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Purification and characterization of circulating *Onchocerca volvulus* antigens from epileptic and non-epileptic onchocerciasis patient sera

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ABSTRACT

Studies conducted during the past 25 years to investigate the possible relationship between onchocerciasis and epilepsy have led to contradictory results. In the present study aimed at contributing to the investigation of a possible relationship between onchocerciasis and epilepsy, we proceeded to purify and characterize circulating *O. volvulus* antigens from sera of onchocerciasis patients with and without epilepsy. Out of 539 onchocerciasis patients included in the study, sera from 78 epileptics and 20 non epileptics with high antigen titres were separately pooled and subjected to affinity purification using immunosorbent columns prepared using human and rabbit anti-*O. volvulus* IgG antibodies. Eluates of purified circulating *O. volvulus* antigens were concentrated, and then the protein contents were determined using the Bradford method. The antigenicity of the purified antigens was evaluated in a direct ELISA using onchocerciasis patient sera. Finally, the molecular composition of the purified proteins was determined by SDS-PAGE. The purified antigens were highly antigenic and there was no significant difference in the reaction profiles of the two groups or categories of patients. SDS-PAGE analysis showed that the purified antigens ranged from 31.63 to 102.40 KDa and there was no difference in the molecular composition of antigens purified from sera of the two classes of patients. Based on this antigen profiling between epileptic and non-epileptic onchocerciasis patients, we cannot conclude with certainty whether onchocerciasis is really a cause of epilepsy in areas where it is hyperendemic as predicted by some epidemiological studies.
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Keywords: Antigen-detection ELISA, Immunoadsorbent columns, Affinity chromatography, Antigenicity, SDS-PAGE.

INTRODUCTION

Onchocerciasis or "river blindness" is a parasitic disease caused by the filaral worm *Onchocerca volvulus*, and transmitted by the bite of a blackfly, *Simulium*. It causes serious cutaneous lesions and can involve in its final phase, an irreversible blindness. It is estimated that about some 40 million people in the world currently suffer from the disease, among which the majority are in tropical Africa (Pion et al., 2009). Studies carried out in several African countries indicate, that

onchocerciasis hyperendemic zones also have a high prevalence of epilepsies (World Health Organization, 1999).

Epilepsy is an affection due to an abnormal, sudden and excessive electric discharge which comes from an injured area of the brain, called the electric focus. This cerebral attack is a type of neurological disease comparable with an electric storm occurring in the brain, and which can be more or less violent, localised and frequent (Tecoma and Corey-Bloom, 2009). According to Pion et al. (2009), epilepsy is particularly common in tropical areas and one main reason for this is that many endemic infections have neurological consequences. There are no geographical, social or racial boundaries to the disease and everyone can suffer from it. In addition, the medical, social and demographic burden of epilepsy remain substantial in tropical countries where it is often seen as a contagious condition and where the aetiology is often undetermined. The causes of the disease are multiple, including those due to infections affecting the brain such as the presence of *Taenia solium* metacestodes in the brain, leading to neurocysticercosis which is one of the major causes of epilepsy in areas where cysticercosis is endemic (De Bittencourt et al., 1996; Carpio et al., 1998).

For several decades, field researchers have reported some overlapping between the geographical distribution of epilepsy and onchocerciasis. To this effect, the question of the existence of a causal relationship between onchocerciasis and epilepsy has been the subject of many studies, essentially based on epidemiologic surveys. However, the results obtained up to date are controversial and many unclarified points persist. The kind of relationship that exists between these two diseases, as well as the epileptogenic nature of *O. volvulus*, need to be explained. We undertook the present study to purify and characterise *O. volvulus* antigens from epileptic and non-epileptic onchocerciasis patients, so as to certify whether or not the antigen profiles differ in the two groups of onchocerciasis patients, as this may give us a clue as to the role of onchocerciasis in the development of epilepsy in areas where the disease is hyperendemic.

MATERIALS AND METHODS
Study subjects

Subjects included in this study comprised epileptics and non-epileptics from the onchocerciasis endemic localities of Batibo-Widikum in the North-West region and Bangoua in the West region of Cameroon. After an information campaign by medical authorities only those from whom we had informed consent were included in the study. Patient identification was done on the basis of clinical signs of the two diseases. It is necessary to note here that an interview was carried out for the purpose of obtaining information on the age of the patient, age at which epileptic seizures began, their manifestations, their frequency, and possibly the responsible factors. A total of 539 onchocerciasis subjects comprising 387 epileptics from Batibo-Widikum and 54 from Bangoua, as well as 98 non-epileptics from Bangoua were included in the study.

Blood collection and sera preparation

Five millilitres (5 ml) of blood were collected in dry centrifuge tubes by veno-puncture at the level of the arm. The blood was transported to the laboratory and allowed to clot overnight at +5 °C in a refrigerator, then centrifuged at 1500 rpm for 15 minutes and the supernatant (serum) collected and stored at -20 °C until use.

Negative control sera for this study consisted of sera from 65 European children who had never been exposed to onchocerciasis and showed no symptoms or signs of epilepsy.

Identification of *O.volvulus* seropositive patient sera by antigen detection ELISA (sandwich-ELISA)

The Sandwich-ELISA technique was essentially carried out as previously described (Tume et al., 1997), using *O. volvulus*-specific anti-Oncho-C27 IgG antibodies, with all incubations done at 37 °C on an orbital shaker. The anti-Oncho-C27 IgG, was diluted to a final concentration of 1 µg/ml in 0.1M Carbonate/Bicarbonate buffer pH 9.6, and 100 µl dispensed into each well of microtitre plates, and incubated for 60 minutes at 37 °C. Plates were emptied of their contents, washed

with Phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween-20 (PBS-T), and blocked by incubating for 45 minutes at 37 °C with 150 µl/well of blocking buffer (0.01% New Born calf serum in PBS-T). The contents of each plate were again discarded and replaced with 100 µl/well of patient (test) or control sera. Plates were incubated for 60 minutes at 37 °C, then emptied and washed thoroughly as above. One hundred microlitres (100 µl) per well of biotinylated anti-Oncho-C27 IgG antibodies diluted 1000-fold in blocking buffer were introduced, and then the plates were incubated for 60 minutes at 37 °C. They were emptied and washed thoroughly again as above. One hundred microlitres (100 µl) of peroxidase-conjugated streptavidin diluted 3000-fold in blocking buffer were then dispensed per well, and incubated for 30 minutes at 37 °C. The plates were once again thoroughly washed and further incubated in the dark at room temperature with 100 µl/well of peroxidase substrate (O-phenylene diamine), prepared according to the manufacturer (Sigma). The incubation lasted for about 20 minutes during which time a significant colour change of the substrate occurred in serpositive wells. Reactions were stopped by adding 25 µl/well of 4M Sulphuric acid (H_2SO_4), and the absorbance values determined spectrophotometrically at 492 nm using an automated ELISA plate reader.

All the above incubation steps, except that of the substrate were carried out at 37 °C with gentle shaking on a rocking platform.

The cut-off value was established as the mean plus 2 times the standard deviation of the absorbance values for the 65 negative control sera ($X_{Neg} + 2SD$).

Seropositive sera from epileptics were pooled into one lot and those from non-epileptics into another lot, then labelled and stored at -20 °C.

Purification of *O. volvulus* circulating antigens by affinity chromatography

The immunoadsorbent columns were prepared using the inert matrix Sephacryl S-100 gel, onto which human anti-*O. volvulus* IgG on the one hand, and rabbit anti-*O. volvulus* IgG antibodies on the other, were coupled. The activation and the coupling were carried out as previously described (Sanderson and Wilson, 1971; Wilson and Nakane, 1976; Tume et al., 1997). Purification of the circulating *O. volvulus* antigens was made from the onchocerciasis-epileptic and onchocerciasis-non-epileptic sera using the two types of immunoaffinity columns. In each case, 10 ml of the pooled serum were thawed and divided into two equal volumes (5 ml each), one for purification through the human anti-*O. volvulus* IgG column and the other through the rabbit anti-*O. volvulus* IgG column. The affinity chromatography was done as previously described (Ngu et al., 1989). The bound *O. volvulus* antigens were eluted using acetate buffer (1.5M Acetic acid, 0.5M NaCl, pH 2.5). The protein-containing eluates were pooled and dialysed against 2 litres of PBS with two buffer changes, and then concentrated using sephadex G25 powder.

The protein concentration of each antigen preparation was determined using the Bradford method (1976), and the samples were aliquoted and stored frozen at -20 °C for subsequent characterisation .

Characterization of circulating *O. volvulus* antigens
Comparative antigenicity of purified O. volvulus antigens

The antibody detection ELISA technique used was essentially carried out as previously described (Tume et al., 1997), with some modifications. The purified antigen, in each case was diluted to a final concentration of 1 µg/ml in 0.1M Carbonate/Bicarbonate buffer pH 9.6, and 100 µl dispensed into each well of microtitre plates, and incubated for 45 minutes at 37 °C. Plates were emptied, washed with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20 (TBS-T), and blocked by incubating for 45 minutes at 37 °C with 150 µl/well of blocking buffer (0.01% New Born calf serum in TBS-T). The contents of each plate were discarded again and replaced with 100 µl/well of either control sera, onchocerciasis-epileptic sera or onchocerciasis-non epileptic sera, each diluted 1000-fold in blocking buffer above. Plates were incubated for 60 minutes at 37 °C, then emptied and washed thoroughly as above. One

hundred microlitres (100 µl) of peroxidase-conjugated goat anti-human IgG antibodies diluted 3000-fold in blocking buffer was then dispensed per well, and incubated for 15 minutes at 37 °C. The plates were once again thoroughly washed and incubated at room temperature with 100 µl/well of peroxidase substrate (O-phenylene diamine), prepared according to the manufacturer (Sigma). After colour development, the reactions were stopped by adding 25 µl/well of 4M Sulphuric acid (H_2SO_4) and the absorbances read at 492 nm using an ELISA plate reader.

All the above incubation steps, except that of the substrate were carried out with gentle shaking on a rocking platform.

SDS-PAGE analysis of purified antigens

This electrophoretic analysis was done as previously described (Towbin and Gopdon, 1979; Tume, 1993). Briefly, a 15% reducing acrylamide gel was introduced into an electrophoresis tank containing the migration buffer (25 mM Tris/192 mM glycine/0.1% SDS). Four volumes of purified antigens from the two patient categories and the two types of affinity columns were each mixed with one volume of 5X concentrated Gel Loading Buffer (GLB) then heated for 5 minutes at 95 ºC. The samples were loaded into the gel wells alongside the Molecular Weight protein markers (Fermentas) and the electrophoresis carried out for 2 hours at 90 mA. The gel was then removed and stained with Coomassie Brillant blue R-250 and then destained as previously described (Towbin and Gopdon, 1979 and Tume, 1993).

The relative mobilities of the Molecular Weight standards and the protein bands for each purified antigen sample were then calculated. A standard curve of the relative mobilities versus $\log_{10}$ of molecular weight of standards was drawn and used to determine the corresponding molecular weight of each purified protein band.

Statistical analysis

The data obtained were analyzed using the software SPSS 10.0 for Windows, and the data expressed in the form of Mean ± Standard Deviation. All the statistical analyses were done using the Student t test to the threshold of 5%.

RESULTS

Detection of circulating O. volvulus antigens in patient sera

Table 1 presents the seroprevalence of *O. volvulus* antigens in the study subjects per locality. Seropositivity to *O. volvulus* antigens was defined relative to a threshold or cut-off value, here defined as the mean absorbance of negative controls, plus 2 times the standard deviation ($X_{Neg} + 2SD$). Based on this, all test subjects whose optical densities are greater than this threshold of detection are declared *O. volvulus* seropositive and all those with the optical density below the threshold are declared seronegative.

According to Table 1, 19.12% of sera from epileptics from Batibo-Widikum tested positive for circulating *O. volvulus* antigens, whereas in Bangoua with a mixed population of epileptics and non epileptics, only 7.4% of epileptics were seropositive. Globally, of the 539 onchocerciasis patients comprising 441 epileptics and 98 non-epileptics included in the study, 78 (17.7%) of the epileptics and 20 (20.4%) of the non-epileptics were seropositive for *O. volvulus* antigens, giving a total prevalence of circulating O. volvulus antigens in the study population of 18.2%. No significant difference (P>0.05) in seroprevalence was observed between epileptics (17.7%) and non epileptics (20.4%). Also, the mean absorbances characteristic of the antigen levels in the two study populations did not show any significant difference (P>0.05).

Characterization of circulating O. volvulus antigens

Antigenicity of purified O. volvulus antigens

Table 2 presents the antigenicity of purified *O. volvulus* antigens with sera from onchocerciasis patients presenting with or without epilepsy. Antigenicity of *O. volvulus* antigens was defined relative to a threshold or cut-off value, here defined as the mean absorbance of negative controls, plus 3 times the standard deviation ($X_{Neg} + 3SD$).

Table 1: Frequency of seropositivity for *O. volvulus* antigens according to epilepsy status.

Village	Number of subjects and status		Number of positives per status		Percentage of positives per status (%)		Mean of optical density (X ± SD) per status		Cut-off value (X$_{Neg}$ + 2SD)
	E$^+$	E$^-$	E$^+$	E$^-$	E$^+$	E$^-$	E$^+$	E$^-$	
Batibo-Widikum	387	/	74	/	19.1	/	0.645 ± 0,015		NA
Bangoua	54	98	04	20	7.4	20.4	0.545 ± 0.013	0.590 ± 0.085	NA
sub-totals	441	98	78	20	17,7	20.4	/	/	NA
TOTAL	539		98		18,2		/		NA
Negative controls	65		NA		NA		0.127 ± 0.032		0.191

E$^+$: Epileptics; E$^-$: Non epileptics.
X$_{Neg}$ + 2SD: Mean absorbance of negative controls plus 2 times the Standard Deviation.

Table 2: Antigenicity of purified *O. volvulus* antigens with sera from onchocerciasis patients with different epilepsy status.

Village	Number of subjects and status		Number of positives per status		Percentage of positives by status (%)		Mean of optical density (X ± SD) per status		Cut-off value (X$_{Neg}$ + 3SD)
	E$^+$	E$^-$	E$^+$	E$^-$	E$^+$	E$^-$	E$^+$	E$^-$	
Batibo-Widikum	200	/	200	/	100	/	1.338 ± 0.883		NA
Bangoua	50	55	50	55	100	100	1.450 ± 0.849	1.381 ± 0.843	NA
sub-totals	250	55	250	55	100	100	/	/	NA
TOTAL	305		305		100		/		NA
Negative controls	65		NA		NA		0.198 ± 0.053		0.357

E$^+$: Epileptics; E$^-$: Non epileptics.
X$_{Neg}$ + 3SD: Mean absorbance of negative controls plus 3 times the Standard Deviation.

Table 2 shows that, 100% of epileptics from Batibo-Widikum presented with circulating *O. volvulus* antibodies in their sera. In Bangoua with a mixed population of epileptics and non epileptics, 100% of epileptics were seropositive for circulating *O. volvulus* antibodies. Globally, 100% of epileptics in the entire study population have been, exposed at least once, to *O. volvulus* worms during their lifetime.

SDS-PAGE

Electrophoretic separation of the various protein components of the antigens immunosorbed from sera of the two patient categories showed that the purified *O. volvulus* antigens were composed of proteins ranging in molecular weight from 31.63 to 102.40 KDa, and there was no difference in the protein profiles of circulating *O. volvulus* antigens purified from the two patient categories and using the two immunosorbent columns (results not shown).

DISCUSSION

The aim of this study was to contribute to the understanding of the relationship between onchocerciasis and epilepsy by evaluating the profile of antigens purified from epileptic and non-epileptic onchocerciasis patient sera, as well as the antigenicity of these purified antigens vis a vis onchocerciasis patient sera from epileptics and non epileptics.

Our results showed no significant difference in the levels of seroprevalence of *O. volvulus* antigens between the sera of epileptic and non-epileptic onchocerciasis patients. These findings correlate with those of Kaiser et al. (2011) in which they found no significant difference in the skin microfilarial load of epilepsy patients compared to the non-epileptic controls. Indeed, studies of Kabore et al. (1996), Druet-Cabanac et al. (1999) and Farnarier et al. (2000) undertaken in different geographical areas that are both hyperendemic for onchocerciaisis and with high epilepsy prevalences did not conclude that there is a relationship between the two diseases.

However, other studies including those of Ovuga et al. (1992), Kipp et al. (1994), Kaiser et al. (1996) and Newell et al. (1997) supported the existence of such a relationship. In addition to these conflicting findings, we do not have any information on the exact chronological order of occurrence of the two diseases among the study populations. In fact, if onchocerciasis was the first disease affecting these patients, its implication as the aetiological agent of epilepsy could be ascribed. On the other hand, if epilepsy was the first disease to affect these patients, then the possible role of onchocerciasis in epilepsies could be highlighted by an increase in the severity of seizures. The existence of no significant difference in the levels of seroprevalence between the two groups of patients (17.7% prevalence among epileptics and 20.4% among non-epileptics) suggests that there may exist no correlation between their onchocercal infection and the development of epilepsy. Although in a study using magnetic resonance imagery (MRI) and cerebrospinal fluid examination (Kaiser et al., 2000), no *O. volvulus* parasites were found in the brains of epileptics from an onchocerciasis endemic area, it is likely that antigenic components rather than the intact parasites may be at the origin of epilepsy in some patients.

In a recent review, Wagner and Newton (2009) raised the interesting hypothesis that immunological responses to helminths including *O. volvulus*, might be involved in the pathogenesis of epilepsy. This correlates very well with findings from previous studies which had shown that immunological mechanisms including the production of some cytokines are implicated in the occurrence of certain epilepsies (Newton and Forster, 1996). Moreover, it was equally shown that cytokines play a role not only in epilepsies (Aarli et al., 2003) but also in onchocerciasis (Ward et al., 1988; King and Nutman, 1991; Soboslay et al., 1992; Chan et al., 1993). Therefore, the implication of onchocerciasis in occurrence of epilepsies could not only be of direct nature through the

various ways by which circulating *O. volvulus* antigens or *O. volvulus* microfilariae would induce a hyperexcitability of a more or less extended neuron population (Molyneux et al., 1989; Newton and Foster, 1996), but also of indirect nature through the immunological mechanisms involving IgE (Buttner et al., 1982) and cytokine production ((Molyneux et al., 1989; Finkelman et al., 1991; Modlin and Nutman, 1993; Newton and Foster, 1996).

Our study also showed a very low prevalence of *O. volvulus* antigens within the epileptic sera. This low prevalence rate suggests that either the epilepsy in seronegative epileptics may have a different aetiological origin or that onchocerciasis may not be a cause of epilepsy. Given that one major occupation of people in the Batibo-Widikum area is pig rearing, it is likely that neurocysticercosis could be at the origin of their epilepsy; alternatively, their epilepsy could be of a mixed aetiology arising from combined infection with *O. volvulus* and *Taenia solium*. However, even though the connection between onchocerciasis and epilepsy may be influenced by the presence of other endemic diseases known to be a cause of epilepsy especially cysticercosis (Kaiser et al., 2010; Katabarwa et al., 2008), a study by Kaiser et al. (2008) in areas where a relationship between onchocerciasis and epilepsy had been found, do not support the hypothesis that neurocysticercosis could be the cause of onchocerciasis-associated epileptic seizures.

We also observed no difference in the banding pattern of purified antigens from epileptic and non-epileptic sera. This observation shows that there may exist no difference in the antigens found in the two groups of patients, and thus suggests that if there exists a difference in the molecular composition of the *O volvulus* antigens circulating in the sera of the two groups of patients, this difference might not be at the macromolecular level but rather at the level of the antigenic epitopes constituting the antigen molecules.

This study thus showed that there is no significant difference in antigen levels circulating in the sera of epileptics and non-epileptics, and also that there is no difference in the molecular composition of these antigens. Also, a very low prevalence of *O. volvulus* circulating antigens was observed among the sera of epileptic patients.

On the basis of the *O. volvulus* antigen profiling between epileptic and non-epileptic onchocerciasis patients obtained in this study, we cannot conclude with certainty whether onchocerciasis is really a cause of epilepsy in areas where it is hyperendemic as predicted by epidemiological studies. Further research is thus needed for a better understanding of the neurological pathogenicity in onchocerciasis.

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