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Primary Structure of and Immunoglobulin E Response to the Repeat Subunit of gp15/400 from Human Lymphatic Filarial Parasites

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We have isolated and sequenced clones encoding the repeated subunit of the surface-associated glycoprotein gp15/400 from the two nematode species predominantly responsible for lymphatic filariasis in humans: *Brugia malayi* and *Wuchereria bancrofti*. The amino acid sequence of the 15-kDa subunit, derived from the nucleotide sequence of the gene fragment from *B. malayi*, is identical to that previously reported for *B. pahangi*, whereas the derived *W. bancrofti* protein sequence differs in only 7 of 132 residues. The identity of the protein in the two *Brugia* species allowed us to use a recombinant from *B. pahangi* to examine the serological response of adult Indonesian subjects infected with *B. malayi*. The polymerase chain reaction-amplified subunit was expressed in *Escherichia coli* via the pDS56/RBS11 plasmid and purified by nickel-chelating chromatography. A significant proportion of individuals produced antigen-specific immunoglobulin E (IgE). This was most pronounced in the individuals with elephantiasis, with 14 of 15 showing elevated titers and a mean of 3.2 ng of specific IgE ml⁻¹. Only 2 of 15 microfilaremic individuals possessed elevated titers of specific IgE, with a mean of 0.045 ng ml⁻¹ for the group as a whole. Asymptomatic amicrofilaremic residents showed approximately equal numbers of responders (defined as having a value in the radioimmunoassay greater than two standard deviations above controls) and nonresponders, with a group mean of 1.2 ng of antigen-specific IgE ml⁻¹.

As in most helminth infections, lymphatic filariasis is accompanied by high levels of immunoglobulin E (IgE) and eosinophilia (11, 15, 26, 41). Hypersensitivity responses to microfilariae most probably contribute to the pathogenesis of tropical pulmonary eosinophilia, but the role of IgE in other clinical manifestations of disease is unclear (28, 30). IgG4 may act as a blocking factor, particularly in microfilaremic individuals, to prevent antigen-specific degranulation of basophils and also presumably of mast cells and eosinophils (29). Associated with this phenomenon is the observation of parallel specificities between IgE and IgG4 (12), which probably occur because synthesis of both isotypes is dependent on interleukin-4 (9, 13, 20).

Relatively little is known about antigens which elicit IgE in filariae. A number have been defined by isotype-specific Western immunoblots (12), and recently a 23- to 25-kDa protein from *Brugia malayi* has been purified and shown to induce IgE synthesis from peripheral blood lymphocytes of patients with tropical pulmonary eosinophilia or lymphatic filariasis (19). We have previously described a complex of proteins in *B. malayi* identified by surface labeling which form a regular series of ascending molecular mass separated by approximately 15 kDa per step (22). Molecular cloning and pulse-chase studies demonstrated that this glycoprotein complex is encoded by a single gene in *B. pahangi* and is derived from processing of a precursor of approximately 400 kDa (39). The proprotein is thus composed largely of multiple direct repeats of 132 amino acids, which are presumed to represent the mature functional molecule. Although no putative biological function was evident from sequence simi-

larities, the protein, termed gp15/400, is homologous to an allergen which has been described from *Ascaris suum* and *A. lumbricoides* (5, 24). This allergen, termed ABA-1, is the most abundant protein species in parasite body fluid and is released during culture of larvae in vitro (14, 38).

In this study, we have determined the primary sequence of the repeated subunit of gp15/400 from the two major parasite species responsible for human lymphatic filariasis, *Wuchereria bancrofti* and *B. malayi*, and examined the IgE response of an Indonesian study population to this repeat unit (p15) expressed in *Escherichia coli*. Because of the predominance of the IgG4 subclass in the spectrum of antifilarial antibodies (17, 31) and its demonstrated role as a blocking factor for histamine release (29), we also assessed the levels of specific IgG4 to p15 in the same individuals.

MATERIALS AND METHODS

Parasites and DNA isolation. *B. malayi* adults were recovered from the peritoneal cavity of Mongolian gerbils (*Meriones unguiculatus*) infected more than 3 months previously and obtained from TRS Labs, Atlanta, Ga. *B. pahangi* parasites were kindly provided by D. A. Denham, London School of Hygiene and Tropical Medicine. The strains used are those in general laboratory use and originate from Malaysia. *W. bancrofti* infective larvae were dissected from mosquitoes that had fed on a volunteer subject in Jakarta, Indonesia. Genomic DNA was prepared from all species by standard techniques (21).

PCR amplification, identification, and sequencing of the repeat gene fragments. The repeating unit for gp15/400 was amplified from genomic DNA of *B. malayi* and *W. bancrofti* by polymerase chain reaction (PCR) with oligonucleotide

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primers derived from the sequence of the *B. pahangi* cDNA as previously described (39). The primers were 5'-TGGCT TACGGATGCCCAAAGG-3' (forward) and 5'-ACTTA GATGCGTCCGAAAATAG-3' (reverse). *B. pahangi* cDNA was also used as a positive control for the reactions. A standard 100- μ l PCR was used (21), with 200 ng of genomic DNA, 500 ng of each primer, and 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min. For *W. bancrofti* DNA, amplification was improved by lowering the annealing temperature from 54 to 50°C. Reaction products were run on agarose gels, blotted, and hybridized with the *B. pahangi* cDNA clone BpL3 (39) labeled with [³²P]dCTP by random oligonucleotide priming (8). Hybridization and washes were performed at high stringency as previously described (39). PCR amplifications were also performed with primers modified to include 5' *Bam*HI and *Hind*III restriction sites to facilitate subcloning. The PCR-generated fragments with an approximate size of 400 bp were digested with *Bam*HI and *Hind*III, purified on GeneClean (Bio 101), subcloned into M13mp19 and M13mp18, and subjected to dideoxynucleotide sequencing on both strands (35).

Expression and purification of the 15-kDa subunit. The *Bam*HI-*Hind*III-linked amplified repeat unit from *B. pahangi* was ligated to pDS56/RBS11 (37) for expression in *E. coli* TG2 cells. The expressed protein therefore contains one full repeat unit with an additional 10 amino acids at the N terminus, i.e., MRGS(H)₆. Cells from a positive transformant (BpL4) were grown to an optical density of 0.1, expression was induced with 2 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and the cells were grown for a further 5 h and harvested by centrifugation (37). Large-scale purification of the protein, termed p15, was performed by nickel-charged chelating Sepharose chromatography at room temperature as follows. Chelating Sepharose (Pharmacia) was charged with 1% NiSO₄ and washed with distilled water. The bacterial cell pellet was freeze-thawed and solubilized for 1 hour in 6 M guanidine hydrochloride–0.1 M Na₂PO₄ (pH 8.0) (solution A). The suspension was centrifuged for 10 min at 10,000 $\times$ g, and the supernatant was loaded onto the column, which was extensively washed with solution A followed by 8 M urea in 0.1 M Na₂PO₄–10 mM Tris (pH 8.0) (solution B) and then by the solution B buffered at successively lower pH values with HCl. The histidine-tailed p15 protein eluted at pH 4.5 and was dialyzed against phosphate-buffered saline (PBS) at 4°C to remove the urea before the protein concentration was estimated (2). A 1-liter volume of *E. coli* TG2 cells gave 18 mg of purified p15. Cell lysates and purified p15 were run on 7 to 25% gradient sodium dodecyl sulfate (SDS) gels, blotted, and reacted by standard procedures (21) with serum from individuals with a filarial infection. A serum pool constructed from sera of four individuals with elephantiasis was used for this purpose; it was diluted 1:200 in Tris-buffered saline (pH 8.0)–5% dried milk powder–0.2% Tween 20.

SPRIA. The purified recombinant fusion protein, p15, was used in a solid-phase radioimmunoassay (SPRIA) (1) as previously described for total parasite antigen (16) to determine levels of specific IgE and IgG4 in human sera. For the IgE determinations, plasma samples were depleted of IgG subclasses by overnight incubation with an excess of protein G-Sepharose beads (16). The recombinant protein was coupled to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) at a ratio of 100 μ g of p15 to 100 mg of Sepharose. For each assay, 0.5 mg of p15-Sepharose was mixed with 50 μ l of plasma (1:2 dilution for IgE and 1:20 for IgG4) in a final volume of 300 μ l of PBS–0.3% bovine serum

albumin–0.1% Tween 20 (PBS-AT) overnight at room temperature. Subsequently, the samples were washed five times with PBS–0.1% Tween 20 (PBS-T) and mixed with 250 μ l of PBS-AT plus 4.5% bovine serum and 0.5% sheep serum, and 20,000 cpm of ¹²⁵I-labeled polyclonal sheep anti-human IgE (CLB: 19-F1) or IgG4 (CLB: MH-164/1), from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), was added. Following overnight incubation at room temperature and four washes with PBS-T, the samples were counted and the results were expressed as percent binding of the added label. For IgE, the results were converted to nanograms per milliliter by plotting against a nonlinear regression curve of reference serum. The reference curve was constructed by incubating Sepharose-coupled sheep anti-IgE (CLB: 19-F2) with serial dilutions of reference serum under conditions identical to those described above. Background binding of a panel of four control serum samples was determined at 0.3% $\pm$ 0.2% (standard deviation [SD]) for IgE and at 0.7% $\pm$ 0.3% for IgG4. The intratest variation of the assay has been determined as 10%; i.e., a serum sample with a value of 2.0% binding would, on repeated assay, fall between 1.8 and 2.2% (1). Statistical comparison of the mean values between groups was performed by the Mann-Whitney U test.

Study population. Serum donors were from Rengat, Sumatra, an area where *B. malayi* is endemic. They were classified according to clinical and parasitological status, as previously described (42). For this study, we used sera from 45 adult males (aged 15 to 75 years), 15 in each group, classified as (i) asymptomatic amicrofilaremic, (ii) microfilaremic, and (iii) those with elephantiasis.

RESULTS

PCR amplification, cloning, and sequence analysis. gp15/400 in *B. pahangi* is encoded by a single gene composed predominantly of direct linked repeats of 396 bp (39). We therefore used PCR with the oligonucleotide primers outlined in Materials and Methods to isolate the repeating gene fragment from genomic DNA of *B. malayi* and *W. bancrofti* and included DNA from *B. pahangi* as a control. A series of fragments which were multiples of approximately 400 bp were obtained in all three species (Fig. 1A). Since the oligonucleotides prime DNA synthesis in opposite directions from a single point in the sequence (Fig. 1C), this suggested that the basic organization of the gene (i.e., as direct linked repeats with no intervening sequences) was conserved in *B. malayi* and *W. bancrofti*. The amplified fragments were hybridized with the cDNA probe BpL3 (39) and washed under high-stringency conditions. The results indicated that the fragments are homologous to the cDNA sequence (Fig. 1B). A faint band of approximately 600 bp was amplified in the *W. bancrofti* sample, presumably as a result of the slightly reduced annealing temperature, but did not hybridize with the cDNA probe. The 400-bp gene fragment from each species was subcloned into M13mp19 and sequenced on both strands.

The actual size of the fragments was 396 bp in all three cases, and the nucleotide and derived amino acid sequences are shown in Fig. 2, along with that recently identified for *Dirofilaria immitis* (7, 32) for comparative purposes. The nucleotide sequence of the *B. pahangi* repeat showed one redundant change from that reported for the cDNA (39), indicating that the PCR amplification had been faithful (the nucleotide sequences of the repeats need not be identical). That of *B. malayi* showed only two changes, both redundant,

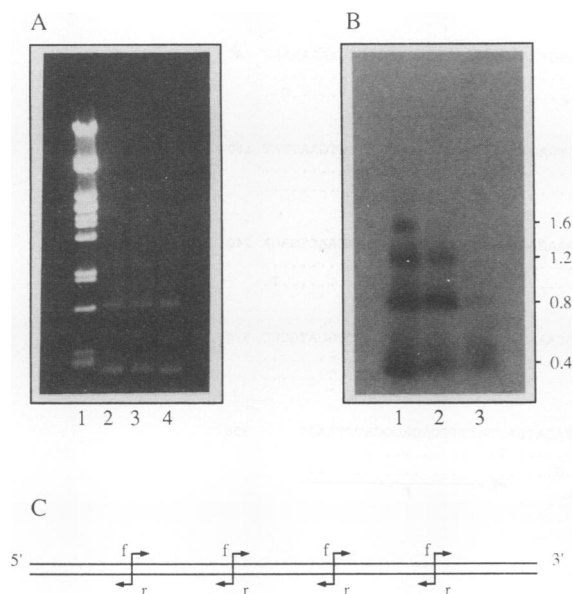


FIG. 1. PCR amplification of gp15/400 repeat units. (A) Ethidium bromide-stained amplified products from genomic DNA of *B. pahangi* (lane 2), *B. malayi* (lane 3), and *W. bancrofti* (lane 4). Lane 1 shows lambda DNA digested with *Pst*I. Products were resolved on a 1% agarose gel. (B) Southern blot of PCR products hybridized with cDNA BpL3 and washed under high-stringency conditions. Lanes: 1, *B. pahangi*; 2, *B. malayi*; 3, *W. bancrofti*. The sizes of the amplified fragments are shown in kilobases. (C) Orientation of PCR primers, detailed in Materials and Methods. Abbreviations, f, forward; r, reverse.

and the *W. bancrofti* nucleotide sequence displayed 21 substitutions. These results may be slightly biased by the PCR amplification, which uses two 22-nucleotide primers, although the *B. malayi* sequence shows a single substitution in one of the complementary sites, which therefore presumably represents a parent strand. Hence, at the level of the derived amino acid sequence, without inclusion of the stretches dictated by the PCR primers, the *B. pahangi* and *B. malayi* repeats appeared identical and the *W. bancrofti* repeat showed 7 amino acid substitutions (94% identity). The consensus amino acid sequence for the *D. immitis* repeat, consisting of 133 residues, was considerably different, with 57% identity and a single deletion and compensating insertion (Fig. 2b).

Expression and purification of p15. To express a single repeat unit of the *Brugia* gp15/400, we generated an amplified repeat fragment by PCR and ligated it to the expression plasmid pDS56/RBS11 (37). Following transformation of *E. coli* with the *B. pahangi*(pDS56/RBS11) construct, cell lysates of several transformants were run on SDS gels, blotted, and reacted with human filarial infection serum to check for correct orientation of the inserts and for expression of the fusion protein. A large-scale preparation was performed with one of the clones that proved positive, and the protein was purified by nickel-chelating chromatography as described in Materials and Methods. The results are shown in Fig. 3. Figure 3A demonstrates that a protein of 15 kDa (the expected molecular mass of a molecule of 142 amino acids, composed of 132 residues encoded by the gene fragment and 10 plasmid-encoded residues appended to the N terminus) is abundantly expressed and purified to homogeneity on the

nickel column. Moreover, Fig. 3B shows that the protein is recognized by pooled sera from Sumatran individuals with elephantiasis due to infection with *B. malayi*. A small degree of background binding to bacterial protein was observed, but this was absent in the purified preparation, which was thus suitable for subsequent serologic testing.

IgE responses to p15 in brugian filariasis. We used purified p15 coated to Sepharose CL-4B in SPRIA for determination of antigen-specific IgE in individual sera from members of the study group. Since IgG4 dominates the spectrum of antifilarial antibodies (17, 31) and can act as a blocking factor for antigen-specific IgE-mediated histamine release (29), we also determined specific IgG4 to p15 by the same method. Data were determined for 15 adult men in each clinical group (asymptomatic amicrofilaremic individuals, microfilaremic patients, and those with elephantiasis) and are presented in Fig. 4. Elevated levels of IgE to p15 (i.e., values greater than the mean plus 2 SDs of control sera) were observed in all but one of the individuals with elephantiasis, with a mean value of 3.2 ng of specific antibody ml⁻¹ (SD, 3.4). Conversely, all but two of the microfilaremic subjects were negative, with an overall mean score of 0.045 ng ml⁻¹ (SD, 0.12). The asymptomatic amicrofilaremic individuals fell into two groups (eight subjects had elevated levels of IgE and seven were negative) with an overall mean of 1.2 ng ml⁻¹ (SD, 2.0). The sharpest distinction therefore lay between the first two clinical groups, since patients with elephantiasis had much higher values of IgE to p15 (on average 70-fold higher) than the microfilaremic patients ($P < 0.001$). The difference in IgE values between the group with elephantiasis and the asymptomatic amicrofilaremic subjects was also significant ($P = 0.01$), and difference between the asymptomatic amicrofilaremic subjects and the microfilaremic subjects was just significant ($P = 0.04$).

The data for IgG4 binding did not show such a sharp distinction, and a fairly heterogeneous response was observed in each group (Fig. 4B). Since we did not have access to reference serum, the data are expressed as percent binding to the antigen-coated Sepharose beads and are therefore presented for comparative means. In contrast to the almost uniform negativity among microfilaremic patients in terms of antigen-specific IgE, 9 members of this group of 15 showed elevated IgG4 levels. The group with elephantiasis showed a mean of 4.4% (SD, 4.7), the group of microfilaremic subjects had a mean of 3.1% (SD, 3.0), and the asymptomatic amicrofilaremic subjects had a mean of 2.9% (SD, 5.8). The high SD in the last group was indicative of a particularly heterogeneous distribution. Values for IgG4 were not significantly different among groups. Figure 5 shows a comparative view of antigen-specific IgE and IgG4 in the same individuals. In the group with elephantiasis, 10 of 15 individuals showed high levels of both IgE and IgG4, but in the other groups there was no positive correlation between the two isotypes. These results also highlight the particularly heterogeneous distribution of specific antibodies in asymptomatic amicrofilaremic subjects. Attempts to demonstrate p15-specific histamine release from peripheral blood basophils of normal donors passively sensitized (33) with sera from Sumatran patients failed, as did control experiments with total-parasite extracts.

DISCUSSION

We show here that the primary structure of the repeated subunit of gp15/400 is very highly conserved in the lymphatic

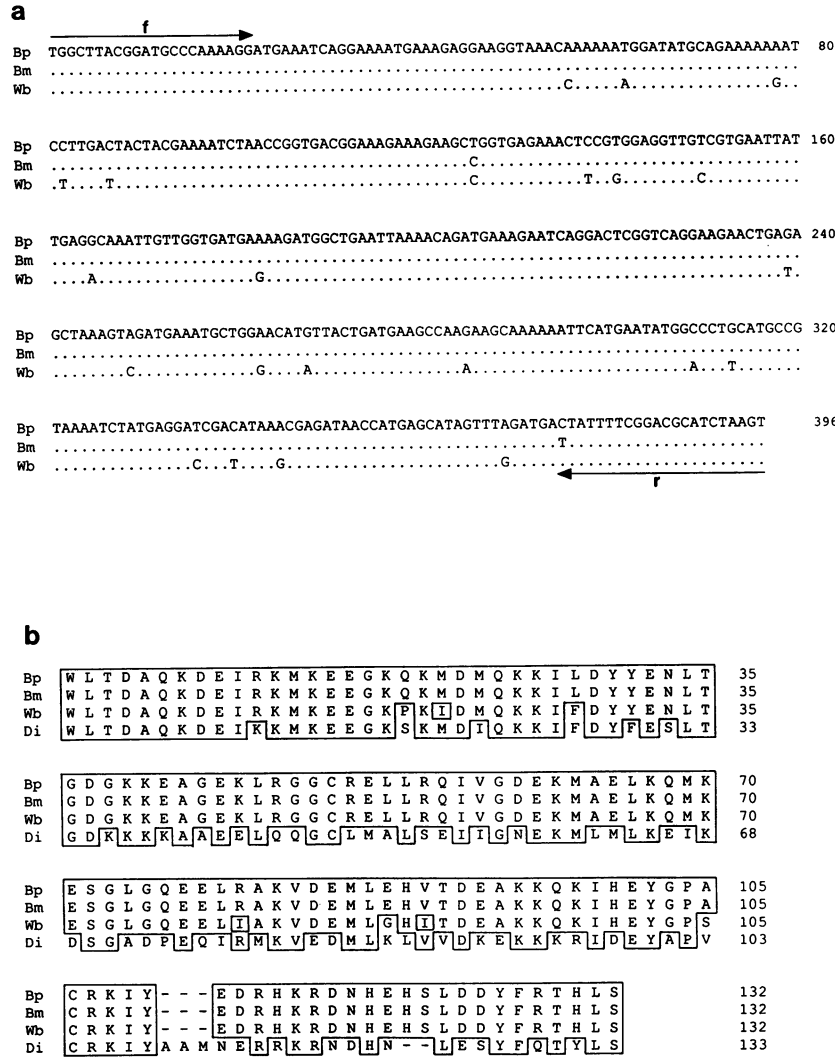


FIG. 2. Sequence of the repeat unit of gp15/400. (a) Nucleotide sequence from *B. pahangi* (Bp), *B. malayi* (Bm), and *W. bancrofti* (Wb). Residues identical to the sequence from *B. pahangi* are indicated by dots, and the positions of the forward (f) and reverse (r) primers are indicated. (b) Derived amino acid sequence of the repeat units. That recently determined for *D. immitis* (28) is shown for comparison (Di). Residues identical to the *B. pahangi* sequence are boxed, and gaps in the alignment are indicated by dashes.

filariae, and the identical amino acid sequence between the two *Brugia* species is quite striking. Strictly, multiple repeats should be sequenced from all species to gain precise knowledge of the overall conservation of the protein, since it is not clear whether all of the repeats, estimated at about 20 per protein (7, 32, 39), are identical, but the present data indicate a high degree of conformity. This result is mirrored by a similar analysis of the cuticular antigen gp29, in which the derived amino acid sequence of the protein from *B. malayi* and *B. pahangi* was determined to be identical, and only 7 of 223 substitutions were observed in the *W. bancrofti* homolog (6). Collectively, the results are consistent with the classification of the lymphatic filariae and the creation of a new genus (*Brugia*) by Buckley (3) on the basis of morphology. As expected, the sequence of the gp15/400 repeat from *D. immitis* is quite divergent from those of the lymphatic filarial species. These data show that diversity in the structure of lymphatic filarial antigens sequenced to date is extremely limited, both between closely related species

and (presumably) between different strains of the same species. An advantage of antigenic conformity is that defined preparations of immunogens may well have a broad spectrum of efficacy against different strains and species of filariae. Such cross-protection has already been observed following immunization with irradiated infective larvae (36) and parasite extracts (4). Given that immunization of mice with ABA-1 confers approximately 50% protection against challenge with *Ascaris* species (25), it will be interesting to assess the immunogenicity of gp15/400 in experimental models of filariasis. A more likely limitation in the effectiveness of defined preparations as vaccines is the genetic variability of the immune response among individuals. In this context, the specificity of the antibody response to gp15/400 and the *Ascaris* homolog ABA-1 in mice has already been examined. Using H-2 congenic mice on BALB and B10 backgrounds, Tomlinson et al. (38) determined that the IgG and IgE

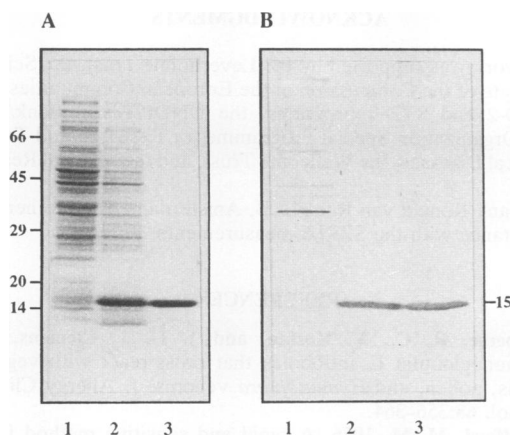


FIG. 3. Expression, purification, and antigenicity of p15. (A) Purification of p15. Lanes: 1, *E. coli* TG2 lysate; 2, lysate of TG2 + pDS56/BpL4; 3, purified p15. Proteins were resolved on an SDS-7 to 25% polyacrylamide gel and stained with Coomassie blue. Molecular mass markers are shown in kilodaltons. (B) Western blot of the preparations illustrated in panel A. Transferred proteins were reacted with a serum pool from individuals with elephantiasis, and antibody binding was visualized by using peroxidase-conjugated second antibody (21).

responses to ABA-1 were both restricted to those of the *H-2^s* haplotype, whereas the response to native gp15/400 of *B. malayi* appeared to be controlled by factors outside the major histocompatibility complex (18). The regulation of the antibody response to these related proteins is therefore completely different in inbred mouse strains, but this is perhaps not surprising: the repeat unit of ABA-1 has recently been cloned and shows only 45% amino acid homology to gp15/400 from *Brugia* species (13a).

Tropical pulmonary eosinophilia is characterized by extremely high levels of IgE (30), and attendant disease is thought to result from immediate hypersensitivity reactions and eosinophilic inflammation (34). Aside from this syndrome, it is unclear whether IgE is essential for the development of filarial immunopathology. Eosinophilic granulomatous infiltration is characteristic of lymphatic lesions, but intact worms may cause lymphedema in the absence of cellular infiltration, and type III immune-complex-mediated hypersensitivity most probably also contributes to exudative inflammation (40). In our previous study with the Sumatran population, total IgE and, in particular, the IgE/IgG4 ratio assayed against total parasite antigen were greatly elevated in individuals with elephantiasis compared with those in asymptomatic microfilaremic individuals (16). Asymptomatic amicrofilaremic subjects presented a heterogeneous group, and this result is also seen when p15-specific antibody responses are assayed (Fig. 4 and 5). Native gp15/400 has a single N-linked oligosaccharide per 15-kDa subunit (39), and although we do not know the significance of this determinant in the evocation of allergic responses, the present data show that the peptide backbone alone will induce significant levels of IgE.

The relative role of IgE in immunity is likewise unclear, although experiments with *B. pahangi* infection of rats showed that an early specific IgE response during the prepatent period was the sole predictive indicator of animals destined to resist infection (i.e., those that developed microfilaremia [10]). The high levels of IgE seen in a proportion of amicrofilaremic asymptomatic individuals may be indica-

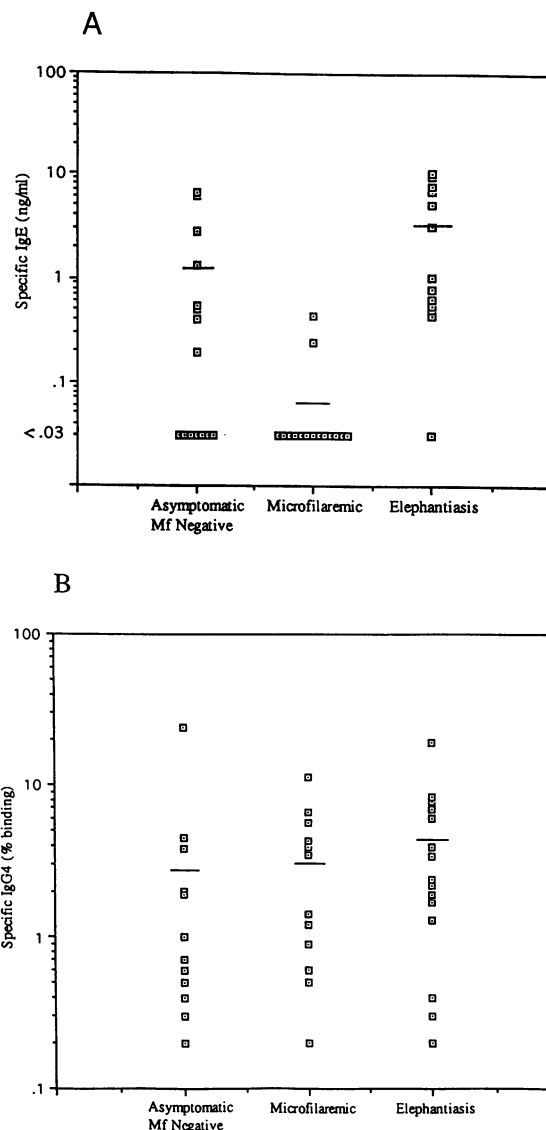


FIG. 4. Detection of p15-specific IgE and IgG4 by SPRIA. Each point represents the value for an individual, and the bars represent the mean values for each classified group. (A) Antigen-specific IgE, measured in nanograms per milliliter. (B) Antigen-specific IgG4, expressed as percent binding.

tive of protective immunity, but the almost uniform elevation of this isotype in patients with elephantiasis suggests that it may not be possible to segregate immunity due to IgE-mediated mechanisms from the onset of immunopathology. Longitudinal studies with human populations may help to resolve these questions.

A significant association has been recorded between responsiveness to a selected number of purified allergens and HLA-DR loci (23, 27). We are in the process of typing the Sumatran study population and will therefore soon be able to generate data on whether recognition of p15 is major histocompatibility complex restricted. This will be complemented by direct histamine release assays to confirm that p15 is functionally allergenic, and it may shed light on the potential involvement of this molecule in the pathogenesis of disease.

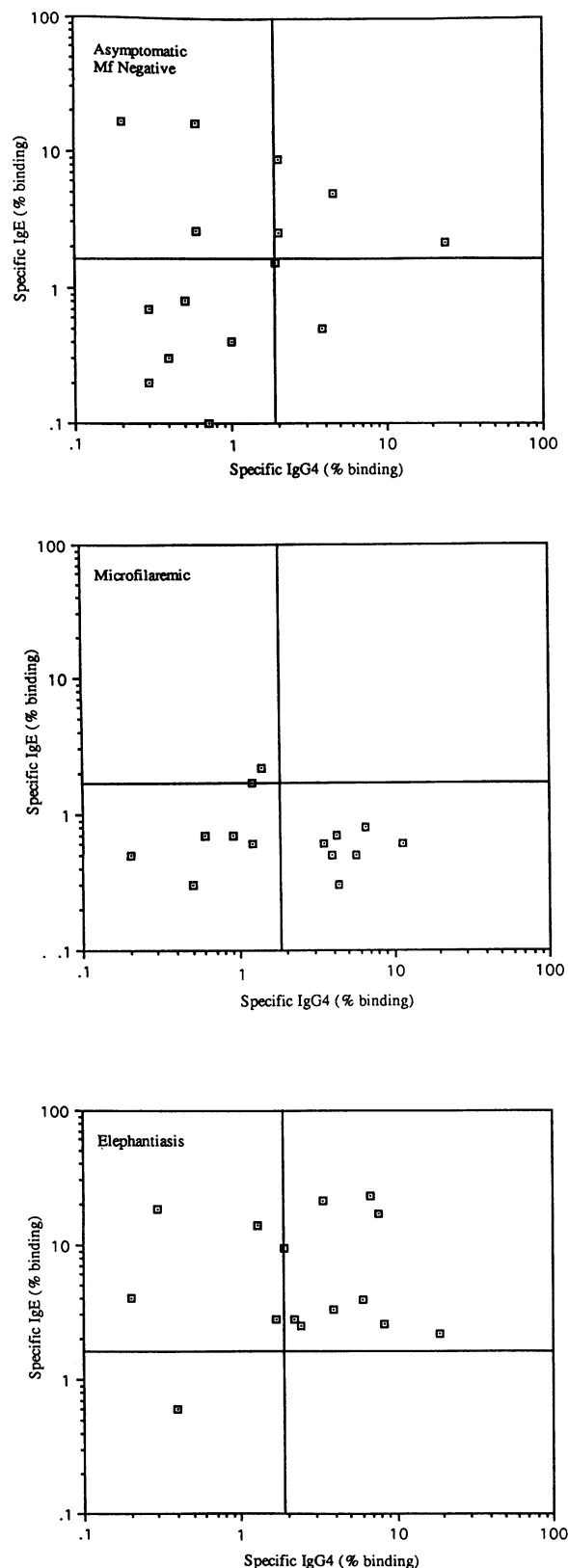


FIG. 5. Relationship between p15-specific IgE and IgG4 for individuals in each classified group. The horizontal lines represent the median IgE value (1.7% binding) for the total population assayed (i.e., all 45 individuals), and the vertical lines represent the median IgG4 value (1.9% binding) for the total population.

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