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COMPARING ANTIBODY RESPONSES TO ONCHOCERCA VOLVULUS AND NON-PARASITE ANTIGENS IN PLACEBO-CONTROLLED AND IVERMECTIN-TREATED ONCHOCERCIASIS PATIENTS

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ABSTRACT

Serum antibodies to parasite-specific and non-parasite antigens were evaluated using enzyme-linked immunosorbent assay (ELISA). Out of the 470 sera collected, 409 were from residents of an onchocerciasis hyper-endemic area, 55 non-endemic and 6 European normal sera served as control. The patients' age, sex, skin microfilaria densities, dermal and ocular clinical manifestations (colour of optic disc) have been well characterised. The study population had participated in a placebo-controlled (n=191) trial of ivermectin (Mectizan®) treatment (n=218). The parasite antigens are phosphate buffered saline crude extract of adult worms of *Onchocerca volvulus*, a recombinant antigen (Ov1.9) and a monoclonal antibody purified antigen (Cam 1). The non-parasite antigens are deoxycholate citrate extract of optic nerve (nerve-DOC) and commercially available IgA, IgM and IgG were used to assay for rheumatoid factor (Rh-F) auto-antibodies. Generally, antibodies to parasite antigens in onchocerciasis patients were remarkably higher than control group (p<0.05) using exact F-test. There was no significant difference (p>0.05) in antibodies to nerve-DOC and Rh-F in patients compared to control. Antibodies increased with increasing skin snip microfilaria load from 0.69±0.28 with 0mf/mg (n=54) as against 0.80±0.26 for those with 4-20mf/mg. Observed slight negative correlation in IgG antibody levels and severity of disc colour with mean OD values of 0.26±0.22 in those graded as having no optic nerve disease (OND) (disc 1, n=86) and 0.17±0.19 for those with severe changes (disc 3, n=49) was not statistically significant (P>0.05). An age dependent significant decrease (P<0.05) in antibodies were observed with 0.64±0.34 for 15-30yr old (n=48) compared to 0.48±0.35 for those 50yr (n=50) for PBS with a similar trend for IgG to Ov1.9 and Cam1. In conclusion, serum parasite-specific and non-parasite antibodies may not be responsible for the pathology of optic nerve disease. Onchocerciasis patients were apparently not at higher risk of developing rheumatoid arthritis than the control.

Key words: Onchocerciasis; Antibodies; Antigens; Immune responses; Ivermectin.

COMPARER LES REPONSES D'ANTICORPS AU ONCHOCERCAVOLVULUS ET AUX ANTIGENES NON PARASITAIRES DANS LES PATIENTS AVEC L'ONCHOCERCOSE CONTROLLES PAR PLACEBO ET TRAITES PAR L'IVERMECTINE.

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RESUME

Les anticorps sériques contre antigènes parasites spécifiques et non parasitaires ont été évalués en utilisant un dosage immunoenzymatique (ELISA). Sur les 470 sérums collectés, 409 provenaient de résidents d'une région hyper endémique de l'onchocercose, 55 sérums normaux non endémiques et 6 européens ont servi de témoins. L'âge des patients, le sexe, les densités de microfilaries, cutanées, les manifestations cutanées et oculaires (couleur du disque optique) ont été bien caractérisées. La population étudiée avait participé à un essai contrôlé par placebo (n=191) sur le traitement à l'ivermectine (Mectizan®) (n=218). Les antigènes parasites sont phosphate tamponné saline extrait brut de vers adultes de *Onchocerca volvulus*, un antigène recombinant (Ov1.9) et un antigène purifié par un anticorps monoclonal (Cam 1). Les antigènes non parasitaire sont l'extrait de citrate de desoxycholate du nerf optique (nerf - DOC) et on a utilisé des IgA, IgM et IgG disponibles dans le commerce pour doser les auto anticorps du facteur rhumatoïde (Rh - F). Généralement, les anticorps contre les antigènes parasitaires chez les patients atteints d'onchocercose étaient remarquablement plus élevés que le groupe témoin (p>0,05) en utilisant le test F - exact. Il n'y avait pas de différence significative entre les anticorps au nerf - DOC et les facteurs rhumatoïdes (Rh - F) chez les patients par rapport au témoin. Les anticorps augmentaient avec augmentation de la charge microfilair de coupe de peau de 0,69±0,28 avec 0mf/mg (n=54) contre 0,80±0,26 pour ceux avec 4-20mf/mg. On a observé une légère corrélation négative dans les taux d'anticorps IgG et la gravité de la couleur du disque avec des valeurs moyennes de OD de 0,26±0,22 chez celles classées comme n'ayant pas de maladie du nerf optique (OND) (disque 1, n=86) et 0,17±0,19 pour les personnes ayant des changements sévères (disque 3, n=49) n'était pas statistiquement significative (P>0,05). Une diminution significative de l'âge (P<0,05) dans les extraits d'anticorps a été observée avec 0,64±0,34 15 - 30 ans (n=48) compare à 50 ans (n=50) pour PBS avec une tendance similaire pour IgG à Ov1,9 et Cam1. En

conclusion, les parasites spécifiques du sérum et les anticorps non parasitaires ne peuvent pas être responsable pour la pathologie de la maladie du nerf optique. Les patients atteints d'onchocercose n'avaient apparemment pas un risque plus élevé de développer la polyarthrite rhumatoïde que le témoin.

Mots clés: L'onchocercose; Les anticorps; les antigènes; Réponses immunitaires; L'ivermectine.

INTRODUCTION

Current control of onchocerciasis has relied on the mass drug administration of Ivermectin or Mectizan® (1). Lately, more emphasis is placed on operational research, drug screening and diagnostics development, while less attention is focused on basic research on the disease. Study into the immune responses of any infection is for a better understanding of the basis or mechanism of immunopathology including the role of autoimmunity involvement, the diagnostic usefulness and to identify potential vaccine candidate immunogens. In onchocerciasis, humoral and cellular immune reactivity to parasite or the substance they release after death varies from one individual to another, and also show diverse clinical manifestations (2, 3). With time, the larva forms (microfilariae) and adult worm (macrofilaria) aged and die (4). Their fragments elicit host inflammatory responses with bystander effects believed to underlie dermal and ocular changes (5, 6). Immunological mechanisms are believed to play a major role in the broad range of dermal and ocular pathologies complicating *Onchocerca volvulus* infections. These have therefore prompted questions concerning involvement of antibodies to parasites-specific and autoantigens in the pathogenesis of onchocerciasis.

The parasite materials share a lot of biochemical and immunological homology with other parasites, related and unrelated species which are often co-endemic with onchocerciasis. This has posed a major problem in interpreting results of assays using undefined crude antigens, thus making the identification of diagnostic and clinical trends very difficult. The measure of antibody class and subclass response is thought would be able to reveal host-parasite immunological interactions that are involved in pathogenesis, and restricted to discrete clinical entity (7). Thus far, a positive association between skin microfilariae load and IgG3 to low molecular weight adult *Onchocerca volvulus* antigens and changes in immune responses following treatment have been reported while a possible cross-reactivity of parasite with host eye tissue component has been suggested. In this investigation, a measure of serum antibody against parasite crude and recombinant, and non-parasite rheumatoid factor (Rh-F) antigens using enzyme-linked immunosorbent assay (ELISA) were performed to know if there is any clinical trend based on age, sex, mf load and eye pathology (change in optic nerve disc colour). ELISA has been reported to show greater sensitivity and measuring IgG RF and IgA in addition to IgM RF by (8).

Onchocerciasis was listed by (9) to be amongst diseases with possible features of autoantibodies to RA. Although there are formal classification criteria for RA according to the American College of Rheumatology, RFs are not a specific diagnostic tool for RA. However, the presence of high RF titers is predictive for developing RA in non-symptomatic subjects and titers are associated with a more aggressive and destructive course and with the occurrence of extra-articular manifestations in RA patients. Possible involvement of autoimmunity in the pathology of the skin and ocular lesions have been suspected. It has been suggested that autoimmunological reactions resulting from cross-reactivity between parasite antigens and components of eye tissues contribute to development of ocular pathology (10). Assessing the levels of antibodies in groups of clinical and parasitological defined onchocerciasis patients compared to control will show the cause and effect relation.

The aim of this research study is to determine the involvement of parasite-specific and non-parasite auto-antigens in the immunopathology of optic nerve disease, which is one of the major causes of irreversible blindness, skin clinical manifestations and correlation with status of microfilaridermia. Secondly, the study will also determine the likely risk of developing rheumatoid arthritis in onchocerciasis patients compared to control.

MATERIALS AND METHODS

Experimental Design: A total of 470 sera, comparison 409 from onchocerciasis patients, 55 from Fatika, a non-endemic area in Kaduna State, Nigeria and 6 European normals as controls were screened for antibody responses to parasite and non-parasite antigens. The onchocerciasis patients comprised of 218 individuals receiving ivermectin and 191 receiving placebo. The individuals were previously characterized by age, sex, skin mf density and ocular pathology (colour of optic disc). All sera samples were stored at -70°C in deep-freezer in 30µl aliquot and thawed once before use.

Antigens: Adult *O. volvulus*, phosphate buffered saline (PBS)-extracted crude antigen, and a monoclonal antibody purified antigen designated Cam 1 were prepared and provided by Dr. Engelbrecht. The Ov1.9 recombinant antigen described by (11) was supplied to the Immunology Research Laboratory (I.R.L), NITR, Kaduna, by Dr. McKennie of Cambridge University. A deoxy cholate citrate extract of human optic nerve tissue

(nerve-DOC) was kindly provided by the Institute McKennie. For Rh-F assay, commercially available human IgA, IgG and IgM (Calbiochem, U.K.) were used as auto-antigens as described by (8). The PBS extract was used at 1:1000 except for IgA at 1:500.

ELISA Protocol: Assays were performed following the protocol of Engelbrecht *et al.* (1992) with slight modification at the NITR Immunology Research laboratory. Wells of microtitre plates were coated with antigens and stored overnight in the fridge at 4°C. Microplates were washed 3-5 times between each step. All other steps were performed at room temperature ranging from 28-32°C. Sera, antigens, anti-human IgG and anti-mouse horseradish peroxidase conjugates were subjected to pre-titration experiment in checkerboard to obtain the optimal working concentration or dilution. Sera were used at 1:400 for IgG and IgG4, 1:250 for IgG1 and IgG3, and 1:50 for IgA. The PBS extract was used at 1:1000 for IgG, 1:2000 for IgG1, 1:8000 for IgG3 and IgG4 and 1:500 for IgA. Anti-mouse IgG hydrogen peroxidase was used at 1:800. The binding of antigen to antibody was demonstrated with hydrogen peroxide (H₂O₂) in disodium hydrogen phosphate (Na₂HPO₄), citric acid and freshly prepared orthophenylene diamine (OPD) substrate solution at 15µl per well allowed to react for 15 minutes. The enzyme reaction was terminated with 2M H₂SO₄ at 30µl per well and plates were read after 5 minutes in a Dynatech MR4000 ELISA reader.

Data Analysis: The mean optical density (OD) values of cases and control, treated and non-treated, male or female, stratification by skin MFL and subjective grading of optic disc colour were computed using Microsoft Excel spreadsheet. Differences in mean±standard deviation (stdev) were subjected to exact F-test of unpaired data at 0.05 level of error.

RESULTS

Optical density (OD) values of the ELISA tests at 492nm of serum antibodies reaction with the parasite and non-parasite antigens were analysed. The mean and standard deviation of the OD-values were computed for each group and sub-groups.

of Ophthalmology, Cambridge University. Dr. Antibody responses (IgA, IgG, IgG₁, IgG₃, and IgG₄) to PBS-crude extract, Ov1.9 and Cam 1 antigens in onchocerciasis patients were remarkably higher than those of control group (Table 1). Onchocercal sera (n=409) gave higher total anti-IgG titres of 0.75±0.24 compared to 0.62±0.3 and 0.37±0.18 for Ov1.9 and Cam 1, respectively. These values were comparatively higher than the mean values obtained for Fatika and European controls shown on Table 1. Similar trends were observed for the isotype (IgG₁, IgG₃ and IgG₄) antibodies to the three antigens (PBS-extract, Cam1 and Ov1.9). The differences between the IgG₄ responses to PBS-extract and Cam1 antigens were statistically significant (p<0.05). With cut-off points set as mean ± 3stdev of European control for PBS-extract (0.05) and Cam1 antigens (0.06) showed that 375 (91.7%) and 361 (88.3%) were found to be serological positive, respectively. Mean OD values for male were slightly higher than female (p>05). Only slight difference in anti-Rh-F IgG of 0.64±0.16, 0.59±0.15 and 0.41±0.22 for onchocerciasis sera, non-endemic and European controls, respectively were recorded. Therefore, there was no further analysis of the data on the basis of bioclinical and parasitological variables.

In isotype assays, anti IgG₄ titres were prominently elevated in patients with a mean of 0.58±0.33 while absent in controls. IgG₁ antibody titres were slightly high in patients with mean±stdev 0.24±0.23 compared with 0.11±0.15 OD-value for Fatika non-endemic control. European normals (n=6) were however negative. IgG₃ assay was virtually negative irrespective of the test sample. Anti IgA titres were slightly higher in patients with a mean of 0.24±0.17 than controls, 0.19±0.14 for Fatika and 0.0.13±0.07 for European normals. Anti IgG₂, IgE and IgM could not be developed due to high background reactions. Similar trend in antibody responses to PBS-extract were observed for Ov1.9 antigens, except that total IgG titres were comparatively lower- about half the OD-value (0.37±0. 2) as against 0.62±0.3. Overall, there were no significant differences between treated and non-treated groups for IgG responses.

TABLE 1: ANTIBODY RESPONSES TO PARASITE ANTIGENS COMPARED TO CONTROLS

(i) Antigen-Antibody Reaction	PBS IgG	PBS IgG1	PBS IgG3	IgG4	PBS IgA
Onchocerciasis sera (n=409)	0.75±0.24§	0.24±0.23	0.09±0.14	0.58±0.33φ	0.24±0.17
Non-endemic control (n=55)	0.38±0.26§	0.11±0.15	0.01±0.05	0.08±0.22φ	0.19±0.14
European negative (n=6)	0.11±0.05§	0.01±0.01	0.01±0.01	0.02±0.01φ	0.13±0.07
(ii) Antigen-Antibody Reaction	Ov1.9 IgG	Ov1.9 IgG1	Ov1.9 IgG3	Ov1.9 IgG4	Ov1.9 IgA
Onchocerciasis sera (n=409)	0.62±0.3 ^o	0.16±0.15	0.08±0.06	0.38±0.34 ^o	ND
Non-endemic control (n=55)	0.37±0.2 ^o	0.1±0.06	0.05±0.02	0.11±0.19 ^o	ND
European negative (n=6)	0.26±0.11 ^o	0.06±0.01	0.03±0.01	0.06±0.08 ^o	ND
(iii) Antigen-Antibody Reaction	Cam 1 IgG	Cam1 IgG1	Cam 1 IgG3	Cam 1 IgG4	IgA
Onchocerciasis sera (n=409)	0.37±0.18 ^α	0.30±0.19	0.11±0.15	0.44±0.33 ^β	ND
Non-endemic control (n=55)	0.23±0.15 ^α	0.19±0.16	0.08±0.08	0.05±0.19 ^β	ND
European negative (n=6)	0.15±0.07 ^α	0.06±0.03	0.05±0.04	0.00±0.02 ^β	ND

The §, φ, ^o, α, and β signs showed differences between onchocerciasis and non-endemic control were statistically significant (p<0.05) by exact F-test.

The OD values of non-treated group increases with increase in MFL for IgG anti-PBS-extract with mean±stdev of 0.69±0.28 for 0mf/mg (n=54) vs 0.8±0.26 for 4-20mf/mg (n=52). IgG₄ had corresponding values of 0.45±0.32 vs 0.66±0.34. A similar trend was observed in the treated group, where IgG₁ values were 0.19±0.23 (n=47) vs 0.32±0.20 (n=46) for the untreated group as shown on Table 2. Except for IgG response to Ov1.9 in

treated group with 0.56±0.31 vs 0.7±0.3, there was no appreciable change with increase in mf density. Figure 2 shows there is age dependency with significant decrease (P<0.05) in mean OD values for IgG₄ against PBS-extract from 0.64±0.34 for 15-30yr old (n=48) compared to 0.48±0.35 for those 50yr (n=50). Similarly, IgG₄ reactivity to Ov1.9 were 0.47±0.37 vs 0.31±0.32 were comparably not different from those of Cam 1.

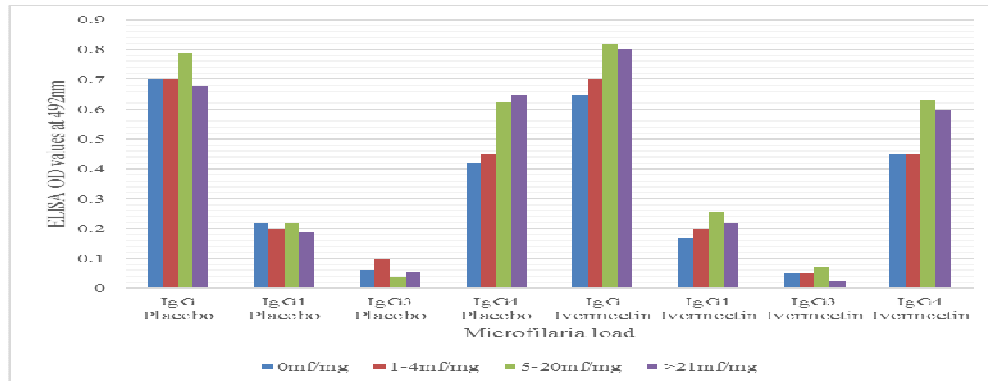


FIGURE 1: ANTIBODY RESPONSES TO MACROFILARIA LOAD IN TREATED AND NON-TREAT SUBGROUPS. ELSA=ENZYME LINKED IMMUNOSORBENT ASSAY, IG= IMMUNOGLOBULIN, MF= MICROFILARIA, NM= NANOMETRE, OD= OPTICAL DENSITY.

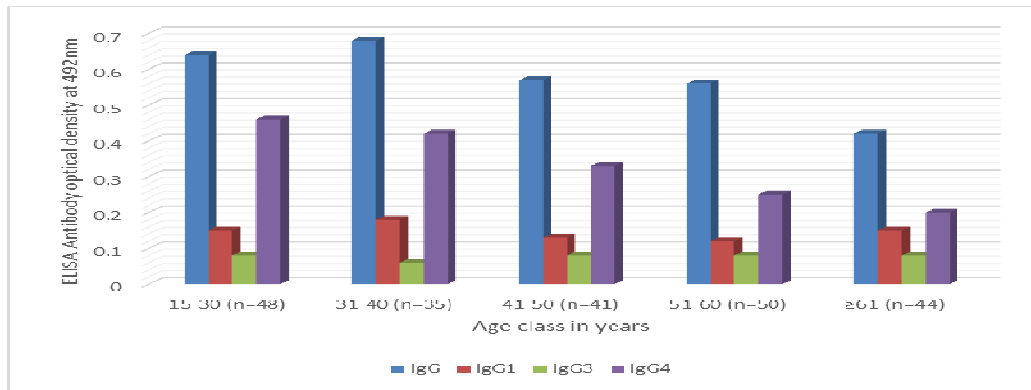


FIGURE 2: ANALYSIS OF ANTIGEN AND ANTIBODY REACTION IN TREATED ONCHOCERCIASIS PATIENTS BY AGE

In the treated group, a slight negative correlation in IgG antibody levels and severity of disc colour with mean OD values of 0.26 ± 0.22 in those graded as having no OND (disc 1, $n=86$) and 0.17 ± 0.19 for those with severe changes (disc 3, $n=49$). There was no significant difference ($p > 0.05$) between cases and control for antibody response to optic nerve-DOC extract (only IgG and IgG4 were evaluated) neither between treated nor untreated groups in antibody levels.

DISCUSSION

Involvement of the parasite and non-parasite specific antibody responses in onchocerciasis has been studied. Active humoral immune response elicited by the antigenic stimulation of dead microfilariae has been demonstrated in this study. High level antibody responses against parasite antigens were more in onchocerciasis patients than in control, and the prominence of IgG than IgA is widely documented. The significant result obtained from the overall assay is observed preferential elevation of anti IgG4 titres in patients' sera. This finding is in agreement with those reported by (3, 12, 13, 14) Most of the IgG were accounted for by IgG4 subclass followed by IgG1 and IgG3. Similar findings were reported in children by (15).

Changes in parasite specific IgE and IgG antibody responses were transiently enhanced at post-diethylcarbamzine (DEC) or Banocide® treatment, and after treatment, parasites possibly release antigens previously hidden from host immune response (16). On the contrary, there was no change in antibodies after ivermectin treatment is a confirmation that the difference may explain possible development of Mazzotti reaction peculiar to DEC treatment, which is very minimal or absent in ivermectin treatment.

It is very likely that the predominant subclass IgG4 may act in blocking hypersensitivity reaction. The clinical status, age and mf density dependency of antibody responses have been documented by other

investigators. Expectedly, reactivity to PBS-extract was more than those of Ov1.9 and Cam 1 antigens, since the formal contains immuno-dominant antigens (13, 17). This may be due to the presence of many epitopes, such as, phosphoryl choline (PC) and other carbohydrate determinants in crude PBS extract. The sensitivity and specificity of IgG4 against PBS-extract was higher than those of Ov1.9, which is an indication that the later stimulates less antibody response (11). Measuring IgG4 response to PBS extract hitherto has been documented to have potential diagnostic value (15). This subclass is non-reactive to phosphoryl-choline (PC), an immunodominant molecule responsible for the majority of cross-reactivity. Although an increase in both IgG and IgM reactivity with Rh-F has been established in loiasis patients with or without glomerulonephritis, the observed slight differences between onchocerciasis cases and controls, deserves further studies to validate if they play a protective role or are just epiphenomena. Onchocerciasis has been listed among the diseases with high risk of detecting Rh-factor by (18). Results obtained from this study did not support the rheumatoid arthritis playing any role in autoimmune disease involving any of the antibody classes (IgA, IgG and IgM). This is in accordance with the held belief that raised levels of IgM, IgG, and IgA RF have been reported in patients with Rheumatoid Arthritis (19). Several groups have reported that a high level of IgA RF is prognostic for a more severe disease outcome (20 and 21).

From all indication, it is obvious that the varied clinical manifestations of onchocerciasis are not due to direct parasite attrition (22). Moreover, it has been established that dead microfilaria elicit bystander response that attract cellular immune mechanism involving cyto-adherence in the process of clearing dead microfilariae with consequence dermal and ocular tissue damage have been documented by (6, 23). It has been shown by (24) that both IgM- and IgG-containing complexes were commonly involved but there was no correlation

between the levels of complexes containing these isotypes. One of the cardinal manifestations of autoimmune disorders is the presence of auto-antibodies and/or self-reacting cells (25), however, the detection of these autoimmune activities are not necessarily associated with clinical findings.

High level antibody responses against parasites antigens showed that IgG was more prominent than IgA. Most of the IgG were accounted for by IgG4 subclass followed by IgG1 and IgG3. This is a possible indication of the predominant subclass IgG4 may act in blocking hypersensitivity reaction (12). The clinical status, age, and mf density dependency of antibody responses have been documented by other investigators (11, 15). Expectedly, sensitivity and specificity of IgG4 against PBS-extract is higher than those of Ov1.9 and Cam 1, which is an indication that they stimulate less antibody responses. Measuring of IgG4 to PBS-extract has been reported to have potential diagnostic value.

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