LOCALIZATION OF EOSINOPHIL GRANULE MAJOR BASIC PROTEIN IN PARACOCCIDIOMYCOSIS LESIONS

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Abstract. Paracoccidioidomycosis is a chronic granulomatous disease caused by the fungus Paracoccidioides brasiliensis. Although eosinophils have long been associated with the immune defense against helminths, the role of eosinophils in the immune response to fungal diseases is not as well studied. The eosinophil granule major basic protein is toxic to helminths and mammalian cells in vitro, and its release has been used as a marker of eosinophil localization and degranulation. To determine whether eosinophil infiltration and degranulation, as evidenced by the deposition of major basic protein, occur in lesions of P. brasiliensis, we used an immunofluorescence technique to localize the P. brasiliensis organisms and eosinophils and major basic protein. Initially, all tissues were stained with polyclonal antibody to major basic protein; subsequently, colocalization of major basic protein and P. brasiliensis by double staining with mouse and rabbit antibodies, respectively, was performed. Nine biopsy tissues from seven patients were analyzed. All nine biopsies showed infiltration of intact eosinophils using both the monoclonal and the polyclonal anti-major basic protein antibodies, along with the presence of P. brasiliensis. Furthermore, using the polyclonal anti-major basic protein antibody, nine of nine tissues showed extracellular major basic protein deposition (granular or diffuse fluorescence staining outside of intact eosinophils). The double staining procedure using the anti-major basic protein monoclonal antibody showed extracellular deposition in five of eight biopsies; in these five biopsies, approximately 60% of the areas containing P. brasiliensis had extracellular major basic protein deposited on the organisms. These observations support the hypothesis that the eosinophil, through toxic granule proteins such as major basic protein, participates in the pathophysiology of paracoccidioidomycosis.

Paracoccidioidomycosis is a deep mycosis caused by Paracoccidioides brasiliensis, a fungus that develops as a yeast at body temperature and as a mycelium at room temperature. Mycelia and spores grow in soil, water, and on plants. The disease is confined to Latin America, with its endemic area extending from Central America to Argentina. The prevalence of the disease in the endemic area has been estimated to be as high as one per 100,000 population.1 Many patients remain undiagnosed and untreated; in endemic areas, up to 60% of the healthy population are skin test positive.2 Paracoccidioidomycosis is a chronic granulomatous disease with involvement of the lungs, lymph nodes, mucocutaneous areas, and other organs.3 The disease most commonly affects males in the 30–49-year-old age group. It presents as either a systemic disease with a rapid course or as a chronic localized mycosis, depending on several factors including the host’s immunocompetence, strain of the parasite, and the environment.4 Current data point to the lungs as the portal of entry.5–7

Eosinophils are considered to be effector cells, which in the presence of antibody or complement, are able to kill parasites. Eosinophils have been associated with defense against parasites, such as Schistosoma mansoni,8,9 Trichinella spiralis,10 Trypanosoma cruzi,11,12 and Onchocerca volvulus.13 The role of eosinophils in the immune response to fungal infections has not been extensively studied. A related disease, coccidioidomycosis, caused by the fungus Coccidioides immitis, may be accompanied by an increase in peripheral blood eosinophils in the range of 3–10%.14 A few case reports on patients with coccidioidomycosis have shown more striking eosinophilia in blood or cerebrospinal fluid.15,16 One report on paracoccidioidomycosis showed peripheral blood eosinophilia in the three patients studied.17

Eosinophils contain several cationic proteins, including major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO).18 Major basic protein is toxic to mammalian cells,19 parasites,8,10,12 and bacteria in vitro.20 This protein is also able to activate basophils, mast cells, and platelets.21,22 Prior studies of diseased tissues have shown extensive MBP deposition in the absence of intact tissue eosinophils.23,24 By using an immunofluorescence technique for the colocalization of eosinophil granule MBP and P. brasiliensis, we tested the hypothesis that eosinophil infiltration and degranulation regularly occur in tissues from patients with paracoccidioidomycosis.

MATERIALS AND METHODS

Tissues. Nine biopsies were obtained from seven patients at the hospital in Botucatu, Brazil. All biopsy specimens were obtained as part of each patient’s diagnostic evaluation. Before admission to the hospital, each patient signed a statement agreeing to all the medical procedures relevant to their diagnostic evaluation. Biopsies were taken from lesions in the oral region, periorbital region, face, neck, palate and tonsils, lip, and vocal cord and were fixed in 10% formalin. All patients had undergone some treatment with antifungal agents (sulfonylamides, itraconazole, or amphotericin B) prior to the time of biopsy. All seven patients responded well to treatment. Table 1 summarizes the clinical histories of the patients.

Immunofluorescence staining with anti-MBP polyclonal antibody. Tissue sections were initially stained with affinity purified polyclonal rabbit antihuman MBP. Staining was performed as previously described.25,26 Briefly, tissues were deparaffinized and trypsin digested to expose antigenic sites. Tissue sections were incubated overnight in 10% normal goat serum to block nonspecific binding of antibody. Tissue slides were washed and either rabbit anti-
MBP or normal rabbit IgG (as a negative control) was applied to the tissue at a concentration of 25 μg/ml and allowed to incubate 30 min at 37°C. After washing, slides were placed in chromotrope-2R to block nonspecific binding of the secondary antibody to eosinophils. After further washing, the secondary antibody, affinity-purified goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Southern Biotechnology Associates, Birmingham, AL), was applied to the tissue at a concentration of 25 μg/ml and incubated 30 min at 37°C. Sections were then washed and a coverslip was applied using 0.1% gelatin in glycerol to prevent fading.

**Immunofluorescence double staining.** Eight of the original nine tissues were double stained using antibodies to both MBP and the *P. brasiliensis* organism (one sample was stained with anti-MBP polyclonal antibody only). Staining was performed, as described above, with the following modifications. Rabbit anti-*P. brasiliensis* was prepared as previously described. The primary antibodies were a mixture of rabbit anti-*P. brasiliensis* (1:30 dilution in normal goat serum) and J150-12A3, a protein A–purified IgG1 mouse anti-MBP monoclonal antibody (100 μg/ml) produced in our laboratory. As negative controls, we used a mixture of protein A–purified normal rabbit IgG (50 μg/ml) and MOPC-31, an IgG1 antibody from a mouse myeloma cell line (100 μg/ml) (Sigma Chemical Co., St. Louis, MO). The secondary antibodies were a mixture of affinity-purified goat anti-rabbit IgG conjugated to rhodamine isothiocyanate (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and affinity-purified goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Southern Biotechnology Associates, Inc.).

**Grocott’s methenamine silver (GMS) staining.** Sections were stained by the Pathology Section Processing Laboratory at Mayo Foundation. Briefly, sections were deparaffinized, hydrated, and oxidized in 10% chromic acid for 10 min. Sections were washed and placed in 1% sodium metabsulfitie for 1 min. After rinsing, sections were placed in preheated methenamine silver solution at 90°C for 5 min. Sections were then placed in the following solutions with distilled water rinses between each: 1% gold chloride for 15 sec, 5% sodium thiosulfate for 3 min, 80% alcohol for 30 sec, eosin counterstain for 30 sec. Finally, sections were dehydrated with 95% alcohol, two changes of absolute alcohol, and two changes of xylene, and mounted.

**Control tissues.** Six facial skin biopsies were taken from six healthy individuals living in the endemic area. The control tissues were from the uninvolved margins of surgical specimens from patients who underwent surgery for different types of skin tumors. Patient ages ranged from 18 to 87 years; five were female and one was male. Immunofluorescence double staining and GMS staining were performed on these tissues as described earlier in this report.

**Photomicrography.** Sections were viewed and photographed with a Zeiss (Oberkochen, Germany) Axioshot fluorescence microscope, using Ektachrome 200 film (Eastman Kodak, Rochester, NY) for fluorescence photographs and Ektachrome 64 film for transmitted light photographs.

**Scoring.** Each 160× tissue field was assigned three separate scores: a score of 0 to +2 for *P. brasiliensis* infiltration, 0 to +3 for eosinophil infiltration, and 0 to +3 for the presence of extracellular MBP. Representative 160× photographs for each score were taken and used as references for the actual scoring of the patient tissues, which was done by two independent observers (Figure 1). *Paracoccidioides brasiliensis* infiltration was scored as zero if no *P. brasiliensis* organisms were present in the field of view, +1 for between one and 30 organisms per field, and +2 for greater than 30 organisms per field. Eosinophil infiltration was scored as zero if no eosinophils were present in the field of view, +1 for fewer than 20 eosinophils per field, +2 for greater than 20 eosinophils but covering less than half of the field, and +3 for numerous eosinophils covering greater than half of the field. Granular/extracellular MBP was scored as zero for none present, +1 for the presence of minimal extracellular MBP, +2 for moderate amounts of extracellular MBP, and +3 for extracellular MBP staining covering greater than half the field. Each tissue was then given a mean score for *P. brasiliensis* infiltration, eosinophil infiltration, and extent of extracellular MBP deposition. The mean of the two observers’ mean scores was calculated.

**Table 1**

Clinical information and scoring of biopsies double stained with polyclonal antibody to *Paracoccidioides brasiliensis* and monoclonal antibody to major basic protein (MBP)*

<table>
<thead>
<tr>
<th>Biopsy ID</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Site of biopsy</th>
<th>Time between onset of disease to biopsy (months)</th>
<th><em>P. brasiliensis</em> infiltration (0–2) Mean</th>
<th><em>P. brasiliensis</em> infiltration (0–2) Max</th>
<th>Eosinophil infiltration (0–3) Mean</th>
<th>Eosinophil infiltration (0–3) Max</th>
<th>Extracellular MBP (0–3) Mean</th>
<th>Extracellular MBP (0–3) Max</th>
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<tr>
<td>A</td>
<td>44</td>
<td>M</td>
<td>Oral</td>
<td>1</td>
<td>1.2</td>
<td>2.0</td>
<td>1.0</td>
<td>1.5</td>
<td>1.6</td>
<td>2.0</td>
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<tr>
<td>B</td>
<td>48</td>
<td>M</td>
<td>Oral</td>
<td>1</td>
<td>1.2</td>
<td>2.0</td>
<td>2.1</td>
<td>3.0</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>C</td>
<td>53</td>
<td>M</td>
<td>Periorbital</td>
<td>5</td>
<td>1.0</td>
<td>1.0</td>
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<td>22</td>
<td>M</td>
<td>Facial</td>
<td>3</td>
<td>1.3</td>
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<td>1.7</td>
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<tr>
<td>E</td>
<td>65</td>
<td>M</td>
<td>Neck</td>
<td>3</td>
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<tr>
<td>F</td>
<td>34</td>
<td>M</td>
<td>Palate and tonsils</td>
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<td>1.3</td>
<td>2.0</td>
<td>2.2</td>
<td>3.0</td>
<td>1.0</td>
<td>1.5</td>
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<tr>
<td>G†</td>
<td>36</td>
<td>M</td>
<td>Vocal cord</td>
<td>6</td>
<td>1.3</td>
<td>2.0</td>
<td>2.2</td>
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<td>1.1</td>
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<td>H‡</td>
<td>Lip ulcer</td>
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<td>2.0</td>
<td>2.1</td>
<td>2.5</td>
<td>0.4</td>
<td>1.0</td>
<td>1.7</td>
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<tr>
<td>I‡</td>
<td>Palate</td>
<td>1.3</td>
<td>2.0</td>
<td>1.7</td>
<td>2.6</td>
<td>0.4</td>
<td>0.5</td>
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<td>Average</td>
<td>1.3</td>
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<td>1.9</td>
<td>2.6</td>
<td>2.6</td>
<td>0.4</td>
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<td>Range</td>
<td>1.0–1.5</td>
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<td>1.0–2.2</td>
<td>1.5–3.0</td>
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<td>1.0–2.5</td>
<td>1.0–2.5</td>
<td>0.4–1.7</td>
<td>1.0–2.5</td>
</tr>
</tbody>
</table>

* Mean is the average of the two observers' mean grades; max is the average of the two observers' maximum grades.
† G, H, and I are consecutive biopsies from one patient on different dates.
‡ NG = not graded, insufficient tissue; this biopsy was stained with polyclonal anti-MBP.
Eosinophils. Eosinophils were observed in direct association with *P. brasiliensis* in approximately 75% of the regions containing *P. brasiliensis*; in other cases, clusters of eosinophils were located nearby but not in direct contact with the *P. brasiliensis*. Extracellular MBP deposition was present in five of the eight biopsies, using the J150-12A3 monoclonal antibody. In these five biopsies, approximately 60% of the regions containing *P. brasiliensis* showed MBP deposition directly on the organisms (Figure 3). The range of scores for eosinophil infiltration was 1.0–2.2 with a mean of 1.9. Extracellular MBP scores ranged from 0.4 to 1.7 with a mean of 1.1 (Table 1).

Staining of the six skin biopsies from six healthy individuals living in the endemic area did not show any eosinophils, extracellular MBP, or *P. brasiliensis* organisms.

**RESULTS**

**Anti-MBP polyclonal antibody stain.** The original staining of the biopsy samples was performed with affinity-purified polyclonal rabbit anti-human MBP. All nine biopsies were positive and showed both intact eosinophils and extracellular MBP (Figure 2). Eight of the nine tissues were stained again with anti-MBP polyclonal antibody for scoring purposes (insufficient tissue remained for one biopsy). The assigned scores for 160× fields in these biopsies ranged as high as 3.0 for both eosinophil infiltration and the presence of extracellular MBP; the mean scores for the eight tissues ranged from 0.4 to 2.4 for eosinophil infiltration and from 0.6 to 1.9 for extracellular MBP.

**Double staining with anti-MBP and anti-*P. brasiliensis*.**

Eight of the nine biopsies were double stained with rabbit anti-*P. brasiliensis* and mouse anti-MBP monoclonal antibody J150-12A3. All eight biopsies showed the presence of *P. brasiliensis*. The number of *P. brasiliensis* organisms varied from 0 to 40 per 160× field. In some tissues, budding around the outer edge of the yeast organism could be seen. The quality of the staining with the rhodamine-conjugated anti-*P. brasiliensis* and with GMS varied somewhat among tissues with regards to the degree of nonspecific staining. Thus, *P. brasiliensis* organisms were more clearly seen with the rhodamine-conjugated anti-*P. brasiliensis* in some biopsy tissues, whereas in other tissues, the organism was more clearly seen with the GMS stain. The range of scores for *P. brasiliensis* infiltration was 1.0–1.5 with a mean of 1.3 (Table 1).

Eight of eight biopsies were positive with the J150-12A3 anti-MBP antibody. All eight biopsies contained numerous eosinophils. Eosinophils were observed in direct association with *P. brasiliensis*.

**DISCUSSION**

Eosinophils and their granule proteins have long been associated with defense against parasites such as *S. mansoni*, *Trichinella spiralis*, *T. cruzi*, and *O. volvulus*. Previous studies on the pathology of the *P. brasiliensis* granuloma have shown several different effector cells: natural killer cells, lymphocytes, cytotoxic T cells, activated neutrophils, activated macrophages, epithelioid cells, giant cells, and B cells. However, eosinophils have not been included among the effector cells. One study on bronchoalveolar lavage fluid from six patients with paracoccidioidomycosis showed an alveolitis with increased numbers of neutrophils but eosinophils were not found. In contrast, two recent studies have associated eosinophilia with the disease. One study of three patients with paracoccidioidomycosis and bone marrow involvement showed peripheral blood eosinophilia in all three

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**FIGURE 2.** Immunofluorescence staining (with polyclonal anti-major basic protein [MBP]) of a neck lesion (biopsy E in Table 1). A, section stained with anti-MBP polyclonal antibody showing eosinophil infiltration and extensive deposition of extracellular MBP. Serial section stained with normal rabbit IgG as a negative control. B, hematoxylin and eosin staining of a section serial to A. (Original magnification ×160.)

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**FIGURE 1.** Scoring standards for infiltration of tissues with *Paracoccidioides brasiliensis*. Tissues were graded in a field-by-field manner by assigning three scores per 160× field: *P. brasiliensis* infiltration: A, +1 (< 30 organisms per field); arrows point to *P. brasiliensis* organisms; B, +2 (> 30 organisms per field); C, +1 (< 20 eosinophils per field); D, +2 (> 20 eosinophils but covering less than half the field); E, +3 (numerous eosinophils covering greater than half the field). Extracellular major basic protein (MBP): F, +1 (minimal extracellular MBP present); G, +2 (moderate amounts of extracellular MBP); H, +3 (extracellular MBP covering greater than half the field). (Original magnification ×160.)
FIGURE 3. Immunofluorescence double staining with anti-major basic protein (MBP) monoclonal antibody and anti-
*Paracoccidioides brasiliensis* polyclonal antibody. A–C, colocalization of eosinophils, MBP, and *P. brasiliensis* in the same neck lesion shown in Figure 2. A, immunofluorescence staining with anti-MBP (J150-12A3) monoclonal antibody showing extensive eosinophil infiltration and degranulation. B, double staining of the same section as in A using anti-*P. brasiliensis* polyclonal antibody. The white arrows point to a few of the *P. brasiliensis* organisms present. Note the deposition of extracellular MBP directly on the *P. brasiliensis* organisms. C, Grocott’s methenamine silver staining of a section serial to A and B. D and E, colocalization of eosinophils, MBP, and *P. brasiliensis* in an oral lesion (biopsy A in Table 1). D, immunofluorescence staining with anti-MBP (J150-12A3) monoclonal antibody. E, double staining of the same section as in A using anti-*P. brasiliensis* polyclonal antibody. Eosinophils and extracellular MBP are in close proximity, but not directly on the *P. brasiliensis* organisms. (Original magnification × 400.)
patients. A second study was a case report of a six-year-old boy with paracoccidioidomycosis who had eosinophilia in the bone marrow and the blood. Another study on the in vitro response of lymphocytes from patients with paracoccidioidomycosis showed an inverse correlation of P. brasiliensis antigen response with eosinophil levels and anti-P. brasiliensis antibody levels.

A related disease, coccidioidomycosis, is endemic in the desert area of the southwestern United States. Previous studies show that coccidioidomycosis is frequently accompanied by an increase in peripheral blood eosinophils in the range of 3–10%. Dissemination to sites such as skin, bones, joints, and the central nervous system occurs in < 1% of exposed persons. Peripheral blood eosinophilia has been suggested as a marker of dissemination of primary coccidioidomycosis and an indicator of a poor prognosis. On the other hand, in this study, all seven patients responded well to treatment with antifungal agents. Biopsies from all of these patients showed eosinophils and extracellular MBP; this may indicate the eosinophils have helped in the host’s defense against the P. brasiliensis.

Extracellular deposition of MBP has been used as a marker of eosinophil localization and degranulation. By immunofluorescence, MBP has been localized extracellularly in tissues and organs whose dysfunction in disease is frequently associated with eosinophil infiltration, such as bronchial asthma, chronic urticaria, and atopic dermatitis. In parasitic diseases, MBP deposition was observed in proximity to degenerating microfilaria in patients infected with O. volvulus and on Schistosoma eggs in granulomas of patients infected with S. mansoni.

Assessment of eosinophil involvement in disease should not be based simply on the number of eosinophils present because intact eosinophils may not be evident; i.e., tissues should be examined for evidence of eosinophil degranulation. Hematoxylin and eosin staining often fails to show the presence of eosinophil granules and extracellular granule proteins. Previous studies on cardiac tissue have shown the advantages of immunofluorescence staining over light microscopy for accurately visualizing eosinophils and their granules or extracellular proteins (Chau E and others, unpublished data). Our immunofluorescence double-staining method has shown a clear association between the presence of P. brasiliensis, infiltration of the lesion by eosinophils, and MBP deposition on the P. brasiliensis. Based on prior studies, other eosinophil granule proteins (EDN, ECP, and EPO) are likely also released in this disease.

Knowledge of the presence of eosinophils and eosinophil proteins furthers our understanding of the pathology of the P. brasiliensis lesions and the overall immune response of the patient. Increased eosinophils in the blood or tissue lesions may be a prognostic indicator and may provide another possible target for disease treatment.

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