

Localized Eosinophil Degranulation Mediates Disease in Tropical Pulmonary Eosinophilia

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To explore the mechanisms underlying the eosinophil-mediated inflammation of tropical pulmonary eosinophilia (TPE), bronchoalveolar lavage (BAL) fluid, serum, and supernatants from pulmonary and blood leukocytes (WBC) from patients with acute TPE ($n = 6$) were compared with those obtained from healthy uninfected individuals ($n = 4$) and from patients with asthma ($n = 4$) or elephantiasis ($n = 5$). Although there were no significant differences in the levels of interleukin-4 (IL-4), IL-5, IL-13, eotaxin, granulocyte-macrophage colony-stimulating factor, RANTES, or eosinophil cationic protein, there was a marked increase in eosinophil-derived neurotoxin (EDN) both systemically and in the lungs of individuals with TPE compared to each of the control groups ($P < 0.02$). Moreover, there was a compartmentalization of this response, with EDN levels being higher in the BAL fluid than in the serum ($P < 0.02$). Supernatants from WBC from either whole blood or BAL cells were examined for chemokines, cytokines, eosinophil degranulation products, and arachidonic acid metabolites. Of the many mediators examined—particularly those associated with eosinophil trafficking—only EDN (in BAL fluid and WBC) and MIP-1 α (in WBC) levels were higher for TPE patients than for the non-TPE control groups ($P < 0.02$). These data suggest it is the eosinophilic granular protein EDN, an RNase capable of damaging the lung epithelium, that plays the most important role in the pathogenesis of TPE.

Tropical pulmonary eosinophilia (TPE), an unusual manifestation of human lymphatic filarial infection, is characterized by an eosinophilic pulmonary infiltrate and marked elevations of immunoglobulin E (IgE) and circulating eosinophils in the serum, all felt to be mediated by immunologic hyperreactivity to filarial parasites or their antigens. Although 129 million people worldwide are infected with lymphatic filariasis, <0.01% develop TPE (20). It is unclear what factors predispose patients to the rare, localized, and profound immune dysregulation associated with TPE.

Typically, microfilariae circulate in the blood of patients with lymphatic filariasis without significant clinical consequences. In the case of TPE, however, these microfilariae appear to be trapped in the lung on their first pass through the circulation, where they are presumed to initiate an inflammatory response. The role of the filariae (and microfilariae, in particular) in the immune response of TPE has been corroborated by lung biopsy findings (37) and high levels of filaria-specific IgE and IgG found in TPE patients (15, 24). In contrast to the majority of people with lymphatic filariasis, who have a downregulated response to the parasites (24), patients with TPE mount a robust systemic and localized immune response that includes elevations of both polyclonal and filaria-specific IgE and IgG (23), as well as expansion of interleukin-4 (IL-4)- and IL-5-producing T cells (19).

Compartmentalization of the immune response to the lungs has been established by studies of bronchoalveolar lavage (BAL) fluid from patients with TPE. The impressively high levels of eosinophils found in the peripheral blood of TPE patients (>3,000/ μ l) are surpassed in the lungs: levels have

been determined to be 12-fold more concentrated in the epithelial lining fluid of the lungs than in the systemic circulation (26). Eosinophils are the predominant effector cell in the BAL fluid of patients with TPE, and unlike the rare eosinophils in the BAL fluid of normal lungs, the pulmonary eosinophils in TPE are degranulated and activated (26) and release abnormally high levels of toxic oxygen radicals even after antifilarial treatment (29). Filaria-specific IgE and IgG antibodies are found in both the serum and BAL fluid of TPE patients; however, the lung antibodies recognize a distinct subset of the filarial antigens recognized by the antibodies in the periphery (23), the most dominant being a parasite-derived γ -glutamyl transpeptidase (16).

TPE has some parallels with atopic asthma, although bronchial hyperreactivity occurs much less frequently in TPE than in asthma, and in TPE, the tissue and blood eosinophilia and IgE elevations are more extreme (22). Although many mediators of eosinophilia (e.g., IL-5, granulocyte-macrophage colony-stimulating factor [GM-CSF], and IL-3 [7]), eosinophil trafficking (RANTES, MIP-1 α [3], and eotaxin [14]), and eosinophil activation (LTB4 [21]) have been shown to be expressed at high levels in the BAL fluid of asthmatic patients following allergen challenge, the mediators orchestrating eosinophilic chemotaxis and activation in TPE are largely unknown. The presence of eosinophilic granular proteins—eosinophil-derived neurotoxin (EDN), MBP, and eosinophilic cationic protein (ECP)—in the airways of patients with asthma (2, 15) has led to the hypothesis that these proteins play a role in the destruction of the bronchial epithelium (8); again, their exact role in TPE remains unexplored.

A mild form of interstitial lung disease persists in the majority of patients treated for TPE (29). For the untreated TPE patient, the outcome is more extreme: a progressive interstitial fibrosis. In some diseases associated with intense tissue fibrosis,

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TABLE 1. Clinical summary of study and control populations

| Patient group (<i>n</i>) | No. by sex ^a | Age (yr) | Peripheral eosinophilia cells/ μ l of blood (range) | GM (10^3) BAL eosinophilia cells/ml (range [10^3]) | GM serum IgE (ng/ml) (range) | Range of serum antifilarial IgG (U/ml) | Range of BAL antifilarial IgE (ng/ml of ELF ^b) |
|----------------------------|-------------------------|----------|---|--|------------------------------|--|--|
| TPE (6) | 6/0 | 15–38 | 9,292 (6,286–41,210) | 850 (304–2,490) | 29,222 (6,286–53,333) | 12,800–100,000 | 63.8–864 |
| Normal (4) | 3/1 | 18–26 | 511 (128–924) | 19 (5–175) | 2,769 (175–9,756) | 200–1,600 | 0 |
| Asthma (3) | 3/0 | 19–31 | 1,073 (750–1,320) | 1.4 (1–1.4) | 524 (132–1,035) | 100–800 | 0 |
| Elephantiasis (5) | 3/2 | 18–40 | 732 (444–903) | 3.7 (2–8) | 1,616 (358–1,563) | 200–3,200 | 0 |

^a Male/female.^b ELF, epithelial lining fluid.

the abundance of degranulated eosinophils supports a link between eosinophilic inflammation and fibrosis (6). Knowledge of the early inflammatory mediators of TPE that could potentiate tissue remodeling would further our understanding of the chronic sequelae of TPE.

In this study, having access to cryopreserved BAL fluid and serum material from TPE patients, we investigated many of the cytokines, chemokines, eosinophilic granular proteins, and leukotrienes that have been implicated in the pathology of other eosinophil-mediated lung diseases to determine whether they also play roles in TPE.

MATERIALS AND METHODS

Clinical study. In 1984, the Laboratory of Parasitic Diseases (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and the Pulmonary Branch (National Heart, Lung and Blood Institute, National Institutes of Health) jointly conducted a study in Madras, India, in which sera and cell-free BAL fluid were collected from subjects who fulfilled the diagnostic criteria for TPE and from three control groups (23, 26). The diagnosis of TPE was based on the following criteria: residence in an area of endemicity in southern India, recent onset of symptoms, chest X-ray infiltrates, restrictive defects in lung function, peripheral eosinophilia, high total IgE in the serum, and high titers of antifilarial IgG in the serum (25). Control group samples were obtained from healthy uninfected and nonsmoking individuals (*n* = 4), individuals with asthma who had respiratory disease but no evidence of lymphatic filariasis (*n* = 4), and individuals with elephantiasis but without respiratory disease (*n* = 5). The healthy and elephantiasis control groups had normal chest X rays, chest examinations, and pulmonary function tests. Patients with asthma had a history of wheezing and exertional dyspnea and a clinical response to oral bronchodilators. All of the asthma control group had normal pulmonary function tests at the time of the study. The BAL procedure was performed using 300 ml of saline lavage fluid as described previously (26).

Leukocytes (WBC) were obtained by dextran sedimentation of either whole blood or BAL cells. After several washes in RPMI, the cells were cultured at a concentration of 10^6 /ml for 24 h in RPMI supplemented with HEPES (10 mM), gentamicin (50 μ g/ml), and 10% fetal calf serum. After 24 h, the cell supernatants were collected and stored at -70° C until they were assayed. These samples were available for a subgroup of the TPE and control patients.

ELISA and RNase assays. Stored and previously unthawed frozen BAL samples, sera, and cell-free WBC culture supernatants from the 1984 study were examined by enzyme-linked immunosorbent assay (ELISA) analysis for chemokine and cytokine production, eosinophil degranulation products, and arachidonic acid metabolites.

BAL values were normalized for volume to correct for the dilution of the lavage fluid infused (27). Normalization based on comparing urea or albumin concentrations in the periphery to the BAL fluid did not change the interpretation of the data (28).

Commercial ELISAs were used according to the manufacturer's instructions for IL-13, GM-CSF, IL-3 (Biosource International Inc.), MIP-1 α , LTB4, and eotaxin (R&D Systems Inc.). IL-4, IL-5, and RANTES were measured using antibody pairs and techniques described previously (32). EDN and ECP measurements were performed as described previously (10), and the assay for BAL RNase was performed exactly as described previously (12, 31).

Statistical analyses. Statistical analyses were performed using the Mann-Whitney U test for comparisons of groups and the Wilcoxon signed rank test for paired analyses. When multiple comparisons were made, Bonferroni's correction was applied.

RESULTS

Study population. The clinical data for the study and control populations summarized in Table 1 represent a subset of previously reported individuals (23, 26, 29). As has been shown previously, the serum IgE, eosinophilia, and serum antifilarial IgG levels were significantly lower in the healthy, asthmatic, and elephantiasis control groups than in those with TPE. All individuals in the asthma control group had mild eosinophilia. Only the TPE group had measurable antifilarial IgE levels in the BAL fluid.

BAL and serum analysis. Despite the marked differences in the serum IgE and peripheral eosinophil counts between the subjects with TPE and the subjects in the three control groups, there were no significant differences among the four study groups in the levels of the eosinophilopoietic cytokines (IL-5 and GM-CSF), the eosinophilic attractive chemokine eotaxin, ECP, or the type 2 differentiating cytokines (IL-4 and IL-13) in the serum (Table 2). Levels of RANTES, a known eosinophil chemoattractant, in the sera of TPE patients were significantly increased compared with those of either healthy individuals or the non-TPE group as a whole (Table 2).

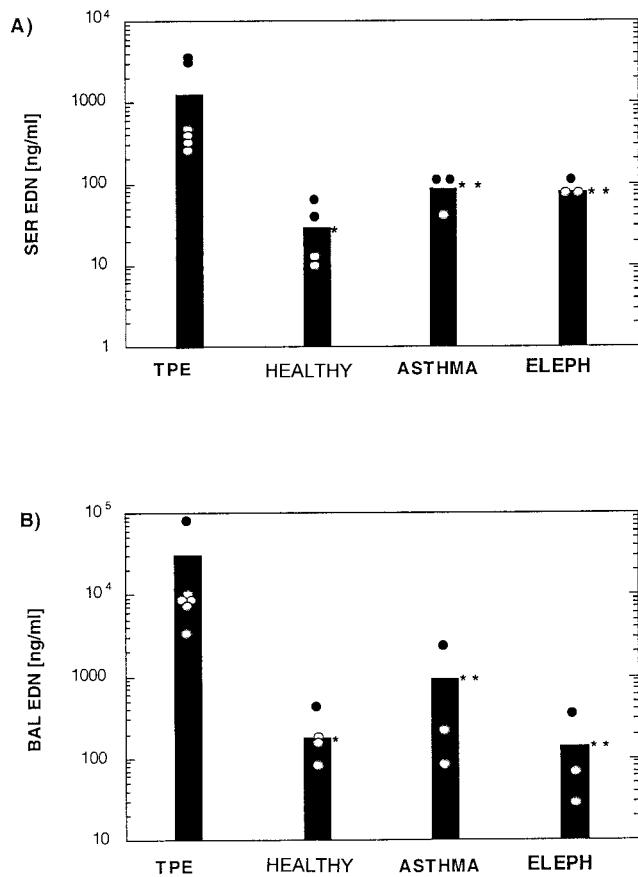
The cytokines (IL-4, IL-5, IL-13, and GM-CSF) and the chemokines (eotaxin and RANTES) were below the level of detection for all BAL samples (data not shown). The eosinophilic granular protein ECP was detectable in all BAL samples, but levels were not significantly different in the TPE group and the other control groups (Table 2).

There was, however, a marked increase in EDN, both systemically and in the lungs of individuals with TPE compared with each of the other control groups ($P \leq 0.02$) (Fig. 1). The geometric mean (GM) levels of EDN for TPE patients (12,155 ng/ml) were on average >33-fold greater than for those with asthma (358 ng/ml), 130-fold greater than for those with elephantiasis (90 ng/ml), and 67-fold greater than for healthy individuals (181 ng/ml). Similarly, the GM of EDN in the sera of TPE patients (706 ng/ml) was 7-fold greater than that of asthma (85 ng/ml) or elephantiasis (90 ng/ml) patients and 32-fold greater than that of healthy individuals (22 ng/ml). Indeed, the TPE patient BAL fluid had RNase levels that were significantly greater than those of normal control samples ($P < 0.05$), and there was a trend toward a positive relationship ($P < 0.06$) between the BAL EDN levels and the BAL RNase values.

Moreover, there was a compartmentalization of this response, with EDN concentrations being higher in the BAL fluid than in the serum when paired BAL fluid and serum EDN levels were compared for each TPE patient ($P < 0.02$) (Fig. 2A). For the elephantiasis group, the GMs of the BAL fluid and serum EDN levels were very similar (89.9 ng/ml for BAL

TABLE 2. Selected levels of serum and BAL cytokines, chemokines, and eosinophil degranulation products in TPE and other clinical groups

| Group | GM value (range) | | | | | BAL ECP (ng/ml) | |
|----------------------|------------------|--------------|---------------|-----------------|------------------------------------|-----------------|----------------------------|
| | IL-4 (pg/ml) | IL-5 (pg/ml) | IL-13 (pg/ml) | Eotaxin (pg/ml) | RANTES (pg/ml) | | |
| TPE | 106 (<78-489) | 88 (<68-331) | <78 (<78) | 87 (24-581) | 58,891 (36,212-162,543) | 393 (105-2,163) | 1,029 (714-1,283) |
| Healthy | 102 (<78-224) | <68 (<68) | 93 (<78-147) | 87 (20-244) | 14,056 ^b (7,660-21,054) | 197 (<78-314) | 1,016 (640-1,280) |
| Asthma | 325 (<78-1,250) | <68 (<68) | <78 (<78) | 179 (78-617) | 19,188 (6,033-57,198) | 152 (129-234) | 796 (408-1,245) |
| Elephantiasis | 142 (<78-250) | 91 (<68-151) | <78 (<78) | 238 (116-588) | 26,451 (13,826-43,372) | 151 (116-232) | 710 ^c (587-894) |
| Non-TPE ^a | 166 (<78-1,250) | 76 (<68-151) | 83 (<78-147) | 128 (20-617) | 19,727 ^b (6,033-57,198) | 164 (<78-314) | 885 (408-1,280) |

^aThe non-TPE group represents a pool of all control groups (healthy individuals and asthma and elephantiasis patients).^b*P* < 0.014 compared with TPE group.^c*P* = 0.03 compared with TPE group.^d*P* = 0.045 compared with TPE group.FIG. 1. TPE is associated with increased levels of EDN in serum (SER) (A) and in BAL fluid (B). Each individual's value is represented by a circle. The top of the bar represents the GM for each group. *, *P* = 0.01; **, *P* = 0.02. ELEPH, elephantiasis.

versus 90.2 ng/ml for serum EDN). In the ECP analysis, there was a 10-fold difference between BAL fluid and serum ECP levels for all TPE patients studied; however, this difference was not statistically significant (Fig. 2B). There was no statistical correlation between EDN levels and eosinophil levels in the sera or BAL fluid of all groups (data not shown), suggesting that the EDN levels were more reflective of eosinophil activation than of total numbers of eosinophils.

WBC and BAL fluid culture supernatant analysis. Because a limited number of WBC and BAL fluid culture supernatants were available for analysis, a preliminary analysis of material from patients who had been recently treated for TPE was performed for the many mediators associated with eosinophil production, recruitment, and activation (including RANTES, IL-13, GM-CSF, LTB4, MIP-1 α , IL-4, IL-5, eotaxin, ECP, and EDN) (data not shown). After the analysis, we chose to look at ECP, EDN, MIP-1 α , and LTB4. In this analysis, the asthma and elephantiasis patients and healthy controls were pooled into one group that was categorized as "non-TPE."

Only EDN (in BAL fluid and WBC culture supernatants) and MIP-1 α (in WBC cultures) were significantly higher for TPE patients than for the non-TPE control groups (*P* ≤ 0.02) (Fig. 3). The contrast in EDN levels in the BAL fluid culture supernatants between the TPE and non-TPE groups was striking (GM, 151 versus 2.5 ng/ml) (*P* = 0.006); in the peripheral

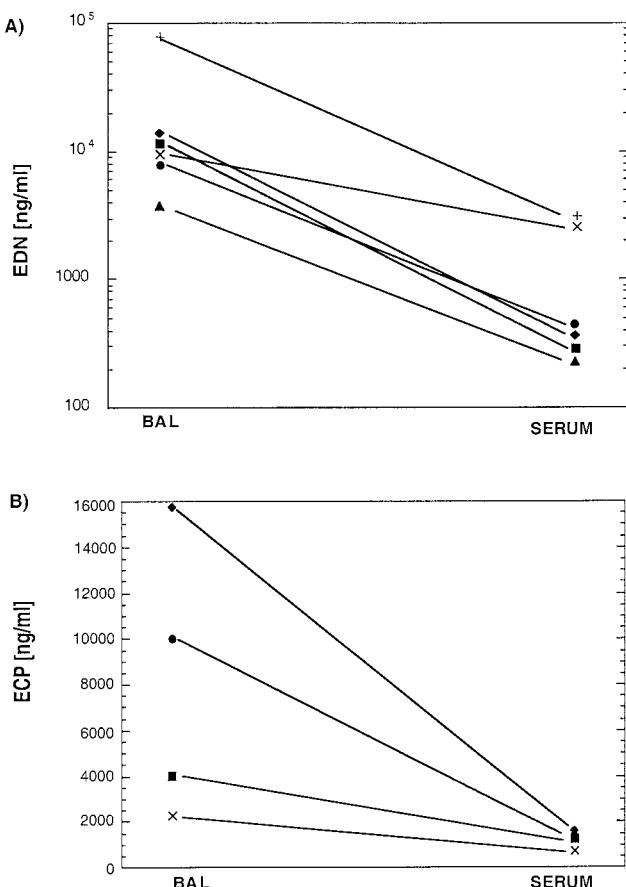


FIG. 2. EDN (A) and ECP (B) are compartmentalized to the lungs of patients with TPE. Each line represents the levels of the eosinophilic granule proteins for an individual patient with TPE. For the ECP analysis, only four paired BAL and serum samples were available.

blood white cell (PBWC) culture supernatants, the levels for the TPE group were 2.7-fold higher than those for the non-TPE group (GM, 121.4 versus 44.4 ng/ml). There was no significant difference in ECP or LTB4 for the TPE group versus the non-TPE group for the BAL fluid and PBWC culture supernatant analyses. For MIP-1 α , there was a significant difference between the TPE and the non-TPE groups for the PBWC culture supernatant analysis (GM, 251 versus 43.65 pg/ml; $P = 0.02$) but not for the BAL fluid culture supernatant analysis.

DISCUSSION

The purpose of this study was to uncover the cellular mediators coordinating the eosinophil-rich inflammatory response of acute TPE. Although an expansion of IL-4- and IL-5-producing T cells may initiate and maintain the exuberant peripheral eosinophilia and IgE response seen systemically, this study highlights the role of the activated and compartmentalized eosinophil in the lungs of patients with TPE. It is also clear from our study that the eosinophilic granular protein EDN, an RNase capable of damaging the lung epithelium, plays a significant role in the pathogenesis of TPE.

The findings of this study corroborate previous observations that the immune response to *Wuchereria bancrofti* and *Brugia*

malayi is compartmentalized to the lungs in TPE (16, 23, 26). Despite the massive eosinophilia in the lungs and systemically, the significantly higher EDN levels in the BAL fluid (compared with the serum) suggests that it is predominantly in the lungs that the eosinophils are activated and degranulated in TPE. In contrast, control patients with lymphatic filariasis (but without lung disease) had nearly equivalent levels of EDN in the BAL fluid and in the serum, as did those with asthma (lung disease in the absence of peripheral eosinophilia). Significantly, the high levels of EDN were not merely a reflection of the massive eosinophilia in TPE: in our study, neither EDN nor ECP levels were significantly related to the level of eosinophilia in the sera or BAL fluid.

EDN levels in the sera of TPE patients were dramatically higher than those in the sera of the comparable control group who had lymphatic filariasis, suggesting that both antigenic stimulation and eosinophil activation are present locally and systemically in TPE patients. Compared with asthmatic patients, patients with TPE had remarkably higher levels of EDN in the BAL fluid, presumably reflecting the intensity of the eosinophilic response. Quiescent and active asthmatics typically manifest a mixed BAL cellular infiltrate of neutrophils and lymphocytes and only a mild eosinophilia (17, 18), whereas in TPE, the eosinophil is the predominant effector cell.

Interestingly, the concentration of EDN—but not of ECP—was found to be significantly elevated in TPE patients for both the serum and BAL samples compared to the three control groups in our study. These data suggest that the release and production of the granular proteins are regulated differently. While it is unclear why EDN would have a special function in TPE, there are studies suggesting that eosinophilic granular proteins are under differential regulatory control, most notably following treatment for filarial infection (10). EDN has also been shown to have its own promoter, which is regulated by the transcriptional factor C/EBP, indicating that there may be differential transcriptional regulation of the eosinophil granule proteins (ECPs) (5). Although it is not surprising that EDN, an eosinophilic granular protein, is elevated in this disease characterized by hypereosinophilia, it is perplexing that EDN, but not ECP, was increased in the lungs and blood of TPE patients in our study. Among the eosinophilic granular proteins, EDN and ECP show the most similarities: both are cytotoxic and helminthotoxic, and both belong to the same RNase A gene superfamily (30).

Eosinophilic granular proteins are helminthotoxic in vitro (11) and are elevated in filarial infections (33, 34). Filaricidal activities, however, are not equivalent among the effector proteins: per mole, ECP has greater in vitro microfilarial toxicity against *B. malayi* than EDN (11), and MBP has been shown to have the greatest helminthotoxic activity of the eosinophilic granular proteins, due to its higher abundance in the granules than the other granular proteins (1, 11). In one of the few studies of filarial infections and eosinophilic granular proteins published, both ECP and EDN levels in serum were found to be significantly higher in patients with bancroftian filariasis and onchocerciasis than in a control group from an area where the diseases were not endemic and without filarial disease (33). In our study, where the lymphatic filariasis control group was much smaller, there was no such difference in the levels of the eosinophilic granular proteins in the sera compared with

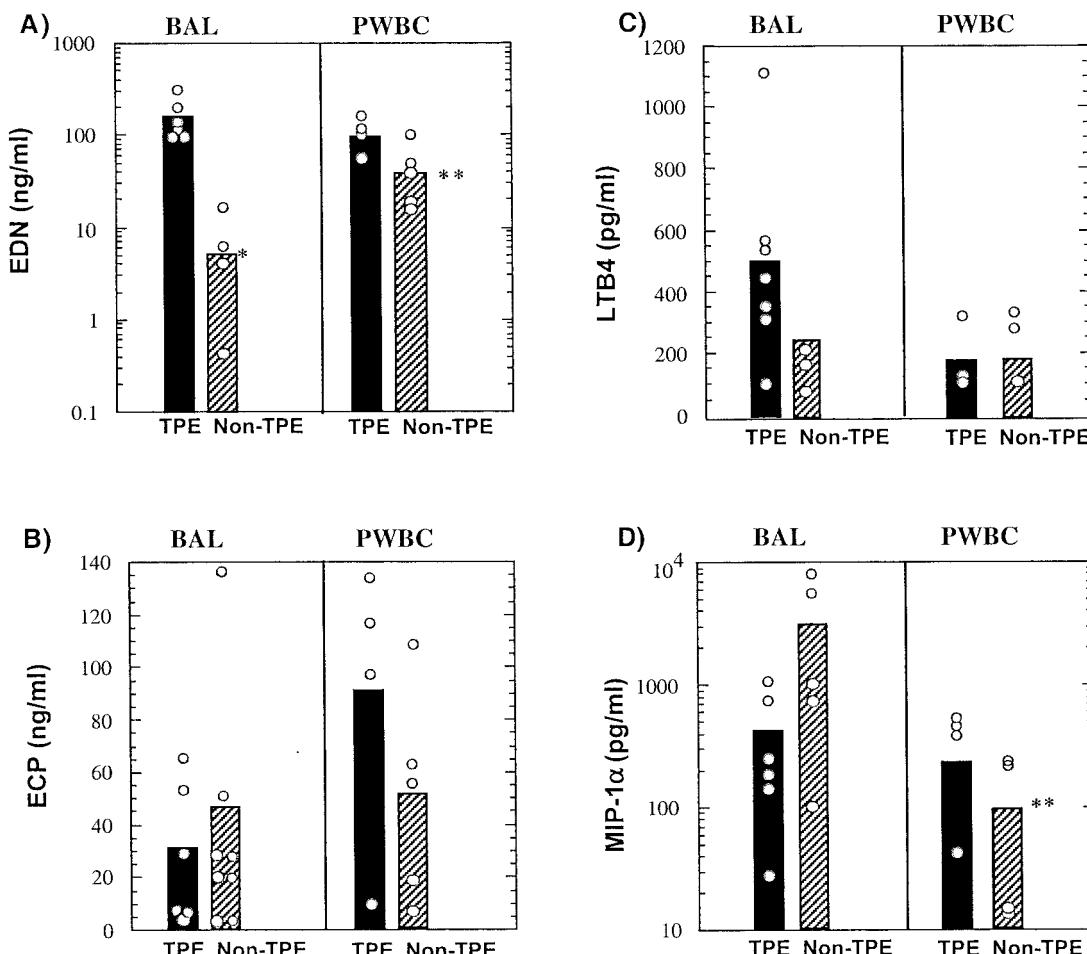


FIG. 3. EDN (A), ECP (B), MIP-1 α (C), and LTB4 (D) levels in BAL fluid and PBWC culture supernatants in patients with (solid bars) and without (hatched bars) TPE. Individual values are represented by circles. The top of the bar represents the GM for each group. The non-TPE group is a pool of three healthy individuals, one elephantiasis patient, and one asthmatic patient. *, $P = 0.006$; **, $P = 0.02$. For the PBWC supernatant analyses, the number of TPE samples was limited to four, as were the non-TPE samples.

a healthy control group; however, it is noteworthy that the healthy control group in our study lived in an area where filariasis is endemic.

In addition to different cytotoxic capabilities among the eosinophilic proteins (1, 11), there is also evidence of different host defense properties. Of the eosinophilic effector proteins, MBP is unique in its ability to stimulate IL-8 release from neutrophils *in vitro* (25). Although both EDN and ECP are RNases, they may have unique effector functions. *In vitro*, EDN alone has activity against respiratory syncytial virus (30). ECP, but not EDN, can stimulate histamine release from rat peritoneal mast cells (39). Surprisingly, the evolutionarily conserved RNase activity shared by ECP and EDN (38) is not responsible for the helminthotoxicity of ECP (11) or for the antiviral activity of EDN (30).

Whether there is selective release of eosinophilic granular proteins is controversial (13). Eosinophils require IgE or IgA stimulation for the release of EDN *in vitro*, whereas IgA (but not IgE) is necessary for the release of ECP (4, 35). Whether this relationship is relevant *in vivo* is unproven, however. Furthermore, in TPE, high levels of IgE in the serum suggest a profound dysregulation of the typically well-regulated IgE re-

sponse (19). It is interesting to speculate whether the extreme elevation of IgE in TPE has an impact on the specific release of EDN.

There are many potential explanations as to why there was not a significant increase in the other mediators measured. In the cases of IL-5 and IL-13 in the serum and all the cytokines and chemokines in the BAL fluid, the minimum levels of detection for the assays were not sensitive enough to detect a change. The majority of cytokines are produced locally, and measurement of levels in a diluted BAL sample may not offer enough sensitivity to detect subtle local increases. Instability of the proteins after many years of storage may be another factor affecting detection. MIP-1 α was the only chemokine found to be significantly elevated in TPE patients compared with controls; however, this relationship was found only in the supernatant of the PBWC culture and not in the supernatant of the BAL fluid WBC culture. As chemokines are driven by concentration gradients, it is likely that the increased MIP-1 α is not important in the pathogenesis of TPE.

The specific regulation of EDN and its role in TPE need to be further elucidated. Notably, alpha interferon (IFN- α) also blocks the IgE-dependent release of EDN and the IgA-depen-

dent release of ECP in the activated eosinophil in vitro (4). Clinically, IFN- α has proven effective in the treatment of idiopathic hypereosinophilic syndrome (9). As EDN likely plays a cytotoxic role in TPE, agents such as IFN- α could be beneficial in both the acute and chronic phases of the disease.

The currently available treatment with diethylcarbamazine, while effective in ameliorating the acute symptoms, leaves a majority of patients with subtle lung abnormalities long after treatment (29). In a certain number of patients (12 to 25%) (36), the dose of diethylcarbamazine is inadequate for cure, leaving the patients vulnerable to relapses and to chronic fibrosis. Priority should be given to the development of combined antifilarial and anti-inflammatory treatment strategies that target the acute eosinophil-mediated syndrome of TPE and prevent the serious fibrotic sequelae seen in the disease.

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