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Project Title: Mechanisms of Survival of *Onchocerca volvulus* in the Human Host.

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EXECUTIVE SUMMARY

Onchocerciasis or 'river blindness' afflicts about 17 million people in tropical endemic countries. The aim of this project was to determine the role of surface molecules of Onchocerca volvulus, the parasitic nematode that causes the disease, in evading host immunity. This information would provide a rational basis for the design of new control measures.

Accordingly, we have identified and characterised the surface sugars and proteins on five worm stages occuring in humans. Between 2 and 10 polypeptides were demonstrated on 5 worm stages which also displayed a variety of sugars. Both the polypeptide and sugar compositions varied from one worm stage to another; although we found common components that were shared by all stages, there were also stage specific antigens unique to defined worm stages. The changing composition of the worm's surface antigens was seen as a means of escaping host immunity. Furthermore, the worm stages appeared to be protected from host immune attack by coatings of human-like proteins (collagens and laminin).

A surface protein, identified with a specific monoclonal antibody was shown to mediate the killing by patients' leucocytes of immature microfilariae (mf) of O. volvulus. Considering that antigens released by dead mf are responsible for disease manifestations this protein is being examined further for a role in immune protection.

Further studies of the surface antigens led to a new diagnostic test for 'river blindness'. The new test detects excreted O. volvulus antigens in patients' urines using specific monoclonal antibodies which we developed to surface components. Once tested on a large scale this test could be applied in rural areas for the detection of prepatent and/or low level infections whose treatment are more tolerated than heavier infections.

In conclusion, this study has improved our understanding of host-parasite interactions and proposed new tools for the control of onchocerciasis.

4.1. RESEARCH OBJECTIVES:

The parasitic nematode, Onchocerca volvulus is a major public health hazard which affects about 17 million people in Africa, Central America and the Yemen. Currently, there is no satisfactory method for eradicating the disease. There is no preventive vaccine. Available drugs like diethylcarbamazine and suramin are known to induce adverse reactions in patients. Ivermectin, the newly developed drug, kills mf but has little effect on adult worms (Goodwin, 1984). Consequently, adult O. volvulus may persist for as long as 10 years in humans with apparently functional immune systems. The mechanism by which O. volvulus evades host immunity is poorly understood, but by analogy with other parasites, one may postulate that the surface of the worm plays an important role in the process (Cohen, 1982). It was proposed that factors such as variations in worm surface, antigen disguise and inhibition of host immunity are possible mechanisms used by O. volvulus to escape host immune attacks. The overall aim of the present project was to determine the role played by surface antigens and other molecules of O. volvulus in evading host immune systems. Specificially we proposed to:

a) identify the surface molecules (including protein and carbohydrate antigens) on each of the developmental stages of O. volvulus.
b) isolate the dominant and/or stage specific antigens of developmental stages of O. volvulus and characterise these biochemically.
c) determine the role of the isolated molecules on the immune response of the host.

In accordance with the provisos of the USAID reviewers, we were limited to the first of the specific objectives listed above. However, our results show that we went beyond the first objective

4.2. RELEVANCE TO DEVELOPMENT:

Onchocerciasis has adverse socio-economic consequences. Apart from disabling and disfiguring its victims, it may render them blind thereby decreasing their productivity whilst increasing their dependence on scarce family resources. Large scale evacuation of fertile lands near high onchocerciasis transmission areas has been

documented in West Africa and associated with a fall in agricultural productivity, the main source of income of the afflicted rural masses. Since onchocerciasis is endemic in Tropical Arrica, Latin America and the Yemen, any contribution to its control is likely to have a positive international impact. New control measures may include effective drugs, vaccines and diagnostic methods.

4.3. INNOVATIVE ASPECTS:

At the onset of this project, few laboratories were concerned with the problem of the mechanisms of survival of O. volvulus in man. We chose to investigate the cuticle because it is the major organ of contact between the parasite and the host immune system. As such the cuticle was seen as a decisive component in the host-parasite interplay (Maizels et al, 1982; Politz and Philipp, 1992). Our choice has since been vindicated by the significant growth in the number of publications on this topic (for reviews see Betschart, 1990). Our proposal to investigate host immune responses with isolated or specifically labelled O. volvulus molecules has also gained wide currency with the application of recombinant DNA and hybridoma technologies (see results).

4.4. ASSISTANCE FROM OTHER ORGANIZATIONS:

The successful completion of this project was facilitated by the collaboration of many organizations. First of all, the A.I.D. Mission in Yaounde was always helpful and cooperative, in solving management problems and maintaining linkages between our laboratory and A.I.D. Office in Washington.

The National Research Council, acting through the Board on Science and Technology for International Development (BOSTID) organised training workshops in which we participated. In November 1991, BOSTID sponsored an International Biotechnology Workshop at our Centre. Not only did this workshop help to train our researchers in many techniques that would be needed in future, it launched our Centre internationally.

Research and/or Training grants were received from other international organisations, including 1 Fellowship from W.H.O. (Geneva), 2 training grants from New England Biolabs, 3 scholarships from the International Program in Chemical Sciences (IPICS, Uppsala). Besides, IPICS has been supporting the purchase of reagents and small equipment items for our group since 1988.

5. METHODS AND RESULTS:

This Section is based on five publications, derived from this project and referred to by their Roman Numerals (see Section 7).

5.1. SURFACE SUGARS OF ONCHOCERCA volvulus (I):

Lectin-binding assays were used to determine the occurence of exposed sugar moieties on infective larvae (L_3), adults (male and female) nodular and skin microfilariae and eggs of O. volvulus. The various parasite stages were isolated and reacted alive with fluorescein isothiocyanate-labelled lectins of diverse specificities. After washing off excess unreacted lectins with buffers the parasites were examined under a fluorescence microscope. These studies enabled us to detect D(+)-galactosyl, D(+)-mannosyl and N-acetyl galactosamine residues on the worm stages. However, the combination of surface sugars detected varied considerably between the parasite stages. Additional studies suggested that some of the sugars were constituents of antigenic glycoproteins (II, III). The data in I also revealed marked differences between O. volvulus and other filarial worm stages with respect to lectin binding and in keeping with the known specificities of these antigenically related parasites.

5.1 SURFACE POLYPEPTIDES (II & III):

Several approaches were employed to identify and characterize the cuticle-associated polypeptides of the worm stages described under 5.1.

Viable parasites were radio-iodinated in the presence of the catalyst, iodogen. Their surface components were then solubilised with detergent for analysis by SDS-PAGE and autoradiography directly or after immunoprecipitation with specific antisera. In

the first phase of our work, sera from patients with different densities of skin mf were used in the immunoprecipitation experiments. No difference was found in the antigen pattern, recognised by sera from different patient groups. However, the number and molecular sizes of polypeptides detected varied from one stage to another. All worm stages contained polypeptides that failed to react with human immune serum and were thus considered to be non-immunogenic in man. These non-reactive polypeptides seemed to represent most of the radiolabel-incorporated by female worms and infective larvae. A few antigens appeared to be stage specific, occuring only on one of the worm stages studied, but a majority of them were shared by several stages. Thus like the surface sugars the polypeptide antigen profile seemed to vary from worm stage to stage. This implied that immune responses mounted against one stage would not be effective against subsequent stages.

Indirect immunofluorescent antibody tests (IFAT) with specific antisera revealed the presence of connective tissue components tightly bound to the adults; nodular and skin mf. Thus collagen type IV was detected in adult females, type VI on skin mf and laminin on nodular mf. These observations confirmed the view that host antigens may disguise the parasites thereby protecting them from immune attack and prolonging their survival: a finding which was further corroborated by the failure of onchocerciasis sera to react with skin mf in the IFAT.

Electron microscopic studies indicated that radioiodination catalysed by IODOGEN may target antigens in the sub-epicuticular layers i.e. in the basal, cortical and hypodermal layers of the cuticle (III). To identify surface antigens we utilised biotinylation as an alternative labelling method since biotin is not expected to penetrate the epicuticle.

Biotinylation revealed polypeptides of 97, 61, 58, 53 and 50kd on adult males, but not females or microfilariae, suggesting that a majority of the iodogen-labelled antigens were not necessarily exposed to the surface. Taken together with the preceeding results, we now believe that the epicuticle of all other stages except adult males is poorly antigenic. Under certain conditions the surface may harbour exported antigens that can be demonstrated

by labelling. Such antigens may play a role in mediating the attack of the parasites by immune cells (V).

HUMORAL IMMUNE RESPONSES TO A MAJOR CUTICULAR ANTIGEN (IV)

During the course of the studies reported in II and III, we noted that antigens with the apparent molecular weight range of 67-68kd were frequently recognized by sera from all patients' groups. The same antigens were present in the five worm stages studied. A polyclonal rabbit antiserum to a cuticular fraction designated F5 also recognized this antigen(s). The rabbit anti-F5 was used to screen a cDNA expression library for expressed O. volvulus antigens. One of the clones isolated expressed a polypeptide of molecular weight 67kd. Its parent proteins were isolated from adult female worms by affinity chromatography and used in serological analysis in parallel with the corresponding recombinant protein (rp67). The results obtained showed that in patients' sera there were high concentrations of IgG4 and IgE antibodies to both the native p67 and rp67. The p67 was however, more specific in detecting anti-onchocercal antibodies. Partial nucleotide sequence data (Sakwe & Titanji unpublished) have indicated that rp67 is an analogue of the major Onchocerca antigen described by Donelson et al (1988). Further studies are in progress to determine the role of rp67 in host-parasite interplay and in immunodiagnosis.

5.4. APPLICATIONS OF SURFACE-ASSOCIATED (CUTICULAR) ANTIGENS OF O. volvulus

The results presented in this section went beyond the provisos of the USAID reviewers, as they addressed the problems of diagnosis and immune protection.

5.4.1. AN IMMUNODIAGNOSTIC ASSAY FOR ONCHOCERCIASIS (V)

Using the rabbit anti-F5 antibodies we had earlier demonstrated the presence in onchocerciasis sera and urines of cuticular antigens, which we proposed as targets for immunodiagnosis (Titanji & Ngu, 1986). During the reporting period the F5 antigens were used to raise a pannel of monoclonal antibodies termed MOVs. The MOVs were selected on the basis of

non-reactivity with human skin, Ascaris lumbricoides and Loa Loa antigens and reactivity with O. volvulus cryocuts. The first batch of 8 MOVs were then evaluated in an antigen capture assay for onchocercal antigens in urine. In this assay the rabbit anti-F5 was immobilised on polyvinyl microtitre wells and used to trap antigens present in urine or patients' sera. After eliminating excess unbound antigens, the bound antigens were then reacted with the MOVs. In the final step the presence of the MOVs were revealed by an anti-mouse globulin enzyme conjugate which catalysed a colorimetric reaction with appropriate substrates. The monoclonal antibody, MOV1, emerged in this assay system as the most specific for the detection of onchocerciasis. Urine was a better specimen for the assay than sera. With a 98% sensitivity and over 90% specificity the developed test compared favourably with others described in the literature.

5.4.2 CELL-MEDIATED AND MONOCLONAL ANTIBODY-DEPENDENT KILLING OF ONCHOCERCA volvulus MICROFILARIAE (VI).

Following the acquisition of the hybridoma technology and its application in Section 5.4.1, another set of 32 monoclonal antibodies were prepared, this time against surface extracts of females, microfilariae and L3-infective larvae of O.volvulus. The same selection criteria were used for this set of MOVs as was described above (Section 5.4.1.). Particular attention was paid to MOVs that reacted with the cuticle. In order to investigate the roles of these MOVs, cytoadherence and cytotoxicity experiments were conducted by incubating microfilariae and patients' leucocytes in the presence of the MOVs.

Our results showed that cytoadherence to mf was selectively enhanced by monoclonal antibodies that recognized a 27kd antigen. The cytoadherence was more dependent on the presence of human complement than the killing reaction which occurred almost to the same degree in the absence of complement. Both reactions required the monoclonal antibody MOV32. These results suggested to us that target antigen of MOV32 is probably involved in mediating the killing of mf by leucocytes in vivo. There is thus an obvious

interest to characterise this target antigen further as a possible vaccine component against onchocerciasis.

6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The most potentially useful aspect of this study is the diagnostic test which we are now evaluating on a larger sample. If our results are confirmed we believe that the method would be adopted for use in rural hospitals and dispensaries in the endemic areas. Already reference is being made to this laboratory to diagnose difficult cases of onchocerciasis in the country.

This project has also strengthened the research capacity of our department by introducing a variety of state-of-the-art techniques: radio-isotopic methods, hybridoma and recombinant DNA technologies, etc. Within the context of the project a number of young researchers have been trained. These include W.F. Mbacham, J.P. Muluh, Evehe Marie-Solange and Amos Sakwe. The first three of these have already obtained their doctorates: the last, Mr. Sakwe, will graduate in early 1993 with a doctorate in biochemistry as well. The P.I. has been promoted to the rank of Associate professor from Senior Lecturer, and has been appointed as the head of the country's only Biotechnology Center.

The equipment items provided by the project are vital for running the Centre's research and teaching programs. Two of our trainees have been employed as teachers in the department. Overall, this project has contributed significantly in expanding the scientific capacity and scope of the host laboratory which hither-to-fore dealt only with simple serological studies.

7. PROJECT ACTIVITIES/OUT-PUTS

7.1 MEETINGS ATTENDED by the P.I.

- P.S.T.C.Conference on Biotechnology for Health and Agriculture. Washington 6-9th June, 1988.
- International Congress on Malaria and Tropical Medicine. Amsterdam, September, 1988.
- International Epidemiology Conference held in Harare, Zimbabwe on 7th-11th August, 1989.

- Onchocerciasis Conference held in Leiden, Netherlands in September 1989.
- International Symposium on the Biochemistry and Molecular Biology of Nematode Cuticle, held in Basel, Switzerland in September, 1989.
- 7th International Congress of Parasitology held in Paris in 1990.
- First International Congress of the Federation of African Immunologists, held in Harare, in June 1992.
- International Congress of Infectious Diseases held in Nairobi, 1992.
- Local Meetings in Cameroon: Cameroon Biosciences Annual Conferences (Ngaoundere, 1989, Dschang 1991); Cameroon Biochemical Scoiety Meeting held in Yaounde, November 14,1992.

.7.2 LIST OF TRAINING WORKSHOPS

- Advanced course on PCR and Molecular Biology techniques held at the Catholic University of America, Washington DC, Sept.20-28, 1990. (Attended by V.P.K.TITANJI).
- PCR/Probe and Recombinant DNA Technology: International workshop organised at the Biotechnology Centre, Yaounde University with the assistance of USAID/BOSTID and CUA, November 11-22, 1991. (Attended by B.ATOGHO, P.N.NDE, M.S.EVEHE, W.F.MBACHAM, J.P. MULUH & V.P.K. TITANJI).
- Biotechnology Course at Smiths College, Massachussetts. (Attended by A.M.SAKWE and W.F. MBACHAM).

7.3 LIST OF PUBLICATIONS FOR THE ENTIRE PROJECT: (See Appendixes).

I. TITANJI, V.P.K., MBACHAM, W.F., LEKE, R.G.F. (1987). Lectin-binding properties of Onchocerca volvulus developmental stages. Annals Universitaire des Sciences de la Sante, 4(2),396-401.

II. TITANJI, V.P.K., MBACHAM, W.F. (1990). Identification of the surface polypeptide antigens of the infective larvae of Onchocerca volvulus. Transactions of the Royal Society of Tropical Medicine and Hygiene, 84, 696-700.

III. TITANJI, V.P.K., MBACHAM, W.F. and SAKWE A. (1990). Identification of different radio-labelled antigens of the developmental stages of Onchocerca volvulus. Acta Tropica, 47, 307-321.

IV. TITANJI, V.P.K., MBACHAM, W.F. and SAKWE, A.M. (1991). Application of defined polypeptides and monoclonal antibodies to the diagnosis of human Onchocerciasis. Abstract No.37 (6 pages). Second CEC Filariasis Meeting held in Amsterdam in 1991.

V. MBACHAM, W.F., TITANJI, V.P.K., THUNBERG, L., HOLMDAHL, R., and RUBIN, K.(1992). A monoclonal antibody-based immunodiagnostic assay for onchocerciasis. Tropical Medicine and Parasitology, 43, 83-90.

VI TITANJI, V.P.K., NDE, P.N. and MBACHAM, W.F. (1992). Cell-mediated and Monoclonal antibody dependent killing of Onchocerca volvulus microfilariae. Scandinavian Journal of Immunology, Supplement 11, pp.

7.4. PATENTS AND PRIZES

No patents were registered, but the P.I. was awarded the 1992 International Society of Infectious Diseases PRIZE (for the Africa, W.H.O. Region), during the ISID Meeting in Nairobi.

8. PROJECT PRODUCTIVITY

Did the project accomplish all the proposed goals? Yes.

9. FUTURE WORK

We have won the contract for a continuation project PSTC No.10 080/936 5600 entitled 'Molecular Cloning of the Surface antigens of Onchocerca volvulus'. The full details of this project were submitted to USAID Washington to which the reader is referred.

10. LITERATURE CITED

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A monoclonal antibody-based immunodiagnostic assay for onchocerciasis

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Abstract

Five murine monoclonal antibodies raised against *Onchocerca volvulus* cuticular extracts and termed MOVs (1–4 and 6) were selected based on reactivity with *O. volvulus* cryosections, and non-reactivity with cryosections of human skin and/or nodular tissue. Two others MOVs 5 and 7 reacted with both. Using the peroxidase-anti- peroxidase (PAP) histochemical method, the target epitopes of MOV 1 were located in the cuticle's basal and cortical layers, those of MOV 2 in the cortical layer; whilst MOV 3–7 stained the basal layer. A sandwich ELISA was then developed The trapping polyclonal antibody was raised in rabbits utilising the same antigens as for preparation of the MOVs. Once captured on microtiter plates, target antigens were identified by the sequential binding of a MOV, followed by a goat anti-mouse globulin/peroxidase or alkaline phosphatase conjugate that catalysed a colorimetric reaction in the presence of appropriate substrates. In this system, MOV 1 emerged as the most specific and potent reagent capable of recognizing antigens of *Onchocerca sp.* with a minimal detection limit of 78 ng per test. MOV 1, failed to react with extracts of *Loa Loa, Ascaris lumbricoides* and *Ascaris suum* in the test. The developed assay relied on the use of MOV 1, required only 1 ml of urine or 0.05 ml serum. About 97.8% of the 47 urines and 50% of the 20 sera from patients studied gave positive results. Only 1 (3%) of 32 control urines and up to 80% of the 10 control sera studied tested positive, suggesting urine as a better specimen source. The test was highly reproducible with intra- and interassay variations of 6% and 15% respectively. In conclusion our results show that *O. volvulus* cuticular components are released into urine in significant quantities to reveal current infection, and that some of these antigens recognised by MOV 5 and 7 may serve as a camouflage to protect the worm from host immune responses.

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Introduction

Estimates of the prevalence of onchocerciasis vary between 17 and 50 million infected people within the endemic areas of Africa and Latin America that harbour a risk population of about 80 million. As a leading cause of blindness, onchocerciasis is also an aggravating factor of the economic backwardness of the endemic countries (WHO, 1987).

Accurate diagnosis of onchocerciasis in subjects with slight infections is difficult to achieve through examination of skin biopsies which often do not contain microfilariae. If diagnosis is established early, treatment can follow without evoking dangerous reactions that tend to develop in heavily infected patients with high loads of skin mf. Patient follow-up during treatment requires accurate diagnostic tests (WHO, 1983). However, such tests are currently lacking.

Generally, it is agreed that the detection of worm products in body fluids is more likely to reflect current infection than specific antibody concentration which may correspond to either present or past infection (Hamilton and Scott, 1984; Haque et al., 1982; Titanji and Ngu, 1986). Thus several studies have been devoted to the detection of onchocercal antigens in body fluids as a more reliable diagnostic method. In 1980, Paganelli et al. reported a significant increase in circulating Clq-binding immune complexes (IC) in onchocerciasis. However the presence of ICs was not diagnostic of the disease, although it indicated the existence of circulating antigens that subsequently became the targets of numerous immunodiagnostic tests (Ouaissi et al., 1981; Des Moutis et al., 1983; Titanji and Ngu, 1984; 1986; Hamilton, 1985; Petralanda et al., 1988).

In our laboratory, a diagnostic antigen fraction termed F5, was isolated from the cuticle of adult female worms and used to develop an immunoradiometric assay that detected these same antigens in 95% of the patients' urines (Titanji and Ngu, 1984, 1986). The aim of this investigation was to develop an immunoenzyme assay for easier application in endemic areas. Thus monoclonal antibodies have been developed and characterised against fraction F5 antigens. A preliminary abstract of this investigation has appeared (Mbacham et al., 1988).

Materials and methods

Patients: Subjects were physically examined by a physician for nodules and skin lesions. Skin snips were taken from the iliac crest, calf muscles and shoulder, deposited in RPMI culture medium and subsequently examined under the microscope for emergent microfilariae (mf).

Parasites: Nodules were excised under local anaesthesia, from patients attending the onchocerciasis clinic, Batchenga in the Sanaga river basin of Cameroon and frozen at −70 °C for cryosections or used directly to isolate adult worms by collagenase digestion as previously described (Engelbrecht and Schulz-Key, 1984; Titanji et al., 1990). *Ascaris lumbricoides* was obtained from an 11-year-old boy in Yaounde, washed abundantly in PBS pH 7.2 and used for the extraction of antigens.

Antigens: O. volvulus antigens were prepared by extracting the worms in buffers, of varying ionic strengths and pH as described by Titanji et al. (1985). The antigenic fractions termed, F1, F2, F3, and F4 were combined with part of F5 (SDS-extract) to obtain "total extracts" (TE) of *O. volvulus*. The low molecular weight antigens (12–25 KDa) from *O. volvulus* were eluted from a preparative 11% homogenous polyacrylamide gel on which 5 mg TE protein had been separated using the buffer system of Laemmli (1970). Antigens were extracted from *Ascaris sum*, *A. lumbricoides*, *Parafilaria bovicola*, and *Onchocerca tarsicole* as described (Marcoulis et al., 1978).

Sera: Blood collected by venepuncture from onchocerciasis patient volunteers were processed for sera as previously described (Titanji et al., 1985). The sera were frozen in aliquots of 200 µl and thawed only once for use in the experiments.

Urines: Urines (10 ml) from patients fasted overnight were dialysed against 20 volumes of 10 mM Tris-HCl pH 8.0 followed by one change of distilled water for 6 hours. The dialysates were lyophilised and the residues obtained redissolved in 200 µl of distilled water. The solutions obtained was sored at −20 °C until thawed only once and used for antigen assay.

Control sera and urine: Blood and urines were collected from European subjects with no symptoms of parasitic infections or from healthy residents of Yaounde with no *O. volvulus* infections. The control samples were treated and preserved as described above.

Rabbit antisera: Rabbits (Mankon-strain, Cameroon) were bled for pre-immune sera. Subsequently, 1 mg of *O. volvulus* F5 antigen dissolved in 1 ml of PBS and emulsified with an equal volume of Freunds' Complete Adjuvant was injected intramuscularly into the calf muscles of the rabbits. The rabbits were given boosters of 250 µg of F5, in Freunds' incomplete adjuvant, weekly for four weeks and then bled, one week after the last booster injection. The blood obtained was used for the preparation of immune sera that were stored at −20 °C.

Normal European skin; was obtained from a non-parasitised 42-year-old female Swede undergoing plastic surgery at the University Teaching Hospital, Uppsala, Sweden. It was cryosectioned and used for immunohistochemistry.

Preparative methods

Monoclonal antibodies: F5 antigen was emulsified 1:1 with Freund's complete adjuvant (FCA). Five BALB/C mice were each innoculated intradermally into their hind foot-pads with 25 µg of F5 antigens. The mice were killed 11 days later by asphyxia in ether vapour. The popliteal and inguinal lymph nodes were dissected out and used for the isolation of lymphocytes (Holmdahl et al., 1985).

Lymphocytes were fused with SP2/0 mycloma cells (Mount Sinai School of Microbiology, USA) in the ratio of 1:5 using 50% (w/w) PEG (Sigma, M. Wt. 1500) in serum free Dulbeco's Minimal Essential Medium (DMEM) supplemented with 100 U.ml^{-1} penicillin, 100 µg.ml^{-1} streptomycin and 10 µmol glutamine (PGS-SIGMA). Hybridomas were grown as previously described (Galfre and Milstein, 1982; Holmdahl et al., 1985).

After 10 days of cultures, IgG producing hybrids were selected on the basis of (i) positive reactions in an alkaline phosphatase based Enzyme Linked Immunosorbent Assay (ELISA) with the immunogen (F5); (ii) non-reactivity with *Ascaris sp.* antigens in a similar assay; (iii) positive reactions with *O. volvulus* sections in an immunohistochemical peroxidase anti-peroxidase staining method (see below) and (iv) non-reactivity with human skin in the same immunohistochemical staining procedure.

Hybrids fulfilling the selection criteria were cloned twice by limited dilution, at 5.0 cells ml^{-1} (Galfre and Milstein, 1982; Holmdahl et al., 1985) and grown in DMEM supplemented with antibiotics containing 3% FCS for 3 weeks. The monoclonal antibodies obtained were purified by affinity chromatography on Protein A-Sepharose 4B columns (Pharmacia) as described by the manufacturers. In the text that follows these monoclonal antibodies are referred to as MOV i.e. *Monoclonal* antibodies to *O. volvulus*.

Characterisation of MOVs: The titers and reactivity of MOVs with other helminths were determined in a direct ELISA. Isotypes, were determined in a sandwich ELISA with subclass specific reagents (Jackson Laboratories, USA). These ELISAs are described below.

Identification of target antigens: These were identified after isolation by affinity chromatography on MOV- Sephacryl columns. MOV 1 or MOV 2 (200 µg), was coupled to periodate activated Sephacryl S-300 (Pharmacia) according to the method of Wright and Hunter (1982). F5 antigens radioiodinated by the iodogen method (Titanji et al., 1990) and containing (300–500) x 10^3 cpm of trichloroacetic acid precipitable radioactivity were purified on the MOV-Sephacryl S-300 columns equilibrated and eluted with phosphate buffered saline pH 7.2. After sample application, elution was interrupted and the columns incubated for 12–14 hours at 4 °C. Subseqently they were washed with PBS until the absorbance at 280 nm of the effluent was zero. Elution was continued with 0.1 M sodium acetate buffer pH 4.0 containing 0.5 M NaCl. Fractions of 1 ml were collected and neutralised with a few drops of 1 M Tris. Peak radioactive fractions were pooled and 100 µl of 1% BSA added to them as carrier protein. The samples were further desalted by chromatography on a Sephadex G25 (PD-10 column- Pharmacia) equilibrated and eluted with PBS. The proteins in the void volume were analysed on a 6–20% gradient SDS-PAGE (Laemmli, 1970). The stained gels were then dried and autoradiographed according to the method of Westerman (1985). A Sephacryl column with coupled rabbit antibodies to F5 served as positive control to purify F5 antigens immunogenic in rabbits. Unsubstituted Sephacryl S-300 columns served as negative control to demonstrate that the binding to affinity columns was specific.

Enzyme immuno-assays

ELISA: Monoclonal antibody isotypes and titers were determined as previously described (Holmdahl, et al., 1985) except that F5 or other helminth antigens were used to sensitise plates at 10 µg.ml^{-1} and conjugates used were goat anti-mouse antibodies linked either to alkaline phosphatase or horse radish peroxidase (Sigma).

Non-competitive inhibition ELISA: This was used to detect antigens in urines and sera. MOVs were serially diluted and mixed with an equal volume of acid treated sera (WHO, 1983) or dialysed urine, to final concentrations of 10, 1, 0.1 µg.ml^{-1} respectively etc. The mixtures were incubated for 24 H at 4 °C and then transfered to plates presensitised with F5 antigens. The MOVs bound to antigens of the urine samples were detected with an anti-mouse (IgG) conjugated to alkaline phosphatase.

The two-site or sandwich immunoenzymatic assay: This assay was used to (i) detect *O. volvulus* antigens in sera and urines, (ii) construct a standard graph using F5 as antigen source, (iii) determine the reactivity of the MOVs with *Ascaris sp.*, *L. loa* and *O. tarsicola* antigens.

In this assay, 50 µl of rabbit anti-F5 antiserum (titer, 1:10⁴) were diluted 100-fold, applied to ELISA plates and incubated for 12–14 H at 4 °C. Antigen source was either urine, sera, other nematode antigens (10 µg.ml⁻¹) and F5 (10 µg.ml⁻¹) as positive control. Plates were further incubated overnight at 4 °C, and washed. Bound antigens were reacted with 50 µl of 10-fold serially diluted MOVs (for calibrations) or MOV 1 at 10 µg.ml⁻¹ (for the test) for 2 H at room temperature (25 °C). Bound MOVs were detected by second antibody conjugates. Normal European serum and urine samples as well as African endemic controls urine samples (n = 30) served as controls. The assay was done four times and the average values for each sample was calculated and then plotted on a scatter diagram. The precision and reproducibility were calculated from internal positive standards (n = 6) included in each assay.

Revelations of ELISAs: Alkaline phosphatase reactions were used in the selection and preliminary characterisation of monoclonal antibodies. The enzymatic reactions were performed with *p*-nitrophenyl phospate dissolved at 0.5 mg.ml⁻¹ in 10 % diethanolamine buffer, pH 9.8 as substrate. The washing and dilution buffer was 10 mM Tris buffered saline, pH 7.4 containing 0.05 % Tween 20.

Peroxidase based reactions were used for the further characterisation of MOVs with comparable results. The substrate for the reaction was in this case, *o*-phenylene diamine (OPD) dissolved at 0.4 mg/ml in 10 mM citrate buffer pH 4.5 and containing 0.006 % of H_2O_2. The washing and the sample dilution buffer was PBS, pH 7.4 containing 0.05 % Tween 20. Reactions were stopped with 50 µl of 0.1 M H_2SO_4. Optical densities at 490 nm were read from a Titertek multiscan ELISA-reader. Data from individual patients (microfilariae/snip) and serum antibodies (1:1 000) were correlated with antigens in urines.

Immunohistochemical staining: the peroxidase anti peroxidase (PAP) method

For immunohistochemical staining, 6 µm thick cryosections of onchocercal nodules and normal human skin were mounted on microscope slides, air dried for 30 min and then fixed in absolute acetone (−20 °C) for 5 min. Staining was done with 50 µl of hybridoma culture supernatants per section according to the manufacturers' instructions (Dakopatts, Denmark). A murine monoclonal antibody to HLA-DR (Dakopatts) antigens was used as positive control (dilution, 1:2 000).

Protein was determined according to the method of Read and Northcote (1981), with bovine serum albumin as standard.

Statistical calculations: Data from the assays were analysed for coefficients of variations as described by Hamilton (1985) and Morrow (1981). The Student t-test was used to calculate the significance of differences between paired experimental groups.

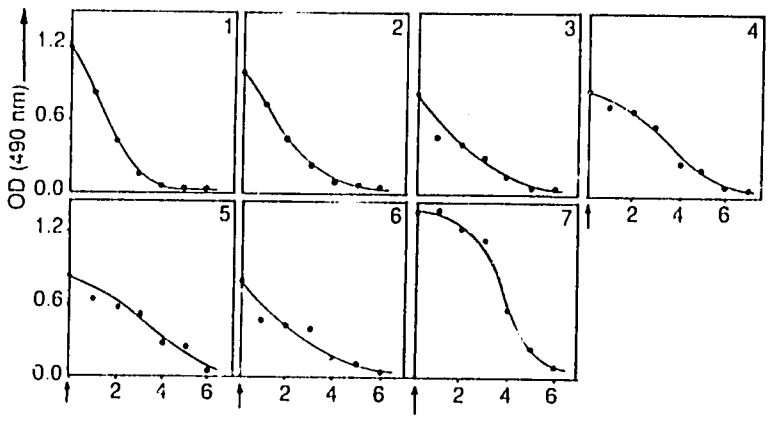

Fig. 1 Binding properties of monoclonal antibodies raised to *O. volvulus* F5 antigens (MOV). Arrow indicates the starting concentration of the MOVs which was 100 µg.ml⁻¹. ELISA plates were coated with F5 antigens as described under Materials and methods.

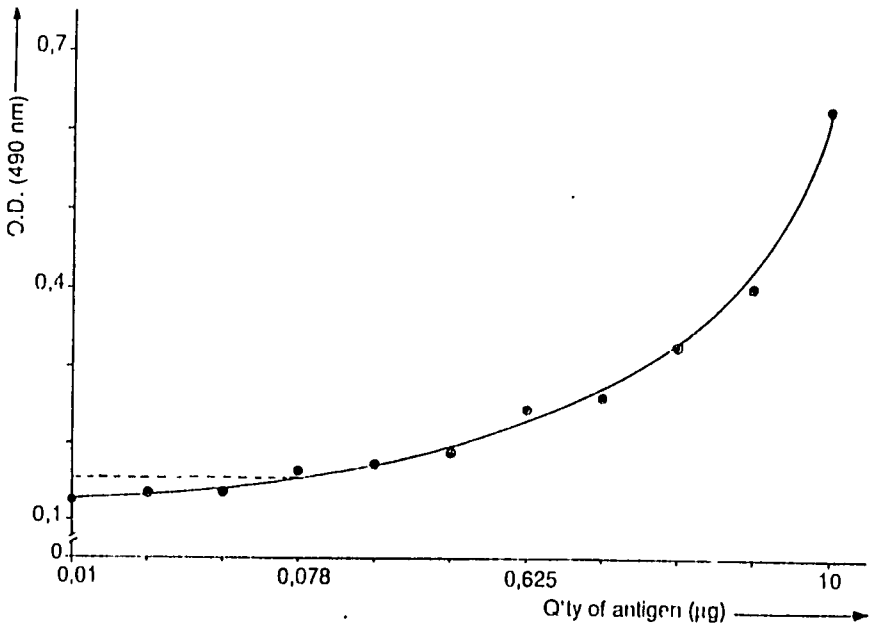

Fig. 2 Standard graph for the detection of *O. volvulus* antigens by MOV 1. The broken line represents the background + 2 standard deviations. In calibrating the system F5 antigens were 2-fold serially diluted down from 10 µg.ml⁻¹ in 0.1 % BSA and applied to plates coated with rabbit antibodies to F5. MOV 1 was aliquoted at a fixed concentration of 10 µg.ml⁻¹ and subsequent reactions were perfromed as described.

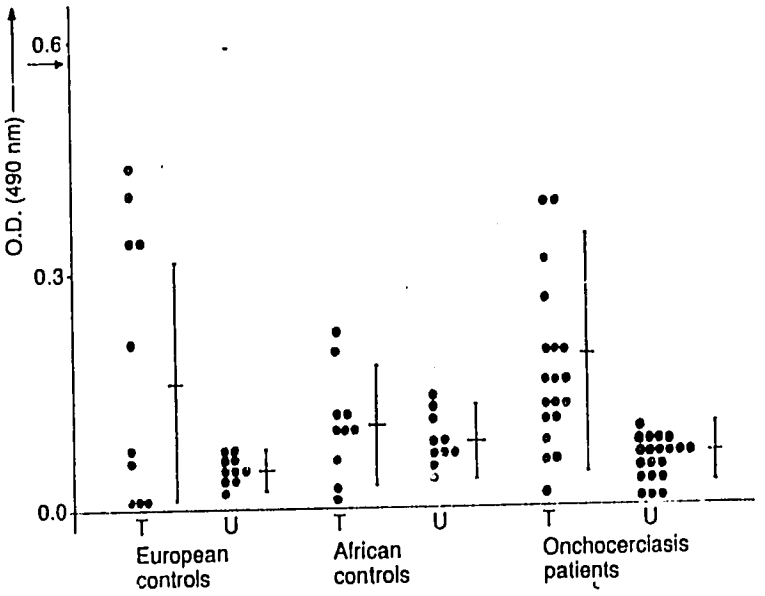

Fig. 3 Immunoenzymatic assay of circulating *O. volvulus* antigens. T – treated 0.1 M glycine, U – untreated. Arrow is the optical density of the positive control (F5 antigens, 1 µg). Horizontal bars represents the means while the vertical bars represent 1 standard deviation about the mean.

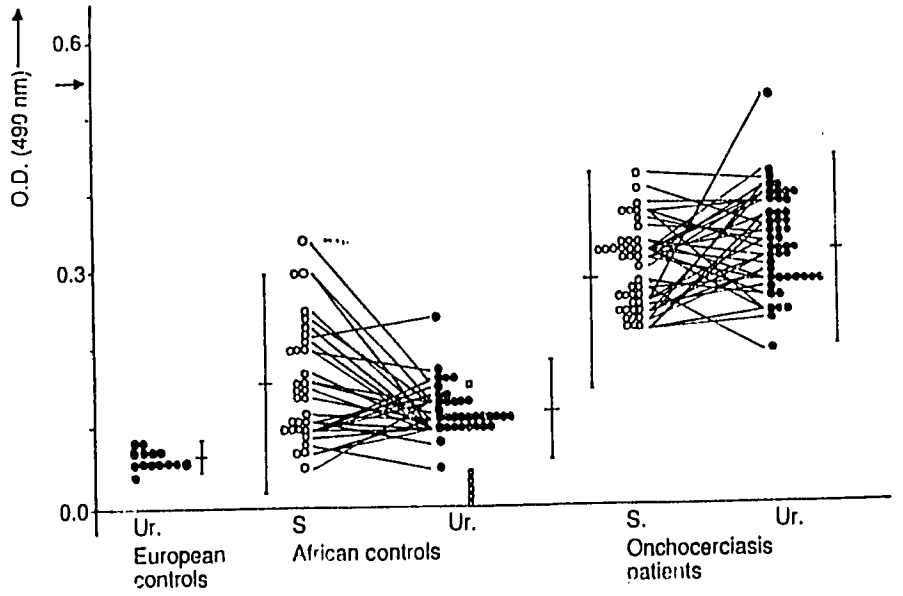

Fig. 4 Immunoenzymatic assay of *O. volvulus* antigens excreted into urine. Ur. – urines; S – sera; Arrow, O. D. of positive controls (F5 antigens, 1.0 µg). ●, antigenuria; ○, antibody concentration; □, antigenuria in active ascariasis (n = 6, 0.04 ± 0,05). Horizontal bars are the means while the vertical bars represent 2 standard deviations from the mean. The points on the diagram are means of 4 determinations agreeing within 6 %.

Results

Properties of MOVs

Subclass and binding characteristics

Of the hybridomas obtained after fusion, seven out of 15 were selected for further study as described under Materials and methods. Those which were excluded reacted either with *Ascaris sp.* or uniquely with human skin. All seven MOVs retained were of the IgG_1 subclass except for MOV 1 which was of the IgG_{2b} subclass. The titers of these MOVs, after affinity purification on Protein A columns, varied between 10^5 and 10^7 from starting concentrations of 100 µg.ml^{-1} (Fig. 1). Although titration of the MOVs started at the same initial protein concentrations, different binding profiles were obtained probably reflecting structural diversity of the target epitopes (Fig. 1). Biotinylation of the MOVs did not change their titers nor binding characteristics and could thus be used to amplify the reactions (results not illustrated).

Immunodiagnostic tests for onchocerciasis

Evidence for the presence of MOV target antigens in urines and sera was demonstrated using a non-competitive inhibition ELISA. In experiments, not illustrated, antigens present in patients' urines or sera were shown to inhibit the ELISA reactions by 71 % and 75 % respectively. Since direct inhibition ELISA is somewhat insensitive and restricted by the range of optical densities that can be obtained in a given assay, we turned to a sandwich assay which is more sensitive and flexible.

Two-site or sandwich immunoenzymatic assay: Immobilised, affinity purified rabbit anti-F5 antibodies were used as the solid phase antibodies to capture antigens in the body fluids. MOV 1, 2, 3 to 7 were then used to detect the captured antigens. MOV 1 was the most reactive, followed by MOV 2 and 3 (results not illustrated). MOV 4, 5 and 6 did not react in the assay system and so were omitted from further characterisation. Based on the above MOV 1 was used in sub-

Table 1 Detection of antigenuria in distinct patients groups.

Zone	Patient group	Number of patients tested	Antigenuria OD ± SD	Antibody mean OD ± SD
Hyperendemic (SA'A)	snip + Ab +	29	0.33 ± 0.06[a]	0.29 ± 0.05[e]
	snip − Ab +	13	0.32 ± 0.07[b]	0.30 ± 0.06[f]
Endemic control (Yaounde)	snip − Ab +	17	0.12 ± 0.02[c]	0.22 ± 0.06[g]
	snip − Ab −	17	0.13 ± 0.04[d]	0.09 ± 0.02[h]

The differences between a and c, a and d, b and c, b and d are statistically significant ($p < 0.01$). The differences between a and b and c and d are statistically insignificant.

sequent assays. Figure 2 represents a standard graph in which MOV 1 was used to detect added antigen. It can be seen that the assay detected at least 78 ng of crude F5 per test.

The sandwich assay was then applied to detect antigens in acid-treated sera. Pretreatment of sera with acid was required to dissociate immune complexes and release bound antigens (WHO, 1983). Figure 3 shows that, circulating antigens could be detected in 50 % of the sera from skin snip-positive onchocerciasis patients. Endemic control sera were non-reactive. The difference between optical density readings of the test and control sera'T (Fig. 3) was statistically insignificant ($p < 0.5$). Surprisingly there were more false positives among European sera than African control subjects (Fig. 3) probably reflecting non-specific interactions with serum factors.

When urines were used as a source of antigens, the assay was significantly specific. It detected excreted antigens in 97.8 % of proven cases of onchocerciasis ($0.001 < p < 0.01$) with respect to endemic control urines). Only one patient had optical density readings above 2 standard deviations of the mean of control urine sample-readings from Yaounde (Fig. 4)

Previous studies (Titanji et al., 1985) has shown a high prevalence of onchocercal antibodies in sera from snip negative subjects resident in Yaounde, a non endemic onchocerciasis area. It was thus interesting to examine the onchocercal antibody serum titres and antigenuria of subjects from Yaounde. The results obtained are summarized in Figure 4 and Table 1. It can be seen that the sandwich assay clearly discriminates between exposed subjects and prepatent cases. Snip-negative, sero-positive subjects from Sa'a a hyperendemic, high transmission village had O.D. readings for antigenuria that were significantly higher than the values obtained for subjects with similar profiles from Yaounde (Table 1). Based on these observations, the snip- negative but sero-positive subjects from Sa'a were deemed to be probably prepatent cases of onchocerciasis whereas similar subjects from Yaounde were assumed to have been simply exposed to the disease.

The specificity of the sandwich assay was calculated to be 96.7 %, whereas its predictive value for the active disease stood at 98 %. We calculated an intra-assay variation of 6 % (n = 6) and an inter-assay variation of 15 % (n = 5). There was no correlation between the parasitic load (mf/snip) and the ELISA readings.

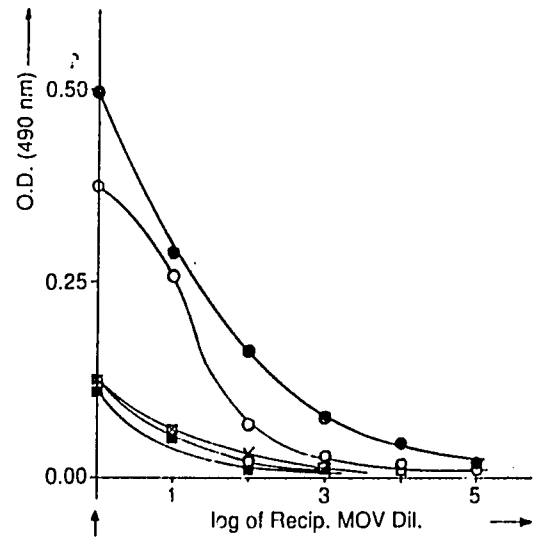

Fig. 5 Reactions of nematode antigens and MOVs. ●, *O. volvulus*; ■, *L. loa*; □, *A. suum*; ○, *O. tarsicola*; x, *A. lumbricoides*. Rabbit antiserum to F5 was coated on plates and used to trap respective parasite antigens present at final concentrations of 10 μg.ml^{-1}. Arrow = starting concentration of the MOVs (100 μg.ml^{-1}).

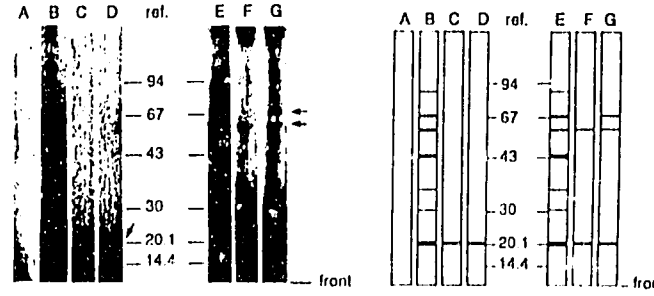

Fig. 6 Identification of target antigens to MOV 1 and 2. Labelled F5 antigens 85.10^3 cpm (B) and (8–10) x 10^3 cpm (C-G) were analysed on an 8–20 % acrylamide gel and autoradiographed for 3 days (B, C, D) and 8 days (E, F, G). C and F contain antigens eluted from MOV 2 affinity columns whereas tracts D and G were from MOV 1, affinity columns antigens in tract E were eluted from a Rabbit anti-F5 affinity column.

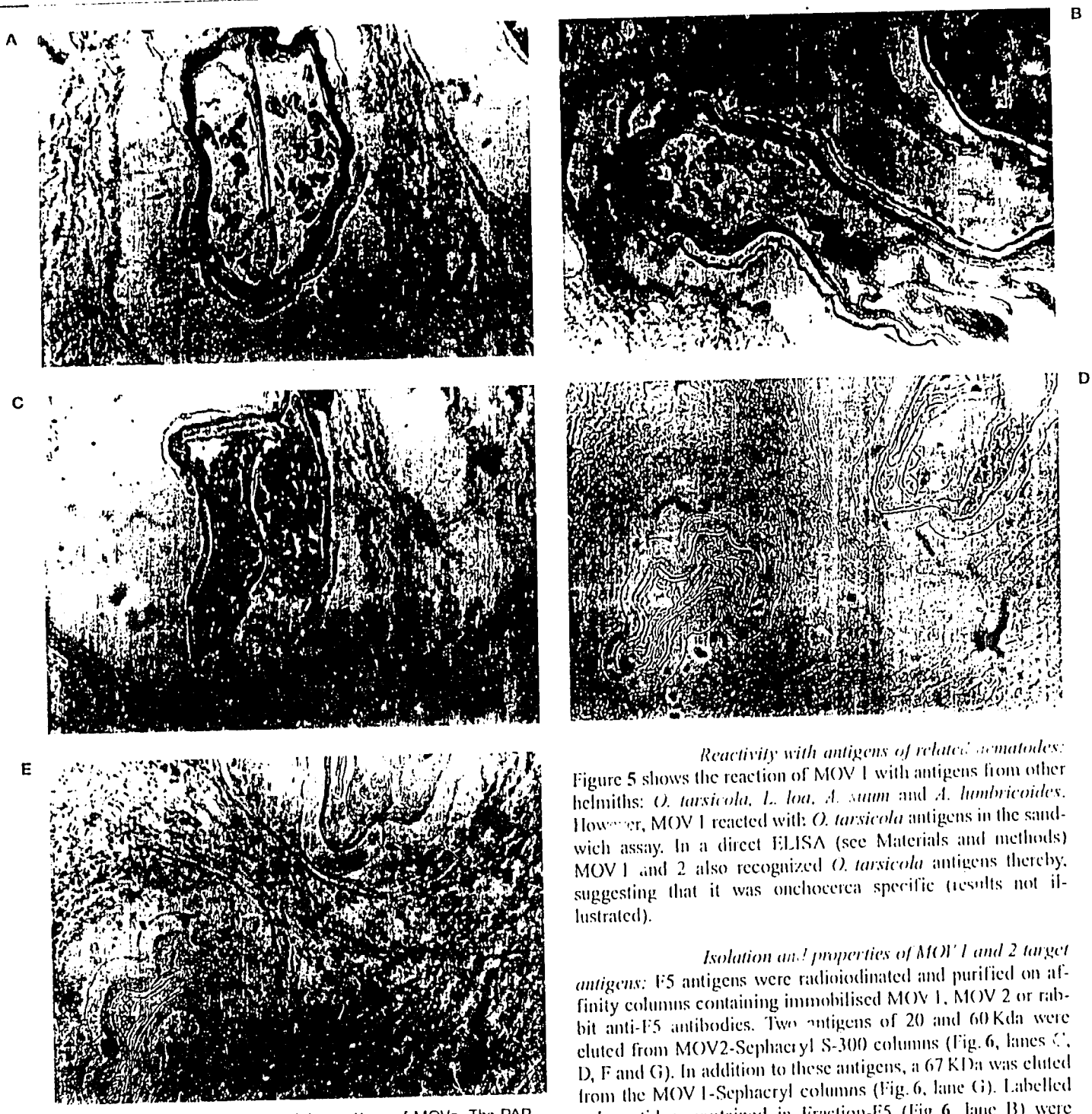

Fig. 7 Immunohistochemical staining pattern of MOVs. The PAP-based technique was used. **A** Staining of the basal layer of an *O. volvulus* worm by MOV 1, 3–7. **B** Staining of the cuticle by MOV 1 and 2. **C** Staining of the uterine contents by MOV 3, 4 and 7. **D** Negative control in which the worm sections were reacted with normal swine serum. **E** Positive control-murine monoclonal antibody against HLA-DR antigens was reacted with nodular cryosections. Note the infiltration of these nodular tissues by immune cells and the apparent lack of these on or around the worm.

Reactivity with antigens of related nematodes: Figure 5 shows the reaction of MOV 1 with antigens from other helminths: *O. tarsicola*, *L. loa*, *A. suum* and *A. lumbricoides*. However, MOV 1 reacted with *O. tarsicola* antigens in the sandwich assay. In a direct ELISA (see Materials and methods) MOV 1 and 2 also recognized *O. tarsicola* antigens thereby, suggesting that it was onchocerca specific (results not illustrated).

Isolation and properties of MOV 1 and 2 target antigens: F5 antigens were radioiodinated and purified on affinity columns containing immobilised MOV 1, MOV 2 or rabbit anti-F5 antibodies. Two antigens of 20 and 60 Kda were eluted from MOV2-Sephacryl S-300 columns (Fig. 6, lanes C, D, F and G). In addition to these antigens, a 67 KDa was eluted from the MOV 1-Sephacryl columns (Fig. 6, lane G). Labelled polypeptides, contained in Fraction-F5 (Fig. 6, lane B) were identical to those contained in the eluate from the rabbit anti-F5-Sephacryl S-300 column, indicating that all of F5 antigens were immunogenic in rabbits (Fig. 6, lane E). The rabbit anti-F5 columns serving as a positive control thus yielded antigens with apparent molecular sizes of: 20.0, 28.5, 34.0, 47.5, 60.0, 67.0, 87.0, and 148 KDa (Fig. 6, lane E). Similar results, not illustrated, have recently been obtained by Western blotting using MOVs and antiserum described above (Mbacham and Titanji, unpublished observations). Susceptibility to radioiodination suggested the protein nature of the target antigens.

Distribution of target antigens in parasite tissues and nodules: In order to localize the target antigens, *O. volvulus* female worm cryosections were stained with the MOVs as described under Materials and methods. Figure 7 shows the staining patterns observed. MOV 1 stained the basal layer and cortical layers. MOV 3–7 reacted with the basal layer of the female worm (Fig. 7A), and MOV 2 stained the cortical layers of the worm (Fig. 7B). MOVs 3, 4 and 7 also recognised epitopes of the uterine contents of the worm (Fig. 7C). Figure 7E showed murine monoclonal antibodies to HLA-DR antigens to react with immune cells, but not with nodular tissue confirming the specificity of the histochemical reactions in Figs. 7A–D.

Discussion

Previous studies have shown antigens of the cuticular fraction termed F5, to be candidates for the immunodiagnosis of onchocerciasis since these antigens could be detected both in patients' sera and urines (Titanji and Ngu, 1984; 1986). The present study confirms and extends these results through the development and use of monoclonal antibodies in an enzyme immunoassay (Table 1, Figs. 2–4). The test described herein compares favorably with others in terms of specificity, simplicity and safety. It has a minimal detection limit of 78 ng crude antigen per assay (Fig. 2) and requires a minimum of 1 ml of urine. Apparently, the assay can discriminate between exposed and prepatent infections (Fig. 4 and Table 1). This makes it more specific than the detection of specific antibody which is prone to yielding false positives when applied to endemic control subjects (Fig. 4, Table 1; Titanji et al., 1985).

The sensitivity of the present assay approaches 98%, a considerable improvement when compared with other methods which detect antigens in about 76–86% of the body fluids examined (Ouaisi et al., 1981; Haque et al., 1982; Des Moutis et al., 1984; Petralanda et al., 1988; Schlie-Gùzman and Rivas-Àlcala, 1989; Chandrasheka et al., 1990). The use of an enzymatic reaction instead of radioactivity to reveal the presence of antigens in this assay clearly favours future application in remote endemic areas. The diagnostic monoclonal MOV 1 under optimal conditions discriminated between onchocercal and other helminthic antigens (Fig. 5). Ascaris infected patients constituting 30% of Yaounde subjects (Titanji et al., 1985) tested negatively as compared to onchocerciasis subjects. It is possible that helminth antigens present in urine did not contain the epitopes detected by MOV 1 in the crude *A. lumbricoides* extracts; urines from children with active ascariasis tested negative (see Results).

In keeping with previous investigations (Ouaisi et al., 1981), we found antigen detection to be easier in urine than in serum. When the present method was applied to acid treated sera, circulating antigen could be detected only in 50% of the investigated sera (Fig. 3). This result agreed with those of Steward et al. (1982) who found IgG/IgM immune complexes in 50% of onchocerciasis sera. The reaction of MOV 1 with European sera could be attributable to the non-specific interference of serum factors (Hamilton, 1985).

No correlation was found between worm burden (mf/snip) and urine antigen concentration in this study. The processes leading to the release of MOV 1 target antigens into urine are probably complex and not proportionately related to worm burden. However Schlie-Guzman and Rivas-Alcala using polyclonal antibodies found a good correlation between skin microfilarial counts and antigen concentration in patients' urines. The use of different types of antibodies may explain the discrepancy between the cited studies.

The target antigens of MOV 1 purified by affinity chromatography were shown to be polypeptides of 20, 60 and 67 KDa, similar in sizes to other previously described antigens (Philipp et al., 1984; Cabrera and Parkhouse, 1986). In the absence of additional structural information it remains to be seen whether the targets of MOV 1 are identical to known antigens. MOV 1 reacted with *O. tarsicola* extracts which could serve as alternative sources for its target antigens.

MOV 5 and 7 recognised epitopes both in the basal layer of the worm and the epidermis of the human skin suggesting antigenic similarities between the two. Following the rigorous procedure in antigen extraction, it is unlikely that these MOVs were raised to human tissue contaminants. In a recent study (Titanji et al., 1990), antibodies to collagen type VI and laminin were shown to react with cryosections of *O. volvulus*. Antigenic similarity between the parasites' cuticle and host connective tissue may be part of an immune evasion mechanism favouring the survival of *O. volvulus* in the immune competent host.

In conclusion, this study has demonstrated the use of cuticular antigens in the diagnosis of onchocerciasis with urine as the better source of antigens. Attempts to improve further on the test sensitivity may require employing a cocktail of monoclonal antibodies raised to antigens excreted into urine. The sharing of common antigenic epitopes between *O. volvulus* and the human skin may play a role in host-parasite interactions in onchocerciasis and should be investigated further.

Acknowledgments

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Cell-Mediated and Monoclonal Antibody-Dependent Killing of *Onchocerca volvulus* Microfilariae*

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To identify the target antigens implicated in the adherence and killing of microfilariae (mf) by leucocytes, we incubated nodular mf and leucocytes in the presence of anti-cuticular monoclonal antibodies (MOVs) and fresh serum. Leucocyte donors were patients with a mean age of 37 years (with 0–1 mf/snip), who had lived in the endemic village studied for at least 10 years. After 16–20 h of incubation, up to 74% of the mf could be seen with 10 or more cells adhering to them. By 36–40 h up to 54% of the mf had been killed by the leucocytes in the presence of a cocktail of monoclonal antibodies termed MOV GIV. The degree of killing in control experiments with the monoclonal antibody MOV 1 remained lower ($P < 0.05$), ranging from 0.0 to 4.5% of mf with initial viability of 90–95%. Western blotting revealed MOV GIV prominent target antigens of 10.5, 18.0, 23.5 and 27 kDa in crude surface extracts of female *O. volvulus*. The detected antigens may play a role in host protection.

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Several studies have shown that leucocytes from onchocerciasis patients can adhere to and kill microfilariae (mf) of *Onchocerca volvulus* in the presence of antibody and complement [1, 2]. These reactions may result in the reduction of skin mf density or the exacerbation of inflammatory symptoms in the patients [3].

Eosinophils and neutrophils have been implicated in the attack of onchocercal mf, but the antigens that mediate this attack are still unknown. Herein, we show that murine monoclonal antibodies (MOVs) raised to cuticular antigens of *O. volvulus* (MOV GIV) promote the killing of *O. volvulus* mf by leucocytes in the presence of fresh normal human serum (NHS). The target antigens of the MOVs used in these experiments are probably implicated in the killing reaction.

* A preliminary account of this work was presented at the 2nd CEC Filariasis Meeting in Amsterdam, September 1991.

MATERIALS AND METHODS

All manipulations were done under aseptic conditions. Monoclonal antibodies (MOVs) were prepared as previously described [4–6].

Peripheral blood leucocytes were isolated by layering 20 ml of heparinized blood diluted twofold with phosphate-buffered saline (PBS) over 15 ml of lymphoprep in a gradient bottle. The bottle was then centrifuged at 800 *g* for 15 min in a Beckman desk centrifuge with a swing out rotor. The leucocytes which banded in the interphase between the plasma-PBS and lymphoprep were aspirated, washed three times with 40 ml × 3 of PBS, and suspended in RPMI-1640 containing antibiotics and serum as described below.

Microfilariae (mf) were isolated from *O. volvulus* as previously described [7]. Viability ranged between 90 and 98% at the start of the experiment. Since it was impractical to handle blood from all patients at once, they were studied in groups of 9–10 for each experiment. Some patients gave blood more than once. The 14 patients who donated blood for this study comprised 6 males and 8 females aged between 18 and 58 years (mean age 37 ± 12). They had been residing in the hyperendemic village of Sa'a in the River Sanaga basin for at least 10 years. Onchocerciasis was diagnosed by demonstration of mf in skin snips [8] and/or onchocercal antigen in the urine [6].

FIG. 1. Cytoadherence of leucocytes to *O. volvulus* microfilariae. Bar represents 0.1 mm.

In a typical killing assay [2], 50–75 mf were incubated per well in flat-bottom microtitre plates (Nunc, Roskilde, Denmark) with 1×10^5 leucocytes in 0.2 ml RPMI, containing 2% L-glutamine, 12 mM HEPES (pH 7.4), 10% newborn calf serum (Sigma, St Louis, MO), 25% vol/vol freshly prepared normal human serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml monoclonal antibody. The incubation mixture was maintained at 37 C in a humid and CO_2-enriched atmosphere prepared by burning a candle in a desiccator essentially as described by Trager [9]. At the end of the incubation, the mf were examined and all those bearing more than 10 cells were counted as showing adherence. Stretched and immotile mf were considered dead. Western blotting was done as described in the Biorad brochure except that the revelation was done by the peroxidase anti-peroxidase method (PAP) as previously described [10].

RESULTS AND DISCUSSION

After 16–20 h of incubation, the reaction mixtures were observed directly under a Wilowert inverted microscope at a magnification of ×400. Intense reactions of the leucocytes on mf could be observed (Fig. 1). In the presence of a cocktail of monoclonal antibodies, designated MOV GIV (comprising MOV 25, 29, 30 and 32), up to 74% of the mf could be seen with 10 or more cells adhering to them. Some of the mf were covered with large clusters of cells, while others were stretched, sluggish or immobilized by the adherent cells. The degree of cellular adherence observed in control reactions with monoclonal antibodies MOV 1 or normal mouse serum (NMS) were significantly lower (Fig. 2). Other monoclonal antibody cocktails, MOV GI (MOV 13, 14, 15), MOV GII (MOV 16, 17, 18, 19) and MOV GIII (MOV 20, 21, 22, 23) directed respectively against L3, mf, and a fusion protein P67 (unpublished results), were equally inactive in the assay.

After 36–40 h of incubation up to 54% of the mf were killed by the leucocytes in the presence of MOV GIV. The attacking cells remained clustered on the dead and degenerating mf. The degree of killing in the control experiments with MOV 1 or NMS remained significantly lower ($P < 0.05$) ranging from 0 to 4.5% (Fig. 2). These observations indicated that the cellular killing reactions were specific and required the presence of MOV GIV. Experiments with the components of MOV GIV applied individually in the incubation medium revealed MOV 32 (IgG_{2b}) to be most active in killing, with minimal killing activity displayed by MOV 25, 29 and 30. All MOV GIV components were active in promoting cytoadherence to the mf (Fig. 3). It should be pointed out that both the cytoadherence and killing reactions were complex, varying in efficiency as a function of the incubation time and individual patients. However, the trends described above were seen reproducibly.

Previous studies had shown that complement is important for cytoadherence to mf [1, 2]. We confirmed this by the observation of a 3–8-fold reduction in cytoadherence and about a twofold decrease in killing activity when heat-inactivated serum replaced fresh serum (Fig. 3). In Western blotting experiments, the target antigens displayed components of 10.5, 18, 23.5 and 27 kDa (Fig. 4) with the last antigen recognized by all of the MOV GIV components. Immunoperoxidase staining of cryosections [6] indicated that the target antigens of MOV GIV were located mainly in the basal and cortical layers of the cuticle, with some found in the hypodermis as well (results not shown).

Cellular adherence [1, 2, 11–13] or killing [1, 2] of *O. volvulus* and *Brugia malayi* [12, 13] worm

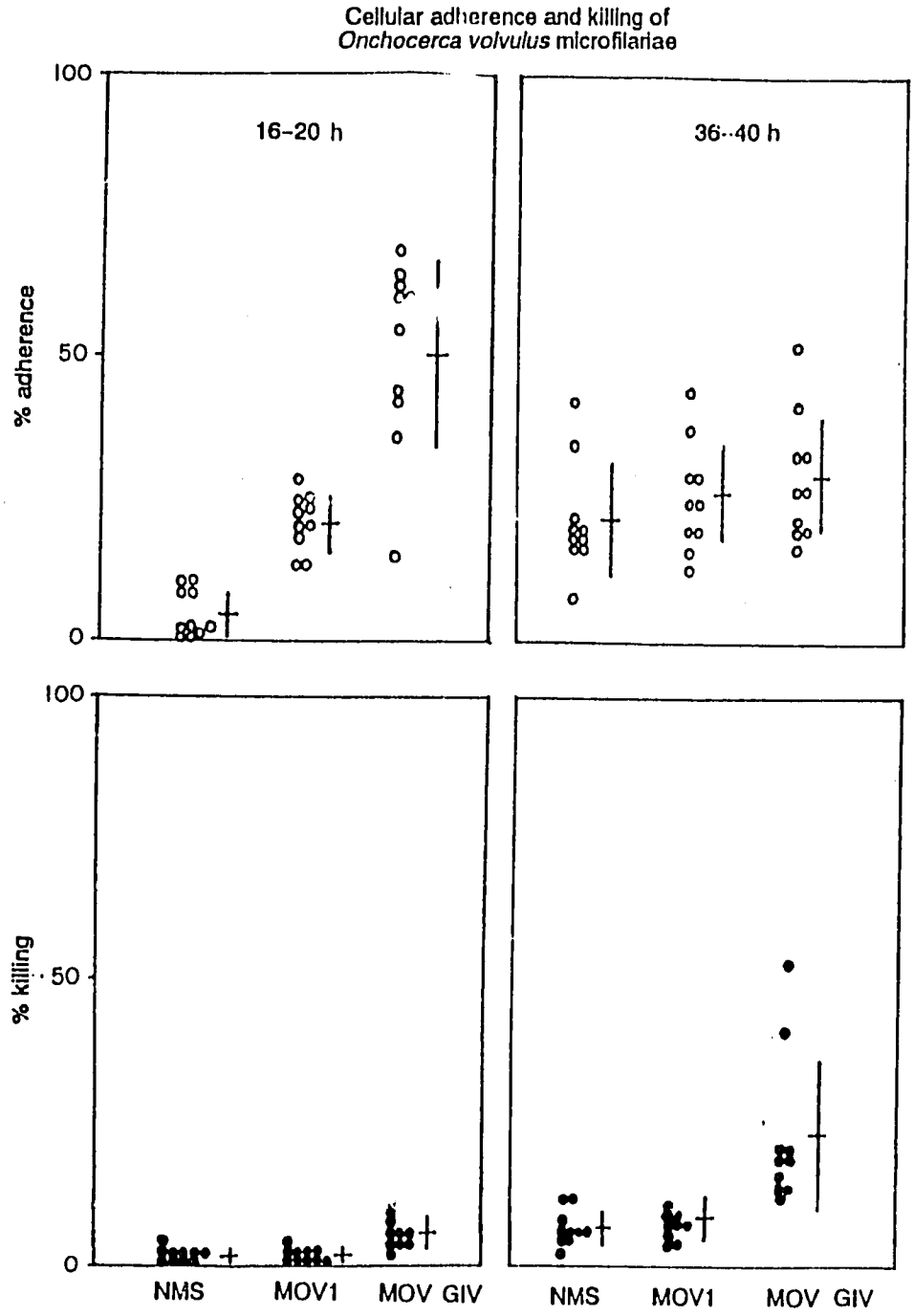

FIG. 2. Cellular adherence and killing of *O. volvulus* microfilariae. o, Cytoadherence of > 10 cells; ●, dead mf (stretched and immotile); MOV, monoclonal antibodies raised to cuticular antigens of *O. volvulus* females; NMS, normal mouse serum.

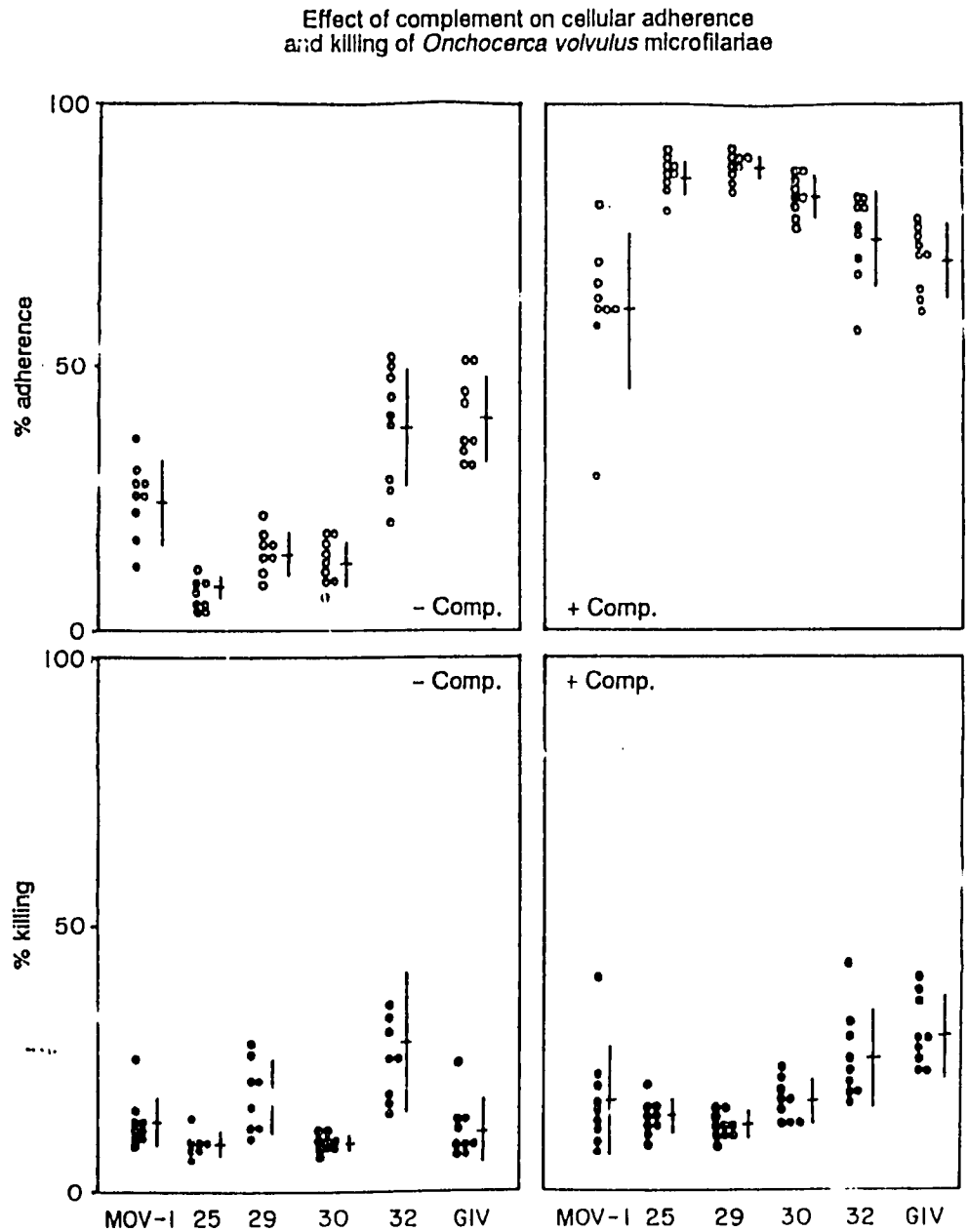

FIG. 3. Effect of complement on cellular adherence and killing of *O. volvulus* microfilariae. o, Cytoadherence of > 10 cells (40–44 h); ●, dead mf (40–44 h); MOV, monoclonal antibodies to cuticular antigens of *O. volvulus* females; Comp., complement.

stages by immune cells have been demonstrated previously in the presence of polyclonal antibody [1, 2, 11, 13] and monoclonal antibodies [12] but the antigens involved in these reactions were not identified. This investigation identifies some of the onchocercal antigens that mediate mf clearance by immune cells. Earlier we had postulated that cuticular antigens would play an important role in host–parasite interactions [8]. Further experiments to determine the protective role of the identified antigens are required.

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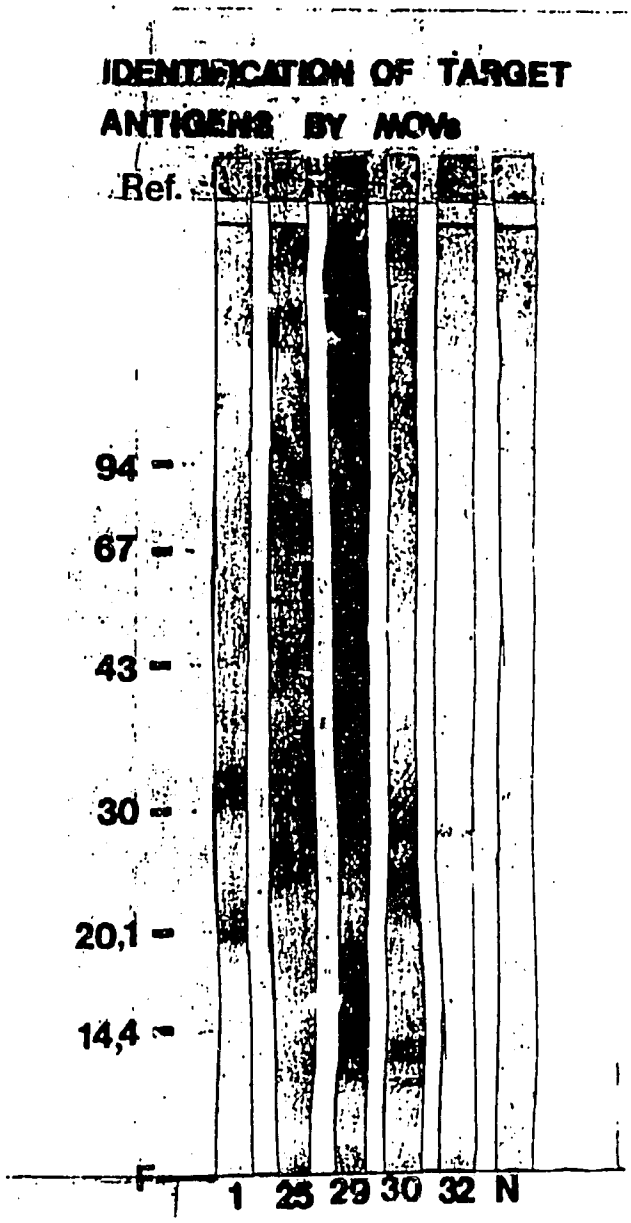

FIG. 4. Identification of target antigens of MOVs. Ref., reference proteins (Pharmacia, Uppsala, Sweden); N, normal mouse serum; F, front; MOVs (1, 25, 29, 30 and 32), monoclonal antibodies to cuticular antigens of *O. volvulus* females.

facilities to prepare monoclonal antibodies, Drs R. Tata, F. Soh and J. Ticha for examining patients and performing nodulectomies, and Mr T. Pogonka for advice on cell isolation.

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Identification of different radiolabelled antigens of the developmental stages of *Onchocerca volvulus*

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By using radioiodination methods which are thought to label preferentially the surface followed by SDS-PAGE and autoradiography, components of different developmental stages of *O. volvulus* have been identified. Between 2 and 10 polypeptide antigens were revealed on infective larvae (L3), females, males, eggs, nodular and skin microfilariae by using immunoblotting assays with human onchocerciasis sera. Antigen recognition did not vary with the density of skin microfilariae in the patients from whom the sera were obtained. Some of the antigens seemed to be stage specific; for example, antigens of 31 kDa which were detected only on skin microfilariae, or the 67.5 and 25 kDa components that occurred on the adult females, but were absent from adult males. Some of these antigens were also identified as glycoproteins. A 68 kDa glycoprotein was found in adult females, males and nodular microfilariae. Two glycoproteins of 74 and 45 kDa were found on egg shells, and a 18.5 kDa glycoprotein was recovered from L3. Type VI collagen was found with a specific antiserum on skin microfilariae, but not on eggs and females. Laminin was found on nodular mf. It is concluded that the changing antigenic profiles of the worm stages and the coating of these worms with connective tissue epitopes contribute to the evasion of host immunity.

Key words: *Onchocerca volvulus*: Onchocerciasis; Iodination; Antigens

Introduction

The nematode *Onchocerca volvulus*, which causes blindness, affects at least 17 million people worldwide (WHO, 1987a). It is thus considered to be an important public health hazard in the countries of Africa and Latin America where it is endemic. It causes not only significant morbidity among the affected populations, but also has detrimental effects on agricultural production, because farmers are forced to migrate from high-transmission areas (WHO, 1987b).

Current efforts aimed at developing accurate diagnostic methods, new drugs and vaccines for onchocerciasis have been complicated by the multiplicity of worm stages that are generated in the human host once infestation has been initiated by blackflies of the genus *Simulium*. Third and fourth stage larvae (L_3, L_4), adults, microfilariae (mf) and eggshell components may be present, and all these stages may have distinct biochemical and immunological properties. By analogy with other parasitic infections (Cohen, 1982), such variations of the worm's antigenic profile may have far reaching implications for worm survival in the host. As yet, however, this has been inadequately investigated in *O. volvulus* (Titanji and Mbacham, 1988).

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As the first parasite tissue which comes into direct contact with the host's immune system and the parasite, the cuticle is likely to be an important site for host-parasite interaction (Maizels et al., 1982). Therefore considerable research effort has been devoted to the characterisation of the surface antigens of *O. volvulus*. Using the Bolton-Hunter reagent as a catalyst, Philipp et al. (1984) found no difference between adult male and female *O. volvulus*, both of which contained a 20 kDa diagnostic antigen. Subsequently Taylor et al. (1986), studying the surface of adults, eggs, mf and L_3 of *O. volvulus*, confirmed the findings of Philipp et al. with respect to adult worms. Furthermore they suggested that skin and nodular mf were non-reactive with hyperimmune sera tested, and exhibited no surface sugars that could be identified by lectin binding methods. These studies also revealed the presence of 7 polypeptides on L_3's, but no information was given on their antigenic nature. Since then we have reported on the presence of exposed sugars on all of the five developmental stages of *O. volvulus* that were recovered from human tissues and *Simulium* flies, suggesting that these vary from stage to stage. Their possible antigenic nature remained, however, to be determined (Titanji et al., 1987).

The aims of the present investigation were: (a) to identify antigens which are accessible to so-called surface iodination in those *O. volvulus* stages that previously have not been extensively studied i.e. L_3's, eggs, nodular and skin mf; (b) to determine whether the recognition of these components by patient immune sera depends on the skin mf density at the time when the sera are collected; (c) to further characterise these antigens biochemically.

Materials and Methods

Reagents

Carrier-free ^{125}I was purchased from Amersham International Ltd., U.K., and Iodogen (i.e. 1,3,4,6-tetrachloro-3α, 6α-diphenylglucoluril) from Sigma. Collagenase from *Clostridium histolytica*, and lactoperoxidase, were products of Boehringer, Mannheim, F.R.G. Pansorbin was from Calbiochem. Culture media, RPMI-1640 with or without Hepes buffer were obtained from Gibco, U.K. Antibodies to the human connective tissue components, collagen types IV and VI, laminin, and undulin were bought from Heyle, F.R.G. Lentil-lectin sepharose-4B was from Pharmacia. All other reagents were of the highest purity obtainable from Sigma, Merck and Serva companies.

Parasites

All parasite isolations were carried out at 25–28°C under sterile conditions. Adult *O. volvulus* males and females were isolated from onchocercomata as described previously (Engelbrecht and Schulz-Key, 1984). Microfilariae released from minced nodules or from skin snips were purified through Percoll gradients according to the method of Chandrashekar et al. (1986), with modifications (Titanji et al., 1987). Infective larvae (L_3's) were dissected out of the heads of blackflies, obtained while feeding on volunteer fly-catchers on the banks of the River Mungo in Cameroon. All isolated parasite stages were shown by microscopic examination to be free of

contamination by host tissue. Isolated parasites were maintained for 24–48 h at room temperature in RPMI-1640 containing 0.16 $mg \cdot ml^{-1}$ gentamycin and 10% normal human serum or fetal calf serum. Parasite viability exceeded 90% as judged by motility, observed under the microscope (mf and L_3), or by eye (adult worms). Before use in labelling experiments the parasites were washed with 3 changes of phosphate buffered saline (PBS, pH 7.2 containing 8.18 g NaCl, 1.15 g $NaHPO_4.2H_2O$ and 0.2 g KH_2PO_4 per litre).

Onchocerciasis sera from patients

Sera were isolated from the blood of subjects examined in Batchenga, a village hyperendemic for onchocerciasis, which has been described previously (Titanji et al., 1985). After examination, two snips were taken from the iliac crest of each patient using a scleral punch and deposited in prelabelled microtitre wells, containing 0.15 ml RPMI-1640 with 0.16 $mg \cdot ml^{-1}$ gentamycin. The snips were then incubated for 24 h in 5 $mg \cdot ml^{-1}$ collagenase dissolved in PBS. The total number of mf obtained from each snip was determined after microscopic examination. On the basis of the results obtained, the 74 patients examined were classified into 4 groups as follows: groups I (0 mf), II (1–10 mf), III (11–50 mf) and IV (more than 50 mf). There were 11, 13, 20 and 30 subjects in groups I–IV, respectively. For some analyses all sera of one group were pooled.

Rabbit antisera to components of female O. volvulus

The antigens were obtained by incubating intact living females in 10 mM Tris-HCl, pH 8, containing 1% sodium deoxycholate and 1 mM freshly prepared phenylmethylsulfonyl fluoride: 0.5 ml buffer was used per worm and incubation was carried out with gentle rocking at 4°C for 3 h. The pooled extracts were dialysed against the extraction buffer and then purified by affinity chromatography on Sepharose-4B columns containing immobilised human antibodies to *O. volvulus*. Each column contained a 0–50% $(NH_4)_2SO_4$ fraction of pooled sera (from one of the patient groups I–IV) covalently linked with CNBr-activated sepharose (Marcoulis et al., 1978; Mbacham and Titanji, 1987). The antigens eluted with 8 M urea were dialysed against PBS, lyophilised and reconstituted in 2 ml of distilled water.

100 μg antigen emulsified with an equal volume of Freund's Complete Adjuvant was injected intramuscularly in two New Zealand white rabbits. Booster injections of 50 μg antigen emulsified in Freund's Incomplete Adjuvant were given together with another 50 μg non-emulsified antigens, 14 days after the first injection and subsequently weekly for a total of 4 weeks. The rabbits were bled 7 days after the last injection to obtain sera. The presence of antibodies to *O. volvulus* was confirmed by immunofluorescence and immunoelectronmicroscopical tests (see below).

Radioiodination of O. volvulus worm stages

Viable parasites were labelled with carrier-free ^{125}I essentially according to the method of Baschong and Rudin (1982) and Marshall and Howells (1985). Iodogen solution at 0.2 $mg \cdot ml^{-1}$ in chloroform was dispensed in 0.2 ml aliquots into 5 ml conical glass tubes and evaporated in the dark at 22–25°C under a fume-hood. The

iodogen-coated tubes were then kept over silica gel in a vaccuum desiccator until used.

For radioiodination, the coated tubes were rinsed once with 1 ml PBS. Parasites at different stages were then added to separate reaction tubes, suspended in PBS. Several parasite stages could be labelled simultaneously. The number of parasites per tube was as follows: 1 adult female; 4–5 adult males; $(5–100)10^3$ mf or eggs and about 700 L_3. The tubes were placed on ice and 0.5–2 mCi (5–10 µl) ^{125}I was then added. The tubes were incubated with gentle intermittent swirling for 10 min and the reaction stopped by transferring the parasites to fresh tubes with a pair of forceps (for adults), or by addition of cold PBS followed by centrifugation at 2000 rpm for 10 min and resuspension in PBS (for mf and L_3's). The labelled parasites were further washed by changing the PBS three times, and then checked for viability, which turned out to be 100% for adults and more than 90% for L_3 and mf.

Lactoperoxidase labelling of adult female worms was done as described previously (Titanji and Ngu, 1986).

Solubilisation of radioactively labelled components

Radiolabelled components were solubilised by incubating the worms at 4°C for 1 h in 0.5–1 ml 10 mM Tris-HCl, pH 8, containing 1% (v/v) detergent (sodium deoxycholate, Triton-X100, Nonidet NP40 or Tween 20) and the following proteolytic inhibitors: leupeptin (5 µg/ml), pepstatin (5 µg/ml), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (25 µg/ml) and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Aliquots (50 µl) of the solubilised extracts were used for protein assays (Read and Northcote, 1981). Serum albumin (5 µl) was added as a carrier protein. The sodium deoxycholate extracts were further chromatographed on Sephadex-G25 (PD10 columns, Pharmacia) equilibrated and eluted with 10 mM Tris-HCl, pH 8.0, with 0.1% (w/v) sodium deoxycholate. Fractions of 1 ml were collected at a flow rate of 30 $ml \cdot h^{-1}$ and assayed for radioactivity in a Beckman G4000 Gamma Counter. The radioactive peaks eluted with the void volume from each of the PD-10 columns were pooled and stored for at most 1 month in aliquots of 0.2 ml at −20°C. They were subsequently thawed once and analysed for the presence of antigens.

Trichloroacetic acid (TCA) precipitable proteins were determined by adding 50 µl of the desalted extracts to 1 ml 20% (w/v) TCA, and centrifuging the mixture at 2000 rpm for 10 min after incubation on ice for 20 min. The supernatant was discarded and the radioactivity of the pellets determined as described above. For better precipitation of the proteins, 100 µl normal rabbit serum was added to the extract prior to TCA.

Immunoprecipitations

Frozen antigens were thawed and cleared by mixing them with an equal volume of normal European serum (from Sweden) and then adding 25% of its volume of 10% (w/v) washed Pansorbin (Calbiochem). After incubating the mixture at 4°C for 1 h it was centrifuged at 2500 × g for 20 min at 4°C in order to remove the precipitate formed. The final supernatant obtained was used in the immunoprecipitation reactions according to the method of Kessler (1975) with modifications (Titanji and Ngu, 1986).

In a typical reaction 100 μl of a four-fold diluted test or control serum was incubated with 100 μl of radioiodinated surface extract in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% (w/v) NaN_3 overnight (12–14 h) at 4°C. Pansorbin was subsequently added, and immune complexes isolated and analysed by SDS-PAGE and autoradiography.

Affinity chromatography

Immunosorbent chromatography of solubilised radiolabelled antigens was done using 2 ml of anti-*O. volvulus* (Ig)-Sepharose-4B packed in Pasteur pipettes as described elsewhere (Mbacham and Titanji, 1987; see also above). The antibodies were obtained by isolating the 0–50% $(NH_4)_2SO_4$ fractions from patient groups I–IV and normal European sera. The antigens eluted with 8 M urea were analysed by SDS-PAGE and autoradiography under reducing conditions (Laemmli, 1970).

Glycoproteins were isolated from the radioiodinated extracts on columns of lentil-lectin Sepharose-4B (1 ml, packed into Pasteur pipettes). The conditions for the experiments were as recommended by the manufacturers. After eluting the glycoproteins with 0.2 M α-D-mannose, they were further purified on the immunosorbent columns and analysed as described above.

Indirect Immunofluorescent Antibody Test (IFAT)

Skin or nodular mf or eggs (100–300), were dispersed into Eppendorf tubes and washed thrice by centrifugation at 2000 rpm for 10 min in PBS. Test sera at various dilutions 1:1, 1:10, 1:25, 1:50 were added to a final volume of 0.2 ml, and the suspension incubated at 25–28°C for 1 h. The reactions were stopped by washing the parasites thrice in PBS. Thereafter a fluorescein-labelled second antibody (rabbit anti-human IgA, IgM & IgG) was added to the washed parasites which were further incubated and washed as above. The final pellets were taken up in 25 μl buffered glycerol, mounted on a slide and observed under a Nachet fluorescence microscope at 100 × magnification. For L_3's the incubations and washes took place in 0.1 ml PBS on a microscope slide (Titanji et al., 1987).

Immunoelectronmicroscopy

The immunocytochemical experiments were done on ultrathin sections from worm material processed and embedded in Lowicryl K4M according to standard procedures (Kiefer et al., 1986).

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed under reducing conditions in gel slabs using the buffer system described by Laemmli (1970). The gels were dried and autoradiography was carried out at −70°C for 1–3 weeks using AGFA Safety Curix RPI films. Protein determination was carried out according to the method of Read and Northcote (1981) with bovine serum albumin as a standard.

Results

Conditions for the identification of radiolabelled antigens

In preliminary experiments it was observed that iodogen catalysed the radioiodination of more antigenic components on female worms than did lactoperoxidase (Fig. 1). Neither of the catalysts killed the worms, as evidenced by micro- and macroscopic movements which persisted after radioiodination of the worm stages. There was no qualitative difference in the SDS-PAGE patterns of antigens solubilised by 1% (w/v) Tween-20, Triton-X-100, Nonidet NP40 or sodium deoxycholate (NaDoc). However, different yields of proteins were obtained with each detergent (Table 1). NaDoc was chosen for further work because it is dialysable and provided the best compromise between the quantity of total radioactivity eluted and the % TCA precipitable radioactivity (Table 1). None of the aforementioned detergents completely dissolved the cuticle, but incubation in 65 mM Tris-HCl, pH 6.8, 3% SDS, 5% 2-mercaptoethanol (ME) at 37°C for 1 h solubilized the cuticle.

Fig. 2 shows the results of the immunoprecipitation experiments with female radiolabelled antigens using different human immune sera and staphylococcal protein-A as the immunoadsorbent. Less than 7% of the radioactively labelled material could be precipitated. The concentration of the precipitated radioactively labelled proteins rose with the load of skin mf up to an intermediary load of 11–50 mf per snip and seemed to fall thereafter. The results suggest that the concentration of antibodies directed against the labelled solubilized proteins were essentially of the same order of magnitude in different sera. This method gave identical antigen patterns on SDS-

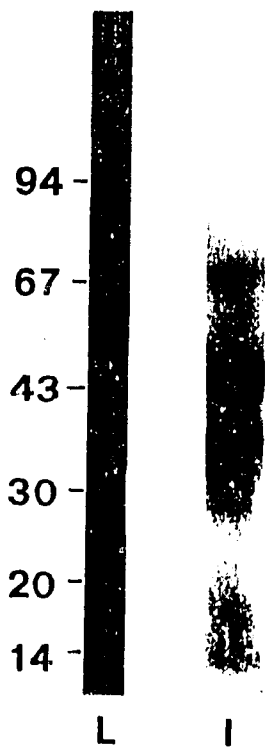

Fig. 1. Autoradiograph of radioiodinated polypeptides extracted from female *O. volvulus*. Worms labelled with lactoperoxidase (L) or Iodogen (I) were solubilised and the extracts analysed by SDS-PAGE.

TABLE 1

Efficiency of different detergents to solubilize radiolabelled components of *O. volvulus* female worms

Parameter	Solubilising agents					
	PBS	NaDoc.	TX-100	NP40	Tween 20	βME
% radioactivity solubilised	ND	50	59	ND	29	90
% TCA precipitable protein	23	70	54	52	97	ND

(NaDoc = sodium deoxycholate; TX = Triton X-100; NP 40, Nonidet P40; T20 = Tween 20; PBS = phosphate-buffered saline).

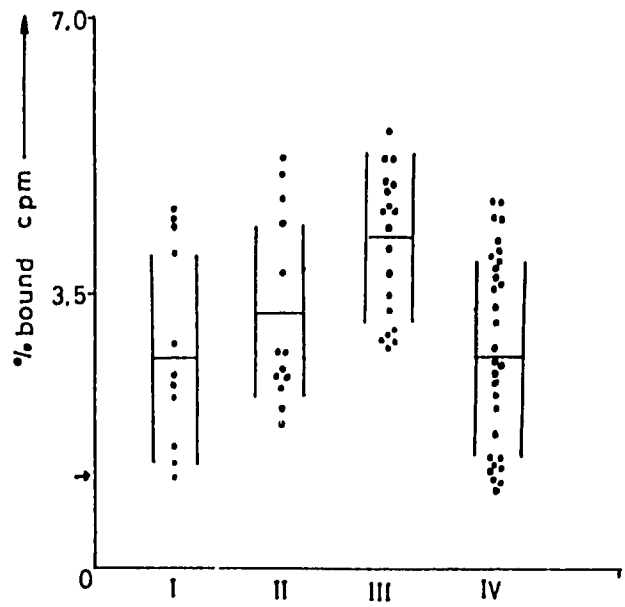

Fig. 2. Immunoprecipitation of radiolabelled female *O. volvulus* extracts with human sera. The immune complexes were isolated with staphyloccocal protein A, washed and counted in a gamma counter. % cpm bound = cpm in the immune precipitate divided by the total TCA precipitable counts added to the reaction × 100. Roman numerals refer to the different groups of patients with different numbers of mf per skin snip: I = 0 mf; II = 1–10 mf; III = 11–50 mf; IV = over 50 mf.

PAGE when compared with eluates from immunosorbent columns containing human antibodies to *O. volvulus*, indicating that antibody isotype was not crucial for the identification of these antigens.

Comparison of the radiolabelled polypeptides and antigens of the O. volvulus developmental stages

The specific radioactivity of solubilized, TCA precipitable material varied between the different developmental stages, being lowest in L3 and highest in females (Table 2). When the labelled and extracted polypeptides and immunoprecipitated antigens were analysed under reducing conditions by SDS-PAGE and autoradiography distinct patterns were obtained for each of the worm stages studied (Table 3). Since it

TABLE 2

Comparison of the specific activity of the radioiodinated proteins in the extracts of different *O. volvulus* stages

O. volvulus stage	% TCA precipitable protein (mean ± S.D.)	Protein solubilised per worm	Specific radioactivity ($cpm \cdot mg^{-1} \times 10^6$)
Adult female	70.0 ± 6.5	385 mg/worm	33.08
Adult male	72.6 ± 5.7	ND	ND
Eggs	60.8 ± 3.5	106 ng/egg	12.76
Nodular mf	52.6 ± 4.0	2.75 ng/mf	7.88
Skin mf	88.6 ± 2.8	8.47 ng/mf	5.96
Infective larvae (L_3)	58.3 ± 2.4	582 ng/L_3	2.16

TABLE 3

Radioactively labelled polypeptides of *O. volvulus* worm stages

No.	kDa (±SD)	Female	Male	Eggs	Nmf	Smf	L_3
1	159.5 ± 0.3	+↓	−	−	−	−	−
2	148.0 ± 0.3	−	−	−	−	+↓	−
3	130.0 ± 0.2	−	−	−	−	−	+
4	114.0 ± 0.2	+	−	−	−	−	−
5	99.0 ± 0.5	+	−	−	−	−	−
6	82.0 ± 1.0	−	−	+	−	+↓	−
7	74.0 ± 1.0	−	−	+↓	+	+↓	−
8	68.0 ± 0.3	−	+↓	−	+↓	+	+
9	67.5 ± 0.7	+↓	−	−	−	−	−
10	55.0 ± 0.2	−	−	+	+↓	−	−
11	51.0 ± 0.1	−	−	+	−	+↓	+
12	45.0 ± 0.3	+↓	−	+↓	−	+	−
13	43.0 ± 1.0	−	−	−	−	+↓	−
14	37.5 ± 1.0	−	+↓	−	−	−	−
15	34.0 ± 0.6	+	+↓	+↓	+↓	+↓	+
16	31.0 ± 0.6	+	−	+	−	+↓	−
17	26.0 ± 1.0	−	−	+↓	+↓	+↓	+
18	25.0 ± 0.1	+↓	−	−	−	−	−
19	20.0 ± 0.3	−	−	+↓	+	−	−
20	19.0 ± 1.0	+↓	+↓	−	−	+↓	−
21	18.5 ± 0.4	−	−	−	−	−	+↓
22	17.5 ± 0.8	−	−	−	+↓	+	−
23	14.0 ± 0.5	+	+	+↓	+↓	+↓	+↓

Arrows indicate polypeptides which could be immunoprecipitated by using hyper-immune sera pools.

was technically not feasible to study all worm stages simultaneously, each stage was studied on a separate occasion, and comparative gels bearing several stages at once were subsequently run to confirm the data (not shown).

The highest number of labelled polypeptides were found in adult females and eggs, which each showed 10 components, and skin mf with 11. Extracts from nodular mf and L_3 revealed 8 and 7 polypeptides, whereas extracts of adult male worms contained at least 5 distinct polypeptides. Table 3 summarizes the calculated molecular sizes of the polypeptides detected, which were usually observed in all isolates from a particular stage. It should be pointed out that several polypeptides not reported in

the table appeared in some, but not all adult worm isolates. Thus in three separate isolates of adult male worms polypeptides with apparent molecular weights of 93, 76.6 and 53.4 kDa were identified. Similarly, components with apparent molecular weights of 303, 167.4 and 136 kDa appeared occasionally in some preparations from female worms. These variable components may have been host-tissue components or variants between different worm strains. It is significant that none of them could be precipitated by hyper-immune sera from patients (Table 3).

In order to identify the polypeptides which were antigenic, the extracts of the worms were reacted with hyper-immune serum pools from patients grouped according to their skin mf densities (Fig. 2). The immune complexes formed were isolated and analysed by SDS-PAGE and autoradiography. Table 3 shows that many of the radiolabelled polypeptides were immunoprecipitated by the human sera. Ten out of 13 polypeptides were immunoprecipitated in extracts of skin microfilariae. Only 2 polypeptides out of seven could be immunoprecipitated from the extract of infective larvae (L_3). 4–6 antigens were immunoprecipitated in extracts from females, males, eggs and nodular mf. Some of the antigens occurred only in one stage, indicating stage specificity; for example, the 82, 51, and 31 kDa components in skin mf. On the other hand, two antigens (67.5 and 25 kDa), present in adult females, were apparently absent from adult male worms, which suggests the presence of sex-specific antigens in *O. volvulus* worm stages.

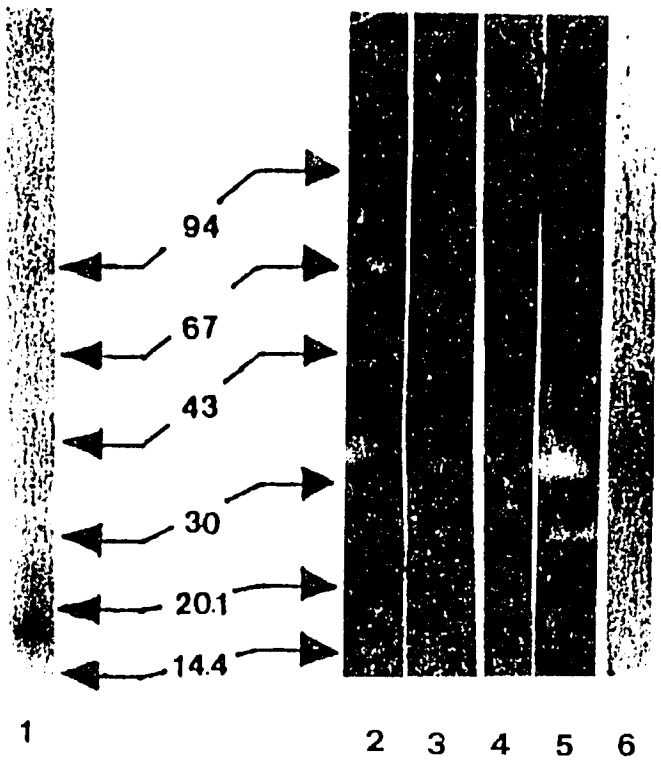

Fig. 3. Glycoprotein antigens of *O. volvulus* isolated by sequential affinity chromatography on lentil-lectin and immunosorbent columns and then analysed by SDS-PAGE and autoradiography. Arrows show the positions of molecular weight markers. Following worm stages were studied: infective larvae (1) females (2), males (3), eggs (4), nodular microfilariae (5). Lane 6 is a control lane containing eluates of radioiodinated surface extracts purified through unsubstituted Sepharose-4B columns. About 4000–8000 cpm of labelled protein were applied to each lane. Dried gels were autoradiographed for 21–24 days.

Since similarity in the molecular size of polypeptides does not necessarily imply immunological identity, experiments were conducted to determine the extent of cross-reactivity between the adult female proteins and those of males and mf. For these experiments female worm components that are immunogenic in man were isolated by immunosorbent chromatography and then used to raise antibodies in rabbits. The rabbit antibody (anti-FSP) showed in immunocytochemical analysis a reactivity towards cuticular epitopes (Fig. 5). When the anti-FSP was allowed to react with radioiodinated extracts of female worms and nodular mf, an identical pattern of precipitated proteins was obtained. However, the anti-FSP cross-reacted with only one 67–68 kDa male antigen (data not shown). These results were consistent with the presence of sex-specific antigens in male and female *O. volvulus*.

Glycoprotein antigens

Radioiodinated worm extracts were sequentially purified by affinity chromatography on lentil-lectin Sepharose-4B, followed by immunosorbent chromatography on a Sepharose-4B column containing immobilized human antibodies to *O. volvulus*. The final eluate obtained from the immunosorbent columns with urea was analysed by SDS-PAGE followed by autoradiography. Radiolabelled glycoproteins were isolated from all stages studied; a 68 kDa glycoprotein antigen was isolated from female and male worms as well as nodular mf, two glycoproteins of 74 and 45 kDa were found in extracts from eggs and one of 18.5 kDa in L_3 (Fig. 3). Owing to the difficulty of obtaining enough material, the glycoproteins of skin mf were not investigated.

Immunofluorescence studies

Indirect fluorescent antibody test (IFAT) was employed to investigate the presence of antigens that could not be easily identified by the radioiodination method used above, and to identify human connective tissue components that may have been tightly associated with the cuticles. Onchocerciasis hyperimmune sera contained antibodies to the worm's egg-shells (Fig. 4). The antibody titres varied from 1:250 in group II patients to $1:10^4$ in group IV patients. No difference was found between the fluorescence patterns on embryonated and non-embryonated eggs. Furthermore, anti-egg shell antibodies appeared in all patient groups, including those without skin mf. Antibodies to mf were rare among the group of patients tested. Only 2 out of a total of 74 sera contained antibodies to the cuticles of nodular mf, demonstrable by the IFAT. One of these patients, a 12 year-old boy, had generalised pruritus, but no palpable nodules and 14 mf per snip. The other, a 26 year-old man, had a generalised skin rash and several palpable nodules. Judging by their clinical presentations, they were similar to many other patients, and it is not known why they had antibodies to nodular mf cuticles, whereas the other patients did not.

We were not able to demonstrate any antibodies in patient's sera directed against infective larvae using the IFAT method. However, this was not surprising in view of the extremely weak reactions observed with L_3 components (Table 3).

IFAT was also employed to examine the possible presence of connective tissue components on the cuticles of selected worm stages. As indicated in Table 4, the basal layer of adult female cuticles was showing fluorescence with rabbit antibodies to collagen type IV, whereas eggs, nodular and skin mf were unreactive. On the other

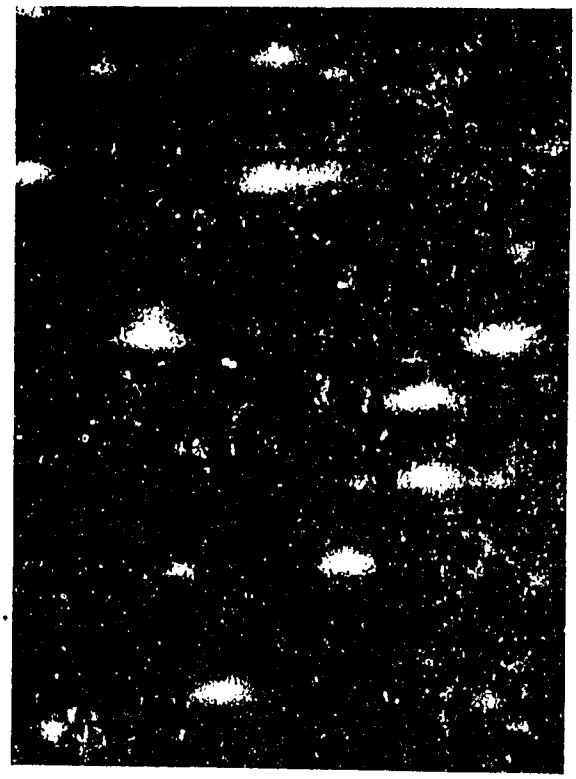

Fig. 4. Indirect fluorescent antibody test with human onchocerciasis serum. A mixture of fluorescent microfilariae and eggs are seen. A control assay using normal European serum showed no fluorescence (data not shown).

hand, type VI collagen was found on skin mf, was hardly available on nodular mf, and was absent from eggs and adult females. Laminin was present on both nodular tissue and purified nodular mf.

Discussion

In this study the patterns of radioactively labelled polypeptide antigens of five distinct developmental stages of *O. volvulus* were investigated. The simultaneous identification of these components in worm isolates from the same village minimised geographically related differences. The present findings, therefore, confirm and extend earlier results (Philipp et al., 1984; Forsyth et al., 1984; Cabrera and Parkhouse, 1986; Titanji et al., 1987). Noteworthy is the first isolation of antigens of L_3-infective larvae, eggs and skin microfilariae (mf). Although L_3 polypeptides were described by Taylor et al. (1986), their antigenic components were not mentioned. In that study mf seemed to be non-reactive with host antibody, which contrasts with our data. The reasons for this discrepancy remain to be determined; the possible effects of parasite strain differences and isolation methods are proposed as possible explanations. The presence of antibodies to the radiolabelled polypeptides in all the sera studied, regardless of the skin mf density, may be explained if it is assumed that the patients had already been exposed to all of the worm stages. Since they were all

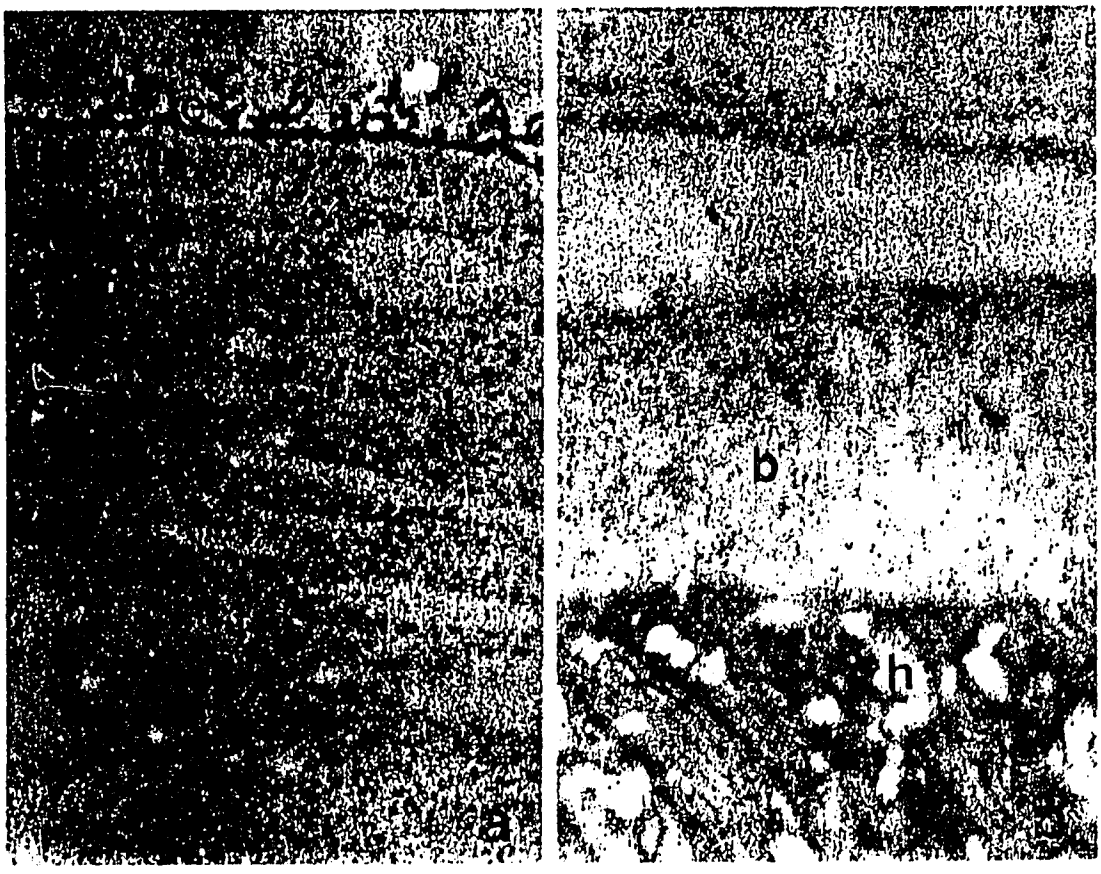

Fig. 5. Immunoelectronmicroscopy of the cuticle of adult female *O. volvulus* labelled with the rabbit anti-FSP serum (dilution 1:200) (a) Corticol region, e = epicuticle, c = cortical zone, × 22 000. (b) Basal region, h = hypodermis, b = basal zone, 37 000.

TABLE 4

Reactivity of different antisera with *O. volvulus* cuticular structures in the IFAT

Rabbit antiserum	*O. volvulus* stages					Nodular tissue
	Adult females	Nodular mf	Skin mf	Eggs	Infective larvae	
Anti-collagen IV	+*	–	–	–	–	+
Anti-collagen VI	–	±	+	–	–	–
Anti-Laminin	–	+	–	–	–	+
Anti-undulin	–	–	–	–	–	–
Anti-FSP	+ +	–	–	–	–	–
Normal rabbit serum	–	–	–	–	–	–
PBS control (reagent blank)	–	–	–	–	–	–

*The basal layer of the cuticle was positive.

living in Batchenga, which is a hyperendemic, high transmission area (Titanji et al., 1985), low skin mf density may simply be an indication of light infection.

In agreement with the published data cited above, the number of radioactively labelled polypeptides detected was rather small. On the basis of the apparent molecular weights determined from one dimensional SDS-PAGE, 23 radioactively labelled polypeptides were found to be distributed among the five developmental stages studied, with about 20 of them being reactive with host antibodies. This estimate may be lower than the true number of polypeptides, because of the limitations of SDS-PAGE and the fact that size identity is not necessarily concomitant with structural and/or immunological identity. Noteworthy is the presence of antigens on eggs, mf and L_3 that have not been described previously (Taylor et al., 1986). By employing a specific rabbit antiserum, polypeptides of female and male worms were shown to be immunologically distinct. This distinction is compatible with the existence of sex-linked differences, which has also been described for *Brugia pahangi* (Sutanto et al., 1985).

Using the Bolton-Hunter reagent to catalyse surface radioiodination, Philipp et al., (1984) found no differences between male and female adult *O. volvulus*. The difference between their result and that described here may be due to the fact that the Bolton-Hunter reagent is known to catalyse the labelling of internal as well as surface structures, whereas iodogen is believed to catalyse preferentially the labelling of surface structures (Baschong and Rudin, 1982; Marshall and Howells, 1985). Further experiments are in progress to study the localization of the iodinated proteins and verify whether they are surface specific.

Previous studies from this and other laboratories have identified exposed sugars on various stages of *O. volvulus* (Taylor et al., 1986; Titanji et al., 1987). However, the possible association of these sugars with antigenic polypeptides had not been determined. The successful isolation of radiolabelled polypeptides from different worm stages by sequential affinity chromatography indicates that some of the glycoproteins are located close to the surface. The IFAT also revealed the presence of connective tissue components, collagen type IV and laminin on some *O. volvulus* stages. This was in agreement with earlier studies (Titanji et al., 1988). The presence of type VI collagens on skin mf is reported for the first time. The association of connective tissue components with various worm surfaces is not likely to have been fortuitous, in view of the rigorous parasite isolation and purification procedures utilized. Since parasites coated with host antigen would not be recognised by the host as being foreign (Cohen, 1982), this may be also part of a mechanism that enables the parasite to evade human immune responses. Since some of the properties of cuticular and vertebrate collagens are shared (Titanji et al., 1988), this could also be a mechanism of immune evasion. Petralanda et al., (1988) proposed in fact the involvement of cuticular collagens in autoimmune complications of onchocerciasis.

Ottessen (1984) identified immunosuppression as the main immune evasion mechanism in onchocerciasis. The results here suggest that other mechanisms also exist; antigenic variation among worm stages, coating of the parasite with host-like antigens, and the low reactivity of surface components with host antibodies. The detection of type VI collagen epitopes on the surface of skin mf is particularly significant as a potential protective coat, since this parasite stage survives for a long time in the skin of immunocompetent individuals and is the cause of skin disease associated with onchocerciasis. The identification of 10 polypeptides of skin mf after iodogen-cata-

lysed radioiodination showed that some components of mf are accessible to the reagent, though they may not necessarily be on the surface. It is possible that this catalyst labelled sub-surface components of the skin mf as well (Baschong and Rudin, 1982).

The stage-specific antigens described here are candidates for use in the differential diagnosis of microfilaraemia and adult worm infections, with potential applications in clinical trials of drugs active against the different parasite stages. The appearance of anti-egg shell antibodies in snip-negative patients could be used for detection of light infections if these antigens turn out to be *O. volvulus* specific. In conclusion it seems that the human host mounts a humoral immune response to a limited number of cuticular components, and this may play a role in the survival of the parasite and the pathogenesis of onchocerciasis, and may be of use in the diagnosis of infection in the human host.

Acknowledgments

This work was supported by grants from the United States Agency for International Development (USAID/PSTC), and the University of Yaounde (Grant FS 83 017). We thank Drs. Nasah and Tata for performing nodulectomies, Dr. W. Rudin for the immunoelectronmicroscopical analysis and Dr. Bruno Betschart of the Swiss Tropical Institute for reading the manuscript.

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ABSTRACTS AND RECOMMENDATIONS

2nd CEC Filariasis Network Meeting

Amsterdam, Wednesday 11th - Friday 13th September 1991

Application of defined polypeptides and monoclonal antibodies to the diagnosis of human onchocerciasis

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Introduction

As a leading cause of blindness and being a debilitating disease, onchocerciasis continues to be an aggravating factor of socio-economic backwardness among its 17 million victims resident in the endemic, tropical countries of Africa, Central America and the Yemen (WHO, 1984). In Cameroon alone, 10% or 1.2 million of the population are affected. Accurate diagnosis of the disease is required to monitor the efficacy of vector control measures, mass chemotherapy with ivermectin and fundamental research aimed at developing new drugs.

The demonstration of microfilariae (mf) of *O. volvulus* in skin biopsies, though a definitive method of diagnosis is insensitive, subject to sampling errors and prone to yield false results in prepatent and/or occult infections. Consequently, considerable efforts have been made towards developing more sensitive immunologic methods for the detection of specific anti-parasite antibodies and/or parasite antigens in patients' body fluid (WHO, 1984; Des Moutis et al. 1983; Schlie-Guzman & Rivas-Alcala 1989; Lobos et al. 1991). The aim of this investigation was to test the efficacies respectively of a monoclonal antibody target to a cuticular antigen and an antigen isolated from the same source as diagnostic tools.

Materials and Methods

Parasites and Patients. The methods for the isolation of *O. volvulus* adult worms, the extraction of antigens, the preparation of polyclonal antibodies to Fraction 5 (F5) and the classification of patients have been described prevlously (Titanji et al. 1990).

Monoclonal Antibodies were raised to cuticular extracts of *O. volvulus* adult worms according to a modification of the method of Holmdahl et al. 1985, and selected by eliminating all clones that reacted with *Ascaris lumbricoides, Loa loa*, and cryocuts of human skin and onchocercal nodular tissues in indirect fluorescent antibody tests (IFAT). One of the monoclonal antibodies designated MOV1 turned out to be *Onchocerca* specific and was retained for use in further experiments.

Defined Polypeptide Antigens. A polypeptide rabbit antiserum to antigen F5 extracted from *O. volvulus* (Titanji et al. 1990) was used to screen a cDNA expression library (Donelson et al. 1988) essentially according to the method of Huyhn et al. (1986). One of the clones isolated, expressed a polypeptide of apparent molecular weight of 67,000. Since preliminary Western blotting experiments with total worm extracts had shown frequent recognition of a 67000 antigen by a panel of hyperimmune onchocerciasis sera, the positive clone was retained for further study. Lysogens were prepared from the recombinant clone and the corresponding antigen isolated after separation by SDS-PAGE and identification by Western blotting. Bands corresponding to the immunostained P67 antigen were cut out of the nitrocellulose sheets, homogenised and emulsified in a 1:1 mixture of Freund's Complete Adjuvant. The mixture was then injected into rabbits to produce an antiserum to P67. This antiserum was used subsequently to construct an affinity column (Mbacham & Titanji 1988) that was used to isolate the parent proteins from total worm extracts. Herein, P67 will refer to the native antigen from worms and rp67 to the recombinant proteins from *E. coli.*

Detection of Specific Antibodies to O. volvulus. Enzyme linked immunosorbent assays (ELISA) for the detection of specific antibodies were performed essentially as described by Holmdahl et al. 1985 except that polyvinyl microtiter plates were precoated either with P67or rp67 at 0.1 µg/ml. The bound human antibodies were detected with the help of an anti-human globulin/peroxidase conjugate.

Results and Discussion

Detection of antibodies to O. volvulus. In order to test if P67 was suitable for the diagnosis of exposure to *O. volvulus* infection, we carried out a direct ELISA for IgG4. Figure 1a shows that all subjects from the hyperendemic village had developed high levels of IgG4 antibodies to P67. By contrast, the serum concentrations of these antibodies were at least one order of magnitude lower than control subjects from the hypoendemic area of Yaounde. Of particular interest were the high serum antibody concentration detected in the sera of subjects whose skin snips were negative for mf. Such subjects with no skin mf, but who have developed specific antibodies to P67 are likely to be exposed or may

have contained their infection. P67 may be a likely candidate antigen for the detection of prepatent onchocerciasis.

In order to produce substantial quantities of P67, recombinant DNA techniques were used. A clone producing a recombinant protein detected by antibodies to P67 was isolated from a cDNA library and partially purified and designated rp67 (see Materials and Methods). Figure 1b shows that rp67 recognised IgG4 antibodies in the same group of patients as well. However, it was less specific than P67 in that the native antigen preparation, reacted with both endemic and control sera.

To study the differences in the specificity of P67 and rp67, both preparations were analyzed by immunoblotting using immune sera. Figure 2a shows immunoblots of affinity-purified P67. The native P67 antigen isolated from adult worms was heterogeneous, containing at least 5 distinct polypeptides with apparent Mr of 67, 85, 108, 154 and 203 KD. The gel patterns obtained with partially purified rp67 are shown on Fig.2b. The proteins expressed in *E. coli* were of relatively lower molecular weights: 30, 37, 46, 57, 84 and 87 KD.

These proteins also reacted with antisera to cuticular fractions, indicating their histologic origin in the worms. The heterogeneity of the P67 and rp67 preparation suggested that these proteins are rather unstable. Immunoblotting experiments (not illustrated) indicate the presence of P67 related components in other worm stages, *viz* adult males, nodular mf and eggs of *O. volvulus*. It would thus appear that P67 is a dominant onchocercal antigen, represented in a wide range of polypeptides with distinct reactivities (Fig.2b); the low molecular weight range being less specific.

Immunodiagnosis of Active Onchocerciasis Generally, it is accepted that the detection of parasite products in body fluids is a more reliable index of active infection than the assay of specific antibodies. An alternative approach in our laboratory has been to develop a test for *O. volvulus* antigens in urine. Figure 3 summarises the results obtained using our developed sandwich ELISA. Onchocerciasis patients from hyperendemic villages had higher ELISA scores for antigenuria, than normal control subjects from Yaounde. The assay detected at least 80ng of crude antigen per test and required only 1ml of urine. With a cut-off point for negative results set at mean +2 standard deviations of the control scores, the test had a sensitivity of 98%, which compares favourably with previously reported values of 76% -86% (Des Moutis et al. 1983, Schlie-Guzman & Rivas-Alcala 1989).

Conclusions

This study presents two approaches for the immunodiagnosis of onchocerciasis, namely antibody and antigen detection. Both methods were highly sensitive. More work is needed on their specificities. Urine antigen detection is probably a more reliable method; its predictive value in prepatent cases merits further investigation. P67 and related proteins are widely recognized by onchocerciasis patients and may be important in pathogenesis of the disease. Its role too deserves further investigation.

Acknowledgements

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FIG.1 IgG_4 Responses in Defined Clinical Groups of Onchocerciasis Patients

Fig.1a

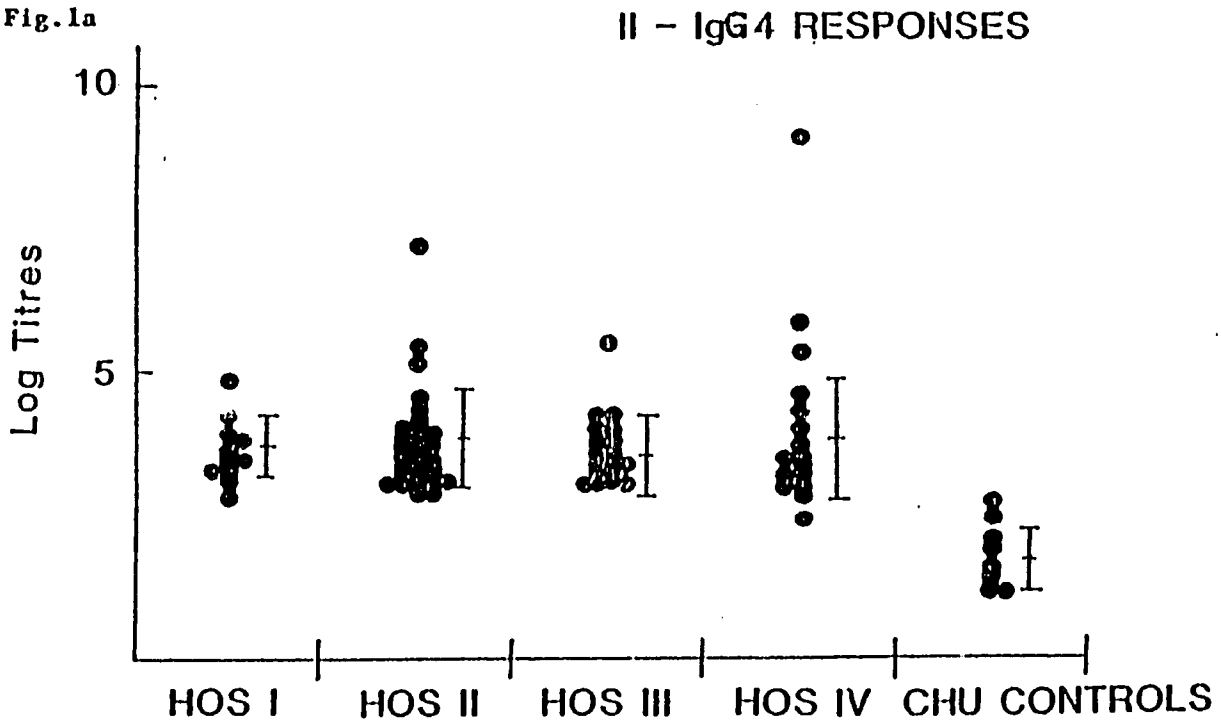


Fig.1b

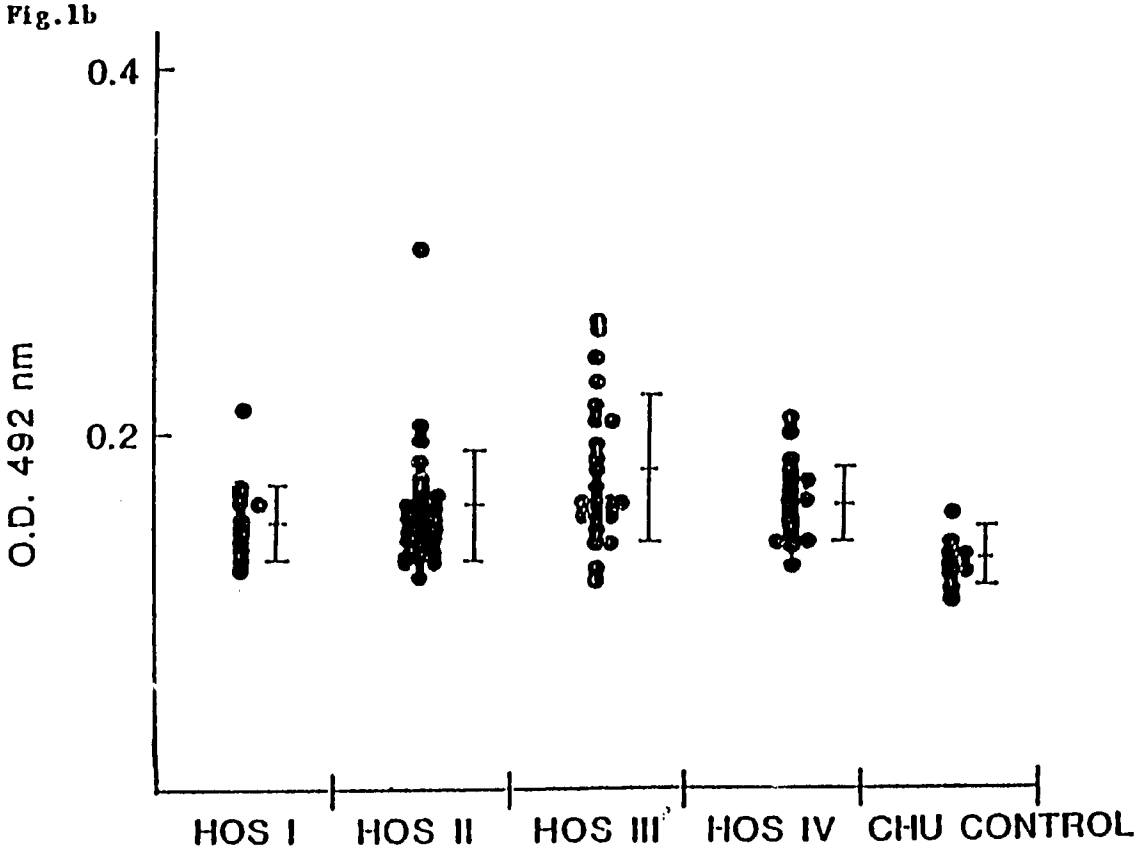
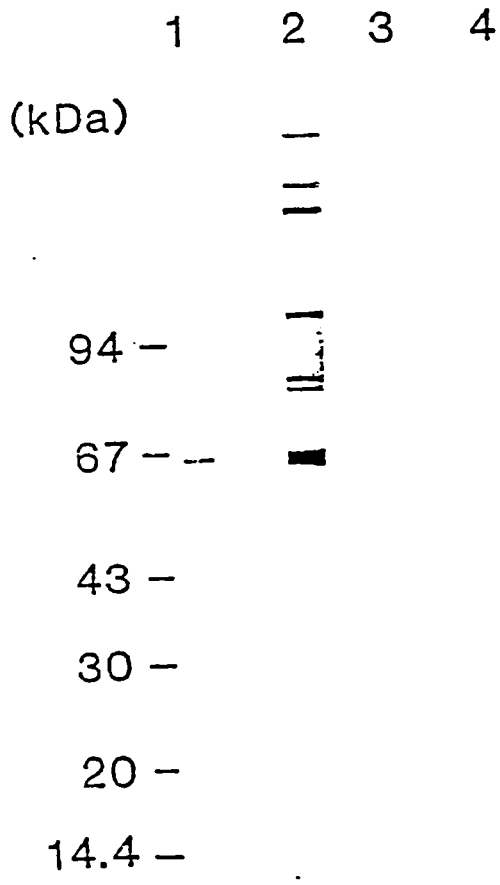
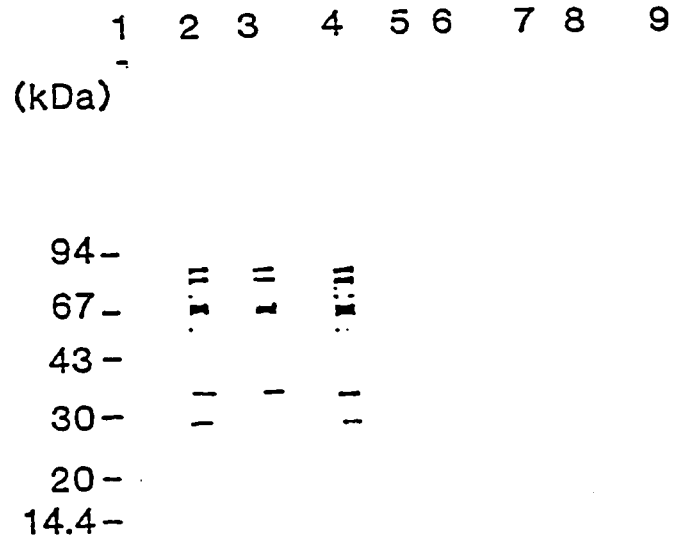


Fig. 1 The native (P67) - a) and the recombinant (rp67) - b) antigen were used in coating the microfilter plates. HOS I, 0mf; HOS II, 1-10mf; HOS III, 11-50mf; HOS IV, 50mf/antp; CHU healthy Controls from the University Teaching Hospital, Yaounde.

FIG.2 Immunological Identification of Native (P67) and Recombinant (rp67) antigens of Onchocerca volvulus





a) Worm extracts from O. volvulus were separated by SDS-PAGE transferred unto NC paper and reacted with Normal rabbit serum, Rabbit anti-clone, Normal human serum, and an onchocerciasis serum pool, respectively in lanes 1,2,3, and 4.

b) Western blotting with E. coli lysates containing recombinant antigen rp67 Lanes 1 - Normal Rabbit S, 2 - Rabbit -clone rp67, 3- Rabbit -F5 (cuticle), 4 - Rabbit -surface proteins ( ), 5 - NHS, 6-9 are human onchocerciasis serum I-IV respectively.

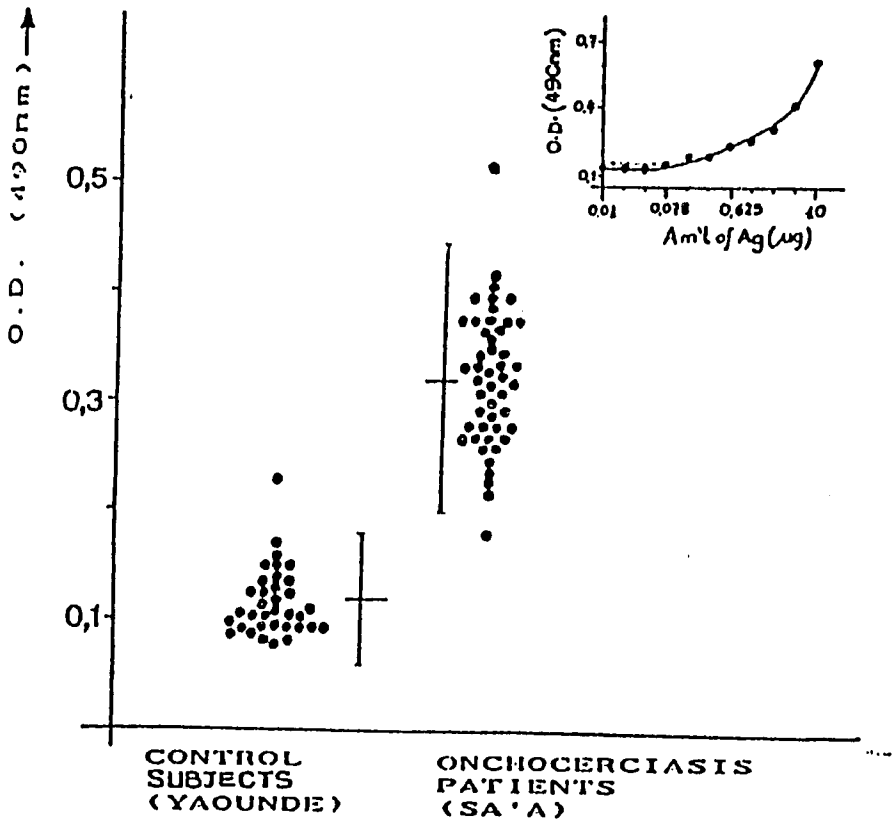


Fig.3 **An immunoenzymatic assay for the detection of O. volvulus antigens in onchocerciasis patient urines.**

The horizontal bars represent the means while the verticle bars represent ± 2 SD. The insert is the standard graph for the detection of antigens in this sandwich assay.

ARTICLES ORIGINAUX
ORIGINAL ARTICLES

Lectin-binding properties of Onchocerca volvulus developmental stages

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Summary :

FITC-lectin binding assays were used to probe the surface sugars of ***Onchocerca volvulus.*** *L_3-infective larvae bound Con A, DBA and LCH, but not PNA, SBA, WGA nor UEA. Adult females had a similar binding specificity as L_3 except that they bound WGA as well. LCH and DBA were the only lectins bound by male worms. Nodular microfilariae only bound LCH and WGA whereas skin microfilariae bound Con A and WGA. In addition to LCH and Con A, eggs bound WGA. Limited proteolytic treatment of the parasites before lectin binding revealed new sites for PNA and LCH on nodular and skin microfilariae respectively. No binding of UEA was observed indicating the absence of fucose on the parasites. The results are consistent with the presence of exposed D(+)-galactosyl, D(+)-mannosyl and N-acetyl galactosamine residues on O.* ***volvulus.*** *We conclude that O.* ***volvulus*** *displays considerable variations in surface sugar specificity during its development in man.*

Résumé :

La composition en glucides de surface cuticulaire, des différents stades évolutifs, chez l'**Onchocerca volvulus** a été mise en évidence par la technique de fixation des lectines conjuguées à l'isothiocyanate de fluorescéine. Les résultats montrent la présence des résidus D(+)-galactosyl, D(+)-mannosyl et N-acetyl galactosamine sur les larves infestantes (L_3), les vers adultes (Males et Femelles), les œufs et les microfilaires isolés des nodules et de la peau. Toutefois, la répartition des différents types de glucides n'est pas la même d'un stade évolutif à l'autre. Nous pouvons conclure que la composition des glucides de surface de l'**Onchocerca volvulus** varie considérablement pendant son cycle évolutif chez l'homme.

KEY WORDS :

Lectins, surface sugars of O. ***volvulus,*** *L_3, eggs, skin and nodular mf, adult worms.*

MOTS CLEFS :

Lectines, glucides de surface de l'**O. volvulus**, L_3, œufs, mf nodulaires et dermale, les vers adultes.

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Introduction

Current interest in the study of filarial parasites has focused on the properties of the cuticle because of its importance in host-parasite interactions (Maizels, et al., 1982). Previous studies on filarial surface properties were devoted to the characterization of protein antigens, presumably because they could be easily detected by immunoradiometric methods. Thus radioiodinated protein antigens were identified on the surface of **Onchocerca volvulus** *adult worms (Philipp, et al., 1984) and* **O. gibsoni** *microfilariae (Forsyth, et al., 1984 ; Taylor, et al., 1986).*

To date little is known about the surface sugars of **O. volvulus**, *although lectin-binding studies have been used recently to probe for the types of sugars expressed on* **Brugia malayi**, **Dirofilaria immitis** *microfilariae and* **O. gibsoni** *eggs (Forsyth et al., 1984 ; Devany, 1985) Considering the antigenic nature of surface saccharides and their potential roles in host-parasite inter-play (Howells, 1981) we decided to investigate the lectin-binding properties of the developmental stages of* **O. volvulus** *as a first step towards the characterization of the surface sugars of the parasite.*

Materials and methods

Reagents : *Proteinase E, Fluorescein-isothiocyanate-labelled lectins (Table 1) and their inhibitory sugars were purchased from Sigma : collagenase was from Boerhinger. All other reagents were of the highest purity provided by Merck, Serva and Sigma companies. Phosphate buffer saline (PBS) used below contained at pH 7.2 : 8 gNaCl ; 0.2 g KCl ; 1,15 g $Na_2HPO_4 . 2H_2O$; 0.2 g KH_2PO_4 ; 0,1 g $MgCl_2 . 6H_2O$ and 0.1 g $CaCl_2$ per litre.*

Parasites : *All manipulations were carried out aseptically. Nodules were excised from onchocerciasis patients at a dispensary in Batchenga (Cameroon) and transported to the laboratory in RPMI-1640 culture medium supplemented with 0.03 % gentamycine. Adult* **O. volvulus** *were then isolated according to the method of Engelbrecht and Schulz-Key (1984). Viability was 60-70 % among adult females which could survive for at least 6 days when maintained in RPMI-1640 containing 0.03 % gentamycine and 10 % normal human serum at room temperature.*

Microfilariae (mf) were isolated from nodules according to a modification of the method of Chandrashekar et al., (1984). Briefly, nodules were minced in 3 volumes of Hanks Balanced Salt Solution (HBSS) containing 0.03 % gentamycine and incubated at (27-29 °C) overnight to permit maximum recovery of mf. Tissue clumps were removed and the suspension filtered through two layers of cheese cloth and then centrifuged at 2000 rpm for 20 min. The pellet containing mf and blood cells was suspended for 10 min. in 10 ml lysis buffer containing 155 mM NH_4Cl and 10 mM $NaHCO_3$. The suspension was then recentrifuged as above, and the final pellet taken up in 3 ml HBSS and then loaded on a 30/40 % discontinuous Percoll gradient made in HBSS (without phenol red). The gradient bottles were then centriguged in a swing out rotor (Sorvall-R II) at 1500 g for 30 min.

Dead mf and tissue debris with a few trapped motile mf were found at the top of the 30 % Percoll layer, whereas most of the motile mf were either in the 40 % Percoll fraction or at the bottom of the tube, freed of cellular contamination. The mf were washed twice with HBSS and maintained at 27-29 °C in RPMI-1640 containing 0,03 % gentamycine and 10 % NHS with daily changes of medium. Viability exceeded 90 % within the first 48 hrs and gradually declined to 50-60 % within 4-5 days.

Skin mf were obtained by incubating skin snips in HBSS containing 0.03 % gentamicyne and 10 % NHS for 24-48 hrs. The skin mf were stored in culture medium as described above.

Infective L_3 larvae, were dissected from infected blackflies. In some experiments the isolated parasite stages were fixed in PBS containing 10 % formalin before lectin binding.

Lectin-binding studies

Initially we used the method of Devany (1985) to study the lectin binding characteristics of nodular and skin mf. Subsequently this method was replaced by a microscope slide technique which we developed to permit the investigation of limited quantities of parasites (L_3 and skin mf) as well as to eliminate the need for multiple centrifugations during the washing procedure (Devany, 1985).

Microfilariae (20-100) were deposited on a marked spot on microscope slide, air dried and fixed with absolute ethanol for 10 min. PBS containing the appropriate lectin at 150 ug per ml was applied to the spot and the slide incubated at 27 °C for 1 hr in a humid chamber. A control experiment with 0.1 M of the corresponding inhibitory sugar (Table 1) was always done simultaneously at a different spot on the same slide. After incubation excess FITC-lectin was withdrawn with a Pasteur pipette under the microscope. The mf were then washed twice by adding 0.2 ml PBS to the spot, incubating for 5 min and then aspirating the buffer. Finally a drop of glycerol was applied to the spots and the mf examined under a Nachet fluorescence microscope at a magnification of 100 X. This technique was also used for the binding of FITC-lectins to L_3 infective larvae, except that only a few unfixed larvae were used for each experiment. Using nodular mf we confirmed that the slide technique gave identical results with the method of Devany (1985). However, we preferred the slide method in subsequent studies because of its simplicity.

For the investigation of adult worms, they were dispensed individually into test tubes and incubated at room temperature with 1 ml of the appropriate lectin at a final concentration of 150 ug ml^{-1}. Control experiments in the presence of inhibitory sugars were done simultaneously. At the end of the incubation the worms were washed four times with 5 ml of PBS and then examined for fluorescence as describe above. Only viable adult worms were used.

Treatment of microfilariae with proteolytic enzymes

In an attempt to open up new binding sites, mf were treated with collagenase, or pronase E. The proteolytic enzymes were dissolved in PBS at a final concentration of 0.01 % (w/v), and 2 ml of the solution used to incubate 5 000 – 10 000 mf for 30 min at 25-29 °C. The incubation was stopped by adding 5 ml cold PBS and centrifuging the mf at 2000 rpm for 10 min. The mf were then resuspended in 5 ml PBS and centrifuged. This washing procedure was repeated three times and the mf used directly in the binding assay or fixed in PBS containing 10 % formol for subsequent use. Identical results were obtained for both fixed and viable parasites (not illustrated).

Results

Lectin-binding characteristics of O. volvulus developmental stages

Table 1 shows that only four out of seven FITC-lectins tested could bind to ***O. volvulus*** *worm stages that had not been subjected to proteolysis. The lectins that bound to the worm stages were Concanavalin A (ConA), Lentil lectin (LCH), wheat germ agglutinin (WGA) and* ***Dolichos biflorus*** *agglutinin (DBA). Peanut agglutinin (PNA), soy bean agglutinin (SBA) and* ***Ulex europeaeus*** *agglutinin (UEA) did not bind to intact parasites under our conditions of assay. The binding of the lectins appeared to be specific since this was always abolished by adding the appropriate inhibitory saccharide in the medium.*

In positive reactions fluorescence was usually seen all over the parasites. The ridges of adult females showed very weak fluorescence, in contrast to "forrows" that were intensely fluorescent.

Table 1 also reveals important differences among the lectin-binding characteristics of the developmental stages of **O. vovulus**. *Thus nodular mf appeared to have sites for LCH and WGA in contrast to skin mf which could bind ConA and WGA, but not LCH. L_3 and adult females displayed similar surface properties, with the sole difference that in addition to ConA, LCH and DBA, the latter also bound WGA. In contrast to adult females, male worms could only bind LCH of all the lectins tested. The lectin most frequently bound was LCH which showed positive reactions with four of the parasite stages tested, followed by ConA and WGA each of which bound three stages. The FITC-lectin-binding characteristics of the eggs were very similar to those by adult females except, that the former did not bind DBA. Taken together our data indicate considerable variation in the set of sugars expressed on the respective* **O. vovulus** *worm stages studied.*

Effect of proteolysis on lectin-binding to microfilariae

In an attempt to open up new binding sites, skin and nodular mf were treated with proteolytic enzymes before lectin binding. The results given in Table 2 showed that, collagenase opened up new sites for LCH and PNA on skin mf and nodular mf respectively, whereas pronase E revealed new LCH binding sites on skin mf. Under no circumstances were we able to demonstrate the binding of UEA to **O. vovulus** *developmental stage studied : a fact which excludes exposed ∞–L–(–)Fucose residues on these parasites. The movements of the mf were reduced considerably by the proteolysis, but well over 80 % of them were still viable as judged by occasional serpentine movement.*

Discussion

The present investigation revealed distinctions among the lectin-binding characteristics of **O. vovulus** *developmental stages studied. Adult females and L_3 seemed to have similar lectin-binding properties that contrasted with those of adult males, nodular and skin mf (Table 2). Given the antigenic nature of filarial surface sugars (Howells, 1981), it is tempting to speculate that the observed differences reflect changes in surface antigen specificities among the developmental stages. Such changes may be important for* **O. vovulus** *worm survival in man since immune responses mounted against L_3 and adult females, for example, would be ineffective in blocking the development of mf. Clearly, additional studies are necessary to clarify the importance of the immune reactivity of surface sugars in* **O. vovulus** *infection.*

The results in Table 1 are consistent with the presence of exposed D(+)galactosyl, D(+)mannosyl, N-acetyl-D-galactosamine and N-acetyl galactosamine residues on the various developmental stages tested. N-acetyl-D-galactosamine and D(+)galactose are presumably not accessible on intact mf, but become exposed after limited proteolysis, indicating that some of the sugars may be buried into the structure of the cuticle (Table 2).

Although the sugar-binding specifities of Lentil lectin (LCH) and Concanavalin A (ConA) are similar, the latter bound to nodular, but not to skin mf and vice versa (Table 1). Similary, adult males could bind LCH, but not ConA. The reason for this apparent discrepancy remains to be determined, although differences in the affinities of the lectins for their binding sites could possibly explain the results obtained.

While this study was in progress Taylor et al. (1986) reported lectin binding characteristics of ***O. vovulus*** *worm stages that differed considerably from our findings (Table 1 and 2). According to these investigators neither nodular nor skin mf could bind to any of the FITC-lectins tested by them, a result which they attributed to the lack of exposed carbohydrates on these parasite stages. In contrast to their findings we found that LCH and ConA did bind to nodular and skin mf respectively, whereas WGA bound to both types of mf (Table 1 and 2). It is possible that different strains of* ***O. vovulus*** *from geographically remote regions may display different surface properties.*

Our results (Table 1 and 2) also suggest a marked difference between the lectin-binding properties of ***O. vovulus*** *and those reported for other filariae. Whereas the cuticle of* ***O. vovulus*** *mf revealed binding sites for LCH and ConA, those of* ***O. gibsoni, B. malayi*** *and* ***D. immitis*** *did not (Forsyth et al., 1984; Devany, 1985). Taken together the present findings indicate considerable variation in the surface sugar specificity during the morphogenesis of* ***O. vovulus*** *in man.*

Conclusion

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TABLE 1 – Lectin-binding properties of ***O. volvulus*** *stages.*

FITC LECTIN	F/L	Inhibitory* sugar used	Sugar** specificity	Lectin binding in the absence of inhibitors					
				L_3	adult female	adult male	N.mf	s.mf	eggs
ConA (102)	1.9	N-acetyl-D-Glucosamine	α-D-Man, α-D-Glc	+	+	–	–	+	+
DBA	2.0	N-acetyl-D-Galactosamine	α-D-Gal NAc	+	+	(+)	–	–	-
LCH (49)	4.1	N-acetyl-D-Glucosamine	α-D-Man, α-D-Glc	+	+	+	+	–	+
PNA	5.3	D(+)Galactose	ß-D-Gal-NAc	–	–	–	–	–	–
SBA	1.7	D(+)Galactose	D-Gal NAc	–	–	–	–	–	–
UEA_1	2.1	-L(–)Fucose	α-L(–)Fucose	–	–	–	–	–	–
WGA	2.2	N-acetyl-D-Galactosamine	$(D\text{-}Glc\ NAc)_2$, Neu NAc	–	+	–	+	+	+

(+) indicates positive reactions whereas (–) indicates negative reactions with no fluorescence. When the corresponding inhibitory sugar was included in the test no fluorescence could be observed (results not shown). All other details are given in the text.
** Information from Sigma information sheets.*
*** The sugar binding specificities were as described earlier (Devany 1985). See also catalogue 1986. F/L = moles fluorescin isothiocyanate per mole lectin. N.mf = nodular microfilariae, s.mf = skin microfilariae, Neu-NAc = N-Acetyl neuraminic acid, Glc = glucose, man = mannose, Gal-NAc = N-acetyl galactose.*

TABLE 2 – Effect of limited proteolysis on lectin binding to microfilariae.

FITC-LECTIN	Lectin binding after enzyme treatment and in the absence of inhibitory sugars			
	COLLAGENASE		PRONASE E	
	Nmf	Smf	NMf	Smf
Con A	–	+	–	+
DBA	–	–	–	–
LCH	+	+	+	+
PNA	+	–	–	–
SBA	–	–	–	–
UEA_1	–	–	–	–
WGA	±	±	±	±

(+) indicates positive reactions ; (–) indicates negative reactions with no fluorescence observed (±) indicates weak fluorescence. All other details are given in the text and the legend of Table 1.

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Identification of the surface polypeptide antigens of the infective larvae of *Onchocerca volvulus*

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Abstract

The surface polypeptide antigens of third-stage infective larvae (L_3) and adult males of *Onchocerca volvulus* have been compared after iodogen-catalysed radioiodination, separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis in β-mercaptoethanol followed by autoradiography. L_3 surfaces contained polypeptides with apparent molecular weights of 14, 18·5, 26, 34, 51, 68 and 130 kDa, whereas the adults contained 14, 19, 26, 34, 37·5 and 68 kDa components. By immunoprecipitation with patient's sera, only the 14 and 18·5 kDa components were shown to be antigenic on the L_3, the other components being unreactive with patients' antibodies. Under similar conditions, 4 of the 6 adult male polypeptides reacted with patients' antisera, confirming their antigenic nature. Lentil lectin and immunosorbent chromatography of the surface components revealed the 18·5 kDa and 68 kDa antigens of L_3 and adult males respectively to be glycoproteins. The apparently weak reactivity of L_3 surface components with host antibody may be part of an escape mechanism that favours the establishment of *O. volvulus* in human hosts.

Introduction

Recent studies of the blinding nematode, *Onchocerca volvulus*, have focused on the antigenic properties of the cuticle of its developmental stages, since these comprise the primary contact organs between the parasite and the immune system of the host (Phillip *et al.*, 1984; Taylor *et al.*, 1986; Carbrera & Parkhouse, 1986; Titanji & Ngu, 1986; Titanji *et al.*, 1988). However, because of their scarcity, the infective third-stage larvae (L_3) which initiate infection and have therefore been considered to be the logical targets of onchocerciasis vaccines (WHO, 1987), have received inadequate attention. Taylor *et al.* (1986) were the first to describe 7 polypeptides on the surface of *O volvulus* L_3 after radioiodination, but they did not identify which of these components were antigens. We, like others, identified the surface sugars of *O. volvulus* L_3, but their antigenicity remained unknown (Taylor *et al.*, 1986; Titanji *et al.*, 1987). However, antibody-dependent immune cell adherence to L_3 has been studied, using cells from various onchocerciasis patient groups, by Leke *et al.* (1988).

The paucity of information on the antigenic nature of the L_3 surface components prompted us to study this parasite stage using the iodogen-catalysed iodination method (Marshall & Howells, 1986), to identify the surface polypeptides that elicit antibody responses in human hosts, and to compare them with the adult male worms whose surface properties have been extensively studied (Taylor *et al.*, 1986).

Materials and Methods

Reagents

RPMI 1640 medium (Gibco, UK), ^{125}I (Amersham International, UK), and lentil lectin sepharose-4B (Pharmacia, Uppsala, Sweden) were used. All other reagents were of the purest grade obtainable from Sigma, Serva or Merck.

Parasites

L_3 were dissected into RPMI 1640 medium from the head of blackflies (*Simulium*) previously infected while feeding on volunteer fly catchers*. They were identified as *O. volvulus* at the Kumba Medical Research Station. About 700 L_3 were used. Adult male worms were obtained by collagenase digestion of nodules (Engelbrecht & Schulz-Key, 1984) and maintained in RPMI 1640 medium. To limit bacterial infection all culture media contained 0·16 mg/ml of gentamycin and manipulations were carried out under sterile conditions.

Patients and sera

Patients were examined at a dispensary in Batchenga in the Sanaga river basin of Cameroon. Skin snips were taken from the calf muscles, iliac crest, and shoulders and incubated for 24 h at 25–28°C in RPMI 1640 medium with gentamycin. The microfilariae released after incubation were counted. The remaining skin snips were further digested for 24 h in collagenase (5 mg/ml in phosphate-buffered saline [PBS] containing 10 mM phosphate buffer at pH 7·4 and 140 mM NaCl) to release any trapped microfilariae. The 20 patients used in this study had 10–15 microfilariae per skin snip. Preliminary experiments with adult worm antigens confirmed this group to be the most reactive with surface antigens. Blood samples obtained by venipuncture were processed for sera as previously described (Titanji *et al.*, 1981). The sera were stored in 0·5 ml portions at −20°C until thawed and immediately used in the experiments.

Radiolabelling and solubilization of surface polypeptides

Only viable motile parasites were used. These were labelled with carrier-free ^{125}I essentially as described by Baschong & Rudin (1982). Iodogen (1,3,4,6 tetrachloro3α,6αdiphenylglycoluril (Sigma was dissolved in chloroform to a final concentration of

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*Volunteer fly catchers had access to ivermectin treatment after the experiment and were exposed only to newly hatched flies unlikely to have been previously infected with *O. volvulus*.

0·2 mg/ml. Aliquots of 0·2 ml of the solution were dispensed into 5 ml test tubes and allowed to evaporate in the dark at 22–25°C in a fume cupboard. The tubes were then stored dry over Blue-Gel® crystals in an evacuated dessicator.

For labelling, the iodogen-coated tubes were rinsed with 1 ml of PBS. In a typical experiment, 4–8 adult males or about 700 L_3 were put into separate tubes and washed thrice in PBS. L_3 were washed by centrifugation at 2000 rpm for 10 min; adult males were washed by aspiration of the PBS with Pasteur pipettes. The parasites were suspended in 1 ml of PBS, and 2 mCi (10 µl) of carrier-free ^{125}I were added. The tubes were placed on an ice bath and incubated with gentle intermittent agitation for 10 min. The reaction was stopped by transferring the adult males to tubes containing fresh PBS. L_3 were centrifuged and washed with 3 changes of PBS as described above. The parasites were separately transferred to fresh tubes for solubilization. After labelling, the adult worms were 100% viable and the L_3 were about 90% viable, as judged by their motilities under a microscope.

Surface polypeptides were solubilized by incubating the labelled parasites in 0·5–1 ml 10 mM Tris-HCl (pH 8) containing 1% sodium deoxycholate and the following proteolytic inhibitors: leupeptin, pepstatin and N-α-*p*-tosyl-L-lysine chloromethyl ketone at final concentrations of 5, 5 and 25 µg/ml respectively. For maximal inhibition of proteolysis, 10 µl of 200 mM phenyl methyl sulphonyl fluoride freshly made in acetone were added to the reaction mixture every 10 min for one hour during the entire solubilizing process. This and subsequent steps were carried out at 4°C unless stated otherwise. Aliquots (50 µl) of the solubilized components were taken for protein determination and the remaining extracts were adjusted to contain 300 µg/ml bovine serum albumin as carrier protein and then desalted on Sephadex G-25 (PD-10 columns from Pharmacia) by equilibrating and eluting with 10 mM Tris-HCl (pH 8) containing 0·1% sodium deoxycholate. The radioactive peaks eluted with the void volume of the column were pooled, divided in 200 µl aliquots and stored at −20°C to be thawed once only before use in experiments. The maximum storage period was 1 month.

To determine the amount of radioactivity precipitable by trichloroacetic acid (TCA), 50 µl of the desalted samples were mixed with 100 µl of normal rabbit serum followed by 1 ml of 20% TCA. After 20 min at 4°C the mixture was centrifuged (2000 rpm for 10 min) and the supernatant discarded. The pellets were then counted in a Beckman 4000 gamma counter and the percentage of TCA-precipitable counts calculated by comparison with the initial radioactivity of the sample. About 60–70% of the solubilized polypeptides were TCA-precipitable.

Immunoprecipitation

Immunoprecipitation was done according to a modification by TITANJI & NGU, 1986) of the method of KESSLER (1975). Frozen antigens were thawed and cleared by mixing with an equal volume of European normal serum, and then adding one-quarter volume of 20% washed Pansorbin® (Calbiochem). After incubating the mixture at 4°C for one hour, it was centrifuged at 2500 *g* for 20 min at 4°C). The precipitate was discarded and the supernatant used in the immunoprecipitation reaction.

In a typical reaction, 100 µl of labelled antigen containing $100–150\times10^3$ cpm with a specific activity of $2·2–3·3\times10^3$ cpm per µg of protein were added to 100 µl of serum, diluted four-fold with 50 mM Tris-HCl (pH 7·4, containing 150 mM NaCl, 5 mM ethylenediaminotetraacetic acid and 0·02% NaN_3). The mixture was then incubated at 4°C for 12–14 h. Immune complexes formed were isolated by staphylococcal immunosorbents and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as previously described (TITANJI & NGU, 1986). Sera from onchocerciasis patient groups (see above) and uninfected European controls were used in the experiments.

Isolation of surface antigens by immunosorbent and lectin chromatography

For some studies, labelled and desalted surface polypeptides were purified by sequential chromatography on 1 ml lentil lectin Sepharose 4B columns equilibrated as described by the manufacturers. The glycoproteins eluted with 0·2 M α-D-mannose were further purified on immunosorbent columns made by linking a 0–50% $(NH_4)_2SO_4$ fraction of pooled onchocerciasis sera to sepharose 4B (MBACHAM & TITANJI, 1987). Antigens were eluted from the immunosorbent columns by boiling in the sample buffer of LAEMMLI (1970) for 5 min. In a few experiments the labelled antigens were purified directly on the immunosorbent columns without prior exposure to the lentil lectin columns.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate was performed essentially according to the method of LAEMMLI (1970) using a Protean II Biorad® apparatus. After drying in acetone (WESTERMAN, 1985) the gels were placed on Agfa Safety Curix RPI® films and autoradiographed in a cassette with intensifying screens at −70°C for 1–3 weeks. Protein was determined according to the method of READ & NORTHCOTE (1981).

Results

Surface polypeptide antigens of O. volvulus *infective larvae and adult males*

In preliminary experiments (results not shown) we found no difference between the antigen profiles of adult worms by immunosorbent chromatography and the pattern obtained by using the staphylococcal immunoprecipitation method of KESSLER (1975). This was also the case with L_3 antigens. Therefore in subsequent experiments we chose the staphylococcal immunosorbent precipitation because of its simplicity (see Materials and Methods section). Using labelled adult worm antigens, the most reactive sera were found to be those from patients with low to moderate microfilarial density (i.e., 1–50 microfilariae per snip). These were then pooled and used in experiments with L_3 for which antigen shortage precluded the examination of individual sera.

Fig. 1 illustrates typical gel patterns obtained when surface antigens were respectively isolated from L_3 and adult male worms respectively and then analysed by SDS-PAGE under reducing conditions followed by autoradiography. It can be seen that these worm

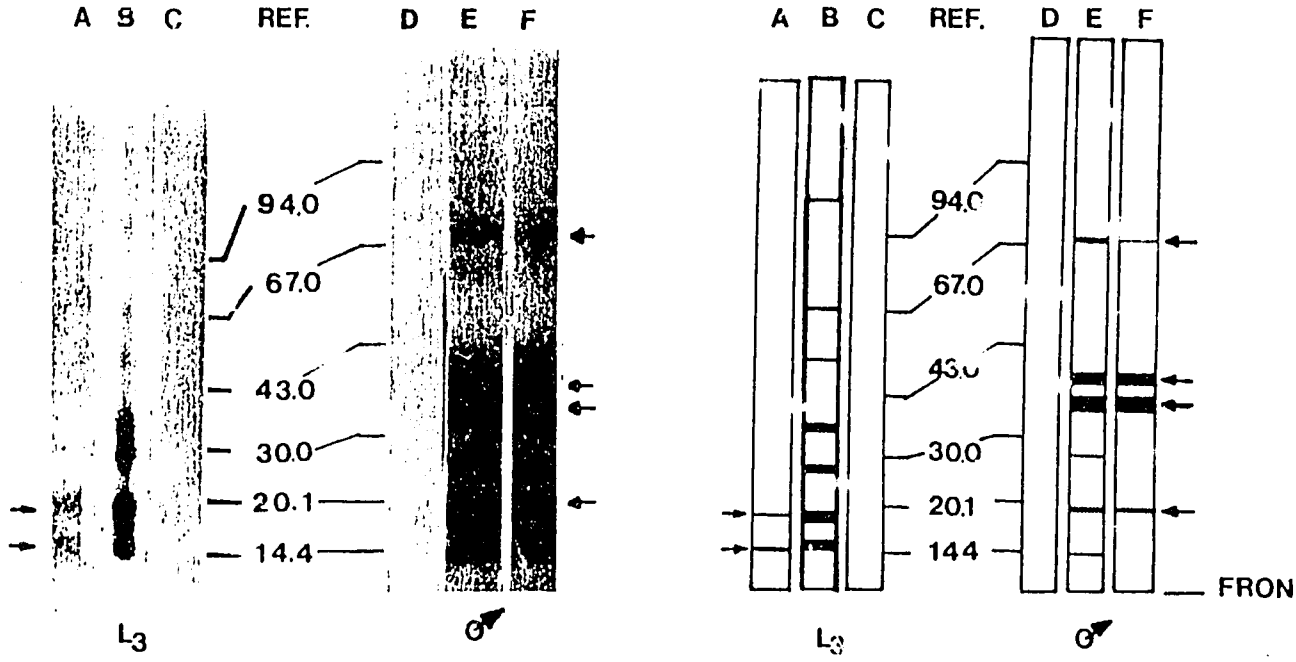

Fig. 1. Polypeptides and antigens of infective larvae of *O. volvulus* (L_3, lanes B and A respectively) and adult males (lanes E and F respectively). Lane B: polypeptides from L_3 (25 000 cpm), autoradiographed for 10 days. Lane E: polypeptides of adult males (23 000 cpm), autoradiographed for 13 days. The antigens (lanes A and F) contained 5000–8000 cpm and were autoradiographed for 21–28 days. Lanes C and D represent control lanes in which normal human sera were used in the immunoprecipitations. REF. are reference molecular mass samples ($\times 10^{-3}$) from Pharmacia. Autoradiographs are shown on the left, and line-diagrams of the gels on the right.

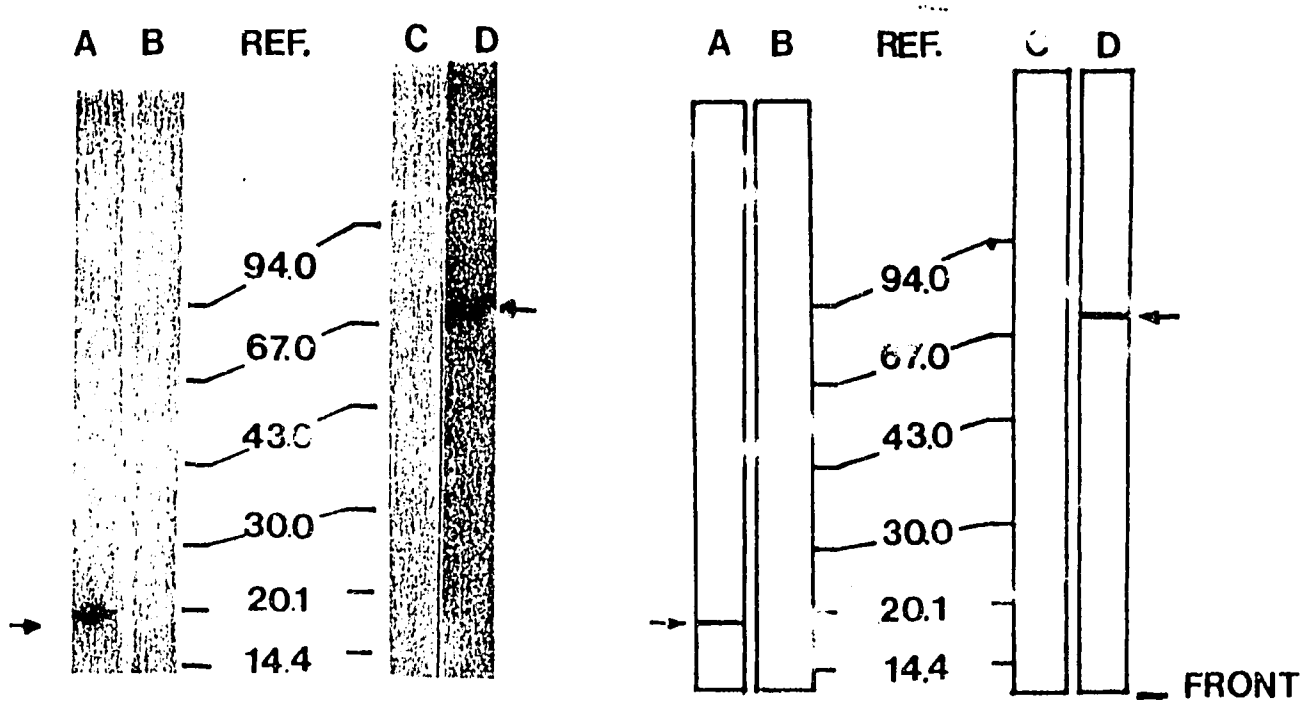

Fig. 2. Glycoprotein antigens of *O. volvulus* third-stage larvae (L_3) and adult males. The glycoproteins were isolated by immunosorbence followed by lectin affinity chromatography and then analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Autoradiographs of the gel patterns (left): lane A, infective larvae glycoprotein (5600 cpm, exposed for 28 days); lane D adult male glycoproteins (48000 cpm, exposed for 21 days); lanes B and C, eluates of control columns containing unsubstituted sepharose-4B [an eluate volume equal to the appropriate experiment (A and D) was applied to the gels]. REF. are reference molecular mass samples ($\times 10^{-3}$) from Pharmacia. Autoradiographs are shown on the left, line diagrams of the gels on the right.

stages contained exposed polypeptides that were easily labelled by the iodogen method. On the L_3 at least 7 distinct polypeptides with apparent molecular masses (expressed as means of 4 determinations±standard deviation) of 14·5±0·5, 18·5±0·4, 26·0±1·0, 34·0±0·6, 51·0±0·1, 68·0±0· and 130·0±0·2 kDa were identified (lane B). Only of these, a 14·5 and a 18·5 kDa antigen, were precipitated by immune sera from onchocerciasis patients (lane A).

Fig. 1 (lane F) clearly shows that the surface of male worms contained a larger number of antigens that reacted with patients' sera than the L_3 (Fig. 1, lane A). Of the 6 components identified on adult males with apparent molecular masses of 14, 19, 26, 34, 37·5 and 68 kDa (Fig. 1, lane E), only the first and third components were not precipitated by patients' sera under the conditions used, the remaining components giving intense radioactive bands after immunoprecipitation. This suggests that the established adult worm stage provoked a more pronounced humoral immune response than the infective larvae, the existence of which in the host is relatively short-lived.

Identification of antigenic glycoproteins

In a previous study it was shown that L_3 could bind concanavalin A (con A), *Dolichos biflorus* agglutinin (DBA), and lentil-lectin (LCH), indicating the presence of α-D-mannosyl, α-D-galactosyl and α-D-glucosyl residues on the surfaces. In that study adult males were shown to bind only LCH (TITANJI *et al.*, 1987). It was therefore interesting to investigate whether these exposed sugars were linked to proteins. Thus the labelled surface components were purified by chromatography on an LCH-sepharose 4B column. The glycoproteins eluted with the competing sugar, 0·2 M α-D-mannose, were further purified on a sepharose-4B immunosorbent column containing human antibodies to *O. volvulus* (see Materials and Methods section). Fig. 2 (lane D) shows a 68 kDa component from the male worm surface to be an antigenic glycoprotein since it was retained by the lectin and immunosorbent columns. This was also the case for an 18·5 kDa component identified on the surface of L_3 (Fig. 2, lane A). Thus at least some of the exposed sugars were protein-bound.

Discussion

This study presents the first identification of the surface antigens of L_3 of *O. volvulus*. As the infective stage, L_3 have often been suggested as suitable targets for vaccine development, L_3 inactivation, it has been proposed, would effectively prevent the transmission of onchocerciasis (WHO, 1987). Our data show that, at least for the group of patients studied, the humoral immune reaction to the L_3 surface was somewhat weak. Although the other surface polypeptides could be easily identified after SDS-PAGE and autoradiography, immunoprecipitates contained only a 14·0 and an 18·5 kDa component. The low antibody-combining capacity of L_3 surface polypeptides when tested with patients' sera could not be explained by our use of staphylococcal immunosorbent which is specific for immunoglobulin G_1(IgG_1), IgG_2 and IgG_4 but not for IgG_3. A similar low yield of L_3 surface antigens was obtained when the surface polypeptides were chromatographed on anti-*O. volvulus* immunosorbent columns containing all IgG isotypes. To explain these findings, we suggest that the L_3 surface is poorly immunogenic in man as a means of avoiding host immunity before more effective escape mechanisms such as immunosuppression come into force. Alternatively, the transient nature of the L_3 may prevent them from eliciting a strong antibody response. There have been preliminary reports of antibody-dependent immune cell adherence to L_3 *O. volvulus* but hardly any of the killing of these larvae by immune cells from putatively immune patients (LEKE *et al.*, 1988). The identified components should be studied further for a possible role in protective immunity.

There are both similarities and differences between the present and previous results. Like TAYLOR *et al.* (1986), we identified 7 components on the L_3 of Cameroonian *O. volvulus*, but the apparent molecular sizes of some of these were different, probably reflecting a difference between the parasite strains from Sierra Leone and Cameroon. An 18·5 kDa antigen appeared only on the L_3 and was absent from adult male worms. Preliminary data suggest that this antigen is also absent from females, eggs and microfilariae, making it a good candidate for stage-specific diagnosis of early infection (Titanji & Mbacham, unpublished observations). Although the 14 kDa component was present in the labelled surface extracts of both stages, it could not be found among the surface antigens of the adult worms. Further experiments to establish the identity of these components of similar size (CARBRERA & PARKHOUSE, 1986) are clearly indicated.

Our data also showed that some of the exposed sugars previously identified on the *O. volvulus* form parts of antigenic glycoproteins, among which are the 18·5 kDa and 68 kDa antigens on L_3 and adult males respectively. This suggests that attempts to generate large quantities of *O. volvulus* surface antigens by recombinant deoxyribonucleic acid techniques should take into account the possible contribution of carbohydrate moities to the immunogenic properties of these glycoproteins.

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