Mononuclear Cell Pulmonary Vasculitis in NZB/W Mice

II. Immunohistochemical Characterization of the Infiltrating Cells

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NZB/W mice spontaneously develop pulmonary lymphoid hyperplasia and vasculitis in an age-related fashion. The cellular infiltrates and pattern of involvement bear similarity to various forms of pulmonary vasculitis in humans. In this study the authors used monoclonal antibodies and the avidin-biotin immunoperoxidase technique to analyze the pulmonary mononuclear cell infiltrates of female NZB/W mice at various ages and levels of disease activity. T cells, T-cell subsets, B cells, and Ia-bearing cells were localized with this technique. Most cells within the infiltrates were T cells that expressed the Lyt-1 phenotype, whereas cells expressing Lyt-2 were rarely observed. Cells reacting with a monoclonal antibody which recognizes cells of the B-lineage and cells expressing Ia antigens were also observed. Before the development of vasculitis, B and T cells were randomly distributed throughout the lesion. In older animals with vasculitis, T cells expressing Lyt-1 were associated with vessel lumen and were primarily responsible for the vascular infiltration, to the apparent exclusion of other lymphoid cell types. The B cells and Ia$^+$ cells were localized at the periphery of the lesions. (Am J Pathol 1986, 123:204–211)

NEW ZEALAND mice, most notably the (NZB × NZW) F_1 hybrid (NZB/W), have been used as a model for the study of human autoimmune disorders and immune complex disease.[1] There is a close similarity between the progressive autoimmune disease developing in these animals and human systemic lupus erythematosus (SLE). Lymphocytic infiltration of several organs has been reported in the other autoimmune mice strains, ie, MRL, BXSB, NZB and NZB/W mice.[1–4]

In a previous paper, we reported the spontaneous occurrence in NZB/W mice of a pulmonary vasculitis which shows similarities to a variety of human pulmonary vasculitides.[5] Before the development of angiodestructive lesions in these animals, perivascular and peribronchiolar lymphoid hyperplasia are observed. In older animals, severe focal disease, characterized by infiltration of the vessel wall by mature lymphocytes and occasional plasma cells and histiocytes, is seen. This ultimately progresses to transmural infiltration, with near total occlusion of the vessel lumen. Although many studies have examined the contribution of peripheral lymphoid cell populations to disease pathogenesis in NZB/W mice, few have characterized the *in situ* nature of the cellular infiltrates. The pathogenesis remains obscure. In this study we analyzed these pulmonary infiltrating cells with monoclonal antibodies directed toward cell-surface antigens. The observation that cells bearing the Lyt-1$^+$ marker are present in the vascular infiltrate to the near total exclusion of other cell markers indicates that these cells may be important in the pulmonary vasculitis in NZB/W mice.

Materials and Methods

Animals

F_1 hybrids of New Zealand Black (female) by New Zealand White (males) (NZB/W) mice were obtained from breeding colonies at the National Jewish Hospi-

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tal/National Asthma Center. Only female mice were examined in these studies.

Monoclonal Antibodies

To identify the surface phenotype of mononuclear cells contained within each inflammatory focus, we used five monoclonal antibodies. For identification of total number of T cells, a rat IgG anti-Thy-1 (c22/22.7.1.1) obtained from Dr. Ian Trowbridge, Salk Institute, San Diego, California, was used.[6] A rat IgG anti-Lyt-1 (53-7.3) which identifies T cells with helper activity/delayed-type hypersensitivity responses and a rat IgG anti-Lyt-2 (53-6.7) which identifies T cells with the cytotoxic/suppressor phenotype were obtained from Dr. L. Herzenberg, Stanford University, Stanford, California.[7] The antibodies from the supernatants produced by these hybridomas were isolated by precipitation with 50% saturated ammonium sulfate (SAS) followed by chromatography on DEAE cellulose. To identify Ia-bearing cells within the infiltrates, we used the rat IgG monoclonal antibody M5/114 (obtained from Dr. T. A. Springer, Harvard Medical School, Boston, Mass).[8] B-cell identification was made with the use of the IgM monoclonal antibody, RA3-2C2 (ATCC, Rockville, Md), which has been shown to identify cells of B-cell lineage.[9] These two antibodies were concentrated from hybridoma culture supernatants by precipitation with SAS and after dialysis against 0.01 M phosphate-buffered saline (PBS), pH 7.6, were used without further purification.

Biotin-Avidin Immunoperoxidase Staining of Lungs

At 6, 9, 10, and 12 months of age, groups of 6–8 mice were anesthesized by an intraperitoneal injection of sodium pentobarbital. Single lobes of lung were snap-frozen in acetone/dry ice and stored at −70 C until sectioned. Four-micron serial sections were fixed by brief immersion in cold acetone and either stained immediately or stored at −70 C. At the time of staining, the frozen sections were fixed in cold acetone for 10 minutes. Sections were then rinsed for 10 minutes in PBS with three changes of buffer. To inactivate endogenous peroxidases, we placed sections in 0.3% H_2O_2 in methanol for 30 minutes. After washings with PBS, the sections were flooded with diluted normal goat serum (Vector, Burlingame, Calif) and incubated for 15 minutes in a humidified chamber. Excess serum was gently blotted from the slides, and the appropriate dilution of the primary monoclonal antibody was applied to the tissue and allowed to incubate for 1 hour. For each monoclonal antibody, the optimal dilution had been determined with the use of NZB/W spleen sections. After the 1-hour incubation, the slides which had been incubated with the anti-Thy-1, Lyt-1, Lyt-2, or Ia were again washed in PBS and incubated for 30 minutes with goat anti-rat IgG (noncross-reacting to mouse IgG, Cappel Laboratories, West Chester, Pa). For the anti-B-cell antibody, the sections were incubated with a goat anti-rat IgM (affinity-purified, μ-chain specific, Cappel) which had been passed through a mouse IgM Sepharose column for removal of cross-reactivity to mouse IgM. Both the goat anti-rat IgG and IgM antibodies were biotinylated as previously described.[10] Avidin and biotinylated horseradish peroxidase complex (ABC reagent, Vector) was then applied to the slides and incubated for 30 minutes. The slides were reacted with a substrate solution of 20 mg/dl 3,3′ diaminobenzidine tetrahydrochloride (Baker Chemical Company, Phillipsburg, NJ) in PBS containing 0.005% H_2O_2. To improve contrast, the sections were incubated with 0.5% $CuSO_4$ in 0.8% NaCl for 5–10 minutes. Sections were counterstained with 1% methyl green or hematoxylin. Finally, sections were dehydrated in graded ethanols and xylene and mounted. Each series of sections included a control in which the primary antibody was omitted. Serial sections from the lungs of individual NZB/W mice were examined for staining with each monoclonal antibody. If a cell had a rim of brown stain around the nucleus it was counted as a positive cell.

Results

Representative sections of each lobe were examined in NZB/W mice at various ages. As previously reported, the severity of the lesions was age-related.[5] The first observable change detected in hematoxylin and eosin (H&E) sections was observed at 4 months of age, when mild perivascular and peribronchial lymphoid hyperplasia was noted. By 8 months of age, all mice had moderate to severe perivascular and peribronchiolar lymphoid hyperplasia. The cells consisted of mature lymphocytes, plasma cells, and histiocytes. The earliest vascular changes were seen at 8 months of age. In half of these animals, there was infiltration of the tunica intima by mononuclear cells. With age, there was progression to transmural infiltration, and in severe cases to total occlusion of the vessel lumen. By 12 months of age, all mice showed extensive multifocal disease, with obliteration of at least one vessel per lobe. These changes were not noted in age-matched BDF_1 control mice.

To identify cell populations and to determine the spatial architecture of the mononuclear cells within the

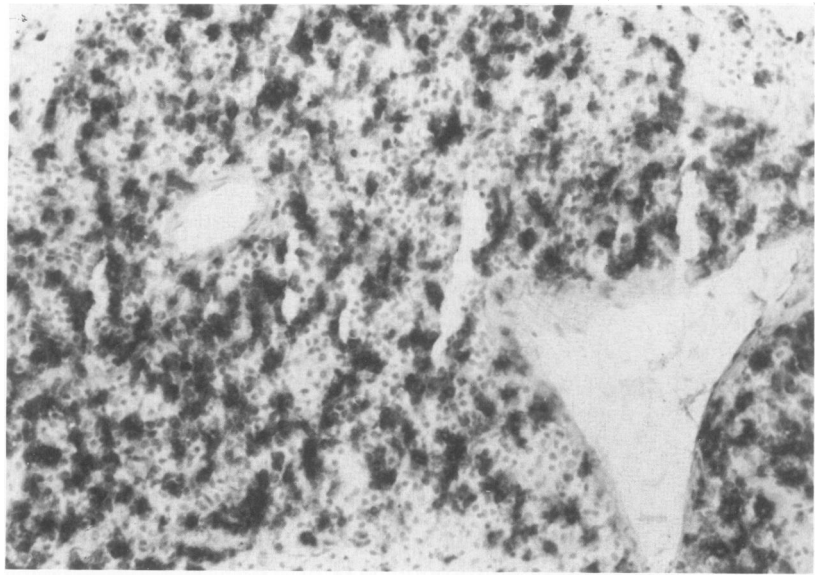

Figure 1—Ten-month-old NZB/W animal. Perivascular mononuclear cell infiltrate stained with ABC technique with the use of monoclonal antibodies that recognize Thy-1$^+$ cells. Note that the Thy-1$^+$ cells are randomly distributed without compartmentalization. (×85)

lesions of NZB/W mice, an ABC technique using monoclonal antibodies directed to cell surface antigens was used. Serial frozen sections were stained with H&E and antibodies to Thy-1, Lyt-1$^+$, Lyt-2$^+$, B cells, and Ia. In our previous report we found that from 4 to 8 months of age the only distinguishing pathologic feature in NZB/W mice was mild to moderate perivascular and peribronchiolar lymphoid hyperplasia. In this study, surface marker analysis demonstrated that such hyperplasia is composed predominantly of T cells, B cells, and Ia$^+$ cells. T subset discrimination using anti-Lyt-1 and anti-Lyt-2 antibodies revealed that Lyt-1$^+$ cells predominated, while only an occasional Lyt-2$^+$ cell was seen. The spatial distribution of these cells within the infiltrates was unremarkable. B and T cells were randomly distributed in both perivascular and peribronchiolar infiltrates, with no evidence of compartmentalization (Figure 1). Most Ia reactivity paralleled the B-cell distribution.

By 9–10 months of age, a distinct change in the spatial distribution of the angiocentric B and T cells was noted. Sections from these animals now showed T cells

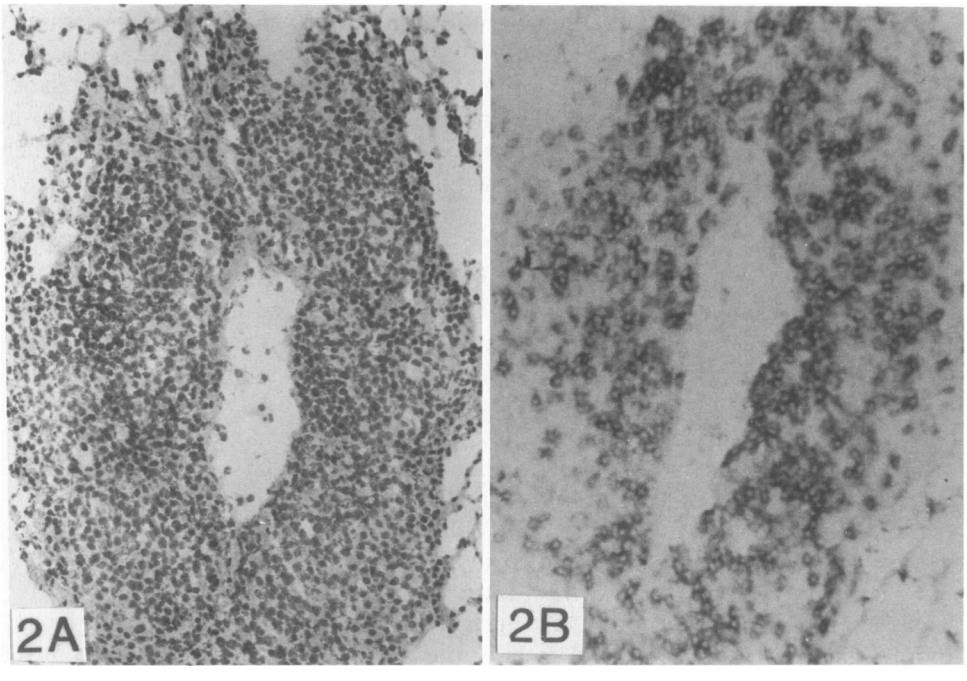

Figure 2—Ten-month-old NZB/W animal. **A**—Perivascular mononuclear cell infiltrates stained with hematoxylin and eosin. **B**—Serial frozen section stained with anti-Thy-1 by the ABC technique. Note that the Thy-1$^+$ cells are now arranged in an organized fashion with the majority of positive cells closest to the lumen. (×85)

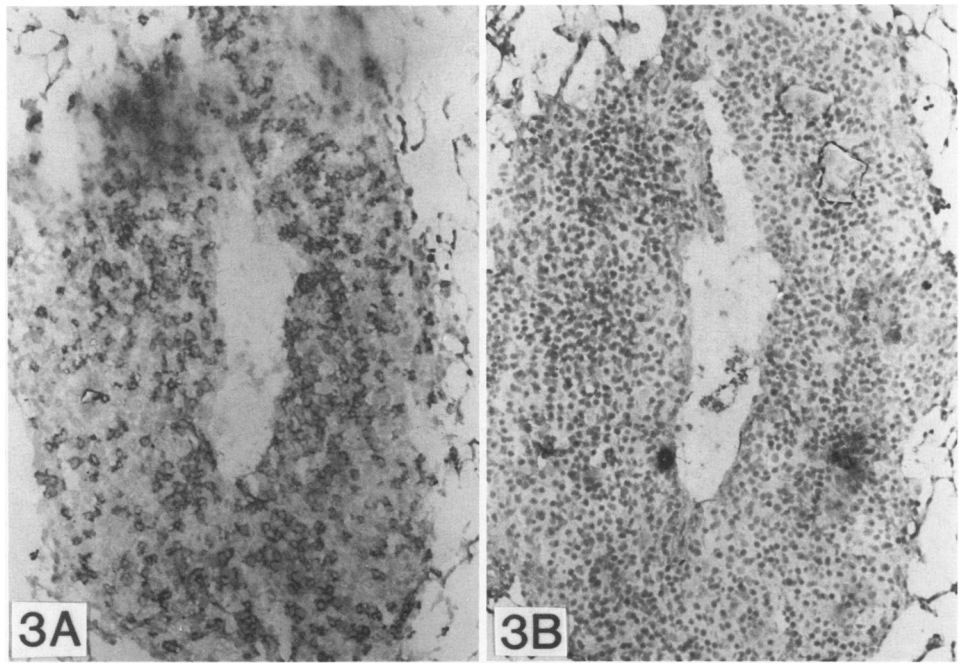

Figure 3A—Serial frozen section of perivascular infiltrate stained for Lyt-1 by the ABC technique. When compared with Figure 2B, note that the majority of the Thy-1$^+$ areas are also positive for Lyt-1. **B**—Same lesion stained for Lyt-2 by the ABC technique. There is virtual absence of Lyt-2$^+$ cells. (×85)

arranged in an intimate perivascular fashion, with the majority of T cells aggregated in immediate contact with the tunica adventitia (Figure 2A and B). This was found in vessels with and without evidence of vasculitis. As before, nearly all of these cells expressed Lyt-1 (Figure 3A and B). The B cell distribution also showed changes relative to younger animals. The B cells were now present primarily at the periphery of the infiltrates, surrounding the perivascular T cells (Figure 4). The majority of the Ia$^+$ reactivity was found to reside in the B-cell compartment (Figure 5).

As we previously reported, older mice showed evidence of pulmonary vasculitis characterized by mononuclear cell infiltration and fragmentation of the limiting elastic membranes.[5] These lesions, which varied from mild to severe, occluding vasculitis, were examined by the ABC technique. In both early lesions (Figure 6A and B), evidenced only by intimal thickening, and ad-

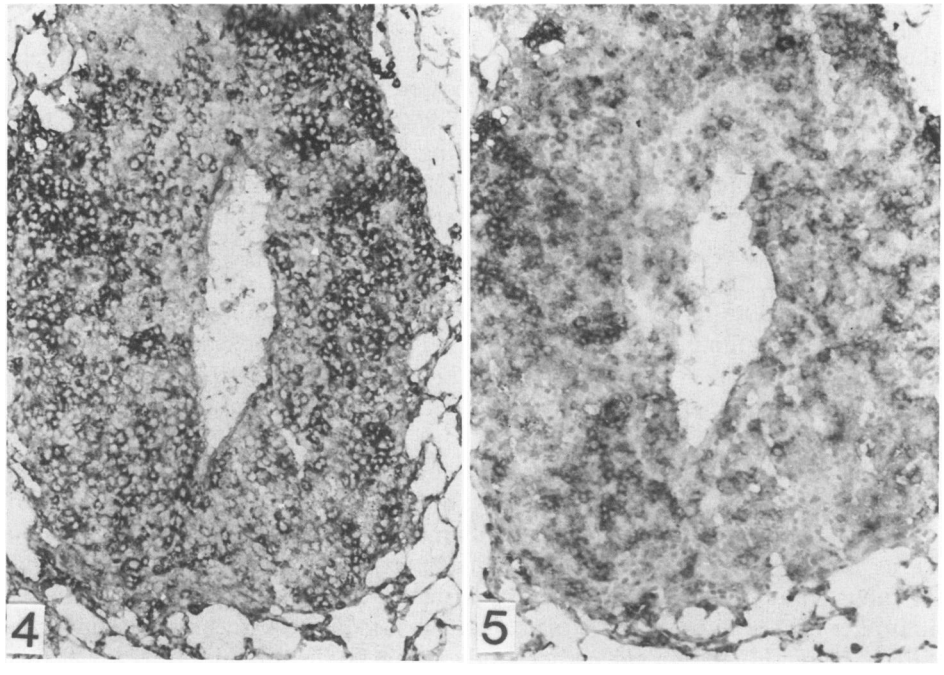

Figure 4—Serial frozen section of the same lesion as seen in Figures 2 and 3 stained for B cells by the ABC technique. The majority of B cells are located at the periphery of the lesion and appear to occupy a different compartment relative to the T cells. (×85) **Figure 5**—Frozen section stained with anti-Ia by the ABC technique. The majority of Ia reactivity resides in the B-cell compartment. (×85)

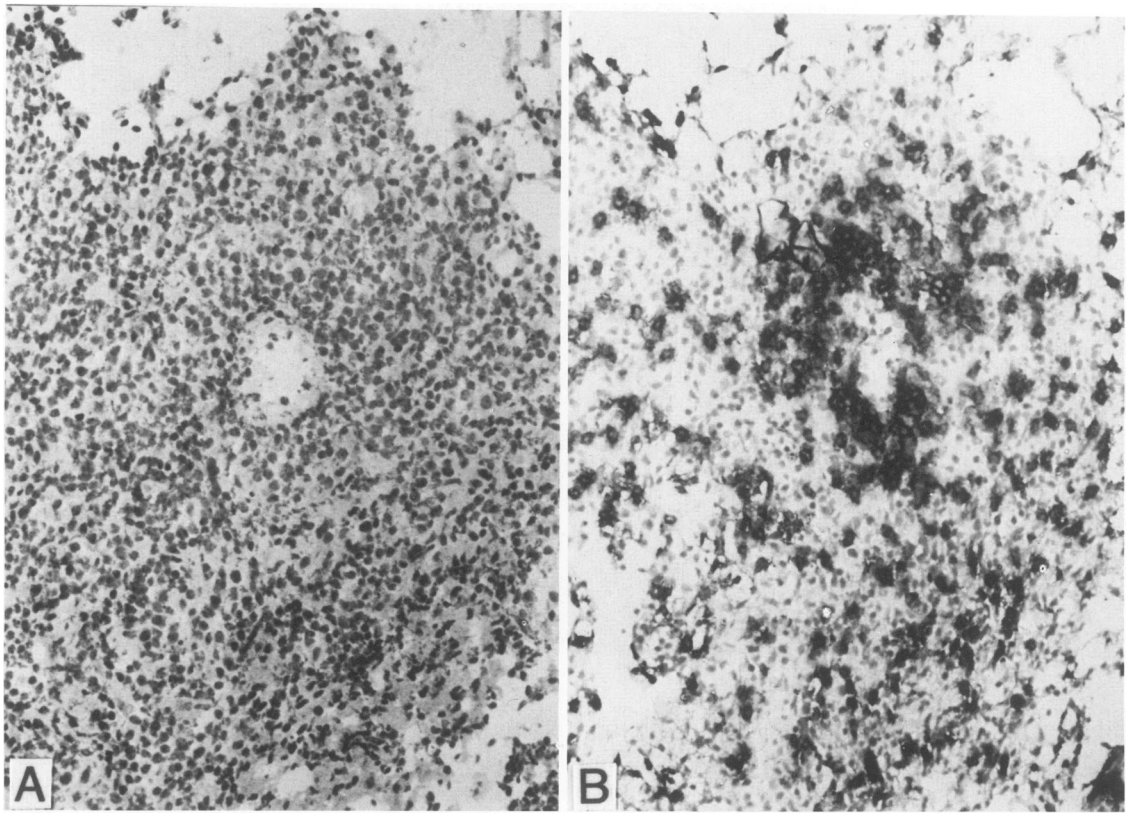

Figure 6A—Early perivascular lesion stained with H&E showing mild intimal thickening due to infiltrating mononuclear cells. **B**—Serial frozen section stained with anti-Thy-1, identifying T cells in association with the tunica intima. (×85)

vanced lesions (Figure 7A and B), most of the infiltrating mononuclear cells were identified as Thy-1$^+$, Lyt-1$^+$ cells. With the onset of vasculitis and cellular infiltration, nearly all of the vascular cellular infiltrates were Thy-1$^+$, Lyt-1$^+$ cells. Ia reactivity continued to reside in the peripheral B-cell compartment.

In animals 10 months old and older, lesions were present in varying stages of severity (Figure 8). The separation of the B and T cells into different compartments was observed regardless of the size or type of vessel involved. Similarly, Thy-1$^+$, Lyt-1$^+$ cells were always identified as the vascular infiltrating cell. In contrast, peribronchiolar lymphoid infiltrates continued to show a random distribution of B and T cells regardless of the age of the animal.

Discussion

A well-described feature of the natural history in NZB and NZB/W strains is the occurrence of lymphocytic infiltrates in multiple organs, including the liver, kidney, and lung.[1,11] These infiltrates often mimic those of human autoimmune disorders. For instance, the infiltrates in the salivary glands resemble those seen in Sjögren's syndrome.[4] In an earlier study using immunofluorescence, B (immunoglobulin positive cells) and T cells (thymus antigen-positive cells) were demonstrated in the organs of NZB/W mice.[11] The authors speculated that the earlier appearing B cells may give rise to the initial damage in these organs and that T cells enter the lesion once this process has been initiated. In a more recent study, an *in vitro* assessment of the pulmonary lymphocyte population in NZB/W mice was made.[12] T and B cells could be recovered from NZB/W and C57BL/6 mice in lung lavage and from the interstitium, with T cells accounting for a significant proportion of the total cells.

As we previously reported,[5] the histopathologic findings of the pulmonary vascular lesions in NZB/W mice can be divided into two distinct pictures, lymphoid hyperplasia and vascular infiltration. Young mice develop florid perivascular and peribronchial lymphoid hyperplasia, which in all cases precedes vasculitis. This is followed at approximately 8 months of age by an eventual transmural infiltration of small to medium-sized vessels by mononuclear cells and ultimate obliteration of the vessel lumen. The elastic membranes are often fragmented. In this report we employed immunohistochemical staining techniques with monoclonal antibodies directed against various lymphocyte surface

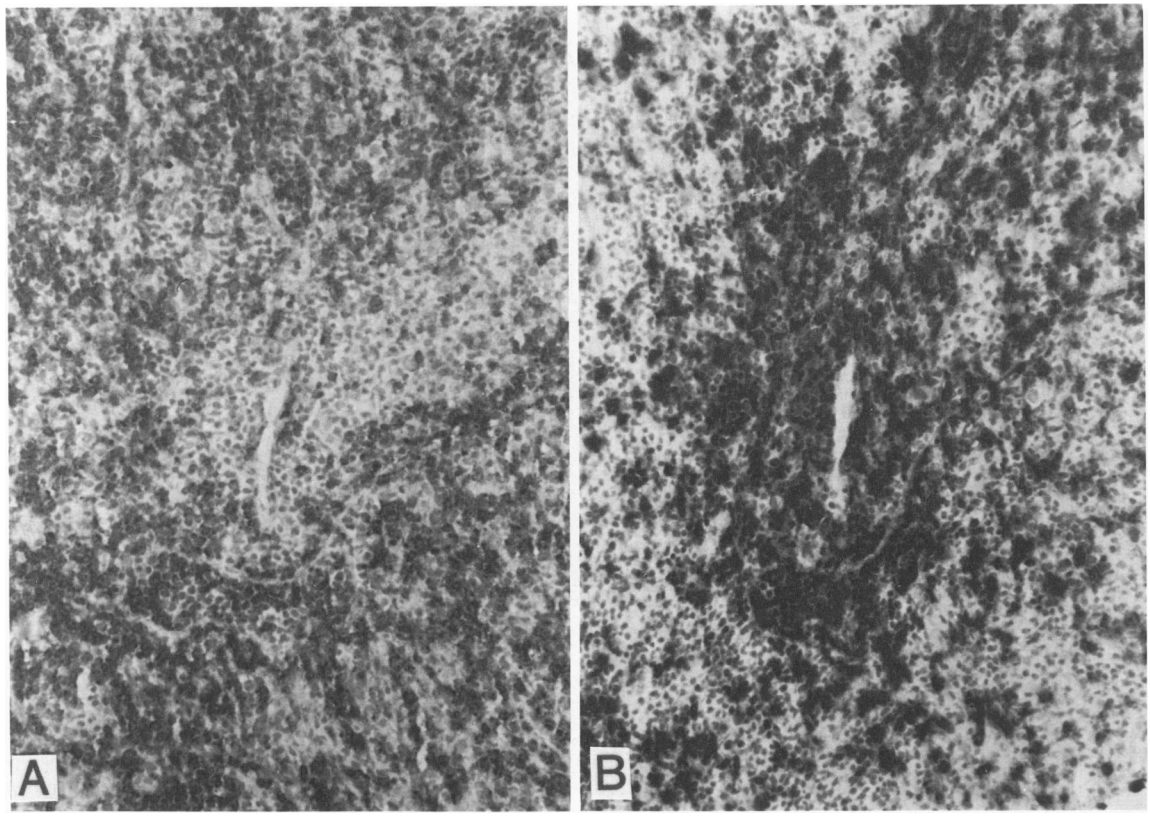

Figure 7—Advanced perivascular infiltrate with near total occlusion of vessel lumen by infiltrating mononuclear cells. **A**—The section is stained for B cells and shows that the B cells are located at the periphery of the lesion and that the majority of the infiltrating cells are not B cells. **B**—Serial section stained for Thy-1$^+$ cells. Virtually all the infiltrating cells and the cells immediately perivascular are T cells (×85)

markers to identify and localize *in situ* the lymphocyte populations infiltrating the lungs of NZB/W female mice.

Two immunohistologic patterns were noted during the development of vasculitis in the NZB/W mouse. Both T and B cells were detected in the lymphoid hyperplasia that preceded the vasculitis. The spatial distribution of these cell types was random throughout the lesion with no compartmentalization of one cell type. T cells within these lesions expressed only Lyt-1 to the exclusion of the Lyt-2 phenotype. This was probably not an artifact of the staining procedure, because the same antibody identifies Lyt-2$^+$ cells in spleen sections of NZB/W mice. Ia$^+$-cells were also identified in these lesions, their distribution paralleling that of the B cells. With increasing age and the development of vasculitis, a different spatial pattern emerged. The infiltrating cells responsible for the vasculitis expressed the Lyt-1 phenotype, whereas the Ia$^+$ and B cells were peripherally located near the tunica adventitia. Lyt-2$^+$ cells were still not identified.

One of the hallmarks of T-cell processes is their propensity for vascular involvement. For example, in angiocentric lymphoma, the predominant lymphocytic component is a mature T cell.[13] In addition, in a single case report of an angiocentric immunoproliferative lesion of the lung, vasculitis was seen in which the infiltrating cells were uniformly positive for Leu 3.[14] In another of the angiocentric lymphomas, lymphomatoid granulomatosis, depressed T-cell function and altered helper/suppressor ratios have been reported.[15]

T cells are observed in several other inflammatory foci of induced or naturally occurring autoimmune diseases. In Sjögren's syndrome, OKT4$^+$ cells predominate in the peripheral blood and at the site of inflammation.[16,17] In Graves' disease and Hashimoto's thyroiditis, the predominant T cells are of the suppressor/cytotoxic phenotype.[18] In experimental allergic encephalomyelitis of the mouse, Lyt-1$^+$ cells play a significant role in mediating the disease.[19] Recently, T-cell clones have been isolated from the thyroid glands of patients with Graves' disease and found to recognize autologous thyroid cells.[20] In addition, T-lymphocyte lines for islet antigens were isolated from the spleen and pancreas of BB rats, in which insulin-dependent diabetes mellitus spontaneously develops.[21]

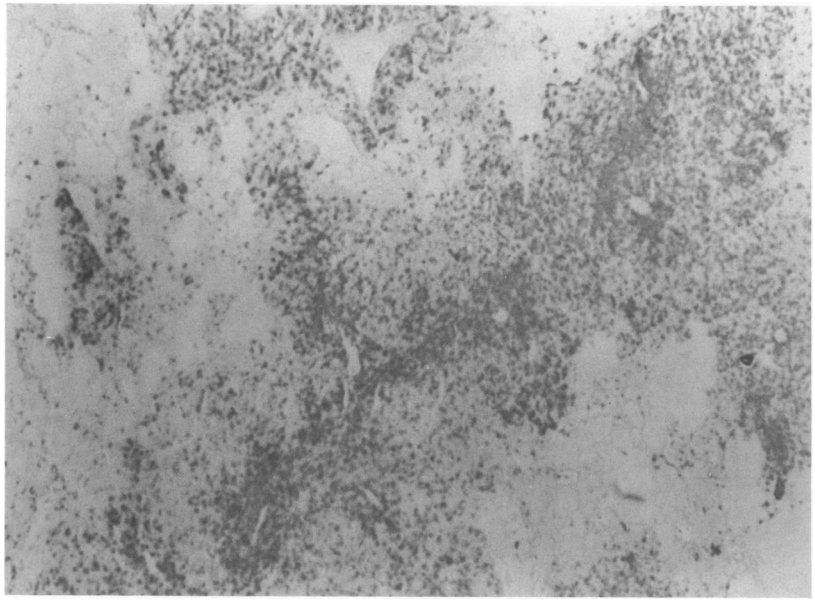

Figure 8—Low-power view of a lung section from a 14-month-old NZB/W animal stained for Thy-1 and showing severe multifocal disease. Note that multiple vessels show T cells arranged in a predominantly perivascular location. (×40)

Interestingly, in experimental autoimmune uveitis in rats, T helper/inducer cells predominate in the lesions during the early stages of the disease, whereas in the later stages there is an increase in the relative number of suppressor/cytoxic cells.[22] The authors suggest that these different stages may reflect the normal kinetics and regulation of an inflammatory response in experimental autoimmune disease. In our study, there were few, if any, Lyt-2$^+$ cells in the infiltrate at any time during the two phases of the disease. The absence of Lyt-2$^+$ cells, which are known to be involved in immunoregulatory activity, may allow the persistence of this ongoing chronic inflammatory process and lead to uncontrolled proliferation of antigen-reactive cells.[23]

The factors that initiate the development of pulmonary lymphoid hyperplasia and subsequent vasculitis are unknown in these mice. It is tempting to speculate on the sequential development of these two events. The initial hyperplasia observed in the lungs of younger NZB/W mice could result from either a generalized benign lymphoproliferation or be triggered by a specific environmental factor, virus, etc. In support of a role for environmental factors, it has been reported that NZB mice raised in a germ-free environment have a lower incidence of pulmonary infiltrates than their conventional counterparts.[24] The uncontrolled T-cell activation could lead to a B-cell stimulation. Splenic B cells of NZB/W mice are known to be hyperactive, ie, have a high frequency of immunoglobulin secreting cells.[25] Our own work, showing a high frequency of immunoglobulin-secreting cells within the lungs of older NZB/W mice, supports this.[26] The proliferation of these polyclonally activated B cells may be aided by B-cell growth factors and B-cell differentiating factors, both T-cell–derived lymphokines.[27] In the context of disordered immunoregulation in the NZB/W mouse, vasculitis and the ongoing chronic inflammatory process could develop as a consequence of the failure of Lyt-2$^+$ cells to regulate inflammatory lymphokine production. In MRL-lpr/lpr mice it has been postulated that unregulated production of another lymphokine, immune interferon (IFNγ), plays a key role in the induction of autoimmunity in these mice.[28] IFNγ is known to induce the expression of Ia on B cells and macrophages[29,30] and has recently been shown to induce Ia on nonlymphoid cells, including endothelial cells.[31] In guinea pigs sensitized to myelin basic protein (MBP), brain vascular endothelial cells express Ia antigens before the development of lymphocytic infiltration.[32] In addition, a recent study demonstrated that these endothelial cells can present MBP to MBP-specific T cells.[33] If in the NZB/W mice pulmonary vascular endothelial cells could be induced to express Ia by IFNγ-producing Lyt-1$^+$ cells present in the lymphoid hyperplasia, then these Ia$^+$ endothelial cells might function as antigen-presenting cells to Lyt-1$^+$ effector cells, inciting T-cell–mediated vasculitis. It was difficult in these experiments to determine whether Ia expression occurred on the vascular endothelial cells because the use of frozen sections did not allow optimum evaluation of pulmonary architecture.

Functional studies of isolated lymphocyte preparations are needed for full understanding of the nature of the infiltrating cells in the lungs of these animals. Lyt-1$^+$ cells have been shown to function as effector cells in both delayed-type hypersensitivity and in graft re-

jection.[34,35] The determination of lymphocyte surface markers on the infiltrating cells may provide insight into the pathogenesis of similar disorders occurring in humans.

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