

B CELL RESPONSES TO PARAMYOSIN

Isotypic Analysis and Epitope Mapping of Filarial Paramyosin in Patients with Onchocerciasis

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To examine the fine specificity of the human immune response to filarial paramyosin, the antigenicity of an expressed rcDNA (2.55 kb) of *Dirofilaria immitis* paramyosin was detailed by ELISA. Using sera from patients infected with *Onchocerca volvulus*, we analyzed both the entire paramyosin molecule and six subcloned fragments for their IgG, IgG subclasses, and IgE responses. Patients from both Guatemala (64% positive) and Ghana (100% positive) reacted to paramyosin with specific IgG levels above normal controls. Although there was no anti-paramyosin subclass restriction common to all patients, the IgG3 response in the Ghanaians was significantly greater than that of Guatemalans ($p < 0.001$). IgE anti-paramyosin responses showed positive correlations with IgG2 ($p < 0.001$), IgG4 ($p < 0.002$), and IgG1 ($p < 0.04$) responses. Epitope mapping using the IgG response to the six subclones demonstrated preferential recognition of the amino terminal end of the molecule (nucleotides 1 to 360). IgG2 reactivity was clearly localized to the most amino-terminal 120 amino acids, and the IgG4 antibodies recognized amino acids immediately adjacent to this fragment. These studies examining the fine specificity of anti-filarial immune reactions should provide a method for understanding how parasites either evade or induce host immune responses.

Onchocerciasis, a disease caused by infection with the filarial parasite *Onchocerca volvulus*, affects ~18 million individuals in Africa and Latin America (1). Although infection may be asymptomatic or manifest itself as chronic pruritus only, in its most severe form it may lead to significant skin or eye pathology, including blindness. Although major progress has been made in both chemotherapy (ivermectin) and vector control, disease control by these means is only partial, and the infection continues to be a significant cause of morbidity in the develop-

ing world. Thus, an immunologic approach to preventing disease continues to be an important goal. The study of immune responses to *O. volvulus*, however, has been complicated by broad antigenic cross-reactivity among parasite Ag and, more importantly, by a paucity of available parasite material. rDNA technology is ideally suited to circumvent some of these difficulties. The ability to produce large amounts of individual parasite proteins is of obvious importance, and the lack of post-translational modification in prokaryotic systems assures the absence from these recombinant Ag of the phosphorylcholine and carbohydrate moieties that are responsible for mediating the cross-reactivity found among parasite Ag (2).

Paramyosin is a protein located in the core of muscle filaments of many invertebrate species, including arthropods and mollusks (3), as well as in helminths where it has also been localized to the tegument (4). This molecule not only acts as an immunogen in several filarial infections (5) (our unpublished observations), but has also been implicated as a potential vaccine candidate in murine studies of *Schistosoma mansoni* (6–9) and *Brugia malayi* (10) infections.

Recently, a cDNA clone coding for *Dirofilaria immitis* paramyosin has been sequenced (11) and found to possess a 99% protein homology with *O. volvulus* paramyosin, 92% homology with the paramyosin of the free-living nematode *Caenorhabditis elegans*, and 33% homology with paramyosin from the parasitic trematode *S. mansoni*. Because of the nearly complete identity between *D. immitis* and *O. volvulus* paramyosin, the reactivity of patients with onchocerciasis to paramyosin could be assessed using the recombinant molecule from *D. immitis*. Therefore, in the present study we have analyzed the antibody response to paramyosin by individuals infected with *O. volvulus*, either the savanna form from Africa (Ghana) or the Central American form from Guatemala. In addition, the responses of a group of individuals from Guatemala previously characterized as "putatively immune" to onchocerciasis (12) were also analyzed. IgG, IgG subclass, and IgE responses to whole paramyosin were assessed and, in addition, six subcloned fusion polypeptide fragments of recombinant paramyosin were also examined so that the specific epitopes responsible for inducing the host immune response of each isotype to this molecule could be identified.

MATERIALS AND METHODS

Preparation of recombinant paramyosin

The cDNA for the paramyosin gene of *D. immitis* was isolated from a cDNA expression library constructed in Agt11 as previously

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TABLE I
Study population

Patient Group	n	Age ^a	Sex	Mf/mg Skin ^b
Infected				
Guatemala	45	33 (15–60)	38M/7F	11.2 (1–48)
Ghana	19	21 (15–51)	19M	77.6 (10–226)
Putatively immune				
Guatemala	12	24 (16–63)	10F/2M	0
Normal				
Guatemala	18	28.5 (23–59)	12M/6F	0
North America	15	31 (19–45)	10M/5F	0

^a Median and range.

^b Geometric mean and range.

described (5). The cDNA insert contains one open reading frame (2550 bp) encoding a polypeptide of 850 amino acids (11). The whole paramyosin molecule used in these studies was expressed in the plasmid vector pCG806fx (13), isolated as a fusion protein with MBP³, then cleaved with factor X from MBP and purified as described previously (5, 14).

Construction and Expression of Paramyosin Subclones

Six subclones spanning the length of the paramyosin molecule (Fig. 1) were constructed by cloning either paramyosin restriction fragments or deletions of these fragments (in the case of the two most amino terminal fragments) into the plasmid vectors pCG806fx or its derivative, pNVI (unpublished observation), containing the maltose binding protein gene *MalE* (13). The MBP-paramyosin fusion proteins encoded by these subclones were used to perform the epitope mapping after they were purified on an amylose affinity resin as described previously (5). Fusion products were used in this part of the study because factor X cleavage of the constructs was unsuccessful.

Patient Populations

Sera from patients with onchocerciasis (either from the savanna region of Ghana or from Guatemala) were used to evaluate the reactivity to recombinant paramyosin and the subcloned fusion proteins. These populations have been described in detail elsewhere (12, 15). All of the infected individuals were microfilaria-positive on skin snip examination. The major clinical differences were in the greater severity of the skin changes in the Ghanaian population and the fact that the Ghanaians had significantly greater microfilaridermia. An additional group from Guatemala considered to be "putatively immune" was also studied; the clinical and immunologic differences between this group and the patients with active infections have been described previously (12). Normal individuals (both non-exposed North Americans and Guatemalans living in areas nonendemic for *O. volvulus*) were also evaluated (see Table I for study population).

Measurement of Paramyosin-Specific Antibodies

Quantitation of antibodies to the recombinant paramyosin was performed using ELISA at serum dilutions found to be optimal. Briefly, recombinant-purified paramyosin was used to coat microtiter plates (Immunlon IV, Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4°C in carbonate/bicarbonate buffer, pH 9.6. Optimal concentrations were found to be 0.1 µg/ml for IgG, 1 µg/ml for the IgG subclasses, and 5 µg/ml for IgE. The plates were then washed in PBS containing 0.05% Tween-20, blocked in 5% BSA for 2 h, and washed again.

IgG antibodies. Sera were added at a dilution of 1/100 and incubated overnight at 4°C. After washing, alkaline phosphatase-conjugated Fc-specific anti-human IgG, (Jackson ImmunoResearch, Avondale, PA) was added; plates were incubated for 2 h at room temperature, then washed and developed using *p*-nitrophenyl phosphate. On each plate, a reference pool comprised of sera from filarial-infected patients was used to construct a standard curve to which all individual sera were compared. Data are expressed as arbitrary U/ml as derived from the standard curve.

³ Abbreviation used in this paper: MBP, maltose binding protein.

IgG subclass antibodies. Sera were added at a dilution of 1/20 and incubated overnight at 4°C. After washing, subclass-specific mAb (HP6069 for IgG1, HP6002 for IgG2, HP6047 for IgG3, and HP6023 for IgG4) (16) were added for 2 h at 37°C. Plates were washed, then alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was added for 2 h at 37°C, after which the assay proceeded as above. Again, a standard reference serum for each subclass was included on each plate for standardization, and the data were expressed as U/ml.

IgE antibodies. Patient sera were preabsorbed with Protein G coupled to Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and used at a final dilution of 1/5. After an overnight incubation at 4°C, a monoclonal anti-human IgE (mAb 7.12; kindly provided by Dr. Andrew Saxon, UCLA, Los Angeles, CA) was added for 2 h at 37°C; the plates were washed and incubated for an additional 2 h at 37°C with biotinylated goat anti-mouse IgG (Jackson ImmunoResearch). After washing, streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch) was added for 1 h at room temperature; the plates were washed and developed as above. Again, a reference serum was used on each plate for standardization.

Measurement of IgG, IgG Subclass, and IgE Antibody to Paramyosin Subcloned Fusion Polypeptides

ELISA were also used to quantitate the antibodies to the subcloned fusion polypeptides. The six fusion polypeptides and the control MBP were each used at 5 µg/ml to coat microtiter plates. Sera were used at dilutions of 1/100 for IgG, 1/20 for IgG subclasses, and 1/5 for IgE; the assays proceeded exactly as described above. Because individuals have variable responses to MBP alone, a "response" value above MBP reactivity was determined for each patient by the formula:

$$X = \text{OD (subclone)} - \text{OD (MBP)}$$

For each individual, the relative reactivity to each of the six subclones was determined by expressing the reactivity ("X") to each fusion polypeptide as a percentage of the mean reactivity to all six polypeptides. The "null hypothesis," implying no preferential reactivity, would yield equivalent values (100% of the mean) for each of the six fragments. Responses to the subcloned fusion polypeptides at least 10% above this value were defined as indicating preferential reactivity.

Statistical Analyses

Student's *t*-test was used to compare mean values in different populations using logarithmically transformed data. Fisher's exact test was used to compare response rates between populations, and Spearman rank correlations were used to assess the significance of associations among the variables.

RESULTS

Anti-paramyosin IgG reactivity. Anti-paramyosin-specific IgG levels varied in the two study populations with onchocerciasis, but mean levels for both groups differed significantly from those of the normal controls ($p < 0.001$; Fig. 2). In general, Ghanaian patients tended to have greater IgG anti-paramyosin levels (100% positive, mean response = 125.5 U/ml) than those of the Guatemalans (64.4% positive, mean response = 51.6 U/ml), but the difference between the two groups was not statistically significant.

Anti-paramyosin IgG subclass reactivity. To determine whether the IgG antibody response to paramyosin was restricted to any of the four IgG subclasses, sera positive for IgG antibody to the whole molecule were subjected to analysis of their anti-paramyosin IgG1, IgG2, IgG3, and IgG4 responses (Fig. 3). All individuals had measurable IgG3 antibodies to paramyosin, but the IgG3 response of Ghanaian patients was considerably greater than that of Guatemalans both in magnitude ($p < 0.001$) and in rate of reactivity (19/19 vs 12/29 above "normal levels," $p < 0.001$). Many patients lacked IgG1, IgG2, or IgG4 antibodies to paramyosin, but the response rates

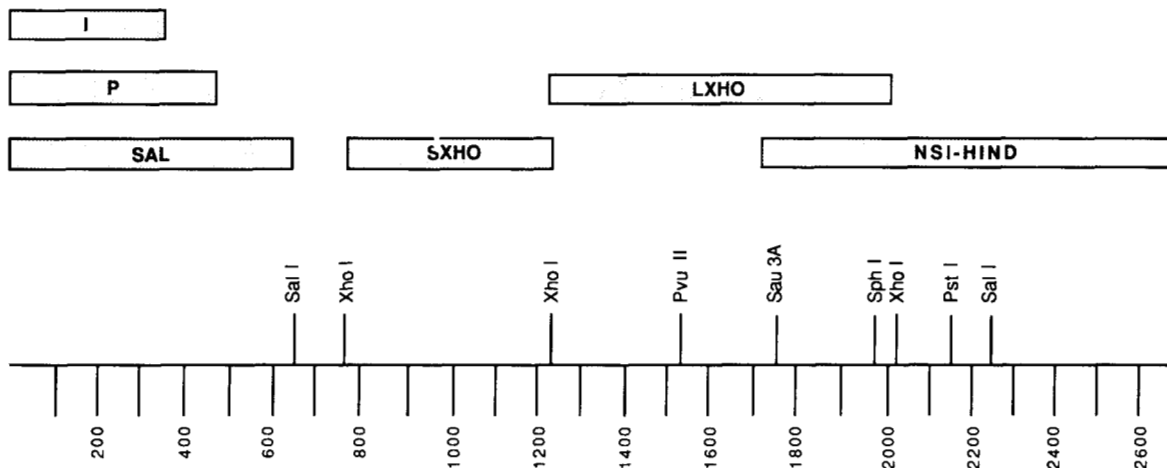


Figure 1. Paramyosin restriction endonuclease sites and subcloned fragments. Schematic representation of cloned *Dirofilaria* paramyosin including the six subcloned polypeptides (shaded boxes) that were fused to MBP and used for epitope mapping.

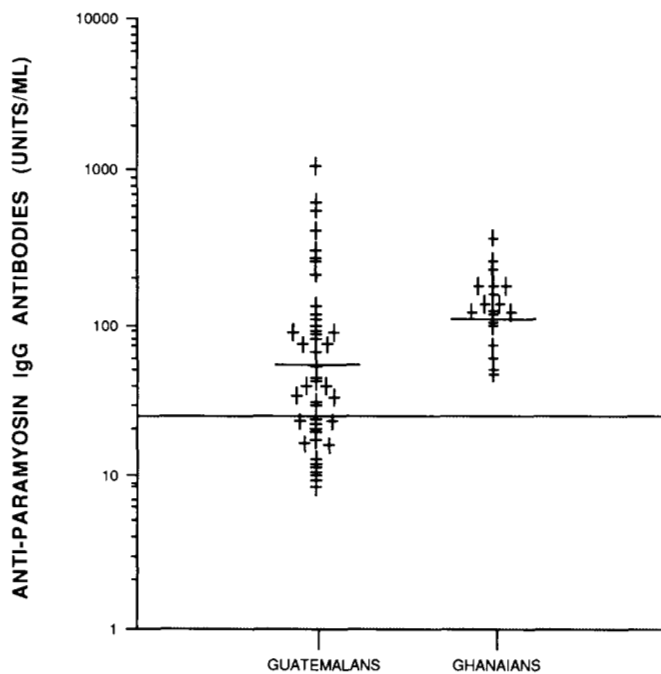


Figure 2. ELISA analysis of anti-paramyosin IgG in *O. volvulus*-infected patients. Each cross-hatch represents an individual patient; the shaded area is the mean + 2 SD of normal controls. Guatemalan and North American normals were grouped ($n = 33$) together, as there was no significant difference in their antibody response (data not shown). The horizontal bars represent the geometric mean for each group.

did not differ between the two groups of infected patients from Guatemala and Ghana (11/29 vs 6/19, $p = 0.446$ for IgG1; 15/29 vs 8/19, $p = 0.361$ for IgG2; 16/29 vs 9/19, $p = 0.407$ for IgG4). When linkages among these subclass responses were sought, only a correlation between the IgG1 and IgG2 anti-paramyosin response ($p < 0.01$) was found.

IgE reactivity to paramyosin. Patient sera were also assayed for their anti-paramyosin IgE levels (Fig. 4). Interestingly, the Guatemalan patients with onchocerciasis reacted at higher levels than did those from Ghana (geometric means of 7.54 U/ml vs 3.21 U/ml, respectively); however, this difference was not statistically significant ($p = 0.085$). Furthermore, the specific anti-paramyosin IgE levels had strongly positive correlations with IgG2 ($p < 0.001$) and IgG4 ($p < 0.002$) responses and were also

correlated to IgG1 ($p < 0.04$).

IgG reactivity to fusion protein fragments of paramyosin. To identify the specific B cell epitopes on the paramyosin molecule, patient sera were assayed by ELISA for reactivity to six subcloned fusion polypeptides (Fig. 5). In general, preferential reactivity was seen with paramyosin fragments at the amino terminal end of the molecule where the three overlapping polypeptides I, P, and Sal occur (Fig. 1). The "I" fusion protein, derived from a small 360-bp sequence and found at the very amino terminal end of the molecule, was a particularly prominent immunogen, with 15/19 Ghanaian and 10/18 Guatemalan patients preferentially reacting to this subcloned fusion protein.

Isotype reactivity to fusion protein fragments of paramyosin. As with whole paramyosin, IgG subclass as well as IgE responses were examined to the six subclones. For this analysis, 17 infected patients (7 Ghanaian and 10 Guatemalan) who had shown above normal IgG anti-paramyosin responses were used (Fig. 6). Again, most preferential reactivity was seen in the amino terminal half of the molecule, but two additional features were also prominent. First, the IgG2 response of the patients was largely to the amino terminal 120 amino acids ("I" fragment), with all but one of the patients studied preferentially recognizing this fragment. Second, unlike the findings with the other three IgG isotypes, very few individuals' IgG4 antibodies preferentially recognized this most amino terminal end of the molecule; rather, there was a clear-cut preferential recognition (by 77% of the individuals) to the overlapping "P" fragment, an observation suggesting that this fragment (on the carboxyl-terminal ends of the "I" and "P" fragments) might represent an IgG4-specific epitope. IgE reactivity to any of the fusion proteins was seen in only 12 of those 17 sera that had shown IgE reactivity to the whole paramyosin molecule. Among these 12, some showed preferential recognition to the amino terminal fragments, but others to the carboxyl-terminal fragment.

Relationship between responses to paramyosin and clinical state. To examine the possibility that responses to paramyosin could be, in part, mediating resistance to infection with onchocerciasis, a detailed comparison was made of the responses to paramyosin by previously studied "putatively immune" individuals (12) and infected

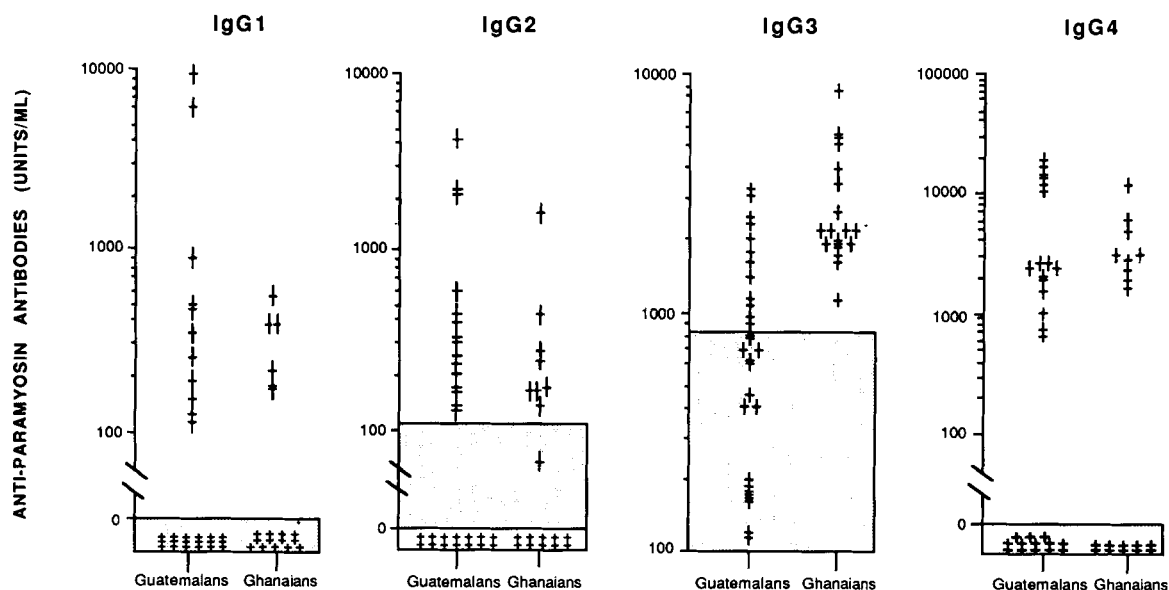


Figure 3. ELISA analysis of IgG subclasses to paramyosin. Each cross-hatch represents an individual patient; the normal range is shown by the shaded area.

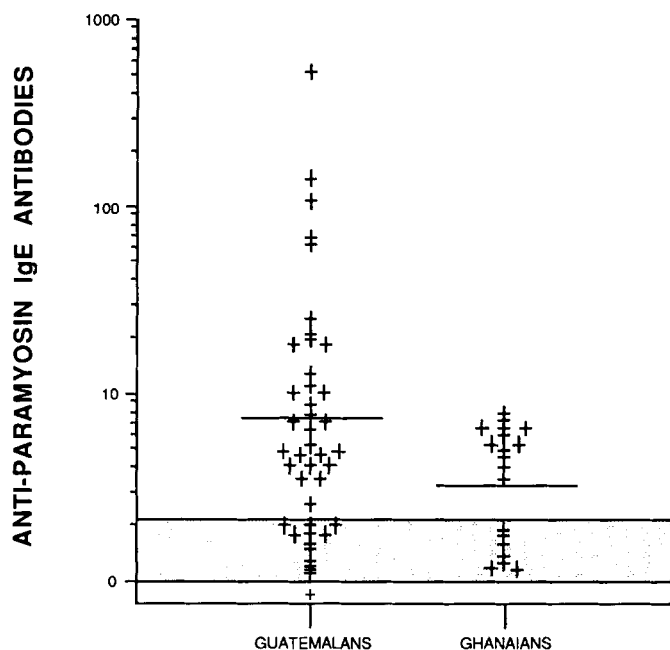


Figure 4. ELISA analysis of anti-paramyosin IgE. Each cross-hatch represents an individual patient; the shaded area is the range of normal controls. The horizontal bars represent the geometric mean for each group.

individuals from the same geographical area hyperendemic for *O. volvulus*. Only when the IgG reactivity to the subcloned fragments was examined was any difference between the two groups seen. Although the pattern of prominent reactivity to the three amino terminal fragments was maintained for both groups, significantly more (67%) of the putatively immune individuals preferentially recognized the LXHO fragment (near the middle of the molecule) than did those who were infected (25%) (data not shown).

When subclass responses to whole paramyosin were examined for their relationship to microfilarial load, IgG3 anti-paramyosin levels were found to be positively correlated to microfilarial levels ($p < 0.05$). When IgE antibody was analyzed for any relationship to microfilarial

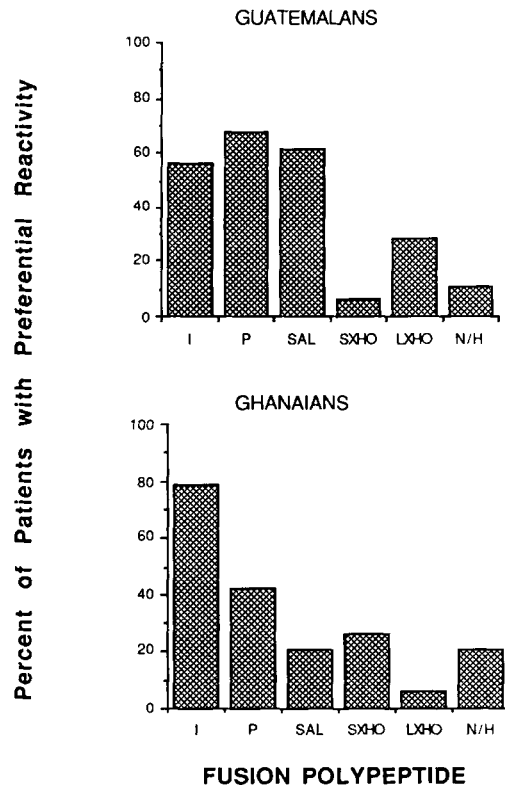


Figure 5. ELISA analysis of IgG specific reactivity to six recombinant fusion protein fragments of paramyosin. Data are expressed as percent of patients who preferentially reacted to each fusion protein as described in Materials and Methods.

load, an inverse correlation was seen ($p < 0.03$) among the patients who were positive for anti-paramyosin IgG, but this relationship did not hold when all patients (both positive and negative for IgG) were included.

DISCUSSION

Paramyosin has been shown to be a potentially important molecule for mediating protective immunity to *S. mansoni* (7-9, 17) and, more recently, for mediating microfilarial clearance in murine *B. malayi* infections

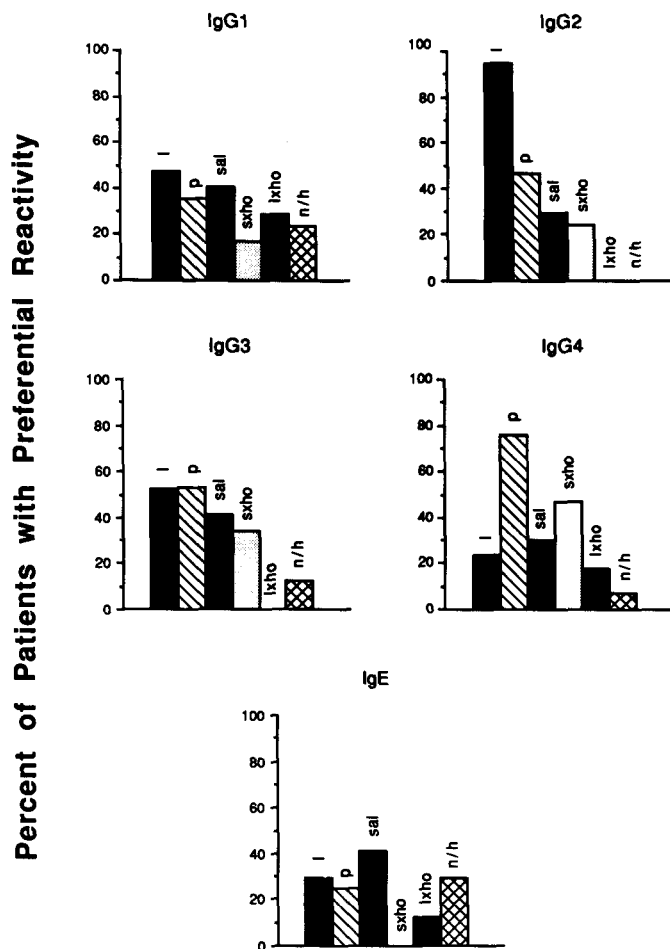


Figure 6. ELISA analysis of IgG subclass and IgE reactivity to six subcloned recombinant fusion protein fragments of paramyosin. Data represent the reactivity of 17 *Onchocerca*-infected patients from both Guatemala and Ghana. Data are expressed as percent of patients who preferentially react to a particular fusion protein; positive reactions were determined as discussed in *Materials and Methods*.

(10). Although two distinct epitopes have been mapped for schistosome paramyosin (7), the immune responses to filarial paramyosins have been less well defined. Using recombinant *D. immitis* paramyosin, a molecule having 99% amino acid homology to *O. volvulus* paramyosin (11), we have examined in detail the antibody response to this molecule using sera from *O. volvulus*-infected individuals and from a group of individuals potentially immune to this infection.

The IgG response to the entire paramyosin recombinant molecule varied from individual to individual, but when the subclass responses were examined, several interesting points emerged. First, among the Ghanaian patients, who as a group had more clinical disease (particularly dermatitis) than the Guatemalans, there was a particularly prominent IgG3 response to paramyosin. This finding parallels observations made in patients with hyperactive onchodermatitis (Sowda), who have previously been shown to have heightened IgG3 responses to onchocercal antigens (18). Second, the levels of these same IgG3 anti-paramyosin antibodies were directly correlated with microfilarial density in the infected patients, an observation providing a possible explanation for the generally higher levels of IgG3 antibodies seen in the more heavily infected Ghanaian study population. Infection with *O. volvulus* also induced paramyosin-specific

IgG4 antibodies in more than half of the patients, and these IgG4 antibodies were found to be associated with parallel rises in IgE. Interestingly, high levels of parasite-specific IgG4 have been found in other filarial disorders (19–21) as well as in schistosomiasis (22), and a specific linkage between IgG4 and IgE has already been proposed (21).

The relationship between isotypic responses to paramyosin and resistance was examined in light of previous findings in other helminth infections (23–26) where the isotype of the antibody produced was found to determine the presence or absence of a resistant state. Although parasite-specific IgE has most commonly been implicated in pathologic responses rather than protective ones (27, 28), the presence of IgE antibodies has also been shown, in some systems, to correlate with resistance to helminth infection (23, 24) and with cellular killing of parasites in vitro (26, 29). Of note, in two human studies of *Wuchereria bancrofti* (30, 31) higher levels of filaria-specific IgE were found in individuals who were “exposed but not infected” (putatively immune) than in infected patients. In the present study, the inverse correlation seen between IgE antibodies and microfilarial levels lends further credence to the concept that these anti-paramyosin antibodies could, in part, be responsible for resisting, or at least controlling, the extent of the onchocercal infection; however, this relationship between anti-paramyosin IgE and microfilarial load was seen only for patients with elevated anti-paramyosin IgG titers. The fact that there may be a “dual recognition” of specific Ag by IgE and IgG or IgG subclasses, as previously demonstrated (21, 32) may explain why patients without elevated IgG responses to a particular immunogen show little or no corresponding IgE. IgG2 has also been associated with eosinophil-mediated parasite killing in schistosome infections (25) and, indeed, in the present study, a positive correlation was seen between IgG2 and IgE levels, again possibly indicating a link between these two isotypes and resistance.

A second important finding in this study was the localization of the actual B cell epitopes of paramyosin by using subcloned fusion polypeptides to identify specific antigenic sites. Such “epitope mapping” is not without its drawbacks for a number of different reasons. First, the immune system almost certainly recognizes isolated fusion peptides differently from the native molecule in the three-dimensional state in which it normally exists; nevertheless, all six of these subcloned fragments have recently been shown to induce, in immunized mice, antibodies that are reactive with native paramyosin (our unpublished observation), and similar B and T cell epitope mapping has been carried out successfully for several other infections including malaria (33, 34), leprosy (35, 36), and schistosomiasis (37). Second, because the fusion polypeptides used in this study were still relatively large pieces of the whole paramyosin molecule (see Fig. 1), it may be possible that even smaller epitopes can later be localized within these polypeptides. Third, because equal protein concentrations of the polypeptides and not equimolar or “equi-epitope” amounts were used in the ELISA assays, the preferential reactivity detected could be somewhat biased by the number of binding sites or differences in antibody affinity or amount; however, because the goal of the present study was only to localize the major antigenic domains of the paramyosin molecule,

finer resolution of these epitopes can await further studies using overlapping synthetic peptides of these important regions.

These potential limitations notwithstanding, the results of the present study were unequivocal in indicating the major area of reactivity in paramyosin to be at the amino terminal end of the molecule, particularly in the first 120 amino acids (corresponding to the first 360 bp), a fact that may be exploitable in future vaccine studies, because this region is more highly conserved among the filarial species than is the carboxyl-terminal end of the molecule (11). Of additional interest was the finding from the comparison between "immune" and infected patients of greater rates of reactivity to the "LXHO" subclone both in the "immune" individuals and in the infected patients with lower microfilarial levels (data not shown). This particular fragment is very similar to one of the peptides from *S. mansoni* paramyosin known to induce IFN- γ by T cells from vaccinated mice (E. Pearce, personal communication).

The examination of the isotypic responses to defined filarial Ag is of obvious importance. Previously, it has been shown that analyzing only the IgG response (and not those of the IgG subclasses) can obscure major differences in antibody reactivity by different clinical groups of patients (20). The majority of these studies on filarial infections has thus far used whole crude Ag preparations to delineate these isotypic responses, but because recombinant Ag are being used more frequently to study immune responses to parasitic infections, the issue of the fine specificity of the immune response will become progressively even more important. By localizing and defining, at the structural level, the specific epitopes that induce a particular response, one should hopefully be able to generalize about the mechanisms parasites have evolved to evade or induce particular host immune responses. Further, by extending these and other studies to the T cell level, the role for paramyosin (and other defined filarial Ag) in inducing lymphokines or T cell subsets that might mediate protection can be addressed directly.

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