GENETIC POLYMORPHISMS OF EOSINOPHIL-DERIVED NEUROTOXIN AND EOSINOPHIL CATIONIC PROTEIN IN TROPICAL PULMONARY EOSINOPHILIA

YAE-JEAN KIM,* V. KUMARASWAMI, EUNHWA CHOI, JIANBING MU, DEAN A. FOLLmann, PETER ZIMMERMAN, AND THOMAS B. NUTMAN

Helminth Immunology Section, Laboratory of Parasitic Diseases, Laboratory of Malaria and Vector Research, and Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; Tuberculosis Research Centre, Chennai, India; Department of Pediatrics, Seoul National University College of Medicine, Seoul, South Korea; Division of Geographic Medicine, Department of Medicine, Case Western Reserve University, University Hospitals of Cleveland, School of Medicine, Cleveland, Ohio

Abstract. Because eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are critical in the pathogenesis of tropical pulmonary eosinophilia (TPE), we analyzed genetic polymorphisms of both in 181 individuals from southern India with varying clinical manifestations of Wuchereria bancrofti infection (including 26 with TPE). Using haplotype frequency analysis, we identified four known (of nine) and two novel haplotypes for EDN (1, 2, 7, 8, 10, and 11). For ECP, five (of seven known) haplotypes (1–5) were identified. Although we found no significant association between frequencies of EDN and ECP polymorphisms and TPE development, we observed a unique pattern of EDN and ECP polymorphism distribution among this population. Genotype TT at locus 1088 of ECP in one TPE patient was not observed in any other clinical group. Although the EDN and ECP polymorphisms appear unlikely to be associated with the development of TPE, further analyses will be more definitive.

INTRODUCTION

Tropical pulmonary eosinophilia (TPE), an unusual manifestation of human lymphatic filarial infection, is characterized by eosinophilic pulmonary inflammatory infiltrates and marked elevations of serum IgE and circulating eosinophils, all believed to be mediated by immunologic hyperreactivity to filarial parasites or their antigens. Although 129 million people worldwide are infected with lymphatic filariasis, fewer than 0.01% develop TPE.1 It is unclear what factors predispose patients to this rare, localized, and profound immunologically dysregulated state, but the findings of association between chitotriosidase 1 (CHIT1) and mannose-binding lectin 2 (MBL2) and susceptibility to human filarial infection have implicated underlying host genetics as partially playing a role.2

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. In contrast to the majority of people with lymphatic filariasis who have a down-regulated T cell response to the parasites, patients with TPE mount a robust systemic and localized immune response3 that includes elevations of both polyclonal and filaria-specific IgE and IgG4 as well as expansion of interleukin-4 (IL-4)- and IL-5-producing T cells.5

Eosinophils are the predominant effector cells seen in the lungs of patients with TPE, and unlike the rare eosinophils in the bronchoalveolar lavage fluid of normal lungs, the pulmonary eosinophils in TPE are degranulated and activated.6 Moreover, a recent study has demonstrated that the localized release of the eosinophil degranulation products eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are critical in mediating some of the pathology seen in TPE.7

The EDN and ECP genes are closely linked on chromosome 14q24-q31,8 and their protein products can be found in the large specific granules of eosinophils.9–11 They both are members of the eosinophil-associated ribonuclease family. EDN (RNase 2) has antiviral activities against both respiratory syncytial virus and human immunodeficiency virus (HIV) in vitro.12,13 ECP (RNase 3) has cytotoxic activity14 and, by itself, can kill microorganisms including bacteria, protozoa, and helminths in vitro.15–17 In addition, ECP can regulate fibroblast activity, modulate airway mucus secretion, and interact with the coagulation and complement system.18 Sequence variations in both EDN and ECP have been identified. For EDN, nine polymorphic sites leading to nine haplotypes were defined, whereas for ECP, seven polymorphic sites leading to eight haplotypes have been observed.19 Another polymorphism in ECP has recently been shown to be associated with allergic symptoms.20,21

With this as a backdrop, we hypothesized that genetic polymorphisms in EDN and ECP might play an important role for the development of TPE. In the present study, we have screened for the polymorphisms of EDN and ECP in a south Indian population and have observed different distribution patterns of haplotypes in this population from those of other groups reported previously.19 We have also found two novel haplotypes (haplotypes 10 and 11) for EDN. Moreover, we have observed homozygosity of a T allele at locus 1088 for ECP that results in a haplotype seen only in a patient with TPE.

MATERIALS AND METHODS

Participants. The study was performed using protocols reviewed and approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board (IRB) and the IRB of the Tuberculosis Research Centre, Chennai, India. Informed consent was obtained from each study participant. Our study population consisted of 181 individuals from south India: 58 normal (N), 55 with asymptomatic microfilaria positive (AMF), 42 with chronic lymphatic dysfunction/
elephantiasis (CP), and 26 with TPE. For three EDN samples in the N group and one ECP sample in the CP group, a polymerase chain reaction (PCR) product could not be obtained; therefore, analysis of these samples was not performed.

**Polymorphism analysis.** Genomic DNA was obtained from peripheral blood by conventional methods. The EDN and ECP genes were amplified by a PCR with primer sets EDN 1f (5′-TCAGGTGGTCCAGACATGTTTAC-3′) and EDN 4r (5′-TAA TGGGTTGAGGAGTTCGACAGTTTAC-3′) and ECP 1f (5′-CCAGGATCCATGCTCGAGCGCGTCG-3′) and ECP 4r (5′-GTCAGTGATGATACAGCAAGAGAG-3′), respectively, derived from the published human sequences (GenBank accession numbers X55987 and X16545). High-fidelity Taq polymerase (Life Technologies, Rockville, MD) was used. The PCR products were purified by ExoSAP-IT (United States Biochemicals Corp., Cleveland, OH); 5 μL of the treated product was used in a sequencing reaction with BigDye terminator chemistry on an ABI3100 DNA sequencer (Applied Biosystems, Foster City, CA). All known and potential polymorphic sites and discrepancies were verified by visual inspection.

Haplotype predictions and reconstructions were determined by using PHASE 1.0 (Isis Innovation Ltd., Summer-town, Oxford, United Kingdom). Sequence data were combined with clinical information for statistical analyses in comparisons between N and the three infected groups (AMF, CP, and TPE) and between TPE and the other two infected groups (AMF and CP).

**Statistical analysis.** Differences in genotypic distributions were tested by contingency table analysis using Fisher’s exact test with SPSS version 11.5 (SPSS Inc., Chicago, IL). For haplotype frequency analysis to test equality of the distribution of haplotypes across patient groups, a Monte Carlo analysis was used. A random contingency table of haplotype pairs by patient groups was generated with the same column and row sum totals as the actual data; this table was then transformed to a contingency table of unique haplotypes cross-classified by patient group. Chi square was calculated for this reduced table. This procedure was repeated 1,000 times, and the 1,000 simulated chi-square statistics provide a reference distribution for the chi-square statistics of the original data.

**RESULTS**

**Identification and confirmation of EDN and ECP polymorphisms.** Among nine observed haplotypes and one hypothetically predicted haplotype for EDN and eight known haplotypes for ECP, we identified six haplotypes (1, 2, 7, 8, and two novel haplotypes) for EDN and five haplotypes (haplotypes 1–5) for ECP (Table 1). For EDN, four polymorphic sites among nine known sites were observed (Table 2). Among them, one synonymous site in a coding region and three sites in noncoding regions were observed (Table 3). All the sites were biallelic. For ECP, four polymorphic sites among seven known sites were observed (Table 2). Among them, one nonsynonymous site in the coding region and three sites in the noncoding region were identified (Table 3). All these sites were biallelic. The results of haplotype frequency analysis in normal individuals in our study were compared with those of an Asian group studied previously that had compared frequencies among different groups

---

**Table 1**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>South Indian n = 110 (%)</th>
<th>Asian† n = 24 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TGGACTACGG 78 (70.9)</td>
<td>12 (50.0)</td>
</tr>
<tr>
<td>2</td>
<td>TGCACTACGG 26 (23.6)</td>
<td>11 (45.8)</td>
</tr>
<tr>
<td>3</td>
<td>TGGACCCAGG 0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>TGGGGTTGCA 0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>CGGACTACGG 0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>TGGACTCCGG 0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>TGGGCTTGCAG 5 (4.5)</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>8</td>
<td>TGGGCTTGGG 1 (0.9)</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>TGGACTAAC 0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>TGCCCAGGG 0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>TGGGCTTACGG 0</td>
<td>0</td>
</tr>
</tbody>
</table>

* EDN = eosinophil-derived neurotoxin; ECP = eosinophil cationic protein.
† Data from Zhang and Rosenberg.‡ For three of the EDN samples, a polymerase chain reaction product could not be obtained; analysis of these samples was therefore not performed.
§ One haplotype 10 was observed in the chronic lymphatic dysfunction/elephantiasis (CP) group.
¶ One haplotype 11 was observed in the CP group.
# Two of haplotype 5 were observed in the tropical pulmonary eosinophilia group.

**Table 2**

<table>
<thead>
<tr>
<th>Locus EDN</th>
<th>Genotype</th>
<th>Normal n = 551 No. (%)</th>
<th>TPE n = 26 No. (%)</th>
<th>AMF + CP n = 97 No. (%)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>5 (9.1)</td>
<td>3 (11.5)</td>
<td>13 (13.4)</td>
<td>0.909</td>
<td></td>
</tr>
<tr>
<td>405</td>
<td>CG</td>
<td>16 (29.1)</td>
<td>12 (46.2)</td>
<td>39 (40.2)</td>
<td>0.397</td>
</tr>
<tr>
<td>416</td>
<td>AA</td>
<td>49 (89.1)</td>
<td>23 (88.5)</td>
<td>91 (93.8)</td>
<td>0.397</td>
</tr>
<tr>
<td>980</td>
<td>AA</td>
<td>49 (89.1)</td>
<td>23 (88.5)</td>
<td>91 (93.8)</td>
<td>0.397</td>
</tr>
<tr>
<td>112</td>
<td>AG</td>
<td>10 (6.2)</td>
<td>6 (11.5)</td>
<td>6 (11.5)</td>
<td>0.62</td>
</tr>
<tr>
<td>ECP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>8 (13.8)</td>
<td>3 (11.5)</td>
<td>12 (12.5)</td>
<td>0.951</td>
<td></td>
</tr>
<tr>
<td>474</td>
<td>AC</td>
<td>11 (20.4)</td>
<td>11 (42.3)</td>
<td>44 (45.8)</td>
<td>0.747</td>
</tr>
<tr>
<td>CC</td>
<td>19 (34.8)</td>
<td>12 (46.2)</td>
<td>40 (41.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1010</td>
<td>CC</td>
<td>1 (1.7)</td>
<td>1 (3.8)</td>
<td>5 (1.2)</td>
<td></td>
</tr>
<tr>
<td>1088</td>
<td>AA</td>
<td>8 (13.8)</td>
<td>3 (11.5)</td>
<td>24 (25.0)</td>
<td>0.355</td>
</tr>
<tr>
<td>TT</td>
<td>0 (0.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* TPE = tropical pulmonary eosinophilia; AMF = asymptomatic microfilaria positive.
† P values are between TPE and AMF + CP groups.
‡ For the reduced table, one sample was not included; therefore analysis of this sample was not performed.
study, for EDN, haplotypes 1, 2, and 7 were seen most commonly among the south Indian normal population in frequencies that were moderately different than previously reported in a different Asian population. We observed EDN haplotypes 1, 2, 7, and 8, with haplotype 1 being the most common haplotype (70.9%). Of note, we did not find haplotypes 3, 4, 5, 6, and 9, which is not surprising since haplotype 5 has been reported to be restricted to Caucasians and haplotypes 3, 4, 6, and 9 restricted to African-Americans. Haplotype 8 was observed in one participant. Of interest, two novel haplotypes, 10 and 11, were observed in the CP group (Table 1). Haplotype 10 was predicted (but not observed) in a previous study, and another novel haplotype has tentatively been given the name haplotype 11. For ECP, we observed four haplotypes (1, 2, 3, and 4) in the normal south Indian group, with haplotype 2 being the most common (40.5%). The frequencies of the haplotypes again differed from those seen previously among Asians. Moreover, we did not observe haplotypes 6, 7, and 8, which have been reported to be restricted to African-Americans, or haplotype 5, also previously found predominantly in African-Americans.

Analysis and distribution of genotypes. Genotype frequency analysis of EDN showed no significant differences in the genotype distribution for all observed polymorphic sites in the EDN gene among the four clinically different groups (N, AMF, CP, and TPE) or between the N group and the three infected (susceptible) groups (AMF, CP, and TPE). There was also no difference between the TPE group and the other two infected groups (AMF and CP) (Table 2). We did not observe polymorphisms at site 1011, a site implicated in the ribonuclease function of EDN. It is known that among the three polymorphic sites (836, 980, and 1011) within the protein-coding sequence, two synonymous polymorphic sites (836 and 980), and one nonsynonymous polymorphic site (1011, in which a C/A mutation results in a His/Asn in at one of the three amino acid residues that form the catalytic site of the RNase) cause EDN to become nonfunctional.

Genotype frequency analysis of ECP showed no significant differences in the genotype distribution for all observed loci in the EDN gene among the four groups studied, between the N group and the three infected groups (AMF, CP, and TPE), or between the TPE group and the other two infected groups (AMF and CP) (Table 2). Of interest, one TPE patient was homozygous for T (compared with A) at this locus, while none of the others studied was homozygous.

Haplotype frequency analysis. Polymorphisms in the EDN gene showed no significant differences in frequencies among the four groups studied, between the N group and the three infected groups (AMF, CP, and TPE), or between the TPE group and the other two infected groups (AMF and CP). Among the nine known haplotypes and one predicted haplotype for EDN, we identified six haplotypes (1, 2, 7, 8, 10, and tentatively 11) (Table 4). Haplotype 1 was the most common haplotype in all groups. Among the eight known ECP haplotypes, five were found in the south Indian population. Haplotype 5 was observed only in one TPE patient (Table 4). This TPE patient was homozygous for a T allele at position 1088; however, there was no obvious difference between this patient and other TPE patients in terms of male gender, high-grade eosinophilia, and abnormal pulmonary function test results.

Sample size analysis. Because the ability to determine statistical significance is directly related to the number of participants, simple calculations were performed to assess how the P value would be changed if the sample sizes were doubled and the observed proportions remained the same. In Table 2 with EDN = 416, the P value changed from 0.40 to 0.23. In Table 4 with haplotype EDN, the simulated P value changed from 0.79 to 0.38, while for haplotype ECP, the P value changed from 0.14 to 0.02. To definitively identify modest differences in proportions of the kind seen in this paper would require a larger study.

**DISCUSSION**

We have explored the role polymorphisms of EDN and ECP might play in the development of TPE. Although we were unable to find any statistically significant relationship in the distribution of genotypes, alleles, and haplotypes for the development of TPE, it is of particular note that we observed haplotype 5 only in a TPE patient, while we did not detect this haplotype in any other group. We also observed two new haplotypes of EDN, 10 and 11, in the CP group. Moreover, we have defined the distribution of haplotype frequencies in this south Indian population, showing it to be different from that of an Asian population in a previous report.

Some evidence suggests that TPE has an underlying genetic basis. It has a minuscule incidence rate1,2,5,26, and biases for male gender1,2,6,25,26 and geography (mostly found in India, Brazil, and southeast Asia).1,6,25,26 Moreover, data from Brazil27 and

### Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>EDN</th>
<th>ECP</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sites</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Coding</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Synonymous</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Nonsynonymous</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Noncoding</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

* For definitions of abbreviations, see Table 1.

### Table 4

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>EDN</th>
<th>Normal No. (%)</th>
<th>TPE No. (%)</th>
<th>AMF + CP No. (%)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18 (23.6)</td>
<td>32 (50.8)</td>
<td>52 (26.3)</td>
<td>0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>2</td>
<td>14 (19.7)</td>
<td>32 (50.8)</td>
<td>52 (26.3)</td>
<td>0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>3</td>
<td>7 (10.0)</td>
<td>32 (50.8)</td>
<td>52 (26.3)</td>
<td>0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>4</td>
<td>2 (2.9)</td>
<td>32 (50.8)</td>
<td>52 (26.3)</td>
<td>0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>5</td>
<td>0 (0.0)</td>
<td>32 (50.8)</td>
<td>52 (26.3)</td>
<td>0.03</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* For definitions of abbreviations, see Tables 1 and 2.
† For three of the EDN samples, a polymerase chain reaction (PCR) product could not be obtained; analysis of these samples was therefore not performed.
‡ For one of the ECP samples, a PCR product could not be obtained; analysis of this samples was therefore not performed.
south India (Gopinath R and others, unpublished data) have identified cases that have occurred among siblings.

In lymphatic filariasis, it has been reported that there are associations between the HH genotype of CHIT1 and the XX genotype of the promoter region in MBL2 and susceptibility to lymphatic filarial infection in this same population, but not with the development of TPE. This finding, however, appears to be population specific in that these same polymorphisms were not associated with disease susceptibility in Papua New Guinea. In a related filarial disease, onchocerciasis, it has been observed that there was an association between HLA-DQ alleles and the level of immune response to parasite antigens and the IL-13 variant Arg110Gln has been shown to be significantly associated with an immunologically hyper-reactive form of onchocerciasis, sowda, a condition often believed to parallel TPE from an immunologic standpoint.

It is well known that TPE patients have extreme peripheral blood eosinophilia and increased IgE as well as filaria-specific IgG, IgM, and IgE localization in the lungs. Histologic examination shows massive pulmonary infiltrations by eosinophils and other polymorphisms (−393C/T, −38C/A, and 124Arg/Thr) in Japanese children and was directly demonstrated in our analysis. The present study clearly shows differences in the distribution of polymorphic sites in this south Indian population compared with other populations (Table 1). Recent studies have shown an ECP 434 (G/C) polymorphism in Swedish students that was associated with development of allergic symptoms and other polymorphisms (−393C/T, −38C/A, and 124Arg/Thr) in Japanese children; however, among the 181 individuals from south India, none of these polymorphisms were seen.

Although TPE is distinguished from other allergic conditions by its elevated antifilarial antibodies and response treatment with antifilarial chemotherapy, there are many similarities in the clinical presentation of TPE and those conditions associated with increased eosinophilia and lung involvement (such as asthma, Churg-Strauss syndrome, the idiopathic hypereosinophilic syndrome, and chronic eosinophilic pneumonia). Although the genetic bases of these other syndromes have not been definitively identified, an association has been demonstrated between an IL-13 variant and atopic dermatitis in three different populations. In addition, there have been many studies linking asthma with multiple different genetic polymorphisms, suggesting that there may well be a molecular but complex (genetic) basis for many of these conditions with lung involvement.

To search for the underlying mechanism in TPE, it is also necessary to consider the filarial worms themselves as an antigen source and host immune responses as a result of exposure to these antigens. Recently, α-glutamyl transpeptidase (α-GT), a major allergen of the lymphatic filarial parasite Brugia malayi, has been implicated in the pathogenesis of TPE. Molecular mimicry between the parasite α-GT homolog and the host membrane-bound α-GT in lung epithelial cells was has been suggested to contribute to the pathogenesis observed in TPE. In a mouse model, Brugia malayi α-GT induced pulmonary inflammation following intranasal challenge, a reaction believed to reflect a breakdown of tolerance against endogenous murine α-GT in the pulmonary epithelium. However, because such autoimmune reactions against pulmonary epithelium α-GT are not universally found in patients with TPE, other factors important in TPE pathogenesis await elucidation.

Compartmentalization of the inflammatory and immune responses in the lungs of patients with TPE suggest that lung epithelial cells and alveolar macrophages are also important effectors in the pathogenesis of TPE. Among the observations on tissue-dependent immune responses in TPE, a direct interaction has been shown between lung epithelial cells and filarial antigenic components, leading to CD-14-dependent activation of NF-κB and production of proinflammatory cytokines. There have also been many studies examining the relationship between non-Indian populations and those in India regarding genetic origin, genetic flow, and genetic influences, focused usually on population migration. There is some evidence that in the south Indian population there is an absence of the HIV-1 protective Δ32 to make Δ ccr5 allele as well as a region of chromosome 20 associated with leprosy susceptibility. Our study results also showed the different distribution of EDN and ECP in this population compared with other groups, although we failed to show significant association with the development of TPE.

In conclusion, as part of continuing efforts to elucidate the mechanism of TPE, we screened the EDN and ECP polymorphisms in a south Indian population. The EDN and ECP polymorphisms are not likely involved in development of TPE. We continue to examine which groups of individuals with what genetic predispositions develop not only a system and skewed immune response, but also a localized and skewed pulmonary inflammatory response when exposed to certain filarial antigens or live parasites.

Received December 6, 2004. Accepted for publication December 28, 2004.

Acknowledgment: We thank Brenda Rae Marshall for editorial assistance

Authors’ addresses: Yae-Jean Kim and Thomas B. Nutman, Helminth Immunology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, 1 Center Drive, Building 1, Room B1-07, Bethesda, MD 20892, E-mail: tnutman@niaid.nih.gov. V. Kumaswami, Tuberculosis Research Centre, Chennai, India. Eunhwa Choi, Department of Pediatrics, Seoul National University College of Medicine, Seoul, Korea. Jianbing Mu, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Disease, National Institutes of Health, NIAID, Bethesda, MD 20892. Dean A. Follmann, Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892. Peter Zimmerman, Center for Global Health and Diseases, Case Western Reserve University, University Hospitals of Cleveland, School of Medicine, Cleveland, OH 44106.

REFERENCES


36. Lin CY, Lu CC, Su HJ, Shen CY, Lei HY, Guo YL, 2002. The association between tumor necrosis factor, HLA-DR alleles,
and IgE-mediated asthma in Taiwanese adolescents. Allergy 57: 831–834.


