Immunoglobulin G Subclass Responses of Children during Infection with *Onchocerca volvulus*

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To characterize the patterns of immunoglobulin G (IgG) subclass and IgE reactivity during the early stages of onchocerciasis, sera were collected from 224 children (age groups, 2 to 5, 6 to 10, and 11 to 15 years) residing in a region of Sierra Leone where *Onchocerca volvulus* is endemic, and these sera were tested by enzyme-linked immunosorbent assay for their reactivity to adult antigens (OvAg) and against four recombinant proteins (OV11, OV27, OV29, and OV16). Over 88% of the samples contained detectable levels of anti-OvAg IgG. In samples from microfilarial (MF)-positive children, IgG4 responses were significantly elevated and constituted on average 39, 35, and 28% of the total IgG responses for the age groups of 2 to 5, 6 to 10, and 11 to 15 years, respectively. For MF-negative individuals, the mean contributions of IgG4 to the total IgG response were 11% (2 to 5 years), 27% (6 to 10 years), and 56% (11 to 15 years). OvAg-specific IgE was detectable in the sera from both MF-negative and MF-positive individuals. To increase the specificity of the response, recombinant antigens OV11, OV27, and OV29 were tested individually or as a cocktail. Nearly 50% of the MF-negative children and 85% of the MF-positive children had detectable levels of IgG against at least one of the recombinant antigens. Only a small portion of the IgG against the recombinant peptides was IgG4. The prevalence of IgG against OV16 in samples from MF-negative children was 51%, and that for MF-positive children was 75%. The general profile of the humoral immune responses mounted by both MF-positive and a large percentage of the MF-negative children during the initial phases of infection with *O. volvulus* is similar to the profile reported for adults harboring chronic *O. volvulus* infections. These results suggest that very quickly after infection, the interactions between parasite and host result in an immunological environment that may contribute to the maintenance of a long-term, chronic infection.

The filarial nematode parasite *Onchocerca volvulus* causes severe dermatological, lymphatic, and ocular pathology in a high percentage of the nearly 20 million infected individuals in Africa and Latin America (48). Much of the pathology associated with onchocerciasis takes place during the chronic stage of infection and is thought to be mediated by immune responses against the microfilariae (MF) that are found in the skin and ocular tissues (24).

The establishment of a chronic filarial infection in humans is accompanied by characteristic cell-mediated and humoral immune responses. The antifilarial humoral immune response is characterized by high levels of immunoglobulin E (IgE) (26, 27, 47), eosinophilia (33), and a parasite-specific IgG response profile that is often dominated by IgG4 (27, 28, 36). Cell-mediated immune responses of *O. volvulus*-infected individuals to both parasite-derived and nonparasite antigens are impaired (24). The degree to which the lymphocyte responses are compromised appears to increase with age (12). Although it may be reasonable to propose that the qualitative and quantitative aspects of the antifilarial response outlined above are important to the regulation of pathology, the precise pathogenesis of onchocerciasis lesions is unknown.

In areas of endemicity, most filarial infections are initiated during early childhood. It is likely that these early interactions between filariae and the human host are important in establishing the immune networks that will dictate the nature of subsequent responses against the parasite and the outcome of infection. Indeed, this is a basic premise behind many of the current strategies for the production of vaccines against filarial parasites. Therefore, it is important to understand the very early events in the ontogeny of the immune responses of children to *O. volvulus* challenge.

Another reason that the early immune responses of children are of interest is that they may be useful as biomarkers of prepatent and low-level infections. The accurate diagnosis of onchocerciasis is particularly important in areas where low levels of infection challenge the limits of sensitivity of traditional measures of parasite density. Definitive diagnosis of *O. volvulus* infection still relies on the detection of the parasite in biopsy material or by visualization of MF during slit-lamp analysis or by the identification of adult parasites in subcutaneous nodules. The numerous attempts to develop sensitive and specific tests for the detection of *O. volvulus* infections have been unsatisfactory because of the broad cross-reactivity...
among filarial and other helminth parasites. Recently, the availability of recombinant *O. volvulus* proteins has afforded an opportunity to design sensitive and specific serodiagnostic assays to detect early and low-level infections in areas of endemicity.

The goals of this study were to determine the IgG subclass profile of children undergoing the early stage of onchocerciasis and to investigate the utility of a set of recombinant proteins in the detection of prepatent and low-level infections. Serum samples were collected from a population of 224 children, aged 2 to 15 years, residing in an area of endemicity in Sierra Leone and were analyzed to determine the IgG subclass and IgE responses against an extract of *O. volvulus* adult antigens (OvAg) and a battery of *O. volvulus* recombinant antigens (OV11, OV27, OV29, and OV16). The qualitative nature of the early responses against the crude parasite extract was found to be similar to the humoral responses reported for chronic filarial infections in adult patients. The profile of the early immune responses to *O. volvulus* recombinant antigens suggested that these responses may serve as markers to identify children with low-level or prepatent infections.

**MATERIALS AND METHODS**

**Study population and clinical evaluation.** A population of 224 children, 2 to 15 years of age, residing in eastern or southern Sierra Leone participated in the study after consent was received from their parents or guardians. Clinical assessment of each child included palpation for nodules and a complete ophthalmologic examination, including a slit-lamp examination. Two skin snips, one from the iliac crest and one from the shoulder, were obtained with a 2-mm-diameter Holth punch (Storz Instruments, St. Louis, Mo.) to determine if the child had microfilaremia. Venous blood was obtained for serum, and the sample was stored and transported at −20°C. Stool and urine samples were also collected and examined for helminth parasites.

**Antigens.** Adult *O. volvulus* parasites were isolated by collagenase digestion (37) of nodules excised from patients at the Njala University College Hospital. The worms were stored at −20°C. Tissues from adult male and female *O. volvulus* worms were disrupted in phosphate-buffered saline on ice with a hand-operated tissue grinder and then spun at 4°C for 20 min at 16,000 × g. The protein concentration of the OvAg extract was determined by the bicinchoninic acid protein assay (Fierce, Rockford, Ill.) with bovine serum albumin as a standard.

*O. volvulus* recombinant antigens OV11, OV27, OV29 (3), and OV16 (29) were expressed as fusion proteins with the maltose-binding protein (MBP) by using the pMAL expression system (New England Biolabs, Beverly, Mass.). Purified MBP was obtained from New England Biolabs. *ELISA.* For the estimation of the amount of IgG and IgG subclasses, wells of polystyrene microtiter plates (Costar, Cambridge, Mass.) were sensitized by placing 50 ng of OvAg in 100 μl of 0.05 M carbonate buffer, pH 9.6, and incubating overnight at 4°C. A serial dilution of a known amount of a positive control serum was placed in a well of each plate to establish a threshold. All values were expressed in units based on extraplotations from an appropriate calibration curve.

The binding of *O. volvulus*-specific IgG was detected with a horseradish peroxidase-conjugated, Fe-specific goat anti-human IgG (Cappel, Durham, N.C.). Antigen-specific IgG was expressed in units obtained by comparing the optical density of the test serum with the optical densities of known amounts of a standard preparation of human IgG (Sigma, St. Louis, Mo.).

For the detection of IgG subclasses, the following horseradish peroxidase-conjugated subclass-specific murine monoclonal antibodies were used at a concentration of 2.5 μg/ml: anti-IgG1 (Bethyl, Montgomery, Tex.), anti-IgG2 (Bethyl), anti-IgG3 (Bethyl), and anti-IgG4 (Zymed Laboratories Ltd., San Francisco, Calif.).

*In vitro assay.* Tissue extracts of adult *O. volvulus* were run on each plate to establish a threshold. All values were expressed in units based on the extrapolations from a standard curve.

**Statistical analysis.** The statistical package SAS, version 6.07, was used to perform all analyses. All tests were two-tailed. A *P* value of <0.05 was considered to denote statistical significance. Assay results are presented as geometric means ± standard errors of the means. Analysis of variance (ANOVA) was performed to determine statistical differences between groups (38).

### RESULTS

The demographic characteristics and parasitological status of the study population of 224 children are outlined in Table 1 and have been described in detail elsewhere (14). Briefly, 53 (23.7%) children were diagnosed by the detection of MF in skin snips as being infected with *O. volvulus*. In general, the prevalence of microfilaremia increased with age from a geometric mean level of 9.8 MF per skin snip in 2- to 5-year-old children to 19.4 MF per skin snip in children 11 to 15 years of age (Table 1). Since the parasitological status of the children varied with age, the results were stratified into the age groups 2 to 5, 6 to 10, and 11 to 15 years. A total of 33 children (9 MF positive and 24 MF negative) were diagnosed as having other parasitic infections. In this subset, 22 were positive for *Acaris lumbricoides*, 15 were positive for *Ancylostoma duodenale*, 3 were positive for *Trichuris trichura*, 2 were positive for *Strongyloides stercoralis*, and 2 were positive for *Schistosoma mansoni*. Infections with these pathogens appeared to have no statistical influence on the anti- *O. volvulus* responses.

In the initial evaluation of the humoral response, we determined the IgG subclass recognition of antigens contained in a crude extract (Fig. 1). Over 88% of the children had detectable anti-OvAg responses for at least one of the IgG subclasses. Total IgG values (the sum of the four IgG subclass responses) in the 2- to 5-year-old group were significantly elevated for the MF-positive children compared with those of the MF-negative individuals (ANOVA, *P* = 0.001). This difference was diminished in the 6- to 10-year-old children, but total IgG levels were still significantly higher in the MF-positive subjects (ANOVA, *P* = 0.04). In the 11- to 15-year-old group, the overall anti-OvAg IgG response in MF-negative children was significantly elevated compared with that of the MF-positive children.

When all of the children in the two youngest age groups were considered, it was shown that the MF-positive children

<table>
<thead>
<tr>
<th>Age group (yr)</th>
<th>n</th>
<th>Prevalence of microfilaremia (%)</th>
<th>Mean no. of MF* (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–5</td>
<td>40</td>
<td>15</td>
<td>9.8 (3–18)</td>
</tr>
<tr>
<td>6–10</td>
<td>159</td>
<td>24</td>
<td>14.4 (2–45)</td>
</tr>
<tr>
<td>11–15</td>
<td>25</td>
<td>32</td>
<td>19.4 (4–42)</td>
</tr>
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</table>

* Geometric mean for number of MF per skin snip.
mounted significantly higher IgG1 ($P = 0.002$) and IgG2 ($P = 0.03$) responses compared with those of MF-negative children (Fig. 1). The IgG1 and IgG2 responses were not statistically different in the 11- to 15-year-old children. The differences between the IgG3 and IgG4 from MF-positive and MF-negative children were not significant for any of the age groups. When the results from only those children who mounted detectable antibody responses to OvAg were considered, there were no significant differences between MF-positive and MF-negative individuals for any of the IgG subclasses.

Forty percent of the MF-negative and 65% of the mf-positive children had detectable levels of anti-OvAg IgG4. In the children 2 to 5, 6 to 10, and 11 to 15 years old who were harboring infections that produced detectable levels of microfilaria, IgG4 made up on average 39, 35, and 28% of the total IgG responses, respectively. For the MF-negative individuals in the 2- to 5- and 6- to 10-year-old age groups, the mean IgG4 levels were 11 and 27% of the IgG response, respectively. The highest levels of IgG4 were observed in the 11- to 15-year-old MF-negative children, for whom IgG4 made up on average 56% of the anti-OvAg IgG response (range, 0 to 87%).

To determine the levels of antigen-specific IgE, samples were randomly selected from the group of children who were MF positive and the results were compared with those for an age-matched set of sera from children who were MF negative. An *O. volvulus*-specific IgE response was detectable in 30 of the 62 children tested (Fig. 2). The highest levels of parasite-specific IgE were detected in sera from the 6- to 10-year-old group. In the sera tested, there were no significant differences in the IgE responses between the different age groups or between the MF-positive and MF-negative patients.

When the IgE and the IgG4 levels from the same individual were analyzed, the results showed that many children mounted a parasite-specific IgE response without having an elevated IgG4 response (Fig. 3). Of the 62 children tested, only about 5% showed an elevation in both IgE and IgG4 responses.

Previous studies have identified four *O. volvulus* recombinant antigens with potential for being useful reagents for the specific diagnosis of onchocerciasis—OV11, OV27, OV29 (3), and OV16 (30). These recombinant antigens were utilized in an ELISA to determine the IgG responses in this pediatric population (Fig. 4). When the sera were tested against OV11,
OV27, or OV29 in separate assays, 43% of the MF-negative children had IgG responses against at least one of the recombinant antigens. In the MF-positive group, 88% were positive for at least one of the recombinant antigens. For the two younger age groups, the MF-negative children had significantly lower responses to OV11 and OV27 (ANOVA, \( P < 0.001 \)), but not to OV29, than did the MF-positive children. The responses of the MF-negative and MF-positive children in the oldest age group were not significantly different for any of the recombinant peptides.

In an attempt to simplify the procedure for assessing the anti-\( O. volvulus \) recombinant IgG responses, we produced a cocktail of OV11, OV27, and OV29 to use in the ELISA (Fig. 5). For the most part, the MF-negative and the MF-positive sera that were negative in the ELISA with the individual recombinant antigens were also negative in the assay with the recombinant cocktail (81% concordance) (data not shown).

Since our results from the assay with the complex OvAg indicated that the IgG4 responses in the children were significantly elevated, we also determined the levels of IgG4 with the recombinant cocktail as antigen. It appears that only a small portion of the IgG4 response is directed against the epitopes contained in OV11, OV27, and OV29 (Fig. 5).

The anti-OV16 IgG response was evaluated for 68 MF-negative and 52 MF-positive children (Fig. 6). Fifty-one percent of the MF-negative and 75% of the MF-positive children were IgG positive for OV16. In the 2- to 5-year-old group, the anti-OV16 IgG levels were significantly elevated (ANOVA, \( P = 0.001 \)) in the sera from MF-positive children compared with those from MF-negative individuals. With increasing age, there were trends toward a decrease in the anti-OV16 IgG in MF-positive children and an increase in the anti-OV16 IgG in the MF-negative children.

**DISCUSSION**

There is a continuing need to develop a sensitive and specific serological test for onchocerciasis. The conventional "gold standard" for the diagnosis of \( O. volvulus \), the detection of MF in small skin biopsies, while highly specific, is inefficient and of...
responses for the MF-negative (Fig. 6). Sera from children in the age groups 2 to 5, 6 to 10, and 11 to 15 years were tested by ELISA to determine the levels of anti-OV16 IgG. Each dot represents a single determination after the subtraction of the response to the MBP fusion partner. The data were expressed according to the OV16 index (see Materials and Methods). The open and stippled vertical bars designate mean values of the anti-OV16 IgG responses for the MF-negative (+) and MF-positive (−) children, respectively. The number within the box below each bar designates the number of individuals who had no detectable response to OV16.

limited utility for the identification of individuals with prepatent or cryptic infections (48). In general, the traditional serological tests for onchocerciasis which have relied on complex and highly cross-reactive antigen preparations have been unsuccessful in accurately detecting early and low-level infections (48). Issues of sensitivity and specificity in the diagnosis of onchocerciasis are especially important for establishing surveillance systems for the recrudescence of infection in areas where vector control and chemotherapy are used to interrupt transmission and for assessing the infection status of children living in areas of endemicity.

Recently, there have been two significant advances in the approaches used for the immunodiagnosis of filarial nematode infections. The first of these is the incorporation of recombinant parasite antigens into diagnostic schemes. Many of these recombinant molecules were identified and characterized for their ability to function in a genus- and/or species-specific fashion. Several studies have applied recombinant parasite proteins to the serological diagnosis of onchocerciasis (3, 4, 13, 15, 22, 29, 30, 31, 34, 40) and lymphatic filariasis (8, 23) and have shown that recombinant antigens can be useful reagents. Although most of these studies used samples obtained from adults, two studies (23, 34) also investigated the responses of children and found that recombinant proteins are also of potential use in the diagnosis of young individuals who are presumably in the early stages of infection.

The results of our assessment of the humoral responses to recombinant OV11, OV27, and OV29 show that 75% of the patently infected (MF-positive) individuals were identified through their IgG responses. When the IgG responses to OV16 are also considered, over 80% of the MF-positive children were antibody positive. For the MF-negative children, the IgG responses to the recombinant antigens indicated that about half harbored prepatent or cryptic infections. Although additional work and longitudinal studies are required to establish and validate the clinical relevance of these responses, it appears that a cocktail of OV11, OV27, OV29, and OV16 will be a useful reagent for the diagnosis of onchocerciasis in children.

It is important to note that 10 of the 53 MF-positive children had no detectable antibody response to any of the four recombinant antigens, whereas these same individuals had low to moderate levels of antibody against OvAg. The antibody-negative children were all from the 6- to 10-year-old age group and had microfilaremia levels that ranged from 5 to 40 MF per skin snip (mean, 17.4 MF per skin snip). These results suggest that when work is being done with pediatric populations, additional epitopes may be needed to serologically identify all infected individuals.

The second major advance in serodiagnosis, the assessment of the Ig isotype and IgG subclass responses produced by individuals infected with O. volvulus or lymphatic filariasis, has allowed for the development of more-specific assays and has provided significant insights into the regulation of the antifilarial immune responses. It has been shown in numerous studies that the levels of total and/or parasite-specific IgE are dramatically elevated in individuals in the chronic stages of onchocerciasis (39, 46, 47) or lymphatic filariasis (19, 27). Of added interest for serodiagnostics, the IgE response can be a more specific measure of infection than can the IgG response (19, 46).

Half of the MF-negative and nearly half of the MF-positive children tested had detectable levels of parasite-specific IgE (Fig. 2). This is the first report of filaria-specific IgE responses in children; thus, it is difficult to know if this elevated IgE response is typical of the early stages of onchocerciasis in all areas. The intensity of transmission, the genetics of the responding population, the history of parasitism, and many other factors could influence the IgE response. If it can be assumed that the elevated level of parasite-specific IgE is a marker for a prepatent or low-level infection in MF-negative children, then it appears that even in the very early phase of onchocerciasis, the host expresses at least some of the characteristics of the immune responses that have been associated with the more chronic stages of filariasis.

Recent studies have demonstrated that the profile of the IgG subclass response may also have important clinical correlates. Studies with either crude parasite extracts (5, 6, 16, 18, 25, 27, 28, 35, 43, 44), partially purified native proteins (5), or recombinant antigens (8, 31, 40, 42) have found that elevated levels of parasite-specific IgG4 are characteristic of the humoral responses of MF-positive individuals who have entered the chronic stage of infection. In contrast to the typical IgG4 response of 2 to 5% of the antigen-specific IgG (11), the humoral responses of individuals with filariasis are dominated by IgG4, with this subclass constituting up to 80 to 90% of the parasite-specific IgG (27).

The anti-OvAg IgG4 responses were elevated (>10% of the total antigen-specific IgG) for 40% of the MF-negative children. This result provides another piece of evidence to suggest that nearly half of the MF-negative individuals in this study were infected with O. volvulus. It is interesting that only 65% of the MF-positive children had detectable levels of anti-OvAg IgG4. This observation is in contrast to results of previous studies which have shown a strong positive correlation between IgG4 levels and MF loads in adults with onchocerciasis (6) or lymphatic filariasis (28, 44). For the subjects in this study, the mean MF load of the IgG4-positive and IgG4-negative children were not significantly different (17.4 ± 7 and 18.4 ± 5 MF per skin snip, respectively). Therefore, it seems unlikely that
the MF load had a significant influence on the level of IgG4 in this pediatric population. It may be that the lack of correlation between IgG4 and MF load is due to the relative immaturity of the immune responses and that firm associations between IgG4 and MF levels are established only later in infection. It is also possible that some of the children who were IgG4 negative and MF positive are individuals who will develop immunity to infection (12, 45). Again, a study in which children are monitored longitudinally will be required to define the clinical significance of IgG4 responses in children.

Of the studies that have focused on the humoral responses of children from areas where filarial nematode infections are endemic, most have been limited in scope because of the small number of subjects sampled (6, 7, 10, 23, 28, 35). A notable exception is a comprehensive study of the antigen-specific IgG responses of a Haitian pediatric population (n = 129) from an area of endemic *Wuchereria bancrofti* (16, 17). Employing the same age groups used in our study, they reported an age-related increase in all IgG subclasses against a complex mixture of filarial antigens. In the older age groups, IgG1, IgG2, and IgG3 levels were significantly elevated in the MF-negative children compared with those of the MF-positive individuals. In addition, the mean level of IgG4 was significantly elevated in the MF-positive 6- to 10-year-old children. This significant difference between IgG4 levels of MF-positive and MF-negative children disappeared in the 11- to 15-year-old group. In the study presented here, we did not observe the magnitude of the age-related change in IgG subclass responses reported for the Haitian population and the differences between the IgG1, IgG2, and IgG3 levels of MF-positive and MF-negative children were minimal. Similar to the Haitian study, in our study we observed a significant elevation in the mean IgG4 levels for MF-negative individuals, but unlike in the Haitian study, the elevation in IgG4 levels was seen in the MF-negative children in the 11- to 15-year-old group. The results of both studies indicate that children are capable of mounting a substantial IgG4 response to parasite antigens. It is unclear whether the differences in the results of these two studies hold any significance for how we view the early stages of immunity to filarial infections. In addition to the obvious difference in parasite species between the two studies, such unknown factors as intensity of transmission, efficiency of infection, rate of parasite development, nutritional status of the children, history of parasitism, host genetics, etc., are likely to have had a significant impact on the immune responses measured.

The IgG4 responses against the OV11-OV27-OV29 recombinant cocktail were relatively low (Fig. 2). In contrast, the humoral responses to the epitopes contained in other recombinant antigens, such as OV33 (31) or SXP-1 (8), were shown to be restricted to the IgG4 subclass. The use of carefully selected recombinant antigens that are strongly and selectively recognized by IgG4 antibodies may significantly enhance the sensitivity and specificity of serodiagnostic assays for filariasis.

Studies have shown that in adult filariasis patients the changes in the levels of filaria-specific IgE and IgG4 parallel each other closely (20, 25). The production of IgE and IgG4 has been shown to be coordinate promoted by interleukin 4 and inhibited by gamma interferon (25, 32). However, this apparent coregulation of IgE and IgG4 does not hold for all cases. Kurniawan et al. (27) reported that during chronic Brugian filariasis, MF-positive individuals had high parasite-specific IgG4 levels and low IgE levels while patients experiencing active pathology showed the reverse relationship. The importance of the coregulation of IgE and IgG4 expression in the serum is still not completely clear but may be tied to the proposed blocking activity of IgG4. There is a strong association between the levels of antigen-specific IgG4 in the serum and the ability to inhibit or enhance the efficiency of IgE-mediated effector functions both in vitro and in vivo (21). The association found by Kurniawan et al. (27) between elephantiasis and high IgE levels is consistent with the notion that the IgE/IgG4 ratio is important.

In this study, most of the children who had elevated IgE responses against parasite antigens did not have elevated IgG4 responses. Elevated IgG4 responses have been associated with repeated and long-term exposure to pathogens (2) and noninfectious agents (1). The results of this study suggest that increases in IgE levels precede increases in IgG4 levels and that these two isotypes are regulated independently. This observation may be important in setting up detection systems to monitor very early infections.

For many individuals who reside in areas of endemicity, constant exposure to the infective-stage larvae results in a persistent, decades-long infection. The initial responses to the parasite are likely to have a significant influence on subsequent reactions during infection. The results of this study show that even before MF can be detected, the host’s immune response is skewed toward the production of high levels of *O. volvulus*-specific IgG4 and IgE. Studies that monitor the fate of children with high and low IgG4 responses may offer significant insight into the pathobiology of filarial nematode infections.

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