

Humoral responses to a secretory *Onchocerca volvulus* protein: differences in the pattern of antibody isotypes to recombinant Ov20/OvS1 in generalized and hyperreactive onchocerciasis

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SUMMARY

The *Onchocerca volvulus* secretory protein Ov20/OvS1 represents a dominant antigen expressed in the infective larvae, microfilariae and adult stages of the parasite. The humoral responses to this protein have not yet been analysed in the polar clinical and immunological forms of onchocerciasis. Analysis by ELISA of class and subclass antibodies to Ov20/OvS1 in persons with the generalized or the hyperreactive form of onchocerciasis revealed similar strong responses of IgG1, IgG4 and IgM antibody levels in both forms of onchocerciasis and significant differences were observed in the IgE and IgA antibody classes. Computation of the ratios of antibodies showed that persons with the generalized form exhibited significantly higher ratios of IgG4 to IgG1, IgG4 to IgE, and IgM to IgE than patients with the hyperreactive form. To investigate the isotype recognition of antigenic sites on Ov20/OvS1 protein, three recombinantly expressed fragments (F1–3) of Ov20/OvS1 were probed using sera which strongly reacted with intact recombinant Ov20/OvS1. Epitope(s) on F1 comprising amino acid residues 1–63 were significantly recognized by IgG1 and IgE, while IgM recognized epitopes on all three fragments. The strongest reaction of IgM occurred with epitope(s) formed by residues 108–171 (F3). In contrast, IgG4 type antibodies were not reactive with either of the three OvS1 fragments, but they reacted with intact Ov20/OvS1 protein. Generalized onchocerciasis, unable to eliminate microfilariae, and hyperreactive onchocerciasis, with a high potency to eliminate or to reduce parasite loads, can be distinguished by a distinct pattern of isotype responses to Ov20/OvS1.

Keywords onchocerciasis, recombinant Ov20/OvS1 protein, humoral immune response

INTRODUCTION

Onchocerciasis, a disease due to infection with the filarial worm *Onchocerca volvulus*, is an important cause of blindness and dermatitis affecting approximately 18 million residents of Africa and Latin America (WHO 1995). A characteristic trait of infections with *O. volvulus* is the wide spectrum of ensuing pathological events. Thus, in patients with comparable exposure to the infective larvae, a high proportion may develop a generalized form of disease. This form is characterized by a low degree of inflammatory processes in the skin, and it can be associated with high densities of microfilariae (mf). In contrast, a small proportion of patients, exhibiting a hyperreactive (sowda) form of onchocerciasis, suffer from severe skin reactions that are usually confined to distinct areas of the body (Anderson *et al.* 1973, Büttner *et al.* 1982). These patients usually show very low densities of mf, a phenomenon thought to result from an active downregulation of parasite loads by the host (Büttner & Racz 1983).

As reviewed by Ottesen (1995), high antibody responses characterize *O. volvulus* infections. Differences in antibody isotypes elaborated among generalized and hyperreactive persons have been reported (Büttner *et al.* 1982, Brattig *et al.* 1994). In helminth infections other than onchocerciasis, the relative amounts of antibody isotypes have been implicated in determining extent of host's pathology as well as survival of the parasites (Grzych *et al.* 1984, Hussain & Ottesen 1986, Khalife *et al.* 1986). Inhibition experiments have shown that IgE, IgG1 and possibly IgG3 antibodies to helminth antigens can, via Fc receptors on granulocytes

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participating in the immune response, mediate a cell-dependent parasite killing (Haque *et al.* 1981, Mehta *et al.* 1982, Khalife *et al.* 1989). On the other hand, IgG4 and IgM antibodies in experiments with schistosomiasis larvae have been demonstrated to prevent effector cell attachment to the parasite therefore being beneficial for parasite's survival (Dunne *et al.* 1987, Hagan *et al.* 1991). In onchocerciasis, a negative correlation of IgG1 and IgG3 antibodies with the parasite load has been discussed as suggestive of a role of these antibodies in the downregulation of mf loads (Dafa'alla *et al.* 1992). On the other hand, a possible enhancement role for the mf survival by IgG4 and IgM antibodies has been suggested (Ndiaye *et al.* 1987; Dafa'alla *et al.* 1992). In addition, IgG4 has been implicated in the modulation of IgE mediated hypersensitivity reactions in filarial infections (Hussain & Ottesen 1986).

Ov20/OvS1 is one of the most extensively studied onchocercal antigens (Bradley *et al.* 1991, Erttmann *et al.* 1995, Tree *et al.* 1995, Jenkins *et al.* 1996). It is a small protein with a molecular mass of approximately 20 kDa, therefore mostly referred to as Ov20 (Tree *et al.* 1995). Its secretion into the host tissue and its high affinity in binding retinol and fatty acids has led to the suggestion that Ov20/OvS1 may facilitate the derivation of nutrients and the production of host modulating molecules by the parasite (Jenkins *et al.* 1996, Kennedy *et al.* 1997, Mpagi *et al.* 2000). The immune responses to Ov20/OvS1 have been studied with regard to immunodiagnosis and protection in *O. volvulus* infection (Bradley *et al.* 1991, 1993, 1995, Erttmann *et al.* 1995, Jenkins *et al.* 1996). Extending in part the work of Bradley *et al.* (1995), the present report investigates the pattern of antibody isotypes elicited by Ov20/OvS1 protein among persons with generalized and hyperreactive onchocerciasis.

MATERIALS AND METHODS

Study population

Sera were obtained during the period 1984–85 from populations residing in areas endemic for onchocerciasis in Liberia, West Africa. These populations have been described in detail elsewhere (Albiez *et al.* 1985, Brattig *et al.* 1987). Because the hyperreactive type of onchocerciasis in West Africa is more frequent in females (Albiez *et al.* 1985), in this study, females aged between 10 and 30 years were considered. Twenty sera were obtained from patients with a generalized form of onchocerciasis having a median mf density of 25.4 mf/mg skin, while another 20 sera were from patients with severe hyperreactive onchocerciasis with a median mf density of 0 mf/mg. The ages of both groups were comparable as indicated by *U*-test ($P = 0.185$).

Production of recombinant Ov20/OvS1 protein and fragments

DNA encoding for Ov20/OvS1 was amplified by polymerase chain reaction (PCR), and the protein was expressed and purified as described previously (Erttmann *et al.* 1995). To obtain the three overlapping peptide fragments of Ov20/OvS1 (F1, F2, and F3), PCR was carried out using cDNA as template with synthetic oligonucleotide primers such that F1 contained the amino acid residues 1–63, F2 residues 45–127, and F3 residues 108–171 of the entire open reading frame of Ov20/OvS1 protein. Expression and purification of the fragments was done as with whole Ov20/OvS1.

Measurement of Ov20/OvS1-specific antibodies

Semi-quantitative analysis of antibody levels to the recombinant Ov20/OvS1 protein was performed using ELISA at serum dilutions found to be at optimum. Briefly, recombinantly purified Ov20/OvS1 was used to coat flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C in carbonate buffer pH 9.6. Optimal concentrations were found to be 5 µg/ml for IgE and 0.5 µg/ml for IgG subclasses, IgA and IgM. The plates were washed three times with PBS containing 0.05% Tween, nonspecific binding sites blocked by 5% bovine serum albumin for 90 min and the plates washed as before.

Patients' sera were added at dilutions of 1:800 (for IgG1), 1:400 (IgG3 and IgG4) and 1:1600 (IgM) in 0.05% BSA/PBS and incubated for 90 min at 37°C. Sera added at dilutions of 1:10 (for IgE) and 1:400 (IgA) were incubated overnight at 4°C. After washing, class- and subclass-specific monoclonal mouse antihuman immunoglobulin antibodies (HP6070A for IgG1 and HP6025 for IgG4, both used at 1:1000, HP6050 for IgG3, clone CIA-E-7-12 for IgE and clone 6E2C1 for IgA, all three diluted 1:500, clone R1/69 for IgM diluted 1:1600 in 0.05% BSA/PBS) were added for 90 min at 37°C. The IgG subclass antibodies were obtained from Calbiochem (La Jolla, CA, USA) while the other antibodies were from DAKO (Glostrup, Denmark). Plates were washed, then alkaline phosphatase-conjugated goat antimouse IgG (Dianova, Hamburg, Germany) was added at a dilution of 1:5000 in 0.05% BSA/PBS and the reaction incubated for 45 min at 37°C. The plates were again washed and developed using *p*-nitrophenyl phosphate (Sigma, Deisenhofen, Germany). On each plate, a reference pool comprised of five strongly reacting sera from onchocerciasis patients was used to construct a standard titration curve to which all individual sera were compared according to Kemeny (1992). Also included was serum pooled from five Africans without filarial infections used as negative control. Data are expressed as arbitrary units (U/ml) derived from the standard curve.

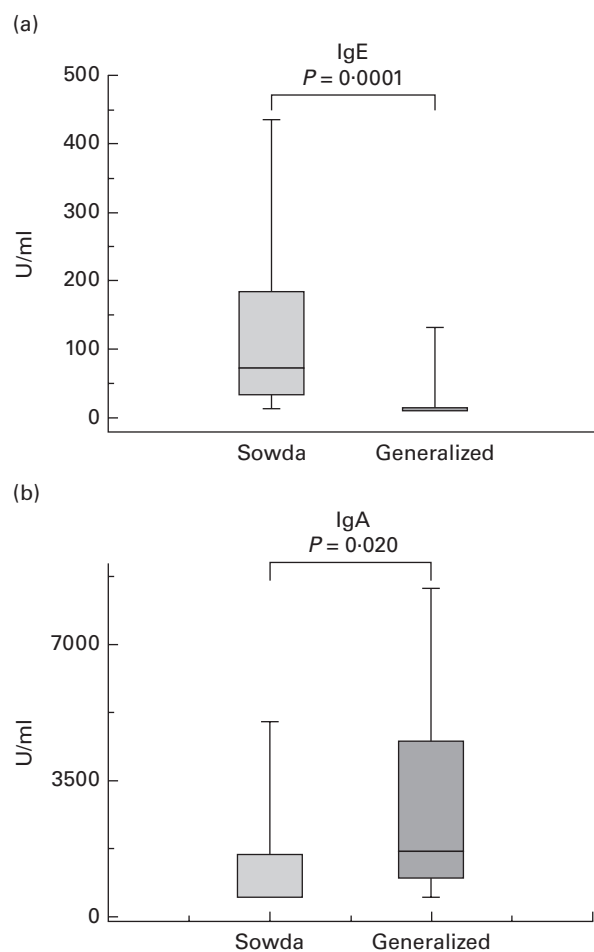


Figure 1 Serum levels of antibody classes (a) IgE (U/ml) (b) IgA (U/ml) in individuals with generalized ($n = 20$) and hyperreactive (sowda) form ($n = 20$) of *O. volvulus* infection.

Sera were probed for IgE without removing IgG antibodies, because in previous experiments no significant differences in IgE units using IgG-depleted and IgG-non depleted sera could be observed in agreement with the observations of Bradley *et al.* (1995). All sera were tested in duplicate. Because IgG antibodies tend to interfere with the binding of IgM (Kemeny 1992), the optimum dilution of sera for testing IgM antibodies was chosen such that IgG subclasses present were at minimum levels.

Measurement of IgG1, IgG4, IGE and IgM antibody reactions to fragments of Ov20/OvS1

ELISA was also used to measure the antibody responses to the recombinant peptide fragments of Ov20/OvS1. The three peptide fragments were used at $0.7 \mu\text{g/ml}$, except at $5 \mu\text{g/ml}$ for IgE antibody, to coat microtiter plates. The

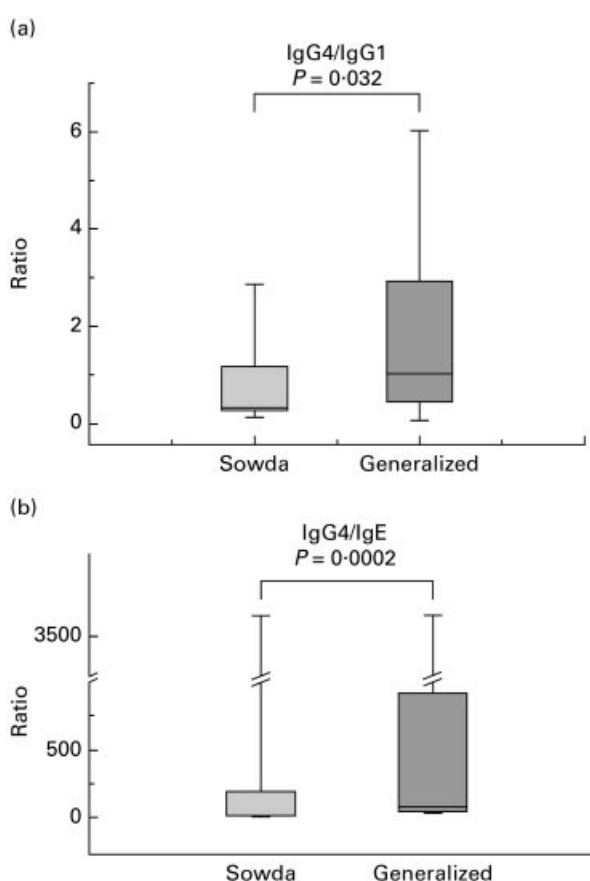


Figure 2 Antibody ratios of (a) IgG4/IgG1 (b) IgG4/IgE in individuals with generalized ($n = 20$) and hyperreactive (sowda) form ($n = 20$) of *O. volvulus* infection.

assays proceeded as described above. Data are expressed as optical densities (OD).

Statistical analysis

The Mann–Whitney *U*-test was used to compare the ages of patients with generalized and those with hyperreactive onchocerciasis and the ratios of antibody isotypes in the respective sera. The Kruskal–Wallis test was used to compare the reactivity of the immunoglobulins of 15 sera to each of the three peptide fragments of Ov20/OvS1 protein.

RESULTS

Classes and subclasses of antibody isotypes against recombinant Ov20/OvS1

The levels of Ov20/OvS1-specific IgG1, IgG3, IgG4, IgE, IgM and IgA antibodies were measured in sera of 40 individuals with onchocerciasis by ELISA and expressed

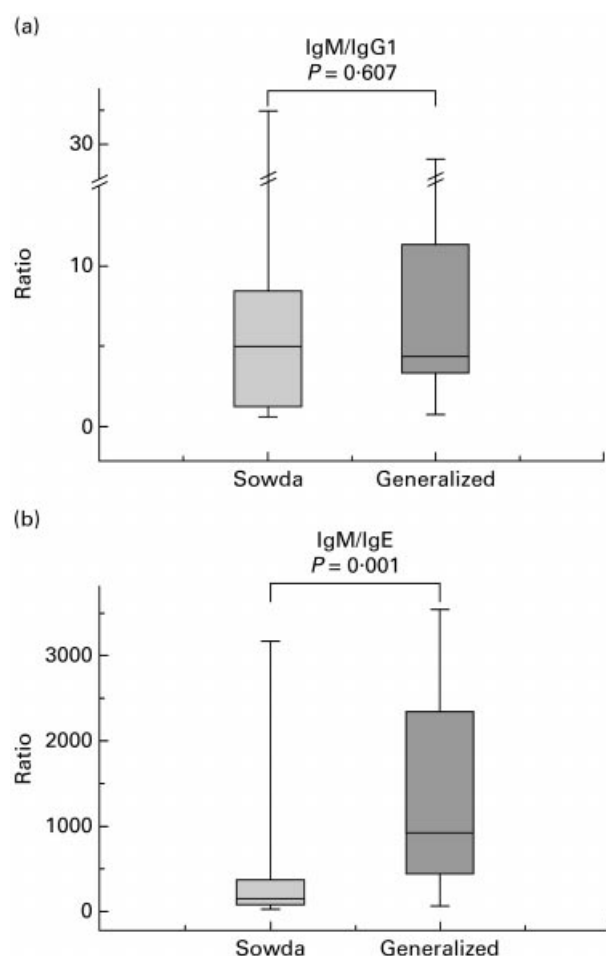


Figure 3 Antibody ratios of (a) IgM/IgG1 (b) IgM/IgE in persons with generalized ($n = 20$) and hyperreactive (sowda) form ($n = 20$) of *O. volvulus* infection.

using arbitrary units (U/ml). High levels of anti-Ov20/OvS1 antibodies were stated for IgG1, IgG4 and IgM. Significant differences between generalized and hyperreactive form of onchocerciasis were only observed for the antibody classes

IgE ($P = 0.0001$) and IgA ($P = 0.020$) (Figure 1a,b). The ratios of immunoglobulin isotype levels were computed and the results are presented in Figures 2 and 3. Individuals with a generalized form of onchocerciasis exhibited higher ratios of IgG4 to IgG1 ($P = 0.032$), IgG4 to IgE ($P = 0.0002$) and IgM to IgE ($P = 0.001$) than individuals with hyperreactive onchocerciasis. No differences between both polar forms of onchocerciasis were observed with respect to the ratios IgM to IgG1, IgG4 to IgG3 and IgM to IgG3 ($P = 0.607$; 0.804 ; 0.188 , respectively).

Class and subclass antibody responses to recombinant fragments of Ov20/OvS1

In order to examine whether patients' antibodies reactive with intact Ov20/OvS1 recognize sequential and/or conformational epitopes and to establish whether the observed isotype pattern also occurs towards similar amino acid sequences, three fragments with overlapping amino acid sequences were expressed and analysed using 15 sera with a known strong reaction to the intact Ov20/OvS1 protein. The fragments F1–3 comprised amino acid residues 1–63 (F1), residues 45–127 (F2) and residues 108–171 (F3) of Ov20/OvS1 protein. As shown in Table 1, F1 was predominantly recognized by IgG1 and IgE isotypes ($P = 0.005$; $P = 0.0001$). In contrast, patients' IgM recognized epitopes on all three fragments, in particular those on F3 ($P = 0.006$). IgG4 antibodies recognized none of the three fragments ($P = 0.650$) as demonstrated by the negligible optical density signals obtained (<10% of the signals of the intact molecule).

DISCUSSION

The onchocercal Ov20/OvS1 antigen, present in the infective larvae, microfilariae and adult stages of *O. volvulus*, is thought to be synthesized in the hypodermal tissue of the parasite. It is secreted by translocation across the cuticle to

Table 1 Statistical analysis of the reactivity of the 15 sera with each of the three peptide fragments of Ov20/OvS1. The optical densities for the intact Ov20/OvS1 molecule are also indicated

Antibody	Optical densities (OD _{405 nm})				Order of reactivity	P-value
	Ov20/OvS1 Mean $\pm$ SD	F1 Mean $\pm$ SD	F2 Mean $\pm$ SD	F3 Mean $\pm$ SD		
IgE	0.256 $\pm$ 0.168	0.128 $\pm$ 0.139	0.006 $\pm$ 0.005	0.021 $\pm$ 0.011	F1 > F3 > F2	0.0001*
IgG1	0.919 $\pm$ 0.558	0.455 $\pm$ 0.383	0.141 $\pm$ 0.097	0.194 $\pm$ 0.126	F1 > F3 = F2	0.005*
IgM	0.510 $\pm$ 0.118	0.318 $\pm$ 0.105	0.245 $\pm$ 0.101	0.374 $\pm$ 0.142	F3 > F1 > F2	0.006*
IgG4	1.496 $\pm$ 0.811	0.082 $\pm$ 0.067	0.067 $\pm$ 0.077	0.142 $\pm$ 0.229	F1 = F2 = F3	0.650

* Significant at 5% level (Kruskal–Wallis and Mann–Whitney *U*-test)

the surface of the worm and into the surrounding tissue of the host (Tree *et al.* 1995, Jenkins *et al.* 1996). This suggests favourable exposure of the antigen to the host immune system. The Ov20/OvS1 antigen can, through elicitation of IL-10, influence the Th1/Th2 balance in persons with onchocerciasis (Bradley *et al.* 1995).

Our present report demonstrated differences in the pattern of antibody recognition of Ov20/OvS1 protein indicating in the polar forms of onchocerciasis variations in the elicitation of B-cell responses by the parasite. In both groups, Ov20/OvS1 elicited pronounced IgG1, IgG4 and IgM, and this is in agreement with a previous study of IgG1 and IgG4 isotypes (Bradley *et al.* 1995). While higher levels of IgA could be documented in generalized onchocerciasis compared to hyperreactive onchocerciasis, IgE was significantly higher in the hyperreactive form. Significantly higher ratios were stated for IgG4 to IgG1, IgG4 to IgE and IgM to IgE in generalized onchocerciasis. This shows that the increase of IgG4 in relation to an increase of IgG1 and IgE as well as the increase of IgM in relation to an increase of IgE antibody isotypes are more pronounced in generalized onchocerciasis. This finding is in accordance with the larger numbers of IgG4-producing plasma cells in the nodules from patients with generalized onchocerciasis compared to those with the hyperreactive form (Büttner, unpublished data). A continuous synthesis of IgM, an antibody predominating in the early humoral responses to parasite infections, has been reported in *Loa loa* infection (Goussard *et al.* 1984).

Data on the fragments suggest that IgG1, IgE and IgM antibody isotypes recognize sequential epitopes on Ov20/OvS1. Fragment F1, comprising amino acid residues 1–63, possesses antigenic sites inducing IgG1 and IgE antibody isotypes. The location of antigenic site(s) for IgE and IgG1 formed by the first 63 amino acids of Ov20/OvS1 peptide strand may be important for a prospective vaccine development because this region is more highly conserved among filarial species than is the carboxyl-terminal end of the molecule (Limberger & McReynolds 1990). IgM recognized antigenic sites on all three peptide fragments, the epitope(s) on F3 inducing the strongest signals, suggesting a high potency of Ov20/OvS1 in eliciting the secretion of IgM antibodies. This finding may also be indicative of a possible interference of IgM in the binding of other antibody isotypes to the Ov20/OvS1.

Since IgG4 did not react with any of the fragments of Ov20/OvS1 protein but with the intact recombinant molecule, this observation suggests that the conformational epitopes formed in the intact Ov20/OvS1 molecule, which appear to be necessary for the recognition of IgG4 antibodies, are lacking on the fragments. The absence of differences in the levels of IgE antibodies between complete sera and IgG-depleted sera may indicate lack of interference

between IgE and IgG4 binding to OvS1. The results revealed with Ov20/OvS1 fragments are in accordance with this interpretation. However, studies in lymphatic filariasis show that IgG4 modulates IgE-mediated hypersensitivity reactions such that the high ratios of IgG4 to IgE have been suggested to be a critical factor in the prevention of severe pathology in filarial infections (Hussain & Ottesen 1986). These findings parallel our results of low ratios of IgG4 to IgE in onchocerciasis individuals with severe pathology.

In conclusion, the predominance of IgG1 and IgE compared to IgG4 in hyperreactive, mf eliminating onchocerciasis is suggestive of an important role of IgG1 and IgE in the immune response to mf.

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