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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY JANUARY 2009 ISBN 1595-689X VOL 10(1)
AJCEM/200899/20905 -http://www.ajol.info/journals/ajcem
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AFR. J. CLN. EXPER. MICROBIOL. 10(1): 26-37

QUANTITATIVE CHANGES IN ANTIBODIES AGAINST ONCHOCERCAL NATIVE ANTIGENS TWO MONTHS POST-IVERMECTIN TREATMENT OF ONCHOCERCIASIS PATIENTS.

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Running Title: Quantitative changes in antibodies.

Abstract:

Serum antibodies to *Onchocerca volvulus* native sodium duodecylsulphate slat extracted antigens and epitopes recognized by three monoclonal antibodies designated Cam8, Cam22, and Cam28 were measured using indirect (sandwich) and competitive enzyme-linked immunosorbent assay (ELISA). Paired serum samples (n=32) were obtained before and two months post-ivermectin treatment. Those with increases of ten percent and above (≥10%) were 16 (50%) for IgG, 13 (40.7%) for both IgG1 and IgG4. Nine (28.2%) for IgM, eight (25%) for IgG3, IgA with four (12.5%) was the least, while IgG2 was not assayed due to cross-reaction. The higher increases in IgG, IgG1 and IgG4 antibodies in females (n=16) than males (n=16) were significant by T-test of unpaired data (P<0.05). Those without onchocercal skin disease, OSD (n=18) had a significant increase of 20.5±29.6%, with pre- and post-treatment values of 0.59±0.15 versus 0.68±0.13 for IgG antibody (P<0.05). Both IgG1 and IgG4 antibodies for those with OSD (n=14) increased by 16.0±24.8%. Only IgG4 antibody increased with the presence of palpable nodule and higher skin microfilarial density. Trend exhibited by Cam 22 and Cam 8 were similar to that of IgG and IgG4, respectively. In conclusion, while IgG1 and IgG4 were both associated with skin diseases, IgG4 assay proved more suitable for onchocerciasis drug screening.

Keywords: Onchocerciasis, ivermectin treatment, antibodies, and antigens.

INTRODUCTION

Onchocerciasis or river blindness is one of the main causes of preventable blindness in sub-Sahara Africa, including Nigeria. Ivermectin® or Mectizan™ (a microfilaricide) is currently used for mass treatment of people in endemic areas. This strategy is preferred to larvicides and adulticides formerly used for fly control, which have been abandoned because of the negative impact on environment. Infections were known to present with diverse clinical manifestations, even within the same organ. The main trust of immunology is to identify immune responses involved in immunopathology and/or immunopathogenesis of the disease, the

molecules (immunogens) that can induce protective immunity for vaccine production, and antigens useful for immunodiagnosis. It has been suggested that it could serve as tool for drug screening and monitoring the efficacy of treatment (1). In onchocerciasis, humoral and cell-mediated immune (CMI) responses are widely reported to vary from one person to another (2). Hence, the possible etiologic role particularly of parasite-specific antibodies has remained poorly understood. Understanding changes in immune responses after treatment in clinically defined patients could explain its secondary effects on pathology.

Changes in polyclonal, and parasite specific antibodies, proliferative T lymphocyte, and cytokine production have been documented after ivermectin treatment (3, 4, & 5). It has been postulated that immunologically mediated destruction of microfilariae could contribute to the pathogenesis of the disease. Dying parasites initiate local inflammatory reactions, with the result of "bystander" tissue damage, which cumulatively determines host pathology (6 &7). Absence of animal model precludes immunological studies; patients' responses to therapy and risk factors for clinico-pathological changes in human infections are evaluated (8). However, Steel et al. (5) had shown that previously recognized and unrecognized parasite antigens were released into circulation.

We report on the effect of ivermectin® on parasite antigen-specific serum antibodies two months post-initial treatment of individuals. This type of study is useful for indirect quantitative assessment of B-lymphocyte anergy (or tolerance) to parasite antigens and to show if changes in antibodies were associated with gender, age, host parasite burden, and/or pathological sequel.

Materials and methods

Sample population

After explaining to the participant in their dialect through an interpreter from the Local Government Area Health

Department, full consent of individuals were obtained before been enlisted for this study. Serum samples were collected randomly from individuals (n=32) comprising 13 males and 19 females that volunteered before initial ivermectin treatment was administered. Paired sera were obtained 2 months post-treatment. The subjects varied in age with a Sample population mean and standard deviation (SD) of 39.9±15.2 and ranges between 15-69 years. Sixteen of them had palpable nodule and the microfilarial density per skin snip was 26.05±35.13 with a range of 0-141. Among them, those with at least one form of skin clinical signs were n=14 and those without were n=18.

Antigens Detail method of preparing sodium duodecyl sulphate (SDS) extracted crude antigen has been described (9). The extract was supplied to the immunology Research Laboratory, NITR, Kaduna as part of a collaborative study by Dr F. Engelbrecht, Heldelberge University, Germany then a Visiting Scientist.

Monoclonal antibodies:

Three monoclonal antibodies (mAbs) designated Cam 8, Cam 22 and Cam 28 raised in mice were prepared by Engelbrecht *et al.*, (9). Only Cam 28 has been characterized and found sensitive to periodate. It reacts with 120 KD molecular weight antigens. These mAbs were provided by Dr F. Engelbrecht.

SEROLOGY

Serum samples were analyzed same day under similar assay conditions. Reactivity of serum antibodies with antigens and the three mAbs were tested with indirect and inhibition (competitive) ELISA, respectively.

Indirect sandwich ELISA

Immunoglobulin (Ig), IgA, IgM, IgG class and IgG1, IgG3 and IgG4 isotypes antibodies were measured using a modified protocol described by Engelbrecht et al., (9). Briefly, microtitre plates were coated with antigens diluted in carbonate/bicarbonate buffer (pH9.6) at 1:100, and incubated overnight at 4°C.

All other steps were performed at room temperature (RT°C). The unspecific site were "blocked" with 200 µl per well of 1-2% bovine serum albumin (BSA) for 1 hour. Serum was added at 1:80, 1:200, and 1:160 for IgA, IgG, and IgM reactivity to SDS extract. Horseradish peroxidase conjugated to rabbit immunoglobulin anti-human IgG (Dako, Denmark) (code P214); IgM (P215) and IgA (P216) were applied at 1:1000, 1:400, and 1:500 diluted.

For isotypes assays serum was added at 1:500 for IgG1, 1:100 for IgG3 and 1:200 for IgG4. Thereafter, monoclonal antibody obtained from Sigma specific for each isotype, IgG1 (clone HP 6001, 1:2000), IgG3 (HP 6050, 1:8000) and IgG4 (HP 6025, 1:8000) were added at 150µl per well. It was followed by goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (BIORAD) at 1:1000 dilution.

Antigen and antibody reactions were detected by addition of freshly prepared substrate solution containing 200µl orthophenylene diamine (OPD) (from Sigma) in 20µl hydrogen peroxide, 0.1M citric acid and 0.2M Na2HPO4 buffer and allowed to react for 15 minutes. The enzyme reaction was terminated with 30µl per wells 2M H2SO4 for 5 minutes. Optical densities (OD) of wells of microtitre plates were measured in a Dynatech ELISA reader (model MR4000) at 492nm-test filter and 630nm-reference filter.

Inhibition (competitive) ELISA

SDS extract was used at 1:1000 and serum at 1:50, for Cam 8, and 1:25 dilutions for Cam 22 and Cam 28. After the serum step, mAb was added at 1:25 dilutions. All subsequent steps were the same for isotype assay.

Assay control

Optimum concentration of antigens, serum, monoclonal antibodies, and conjugates were determined in a series of pre-titration experiments. The final assays were performed in duplicate. Percentage inhibition was based on the difference between individuals mean OD-value and mean±2SD of the 16 internal control blank wells.

Statistical analyses

The means and standard deviation (mean±SD) for absolute and percentage changes over pretreatment values for group or subgroup were tabulated. The differences between pre-treatment and post-treatment values were subjected to t-test of paired and unpaired data.

RESULTS

Changes in ELISA antibodies after treatment

Percentage increases in antibodies were calculated as the difference between baseline (pre-treatment) and follow-up (post-treatment) optical density (OD)-values over the baseline value. Among the antibody classes, the only remarkable increase was in IgG reactivity. Similarly, the IgG subclasses had increases as shown on Table 1. A change of ten percent and above (≥10%) were recorded in 16 individuals (50%), nine (28.2%) and four (12.5%) of the samples for IgG, IgM and IgA responses to SDS extract, respectively. Among the three IgG isotypes, levels were enhanced in 13 (40.7%) patients for both IgG1 and IgG4, and 8 (25%) for IgG3. The mean±SD of IgG, IgG1 and IgG4 increased from 0.60±0.15, 0.42±0.11 and 0.46±0.09 pre-treatment OD-values to 0.68±0.14, 0.45±0.10 and 0.50±0.07 post-treatment OD-values. The increases represented a percentage change of 15.1±24.9%, 11.1±22.5%, and 13.0±25.4%. On the contrary, decreases of ≥10% were recorded in 16 (50%) of the samples for IgA and 8 (25%) for IgM and 7 (22.0%) for IgG3.

Analysis by sex and age

A significant increases in IgG, IgG1, and IgG4 antibodies in female subgroup (n=19) were higher than the male subgroup (n=13) as shown on Table 1 ($P<0.05$). Only IgG antibody showed an age dependent increase of 10.2±15.3% (0.62±0.16 vs 0.66±0.12) for those ≤40 year (n=16). The 19.6±31.1% (0.58±0.14 vs 0.69±0.16) for individuals ≥41 year old (n=16) was significant. However, the differences between the two subgroups (≤40 year and ≥41 year old) were not statistically significant ($P>0.05$).

Analysis of antibody changes by infection status

There were no remarkable differences between pre- and post-treatment IgG and IgG1 levels. Only IgG4 antibody was significantly higher in the subgroup with (n=16) than without (n=16) palpable nodule (Table 2). Presence of palpable nodule had affected IgM antibody increase in those with 10.4±19% as against the 1.0±29.7% for those without nodule. Similarly, only IgG4 antibody level was increased among persons having high skin microfilariae (≥16mf/snip, n=14)) compared to the subgroup with low skin mf (≤ 16mf/snip, n=18). The percentage difference of 21.9±35.8% vs. 5.6±6.1%, respectively was statistically significant by t-test of unpaired data (P<0.05).

Evaluation of binding of mAbs to worm extract

The increased capacity of serum to inhibit binding of three monoclonal antibodies designated Cam 8, Cam 22, and Cam 28 to SDS extract was evaluated. The mean±SD of OD-values before and after treatment were 0.21±0.04 vs. 0.19±0.06) for Cam 8, with 0.39±0.12 vs. 0.34±0.14 for Cam 22 and no change in serum inhibition of Cam 28 (0.31±0.10). Post-treatment changes in serum inhibition of ≥10% occurred in 13 (40.7%) for Cam 8, with Cam 22 having 15 (48%

Effect of onchocercal skin disease on antibody levels

Presence of onchocercal skin disease (OSD) in individuals influenced the levels of antibodies. Table 3 shows that those presenting with OSD (n=14) had increased IgG1 and IgG4 than those without OSD (n=18). The reverse was the case for IgG antibody with a mean+SD percentage change of 20.5±29.5% and 7.7±13.9% for those without OSD compared to those having OSD. A change in IgG mean±SD of OD-values before and after treatment from 0.59±0.15 to 0.68+0.13, was statistically significant by t-test of paired data (P<0.05).

Serum inhibition

Cam 28 had 8 (25%) of the samples (n=32). An age dependent increase in inhibition of Cam 22 was recorded, but

not statistically significant (P>0.05). In addition, the difference was influenced by presence of nodule and number of skin microfilariae. Increase in Cam 22 inhibition was more in subgroup without OSD compared to Cam 8 that had higher increase in the subgroup having OSD.

Table 1: Changes in antibodies two months after ivermectin treatment analyzed by sex.

Mean±SD	IgG	IgG1	IgG4
Pre-dose (n=32)	0.55±0.15	0.39±0.11	0.43±0.10
Post-dose	0.65±0.16	0.44±0.12	0.49±0.08
Percentage changes (%)	20.5±28.4*	19.0±25.4*	18.2±30.6*
Pre-dose, male (n=13)	0.67±0.13	0.47±0.10	0.50±0.05
Post-dose	0.71±0.09	0.46±0.09	0.52±0.04
% changes	9.4±15.11	No change**	4.3±6.8**
Pre-dose, female (n=19)	0.562±0.15	0.39±0.11	0.43±0.10
Post-dose	0.65±0.16	0.44±0.12	0.49±0.08
% changes	18.63±29.16*	18.6±25.5**	18.5±31.0**

Note: *Observed intra difference within a subgroup (pre-dose vs post-dose) and **inter differences between subgroups (male and female) were statistically significant (P<0.05) by t-tests of paired and unpaired data, respectively. SD= standard deviation.

Table 2: Changes in antibodies two months after ivermectin treatment depending on palpable nodule.

Mean±SD	IgG	IgG1	IgG4
Pre-dose nodule –ve (n=16)	0.59±0.17	0.41±0.13	0.46±0.09
Post-dose	0.65±0.16	0.42±0.11	0.49±0.09
Percentage change	13.8±32.3	10.3±28	7.3±12.4
Pre-dose nodule +ve (n=16)	0.62±0.12	0.43±0.10	0.46±0.09
Post-dose	0.70±0.11	0.48±0.10	0.52±0.04
Percentage changes	16.0±14.1*	11.5±15.2	18.1±32.8*

Note: * Intra differences in antibody levels were statistically significant (P<0.05) by t-test of paired data. The –ve = negative and +ve = positive subgroups

Table 3: Changes in antibodies after treatment analyzed by presence of onchocercal skin diseases.

Mean±SD	IgG	IgG1	IgG4
Without OSD (n=18)			
Before treatment	**0.59 ± 0.15**	**0.41 ± 0.11**	**0.47 ± 0.07**
After treatment	**0.67 ± 0.12**	**0.43 ± 0.11**	**0.50 ± 0.06**
% changes	**20.5 ± 29.6***	**7.0 ± 19.7**	**8.0 ± 11.4**
With OSD (n=14)			
Before treatment	**0.63 ± 0.14**	**0.43 ± 0.12**	**0.45 ± 0.10**
After treatment	**0.68 ± 0.16**	**0.47 ± 0.10**	**0.50 ± 0.08**
% changes	**7.7 ± 13.9**	**16.0 ± 24.8***	**18.8 ± 35.4***

Note: OSD= Onchocercal skin diseases. *The intra differences were statistically significant (P<0.05) by t-test of paired data.

Discussion

Antibodies to *O. vulvulus* adult worm extract were quantified in ELISA in same patients' (n=32) paired sera taken before and two months after initial ivermectin dosing. Appreciable increase in antibody levels that were observed in some and not in others only confirmed the variability within an individual, and individual differences in immune response to onchocerciasis infection

(6). The poor performance of IgA followed by IgM and IgG3 antibodies clearly point to non-stimulation or specific tolerance by many infected individuals. Other possibilities include the fact that IgA and IgM have relatively shorter half-life and they are produced in low concentration. While IgM is mainly a primary response compared to the secondary or anamnestic response induced by ivermectin treatment. In addition, the rates at which the three antibodies were catabolised must have by far outweighed the rates of their syntheses. The combination of these factors is strongly believed to underlie the decreases recorded for the three antibodies. Yet, no remarkable trend was exhibited by the observed decline.The enhanced antibody titers in females than the males following treatment

could not be explained. Whether this connoted gender difference in immunological reaction to the drug calls for further investigation. For long, gender related differences to infection, immune responses, and clinical manifestations have all been suspected. Our data clearly showed that parasite materials librated after ivermectin treatment evokes variable secondary or anamnestic responses resulting from antigenic stimulation.

A large number of microfilariae from superficial layers of skin were reported to be sequestered into deeper connective tissues, fats and lymph node following treatment (10). This might be one reason why vigorous responses do not accompany ivermectin treatment. Previous research works undertaken along this line have shown changes in qualitative immunoblot analyses of IgG and IgE antibody to previously recognized and unrecognized antigens, which occurred in 50% and one third of the patients, respectively. Others (3) had observed the same frequency of antibody change two weeks after diethylcarbamazine (DEC) and ivermectin treatment, respectively. From our study, a similar quantitative trend two months post-treatment was established in which 50% of onchocerciasis patients presented with antigen-specific immune response. Whether the observed increases in antibodies provoke concomitant immunity in those individuals remains a matter of conjecture.

On the contrary, the other specter of individuals exhibited immune unresponsiveness or tolerance. Reports showed that acquisition of *O. volvulus* could not occur constantly over time, which strongly emphasize the importance of immunosuppressive processes in man. Suppression of parasite-specific immunity leads to parasite establishment rates, which increase along with the parasite burden (11). Therefore, we can only speculate that there was B-cell tolerance to antigens released after treatment, since not all individuals responded with increased antibodies. Normally, if antigen is present in high enough concentration for a long period it is

possible for immune tolerance be induced following a state of specific immunity (12). In chronic infection, clonal exhaustion may diminish the capacity of immune system to respond following treatment (3). Circulating antigens that form complexes with antibodies in onchocerciasis (1 & 13) were capable of inducing some form of tolerance. All these notwithstanding, what remains unclear are whether the assumed state of tolerance involved T-lymphocytes in clonal elimination or blocking. Already, Steel *et al.,* (5) have shown that T cell proliferative response was enhanced 6 months after ivermectin treatment but reverted back to pretreatment values in 1year. It is very likely that the adult worm extract used contains mostly low molecular weight (LMW) antigens (9). The antigens could be either proteins or polysaccharide-protein complexes that can provoke T-dependent antibody responses.

The remarkable increases in IgG4 antibody associated with infection intensity based on skin microfilaria density and palpable nodule were statistically significant ($P<0.05$). The increase was apparently in agreement with earlier reports correlating the levels of this isotype with parasite burden (14-15). It seems IgG4 is a likely candidate of choice for antibody assays for drug screening. Again, it plays the role of blocking hypersensitivity reaction induced by IgE and preventing microfilaria clearance as well (7, 16, 17 & 18). Earlier work by Whitworth et al., (19) showed there was no reduction in itching 2 months after initial ivermectin treatment. Higher increases in IgG1 and IgG4 antibody level of patients having skin diseases support the fact that hypersensitivity induced by the former is unaffected by immune blockade of the later.

Among the three mAbs, only Cam 22 showed an appreciable overall increase in percentage inhibition. The observed increases in older age (≥41years) and palpable nodule positive subgroups compared with those of younger age (≤40years) and palpable nodule negative subgroups respectively, were not statistically significant ($P>0.05$).

Important inferences from this study are (i) the gender sensitivity to antibody changes, (ii) association of IgG1 and IgG4 with skin disease, and (iii) the potential of IgG4 indirect and Cam 22 competitive ELISA for drug screening. In addition, the studies showed there was selective unresponsiveness to antigenic epitopes defined by the mAbs. In future, this type of study will become difficult to carry out, since most endemic areas would have been covered by the on-going community directed treatment of onchocerciasis with ivermectin**.**

Acknowledgements

We thank Dr. I. Halid, the then Director-General, NITR, for approving fund for this project. We appreciate the inputs from European Economic Community (EEC) grant to Dr. Edeghere. We thank Mr. Peter Damboi the Ophthalmic Nurse, formerly of National Eye Centre, Kaduna, Mal. M.S Mohammed, the Rural Health Superintendent and Mr. James Gazama, Technical Assistant from NITR for their various roles in the project.

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