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Full Length Research Paper

Molecular cloning and differential IgG responses to a histidine-rich antigen (OvL3.C1) of *Onchocerca volvulus* by selected residents of onchocerciasis endemic regions in Cameroon and Ecuador

Alfred N. Amambua*, Alain N. S. Newo, Clotilde S. Sirri, Raymond K Yengo, and Vincent P.K. Titanji

Biotechnology Unit, Faculty of Science, University of Buea, Box 63, Buea, Cameroon.

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In order to further investigate host-parasite interactions in onchocerciasis, a major *Onchocerca volvulus* histidine rich antigen termed OvL3.C1 was isolated from an *O. volvulus* cDNA library using antibodies from putatively immune subjects living in onchocerciasis endemic communities in Cameroon. Analysis of its sequences predicted the protein to be helix-rich with a single transmembrane region. Recombinant OvL3.C1 antigen induced from pBAD-TOPO/Thio vector in *Escherichia coli* was purified as inclusion bodies and further by a combination of Ni^{2+} chelate chromatography and electro-elution. Anti-OvL3.C1 immunoglobulin G (IgG) subclass levels were assessed by ELISA in 15 pairs and 18 pairs of selected and cross-matched infected and putatively immune subjects from Cameroon and Ecuador, respectively. IgG3 and IgG4 levels were shown to be significantly higher in putatively immune (immune protected) subjects. A higher IgG3 level in endemic normal subjects is implicated in parasite killing and the development of the putative immune status while IgG4 has been shown to block onchocercal pathology. OvL3.C1 is a dominant antigen in onchocerciasis which elicits strong responses in subjects expose to both African and South American forms of onchocerciasis. It is therefore an important player in mechanisms of resistance or allergy attenuation in onchocerciasis.

Key words: Onchocerciasis, immunoglobulin G, putative immunity,

INTRODUCTION

Onchocerciasis has adverse impact on the health and quality of life of the infected population. The commonly used control strategies involving vector control and chemotherapy have limitations; they are prolonged, expensive and associated with numerous relapses (Molyneaux and Davies, 1997). Thus complementary control strategies involving the use of vaccines are needed. However, the development of a vaccine against helminthic parasites like *Onchocerca volvulus* is hampered by the complexity of antigenic profiles and the parasites' ability to evade or suppress the immune system (Sher, 1988).

Immunoepidemiological data collected over the past 20 years provide compelling evidence that humans can acquire protective immunity against *O. volvulus* infection (Selkirk et al., 1992; Abraham et al., 2002). However, many gaps remain in our understanding of the relationships linking this acquired resistance to *O. volvulus* and antigens potentially of use as vaccines. Antibody and cellular responses of selected and cross

*Corresponding auhor. E-mail: alfngwa@yahoo.com. Tel: (237) 769 89 75. Fax: (237) 332 22 72.

matched infected and endemic normal subjects have been reported in several endemic regions and in Cameroon to a potentially immunoprotective antigen Ov47 (Ward et al., 1988; Elson et al., 1994; Ghogomu et al., 2002; Titanji et al., 2002). To further identify antigens involve in protective immunity against *O. volvulus*, sera from resistant individuals living in endemic areas (putatively immune subjects) were used to screen a cDNA library of the infective stage of the parasite. Here we report on the identification, cloning and serological characterisation of a recombinant antigen termed OvL3.C1 (Genbank accession number, AJ272105) as an additional dominant marker of immune mechanisms in human onchocerciasis.

MATERIALS AND METHODS

Subjects and sera

Sera from Cameroon and Ecuador were used. The study area in Cameroon was the onchocerciasis endemic area of Mbonge that had been previously described (Ghogomu et al., 2002; Titanji et al., 2002). The study area and subjects from Ecuador have been extensively described by Elson et al. (1994). Subjects included in the study were either putatively immune to or infected with O. *volvulus*. Putatively immune subjects from Cameroon were endemic residents who showed neither skin microfilaria nor onchocercal stigmata after a longitudinal survey of 1300 inhabitants over 36 months. They also must have lived in the endemic zone for at least 10 years. Infected subjects were those who had all signs of onchocercal pathology and skin microfilariae. Sera from 15 putatively immune subjects and their age/sex cross matched onchocerciasis infected pairs were selected, pooled separately and employed for comparative immunoscreening of *O. volvulus* cDNA library. Before use, fifty microlitres of each pool was thoroughly mixed and depleted of *Escherichia coli* antibodies using *E. coli* total cell lysate. Briefly, 2.5% glutaraldehyde solution was added dropwise to 5 mg of *E. coli* cleared lysate to form an immunosorbent gel. Pooled sera were diluted 1:10 in phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA) and mixed with the antigen gel. Mixtures were rocked gently for 1 h and centrifuged. Supernatants were collected and repeatedly depleted of *E. coli* antibodies using fresh immunosorbent until anti-*E. coli* antibodies were undetectable by ELISA.

From a set of 43 onchocerciasis infected and 40 putatively immune sera from Esmeralda Province in Ecuador that were donated by Prof. Jan Bradley (Nottingham University), 18 pairs of age and sex cross-matched infected and putatively immune subjects were selected for comparison with a similar class of subjects from Cameroon.

Screening of *O. volvulus* infective stage (L3) cDNA library

An expression library constructed in Lambda unizap using mRNA from infective larvae (L3) of *O. volvulus* was kindly donated by Prof Steven Williams (Smiths College Massachusetts, USA). The library had a titre of 10^8 to 10^{11} plaque forming units/ml. Screening was carried out using the Clontech kit following manufacturers instructions. After probing of blocked nitrocellulose filters with absorbed antisera, unbound antibodies were washed off with three changes of PBS containing 0.05% Tween-20. Filters were then reacted with rabbit-antihuman IgG followed by biotinylated goat antirabbit IgG (Sigma). Plaques with bound antibodies were detected using avidin-biotinylated horse raddish peroxidase and its substrate solution, 4-chloro-1-naphtol. Recombinant pBluescript-SK (+) was excised and extracted from plaques by IgG from putatively immune serum pool and sent for custom sequencing (MWG-Biotech, Germany).

Preparation of expression construct

Primers specific to the open reading frame of OvL3.C1 and *Taq* DNA polymerise were used to amplify the gene from a pBluescript SK (+) vector by PCR. The Primers were 5' primer: 5'-ATGCAGATGCAGATGGTTGT-3' and 3' primer: 5'-GAATTTTCCCAGCACGTGCT-3'. The PCR was carried out on a Gradient Master Cycler (Eppendorf) programmed as follows: pre-denaturation at 94°C for 4 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, elongation at 72°C for 2 min, with a final elongation at 72°C until 10 min and incubation at 4°C until stop. PCR products were analysed on a 1.5% agarose gel ran at 70 V for 1 h and viewed briefly on a UV transilluminator.

OvL3.C1 amplicons were cut out and purified from agarose gel using Sephaglass band prep Kit (Pharmacia). These were sub-cloned into the pBAD-TOPO-THIO vector (Invitrogen) in a reaction mixture containing 3 µL of fresh PCR product, 1 µL of 100 mM NaCl solution, 1 µL of sterile water and 1 µL of TOPO vector. Following 5 min of incubation on ice, 2 µL of cloning reaction were used directly to transform competent Top10 cells (Invitrogen). Recombinants were selected by colony PCR using OvL3.C1 specific primers as specified above.

Expression and purification of OvL3.C1

Prior to preparative purification, 20 mL cultures were set up from single isolated recombinant colonies and grown to saturation overnight. Sub-cultures of the overnight cultures were grown to an optical density of 0.6 at 600 nm before induction for 5 h with 0.02% arabinose. Following analysis on SDS-PAGE, positive cultures were processed for purification of recombinant proteins by metal-chelate chromatography on Ni^{2+}-sepharose columns (Pharmacia) and then by electro-elution.

Proteins from over expressed cultures were prepurified as inclusion bodies prior to electro-elution according to the protocol described in Sambrook et al. (1989). Following cell lysis, treatment of lysate with 1 µg/mL of Dnase and Rnase each and then centrifugation, the pellet (inclusion bodies) was washed thrice in PBS (supplemented with 20 mM $MgCl_2$, 25% sucrose, 5 mM EDTA, 1% Trixton-X-100). It was then suspended in 1 mL of 50 mM Tris-HCl, pH 8 containing 5 M guanidinium hydrochloride, and briefly vortexed vigorously to facilitate the solubilization of the aggregated proteins. The resultant suspension was incubated on ice for 1 h, centrifuged, added to renaturation buffer (50 mM Tris-HCl, pH 8, 20% glycerol, 1 mM DTT), and stirred gently at 4°C overnight to renature the proteins. The following day, the supernatant was clarified by centrifugation at 12,000 rpm for 30 min at 4°C, analysed by SDS-PAGE and stored at –20°C until further purification by electroelution as described in Sambrook et al. (1989).

Sequence analysis

Computer analyses of the DNA and protein sequences were done using bioinformatics softwares available on the World Wide Web. The amino acid and DNA sequences of the antigen were used to query the nucleic acid databank of nematodes in Wormbase, the general nucleic acid databank of the National Center for Biotechnological Information (NCBI) and European Molecular Biology Laboratory (EMBL) web sites (Wheeler et al., 2004). Following translated blast (tBlastx) based on the Blossum62 algorithm, sequences showing more than 30% homology to the amino acid and nucleic acid sequences of the clone were selected as putative homologues. Primary structures were compared by

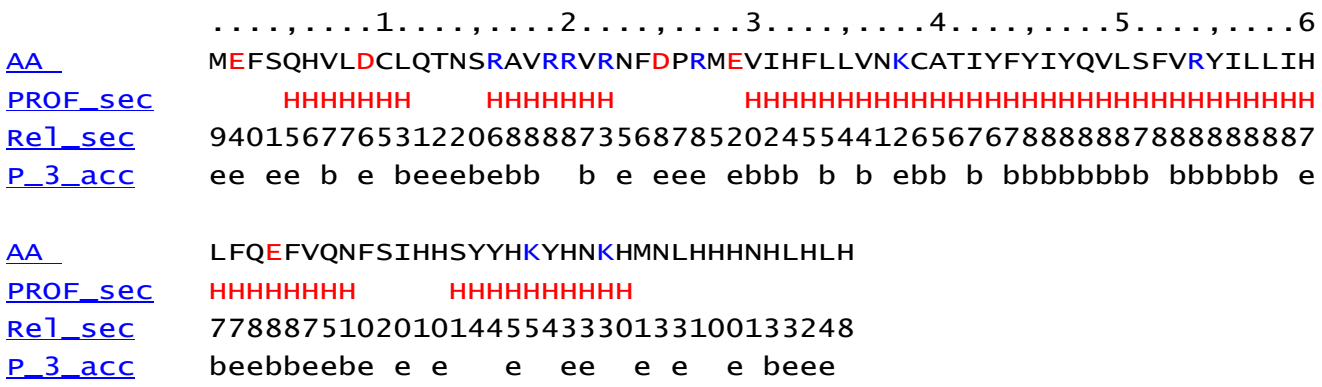

Figure 1. Predicted primary and secondary structures of OvL3.C1. AA: amino acid sequence. PROF_sec: PROF predicted secondary structure: H=helix, E=extended (sheet), blank=other (loop). PROF = Profile network prediction heidelberg. Rel_sec: reliability index for profsec prediction (0=low to 9=high). P_3: PROF predicted relative solvent accessibility (acc) in 3 states: b = 0-9%, i = 9-36%, e = 36-100%.

multiple sequence alignments using ClustalW. Conserved regions within homologues were used to pick out putative functional homologues and analysed by querying the Expasy and Swissprot databases.

Western blot analysis

For SDS-PAGE, *O. volvulus, O. gutturosa, O. ochengi* adult and *O. volvulus* microfilariae antigens were solubilised in sample buffer as described by Lucius et al. (1988). Proteins were separated on a 12.5% uniform SDS-polyacrylamide gel. Western blot procedures were essentially as described by Towbin et al. (1979).

To identify OvL3.C1 parent proteins, anti-OvL3.C1 antibodies were plaque purified and used to probe blots. Briefly, 10 mM impregnated nitrocellulose filters overlaid on XL-1 Blue OvL3.C1 plaques were lifted, washed in Tris buffered saline (TBS) and blocked with 0.2% BSA. They were then incubated overnight at 4°C with putatively immune serum pool previously depleted of *E. coli* antibodies as described above. Bound anti-OvL3.C1 antibodies were eluted using 3 mL of glycine-HCl at pH 2.5 and neutralized with 1 M Tris containing 1 mg/mL BSA. These antibodies were used to probe *Onchocerca* protein blots in standard immunoassay. Immune complexes were detected using 0.25% (w/v) diaminobenzidene (DAB) peroxidase substrate. Development reactions were stopped by washing strips abundantly in distilled water. All blots were stored in the dark prior to photography or scanning for permanent record.

Enzyme-linked immunosorbent assays

Standard micro well enzyme linked immunosorbent assay (ELISA) was performed using purified recombinant proteins and total *O. volvulus* adult worm antigens (Ghogomu et al., 2002). Optimal eagent dilutions and incubation times were determined by chequer-board titration. Indirect ELISA was used to separately determine parasite specific IgE, IgG1, IgG2, IgG3 and IgG4 antibodies present in individual patient, putatively immune and European control sera. Often 50 µL of 5 µg/ml recombinant protein or 2 µg/ml of crude proteins in 50 mM carbonate buffer were added to each well of a 96 well flat bottom Immulon microtitre plate (Dynex Technologies) and incubated overnight at 4°C and then for 1 h at 37°C with 5% non-fat milk in PBST (NFM) to block unbound sites. They were probed with sera diluted 1:100 for the detection of IgG subtypes and then incubated overnight at 4°C. Secondary antibodies were mouse anti-human monoclonal IgGs (Sigma). The conjugate was HRP-goat-antimouse immunoglobulins (Biorad) (1:5000) and development was with o-phenylene diamine (Sigma) in citrate buffer containing 0.02% hydrogen peroxide by incubating for 30 min in the dark. Reactions were stopped with 25 µL of 3M HCl and optical densities read at 495 nm (Denly-well-scan, Dynatech).

Statistical analysis

Statistical analysis of antibody levels between putatively immune and infected subjetcts, as reflected by their optical densities obtained in ELISA, were done using the Microsoft Excel package and the Number cruncher comprehensive statistic software (NCSS) including the power analysis and sample size software for NCSS (PASS). Group means, medians and standard deviations of optical densities were analysed by T-tests. Non-parametric analyses of two samples included the use of Mann-Whitney U or Wilcoxon Rank-Sum tests for difference in Medians and Kolmogorov-Smirnov Test for different distributions. Decisions on significance of differences between two groups were at 95% confidence interval ($p<0.05$) and considered when this agreed for both tests utilized.

RESULTS

Cloning and expression of OvL3.C1 genes in pbad/Thio TOPO vector

Using specific primers, the 288 bp open reading frames of OvL3.C1 was amplified from their lambda-zap isolates and from pbl subclone (Figure 3). Induction of recombinant protein from TOPO-pBAD-thio vector in Top10 *E. coli* bacteria (invitrogen) was strongly controlled and the 28 kda OvL3.C1-thioredoxine His-tagged fusion protein could only be visible after induction with 0.02% of arabinose (Figure 4a). OvL3.C1 fusion protein formed inclusion bodies upon optimum expression and remained insoluble in 100 mm phosphate buffer at pH 7.4. Proteins could be solubilised by lysing cells with phosphate buffer supplemented with 6 M urea or 4 M guanidine hydrochloride, 2% Trixton-x-100, 2% Tween-20, 10 mM

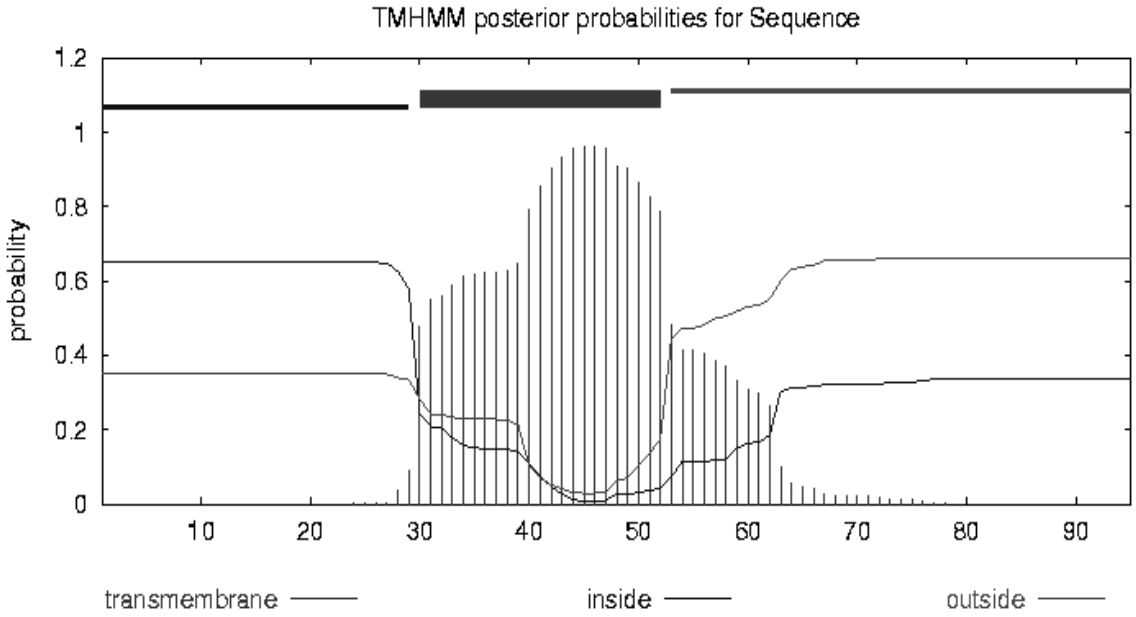

Figure 2. Transmembrane helix prediction for OvL3.C1. Plot of probability of location of secondary folds and trans membrane helix determination.

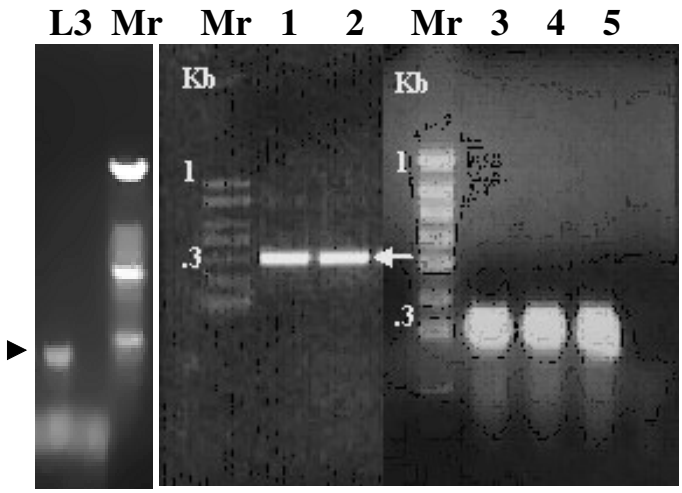

Figure 3. Subcloning of OvL3.C1. Agarose gel electrophoregrams of OvL3.C1-specific PCR on *O. volvulus* L3 cDNA (L3), pBL-OvL3.C1 (1, 2); OvL3.C1 colony PCR on OvL3-Topo-pbad/Thio Top10 *E. coli* transformants (3 to 5).

mercaptoethanol and 20% glycerol. OvL3.C1 bound onto Ni^{2+}-sepharose column eluted at 2 M imidazole with a purity of about 75%. Inclusion bodies were completely soluble only in 8 M urea or 4 M guanidine-HCl (Figure 4b). Highly homogeneous protein (>95%) could be purified by electroelution of inclusion body preparations separated by SDS-PAGE (Figure 4c).

Characterisation of native OvL3.C1 antigens

Blots of *Onchocerca* worm antigens extracted with buffer containing 2-mercaptoethanol and SDS revealed bands of varying sizes upon reaction with plaque purified OvL3.C1-specific antibodies (Figure 5). *O. ochengi* adult females, *O. volvulus* adult females and microfilariae showed reactive bands of 46000 and 94000 while there was a strong reaction with a 32000 band from *O. gutturosa* protein.

Sequence analysis

The OvL3.C1 isolated cDNA had six open reading frames (ORFs). The longest complete ORF translates for a 95 amino acid long histidine-rich protein. This frame is made of 14.7% histidine mainly from residue 72 to 95 at the C-terminus of the protein. Blast analysis of the OvL3.C1 sequence revealed 54% homology to a hypothetical protein from *Plasmodium yoelii yoelii* and 56% homology to a mouse guanine insertion enzyme, tRNA guanine transglycosylase (TGUT).

Secondary structure predictions of the deduced aminoacid sequence using PROF and PHD predictive algorithms revealed an 'all-alpha' molecule with 66.32% alpha helices and 33.68% loops (Figure 1). A continuous helical stretch from residues 39 to 56 is predicted to have a high probability of being a transmembrane region with the histidine rich c-terminus tail located to the outside of the membrane (Figure 2). A single putative N-glycosyla-

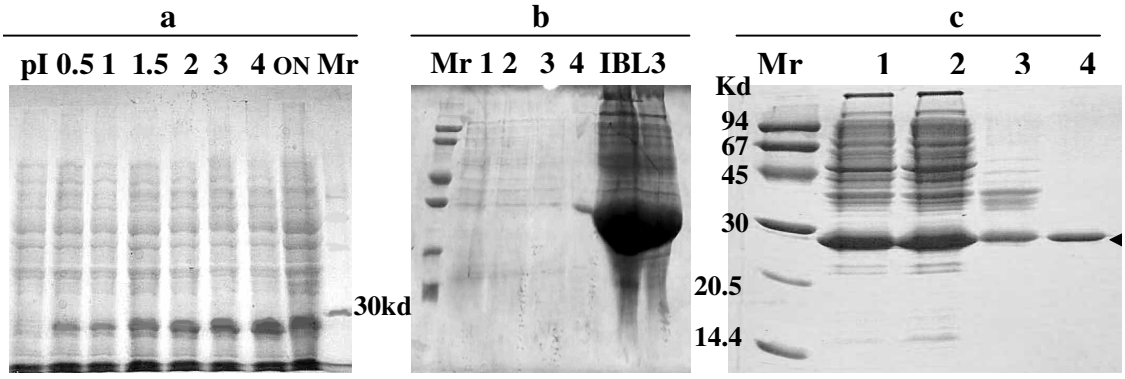

Figure 4. Expression and purification of OvL3.C1 recombinant fusion proteins. **(a)** SDS-PAGE electrophoregrams of time dependent expression of OvL3.C1-Thio pre-induced (pi) and induced (0.5 – 4 h) and overnight (ON). **(b)** Purification of ovL3.C1 inclusion bodies; washes (b1 - b4) and resolubilised inclusion bodies (IBL3). **(c)** Electroelution of partially purified OvL3.C1; cell lysates (c1, c2), purified OvL3.C1-Thio (c4).

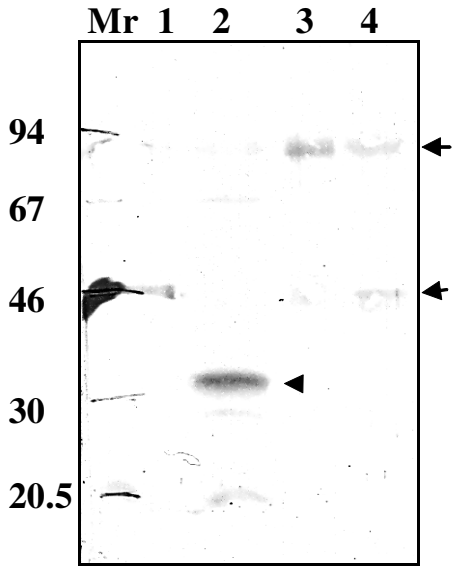

Figure 5. Western blot pattern of OvL3C1 parent proteins. Blots of SDS-PAGE separated *O. ochengi* adult females (1), *O. guttorosa* (2), *O. volvulus* adult females (3) and *O. volvulus* microfilarial (4) protein extracts were probed with plaque purified antibodies from endemic normal serum pool used for immunocreeening and selection. Mr indicates the molecular weight reference proteins.

tion signal defined by the consensus sequence NFSI is located from residues 68 to 71.

IgG isotypic responses of onchocerciasis endemic subjects to OvL3.C1

The anti-OvL3.C1 IgG1, IgG2, IgG3 and IgG4 subclass responses were investigated using two groups of 15 pairs and 18 pairs of matched infected (INF) and putatively immune individuals from Cameroon and Ecuador, respectively. Sera from uninfected inhabitants of nonendemic towns in Cameroon and Europeans (NES) who have never visited Africa were used as controls. Sera from onchocerciasis endemic regions showed a variable degree of IgG responses to OvL3.C1 with mean values significantly higher than those of the uninfected groups. Particularly, the mean OD value of IgG3 antibody responses of endemic normal subjects (putatively immune subjects) against OvL3.C1 was higher than those of the patients from Cameroon (Figure 6a). Endemic normal subjects tend to have a higher IgG4 response to the recombinant antigen though OD values were not significantly different from those of infected subjects ($p<0.05$) (Figure 6b). However, putatively immune subjects from Ecuador had higher IgG4 levels than for infected (Figure 6d) unlike for IgG3 ($p<0.05$) (Figure 6c). IgG1 and IgG2 against the recombinant protein did not show any statistical difference between putatively immune subjects and INF in both the Cameroonian and Ecuadorian foci (data not shown).

DISCUSSION

Lack of animal models and parasite material are major impediments to the knowledge of *onchocerca* biology that may lead to the development of novel control tools against onchocerciasis. To contribute to knowledge on onchocerciasis, we employed recombinant DNA technology to isolate, express and characterize antigens associated with *O. volvulus.* Herein, we describe an anti gen clone (OvL3.C1: AJ272105) of *O. volvulus* that is preferentially recognized by exposed but apparently healthy subjects (putatively immune) from an onchocerciasis endemic zone of Cameroon. As the subjects had no onchocercal lesions despite long term residence (exceeding 10 years), they were deemed to be

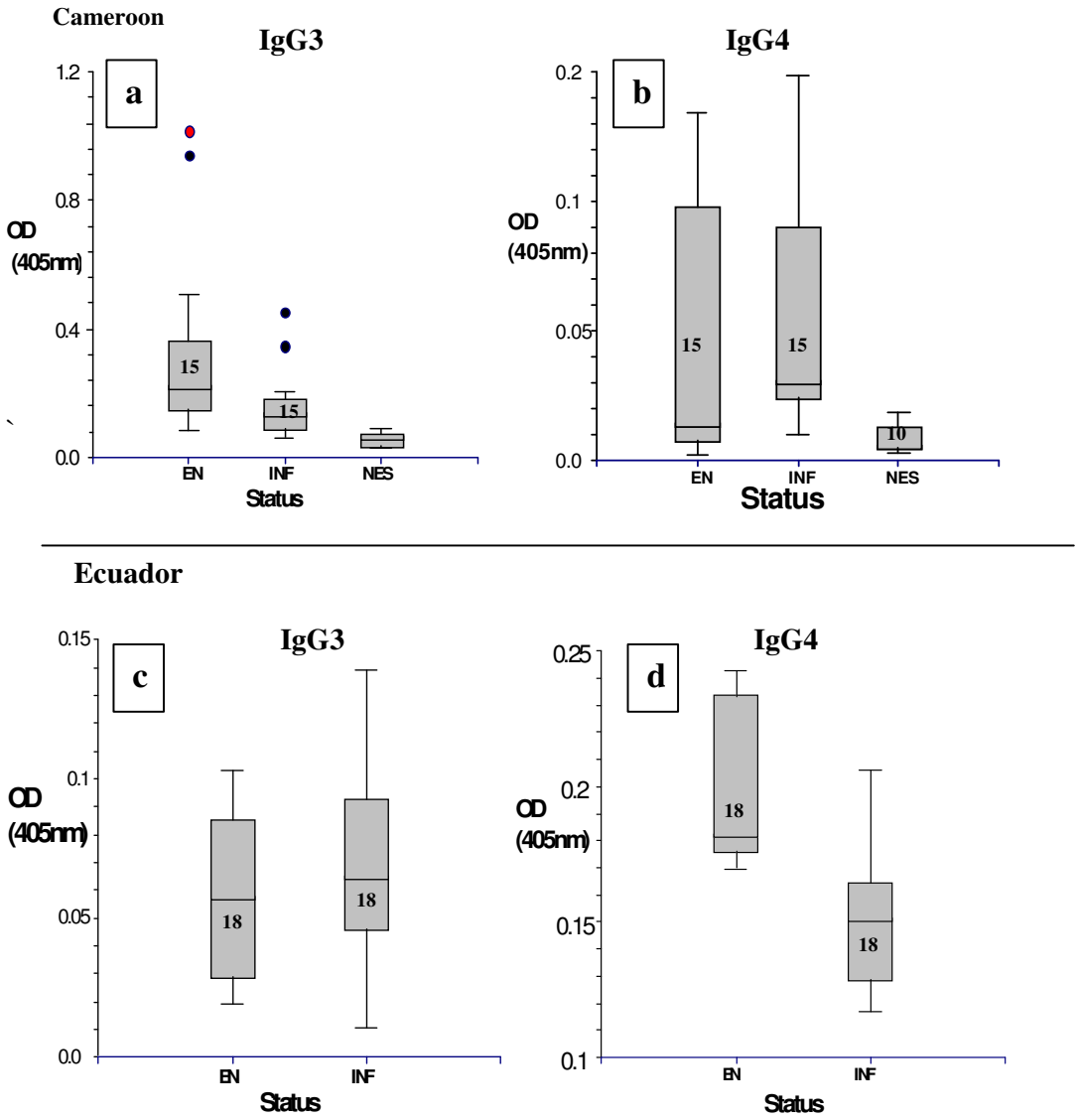

Figure 6. Anti-OvL3.C1 IgG3 and IgG4 responses of endemic normal subjects (EN) and infected (INF) subjects from Cameroon and from Ecuador. Normal European sera (NES) were from European adults who have never visited onchocerciasis endemic areas.

resistant to infection. Thus isolation of OvL3.C1 cDNA from *O. volvulus* infective larva cDNA library based on its preferential recognition by antibodies from these putatively immune subjects indicated that the antigen may be involve in resistance mechanisms in onchocerciasis (Titanji et al., 2002). To better understand the nature of the antigen, further analysis of the amino acid sequence was done using web based sequence analysis tools (Rost, 1996; 2000; Apweiler et al., 2000; Wheeler et al., 2004). The recombinant protein was also expressed in *E. coli* bacteria, purified and used to analyse its reactivity with antibodies in paired infected and putatively immune endemic subjects.

The isolated OvL3.C1 cDNA translates into a major 95 amino acid histidine rich protein and five other open reading frames. Further sequence analysis revealed the 95 amino acid protein to be related to histidine rich protein family particularly from *Plasmodium yoelli* and mouse mitochondrial tRNA-ribosyltransferase. Despite this homology with a mitochondrial protein, the presence of a transmembrane sequence (Figure 1) and an N-glycosylation site suggest that the protein may nevertheless be deployed on parasite surface and available for immune recognition. Given its reaction with antibodies from potentially protected individuals, it may belong to the group of 'vital molecules' important for parasite survival that can be targeted for vaccine production or whose pathways may serve as novel targets for rational drug and vaccine design against onchocerciasis (Lustigman et al., 2002). OvL3.C1 in a

TOPO-pBAD contruct expressed a 28 kda insoluble recombinant fusion protein with thioredoxine in *E. coli*. Purification could therefore be achieved by first solubilising inclusion bodies with detergents followed by electroelution (Figure 4).

To confirm the parasite origin of OvL3.C1, plaque purified OvL3.C1 specific antibodies were used to blot protein extracts from *Onhocerca* spp. microfilariae and adult worm antigen extracts. Recognition of protein bands from 32 to 94 kda in both *O. volvulus* and related nematodes *O. ochengi and O gutturosa*, suggests that the isolated clone may be part of a larger dominant antigen, which may present additional epitopes common to *onchocerca* spp. (Titanji et al., 2002). It is also probable that the difference in native antigen size may have been due to sequence divergence between the species. As shown for a *Trypanosome brucei* antigen by Nolan et al. (1997), it is possible that posttranslational modification OvL3.C1 by N-glycosylation may also have accounted for the dramatic increase in the apparent molecular weight of the native proteins on SDS-PAGE gels. The occurrence of the antigen in different *Onchocerca* species and in stages other than the L3 further underpins the dominance of the antigen in host parasite interplay in onchocerciasis.

In an attempt to further elucidate the association of OvL3.C1 to immunity, we compared the IgG reactivities of selected and characterised putatively immune and infected individuals in Cameroon and Ecuador (Ghogomu et al., 2002; Elson et al., 1994). We found a high degree of variability in the IgG antibody reactivities of endemic subjects to OvL3.C1 recombinant protein. Particularly, the recombinant antigen reacted with IgG3 and IgG4 antibodies in both patients and putatively immune people, suggesting that serological responses of putatively immune subjects compared to patients in onchocerciasis is not always distinctive. However, the proportions of high and low respondents to IgG3 and IgG4 subtypes varied among patients and putatively immune subjects. In Cameroon, it was found that the predominant isotype elicited in putatively immune subjects was IgG3, while patients showed a tendency to have higher levels of IgG4 against the recombinant (Figure 6a). IgG3 responses in putatively immune people had been shown to be important in resistance mechanisms in onchocerciasis (Boyer et al., 1991; Ottessen, 1995; Meyer and Kremsner, 1996). Thus OvL3.C1 may be involved in resistance mechanisms in onchocerciasis. Higher levels of IgG4 in putatively immune people against an antigen as found for OvL3.C1 in Ecuador is uncommon (Kwan-Lim et al., 1990). The IgG4 in patients from Cameroon and putatively immune people from Ecuador may be such as to block IgE induced allergy, implicated in onchocercal pathogenesis (Ottessen, 1995). Contrary to findings in Cameroonian forest foci, putatively immune subjects from Ecuador had significantly higher IgG4 responses against the recombinant protein (Figure 6d). These differences in response between the different onchocerciasis zones may be as a result of differences in genetic factors reported to influence immune responses to filarial infections (Kwan-Lim and Maizels, 1990; Abraham and Greives, 1990).

This study isolated and identified OvL3.C1 as a dominant antigen in onchocerciasis with epitopes that may be involved in immunity against *O. volvulus*. Presence of native OvL3.C1 proteins in microfilariel and adults followed by reactivity of the recombinant protein with patient sera indicate that the antigen has a relevant role in onchocercisis. We therefore envisage characterising the native variants of the protein to further establish its association to immune protective or pathogenic mechanisms in onchocerciasis.

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