PD-ABI-871
3H-24

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18 OCT. 1985

Dr. Joe Stockard,
Tropical Medical Advisor
Agency for INternational
Development,
Washington, D.C. 20523

Dear Dr. Stockard,

Re : AID grant # 3H.24. ONchocerciasis in
the United Republic of Cameroon :
the development of ELISA for immunodiagnosis
P.I. David A. Levy Co-P.I. Jacob L. NGU

Dear Dr. Stockard,

Please find enclosed our Final Report on the above AID grant. I should be grateful if you would contact me if further information is required.

Yours sincerely,

18 OCT. 198

Professeur J. L. NGU.

cc :
- David A. Levy M.D
- USAID Yaounde

REC'D IN R&D/R
NOV 13 1993

PD-ABI-871

Project title: Onchocerciasis in the United Republic of Cameroon: The development of ELISA for Immunodiagnosis.

Project Identification: 3H.24

This is Final Progress Report (#6)

Submitted to: Dr. Alfred Buck, AID Project Officer,
S&T/HEA
SA-18
Agency for International Development
Washington, D.C. 20523

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1 reprint

SPECIFIC AIMS OF THE STUDY

The objective of this study was to develop an enzyme-linked immunosorbent assay (ELISA) and anti-O. volvulus mouse monoclonal antibodies for the immunodiagnosis of asymptomatic O. volvulus infections in Cameroon.

Onchocerciasis is hyperendemic in over 14 villages which are situated along the Sanaga River Basin, a well-known breeding ground for the blackfly vector in Cameroon. Our study was confined to subjects living permanently in only two of these villages, namely, Batchenga and Nachtigal (Fig.1). These hyperendemic foci were strategically selected on the basis of proximity to the laboratory (65km).

During the last 2 years, we conducted thorough clinical, parasitological and serological studies on 250 permanent adult male and female residents of these two villages (Boto, Levy and Ngu, 1985). The residents are largely peasant farmers (94%) with a few students (3%), carpenters (2%) and others (1%). The study group fell into 4 distinct categories based on these clinical and parasitological findings: a) subjects presenting with both skin microfilariae and onchocercomata; b) subjects presenting with skin microfilariae, but no onchocercomata; c) subjects presenting with onchocercomata, but no skin microfilariae; and d) exposed subjects manifesting only localized disease (if any), and no detectable skin microfilariae or palpable nodules. A sample of cohorts representing these distinct clinical and parasitological profiles is illustrated (Table 1). Subjects in category "D" on this table presented with neither skin mf nor nodules. Infection in this group could not be established unequivocally on clinical criteria alone. The intriguing observations regarding these individuals were as follows: they all presented with pruritus and reacted positive in the immediate skin hypersensitivity test in which O. volvulus antigens isolated by immuno-affinity columns were used; they registered the highest titers of serum anti-O. volvulus antibodies we have tested in ELISA and in Ouchterlony double diffusion gels (Boto, Levy and Ngu, 1985). It is unlikely that these individuals got exposed and then recovered from O. volvulus through self-cure. No sterile immunity has yet been demonstrated in any human or animal model of filariasis. We think that the cohorts in category "D" are either at the prepatent phase of O. volvulus infections, or they may harbor unisexual or other asymptomatic forms of the infection. Clearly, this category of subjects stand to gain the most from an immunodiagnostic test which is based on the use of monoclonal antibodies to detect parasite antigens in urine, saliva, plasma or serum. In this final progress report, we describe the success and limitations of our attempts to develop an ELISA methodology and murine anti-O. volvulus monoclonal antibodies to meet this diagnostic need. The studies were conducted partly at The Johns Hopkins University but largely in the W.H.O./TDR laboratory at The University Centre for Health Sciences (C.U.S.S., for short) in Cameroon.

EXPERIMENTAL PROCEDURES

A. The development of ELISA for the detection and/or quantitation of O. volvulus antibodies/antigens.

In the preceding studies, we had used the dog heartworm model to perfect an ELISA for detecting filarial antibodies and antigens (Boto, D'Antonio and Levy, 1984; Boto, Powers and Levy, 1984). This highly sensitive technique was subsequently modified with relative ease for the detection of human anti-O. volvulus antibodies in serum (Boto et al., 1985). The modification involved the use of peroxidase-labeled anti-human IgM (Fc-specific), IgG (Fc-specific), IgE or Ig (A+M+G). The sources of the test antigens of O. volvulus for ELISA were described in detail in progress report #3 (Mar. 15, 1984) and in the manuscripts attached (Boto et al, 1985; Lando et al., 1985). Briefly, these were prepared by the use of collagenase for the selective digestion of human tissues which constitute the nodule. The process results in the liberation of large numbers of viable adult parasites from the nodule (Boto et al., 1985). The antigens were obtained by the sequential passage of the detergent extracts of the worms through a human anti-L. loa antibody affinity column, acid elution from a human anti-O. volvulus antibody column and then collected in the void volume of a rabbit anti-human sephacryl bead column. The resulting preparation showed no significant cross-reactions when tested against L. loa antibodies or ascaris antigens in ELISA. The ELISA played a vital role in our subsequent studies of the antibody responses in onchocerciasis subjects presenting with distinct clinical symptoms (Boto et al., 1985; Lando et al., 1985). In situations of no alternative, we used it to establish non-clinical exposure to O. volvulus in a number of suspected cases referred to our laboratory by clinicians in two major hospitals in Yaounde: The Central Hospital and The University Teaching Hospital (more commonly known as C.H.U.).

The ultimate aim of the study, however, was not focussed on the detection of anti-parasite antibodies. The test that we had developed at this point had to be turned around so that it could detect O. volvulus antigens in the body fluids. The detection of these antigens at high sensitivity and specificity required the use of the monoclonal antibodies as probes.

B. The development of murine anti-O. volvulus monoclonal antibodies.

We considered two philosophically different approaches for developing mouse monoclonal antibodies which detect parasite antigens in the body fluids. One alternative was to use a mixture of soluble proteins that have been extracted from the worms for the immunization of mice. The extract is bound to contain a large number of antigenically distinct molecular species. Because the immune response of the mouse cannot be predicted, there is no guarantee that any of the monoclonal antibodies developed in this manner would be of use in immunodiagnosis. The second alternative, which we followed, was to isolate those O. volvulus proteins which elicit antibody responses in the human host, for the immunization of mice. This latter approach was based on the following rationale:

O. volvulus antigens which exist in soluble form in the human serum or tissues could be favored to elicit antibody responses in the host. Molecules which form integral structures of the worm or which do not detach from the organism cannot be processed by the macrophages and are thus poorly immunogenic, if at all. Xenogeneic monoclonal antibodies developed against the isolated antigens can be expected to detect the same antigens in the body fluids.

The following procedure was followed for the production of anti-O. volvulus monoclonal antibodies:

a. Isolation of O. volvulus proteins which are antigenic in the human host; human anti-O. volvulus antibody affinity columns were used.
b. Immunization of mice with the isolated antigens.
c. Production of mouse hybridomas.
d. Screening of mouse hybridoma culture supernatants for anti-O. volvulus antibodies.

These experiments are schematically illustrated in Fig. 2.

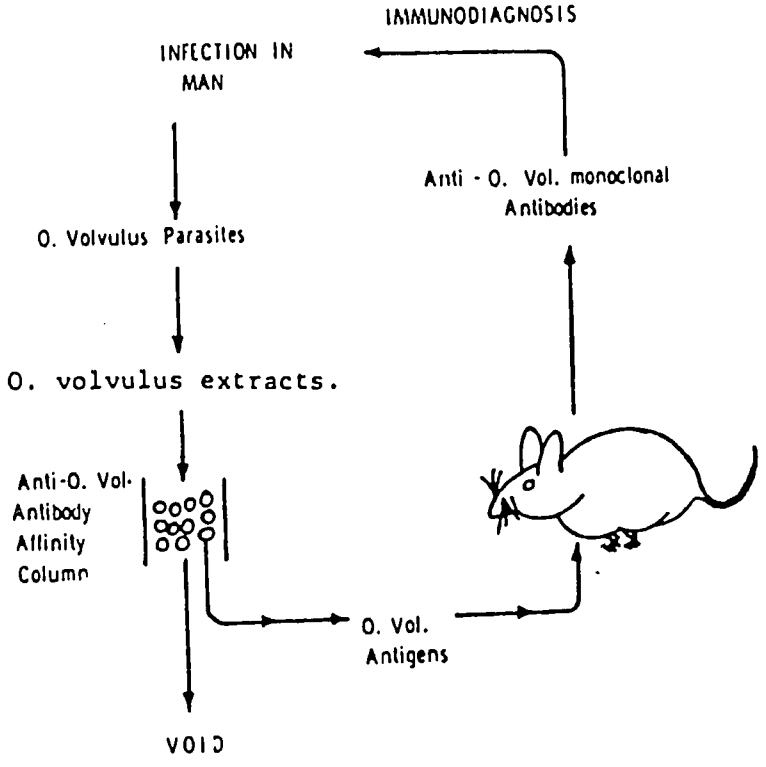

Fig. 2. A scheme for the development of murine anti-O. volvulus monoclonal antibodies for the immunodiagnosis of onchocerciasis.

a. The isolation of O. volvulus antigens for the immunization of mice.

In the original proposal, we had planned to use immunoabsorbent columns to isolate O. volvulus antigens from human serum or urine samples for the immunization of mice. Because the effort would consume valuable time, antibodies and reagents, it was essential to demonstrate the presence of these antigens in substantial amounts in these fluids. In the first place, we fractionated the serum and urinary proteins by SDS-PAGE, blotted them onto nitrocellulose sheets and then stained them with immunoperoxidase reagents to detect parasite antigens. The background of the reaction was too loud to permit antigen identification. The reaction was due to the dominant presence of human Ig in the samples. The adult O. volvulus parasites subsequently became our primary source of proteins from which to isolate the required antigens for the immunization of mice. To do this, the sera of 132 onchocerciasis subjects were examined for high titered anti-O. volvulus antibodies for the construction of immuno-affinity columns (progress report #3, Mar. 15, 1984; Boto et al., 1985). O. volvulus antigens were positively selected by the binding of non-ionic detergent extracts of the adult worms onto the human antibody affinity column, followed by acid elution, dialysis against distilled water and lyophilization. In progress report #4 (15 July, 1984), a similar preparation was shown to be potent in inducing the immediate skin hypersensitivity reaction in those affected, and in binding human anti-O. volvulus IgM, IgE and IgG antibodies in ELISA. When analyzed by SDS-PAGE and coommassie blue staining, the preparation displayed at least 12 discrete protein bands (Fig. 3).

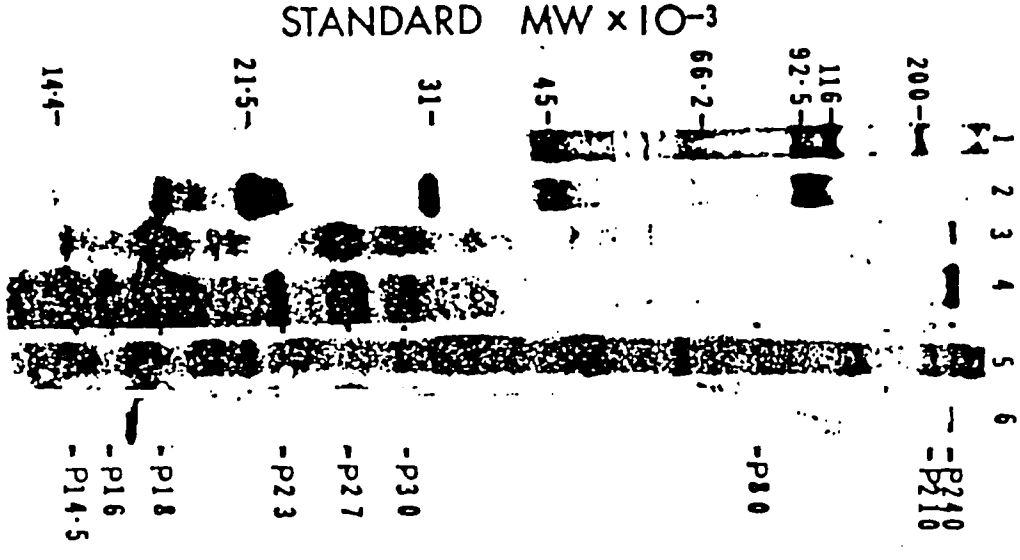

Fig. 3. S.D.S - Polyacrylamide gel electrophoretic analysis of O. volvulus antigens isolated by acid elution from human anti O. volvulus antibody affinity columns (lanes 3 and 4); total extracts of adult O. volvulus worms (lane 5); protein eluted from a rabbit anti-whole human serum Ig affinity col (lane 6); standard molecular weight markers (lanes 1 and 2)

b. The immunization of balb/c male mice with the isolated antigens, the fusion of the immune spleen cells with the P3X63Ag8.652 balb/c myeloma cell line, as well as the screening of the hybridoma culture supernatants for specific antibodies were described in detail in progress report #5 (6 Feb. 1985). For the screening of the culture supernatants, the ELISA which was described in the early section of this report had to be modified for the detection of mouse, instead of human, anti-O. volvulus antibodies. This merely involved substituting peroxidase-labeled anti-mouse Ig (A+M+G) for peroxidase-labeled anti-human Ig. A total of five hybridomas which secrete anti-O. volvulus antibodies were developed in two separate fusions (progress report #5). One of these cell lines ceased Ig secretion after about 1 month in culture. The following cell lines continued to secrete specific antibodies: CAM-1, CAM-2, CAM-3 and CAM-4; they were frozen in liquid nitrogen, pending additional experiments. We were, however, baffled that the anti-O. volvulus antibodies secreted by 3 of the 4 cell lines are all of the IgM class (Table 2), just as those reported by Dr. Andre Capron of Pasteur Institute, Paris.

Table 2. Isotype analysis (ELISA) of anti-O. volvulus antibodies secreted in culture medium by three murine hybridomas. The figures represent O·D· 490 readings of triplicate determinations for each Ig isotype.

Hybridoma cell line	Ig class tested			Ig class detected
	IgA+M+G	IgM	IgG	
CAM-1	0.87 0.70 0.76	0.35 0.37 0.37	0.09 0.06 0.05	IgM
CAM-2	1.57 1.49 1.24	1.35 1.27 1.10	0.11 0.12 0.11	IgM
CAM-4	0.95 0.89 0.89	0.44 0.41 0.45	0.05 0.09 0.07	IgM

The Ig isotype of the anti-parasite antibodies secreted in the culture medium by these cells was tested by ELISA, using peroxidase-labeled rabbit anti-mouse IgM (Fc-specific) or IgG (Fc-specific). We do not know whether the IgM is selected or favored by the nature of the immunogen used, or by any immunological phenomena in mice or by the immunization regimen we used.

C. Experiments pending.

The hybridoma cell lines that have been cryopreserved in liquid N_2 will have to be cloned, and then expanded in vitro or in vivo in the form of ascitis tumors in syngeneic balb/c mice in order to obtain large quantities of homogeneous monoclonal antibodies. Before they can be used for the development of the immunodiagnostic test, their specificties and cross-reactivities have to be defined in terms of the target O. volvulus or other filarial antigens they recognize. The mf, L_3 and the adult parasites could possibly be used to assess the expression of these antigens at distinct stages of metamorphosis of the organism. Once they are fully characterized, the monoclonal antibodies can then be incorporated in either a sandwich ELISA or in a reverse passive hemagglutination test to diagnose asymptomatic onchocerciasis. The project needed another 2-3 years to accomplish the ideal goal of developing a rapid, simple and sensitive immunodiagnostic test.

D. Other benefits of the project to Cameroon

Apart from the supply of reagents and new equipment which made this study possible at C.U.S.S., the following benefits were derived from the project:

i. To the subject population: The project provided funds that were used to hire physicians and technicians who conducted thorough medical, parasitological and serological examinations on the 250 subjects who participated in the study; and for the purchase of anesthetics, antibiotics, etc., for nodulectomy. Nodulectomy is the only method of reducing the adult worm burden in those affected. About 100 cohorts underwent therapeutic nodulectomy in the course of the project. Both the medical exam and nodulectomy were conducted without charge to any subject. Subjects that needed therapeutic remedies for other parasitic infections, notably ascariasis (at least 65% of the total number examined), and to a lesser extent schistosomiasis, were documented and either supplied with prescriptions to be filled elsewhere, or directed to other medical centers with better facilities. Chemotherapy for ascariasis is quite cheap and was occasionally supplied to the subjects when available. These inducements were effective in maintaining a high level of attendance and support from both the cohorts and the local medical assistants at Batchenga dispensary.

ii. Manpower training: Dr. Gabriel Lando (local post-doctoral fellow) and Mrs. Patience Moffaha (research assistant) were trained in a) the derivation of O. volvulus mf or adult parasites from the human host, b) preparation of antigens from them, c) immunochemical techniques, including ELISA, protein blotting methods, immunodiffusion tests, SDS-polyacrylamide gel electrophoresis, etc. One of the manuscripts which are attached (Lando et al., 1985) and another one which is under preparation by Dr. Rose Leke of C.U.S.S., could be considered as rough estimates of the success of the project in this respect. It seems evident that these individuals will be able to push forward certain aspects of the project when new funds for reagents become available. In addition, the project procured an Apple IIe word processor in which Mrs. Regine Ntam, Professor Ngu's secretary, was subsequently well-trained.

iii. Teaching responsibilities. Dr. William Boto, the resident investigator at C.U.S.S., gave laboratory sessions and several lectures in immunology to the 2nd and 3rd year medical classes, as well as in a bi-annual international immunology course sponsored by the W.H.O. in Yaounde, Cameroon. He did not ask or receive extra pay for these services.

LITERATURE CITED

1. Boto, W.M.O., D.A. Levy and J.L. Ngu. (1985). Antibody responses in onchocerciasis subjects presenting with distinct clinical symptoms. (Submitted).

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3. Boto, W.M.O., K.G. Powers and D.A. Levy. (1984). Antigens of Dirofilaria immitis which are immunogenic in the canine host: detection by immuno-staining of protein blots with the antibodies of occult dogs. J. Immunol. 133:975.

4. Lando, G., W.M.O. Boto and J.L. Ngu. (1985). Antigens and allergens of Onchocerca volvulus which are recognized by the antibodies of the infected human host. (submitted).

*supported by U.S.-AID grant 3H.24

Table 1. A sample of Batchenga and Nji populations presenting with distinct clinical and parasitological profiles. Nji is a focus in Nachtigal.

Subject Code	Age (yrs)	$\bar{X}\pm$S.D.	Time of Residence (yrs)	$\bar{X}\pm$S.D	Nodules	Skin mf.	Subject category
N1	41		9		+	+	
N2	46		7		+	+	
C1	32		31		+	+	
C4	51		30		+	+	
C14	32		10		+	+	
C22	40		2		+	+	
C23	60		60		+	+	
C27	60	45±10	60	30±18	+	+	A
C28	45		45		+	+	
C30	40		40		+	+	
C31	59		59		+	+	
C36	35		35		+	+	
8	37		12		+	+	
C40	62		20		+	+	
C48	34		34		+	+	
C49	43		25		+	+	
C51	53		34		+	+	
C20	45		45		+	−	
C32	35		35		+	−	
C44	45		45		+	−	B
C50	19	37±11	19	36±12	+	−	
C57	40		N.D.		+	−	
C2	44		17		−	+	
C3	49		48		−	+	
C5	63		13		−	+	
C6	43		41		−	+	C
C7	21		10		−	+	
C10	M.D.		10		−	+	
C12	60	43±14	60	26±17	−	+	
13	46		46		−	+	
C15	66		20		−	+	
C17	50		22		−	+	
C29	30		30		−	+	
C37	34		34		−	+	
C39	31		8		−	+	
C41	45		25		−	+	
C45	20		7		−	+	
N3	46		2		−	−	
C9	20		4		−	−	
C11	21		20		−	−	
C16	40	33±13	40	20±17	−	−	D
C18	22		5		−	−	
C21	40		15		−	−	
C46	50		50		−	−	

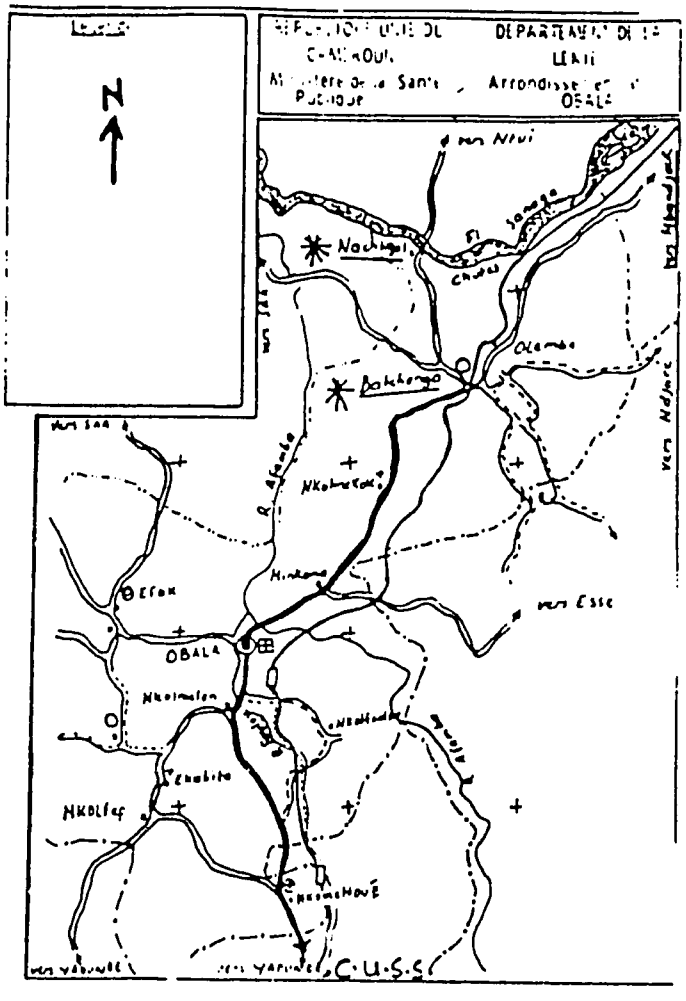

Fig. 1. Map locations of Batchenga (*) and Nachtigal (*) villages relative to Sanaga River Basin and to the Laboratory at C.U.S.S. (65KM away).

Title

Antigens and allergens of *Onchocerca volvulus* recognized by the antibodies of the infected human host.

Running title

Antigens and allergens of *Onchocerca volvulus*

AUTHORS

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FOOTNOTES

1. Supported by grants from the U. S. Agency for International Development.

2. Correspondence: Dr. William M.O. Boto, Department of Immunology and Infectious Diseases, The Johns Hopkins University, School of Hygiene and Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, U.S.A.

3 Abbreviations used in this paper :

O.V. *Onchocerca volvulus* ; mf microfilaria(e) ; L3, infective third stage larvae ; W. bancrofti, *Wuchereria bancrofti* ; B. malayi, *Brugia malayi* ; L. loa, *Loa loa* ; PBS, phosphate buffered saline ; AF-3, *O. volvulus* antigens isolated by immuno-affinity columns ; DS10, a pool of 10 sera obtained from the diplomatic personnel.

ABSTRACT

In a previous communication, we had reported the presence of high levels of serum anti-Onchocerca volvulus IgM, IgG or IgE antibodies in subsets of onchocerciasis subjects who presented with distinct parasitological evidences and clinical symptoms of the infection. In the present study, we describe the profiles of antigens and allergens which are recognized by the IgM, IgG or IgE antibodies of the subjects presenting with the variable parasitological manifestations. The antigens and allergens reacting with these immunoglobulin isotypes were identified by immunoperoxidase staining of O. volvulus proteins which had been transferred from one-dimensional SDS-Polyacrylamide gels onto nitrocellulose sheets. The IgG, IgM or IgE antibodies recognized 19, 13, and 8 discrete antigens or allergens, respectively, on the nitrocellulose blots. These molecules ranged from 10 to 200 kilodaltons (Kd) in size. When the reactivities of antisera from the distinct clinical or parasitological groups were compared, the IgG, IgM or IgE antibodies from microfilariae negative donors recognized the widest spectrum of antigens or allergens at the greatest intensities, in comparison to those of subjects who were microfilariae positive, nodule negative or microfilariae positive and nodule positive. Lastly, all the 8 major allergens recognized by the IgE antibodies were also detected at high intensities by IgM or IgG of the same individual. We conclude that a) serum anti-O. volvulus IgM, IgG and IgE antibodies of subjects presenting with variable parasitological evidences recognize distinct profiles of O. volvulus antigens and allergens, and b) all O. volvulus allergens also elicit IgM or IgG responses in the same human host.

INTRODUCTION

Onchocerca volvulus is a parasitic filarial nematode which causes the disease called onchocerciasis or river blindness in an estimated 20 to 40 million human beings in the world. The vast majority of these afflictions occur in endemic regions of tropical and subtropical Africa, Latin America, and the South Arabian peninsula. The maturation of the parasite involves the development of the microfilariae (mf) into the infective 3rd stage larvae (L_3) in the female blackfly (Simulium damnosum). Once inoculated into a healthy host as the arthropod vector takes another blood meal, the L_3 mature into additional ill-defined developmental stages before molting to become the adult worms. The gravid adult organisms give birth to and shed thousands of the mf in the host during an estimated 15 years of fecundity. Diagnosis is based on the demonstration of the mf in snips of skin obtained from suspected cases (1,2) and on the presence of palpable skin nodules (onchocercomata). These skin nodules contain the adult organisms and the numerous mf that they produce (3).

The major pathogenesis of O. volvulus infections appears to be due to a) the virulence or parasitic mode of life of the mf, and b) the severe hypersensitivity reactions which are induced in the human host. It has long been speculated that the mild forms of the disease variously called "sowda" or "localized" asymptomatic onchocerciasis (4,5,6) are due to the numerical reduction or total elimination of the mf by specific antibodies and/or cellular immune mechanisms of the host. In a previous report, we demonstrated distinct patterns of anti-O. volvulus antibody responses in subsets of subjects presenting with variable clinical symptoms and parasitological profiles of the infection (Boto et al, submitted). The aim of the current

study was to define in molecular terms, the species of O. volvulus antigens/allergens which are recognized by serum antibodies obtained from subsets of subjects manifesting these variable clinical and parasitological patterns of the infection. The significance of these studies is to identify the immune mechanisms which appear to limit or exacerbate the clinical course of O. volvulus infections in the human host.

MATERIALS AND METHODS

Sources of anti-O. volvulus antisera.

Onchocerciasis is hyperendemic in over 14 villages which are situated along the Sanaga River Basin, a well-known breeding ground for the blackfly vector in Cameroon. The subjects who donated the antisera for the studies were identified clinically and by skin snip test for mf. Negative control sera were obtained from the diplomatic personnel within 3 months of their first arrival in Yaounde, Cameroon.

Sources of O. volvulus antigens for ELISA and for immunochemical studies.

Viable adult O. volvulus worms were recovered by the selective digestion of patients' nodules using collagenase (3). The enzyme completely digests human collagen which constitutes the nodule but is without effect on the parasite therein. In some cases, the worms were dissected mechanically from the nodules that had been extirpated from patients within the previous 3-12 hrs. Following exhaustive washing in PBS, 1 gram (wet weight) of the organisms were suspended in 2 ml of extraction buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.02 mM sodium azide, pH 7.39) containing 2 mM phenylmethyl sulfonyl fluoride and

1% triton X-100. The suspension was frozen to -20°C and the resulting ice was crushed to powder by force. The ice cuts and disintegrates the worms completely, whereas sonication will not. Insoluble material was sedimented at 100,000 x g for 1 hr at 4°C. The test antigens for ELISA were prepared by the sequential passage of the supernatant through a human anti-*Loa loa* antibody affinity column, acid elution from a human anti-*O. volvulus* immuno-affinity column and then recovered in the void volume of a rabbit anti-whole human serum antibody affinity column. The procedure rendered the antigens specific for the detection of anti-*O. volvulus* antibodies (Boto *et al*, submitted). The rest of the supernatant of the worm extract was dialyzed against two changes of 15 mM Tris buffer, pH 7.5, containing 75 mM NaCl, and then against 1.5 mM Tris buffer containing 7.5 mM NaCl, pH 7.5. The dialysate was lyophylized and reconstituted in 2 ml of deionized water and then stored at -20°C till used for gel analysis.

The quantitation of anti-O. volvulus antibodies by ELISA.

ELISA for anti-*O. volvulus* IgM, IgG and IgE antibodies was conducted as previously described (Boto *et al*, submitted) using peroxidase labeled goat anti-human IgM (Fc-specific), IgG (Fc-specific) or IgE and O. phenylenediamine / H_2O_2 as the indicator substrate. Peroxidase labeled anti-human IgE Fc portion was not available.

SDS-PAGE of the worm extract.

A slab gel (0.15 cm x 12.5 cm x 24 cm) composed of 10-20% acrylamide gradient and 0.1% SDS was prepared and used according to Laemmli (7). The worm extract was added to sample buffer and then heated in boiling water for 3 min. Two and a half mg of *O. volvulus* proteins were loaded into a single gel lane of 12 cm width. Electrophoresis was conducted at 90 volts for 14 hrs.

Electro-blotting of proteins from SDS-PAGE gels to nitrocellulose sheets.

Protein bands were transferred from the slab gels and immobilized on nitrocellulose sheets essentially as described by Towbin (8).

Immunoperoxidase staining of antigens on nitrocellulose sheets.

Nitrocellulose sheets which had been sensitized with O. volvulus proteins were cut into 0.4 cm strips. The strips were incubated with the test or control sera (9) followed by peroxidase-labeled goat anti-human IgG (Fc-specific), IgM (Fc-specific) or IgE (Cappel Cochranville, PA., U.S.A.). The antibody binding polypeptides were visualized following the addition of a solution of 2.4 diaminobenzidine and hydrogen peroxide to the strips (9).

RESULTS

The test sera were selected from a large lot (80 samples) donated by subjects who presented with the following parasitological and clinical evidences of O. volvulus infections: a) subjects with no skin microfilariae and no palpable nodules; b) subjects with no skin microfilariae and with palpable nodules; c) subjects with skin microfilariae and no palpable nodules; and finally, d) subjects with both skin microfilariae and nodules. Anti-O. volvulus antibodies in these many sera had been previously analyzed by ELISA or by a semi-quantitative immunodiffusion test in gels, as previously reported (9). On the basis of these analyses, the top 3 sera were selected from each subject category and then used in the present studies. The relative titers of anti-O. volvulus IgG, IgM or IgE antibodies in each subset of the selected sera, as determined by ELISA based on immunoperoxidase reactivity, is shown (Table 1). Exceedingly high titers of anti-O. volvulus

IgG antibodies were detected in the sera of exposed subjects who were mf^-, $nodule^-$ (18,750 ± 6,250 $\bar{x}$ ± S.D.) or who were mf^-, $nodule^+$ (1:13,330 ± 1,178). Subjects who were mf^+, or mf^+ $nodule^-$ registered the lowest levels of IgG antibodies. High levels of IgG antibodies thus bear a strong inverse relationship to the presence of skin mf. The levels of IgM or IgE antibodies did not seem to differ significantly among the different subject categories. Finally, the presence or absence of skin nodules seemed to bear little or no special relationship to IgM, IgE or IgG levels. The IgG, IgM and IgE antibody levels in the samples of sera that were to generate the subsequent illustrations of protein blots is shown (Fig. 1).

The profiles of antigens and allergens recognized by the IgM, IgG or IgE antibodies.

The reactivities of the antisera obtained from these subjects were defined in molecular terms by immunoperoxidase staining of *O. volvulus* proteins which had been transferred from polyacrylamide gels onto nitrocellulose sheets. The profiles of antigens detected in these blots by the IgG or IgM or IgE antibodies are illustrated (Fig. 2) The IgG antibodies detected 19 *O. volvulus* antigens in a variety of tests. The most intensely stained molecules ranged from 10-35 Kd. The IgM antibodies appeared to detect a narrower spectrum of antigens, including molecules which were heavily stained at 15, 25, 35, 140 and 190 Kd. Antigens marked at 140 Kd and 190 Kd were frequently poorly stained, if at all, by the IgG. The IgE antibodies detected the narrowest spectrum of *O. volvulus* allergens in comparison to molecules recognized by the IgM or IgG. These predominant allergens are marked at 15, 25, 30, 140, and 190 Kd, with faint bands in between.

Virtually all these allergens which were detected by the IgE antibodies could be identified in protein blots stained with the IgG or IgM antibodies.

We then compared the profiles of antigens and allergens which were detected by the IgG, IgM or IgE antibodies from the sera of subjects representing the various parasitological groups. The IgG antibodies from mf^- nodule $^-$, or mf^- nodule $^+$ donors stained the widest spectrum of antigens at the greatest intensities (Fig. 3). A similar conclusion appeared to hold true for antigens stained by the serum IgM or IgE antibodies from the distinct parasitological groups (Fig. 4). In the illustration of Figs. 3 and 4, one antigen which migrates as a smear at around 20 Kd and another at 35 Kd were stained at high intensities by at least 80% of (5 of 6) sera of mf^- donors, but barely detected by less than 20% of (1 of 6) sera from mf^+ donors. Apart from these two molecules, distinction between the reactivities of sera from mf^- and mf^+ donors were largely quantitative as estimated by the intensities of staining of the various protein bands.

These distinct reactivities of IgM or IgE or IgG antibodies are apparently not due to any remarkable differences in the titers of these antibodies found in the different test sera. According to the results of Fig. 1, the IgM and IgE serum antibody levels are comparable among the various subject groups. We think that the distinct reactivities of sera from these patient groups reveal differential antibody responses of the individuals towards each species of O. volvulus antigens that have been identified on the nitrocellulose blots.

DISCUSSION

Infections due to O. volvulus present with a broad spectrum of clinical manifestations which are often overlapping and difficult to delineate. In the present study, we assigned the exposed subjects to 4 nominal groups on the basis of two criteria which seem to be the most objective for estimating the infection burden. The categories were: presence or absence of skin mf (mf^+ or mf^-) and the presence or absence of palpable skin nodules ($nodules^+$, $nodules^-$) in which the adult parasites dwell.

Detailed serological studies demonstrated the presence of exceedingly high titers of serum IgG antibodies in mf^- subjects. The lowest IgG titers were detected in mf^+ serum donors. The presence or absence of skin nodules which indicate the adult worm burden did not bear any special correlation with IgG, IgM or IgE antibody titers. The distribution of IgM or IgE antibody levels among mf^+ or mf^- parties did not seem different. In a previous study where sera from a much larger number of subjects of each category were used, we found significantly higher levels of both IgM and IgE in mf^+ donors (9).

Distinctions between the reactivities of antisera obtained from the various subsets of subjects were further probed by immuno-staining of nitrocellulose blots of O. volvulus proteins. According to these experiments, the IgG and IgM antibodies of mf^- donors detected the widest spectrum of parasite antigens at the greatest intensities. Antibodies from these subjects appeared to stain additional antigens in comparison to those of mf^+ donors.

A natural speculation that the previous study (9) and the current data provoke is whether the IgG antibodies have anything to do with the absence of mf in exposed subjects who manifest only localized

disease, if any. The study of animal models of filariasis has identified stage-specific immunity which appears to be directed primarily at the mf (10,11). The adult parasites which produce these mf are apparently unaffected by the cell-mediated or antibody-dependent mechanisms which partially protect the host. If antibodies do control the level of mf of O. volvulus, they certainly do not prevent the development of skin nodules. This suggests that they bear no impact on the adult worms. Subsequent reports from our laboratories will assess the effects of antibodies on the developmental stages of O. volvulus.

Perhaps the most intriguing finding of this study is that IgE antibodies detect a number of O. volvulus allergens, all of which were also identified in protein blots stained with IgM or IgG of the same individual. This means that these allergens also elicit IgM or IgG antibody responses in the infected host. The observation provides clues to a) the use of IgG or IgM to isolate the allergens for further analysis, and b) immunological manipulations that could minimize the severe atopic reactions that are normally characteristic of onchocerciasis.

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Table 1. The titers of anti-*O. volvulus* serum antibodies of onchocerciasis subjects presenting with distinct clinical and parasitological profiles.

Ig Isotype	Reciprocal Ig Titer			
	mf − nod. −	mf − nod. +	mf + nod. −	mf + nod. +
IgG	12,500 25,000 25,000	12,500 12,500 15,000	1,250 1,250 6,250	1,250 6,250 6,250
IgM	1,250 1,250 6,250	1,250 1,250 1,250	250 250 1,250	250 250 1,250
IgE	250 250 1,250	250 250 500	250 250 1,250	250 1,250 1,250

LEGENDS

Figure 1. Anti-O. volvulus IgG, IgM or IgE antibody levels in the sera used to stain the protein blots which are illustrated in the subsequent figures. Subjects who were mf^-, $nodule^-$ (O), mf^-, $nodule^+$ (▲), mf^+, $nodule^-$ (□) and mf^+, $nodule^+$ (△), non-infection serum pool from the diplomatic personnel (●)

Figure 2. The profiles of O. volvulus antigens and allergens stained by the IgG, IgM or IgE antibodies of subjects who were mf^-, $nodule^-$ (Panel A) or mf^-, $nodule^+$ (Panel B). The nitrocellulose strips 1,2 or 3 of each panel were stained with the IgG or IgM or IgE antibodies, respectively.

Figure 3. The profiles of O. volvulus antigens detected on protein blots by the IgG antibodies of subjects with or without mf or nodules. Nitrocellulose strip marked "N" was stained with a non-infection serum pool from the diplomatic personnel.

Figure 4. The profiles of O. volvulus antigens and allergens detected on protein blots by the IgM or IgE, respectively, of subjects with or without mf or nodules. Strip marked "N" was stained with a non-infection serum pool.

Table 1. The titers of serum anti-O. volvulus IgG, IgM or IgE antibodies in triplicate samples of onchocerciasis subjects presenting with or without skin mf or nodules.

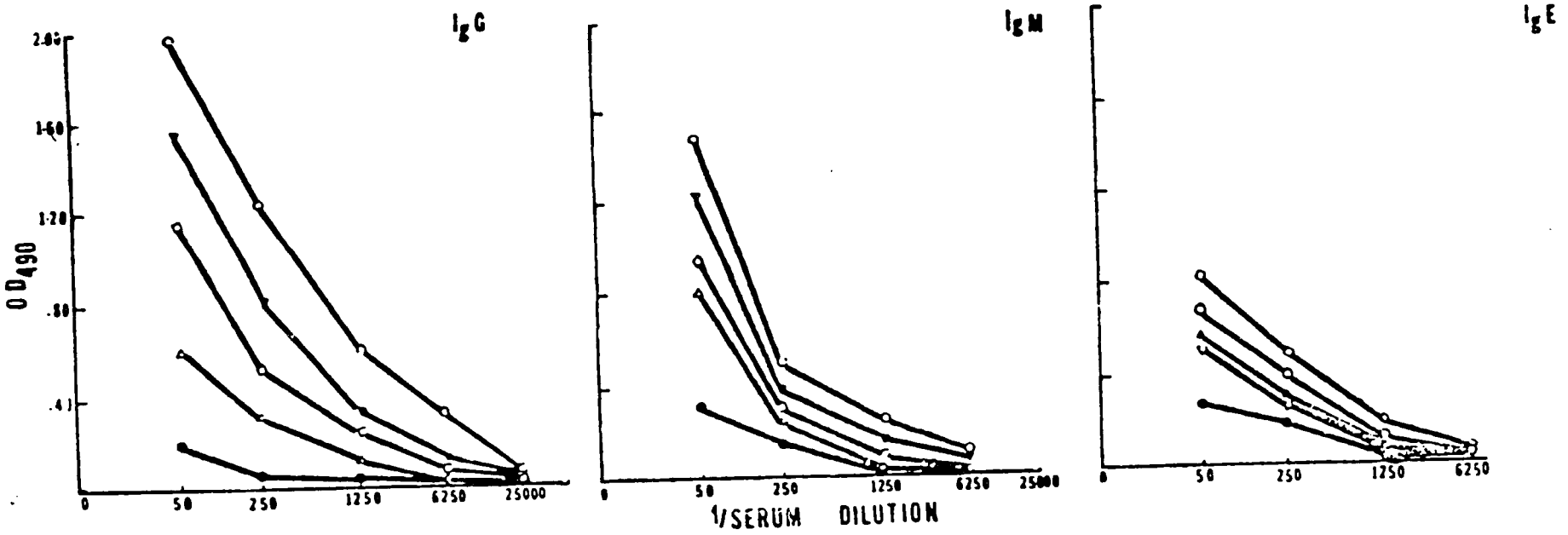

Figure 1. Anti-O. volvulus IgG, IgM or IgE antibody levels in the sera used to stain the protein blots which are illustrated in the subsequent figures. Subjects who were mf^-, $nodule^-$ (○), mf^-, $nodule^+$ (▲), mf^+, $nodule^-$ (□) and mf^+, $nodule^+$ (△), non-infection serum pool from the diplomatic personnel (●)

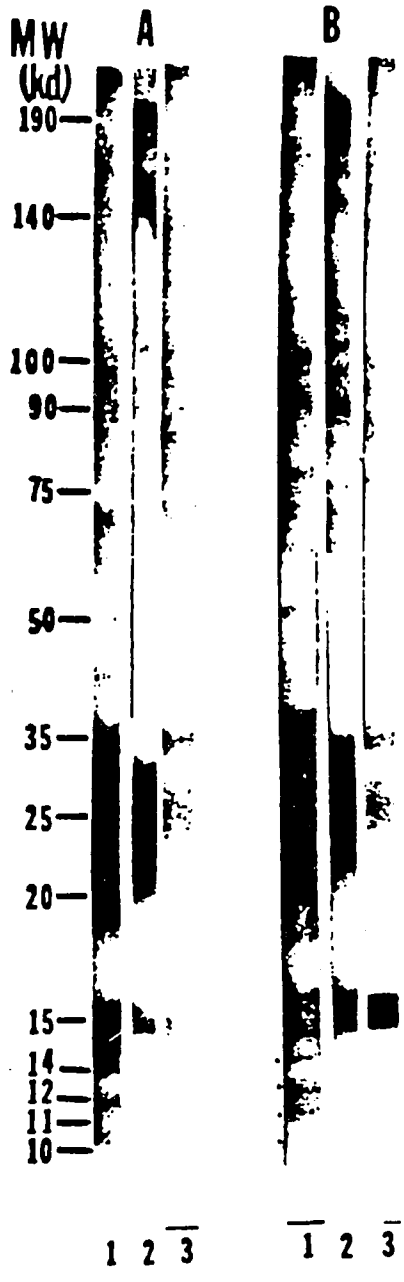

Figure 2. The profiles of O. volvulus antigens and allergens stained by the IgG, IgM or IgE antibodies of subjects who were mf^- $nodule^-$ (Panel A) or mf^-, $nodule^+$ (Panel B). The nitrocellulose strips 1,2 or 3 of each panel were stained with the IgG or IgM or IgE antibodies, respectively.

Figure 3. The profiles of O. volvulus antigens detected on protein blots by the IgG antibodies of subjects with or without mf or nodules. Nitrocellulose strip marked "N" was stained with a non-infection serum pool from the diplomatic personnel.

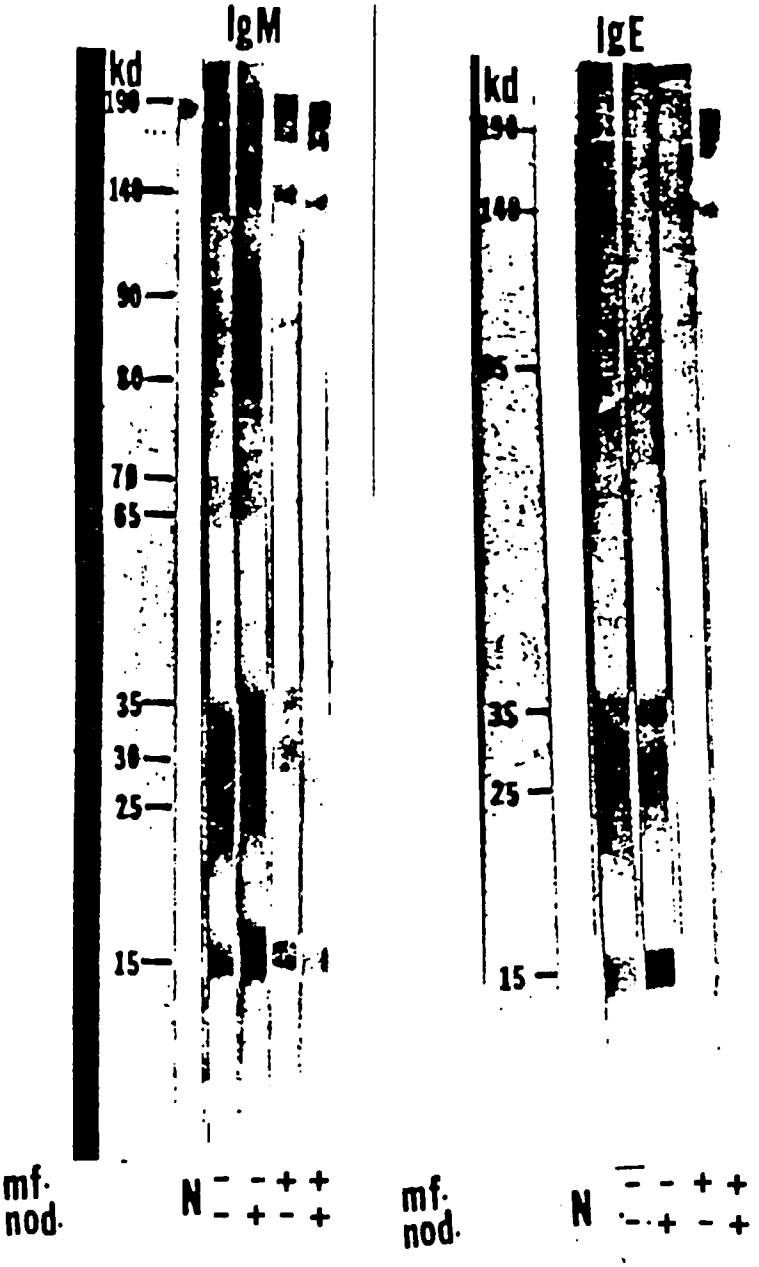

Figure 4. The profiles of O. volvulus antigens and allergens detected on protein blots by the IgM or IgE, respectively, of subjects with or without mf or nodules. Strip marked "N" was stained with a non-infection serum pool.

TITLE

Anti-*O. volvulus* Antibody Responses of Onchocerciasis Subjects Presenting With Distinct Clinical Symptoms.

Running Title: Antibody Responses in Onchocerciásis.

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FOOTNOTES

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2. Correspondence: Dr. William M.O. Boto, Department of Immunology and Infectious Diseases, The Johns Hopkins University, School of Hygiene an Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, U.S.A.

3. Abbreviations used in this paper :

O.V., Onchocerca volvulus ; mf microfilaria(e) ; L3, infective third stage larvae ; W. bancrofti, Wuchereria bancrofti ; B. malayi, Brugia malavi ; L. loa, Loa loa ; PBS, phosphate buffered saline ; AF-3, O. volvulus antigens isolated by immuno-affinity columns ; DS10, a pool of 10 sera obtained from the diplomatic personnel.

ABSTRACT

Onchocerca volvulus is a filarial parasite which causes disease (onchocerciasis or river blindness) in man. In the hyperendemic villages of onchocerciasis in Cameroon, West Africa, we identified subsets of the human population presenting with the following distinct clinical symptoms and parasitological profiles: a) subjects presenting with both microfilarodermia (skin microfilariae) and onchocercomata; b) subjects presenting with microfilarodermia, but no onchocercomata ; c) subjects presenting with onchocercomata, but no microfilarodermia and d) exposed subjects manifesting only localized disease (if any), and no detectable skin microfilariae or palpable nodules.

The present study has examined the patterns of anti-*O. volvulus* antibody responses in individuals manifesting these distinct clinical and parasitological outcomes of the infection. Anti-*O. volvulus* antibodies were quantitated by ELISA and by semi-quantitative Ouchterlony double immuno-diffusion in agarose. Test antigens were isolated by sequential passage of non-ionic detergent extracts of adult *O. volvulus* parasites through a human anti-*L. loa* antibody affinity column and acid elution from a human anti-*O. volvulus* immunoabsorbent column.

High titers of IgG antibodies (1:6,250-1:31,250) were detected in the sera of subjects manifesting only localized disease or asymptomatic infections. The lowest levels of IgG antibodies (≤1:6,250) seemed to associate with the presence of skin microfilariae. In contrast, high levels of IgM antibodies (1:250-1:1,250) or IgE antibodies

(1:250-1:1,250) were found largely in the sera of subjects in whom microfilariae were demonstrated. The presence or absence of skin nodule did not seem to affect specific immunoglobulin levels among individuals presenting with skin microfilariae.

The results of this study demonstrate distinct patterns of antibody responses among subjects presenting with variable clinical and parasitological manifestations of onchocerciasis. We discuss the role that the specific Ig isotypes may play in the pathogenesis of *O. volvulus* infections in the hyperendemic foci.

INTRODUCTION

In established infections, the majority of the adult male and femal *O. volvulus* parasites dwell in raised fibrous, palpable cysts called nodules or onchocercomata in the affected individual. Free-living adult worms and deeply seated impalpable nodules have also been described (1,2). The onchocercomata is largely composed of collagen of host origin. The adult parasites and the numerous microfilariae (mf) they produce can be isolated live by the selective digestion of the nodule using collagenase (3).

Onchocerciasis is transmitted from person to person through the feeding habits of *Simulium sp.* (blackflies). The female arthropod ingests the mf as it takes a blood meal through the skin of an infected subject. The mf develop into infective third stage larvae (L_3) in the vector in 6-7 days. Once inoculated into a healthy host as the fly take another blood meal, the L_3 mature into additional ill-defined developmental stages in 10-20 months, before molting to become the adul worms. Fertilization of sexually mature females by the male worms and the subsequent embryogenesis and hatching of the eggs of *O. volvulus* occur *in utero*. Mature embryos hatch into motile juvenile mf. The gravid female sheds millions of these mf in the host during an estimate 15 years of fecundity. In contrast to those of *W. bancrofti*, *B. malayi* or *L. loa*, mf of *O. volvulus* are not found in peripheral blood: they migrate from the site of birth and dwell just underneath the skin (5,6) and to the regions of the eye where they cause the worst pathology resulting in the loss of sight (6,7,8). Indeed, a demonstration of mf in skin snips as originally reported by O'Neill (9) remains the most definitive diagnostic test for the infection (6,10).

In the hyperendemic rain forest areas of Cameroon, where this study was performed, the disease presents itself with a broad spectrum of dermal pathology and lymphadenitis. The dermatitis which is typified by altered pigmentation, chronic pruritus and papular eruptions can be either "generalized" when it is diffuse and occurs in many parts of the

(5,12). Localized onchocerciasis is often associated with infections in the South Arabian Peninsula, notably in North Yemen, where it is locally known as "Sowda" (6,12,13). However, Sowda occurs in low numbers of cases in East and West Africa, including Cameroon (11,15). Furthermore, in the Republic of Cameroon, there is a minor subset of the adult population (18 years or older) which has lived in the same hyperendemic villages, but without detectable onchocerca stigmata or skin mf. Langham and Richardson (16) described a similar group of asymptomatic subjects in a hyperendemic rubber plantation in Liberia. One intriguing aspect of localized or asymptomatic onchocerciasis is the lack of mf in skin snips sampled from those affected either in North Yemen (12) in Cameroon (10,11,15) or in Liberia (16).

Although several distinct clinical profiles have been clearly delineated in onchocerciasis, the immune response of the human host has not been explored in concert with the pathology of the infection. The present study illustrates variability in antibody responses among onchocerciasis subjects presenting with distinct clinical and parasitological patterns.

MATERIALS AND METHODS.

The study population:

Onchocerciasis is hyperendemic in over 14 villages situated along the Sanaga River Basin, an established breeding ground of blackfly vectors in Cameroon. This study was confined to the adult male and female populations (18 years or older) living permanently in only two of these villages, namely Batchenga and Nji. Younger age groups other than children, tend to be too mobile to be considered permanent residents. All subjects who were examined at Batchenga dispensary had reported for apparent pruritus or other manifestations of onchocerciasis, notably skin lesions, onchocercomata and impaired vision.

Diagnosis of *O. volvulus* infections.

a) The skin snip test for mf : 4 mm skin snips were taken from the knee or buttocks (6,17) and then dropped into 150 ul of RPMI 1640 medium in V-bottom wells of polystyrene microtiter plates. All the mf migrated from the snips into the medium during an overnight incubation at 4 C. The plates were spun at 200 X g for 5 mins. The organisms sedimented at the bottom. One hundred ul of medium were withdrawn. The pellet was agitated in the minimum volume left behind. The total suspension was transferred to a glass slide and the mf were enumerated microscopically at 10X or 40X magnification.

b) The single prick skin hypersensitivity test: the test antigen was isolated by immuno-affinity fractionation of extracts of adult *O. volvulus* worms.

c) Clinical examination: subjects were examined for palpable skin nodules, depigmentation or hypopigmentation, skin thickening, wrinkles, pruritus or papular eruptions as well as ocular damage.

Blood and stool for sera and parasitological studies:

About 20 ml of venus blood were drawn from the arm. Thick smears were made on glass slides and examined for blood-born mf. The rest was

used for the derivation of serum. Negative control sera were obtained from the diplomatic personnel who came to Yaounde, Cameroon, within the last 3 months and who had not been in any part of tropical Africa, Lati America or Yemen before. Stool samples were collected in screw cap plastic vials and then examined microscopically for ascaris eggs.

Sources of *O. volvulus* parasites and antigens :

The adult *O. volvulus* parasites were recovered in viable form by the selective digestion of host tissue from freshly derived nodules, using collagenase (3). In some cases, fragments of the worms were dissected from the nodules. Thirty grams (wet weight) of the organisms were homogenized in 40 ml of extraction buffer (50 mM Tris, 150 mM NaCl 5 mM EDTA, pH. 7.39) containing 1 mM phenylmethylsulphonyl fluoride, 1% Triton X-100, 0.25% sodium deoxycholate. The supernatant obtained following ultracentrifugation of the homogenate was dialysed against 1 liter of binding buffer (15 mM Tris, 75 mM NaCl, pH 7.2, 0.1% tween 20) Antigens for serological studies were isolated by antibody affinity columns constructed from sephacryl S-300 beads.

Sephacryl S-300 beads (Pharmacia) were activated by oxidation with sodium m-periodate as follows (18). Beads were washed extensively in distilled water and then once in 0.1 M sodium acetate buffer, pH 5.0. Oxidation was initiated by resuspending the slurry in 20 mM m-periodate and shaking for 2 hr at room temperature. Higher salt concentration produced beads of noticeable fragility. After reaction, excess periodat was consumed by adding glycerol to 0.16 %. Oxidized beads were washed extensively in distilled water and then thrice in 50X its own volume o- coupling buffer (0.1 M NaHC0 , pH 9.3). After another wash in distille water, the beads could be stored in the acetate buffer for at least 1 month without deterioration in coupling efficiency.

For the preparation of immunoglobulins (Ig) to couple, 26 individual sera were selected from a pool of 132 donated by *O. volvulus* infected subjects as diagnosed clinically and or parasitologically. Each selection detected at least 3 of 5 possible precipitin bands of *O. volvulus* antigens in Ouchterlony agarose double immuno-diffusion gels.

The Ig fractions of a pool of 20 ml of the selected anti-*O. volvulus* antisera, and a similar pool of 10 ml of human anti-*L. loa* antisera and 20 ml of a high titered rabbit anti-whole human serum were obtained by precipitation in ammonium sulfate at 33 % saturation. The individual Ig pellets obtained after centrifugation were reconstituted in coupling buffer 2X the original serum volumes. Sample were dialysed overnight against 1 liter of coupling buffer. The anti-*O. volvulus* Ig (20 ml at 26 mg/ml), or the anti-*L. loa* Ig (20 ml at 12 mg/ml) or rabbit anti-human serum Ig (20 ml at 13 mg/ml) were added to equal volumes of packed sephacryl beads in plastic containers. The suspension was made 0.05 % in Triton X-100 to prevent protein clumping and thus facilitate coupling. Tubes were shaken end-e at room temperature overnight and then washed once in PBS. The couplin efficiency of the different antibody preparations to sephacryl were as follows: *anti-O. volvulus* Ig, 18 mg/ml beads (69.0 %); anti-*L. loa* Ig, 11 mg/ml beads (92%) and anti-human Ig, 12 mg/ml beads (92 %). Unreacted aldehyde groups on the beads were reduced by incubation in 0.15 M sodium borohydride in PBS (18) for 1 hr at room temperature. Th beads were finally washed in PBS.

Twenty mls of *O. volvulus* extracts at 17 mg/ml protein, were shaken with 10 ml of packed anti-L. loa Ig sephacryl for 2 hr at room

temperature. The void volume was recovered and then shaken with 20 mls of the anti-*O. volvulus* Ig beads for 2 hrs at room temperature. The bead suspension was packed into a syringe and washed with binding buffe till drops of the eluate registered no reaction with the Bio-Rad protei dye reagent. Specifically bound antigens were eluted in the first 15 ml of 0.1 M sodium acetate buffer, pH 2.5. The eluate was immediately adjusted to pH 7.4 and then dialysed against 2X1 liter of the binding buffer overnight. The dialysate (about 16 ml) was added to 15 ml of rabbit anti-whole human serum Ig beads and then agitated for 4 hrs at room temperature. The required *O. volvulus* antigens were recovered in the void volume, dialysed against 2X1 liter of saline and then against distilled water ; the dialysate was lyophilized and reconstituted in 6 mls of saline at a protein concentration of 1.22 mg/ml. The *O. volvulus* antigens isolated according to this protocol is herein designated as "AF-3"

ELISA procedure:

Anti-*O. volvulus* antibodies in the sera of the infected subjects were titered using ELISA based on immunoperoxidase reactivity as we described previously (19), with the following modifications: a) block titration of each batch of antigen preparation was essential to establish a concentration range which gave the minimum background of reaction ; this ranged from 5 - 10 ug/ml of protein used to sensitize Polyvinyl chloride microtiter plates; b) peroxidase labeled goat anti-human IgG (FC-specific) or anti-human IgM (FC-specific) or anti-whole IgE (Cappel, Cochranville, P.A.) were used. Enzyme labeled anti-IgE heavy chain was not available. The AF-3 was the standard test antigen for ELISA. Antigens of comparable specificity and sensitivity for IgG or IgM quantitation could also be prepared by sequential absorption of *O. volvulus* whole extracts with anti-*L. loa* and anti-human Ig beads. Such preparation was, however, less sensitive for the detection of specific IgE antibodies.

RESULTS.

Recovery of *O. volvulus* parasites from human nodules:

The collagenous host tissues which constitute the onchocerca nodule car be selectively digested away by collagenase. When we limited it to 4 days post initiation, this procedure released numerous bundles of viab adult *O. volvulus* parasites from the nodules (fig. 1). Viability was checked by the shedding of mf *in vitro*, the contraction of the uterine wall as assessed microscopically and the accummulation of acid metabolites which decreased the pH of sterile growth medium.

Antigens for serological studies:

These were obtained by the sequential passage of detergent extract of the worms through a human anti-*L. loa* immunoabsorbent column, acid elution from a human anti-*O. volvulus* Ig column and then collected in the void volume of a rabbit anti-human serum Ig affinity column. The reactivities of the antigens at these distinct stages of purification were analysed in Ouchterlony agarose double immuno-diffusion gels. *O. volvulus* antigens isolated by a human anti-*O. volvulus* antibody affinity beads demonstrated identical patterns of reactivity with the total detergent extracts of the parasi (fig. 2, Panel A.). Antigens isolated by this procedure alone exhibite slight cross-reactions with human anti-*L. loa* antibodies (fig. 2,

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Panel B), unless the worm extract was first absorbed on an anti-L. loa antibody-affinity beads followed by positive selection using the anti-*O.volvulus* affinity column. The mixture of proteins obtained by the latter procedure (fig. 2, Panel C) was designated as AF-3 for descriptive purposes only. None of the preparations obtained in this wa contained Australian antigen as examined in a standard ELISA test.

Reactivities of AF-3 in the immediate skin hypersensitivity test and in ELISA.

When filter-sterilized and then applied to the forearm in a single prick intradermal test, the AF-3 was potent in inducing immediate skin hypersensitivity reactions in those affected by *O. volvulus* (fig. 3). This is an *in vitro* test for specific IgE antibodies bound to mast cells and perhaps to basophils. The antigen was likewise potent in detecting specific anti-*O. volvulus* IgG, IgM and IgE antibodies, but not anti-*L. loa*, or anti-*D. Perstans* when used to sensitize microtiter plates for ELISA (fig. 4). Reactivity with a pool of 10 sera (DS10) donated by diplomatic personnel in Yaounde was at a minimum. The AF-3 was subsequently applied for the detection of parasite specific antibodies in the sera of subjects in distinct clinical and parasitological groups.

Subjects identified:

The permanent residents of Batchenga and Nji villages, who participated in this study are largely peasant farmers (94%), with a fe students (3%), carpenters (2%) and others (1%). The subjects were examined on parasitological and clinical criteria. The study group fell into four distinct categories based on these clinical and parasitological findings ; a) subjects presenting with both microfilarodermia and onchocercomata ; b) subjects presenting with onchocercomata, but no microfilarodermia and ; c) subjects presenting with microfilarodermia, but no onchocercomata and d) exposed subjects manifesting localized onchocerciasis (if any), but no onchocercomata or skin mf. A sample of individuals representing these classifications is shown (Table 1). Category "d" includes those diagnosed for localized disease and non-clinical infections despite prolonged exposures to *O. volvulus*. All members of this group who were given the immediate skin hypersensitivity test scored positive. Of the 250 subjects examined, only 20 manifested localized or asymptomatic onchocerciasis. No blood-born mf were found in thick smears of blood obtained from the subject category "d".

Anti-*O. volvulus* IgG, IgM and IgE antibody levels among subjects in distinct clinical and parasitological groups.

ELISA titers of anti-*O. volvulus* Ig isotypes are illustrated (fig. 5). High titers of IgG antibodies (1:6,250-1;31,250) were detecte largely in the sera of subjects manifesting localized disease or non-symptomatic infections. The lowest IgG titers seemed to associate with the presence of skin mf. In contrast, high levels of IgE antibodie (>1:50) or IgM antibodies (>1:50) seemed to associate with the presence of skin mf. The presence or absence of palpable nodules did not appear to influence the levels of specific antibodies in the subject groups.

These data which indicate distinct patterns in antibody responses among the clinical groups were confirmed in a semi-quantitative Ouchterlony double immuno-diffusion tests (fig. 6) which measured precipitating antibodies without regard to the relative levels of the

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constituent Ig isotypes in each serum. High levels of precipitating antibodies (1:8 and in some cases 1:16 dilutions) were demonstrated in the sera of subjects manifesting localized disease or asymptomatic infections. The lower limit of sensitivity of Ouchterlony gels is about 40 ug/ml of specific antibody. Sera reacting at 1:8 dilution contain an estimated 320 ug/ml of specific anti-*O. volvulus* antibodies. When lower serum dilutions were compared, intense precipitin reactions were recorded from the sera of these latter two groups. In all cases, only the top precipitating sera of each subject category were selected for this illustration (fig. 6). Anti-*B. malayi* serum of a standard W.H.O. source showed no detectable cross-reactions with the *O. volvulus* antigens used in these tests.

<u>High levels of anti-*O. volvulus* antibodies are strictly associated with infections in the hyperendemic areas:</u>

The considerable IgG and IgE antibody titers present in the sera of residents in the hyperendemic foci were not detected in the sera of urban dwellers who also reported for apparent pruritus (fig. 7). The urban setting from which the latter group was identified is 65 km from the nearest vector breeding ground, and is considered a hypoendemic zone based on the distribution of the parasite within the population as assessed by the skin snip test (Ngu et al., unpublished). It is, however, evident from ELISA test results (fig. 7) that not all the urban dwellers examined were free of *O. volvulus* infections; these town residents do not constitute a negative control group for the infection. Ten % of 28 reporting for pruritus turned out to be skin snip positive for mf and to possess significant levels of anti-*O. volvulus* serum antibodies. The affected minority are probably migrant workers from hyperendemic rural areas of the country. The finding that only 10 % of the urban dwellers reporting with pruritus were exposed to *O. volvulus* supports a previous observation (17) that pruritus *per se* is neither specific nor reliable for the diagnosis of onchocerciasis.

DISCUSSION

Past attempts to establish reliable assay methods for the determination of anti-*O. volvulus* antibodies in human subjects proved unproductive for one major reason : extensive serological cross-reactions occur among the major human and sylvatic filariids, and thus no test antigens specific for the parasite have been isolated. With regard to the present study, concurrent *L. loa* and *O. volvulus* infections can occur in the same individuals in several foci in Cameroon. We countered the issue of cross-reactions between these two major filariae by using immuno-affinity columns to absorb out antigenic determinants which may react with *L. loa* antibodies. Furthermore, microscopic examinations have revealed no evidence of blood-borne microfilariae in subjects involved in this study.

Other reports have indicated strong cross-reactions between *O. volvulus* and ascaris helminth (20). Under conditions where extracts of mf or adult *O. volvulus* worms strongly inhibited the binding of the AF-3 antigen preparation in ELISA, high concentrations (50 ug/ml of protein) of *A. lumbricoides* were ineffective (data not shown). Reactivities towards ascaris may have been eliminated by using low concentrations of the test antigens (5-10 ug/ml) to sensitize microtitre plates for ELISA. Alternatively, absorption of the test antigen on an

anti-*L. loa* immuno-affinity beads could have removed common specificities which have been conserved within the nematode phylum in evolution. Furthermore, we tested for cross-reactions by allowing ascaris antigens to inhibit the binding of antibodies to *O. volvulus* antigens on ELISA test plate. Since a high proportion of the cases of onchocerciasis we studied also shed ascaris eggs in stool, a test for the presence of cross-reacting antibodies, as used in past investigations, would be invalid. It thus appears that the levels of specific antibodies that have been determined in this investigation are exclusively directed at antigens of *O. volvulus*.

The duration and intensity of exposure to the parasite are most likely to affect antibody responses among members of the various clinical groups, and hence the data presented herein. Since the overwhelming majority (94 %) of the cohorts are peasant farmers, the intensity of exposure in job situations is presumably a constant assuming a uniform distribution of the blackfly population. The period of residence, rather than the overall chronological age of the subjects in the hyperendemic villages appears to represent a more accurate approximation of the duration of exposure. Individuals presenting with localized disease or non-clinical infections experienced the shortest exposure of 20 years (Table 1, category d). Individuals presenting with microfilarodermia, but no onchocercomata were exposed for 26 years, while those with both microfilarodermia and onchocercomata were exposed for 30 years. These differences in the duration of exposure of the subject categories seem significant, but the degree to which they may affect the antibody levels among the distinct clinical groups is uncertain. The prepatent period in *O. volvulus* infections is only 10-20 months.

The strength of association between exceedingly high serum titers of IgG with localized onchocerciasis or non-clinical infection (fig. 5) may be interpreted as evidence indicative of a role for antibodies in immunological protection of the host. In contrast, the preferential occurrence of high levels of IgE antibodies in those overwhelmed by infestation and the attendant disease suggests that this Ig isotype does not play a vital role in limiting the disease process or parasitization. On the contrary, hypersensitivity and inflammatory reactions of the host are considered to play a major part in inducing or exacerbating the debilitating clinical symptoms of onchocerciasis (6). The observation that the reagenic serum IgE antibodies exist in high titers in those subjects manifesting the worst pathology and degree of parasitization may be regarded as one evidence in support of this latter view.

The parasitology and immunology of localized and asymptomatic onchocerciasis described herein seem to bear semblance to those of tropical pulmonary eosinophilia which is associated with bancroftian and brugian filariases (21,22) and of occult filariasis in dogs (19,23) Extreme elevation in parasite-specific antibodies and low levels of microfilaremia, if any, are typical features of the latter forms of filariasis. *In vitro* and *in vivo* studies have demonstrated that the occult state in the canine disease is maintained by microfilaricidal antibodies and cell-mediated immune mechanisms of the host (22,24). These antibodies, however, do not seem to affect the viability or the fecundity of the adult filariae which can be demonstrated in the tissues of the host at necropsy (23). By analogy, it appears conceivable that subjects presenting with localized onchocerciasis or asymptomatic infections do harbor the adult parasites which are unaffected by the antibodies ; but the mf which these adults produce could be subject to

destruction by the immune factors of the host, including the antibodies. The subsequent investigations in our laboratories seek to establish the effects of these specific antibodies on the *in vitro* viability of purified mf of *O. volvulus*.

We thank Ms Beverly Greenfield for providing sera from the diplomatic personnel in Yaounde, and Ms. Regine Ntam for typing the manuscript.

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LEGENDS

Table 1. A sample of Batchenga and Nji populations presenting with distinct clinical and parasitological profiles.

LEGENDS

Fig.1. Bundles of adult *O. volvulus* parasites recovered by the selective digestion of human nodules using collagenase enzyme (Boehringer Mannheim, W. Germany).

Fig.2. Agarose gel immunodiffusion analysis of *O. volvulus* antigens. Panel A: t, total worm extract; a, antigen isolated from t by human anti-*O.volvulus* immuno-affinity beads; s, human anti-*O. volvulus* serum pool. Panel B : Cross-reactions between isolated antigen, a, with anti-*L. loa* sera L1-L3; S, human anti-*O. volvulus* serum pool. Panel C: Pre-incubation of the worm extract t, with anti-*L. loa* immuno-affinity beads eliminates cross-reactions of antigen a, against *L. loa* sera L1-L3; s, human anti-*O. volvulus* serum pool.

Fig. 3. Induction of immediate skin hypersensitivity reaction by variable concentrations of AF-3:

Fig. 4. Reactivities of AF-3 with anti-*O. volvulus* IgG, IgM and IgE antibodies in ELISA. Anti-*O. V.*, *anti-O. volvulus serum*. The data were obtained from serum of a single subject carrying localized onchocerciasis. DS10, serum pooled from 10 members of the diplomatic community in Yaounde (negative controls). Each point is the mean of triplicate determinations.

Fig. 5. ELISA titers of anti-*O volvulus* IgG, IgM and IgE antibodies in the sera of onchocerciasis subjects presenting with (F) localized disease, but no skin mf or onchocercomata; (G) onchocercomata but no skin mf, (H) skin mf, but no onchocercomata, (I) skin and onchocercomata. Antibody titer defined as the last dilution of serum giving an O.D. 490 reading greater than that of pooled diplomatic personnel (DS10) when used at 1:50 dilution. Number of subjects shown here : F=11, G=6, H=40, I=40.

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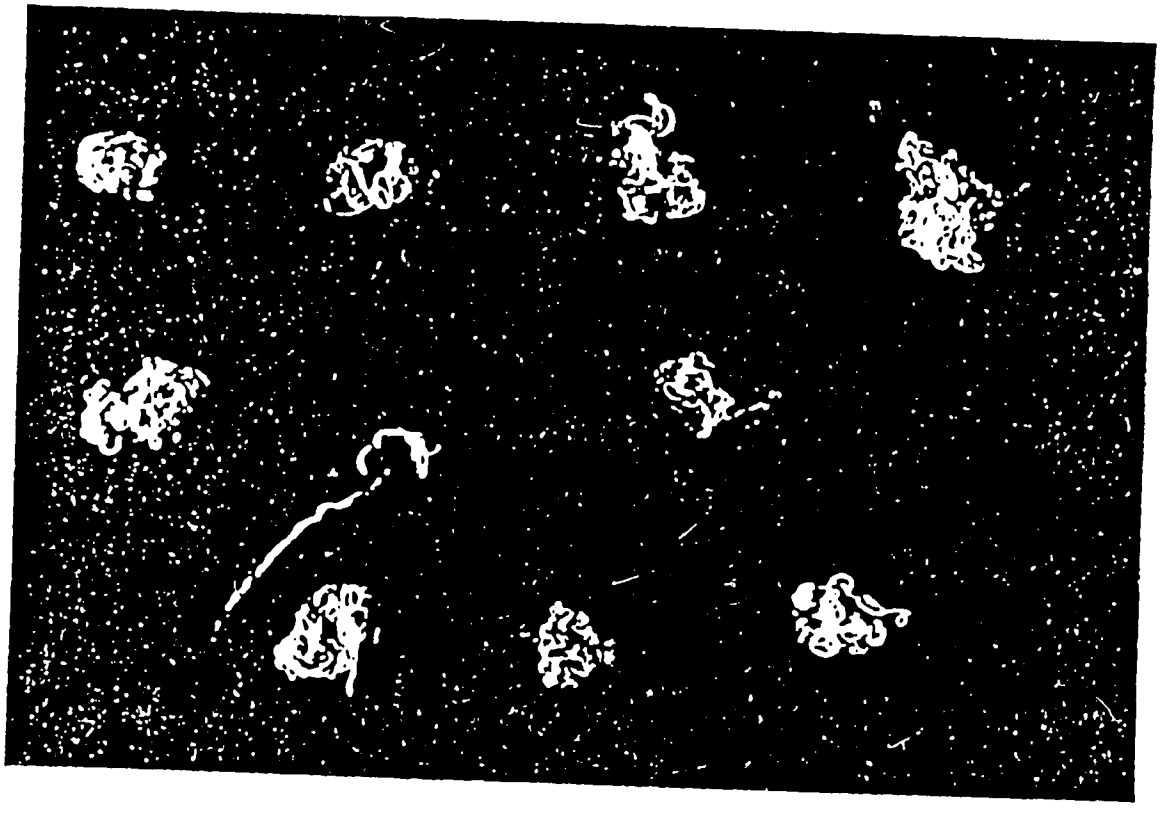
Subject Code	Age (yrs)	$\bar{X}\pm$S.D.	Time of Residence (yrs)	$\bar{X}\pm$S.D.	Nodules	Skin mf.	Subject category
N1	41		9		+	+	
N2	46		7		+	+	
C1	32		31		+	+	
C4	51		30		+	+	
C14	32		10		+	+	
C22	40		2		+	+	
C23	60		60		+	+	
C27	60	45±10	60	30±18	+	+	
C28	45		45		+	+	A
C30	40		40		+	+	
C31	59		59		+	+	
C36	35		35		+	+	
C38	37		12		+	+	
C40	62		20		+	+	
C48	34		34		+	+	
C49	43		25		+	+	
C51	53		34		+	+	
C20	45		45		+	−	
C32	35		35		+	−	
C44	45		45		+	−	
C50	19	37±11	19	36±12	+	−	B
C57	40		N.D.		+	−	
C2	44		17		−	+	
C3	49		48		−	+	
C5	63		13		−	+	
C6	43		41		−	+	
C7	21		10		−	+	C
C10	M.D.		10		−	+	
C12	60	43±14	60	26±17	−	+	
C13	46		46		−	+	
C15	66		20		−	+	
C17	50		22		−	+	
C29	30		30		−	+	
C37	34		34		−	+	
C39	31		8		−	+	
C41	45		25		−	+	
C45	20		7		−	+	
N3	46		2		−	−	
C9	20		4		−	−	
C11	21		20		−	−	
C16	40	33±13	40	20±17	−	−	
C18	22		5		−	−	D
C21	40		15		−	−	
C46	50		50		−	−	



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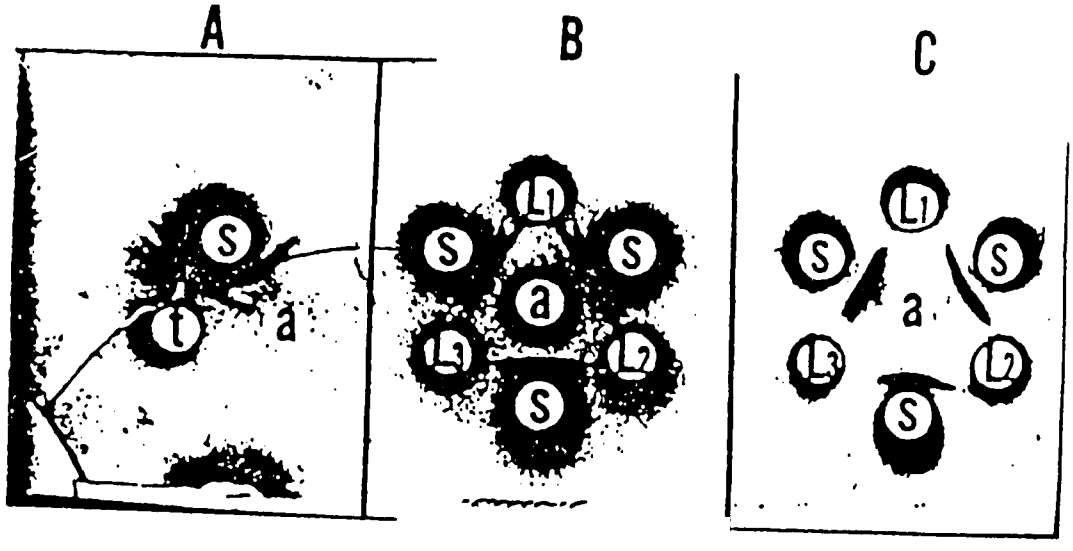

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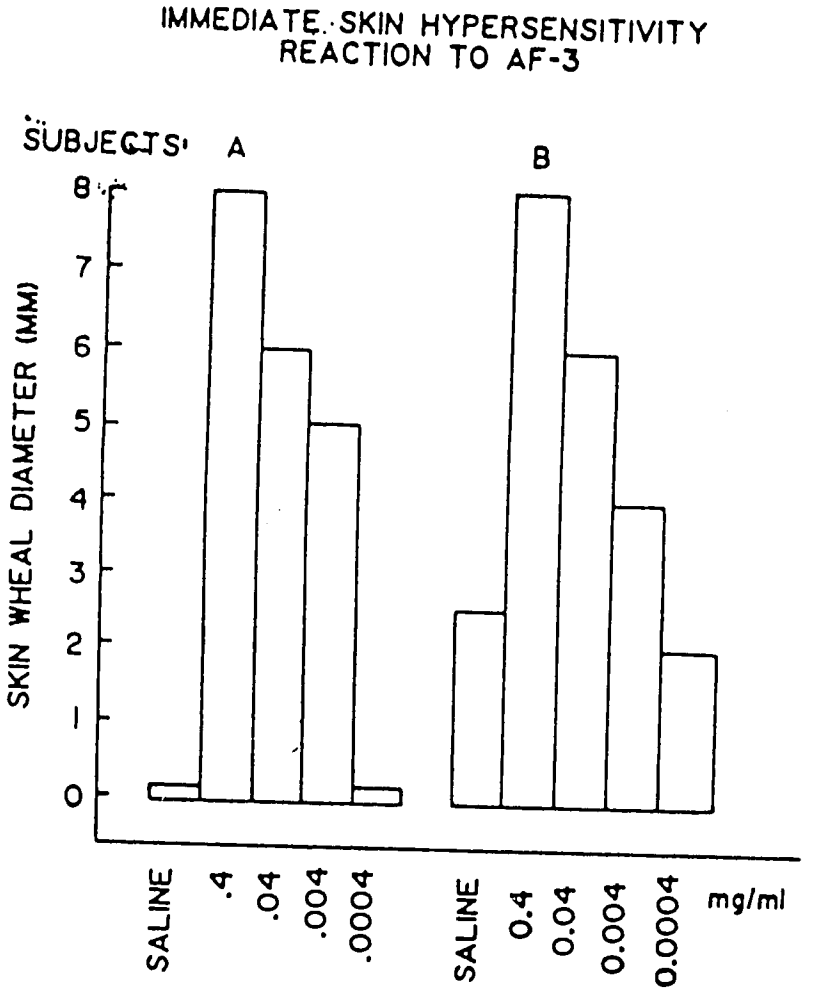

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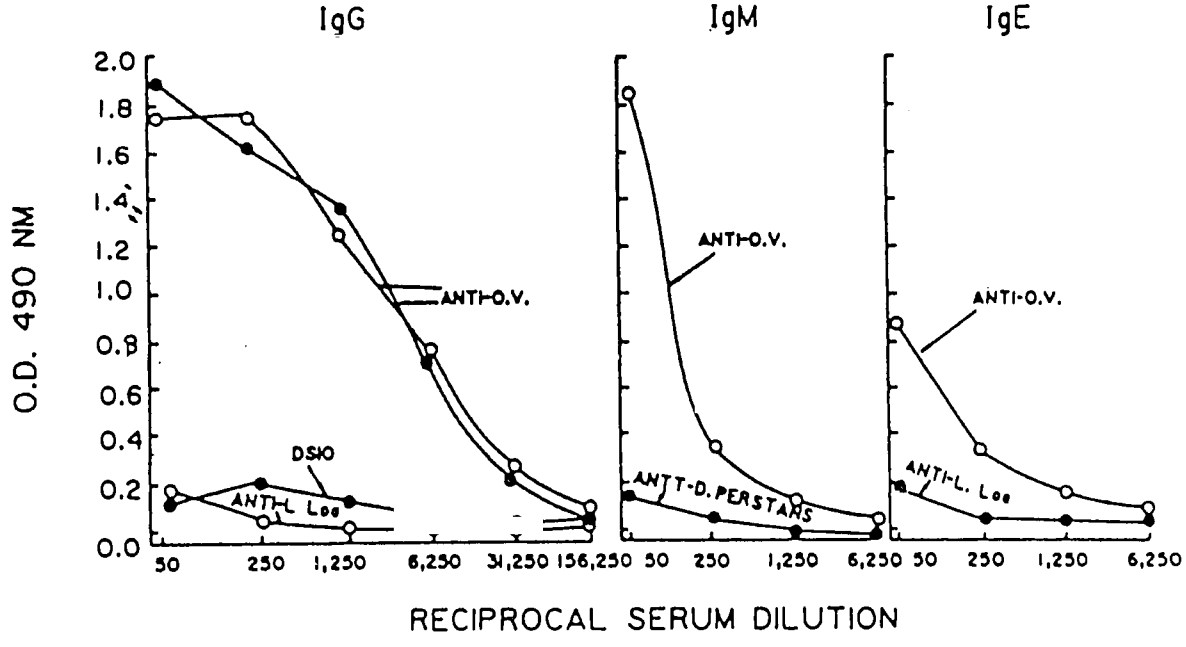

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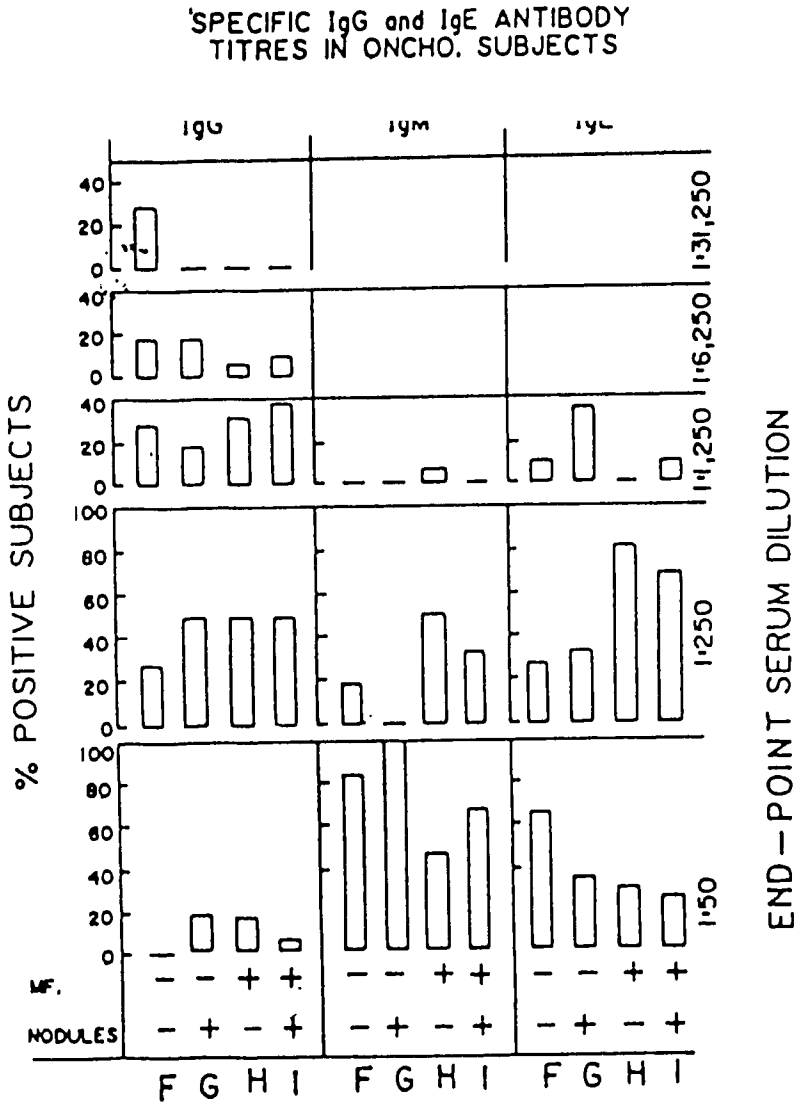

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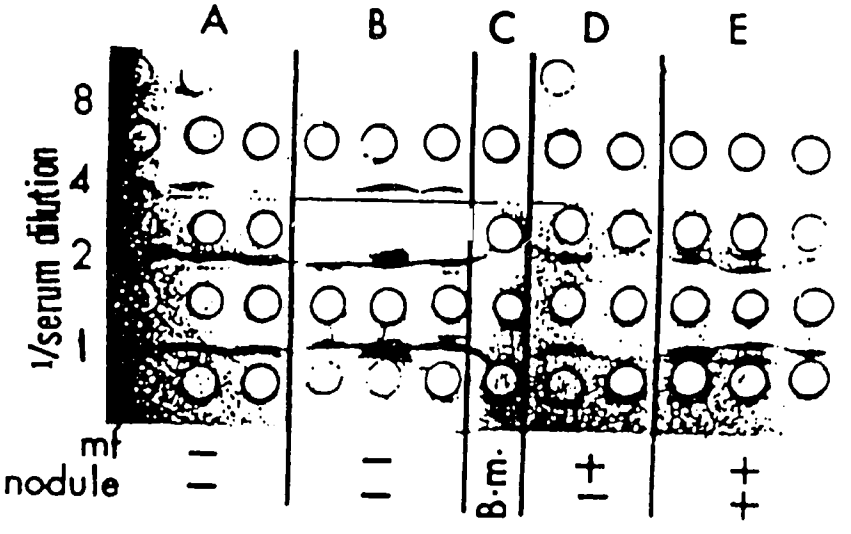

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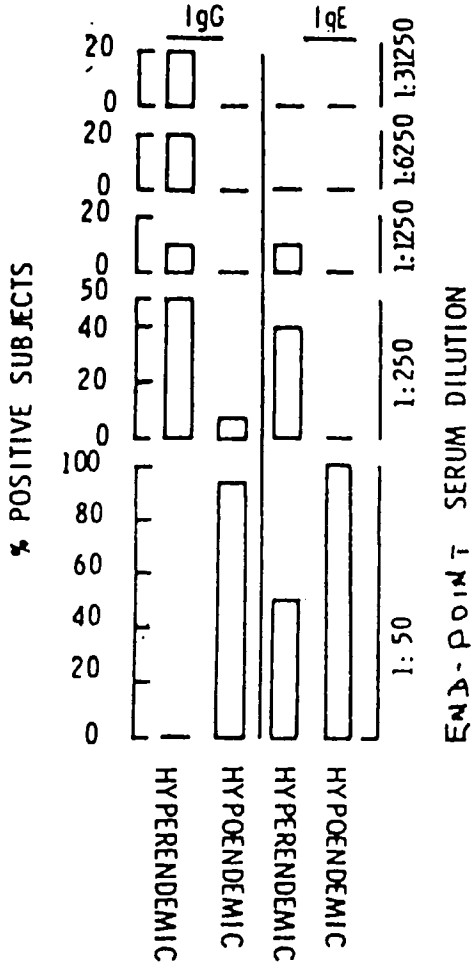

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YAOUNDE 25-7 1985 GUICHET

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DESTINATAIRE Dr Irvin M. Asher
ADDRESSEE Director, Science Program
Agency for Inter. Development
à - at Washington DC 20523 USA

517-2 RECEPISSE D'UN ENVOI RECOMMANDE OU AVEC VALEUR DECLAREE
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Nature de l'objet / Nature of item

Valeur déclarée / Insured value

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Poids — Weight

25 JUIL. 1985

... Irvin M. Asher, Ph.D.
Director, Science Program
Office of the Science Advisor

Dear Dr. Asher,

Re : AID Grant ## 3.H24. Onchocerciasis in Cameroon : the development of ELISA for Immunodiagnosis.

I learnt with much regret that your office has not received regular progress reports on the above referenced grant. According to our records the originals of these reports had been forwarded on a 3-4 monthly basis to Dr. David Levy of the Johns Hopkins University. The original understanding was that, as one of the Principal Investigators, Dr. Levy would then submit them to your office in D.C., with a cover letter and any modifications that he deemed necessary. Copies of these reports were submitted simultaneously to the local AID mission in Yaounde, and I am enclosing a set for you, just for the record since most of them are so long overdue.

As you know, the grant ## 3.H.24 expired on 30th, June 1985. Your letter arrived when we were compiling the sixth and final progress report. This report will assess the overall achievement and difficulties that were realized in the pursuit of the grant goals over the two-year period. I believe that we are allowed a grace period of months in which to submit this report. Please allow me to summarize below the impact that the grant has had on :

a) our efforts to develop a test of greater reliability and application to diagnose prepatent, unisexual and other asymptomatic O. volvulus infections, and b) the training of local man-power to strengthen the research capability of our laboratories at C.U.S.S.

a) The development of ELISA for immunodiagnosis of onchocerciasis : In a manuscript that has been submitted, we have described the first specific, reliable, sensitive, simple and cheap ELISA methodology for the detection of O. volvulus antibodies in human serum. In progress report ##5, we turned the test around to detect mouse anti-O. volvulus hybridoma antibodies in microtiter plates. It should be apparent that the development of the ELISA and the murine hybridoma antibodies constitute major mile-stones in the pursuit of the goals of the grant. The antigens to which the hybridoma antibodies were raized had been strategically selecte and isolated by immuno-affinity procedures that consumed a considerable, but justifiable length of time. If time had allowed, the next phase of our study was to use the ELISA or passive reverse hemagglutination reactions

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and the monoclonal antibodies for the detection of O. volvulus antigens that we suspect to be in urine, plasma or serum of infected individuals. We have not reached this phase of the project yet, not because of any insurmountable technical difficulties, but purely because of time constraints. In retrospect, I believe that we expected to achieve a lot too quickly when we first submitted the grant to you for funding. We produced four hybridoma cell lines between July 84 and Feb. 1985. In order to use their antibodies for the detection of the parasite antigens, these cells had to be cloned and made to produce large quantities of homogeneous monoclonal antibodies, either in vitro or in ascites (refer progress report 1/85). These steps would require an additional 6 months, at least. The cells have been cryopreserved in liquid nitrogen and will be cloned and propagated accordingly.

b). Strengthening Research Capability of C.U.S.S.
This has been achieved through the provision of 1) numerous reagents, apparatus and facilities in the laboratories 2) the training of a local post-doctoral fellow, Dr. Gabriel Lando and the supporting research assistant, Mrs. Patience Moffah in the use of modern immunological, parasitological and other biochemical research techniques, most of which were described in the original grant and some of which were initiated here. The three manuscripts which are being prepared or which have been submitted will ultimately attest to the success of this training program. We are, however, in search of new funds which will assist these local personnel to expand their own research in future.

Sir, most of the points that I have touched upon here will be presented more clearly in the upcoming final progress report.

Re : AID grant ##?? production of O. volvulus antigens by recombinant microbiology.

This grant did not contain any funds to support the work in Cameroon. All the gene cloning is being carried out in the laboratory of Dr. David Levy at the Johns Hopkins. However, the work requires the supply of O. volvulus parasites which can only be obtained from infected populations in Cameroon. My record shows that a certain Dr. Alan Scott, who is cloning the genes at Hopkins, made two requests between 1984 and 1985, for the parasites to be sent over. The record shows that both requests were met promptly. The derivation of these parasites from human tissue is therapeutic in itself, but is laborious field work which requires the use of hired physicians. As long as funds are available for the field work involved, we will continue to supply the necessary material to support this very exciting and potentially rewarding research program.

cc : Joe Stockard,
AFR/TR
Gary Leinen, USAID/Yaoundé
John Daly, SCI
M. Rechcigl, SCI
G. Shivers, SCI.

Professeur J. L. [illegible]

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61

FINANCIAL REPORT

Project N° 3.H24

Title : Onchocerciasis in United Republic of Cameroon
Development of ELISA for Immunodiagnosis

A. *Funds received by CUSS from Johns Hopkins, University School of Hygiene*

1983-84.....	US Dollars	16,350	CFA 8.942.305
1984-85.....	US Dollars	14,000	CFA 6.413.798
TOTAL.....		30,350	15.356.103

B. *Expenditure : Absolutely no contested*

1. Transport.....2.330.000 CFA
2. Transport
   runing costs - car..... 500.000 CFA
   -Land Rover.. 650.000 CFA
3. Telephone
   Telexes Postage ..... 400.000 CFA
4. Equipment.....4.000.000 CFA
5. Equipment maintenance.....1.500.000 CFA
6. Supplies.....2.200.000 CFA
7. Foreign Travel..... -
8. Administrative field
   staff, technical, field
   trips.....3.776.103 CFA

TOTAL.....15.356.103 CFA

BALANCE..... *000*

Project 3.F31

Title : The Production of antigen of *Onchocerca volvulus* by Recombinant Microbiology.

Funds received by CUSS : 0 Dollars US.

Signed : 25/7/5

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25 JUIL. 1985

1067

Dr. Irvin M. Asher, Ph.D.
Director, Science Program
Office of the Science Advisor

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a) The development of ELISA for immunodiagnosis of onchocerciasis : In a manuscript that has been submitted, we have described the first specific, reliable, sensitive, simple and cheap ELISA methodology for the detection of O. volvulus antibodies in human serum. In progress report #5, we turned the test around to detect mouse anti-O. volvulus hybridoma antibodies in microtiter plates. It should be apparent that the development of the ELISA and the murine hybridoma antibodies constitute major mile-stones in the pursuit of the goals of the grant. The antigens to which the hybridoma antibodies were raized had been strategically selected and isolated by immuno-affinity procedures that consumed a considerable, but justifiable length of time. If time had allowed, the next phase of our study was to use the ELISA or passive reverse hemagglutination reactions

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and the monoclonal antibodies for the detection of O. volvulus antigens that we suspect to be in urine, plasma or serum of infected individuals. We have not reached this phase of the project yet, not because of any insurmountable technical difficulties, but purely because of time constraints. In retrospect, I believe that we expected to achieve a lot too quickly when we first submitted the grant to you for funding. We produced four hybridoma cell lines between July 84 and Feb. 1985. In order to use their antibodies for the detection of the parasite antigens, these cells had to be cloned and made to produce large quantities of homogeneous monoclonal antibodies, either in vitro or in ascites (refer progress report #5). These steps would require an additional 6 months, at least. The cells have been cryopreserved in liquid nitrogen and will be cloned and propagated accordingly.

b). Strengthening Research Capability of C.U.S.S.
This has been achieved through the provision of 1) numerous reagents, apparatus and facilities in the laboratories 2) the training of a local post-doctoral fellow, Dr. Gabriel Lando and the supporting research Assistant, Mrs. Patience Moffah in the use of modern immunological, parasitological and other biochemical research techniques, most of which were described in the original grant and some of which were initiated here. The three manuscripts which are being prepared or which have been submitted will ultimately attest to the success of this training program. We are, however, in search of new funds which will assist these local personnel to expand their own research in future.

Sir, most of the points that I have touched upon here will be presented more clearly in the upcoming final progress report.

Re : AID grant #?? production of O. volvulus antigens by recombinant microbiology

This grant did not contain any funds to support the work in Cameroon. All the gene cloning is being carried out in the laboratory of Dr. David Levy at the Johns Hopkins. However, the work requires the supply of O. volvulus parasites which can only be obtained from infected populations in Cameroon. My record shows that a certain Dr. Alan Scott, who is cloning the genes at Hopkins, made two requests between 1984 and 1985, for the parasites to be sent over. The record shows that both requests were met promptly. The derivation of these parasites from human tissue is therapeutic in itself, but is laborious field work which requires the use of hired physicians. As long as funds are available for the field work involved, we will continue to supply the necessary material to support this very exciting and potentially rewarding research program.

cc : Joe Stockard,
AFR/TR
Gary Leinen, USAID/Yaoundé
John Daly, SCI
M. Rechcigl, SCI
G. Shivers, SCI.

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IMMUNOLOGY BIOTECHNOLOGY LABORATORIES (IBL)

(WHO COLLABORATING CENTER FOR RESEARCH AND TRAINING IN IMMUNOLOGY.)

(MOLECULAR BIOLOGY/BIOTECHNOLOGY SECTION.)

PROGRAMME OF ACTIVITIES

IMMUNOLOGY - BIOTECHNOLOGY LABORATORIES (IBL)
(WHO COLLABORATING CENTRE IN IMMUNOLOGY)
FACULTY OF MEDICINE AND BIOMEDICAL SCIENCES
UNIVERSITY OF YAOUNDE 1 - CAMEROON REPUBLIC.
B.P. 4563, NLONGKAK, YAOUNDE -CAMEROON

Tel: (237) 203796 Fax: (237) 203796 or 311224 Telex 8384KN

Historical background.

Research in immunology had been going on for some time at the University Center for the Health Sciences (CUSS), by force of circumstances to a limited degree, but became a major area of activity of the Institution in 1979 following an "Institution Strengthening Grant Award" made to CUSS by the World Health Organization Tropical Diseases Special Programme in Geneva. The main purpose of the award was to strengthen the research capabilities of the Institution in immunology, particularly with regard to research in the six diseases of the "Special Programme" Malaria, Filariasis, Trypanosomiasis, Schistosomiasis, Leishmaniasis and Leprosy. It was decided that the main thrust of research in immunology would be in the Filariasis, particularly Onchocerciasis. The strategic planning decided upon for sustainable growth in this area at CUSS was through:

- promoting the return of Cameroonian graduates in immunology to Cameroon from abroad
- equipping the existing laboratories in CUSS for high quality research in immunology
- providing specific training for Cameroonian immunologists in laboratories in Europe and in the USA
- financing short training courses in the laboratories at CUSS for young scientists from other African countries
- financing the establishment of links between the research group at CUSS and other research groups in developed countries.

This programme of activities was a notable success in that by 1985, there existed at CUSS a group of five active Cameroonian immunologists, with three more in the course of training for a PhD degree in Institutions abroad; short training courses had been held at CUSS with participants coming from a number of African countries: Gabon, Congo, Sierra Leone, Zaire and Benin; worthy advances had been made by the group in CUSS on host immune responses to *O. volvulus* infection, immunopathogenesis of onchocercal lesions, onchocercal derived antigens in immune-complex nephropathy in endemic areas, diagnosis of the infection by antibody detection etc. In recognition of what had been achieved, CUSS was designated a WHO Collaborating Center in 1985.

Around this time it was concluded that to develop sensitive, discriminatory diagnostic tests for the disease, one would have to resort to molecular /biotechnology techniques. This view was considered also true for identifying candidate vaccine subunits, another major preoccupation of the research group. It was therefore planned to evolve in this new area using graduates from the Faculty of Science who would be sent abroad for training in well established and reputable research laboratories.

Research Activities of the Molecular/Biotechnolgy Section

The major areas of research in molecular biology/biotechnology by the scientists of this Center are in the fields of Health Sciences and Agriculture.

Health Sciences.

Rationale:
Onchocerciasis is the main disease in which molecular biology and biotechnology techniques are being employed here for research purposes. It is caused by the parasite nematode *Onchocerca volvulus,* which is transmitted to man by the blood sucking fly vector of the genus *Simulium.* The infection causes many clinical manifestations, the most distressing of which are severe visual impairment, blindness, kidney pathology culminating in renal failure, disfiguring skin lesions and debilitation. More than 40 million people are affected worldwide, particularly in Africa, the Yemen, Central and South America. There are even more than this number at risk from the disease; this includes visitors from onchocerca free areas or countries to endemic regions. As the fly vectors of the disease breed on weeds along the banks of fast flowing streams and rivers, people in endemic zones tend to flee lands bordering these rivers and streams where these flies abound. These, unfortunately, are also the most fertile lands in these countries. More distressing still is the observation made recently that building dams for the generation of electricity in rural areas considered previously to be onchocerca free, provides ideal conditions for the breeding of *Simulium* vectors which migrate from endemic zones not far away. This jeopardizes rural development programmes. The socioeconomic consequences of onchocerciasis are a sequel to increasing disability with age, particularly severe visual impairment and blindness, leaving a young

population to care for increasing numbers of dependent elderly compatriots.

There is no known treatment to reverse established complications of this disease. There is no effective prophylactic medication against the parasite. Efforts to eradicate the vectors using larvicidal chemicals sprayed on their breeding sites have proven expensive and, at best, only partially effective. Areas cleared of vectors have been re-invaded by *Simulium* from neighboring endemic zones, as these flies can cover distances of up to 400 kilometers. For the present, the most feasible containment strategy that may reasonably be advocated is the early detection of infection in humans and their treatment with ivermectin. The main specific areas of our research activity have been the following:

1. **Sensitive, discriminatory diagnostic test.**

An adult *O. volvulus* cDNA library was screened with antibodies raised in rabbits to affinity column purified *O. volvulus* antigens. Clones were selected on the basis of their non-cross-reactivity with antigens derived from other filariasis and human helminths. The proteins coded for by these genes were expressed by subcloning into pmal c vector for the E. coli system. These proteins were evaluated for use as diagnostic reagents for detecting antibodies to *O. volvulus* , using ELISA and a panel of coded sera from the WHO filariasis bank. Antigens of clone OvD 5B had 100% specificity and 87% sensitivity, those of OvD 21B 100% specificity and 63% sensitivity, while **OvD 3B** expressed proteins were **100% specific** and **100% sensitive.** Further evaluation is in progress using 330 sera from an area endemic for onchocerciasis and normal controls (sera from uninfected subjects). A major drawback is that the recombinant proteins are still fused to maltose binding protein (MBP), making the assay tedious because of the many controls (MBP control for each test serum) needed for each batch of sera tested.

Plans are underway to express these genes in the yeast system. Further, the recombinant fusion-free proteins coded by these genes will be used to develop a diagnostic test for onchocerciasis based on identifying and quantifying specific onchocercal antigens in test samples, probably the best indication of worm burden. Another set of genes initially isolatedfor candidate vaccine subunits have been cloned an d expressed in the Yeast system.Two of these genes have sequence homology. One has been expressed intracelluarly(Oncho-C27) and the other as secretory recombinant proteins 9 nch0-C71). Surprisingly, both recombinant proteins have proven highly sensitive (99.99%) wen sed in the ELISA-based diagnostic test for antibodies to *O. volvulus*, using a panel of 330 positive sera and 30 negative controls. The proteins have tested specific for *O. volvulus* and did not react with human, mouse or rabbit antisera to*Loa loa, S. mansoni, Brugia malayi, Ankylostoma intestinale, A. lumbricoides, and T. gambiense.*This is the first time that specific onchocercal antigens for use in immunodiagnosis have been produced by recombinant techniques in the yeast system. The procedure is being engaged to obtain Patent rights for Oncho-C27 andC71.

2. **DNA probes for detecting and distinguishing between savannah and forest strains of *O. volvulus* in flies caught in the wild.**

The international onchocerciasis control programme for the eradication of the *Simulii* vectors from endemic zones, depends for its success on monitoring the fly infection rates particularly after reinvasion of areas previously denuded of these vectors. Further, as it

has been noted that severe eye lesions are more often associated with infection by savannah strains of the parasite than with the forest variety, it is important to determine the prevailing strain of parasite in infected vectors.

Dr. Unnasch's laboratory at the University of Alabama at Birmingham, USA, has isolated and developed two form specific DNA probes: pFS-1 specific for the forest form of the parasite, and pSS-1BT specific for savannah form. In collaboration with others, a third probe, designated pOVS134, specific for *O. volvulus* has been identified. The larvae could therefore be characterized on the basis of differential hybridization to these probes. Extraction of genomic DNA from infective larvae permits the amplification of the 149bp repeat sequence family, of which pFS-1, pSS-1BT and pOVS134 are members, utilizing the polymerase chain reaction (PCR). In adapting PCR for this assay, 17 known examples of the repeat were compared, and a relatively conserved region of 42bp identified. From this region two populations of degenerate oligonucleotides were synthesized for use as primers for PCR, as they are able to direct the amplification of the 149bp repeat family from every *O. volvulus* isolate so far tested, as well as the 149bp repeat sequence from other species of *Onchocerca*. Subsequent steps in the assay permit the identification of the parasite species and its form using the specific DNA probes mentioned above. To ensure feasibility as well as reproducibility of the assay in developing countries, the specific DNA sequences have been adapted for use as single strand hybridization probes based on the bacteriophage M13. This improves consistency of the assay as the specificity of these probes are confined to a rather narrow window of hybridization (conditions of stringency), owing to the fact that all of the clones contain representatives of a divergent repeat family present in the genome of *O. volvulus*. Thus, below a certain stringency of hybridization, non-specific members of the repeat family will hybridize to the probes. The three probes have in the past been tested on DNA extracts of isolated adult worms and skin microfilariae.

In collaboration with Dr Unnasch, we are adapting these probes for use in the field. We have developed an efficient method for extracting DNA from infected flies. Utilizing the polymerase chain reaction (PCR) and the "dot blot" technique, we can detect infection of *Simulii* at a sensitivity of 5 or more microfilariae per fly. This works out to about 1 or 2 infective larvae per vector. The assay can be performed with out using radioactive detection methods; the enzyme avidin biotin is now being routinely employed for revealing hybridization of the probe to the single stranded system. Evaluation of the specificity of two of the probes to geographic strains of the parasite is in progress. Further, it is envisaged that by improving the efficiency of genomic DNA extraction from infected vectors (notable for their tough exoskeleton) we will obviate the need for the PCR step in the assay.

3. High level expression of fusion-free recombinant antigens of *O. volvulus* and their evaluation as candidate vaccine subunits.

In earlier studies, we demonstrated the presence of protective immunity to onchocerciasis in a small group of subjects living in an endemic zone. The mechanisms accounting for protection involved antibody dependent cellular cytotoxicity (ADCC) requiring specific antibodies as well as complement. We produced monoclonal antibodies (mABs) to L_3, characterized them and selected those that mediated ADCC with L_3, skin microfilariae (mf) and mf from onchocerca nodules as targets. These mABs were then used to screen adult and larval onchocerca cDNA libraries constructed in lambda gt 11 using adult RNA. Of the 30 clones isolated, two have been expressed at high levels in yeast, and three others have been subcloned into this system and will be expressed soon. Further selection of the antigens to obtain those most suitable for use in vaccine

formulation will be based on their ability to induce antibodies that will mediate ADCC with L_3 and L_4 as targets *in vitro* and *in vivo,* as well as proliferative response of primed lymphocytes cultured with these antigens.

4. Genetic control of host responses to invasion by infective larvae; HLA association with the different clinical groups of subjects found in onchocercal endemic areas.

Subjects who have lived in zones endemic for onchocerciasis over a long period show a plenum of clinical responses to the infection which cannot be correlated with the degree of exposure, nutritional status of the subject or other concomitant diseases. The responses range from a small minority of subjects who appear to be clinically healthy with no overt manifestation of the infection to the vast majority with differing clinical manifestations. The latter group includes the following sub-groups : generalized onchocerciasis, restricted skin distribution or localized onchocerciasis, apparently healthy skin but subjects have microfilaridemia and onchocercomata, severe eye lesions or blindness which may be associated with any of the foregoing clinical manifestations.

There is overwhelming evidence of cellular and humoral immune responses to parasite antigens in all these subjects. Indeed, most of the clinical manifestations are a sequel to host immune response to the parasite. We are investigating the possibility that the different clinical expressions may be linked to HLA subclasses. More specifically, we wish to determine whether or not there is linkage between HLA Class 11 subclasses and protection to the infection seen in the small group of subjects in endemic zones. If this is shown to be the csae, one may proceed to determine the antigenic moitities which are preferentially associated with the molecules of the associated protective Class 11 subclass (es)before presentation to 'T'cells. This is an alternative approach to isolating candidate vaccine subunits. The possibility of there existing HLA-linked susceptibility to eye lesions, especially choroidoretinitis, may also be similarly studied. Molecular techniques are being employed for determining the HLA Class 11 subclasses.

Agricultural Biotechnology.

Rationale:

In vitro clonal propagation through cell and tissue culture techniques has been used successfully for rapid and large scale multiplication of plants (Murashige T., 1974). The advantage of the method is that it is fast and it requires a small space compared to conventional methods of vegetative propagation. The application of this technology in Africa will be appreciated most in the multiplication of root crops and tubers such as cassava, yams, cocoyams and potatoes which are the staple diet of Africans (Ng, 1986). In yams, for example, the typical selection scheme practised by farmers involves selecting the best yielding or growing yam plants and their propagation by setts obtained from the tubers. The farmers usually set aside 1/4 of each harvest to use as seed for the next planting season. As a consequence of this asexual method, many important species and cultivars rarely produce viable seeds while others are completely sterile (Ammirato et al., 1984). Successful

micropropagation of tubers would require a high rate of survival in the field of plantlets after acclimatization. Any growth regulators which enhance both growth and survival rates would ensure the success of this technique.

Growth regulators which enhance successful micropropagation of tubers.

Hormones are organic compounds naturally synthesized in higher plants and which influence growth and development. Apart from these natural products, synthetic compounds have been developed which correspond to the natural ones. Hormones and these synthetically produced compounds are collectively called regulators and are primarily responsible for the distribution of the compounds which the plant biosynthesizes. In *in vitro* culture of higher plants, regulators, especially auxins and cytokinins are essential for growth. It can be said that *in vitro* culture is rarely successful in the absence of regulators (Pierik, 1987). Preliminary studies carried out in this laboratory have revealed that the yam explants inoculated in an experimental yam establishment medium (i.e. replacement of the cytokinin kinetin and the auxin IAA with 1.0% chloroform extract of the leaves of one of the plants under study) showed bigger leaves, longer stems as well as root formation after three weeks of incubation than those explants inoculated in the normal yam establishment medium. Likewise, cultures with n-butanol extract of the leaves of the same plant have also yielded interesting results; the yam explant grown in medium with n-butanol extract as source of possible growth factors has more nodes than controls, a plus factor in micropropagation since more explants can be obtained for the next subculture. Purification and delineation of the molecular structure of these regulators is in progress.

The present project on the extraction of growth hormones from indigenous plants of Cameroon and their use in the micropropagation of root crops and tubers can be of great value in achieving the goal of food self-sufficiency in Africa. We plan to screen extracts of many rare plants found in the Korup, Africa's oldest rain forest located in the Southwest Province of Cameroon. The forest is said to be richer in plants and animal species than any other African forest for which comparable data is available. Half of the plant varieties of Africa's tropical forest is found in Korup. Even for a rain forest, it has poor soils which it has been suggested induced the evolvement of an ecological environment rich in plants with a diverse range of chemicals of possible medicinal, agricultural and industrial uses. There are, to date, more than 90 such substances which have been identified, 38 of which had not been known previously.

Facilities Available.

The laboratories of this Center are located in the Faculty of Medicine and Biomedical Sciences, University of Yaounde 1. There are four laboratories two of which are mainly for research in molecular biology/biotechnology. The following are the various technical and support services available:

Research Services:
Cell cultures, Recombinant DNA facility and Immunology

Support Services:
Animal House, Library.

Equipment:
Modern laboratory work benches, sinks etc, Water distillation equipment, Lamina flow hoods, Incubators for cell cultures, superspeed centrifuge and ultracentrifuge, PCR equipment, Sterilization equipment, Iso-electric focussing, Fraction columns, fraction collectors and Combi cold chambers/cupboards, ELISA readers, fluorescence inverted and contrast medium microscopes.

** There is the University of Yaounde **BIOTECHNOLOGY CENTER (BTC)** at N'Kolbisson a few kilometers away with which our Center collaborates. The BTC was created, refurbished and equipped by Professor J. L. Ngu (the Head of the WHO Collaborating Center) in 1989 when he was Vice Chancellor of the University of Yaounde. The BTC was the only one in Africa chosen by the WHO Tropical Diseases Research Special Programme for strengthening in molecular biology/biotechnology. The project for development and implementation of diagnostic DNA probes for the various forms of *O volvulus* (described above) was funded by this WHO Special Programme. BTC stands in grounds of about 4000 square meters in surface area and has a secure perimeter fencing. BTC has two storey buildings (ground floor and first floor) with two single-floor outcrop attachments to the major of the two buildings. There are in all 20 air conditioned and wire-mesh-screened laboratories, 10 offices, a library, a seminar/conference room, a room with computer facilities, an air-conditioned animal house, a medical emergency service etc. It has, in addition to what has been listed for the WHO Collaborating Center research support services, facilities for Microbiology including Virology and Parasitology, fermentation facility for large scale growth of micro-organisms, monoclonal antibody production facility, walk-in cold room, manual sequencing facility, modern culture chambers and accessories for Agricultural biotechnology. Support services include library, conference room, administrative block, common facility for media and buffers preparation, water reservoir, stand-by electrical plant, Maintenance workshop, animal house, restaurant.
BTC functions exclusively under the administrative authority of the University of Yaounde, and does not have full-time research staff.

Future Plans and Activities of the WHO Collaborating Center - the Molecular Biology/Biotechnology Section

The major pre-occupation of this Center is to achieve a status of relevance and sustainability in research. This would require a critical mass of molecular biologists working in close collaboration with clinicians and agricultural professional staff. Links with scientists and Institutions in Cameroon and outside Cameroon would have to be established permitting the free exchange of scientists and technical personnel. To achieve the foregoing, it is planned that:

1) The Center will move into functional premises, when funds can be found for this, with all its facilities in the same building. Its Board of Governors would comprise representatives of Donors as well as representatives of African Countries. This would assure relevance, accountability, benefit to a greater number of African countries than has hitherto been the case, and efficiency in management.
   One cannot achieve this level of autonomy and accountability in the case of BTC, as it would require changing the statute of the University of Yaounde, a decision which is not up to us
2) Training will be a major activity of this Center, the trainees being drawn from Cameroon as well as from other African Countries. A list of our staff and students trained here and in some cases abroad as well is appended.
3) Research and commercial exploitation of research results will ensure relevance and sustainabilty. The research topics described above will form the core of research activities of the Center at the beginning, but would be expanded when feasible. Low cost reagents will eventually be prepared at this Center for distribution to other African laboratories. Research will be combined with the offer of certain services, such as HLA typing, DNA probes based assay for onchocerciasis control programmes, to Public Health Services, Research Groups and Hospitals in the region.

Staff of the WHO Collaborating Center

Head: Professor Jacob L. Ngu, MB. BS, MD, FRCPE, FRCP, FCAS.

Molecular Biology/Biotechnology:

Professor J. L. Ngu,

Dr. Gabriel Lando, MSc, PhD
Dr Efigena Capuli MRCVS
Dr. Christopher Tume 'Doctorat 3eme Cycle'
Ms Celine Nkenfou (Doctoral student)
Mr. Joseph Donfack (")
Mr. Sylvain Djoha (")
Ms Martha Mesembe, BSc (Senior technician)
Ms Bridgitte Finjap, BSc (Technician)
Ms Comfort Mbeng ( )

Immunology

Trypanosomiasis:

Professor Tazoacha Asonganyi, MSc, PhD
Ms Georgette Kamongne (Doctoral student)
Mr Atabong (Technician)

Malaria:

Dr Rose Leke, MSc, PhD
Mr. Apollinaire Tsafack (Doctoral Student)

DOCTORATE STUDENTS AND STAFF TRAINED ABROAD AND IN THE LABORATORIES OF THE W.H.O. COLLABORATING CENTER IN IMMUNOLOGY AT C.U.S.S.

NAME	PERIOD OF TRAINING	AREA OF TRAINING	FOREIGN INSTITITION ASSOCIATED WITH TRAINING	FUNDING AGENCIES.
TUME Christopher	September 89-June 1990	Hybridoma technique. Fly Breeding	BMC Uppssala IC London.	WHO/TDR Geneva.
	June 91-December 1992	Molecular Biology	Chiron Corporation University of California S.F.	WHO/TDR EDNA Clark Foundati
Tsafack Appolinaire	1990-1991	Free Radicals. Alternative approach in malaria therapy.	Hebrew University Jerusalem.	USAID. Washington.
Nkenfou Nguefue Celine.	June 91- October 92.	Molecular Biology.	New England Biolabs Boston U.S.A.	WHO/TDR. NEB Foundation.
Messembe Martha.	April 91-April 92.	Molecular Biology.	University of Alabama at Birmingham U.S.A.	WHO/TDR.
Georgette Njuidje	1992-1995	Immunology of Trypanosomiasis Ph.D. Programme.	ILRAD Nairobi Kenya. University of Bristol. U.K	WHO/TDR.
Tawe Wilson	1993-1997	Molecular Biology. Ph.D Programme	Bernard Notche Institute, Germany	DAAD German Republ
Donfack Joseph.	1993	HLA Typing . Molecular technique.	London Hospital Medical College; Immunology/Molecular Biology Research Laboratories U.K.	EEC.
Dr Gabriel Lando	1989-1990	*O. volvulus* form specific probes	University of Alabama at Birmingham	WHO/TDR
Dr Rose Leke	1989	HLA typing. Serological methods	Germany	DAAD
Five Technicians	1989 - 1990 (18 months)	Immunology techniques	Laboratories of WHO collaborating Center at CUSS, Cameroon	The Center
Bridgitte Finjap	1990	Plant micropropagation techniques	Institute of Agricultural Research, Ekona, Cameroon	The Center
Dr. Efigena Capuli	1990	Plant micropropagation techniques	Institute of Agricultural Research, Ekona, Cameroon	The Center

Linkages with other Laboratories

Links have been formed with the following:

USA

1. New England Biolabs. The BTC at N'kolbisson, Yaounde, has been designated a 'sister' laboratory of NEB by the New England Biolabs Foundation. The WHO Collaborating Center's links with NEB is through BTC at N'kolbisson.
2. Drs Phil Barr and Ian Barthurst of **LXR S.F.**, formerly of **Chiron Corporation, Emeryville, S.F.**
3. Dr. James McKerrow, Department of Pathology, UCSF.
4. Dr. T. Unnasch, Dept of Geographic Medicine, University of Alabama at Birmingham.

United Kingdom

1. Professor Roland Levinsky, Dean, Institute of Child Health, University of London.
2. Professor Bottazzo, The London Hospital Medical College, Dept of Immunology/Molecular Biology, University of London.

Cameroon

1. Biotechnology Center (BTC) at N'Kolbisson, University of Yaounde 1
2. Institute of Agricultural Research, Ekona Center, S.W. Province.
3. Centre for Health Technology (CHT), a unit of the 'Automation and control Laboratory' of the School of Engineering of the University of Yaounde 1. CHT is an instrument for providing assistance on technology assessment, procurement and maintenance to the health sector in Cameroon. The CHT is a member of the HealthNet where-in a communications satellite in polar orbit permits African health care professionals to talk with colleagues in different countries and on other continents. The satellite is part of HealthNet, a communications network established by SatelLife, an international non-profit organization. Ground stations send and receive messages which are then relayed by the satellite to other ground stations. This facility puts an end to the isolation of medical and health-related research scientists from their colleagues elsewhere.
4. Clinicians in the Dept. of Medixine, Faculty of Medicine, University of Yaounde1: Professor Kathleen Blackett-Ngu (Cardiology Endomyocardial fibrosis), Dr. J-C Mbanya (Endocrinology-Diabetes mellitus), Dr Dora Mbanya (Haematology - endomyocardial fibrosis),and in the Ministry of Health: Dr. Josephine Mbwagbor (Dermatology - onchocerciasis) and Dr. Grace Fobi (Ophthalmologist-onchocerciasis).

3.H 24
Final

IMMUNOLOGY- BIOTECHNOLOGY LABORATORIES
IBL FACULTY OF MEDICINE & BIOMEDICAL SCIENCES
UNIVERSITY OF YAOUNDÉ 1- CAMEROON REPUBLIC
B.P. 4563, Nlongkak, Yaounde, Fax: (237) 203796 or 311224 Tlx; 8384KN

RESEARCH LABORATORIES:
Head: Professor Jacob L. Ngu MB,BS;MD; FRCP(Edin); FRCP(Lond);FCAS.
WHO Collaborating Centre for Research & Training in Immunology.
HLA Tissue typing :
Mr J. Donfack MSc,
Ms Comfort Mbeng
'Redox Chemistry':
Dr. A. Tsafack
Molecular Biology ;
Dr. G. Lando,PhD
Dr. C. Tume
Ms C. Nkenfou MSc,
Ms M.. Messembe BSc
Agricultural & Veterinary Sciences:
Dr. E. Capuli DVM, MPH
Ms B. Finjap BSc
Immunology:
Dr. Rose Leke ,PhD.,
Prof. T. Asonganyi PhD

Dr. John Daly, PhD,
Acting Director,
Office of Research,
Agency for International Development,
Bureau for Research and Development,
WASHINGTON D.C. USA. 20523-1818

July 24, 1993

Dear Dr. Daly,

I refer to your letter of the 25th of June, 1993, informing me that you have not got in your files my final report on the following grant award which expired on the 31st of December, 1985.: 'Onchocerciasis in the United Republic of Cameroon: Development of ELISA for Immuno-diagnosis' (3.H-24). I am at a loss to explain this hiatus in my file with you, because the signed complete report was sent to Dr. Joe Stockard by registered mail on the 23rd of October, 1985. A copy of the said letter, and I presume documents, were sent to both Dr. A. Levy (Principal Investigator), Johns Hopkins School of Medicine, Baltimore, and USAID Yaounde. Photocopies of the registered mail receipt and letter to Dr. Stockard will be found in the accompanying documents, which are bound volumes of: photocopy of the final report, copy of letter to Dr. Irvine M. Asher dated 25 July, 1985, on the subject of Progress Reports on the said grant award, copy of Financial Report, and a brochure on Immunology Biotechnology Laboratories (IBL).

I seem to have problems with my communications to USAID, Washington. The Progress Reports on the above project had apparently not been received. My letter of July 25th 1985 addressed to Dr. Asher and sent to him by registered mail was to clarify that point. Oddly enough, my joint proposal submission with Professors Golenser and Hamburger of Hebrew University, Jerusalem (C12-028) to USAID, Washington sent in August, 1991 by DHL was rejected because there was no trace of the proposed budget. I do hope that this bound copy of the report sent to your office in 1985, will get to you.

The project ended eight years ago, and the report submitted seemed adequate at the time. Since it will be [illegible] now by your experts, it seems

REC'D IN R&D/R
AUG 13 1993

to me relevant to mention that we have made some significant progress in the intervening years. This progress is summarized on page 4 of the copy of the brochure in the accompanying document. The recombinant antigens are being used in the ELISA test for onchocerciasis.

1. Sensitive, discriminatory diagnostic test.

An adult *O. volvulus* cDNA library was screened with antibodies raised in rabbits to affinity column purified *O. volvulus* antigens. Clones were selected on the basis of their non-cross-reactivity with antigens derived from other filariasis and human helminths. The proteins coded for by these genes were expressed by subcloning into pmal c vector for the E. coli system. These proteins were evaluated for use as diagnostic reagents for detecting antibodies to *O. volvulus*, using ELISA and a panel of coded sera from the WHO filariasis bank. Antigens of clone OvD 5B had 100% specificity and 87% sensitivity, those of OvD 21B 100% specificity and 63% sensitivity, while OvD 3B expressed proteins were 100% specific and 100% sensitive. Further evaluation is in progress using 330 sera from an area endemic for onchocerciasis and normal controls (sera from uninfected subjects). A major drawback is that the recombinant proteins are still fused to maltose binding protein (MBP), making the assay tedious because of the many controls (MBP control for each test serum) needed for each batch of sera tested.

Plans are underway to express these genes in the yeast system. Further, the recombinant fusion-free proteins coded by these genes will be used to develop a diagnostic test for onchocerciasis based on identifying and quantifying specific onchocercal antigens in test samples, probably the best indication of worm burden. Another set of genes initially isolatedfor candidate vaccine subunits have been cloned an d expressed in the Yeast system.Two of these genes have sequence homology. One has been expressed intracelluarly(Oncho-C27) and the other as secretory recombinant proteins 90nch0-C71). Surprisingly, both recombinant proteins have proven highly sensitive (99.99%) wen sed in the ELISA-based diagnostic test for antibodies to *O. volvulus*, using a panel of 330 positive sera and 30 negative controls. The proteins have tested specific for *O volvulus* and did not react with human, mouse or rabbit antisera to*Loa loa, S. mansoni, Brugia malayi, Ankylostoma intestinale, A. lumbricoides, and T. gambiense*.This is the first time that specific onchocercal antigens for use in immunodiagnosis have been produced by recombinant techniques in the yeast system. The procedure is being engaged to obtain Patent rights for Oncho-C27 and C71.

The USAID grant 3.H-24 was a major mile stone in the evolution of the research group at CUSS. It sets the pace for development of human resources, the acquisition of skills and equipment necessary to undertake the state-of-the-art research in this field. Our immediate goal is to develop these diagnostic reagents for use in onchocerca-endemic areas.

Yours faithfully,

J. L. NGU

cc. USAID, Yaoundé

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Immunity to Onchocerca volvulus. Serum mediated leucocyte adherence to infective larvae in vitro

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Summary

Leucocyte adherence to infective larvae of *Onchocerca volvulus* in the presence of serum was evaluated using sera from four clinically distinct groups of patients with Onchocerciasis from an area hyperendemic for the disease. Significant cellular adherence to infective larvae occured for the most part in the presence of sera obtained from subjects with either no microfilaridemia and few or no palpable nodules. These patients had, as well, the highest serum titres of specific anti-*O. volvulus* IgG antibodies. In contrast, sera from subjects with many palpable nodules and heavy skin infiltration with microfilariae (generalised disease) did not mediate significant adherence of leucocytes to infective larvae targets. Further, this group had the lowest serum levels of specific anti-*O. volvulus* antibodies of the IgG isotype. The findings are in keeping with the hypothesis that specific protective immunity may occur in *O. volvulus* infections.

Introduction

Onchocerciasis in Cameroon presents a broad spectrum of immune reactivity and dermal pathology (Ngu and Blackett, 1976). It is not known what accounts for the apparent variability in susceptibility to the infection of peoples equally at risk resulting in only a small percentage of these with demonstrable clinical evidence of resistance to the infection. This host resistance may be innate or adaptive. There is to date no evidence that such protection is immune mediated. In this regard it is intelligible to expect containment of the infection to be most effective when the host's immune reaction are directed against infective (L3) rather than the other larval stages, because of the smaller number of the former than the latter present in the host at any one time. An effective in vitro immunological defense mechanism seen in other helminth infections, schistosomiasis for example, is antibody dependent cell adherence to and subsequent cytotoxicity of larval stages (Butterworth et al., 1974). In human filariasis leucocyte adherence to infective larvae has been reported in *Brugia malayi* (Sim et al., 1982) *Wuchereria bancrofti* (Higashi and Chowdhury, 1970).

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In onchocerciasis, adherence of eosinophilis to infective larvae was observed in the presence of sera from infected patients (Mackenzie, 1980). However no conclusion could be drawn from these observations with regard to immune-mediated protection as no information was provided on the clinical status of these patients.

We report here our findings on serum-mediated adherence of buffy coat cells to infective larvae of *Onchocerca volvulus* in clinically distinct groups of onchocercal subjects from an area hyperendemic for the disease.

Materials and methods

Subjects: Sera were obtained from villagers who had been resident for more than 15 years in areas hyperendemic for onchocerciasis. Particular attention was given to identifying subjects who appeared to be clinically healthy – namely, no overt signs of onchocerciasis, but who had evidence of exposure to the parasite. Subjects were classified on the basis of a thorough physical and parasitological examination, as follows:

1. Skin microfilariae and palpable nodules (overt generalised disease + skin lesions)
2. Skin microfilariae and no palpable nodules
3. No skin microfilariae but palpable nodules
4. No skin microfilariae and no palpable nodules (no overt physical signs of onchocerciasis).

Sera were obtained from negative controls – non-Cameroonian (white) visitors who had not previously lived in areas endemic for filariasis.

Evidence of exposure – Skin Test: All subjects were skin tested with *Onchocerca volvulus* antigen (Onchocerca Supernatant) as previously described (Titanji et al., 1983). A drop of antigen was placed on an area of the forearm that had been cleaned with ethanol. A single prick was made through the solution using a 26G needle. Phosphate buffered saline was used as control.

Elisa test for specific Serum anti-O. volvulus antibodies: The test antigens for ELISA were obtained by the sequential passage of non-ionic detergent column of human anti – *Loa loa* antibody linked to sephacryl S-300 beads (Pharmacia). The antigens were eluted with 0.1 M sodium acetate buffer pH 2.5. These were passed through a human *O. volvulus* antibody affinity column, and then through a rabbit anti-whole human serum antibody affinity column each time eluting antigens with 0.1M sodium acetate buffer pH 2.5. The resulting antigen preparation was tested for specificity to *O. volvulus* and for absence of cross-reactivity with *Loa loa*, *Dipetalonema perstans*, and *Ascaris lumbricoides* antigen preparations using ELISA inhibition assays. ELISA based on immunoperoxidase reactivity was conducted as previously described (Boto et al., 1984) with the following modifications: peroxidase labelled anti-human IgM (Fc

Table 1 Adherence of cells to L3 with serum from different clinical groups, specific antibody titres and Total Immunoglobulin value.

Subjects	Adherence assay after incubation for 3 1/2 hrs.	Specific anti-*O. volvulus* antibodies mean ± SD			Total Ig in I.U./ml Mean ± SD		
		IgG	IgM	IgE	IgG	IgM	IgA
Group A: No palpable nodules None or few skin microfilariae	5 cells or more adhering to infective larvae	9650 ± 11045	500 ± 433	130 ± 97	363 ± 122	374 ± 210	147 ± 76
Group B: Presence of palpable nodule and few or no skin microfilariae	2–5 cells adhering to infective larvae	2383 ± 3024	83 ± 74	383 ± 394	356 ± 73	622 ± 674	98 ± 31
Group C: Presence of palpable nodules and heavy skin microfilariae	Less than 2 cells adhering to infective larvae	807 ± 1609	207 ± 304	229 ± 336	487 ± 228	552 ± 515	131 ± 60

Group A has 10 subjects; B has 6 subjects; C has 14 subjects

specific) IgG (Fc specific) and IgE (Cappel laboratories, Westchester, Pennsylvania) were used.

Total Ig Quantitation: Quantitation of IgG, IgM, IgA was carried out using materials and reagents from Behring Laboratories: Partigen immunodiffusion plates (Nor-partigen IgG-HC, Nor-Partigen IgM, Nor-Partigen IgA) standard serum and partigen dispenser. Five microliters of patient serum was introduced into each well and diameters read 48 hrs later for IgG and IgA and five days later for IgM. Values were read off a reference table. Whenever diameters were greater than those found on the reference chart, the test was repeated with diluted serum.

Cells for Adherence Assay: Buffy coat cells were obtained from normal Cameroonians who had no demonstrable signs of onchocerciasis and were skin test negative. Heparinated venous blood was collected, diluted 1 : 1 with medium (RPMI 1640) and layered over a 2-layer percoll density gradient (62%, 76% gradient). This was centrifuged at 1400 rpm for 20 min at 20°C. Buffy coat cells were harvested, washed three times with medium containing heparin (100 IU/ml). Concentration and viability of cells were assessed using a dye exclusion test (eosin 0,5% in 0,9% sodium chloride).

Adherence Assay: The assay was carried out in flat-bottom microtiter wells. To 4–5 larvae in a well was added: 100 ul of undiluted serum (hyperimmune patient serum or normal human control serum) and 5×10^3 leucocytes in 100 ul of medium. Plates were incubated at 37 °C. Wells were examined using an Olympus inverted microscope, at 1 hr, 3 1/2 hr, 24 hr. Larvae with more than five cells adhering to it were considered positive for the assay. Two sets of negative controls per microtitre plate were assayed simultaneously with subject sample. These were: medium alone, and serum from healthy subjects, in place of serum from patients.

Statistical Analyses: The student T test, and correlation analysis were used.

Results

Three groups of patients were identified on the basis of the numbers of cells adhering to target infective larvae in culture medium to which patient serum had been added (Table 1).

Group A: Many cells adhered to all target larvae. In some cases these appeared as clumps of cells all over the target. In others the cell clumps were confined to the extremities of the worm. In yet another group the cells were fewer but adhered to the worm at several sites. All, except one of the patients had no palpable nodules. Only half of these subjects had skin microfilariae, for the most part sparse in desity.

Group B: subjects showed adherence of a few cells (2–5) to some larvae.

Group C: There was either no adherence of cells to target, or 1 or 2 cells adhered to a few larvae. Most of these patients had palpable nodules and heavy microfilariae infection of the skin.

Intradermal Reaction to O. volvulus antigens: This reaction was positive in all 30 subjects in this study. The difference between the test antigen and the control was at least three millimetres in all cases.

Specific Antibodies: Specific antibody levels are given in Table 1; specific anti-*O. volvulus* IgG was highest in Group A subjects and least in Group C. The difference between these two groups was significant ($P < 0.01$).

Total Ig values: These are high in all groups for IgG and IgM, but not for IgA. There is no significant correlation between total Ig values (IgG, IgM) and specific antibodies (IgG, IgM).

Discussion

Specific antibodies to antigens derived from the worms are produced in patients with *O. volvulus* infections (Ngu and Blackett, 1976). Whether these antibodies are protective or not is not known since they can be detected in high titres in individuals with on-going infection.

In our studies, the group of patients that showed no palpable nodules and no detectable skin microfilariae had the highest specific antibody titres (IgG) and showed the most serum mediated cellular adherence to infective larvae. Group A patients seem clinically protected compared to group C. The significant difference in specific IgG levels between groups A and C suggests that this is the Ig class that is most important in this antibody dependent mechanism.

Experiments on in vitro adherence of cells to infective larvae of *B. malayi* (Sim et al., 1982) *W. bancrofti* (Higashi and Chowdhury, 1970), *B. pahangi* (Chandrashekar et al., 1985) suggest that serum dependent cell mediated mechanisms may be important in the destruction of these worms. Effective protective immunity in hosts would be directed preferentially against infective larvae (L3) than against skin or embryonic microfilariae, since the numbers of L3 involved at any one time is much smaller than those of the others. Further, the time taken by L3 to develop to adult worms (months to years) is sufficiently long for the host to mount an effective immune response. This may account for the apparent protection of some individuals in our study group all of whom had been exposed to the infection as evidenced by a positive immediate hypersensitivity reaction to intradermal injection of *O. volvulus* antigens. Factors governing susceptibility to or protection from overt disease need to be studied.

The presence of microfilariae in patients with *B. malayi* (Sim et al., 1982) and in onchocerciasis as seen in this study may hinder the production of antibodies necessary for adherence and possibly for protection, or the presence of large numbers of microfilariae could mop up antibodies produced.

There is probably a spectrum (Ngu, 1978) whereby in the prepatent period, a patient is able to respond to bites of Simulium and the introduction of infective larvae by producing high levels of antibody (IgG). As the patient increasingly gets overwhelmed with the disease, skin microfilariae increase, and IgG antibody production is suppressed. Such immunosuppression in the presence of skin microfilariae may be related to numbers of microfilariae (induced tolerance).

Cell types involved in antibody-dependent cell cytotoxicity seem to vary in the different reports. Identification of the cell population in our studies and ultrastructural studies to show any damage are being carried out.

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