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Tissue Eosinophilia and Eosinophil Degranulation in Syndromes Associated with Fibrosis

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Eosinophilia has long been associated with endomyocardial fibrosis, but the involvement of the eosinophilia in fibrosis of other organs is unclear. To investigate this question, the authors tested whether tissue eosinophilia and eosinophil degranulation are present in syndromes associated with fibrosis. The authors used an indirect immunofluorescent technique to localize eosinophil granule major basic protein (MBP) in formalin-fixed, paraffin-embedded tissue specimens from 50 patients. Thirty-four specimens were obtained from patients with inflammatory fibrosis: 12 with idiopathic retroperitoneal fibrosis, seven with sclerosing mediastinitis, four with sclerosing cholangitis, and 11 with pulmonary fibrosis. The remaining 16 specimens were obtained from patients with noninflammatory fibrous proliferations: four with keloids, six with scars, three with Dupuytren's contracture and three with dense stromal fibrosis of the breast. Eosinophil infiltration and/or extracellular MBP deposition were observed in 28 of the 34 specimens (82%) from patients with inflammatory fibrosis, including 11 of the 12 cases of retroperitoneal fibrosis, five of the seven cases of sclerosing mediastinitis, all four cases of sclerosing cholangitis, and 8 of the 11 cases of pulmonary fibrosis. In contrast, eosinophil infiltration and MBP deposition were not observed in specimens from the 16 patients with noninflammatory fibrous proliferation ($P < 0.001$). These results indicate that eosinophil infiltration and release of a granule protein, namely MBP, commonly occur in inflammatory fibrotic lesions. (Am J Pathol 1992, 140:521–528)

The association between tissue eosinophilia and fibroplasia is supported by numerous prior observations. For example, eosinophilia and eosinophil degranulation are associated with endomyocardial fibrosis in patients with the hypereosinophilic syndrome[1,2] and with the nodular sclerosing variant of Hodgkin's disease.[3,4] Furthermore, the fibrosis beneath the basement membrane in bronchial asthma is well known.[5,6] Eosinophilia and eosinophil degranulation occur in syndromes associated with fibrosis, such as the toxic oil syndrome[7] and the eosinophilia myalgia syndrome.[8,9] Moreover, eosinophil extracts may stimulate fibroblast proliferation, suggesting that an eosinophil product directly causes fibrosis.[10]

Eosinophil major basic protein (MBP) has been localized to the human eosinophil granule core.[11,12] Major basic protein is toxic to mammalian cells[13] and larval helminths[14–16]; MBP is also able to activate basophils, mast cells, and platelets.[17–19] The release of MBP, both *in vitro* and *in vivo*, has been used as a marker of eosinophil infiltration and degranulation.[20] By using an immunofluorescence technique for localization of eosinophil MBP, we tested the hypothesis that tissue specimens from inflammatory fibrosis show eosinophil infiltration and degranulation. The results indicate that eosinophil infiltration and degranulation commonly occur in tissues showing inflammation and fibrosis.

Materials and Methods

Patients

Fifty patients with syndromes associated with fibrosis were randomly selected for study. All patients underwent excisional or endoscopic biopsy procedures between October 1958 and May 1988. Thirty-four specimens were

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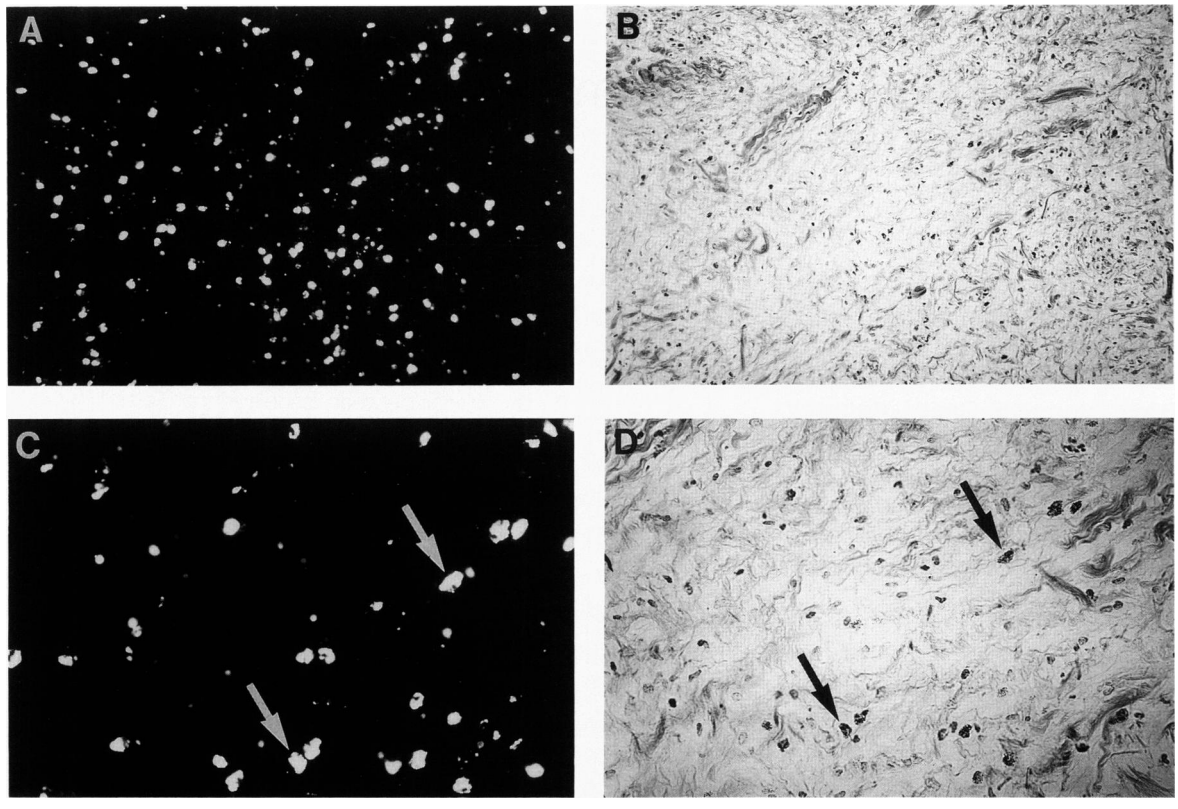

Figure 1. *Infiltration of eosinophils in mediastinal tissue from a patient with early sclerosing mediastinitis.* **A,C:** *Section stained with anti-MBP.* **B,D:** *Same section counterstained with H&E. Note the virtual absence of extracellular MBP deposition in the loose and immature-appearing connective tissues. The score for eosinophil infiltration was 2+, and the score for extracellular MBP deposition was 0. At higher magnification, note a close correspondence between the fluorescent ovals (white arrows in* **C***) and intact eosinophils (black arrows in* **D***). A serial section stained with NRIgG was negative (results not shown).* **A,B,** *×160;* **C,D,** *×400.*

from patients with inflammatory fibrosis, including 12 with idiopathic retroperitoneal fibrosis, seven with sclerosing mediastinitis, four with sclerosing cholangitis, and 11 with pulmonary fibrosis (10 patients with idiopathic pulmonary fibrosis and one patient with drug-induced pneumonitis). These 34 patients ranged in age from 20 to 77 years, with a mean age of 54 years; 18 patients were women and 16 were men. In addition, 16 specimens were from patients with syndromes associated with noninflammatory fibrous proliferations; namely, four with keloids, six with scars, three with Dupuytren's contracture, and three with dense stromal fibrosis of the breast (fibrosclerosis). These 16 patients ranged in age from 8 to 76 years, with a mean age of 53 years; six patients were females and 10 were males.

Immunofluorescence Localization of MBP

An indirect immunofluorescence method was used to detect the presence of eosinophil granule MBP in the paraffin-embedded sections.[21–23] Two 6-μ serial sections were cut from each paraffin block and affixed to glass slides by LePage's Bond Fast resin glue. The sections were deparaffinized in xylene and rehydrated in absolute alcohol, 80% ethanol, and distilled water. The sections were incubated for 1 hour at 37°C in 0.1% trypsin solution to unmask antigenic sites, then incubated overnight in 10% normal goat serum to block nonspecific binding by fluorescein-labeled goat anti-rabbit gamma G immunoglobulin (IgG). The next day the slides were washed and overlaid with equal concentrations of either normal rabbit IgG (NRIgG) or affinity-purified rabbit anti-human MBP. After incubation at 37°C for 30 minutes, the sections were washed and incubated in 1% chromotrope 2R for 30 minutes to block nonspecific binding of fluorescein dye to the eosinophils. After another wash, the sections were overlaid with affinity-purified fluoresceinated goat anti-rabbit IgG and incubated at 37°C for 30 minutes. After a final wash, the slides were mounted with glycerol containing paraphenylenediamine[24] to delay fading of fluorescence emission, coverslipped, and sealed with clear nail polish. The slides were examined with a Zeiss (Carl Zeiss, Inc, Oberkochen, West Germany) standard microscope equipped with standard light illumination, Zeiss IV FL vertical illumination for epifluorescence, and a fluorescein filter system. After examination of the sections by fluorescence, areas of interest were photographed, and the

coverslips were removed. The sections were counterstained with hematoxylin and eosin, and the identical areas were rephotographed.

Affinity Chromatography-purified Reagents for Indirect Immunofluorescence

The specificity of anti-MBP staining in formalin-fixed, paraffin-embedded tissue has been investigated in detail and previously reported.[21–23,25] Briefly, immunoabsorption of anti-MBP serum with MBP removed reactivity, whereas immunoabsorption with unrelated basic proteins did not remove reactivity. To further reduce the possibility of nonspecific staining of other tissue elements, we used affinity chromatography-purified reagents. In the first stage of the assay, an affinity chromatography-purified rabbit anti-human MBP was used to localize MBP, and a staphylococcal protein A–purified NRIgG was used as the negative control; the preparation of these reagents has been described in detail elsewhere.[23] Equal concentrations of NRIgG and the affinity-purified anti-MBP were used in the immunofluorescence procedure. The affinity-purified anti-MBP does not react with normal tissues, including lung,[22] skin,[23] lymph node,[3] kidney,[26] liver,[27] and orbital tissue.[28] In addition, we used an affinity-purified, fluorescein-conjugated goat anti-rabbit IgG, available commercially through Southern Biotechnology Associates (Birmingham, AL), as the second-stage antibody.

Evaluation of Immunofluorescent Staining

The tissue eosinophilia, as seen by immunofluorescent staining of intracellular MBP, and eosinophil degranulation, as seen by extracellular MBP deposition, were evaluated in a blinded protocol by two of the authors (GJG and HN). Scoring of eosinophil infiltration was graded on a scale from 0 to 3+. If there were none or an occasional

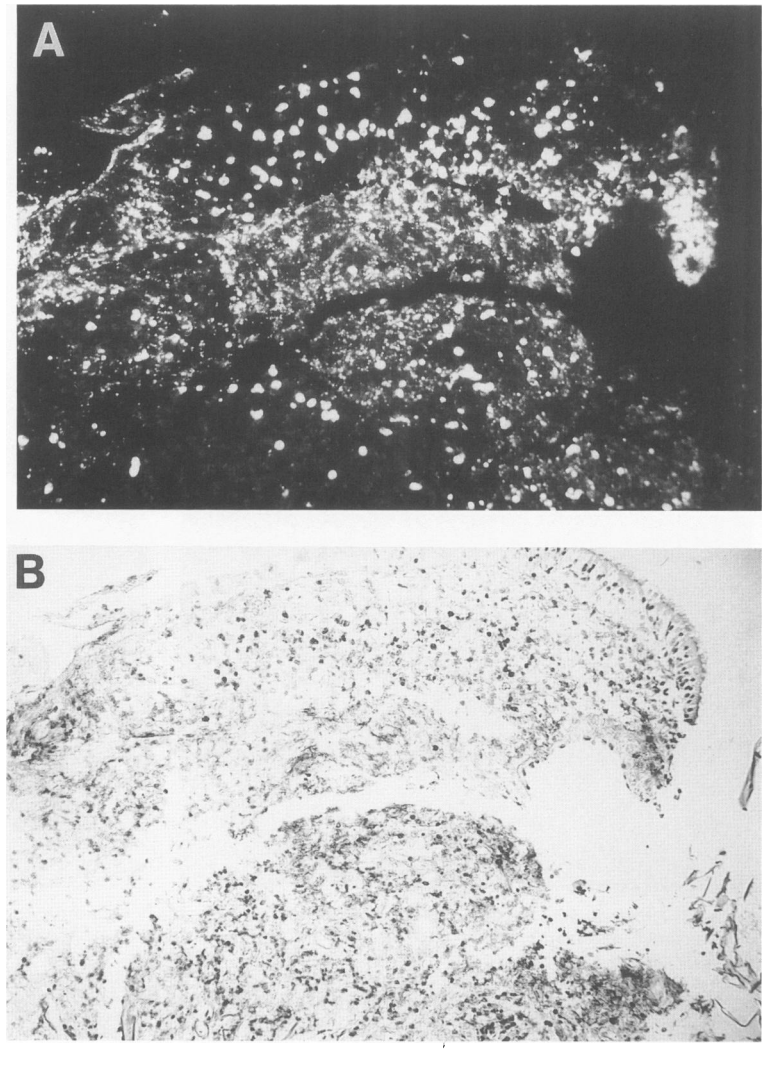

Figure 2. *Coexistence of eosinophil infiltration and extracellular MBP deposition in an area of submucosal scarring in the distal trachea of another patient with sclerosing mediastinitis.* **A**: *Section stained with anti-MBP.* **B**: *Same section counterstained with H&E. Note the extracellular MBP deposition as small, irregular granules in the loose fibrostroma. The scores for eosinophil infiltration and extracellular MBP deposition were both 2+. A serial section stained with NRIgG was negative (results not shown).* **A,B,** ×160.

eosinophil per 160× field, a score of 0 was assigned; if a biopsy specimen contained a few eosinophils per 160× field, it was given a score of 1+. A biopsy specimen containing moderate numbers of eosinophils was scored 2+, and a specimen containing confluent eosinophils was scored 3+. Extracellular MBP deposition as a marker of eosinophil degranulation was graded similarly. If no extracellular MBP staining was present, a score of 0 was assigned; if minimal extracellular MBP deposition (involving less than 10% of the tissue) was observed, a biopsy was assigned a score of 1+. Moderate MBP deposition (involving between 10% and 33% of the tissue) was scored 2+; whereas 3+ was assigned to specimens exhibiting marked deposition of MBP (involving more than 33% of the tissue). Statistical analyses were by chi-square test and by Spearman rank order correlation.

Results

The results of immunofluorescent localization of MBP were divided into three different patterns: 1) infiltration by intact eosinophils into immature connective tissues in the virtual absence of extracellular deposition of MBP (Figure 1); 2) infiltration by intact eosinophils as well as extracellular MBP deposition, often striking, onto fibrous tissue (Figure 2); and 3) extracellular MBP deposition in proximity to dense collagen bundles in the virtual absence of intact eosinophils (Figure 3). In contrast, the complete absence of both eosinophil infiltration and extracellular MBP deposition is illustrated in a specimen of tissue showing fibrosclerosis (Figure 4).

Eosinophil infiltration and/or extracellular MBP deposition were observed in 11 of the 12 specimens from patients with idiopathic retroperitoneal fibrosis, five of seven with sclerosing mediastinitis, all four with sclerosing cholangitis, and 8 of 11 with pulmonary fibrosis (Figure 5A). The intensities of eosinophil infiltration and degranulation in these tissues were related as judged by rank correlation analysis ($r_s = 0.76$, $P < 0.001$). A few specimens of sclerosing cholangitis showed characteristic eosinophil infiltration and extracellular MBP deposition around hepatic bile ducts. Overall, eosinophil infiltration and/or degranulation were observed in 28 of the 34 specimens (82%) from patients with chronic inflammatory diseases associated with fibrosis. In contrast, eosinophil infiltration

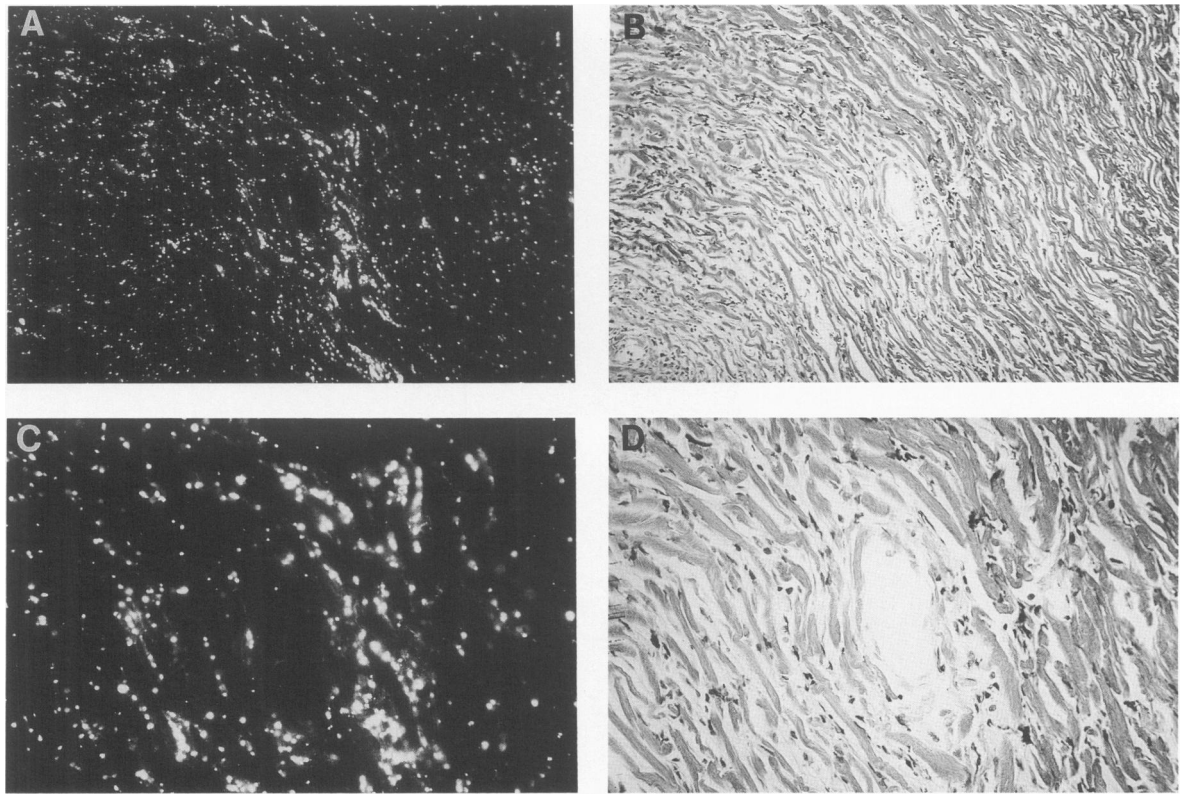

Figure 3. *Extracellular MBP deposition in a zone of diffuse fibrosis with mild chronic inflammatory (lymphocytic) infiltrate from a patient with idiopathic retroperitoneal fibrosis.* **A,C:** *Section stained with anti-MBP.* **B,D:** *Same section counterstained with H&E. Intact eosinophils (as illustrated in Figure 1) are absent in the dense fibrous tissue, and only scattered lymphocytes can be seen. The score for eosinophil infiltration was 0 and the score for extracellular MBP deposition was 2+. At higher magnification* **(C,D)**, *note the punctate extracellular MBP deposition in association with dense collagen bundles. A serial section stained with NRIgG was negative (results not shown).* **A,B,** *×160;* **C,D,** *×400.*

or degranulation were not observed in specimens from patients with noninflammatory fibrous proliferations (0/16) (Figure 5B).

A significant difference in the scores of eosinophil infiltration was detected between specimens from patients with chronic inflammatory fibrous proliferations and specimens from patients with noninflammatory fibrous proliferations ($\chi_2 = 30$, $P < 0.001$). Furthermore, a significant difference in the scores of extracellular MBP deposition was detected between specimens from patients with chronic inflammatory fibrosis and specimens from patients with noninflammatory fibrous proliferations ($\chi^2 = 25.5$, $P < 0.001$). Finally, as expected, a significant difference in the combined scores of eosinophil infiltration and extracellular MBP deposition was detected between specimens from patients with chronic inflammatory fibrosis and specimens from patients with noninflammatory fibrous proliferations ($\chi^2 = 29.9$, $P < 0.001$). Generally speaking, intact eosinophil infiltration was mainly observed in immature fibrous tissues, whereas extracellular MBP deposition mainly occurred in tissues with more advanced fibrosis.

Discussion

The eosinophil granule MBP has been localized by immunofluorescence in a number of tissues and organs whose dysfunction in disease has been associated with eosinophil infiltration and degranulation.[20] Here we tested the hypothesis that eosinophil degranulation, as evidenced by extracellular localization of MBP, and eosinophil infiltration are associated with lesions demonstrating fibrosis and inflammation that are not classically characterized by marked eosinophil infiltration. This hypothesis was based on prior observations showing such associations[1–9] as discussed above. For example, in eosinophilic endomyocardial fibrosis, deposits of eosinophil cationic protein and MBP were observed in endocardial fibrotic tissue.[2] In parasitic diseases, extracellular MBP

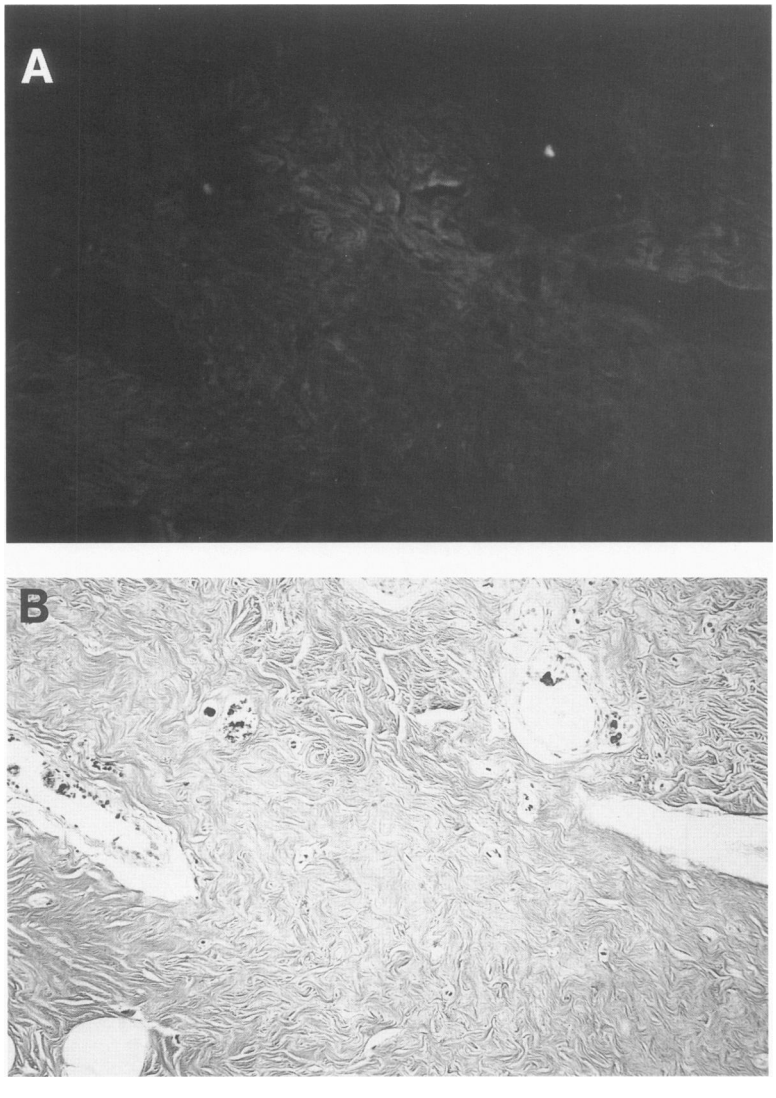

Figure 4. *Absence of both eosinophil infiltration and extracellular MBP deposition in breast tissue from a patient with dense stromal fibrosis as a part of fibrocystic disease.* **A**: *Section stained with anti-MBP.* **B**: *Same section counterstained with H&E. Note the complete absence of MBP staining. The scores for eosinophil infiltration and extracellular MBP deposition were both 0. A serial section stained with NRIgG was also negative (results not shown).* **A,B**, ×*160.*

deposition was observed in fibrotic lymph nodes from patients infected with *Onchocerca volvulus* (Kephart GM, Gibson DW, Connor D, Gleich GJ: unpublished observations).

In this study, we selected two groups of lesions associated with fibrosis. The first group, which we called inflammatory fibrosis, included examples of idiopathic retroperitoneal fibrosis, sclerosing mediastinitis, primary sclerosing cholangitis, and idiopathic pulmonary fibrosis. At affected sites, all of these conditions are associated with varying degrees of fibrosis and histologic inflammation, generally mononuclear in character. Idiopathic retroperitoneal fibrosis (sclerosing retroperitonitis) may have a dense lymphoid infiltrate that is predominantly mononuclear in character, with numerous plasma cells and small numbers of eosinophils at the periphery.[29] Sclerosing mediastinitis commonly is associated with lymphoid follicles; infiltrates of lymphocytes and plasma cells are present at the periphery of the fibrous masses, but eosinophils are generally not prominent.[30] In primary sclerosing cholangitis, the inflammatory infiltrates are primarily lymphoplasmocytic; however, neutrophils and eosinophils may be observed.[31] In idiopathic pulmonary fibrosis, the inflammation is generally mononuclear in character, manifesting as modest infiltrates of lymphocytes and plasma cells in zones of active fibrosis; eosinophils are not a prominent feature.[32] The second (control) group, which we called noninflammatory fibrous proliferations, included examples of keloids, hypertrophic scars, Dupuytren's contracture, and dense stromal fibrosis in breast specimens. Dupuytren's is considered a form of palmar fibromatosis; it is generally not associated with inflammation, although the degree of collagenization and fibroblastic proliferation may be quite prominent.[33]

Our results indicate that eosinophil infiltration and extracellular MBP deposition are significantly greater in specimens from patients with chronic inflammatory diseases associated with fibrosis than in specimens from patients with noninflammatory fibrous proliferations (Figure 5). These results and prior observations[1–9] support the hypothesis that eosinophils migrate into immature connective tissues, presumably responding to chemotactic stimuli, and release their cationic toxins into the connective tissue. Although MBP was localized in this

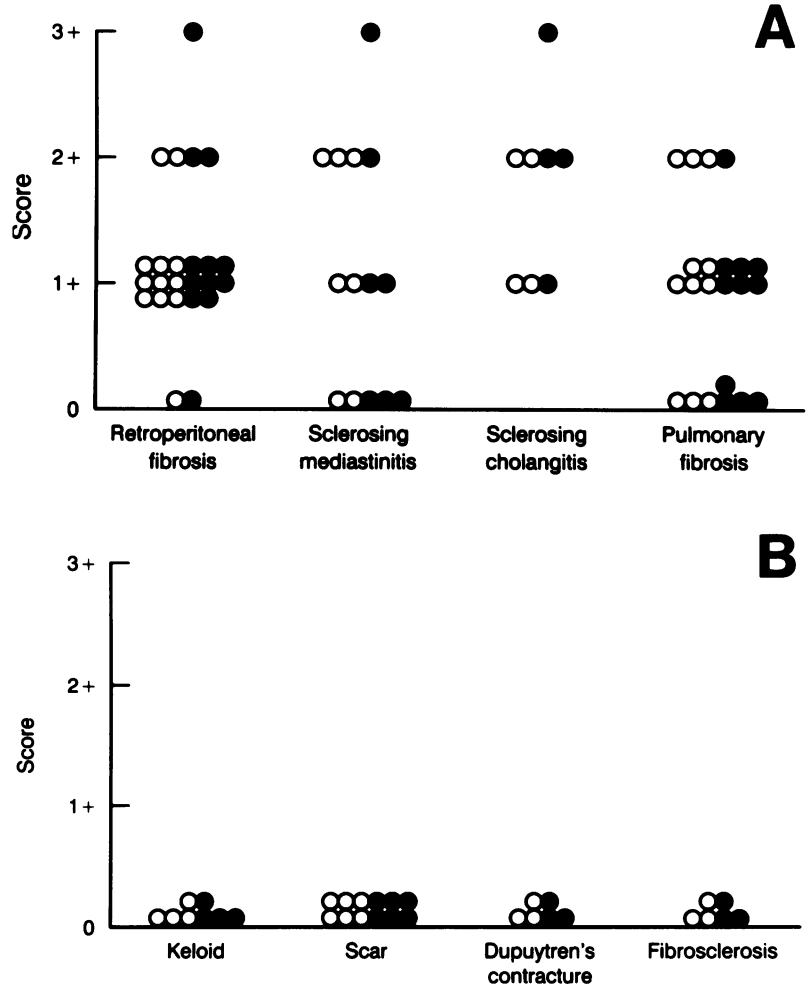

Figure 5. *MBP localization in patients with inflammatory fibrosis and noninflammatory fibrous proliferations.* A: *Eosinophil infiltration (open circle) and/or extracellular MBP deposition (closed circle) were observed in 28 of the 34 specimens (82%) from patients with inflammatory diseases associated with fibrosis.* B: *Eosinophil infiltration and extracellular MBP deposition were not observed in specimens from patients with noninflammatory fibrous proliferations (0/16). In this figure, dense stromal fibrosis of the breast is referred to as fibrosclerosis.*

study, other eosinophil proteins are also likely released, including the eosinophil peroxidase, eosinophil cationic protein, and eosinophil-derived neurotoxin. Whether eosinophils or their products possess the ability to stimulate fibroplasia in these diseases is not known. The results of Pincus and her associates[10] showing that eosinophil extracts stimulate fibroblast proliferation encourage belief that eosinophils possess a fibroblast stimulation factor. Furthermore, our preliminary results suggest that eosinophil-derived neurotoxin (but not MBP, the eosinophil peroxidase, or the eosinophil cationic protein) is able to stimulate fibroblast proliferation.[34] In conclusion, the observations in the specimens from patients with inflammation and fibrosis stand in sharp contrast to those in patients with noninflammatory fibrous proliferations. In the latter, no eosinophil infiltration and degranulation were observed. This observation suggests that eosinophils do not play a role in these noninflammatory forms of fibrosis or that their role is transient.

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