1 hour, dehydrated in an increasing concentration of ethanol series, and embedded in Epon 812. One-micron-thick sections were cut with glass knives and stained with toluidine blue.[14] Selected blocks were retrimmed, and 60–90-μ sections were cut and place on 200 mesh copper grids. Each grid was stained for 10 minutes with uranyl acetate[15] and for 10 minutes with lead citrate.[16] Stained sections were reviewed using a Philips EM300 electron microscope at 80 kv.

Six-micron-thick hematoxylin and eosin (H&E)-stained sections were evaluated under light microscopy for the prevalence of disease by scoring cellular infiltrates around pulmonary vessels (*P*), bronchi (*B*), and interstitium (*I*) in 50 random fields (x160) with the use of all five lobes per animal. The presence or absence of infiltration was scored as 1 and 0, respectively.

Lavage Procedures

A total of 26 male and 24 female MRL/Mp-lpr/lpr mice were studied at 2, 4, and 6 months of age. They were littermates caged with the mice which were sacrificed for histologic studies. After intraperitoneal administration of 0.1 ml (50 mg/ml) pentobarbital, the trachea was exposed through an anterior neck incision and cannulated with a 20-gauge plastic catheter. The catheter was secured with silk sutures. Bronchoalveolar lavage was performed by infusion and gentle aspiration of a 1.5-ml aliquot of normal saline. The procedure was repeated three times, and a total volume of 3 to 4 ml of lavage was recovered from each mouse. Lavage samples were centrifuged at 500*g* for 5 minutes. The cells were suspended in 2 ml of Hanks' balanced salts solution without calcium or magnesium.[11]

Cellular Analysis

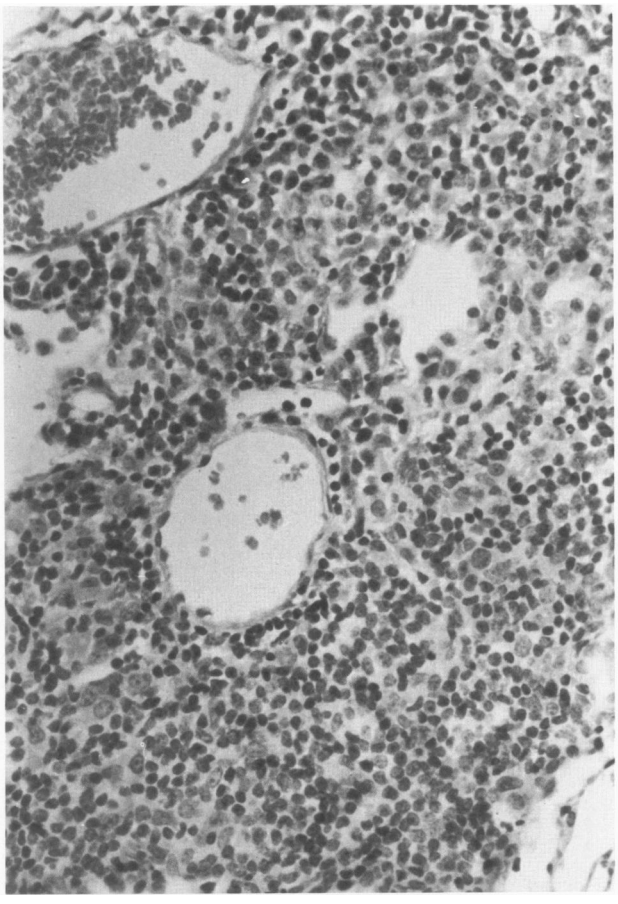
The total number of leukocytes in the BAL samples was determined with the use of a standard hemocytometer. Each cell suspension was placed in a cytocentrifuge, and smears were obtained. The differential count of BAL cells was determined by review of Wright-stained smears and subclassification of 300 consecutive cells.[12]

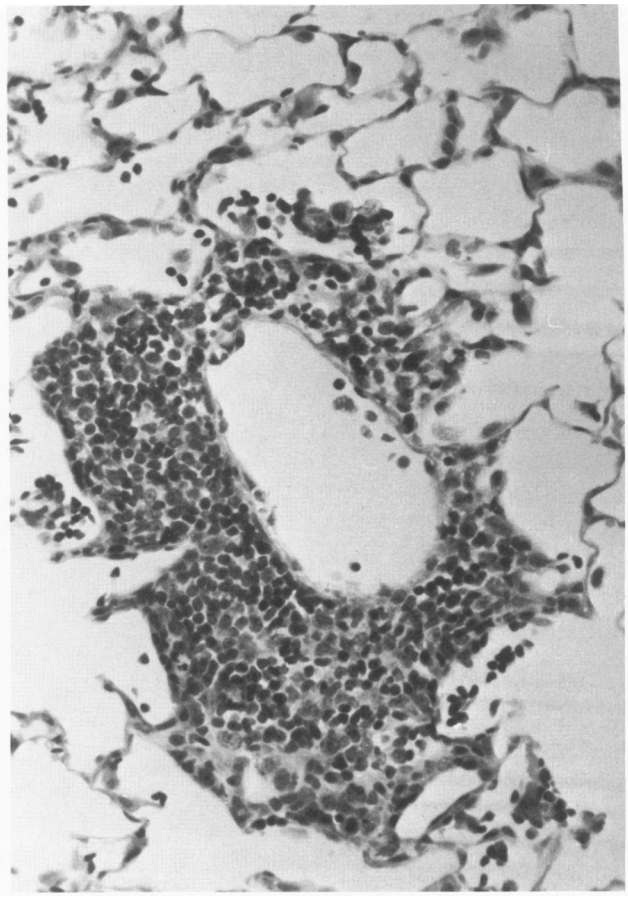
Cyclophosphamide Injection

Twenty-six male and 22 female MRL/Mp-lpr/lpr mice were given subcutaneous injections of 0.1 ml normal saline daily. Twenty-eight male and 25 female 30 day old mice received subcutaneous injections of cyclophosphamide 8 mg/kg/day for 30 days. The lung tissue and BAL fluid were processed as described above.

Statistical Analysis

Mean values ± standard error of mean (SEM) were calculated at each age for the infiltration scores in the lung tissue and for lymphocyte and polymorphonuclear





Figures 1 and 2—Perivascular infiltrate in a 4-month-old MRL/1 female mouse showing mature lymphocytes and some plasma cells. (Figure 1, original magnification, ×530; Figure 2, original magnification, ×550)

cells (cells per 300 cells) in BAL. Age-associated changes in the lung tissue and lavage were evaluated by use of the *t* test. The changes in the infiltration score in the lung tissue and cell count in BAL were likewise compared with the *t* test between saline-treated and cyclophosphamide-treated mice.

Results

Age-Related Changes in Histology

Lung sections from a representative 4-month-old MRL/l female revealed perivascular, peribronchial, and interstitial cellular infiltrate. Perivascular infiltrates consisted predominantly of mature lymphocytes and some plasma cells (Figures 1 and 2). By 6 months of age there was invasion of the vascular muscle wall (Figure 3). Likewise, the peribronchial infiltrates were composed of lymphocytes, but no mucosal ulceration was noted (Figure 4). Pronounced infiltration with lymphocytes and some plasma cells was observed in the interstitial space (Figures 5–7). Electron microscopy confirmed this infiltration to be composed predominantly of lymphocytes and some plasma cells. No polymorphonuclear leukocytes or eosinophils were seen (Figure 8).

In *male* mice, mean perivascular infiltrate scores were 2 ± 1 (mean ± SEM) at 2 months of age. Scores increased significantly to 19 ± 2 at 4 months ($P < 0.001$) and 17 ± 5 at 6 months ($P < 0.001$) when compared with 2 months of age. Mean peribronchial infiltrate scores were 2 ± 1 at 2 months of age with significant increases to 15 ± 2 and 13 ± 4 at 4 and 6 months, respectively ($P < 0.001$). The interstitial infiltrate scores increased from 1 ± 1 at 2 months of age to 3 ± 1, and 3 ± 2 at 4 ($P < 0.001$) and 6 months ($P < 0.05$), respectively (Figure 9). Perivascular infiltrate scores in *females* increased significantly from 4 ± 1 at 2 months of age to 15 ± 3 at 4 ($P < 0.001$) and 27 ± 3 at 6 months of age ($P < 0.001$). A similar significant increase was observed in peribronchial scores from 4 ± 1 at 2 months of age to 12 ± 2 and 18 ± 2 at 4 and 6 months of age ($P < 0.001$). Interstitial infiltrate scores increased from 2 ± 1 at 2 months of age to 4 ± 1 and 4 ± 1 at 4 and 6 months of age (in each instance, $P < 0.001$), respectively (Figure 9).

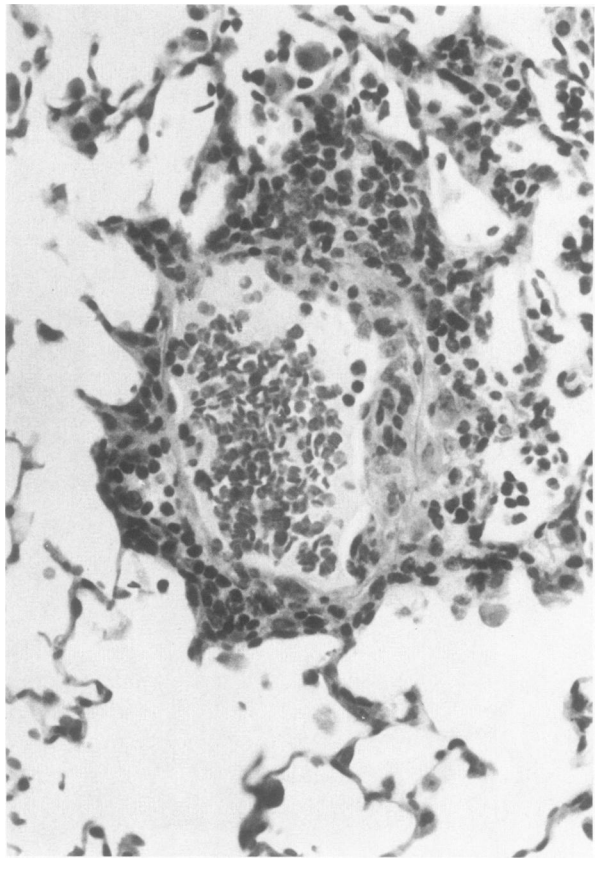


Figure 3—Cellular invasion of vascular muscle wall in a 6-month-old MRL/1 female mouse. (Original magnification, x 650)

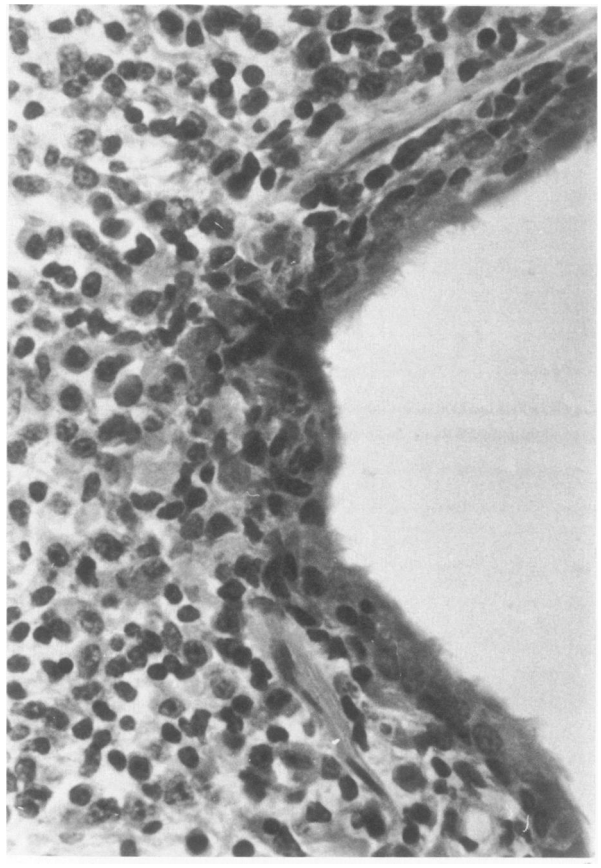


Figure 4—Peribronchial lymphocytic infiltration in a 4-month-old MRL/1 female mouse. (Original magnification, x 1000)

Age-Related Changes in BAL

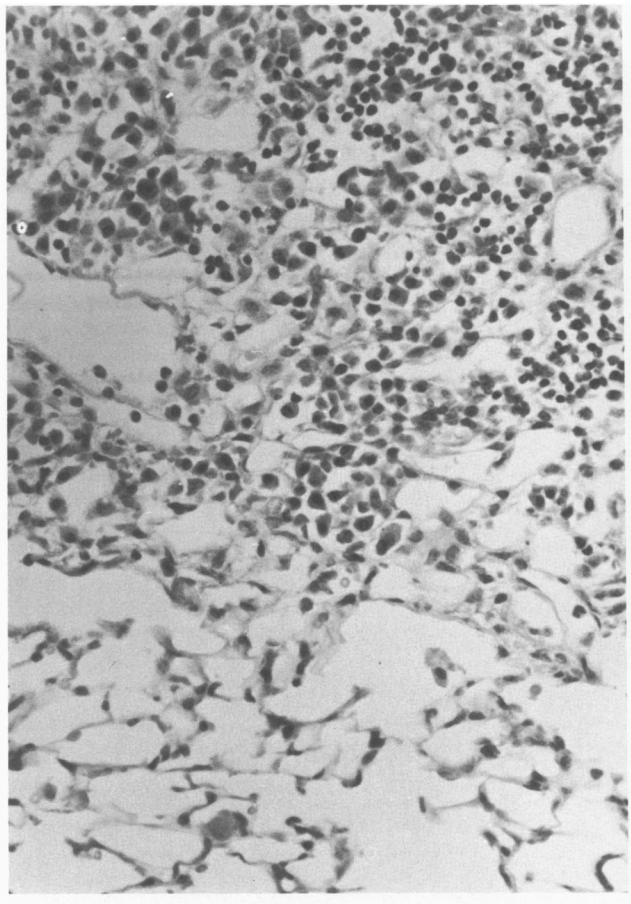
Total Leukocyte Count

The total leukocyte count in 2-month-old *male* mice was 1.5×10^6 cells/ml. At 4 months of age, the value was 1.7×10^6 cells/ml. At 6 months of age the value increased to 1.9×10^6 cells/ml ($P < .05$). In the 2-month-old *female* mice the total leukocyte count was 1.7×10^6 cells/ml. The values were 1.6×10^6 cells/ml and 1.7×10^6 cells/ml at 4 and 6 months, respectively.

Differential Cell Count

The mean *lymphocyte* count in saline-treated 2-month-old *male* mice was 19 ± 10 cells (mean ± SEM). There were significant (when compared with 2-month-old mice) increases in lymphocyte counts at 4 and 6 months of age (112 ± 28 and 90 ± 17, respectively) ($P < 0.01$). In *females* the lymphocyte count increased significantly from 13 ± 6 cells at 2 months of age to 182 ± 22 cells at 4 months ($P < 0.001$) and 94 ± 12 cells at 6 months of age (Figure 10) ($P < 0.001$). Although there was a slight decrease in lymphocyte count between 4 and 6 months of age in both sexes, this decrease was significant ($P < 0.05$) only in the females (Figure 10). *Polymorphonuclear leukocyte* (PMN) counts in *male* mice were 20 ± 5 cells, 19 ± 5 cells, and 6 ± 2 cells at 2, 4, and 6 months, respectively. In comparison, PMN counts in *female* mice were 46 ± 17 cells, 29 ± 15 cells, and 15 ± 6 cells at 2, 4, and 6 months of age. The changes in PMNs in both sexes were not significant (Figure 10). The *macrophage* count in saline-treated *male* mice at 2 months of age was 260 ± 14 cells and decreased significantly to 168 ± 27 cells ($P < 0.05$) at 4 months and to 204 ± 16 cells ($P < 0.05$) at 6 months. In *females* the macrophage count decreased significantly from 240 ± 14 cells at 2 months of age to 88 ± 15 cells ($P < 0.05$) at 4 months and 191 ± 11 cells at 6 months of age ($P < 0.05$) (Figure 10). Although there was an increase in macrophage count between 4 and 6 months of age in both sexes, this increase was significant ($P < 0.05$) only in the females.

Changes in *lymphocyte* counts in the BAL paralleled changes in the histologic parameters in *males* of all ages and in *females* at 2 and 4 months of age. There was a discrepancy between histologic evidence of perivascular inflammation and the lymphocyte count in the



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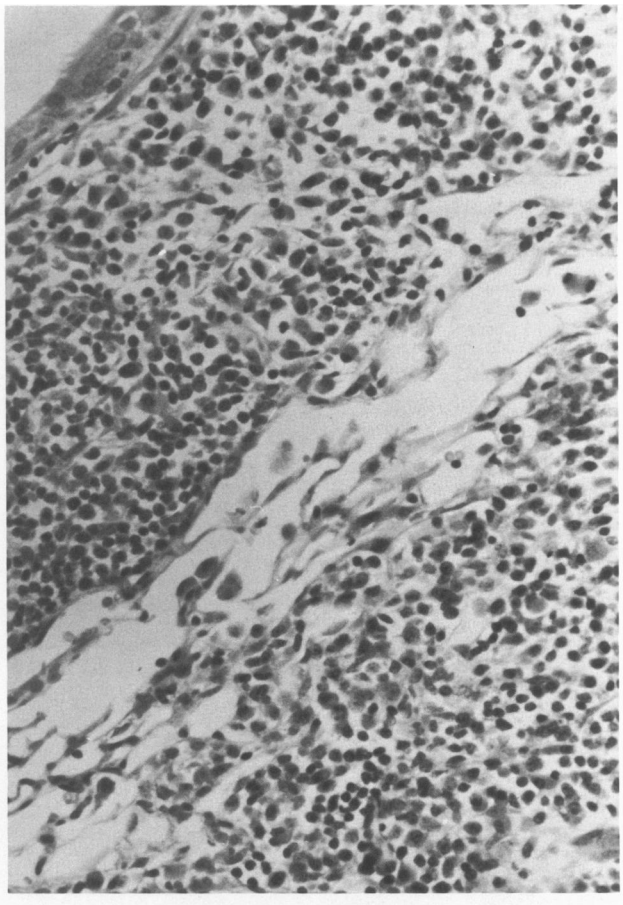

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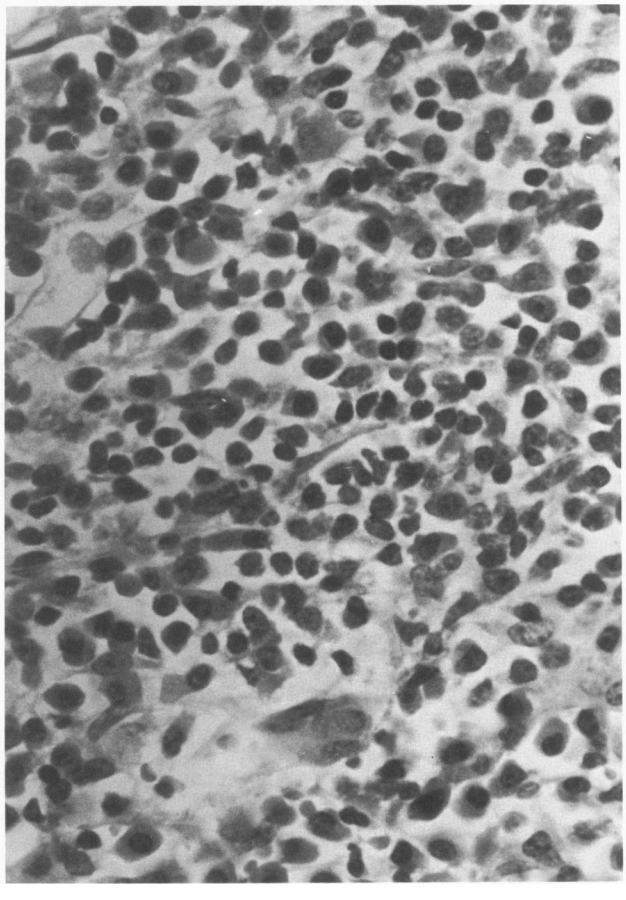

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Figures 5–7—Pronounced infiltration with lymphocytes and some plasma cells in the interstitial space. (Original magnifications: Figure 5, ×560; Figure 6, ×530; Figure 7, ×800)

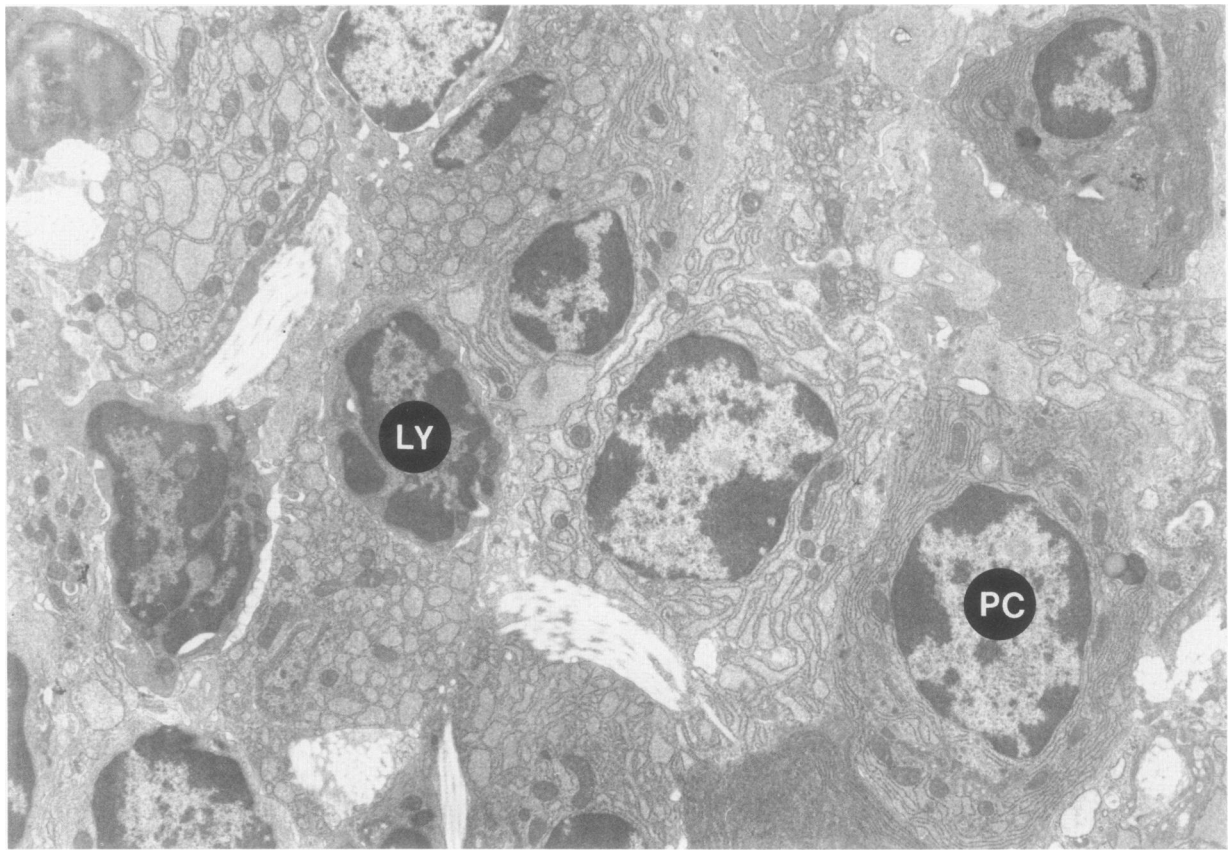

Figure 8—Electron micrograph of intraalveolar and interstitial cellular infiltrate in a 4-month-old MRL/1 mouse. (Original magnification, ×33,000) (*LY*, lymphocyte; *PC*, plasma cells).

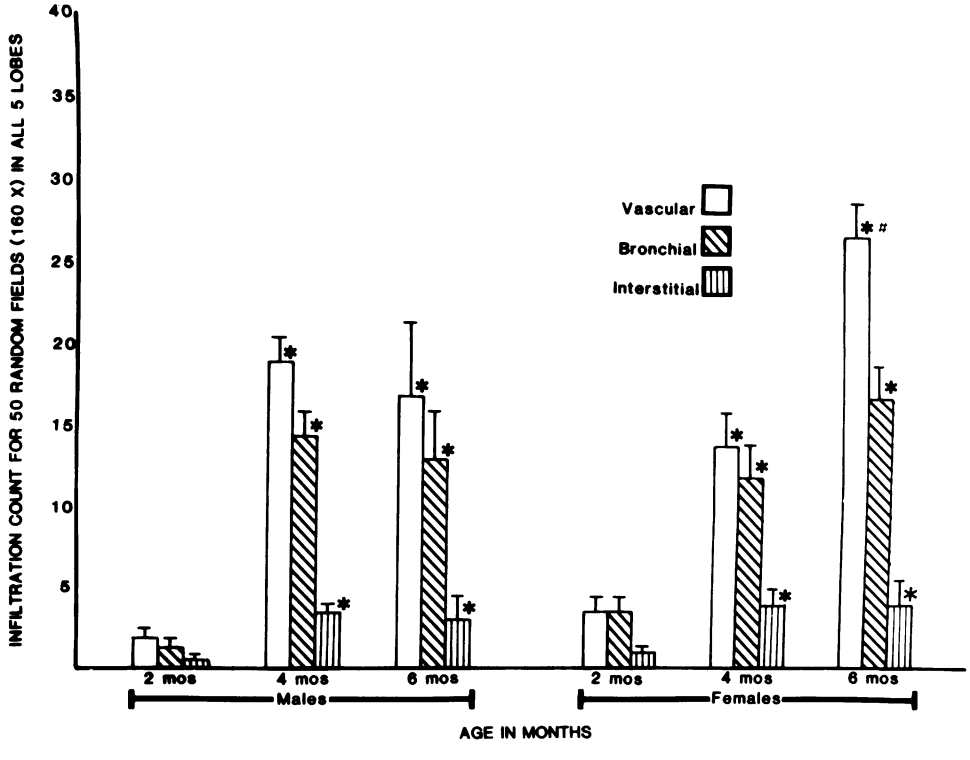

Figure 9—Age-related changes in perivascular, peribronchial, and interstitial scores (mean ± SEM) in male and female MRL/1 mice. *Significant change, compared with 2 months of age. #Significant change, compared with 4 months of age.

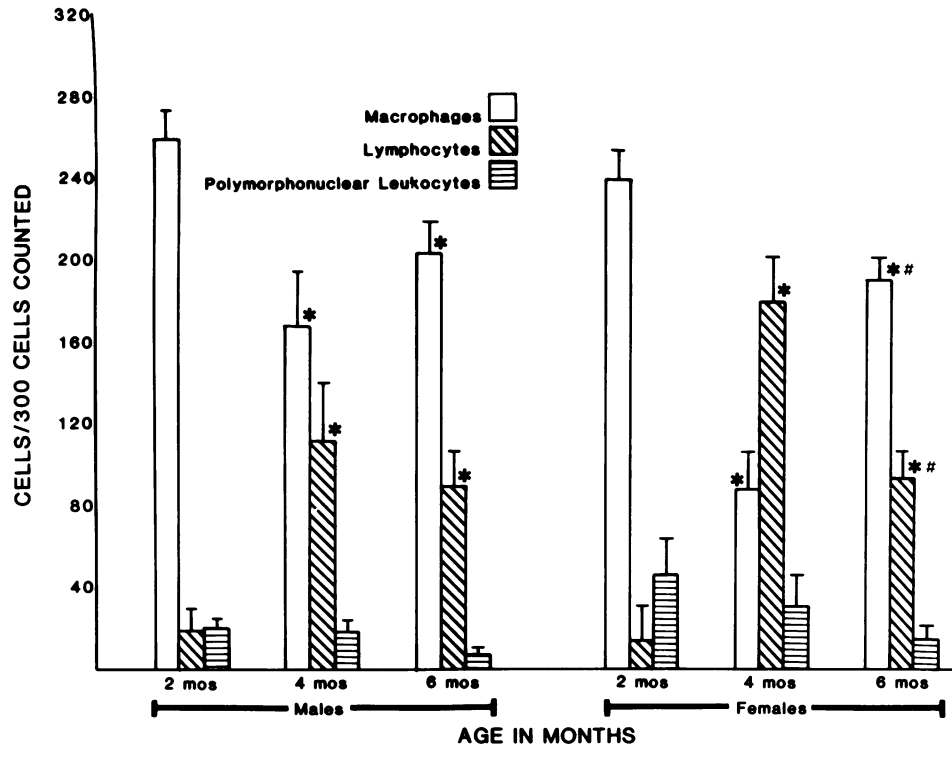

Figure 10—Age-related changes in lymphocytes, polymorphonuclear leukocytes, and macrophages (mean ± SEM) in male and female MRL/1 mice. *Significant change, compared with 2 months of age. #Significant change, compared with 4 months of age.

BAL (ie, perivascular inflammation increased and lymphocyte count in BAL decreased) in the 6-month-old female mice (Figures 9 and 10).

Effect of Cyclophosphamide (8 mg/kg)

Histologic Findings

The mean perivascular, peribronchial, and interstitial infiltrate scores showed a similar trend toward reduction in the cyclophosphamide-treated *male* mice (0.39, 1.06, and 0.06, respectively) when compared with saline-treated mice (1.75, 1.25, and 0.44), respectively (Figure 11). This trend was not noted in *female* mice; ie, counts in cyclophosphamide-treated female mice (6.47, 2.4, and 0.4, respectively) were unchanged, compared with values (3.5, 3.4, and 0.7, respectively) in saline-treated mice (Figure 11).

Total Leukocyte Count in BAL

The total leukocyte count increased from 1.5×10^6 cells/ml in saline-treated mice to 2.1×10^6 cells/ml in cyclophosphamide-treated *male* mice ($P < 0.05$). In *female* mice the total leukocyte count was 1.7×10^6 cells/ml in saline-treated mice, compared with the value of 1.8×10^6 cells/ml in the cyclophosphamide-treated mice.

Differential Cell Count in BAL

The *lymphocyte* count was 2 ± 1 cells (mean ± SEM) in cyclophosphamide-treated *male* mice and 19 ± 10 cells in the saline-treated male mice; this apparent difference was not statistically significant. This trend was not noted in cyclophosphamide-treated *female* mice; ie, lymphocytes in BAL from cyclophosphamide-treated mice were 54 ± 21 cells, compared with 13 ± 6 in the saline-treated animals. The change in *macrophage* and *polymorphonuclear* leukocytes counts was not significant (Figure 12).

To further explore the sex difference, we compared the histologic scores and BAL lymphocyte counts in cyclophosphamide-treated males and females. The histologic scores and lymphocyte counts in *males* was significantly lower ($P < 0.05$) than cyclophosphamide-treated *females* of the same age (Figures 11 and 12).

Discussion

The autosomal recessive gene *lpr* (lymphoproliferation) leads to massive generalized lymphadenopathy in MRL/l mice. Ninety percent of the females die of immune complex glomerulonephritis at 7.3 months of age; males develop similar disease and die at 8.6 months.[7] Earlier reports have mentioned peribronchial, perivascular, and interstitial lymphocytic infiltration and atelectasis in the lungs of these animals.[6-8] These pathologic

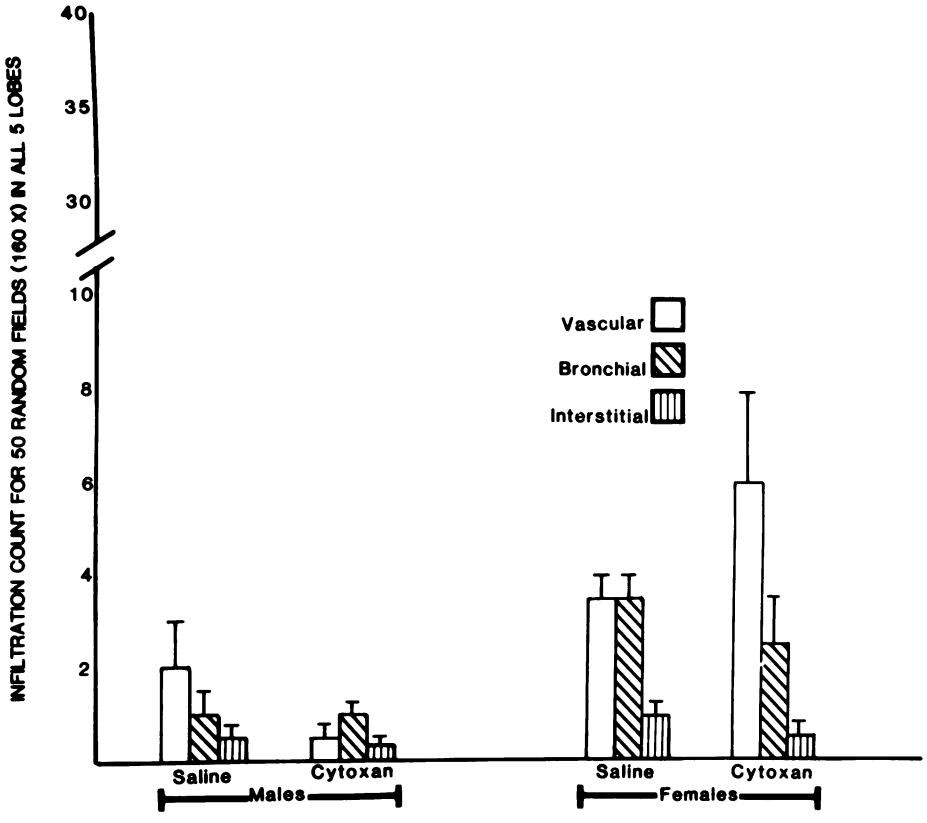

Figure 11—Perivascular, peribronchial, and interstitial infiltrate scores (mean ± SEM) in saline- and cyclophosphamide-treated (8 mg/kg/day for 30 days) male and female MRL/1 mice.

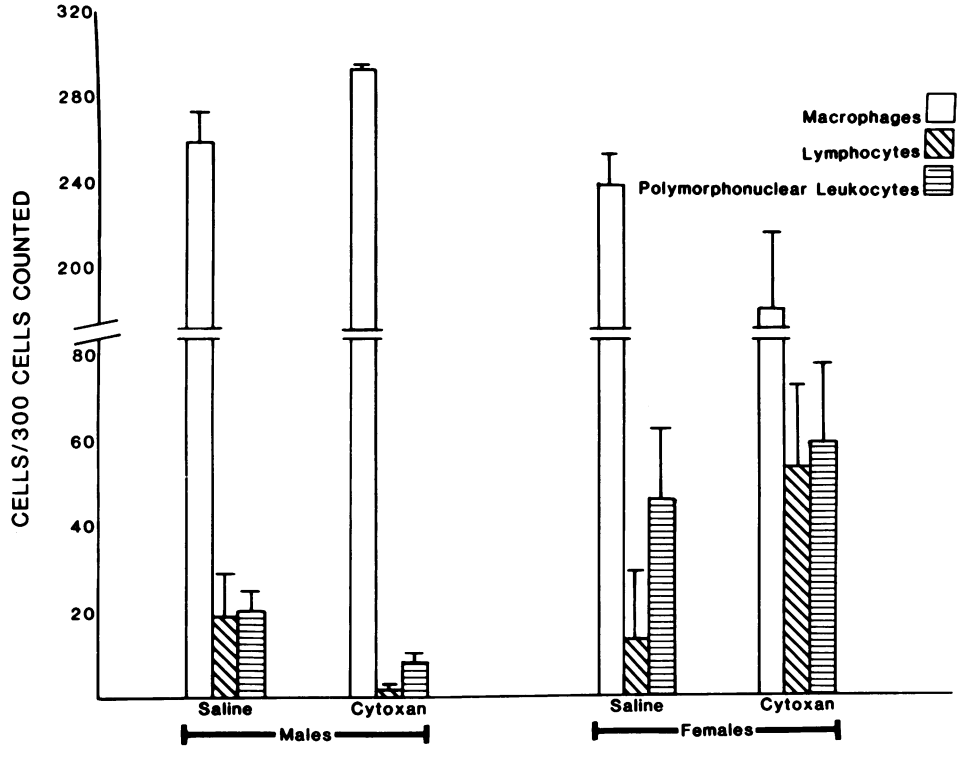

Figure 12—Bronchoalveolar lavage lymphocyte, polymorphonuclear leukocyte, and macrophage counts (mean ± SEM) in saline- and cyclophosphamide-treated (8 mg/kg/day for 30 days) male and female MRL/1 mice.

findings are very similar to changes in the lungs of patients with SLE.[5] Clinical glomerulonephritis has been reported to occur in 3–6-month-old MRL/l males and females.[6] We have previously reported[9] that perivascular lymphocytic infiltration in the lung precedes renal involvement in this murine model of lupus.

The current study defines age-related evolution of pulmonary inflammation in the MRL/l strain and correlates results of histologic assessment of lung disease with BAL. Histologic findings in this animal model of immune-complex disease indicate that the lung is an important indicator of disease. The progression of perivascular infiltrates with age are not unlike those seen in human SLE. However, although we did not observe true vasculitis, we did show vascular wall invasion at 6 months of age in MRL/l females. In addition, the mononuclear infiltration seen in the interstitium is similar to the interstitial pneumonitis seen in human SLE.[4] MRL/l males are reported to have necrotizing polyarteritis of the kidney, heart, and gential organs with higher levels of autoantibodies and circulating immune complexes present.[17] We were unable to demonstrate similar vascular lesion in the lung of this strain.

Bronchoalveolar lavage is used as an investigative tool for study of the activity of interstitial lung diseases.[10] Weinburger et al[11] reported 10% lymphocytes in BAL obtained from patients with collagen vascular disease and pulmonary fibrosis. However, there are no studies correlating serial changes in BAL with pulmonary disease. The availability of murine model of autoimmune disease permitted us to examine animals serially to assess sequential changes in BAL and histologic features during the evolution of pulmonary inflammation. We also utilized these methods to evaluate response to cyclophosphamide therapy.

In this study we have demonstrated that age-related changes in the lymphocyte count in BAL show striking similarity to histologic changes in the lungs of MRL/Mp-lpr/lpr mice. The maximum lymphocyte count was observed at 4 months of age in saline-treated male and female MRL/l mice, and lymphocytes decreased slightly at 6 months of age in both sexes of mice. The increase in lymphocyte count was more marked in females than in males, but this sex difference was not statistically significant. Changes in peribronchial and interstitial inflammation in the 6-month-old mice were not significantly different when compared with changes in the lymphocyte count in the BAL. There was a discrepancy between the BAL lymphocyte count and the perivascular inflammation in the 6-month-old female MRL mice.

The predominant cell types to increase in BAL in our study were lymphocytes. Likewise, the analysis of lung tissue in these animals by electron microscopy revealed that the infiltrates were composed largely of lymphocytes and some plasma cells. Theofilopoulos et al[18] reported that the MRL/Mp-lpr/lpr mouse has normal absolute numbers of B lymphocytes but at a reduced frequency, compared with massive T-lymphocyte proliferation. In addition, lymphocyte cell cultures from MRL/Mp-lpr/lpr lymph nodes have been shown by flow cytometry to be all T lymphocytes.[19] Although in our study lymphocyte subtyping was not done in the BAL, we speculate on the basis of the above information that these were predominantly T lymphocytes.

There was a sex-related difference in histologic features and BAL lymphocyte counts in cyclophosphamide-treated (8 mg/kg/day) mice. There was a trend toward reduction in the histologic evidence of inflammation, which was reflected by decreased lymphocyte count in BAL in male MRL/Mp-lpr/lpr mice. There was no trend toward reduction in the lung inflammation or lymphocyte count in the BAL in treated MRL/Mp-lpr/lpr female mice. The sex-related difference was even more striking when we compared the cyclophosphamide-treated males and females, in that the histologic score and BAL lymphocyte count in cyclophosphamide-treated males was significantly lower than in cyclophosphamide-treated females of the same age. These findings are in agreement with our previous report of a sex-related difference in pulmonary perivascular infiltrates in MRL/Mp-lpr/lpr mice.[9]

In conclusion, MRL/l animal model can be used in the study of the progress of pulmonary inflammation in SLE. There was a striking similarity between age-related changes in histologic evidence of pulmonary inflammation and the lymphocyte count in BAL in the MRL/Mp-lpr/lpr mouse model of autoimmunity. There was a trend toward reduction in the histologic evidence of inflammation lymphocyte count in cyclophosphamide-treated males but not in the females. Further studies are in progress to assess the effect of higher doses of cyclophosphamide and sex hormones on pulmonary inflammation and BAL lymphocyte subpopulations in MRL/Mp-lpr/lpr mice.

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