Preliminary Evaluation of Recombinant *Onchocerca volvulus* Antigens for Serodiagnosis of Onchocerciasis

A. F. OGUNRINADE,¹ R. CHANDRASHEKAR,^{2*} M. L. EBERHARD,³ AND G. J. WEIL²

*Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan, Nigeria*¹;

Departments of Medicine and Molecular Microbiology, Washington University School of

*Medicine and Jewish Hospital of St. Louis, St. Louis, Missouri 63110*²; and *Division of*

*Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333*³

Received 8 January 1993/Accepted 5 April 1993

Serodiagnostic assays for onchocerciasis based on native antigens are hampered by the scarcity of antigen, and they suffer from poor specificity. The present study was designed to evaluate the diagnostic utility of recently described recombinant *Onchocerca volvulus* antigens OC 3.6 and OC 9.3 in enzyme immunoassays. The recombinant proteins were expressed as glutathione *S*-transferase fusions and were tested in several enzyme immunoassay formats to measure immunoglobulin G (IgG) and IgG4 antibodies with sera from patients with onchocerciasis in Nigeria and with various types of control sera. The best results were obtained by measuring IgG4 antibodies to the fusion proteins. Forty of 42 (95%) serum specimens from patients with onchocerciasis were reactive with OC 3.6; the reactivity with OC 9.3 was 81%. Results obtained with sera from experimentally infected chimpanzees suggest that OC 3.6 might be especially useful for detecting prepatent infections in humans, while OC 9.3 mainly detects mature, patent infections. Sera from individuals in Nigeria and the United States residing in areas nonendemic for onchocerciasis were uniformly nonreactive with these antigens in IgG and IgG4 assays, as were sera from patients with bancroftian filariasis, brugian filariasis, loiasis, ascariasis, schistosomiasis, and dracunculiasis. These results suggest that enzyme immunoassays based on the recombinant antigens OC 3.6 and OC 9.3 are useful for the diagnosis of onchocerciasis.

Onchocerciasis is an important cause of blindness and severe dermatitis in sub-Saharan Africa and Latin America (32). At present, diagnosis of onchocerciasis in humans depends primarily on the demonstration of microfilariae in skin snips (32). However, skin-snip examination is not sensitive for the detection of early infections or for the diagnosis of onchocerciasis in individuals with low microfilarial densities in the skin (19, 27). False-negative skin-snip examinations are often seen in children, recently infected adults (especially expatriates), and individuals who develop allergic hypersensitivity to microfilariae ("sowda"). The skin-snip procedure is inconvenient, and special care is needed to ensure that blood-borne viruses are not spread in field studies. A sensitive and specific test for antibodies to the parasite would provide an excellent tool for identifying early infections and also for use in epidemiological surveys (7, 24, 26).

Serodiagnostic assays for onchocerciasis based on native antigens are hampered by the scarcity of antigen, and they suffer from poor specificity (1, 30). We have recently identified and characterized two recombinant *Onchocerca volvulus* antigens, OC 3.6 and OC 9.3, which appeared to be promising reagents for use in the diagnosis of onchocerciasis on the basis of preliminary results obtained by Western blotting (immunoblotting) (4). The purpose of the study described here was to systematically evaluate the use of these recombinant antigens in enzyme immunoassays for onchocerciasis.

MATERIALS AND METHODS

Human sera. Sera were collected from people over 10 years of age who reside in villages in a savannah-type area

near New Bussa in northern Nigeria with an endemic focus of onchocerciasis. The diagnosis of onchocerciasis required either a positive skin-snip examination for microfilariae or the presence of characteristic subcutaneous nodules or onchocercal dermatitis on clinical examination, as described previously (29). Subjects were questioned regarding visual symptoms, but formal ophthalmological examinations were not performed.

Control sera were obtained from residents of Ibadan, Nigeria, which is outside of the onchocerciasis transmission zone (nonendemic area) and from residents of St. Louis, Mo., who have never been exposed to the parasite. Other control sera tested were from patients infected with *Wuchereria bancrofti*, *Brugia malayi*, *Loa loa*, *Ascaris lumbricoides*, *Dracunculus medinensis*, and *Schistosoma mansoni*. These sera were collected in areas that are nonendemic for onchocerciasis in India, Egypt, and Nigeria.

Chimpanzee sera. Five chimpanzees (*Pan troglodytes*) were used in the present study, and all came from Yerkes Primate Center Colony. All five animals were males, and they ranged in age from 6 to 22 years. They were colony-born chimpanzees, and all were free of preexisting filarial infections, as monitored by two preinoculation blood and skin-snip examinations. Third-stage larvae (L₃) were harvested from blackflies (*Simulium yahense*) previously infected in Liberia and shipped via air to the Centers for Disease Control and Prevention laboratories in Atlanta. Two animals were inoculated with 200 L₃ each, two were inoculated with 300 L₃ each, and one animal received 400 L₃. Serum samples were collected at preinoculation and monthly after infection. Skin snips were examined monthly beginning 8 months after inoculation, as described previously (5).

Expression of OC 3.6 and OC 9.3 in pGEX-2A. OC 3.6 and OC 9.3 are recombinant λ gt11 clones with *O. volvulus* cDNA inserts which have been described in detail elsewhere (4).

* Corresponding author.

These cDNA inserts were subcloned into the plasmid expression vector pGEX-2A, which was kindly supplied by Richard Lucius. This plasmid has been constructed to produce fusion proteins with glutathione *S*-transferase (GST) of *Schistosoma japonicum* at the carboxy terminus to facilitate purification (23). Competent *Escherichia coli* JM101 cells were transformed with the ligation mixtures. Primary screening of transformants for expression of the GST-fusion proteins was performed with 2-ml cultures induced with isopropyl- β -D-thiogalactopyranoside (IPTG; Boehringer Mannheim, Indianapolis, Ind.). Bacteria were concentrated by centrifugation and were resuspended in sample buffer for separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10); this was followed by staining with 0.05% Coomassie blue. Transformants expressing the OC 3.6 and OC 9.3 fusion proteins were confirmed by immunoblotting with a pool of serum from patients with onchocerciasis (28).

Recombinant antigens were purified by affinity chromatography with glutathione-agarose (Sigma Chemical Co., St. Louis, Mo.), as described by Smith and Johnson (22). Briefly, a 10-ml overnight culture was inoculated into 1 liter of NZCYM medium (GIBCO Bethesda Research Laboratories, Gaithersburg, Md.) containing 50 μ g of ampicillin per ml (Sigma). Cultures were grown at 37°C with shaking to an optical density at 600 nm (OD₆₀₀) of 1.0. IPTG (final concentration, 0.3 mM) was then added, and the culture was grown for an additional 5 h, after which the cells were pelleted and resuspended in 0.01 M phosphate-buffered saline (PBS; pH 7.4) with 1% Triton-X 100 (TX100; Sigma) (PBS-TX100). The cells were frozen at -20°C overnight. Cells were thawed in cold water and lysed by mild sonication. Cellular debris was removed by centrifugation at 10,000 $\times g$ for 15 min.

Glutathione-agarose beads were swollen in PBS and packed into a small column. The column was washed with 5 bed volumes of PBS to remove the preservative. The gel was then equilibrated with 5 bed volumes of PBS-TX100 buffer. The lysate was passed through a 0.45- μ m-pore-size filter before it was applied to the column. The sample was applied to the column, and the eluent was discarded. The bound fusion protein was eluted with 5 bed volumes of elution buffer (5 mM glutathione [Sigma] in 50 mM Tris-HCl [pH 8.0]); this was followed by dialysis against PBS. The dialyzed protein was concentrated by using a membrane concentrator (Centricon-10; Amicon, Beverly, Mass.), and the protein concentration was measured with a commercial kit (BCA; Pierce Chemical Co., Rockford, Ill.).

Enzyme immunoassay for detection of total IgG and IgG4 antibodies to recombinant antigens. Preliminary studies were carried out to determine the optimal concentration of recombinant antigens to use for the immunoglobulin G (IgG) and IgG4 antibody assays. Briefly, the recombinant antigens OC 3.6 and OC 9.3 and the control antigen GST (100 μ l per well; 0.5, 1.0, or 2.0 μ g/ml in 0.06 M carbonate buffer [pH 9.6]) were incubated in polyvinyl microtiter plates (Dynatech Laboratories, Alexandria, Va.) overnight at 37°C. Plates were washed three times with PBS containing 0.05% Tween 20 (Sigma) (PBS-T) and were blocked with PBS-T containing 5% fetal calf serum (FCS) (PBS-T-FCS) for 1 h at 37°C. Sera (positive and negative pools) diluted 1:25 in PBS-T-FCS were added to the wells, twofold dilutions were made in PBS-T-FCS, and the dilutions were incubated for 2 h at 37°C. Plates were washed three times with PBS-T. IgG antibody binding was detected with horseradish peroxidase-conjugated goat anti-human IgG antibody (Organon Teknica-Cappel, Malvern, Pa.) in PBS-T-FCS. After 1 h of

FIG. 1. SDS-PAGE and immunoblot analysis of expression and purification of OC 3.6-GST (A) and OC 9.3-GST (B). A 10% slab gel was loaded with *E. coli* extract after IPTG induction (lanes 1) and with purified fusion proteins eluted from glutathione-agarose beads (lanes 2). The immunoblots were developed with a pool of serum from humans with onchocerciasis (1:500) and then alkaline phosphatase-conjugated goat anti-human IgG and substrate.

incubation at 37°C, the plates were washed and substrate (*o*-phenylenediamine [Eastman Kodak, Rochester, N.Y.] with H₂O₂) was added. After 10 min at room temperature, the enzyme reaction was stopped with 4 M H₂SO₄. The OD₄₉₀ versus a PBS blank was read with an MR 300 enzyme-linked immunosorbent assay (ELISA) reader (Dynatech Laboratories). Sera were tested separately for antibody reactivity to GST and to recombinant antigens to give a net OD. Because similar results were obtained when 0.5, 1.0, or 2.0 μ g of antigen per ml was used to coat the plates, 0.5 μ g/ml was chosen for later studies to conserve antigen.

All sera were tested at a dilution of 1:100 for the IgG assay. Sera with a net OD of greater than the mean plus 3 standard deviations obtained with a panel of 20 serum specimens from adult residents of St. Louis, Mo., were considered positive. If duplicate wells produced disparate results, the assay was repeated. As quality controls, positive and negative control sera were tested on each plate.

IgG4 antibodies to recombinant *O. volvulus* antigens were detected as described above, except that the serum dilution tested was 1:50 and IgG4 antibody binding was detected with horseradish peroxidase-conjugated monoclonal antibody HP 6023, which binds to the Fc portion of human IgG4 (21).

RESULTS

Expression of OC 3.6 and OC 9.3 in pGEX-2A. The expression and purification of OC 3.6 and OC 9.3 as GST-fusion proteins are shown in Fig. 1. Fusion protein bands with apparent molecular masses of 50 kDa for OC 3.6 (Fig. 1A) and 40 kDa for OC 9.3 (Fig. 1B) were evident in bacterial lysates by SDS-PAGE and Western blotting (lane 1). The fusion partner, GST, has a molecular mass of 26 kDa (23). Fusion proteins were purified from cell lysates by affinity chromatography as described above (lane 2). Typical yields

FIG. 2. Comparison of IgG and IgG4 enzyme immunoassays for antibodies to recombinant antigens OC 3.6 (A) and OC 9.3 (B). OD cutoff values (mean plus three standard deviations) obtained with a panel of sera from individuals in areas nonendemic for onchocerciasis are indicated by the broken lines.

from 1-liter cultures were 0.4 to 0.6 mg for OC 3.6-GST and 2 to 3 mg for OC 9.3-GST.

Relative sensitivities of IgG and IgG4 ELISAs for antibodies to recombinant *O. volvulus* antigens. In preliminary studies, 23 serum specimens from patients with onchocerciasis were tested for their IgG and IgG4 reactivities to OC 3.6 and OC 9.3 by enzyme immunoassay. Although 21 of 23 serum specimens contained antibodies to OC 3.6, the ODs in the IgG4 assay were much higher (Fig. 2A). The difference between the IgG and IgG4 assays was even more dramatic for OC 9.3 (Fig. 2B). In general, human sera had little or no background reactivity with GST in the IgG4 assay, but IgG reactivity with GST was significant and highly variable. Thus, the best signal-to-noise ratios were obtained by measuring the levels of IgG4 antibodies to the fusion proteins. Forty of 42 (95%) serum specimens from patients with onchocerciasis tested in the present study were reactive with OC 3.6; reactivity with OC 9.3 was 81% (Table 1). Assays for total IgG antibodies to these antigens were less sensitive than the assays for IgG4 antibodies to the antigens (Table 1).

Reactivities of control sera with recombinant antigens. The reactivities of various types of control sera with both recombinant antigens are shown in Table 1. Sera from individuals in areas of Nigeria ($n = 24$) and the United States ($n = 20$) nonendemic for onchocerciasis were uniformly nonreactive with both antigens. Sera from patients with bancroftian filariasis, brugian filariasis, loiasis, ascariasis, schistosomiasis, and dracunculiasis were also nonreactive with these antigens.

Reactivities of chimpanzee sera with recombinant antigens.

TABLE 1. Comparative sensitivities and specificities of IgG and IgG4 ELISAs for antibodies to recombinant antigens

Serum source	No. of serum specimens positive/no. tested			
	OC 3.6		OC 9.3	
	IgG	IgG4	IgG	IgG4
Onchocerciasis	27/32	40/42	23/33	34/42
Nonendemic, normal (Nigeria)	0/24	0/24	0/24	0/24
Nonendemic, normal (United States)	0/20	0/20	0/20	0/20
Brugian filariasis	0/10	0/10	0/10	0/10
Bancroftian filariasis	0/10	0/10	0/10	0/10
Loiasis	0/3	0/3	0/3	0/3
Ascariasis	0/5	0/10	0/5	0/10
Schistosomiasis	0/6	0/6	0/6	0/6
Dracunculiasis (pool)	0/1	0/1	0/1	0/1

Skin snips from all five chimpanzees became positive for microfilariae between 11 and 25 months postinoculation. Results of serology studies performed with chimpanzee sera are shown in Fig. 3. Only the IgG results are shown in Fig.

FIG. 3. IgG antibody reactivities to recombinant *O. volvulus* antigens OC 3.6 (closed circles) and OC 9.3 (open circles) in sera from experimentally infected chimpanzees. Arrows indicate when microfilaremia was first detected.

3, because the IgG4 conjugate did not work well with chimpanzee sera. Antibodies to OC 3.6 were first detected 4 to 6 months after infection, during the prepatent period, and they persisted throughout the period of observation. In contrast, antibodies to OC 9.3 were first detected much later, around the time of onset of microfilarial patency.

DISCUSSION

Prior efforts to develop serodiagnostic assays for onchocerciasis have been hampered by the high degree of cross-reactivity observed between *O. volvulus* and other common parasitic nematodes (1). Numerous attempts have been made to improve the specificity of onchocerciasis antibody assays by measuring isotype-specific antibodies to crude worm extracts or by using partially purified antigens that are enriched for relatively *Onchocerca*-specific low-molecular-mass antigens (3, 25, 31). However, none of these assays has been shown to be sensitive and specific for *O. volvulus* infection. Recently, the field has turned to recombinant DNA technology in hopes of producing sensitive and specific assays that are not dependent on scarce parasite materials harvested from patients (2, 4, 12–15). In the present study, we evaluated the diagnostic utility of two recombinant antigens, OC 3.6 and OC 9.3, which were expressed as GST-fusion proteins.

Preliminary studies were performed with sera from patients with onchocerciasis to optimize the assays. Better signal-to-noise ratios were obtained by measuring IgG4 antibodies to the recombinant fusion proteins. There was almost no background IgG4 reactivity with GST, and IgG4 reactivity to the recombinant antigens was much stronger than IgG reactivity. Several groups have shown that measurement of IgG4 antibodies to filarial antigens improves diagnostic specificity (9, 11, 14, 17, 29). IgG4 is a minor IgG subclass that comprises only 5 to 7% of total IgG in U.S. adults (8). Increased levels of IgG4 are produced in response to certain types of chronic antigenic stimuli (e.g., infections with tissue-dwelling helminth parasites), and prominent IgG4 antibody responses have been reported in humans with filariasis (18).

The IgG4 enzyme immunoassays with OC 3.6 and OC 9.3 were evaluated with sera from patients with onchocerciasis and various types of control sera. The sensitivities of these assays (95% for OC 3.6; 81% for OC 9.3) were significantly better than those obtained in prior studies (4, 16), which used different forms of these antigens (different fusion partners) in dot-immunoblot and Western blot assays. Collaborative studies are in progress to evaluate these assays with sera from individuals in other areas of Africa and Latin America endemic for onchocerciasis. Preliminary results are consistent with those obtained in the present study with sera from individuals in Nigeria (15a). Additional studies are also needed to determine whether there is any correlation between total or isotype-specific antibody responses and the age and/or clinical status of patients. The chimpanzee antibody data are interesting in this regard. They suggest that OC 3.6 might be useful for detecting early (prepatent) and mature infections, and that only mature infections would be detected with OC 9.3.

Antibody assays with OC 3.6 and OC 9.3 were entirely specific for *O. volvulus*. Recent studies of a recombinant antigen that is related to OC 3.6 (Ov33) found significant nonspecific IgM and total IgG antibody reactivities to that antigen, while IgG4 was much more specific (14). It is possible that the epitopes responsible for false-positive an-

tibody reactivity to Ov33 are in the amino-terminal portion of the molecule that is missing from the cDNA sequence of OC 3.6 (4).

Efforts are under way to cleave the GST-fusion partner to produce nonfusion recombinant OC 3.6 and OC 9.3 proteins in hopes of further improving these assays. Several other research groups have described recombinant *Onchocerca* antigens with diagnostic potential (2, 4, 6, 12–15), and cooperative studies sponsored by the Filariasis Steering Committee of the Special Program for Research and Training on Tropical Diseases of the World Health Organization are in progress to determine the relative diagnostic values of these antigens (20). This cooperative approach should accelerate the development of a sensitive, specific, and practical antibody diagnostic test that will be useful for the diagnosis of onchocerciasis in countries endemic for the disease.

ACKNOWLEDGMENTS

This work was supported in part by grant AI-22488 from the National Institutes of Health and by the Edna McConnell Clark Foundation. A. F. Ogunrinade is a recipient of a Biotechnology Research Fellowship from the Rockefeller Foundation.

We thank Fanya Liftis for excellent technical assistance.

REFERENCES

1. Ambrose-Thomas, P. 1980. Filariasis, p. 84–103. In V. Houba (ed.), *Immunological investigations of tropical parasitic diseases*. Churchill-Livingstone, Ltd., Edinburgh.
2. Bradley, J. E., R. Helm, M. Lahaise, and R. M. Maizels. 1991. cDNA clones of *Onchocerca volvulus* low molecular weight antigens provide immunologically specific diagnostic probes. *Mol. Biochem. Parasitol.* 46:219–228.
3. Cabrera, Z., and R. M. E. Parkhouse. 1987. Isolation of an antigenic fraction for diagnosis of onchocerciasis. *Parasite Immunol.* 40:261–267.
4. Chandrashekar, R., K. Masood, R. M. Alvarez, A. F. Ogunrinade, R. Lujan, F. O. Richards, Jr., and G. J. Weil. 1991. Molecular cloning and characterization of recombinant parasite antigens for immunodiagnosis of onchocerciasis. *J. Clin. Invest.* 88:1460–1466.
5. Eberhard, M. L., J. W. Dickerson, A. E. Boyer, V. C. W. Tsang, R. Zea-Flores, E. M. Walker, F. O. Richards, G. Zea-Flores, and E. Strobert. 1991. Experimental *Onchocerca volvulus* infections in mangabey monkeys (*Cercocebus atys*) compared to infections in humans and chimpanzees (*Pan troglodytes*). *Am. J. Trop. Med. Hyg.* 44:151–160.
6. Garate, T., F. J. Conrath, W. Harnett, D. W. Buttner, and R. M. E. Parkhouse. 1990. Cloning of specific diagnostic antigens of *Onchocerca volvulus*. *Trop. Med. Parasitol.* 41:245–250.
7. Greene, B. M. 1992. Modern medicine versus an ancient scourge: progress toward control of onchocerciasis. *J. Infect. Dis.* 166:15–21.
8. Hamilton, R. G. 1987. Human IgG subclass measurements in the clinical laboratory. *Clin. Chem.* 33:1701–1725.
9. Kwan-Lim, G. E., K. P. Forsyth, and R. M. Maizels. 1990. Filarial-specific IgG4 responses correlates with active *Wuchereria bancrofti* infection. *J. Immunol.* 145:4298–4305.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680–685.
11. Lal, R. B., and E. A. Ottesen. 1988. Enhanced diagnostic specificity in human filariasis by IgG₄ antibody assessment. *J. Infect. Dis.* 158:1034–1037.
12. Lobos, E., M. Altmann, G. Mengod, N. Weiss, R. Werner, and M. Karam. 1990. Identification of an *Onchocerca volvulus* cDNA encoding a low molecular weight antigen uniquely recognized by onchocerciasis patient sera. *Mol. Biochem. Parasitol.* 39:1603–1605.
13. Lobos, E., N. Weiss, M. Karam, H. R. Taylor, E. A. Ottesen, and

- T. B. Nutman. 1991. An immunogenic *Onchocerca volvulus* antigen: a specific and early marker of infection. *Science* 251:1603-1605.
14. Lucius, R., A. Kern, F. Seeber, T. Pogonka, J. Willenbacher, H. R. Taylor, M. Pinder, H. W. Ghalib, H. Shultz-Key, and P. Soboslay. 1992. Specific and sensitive IgG₄ immunodiagnosis of onchocerciasis with a recombinant 33 kDa *Onchocerca volvulus* protein (Ov33). *Trop. Med. Parasitol.* 43:139-145.
 15. Lustigman, S., B. Brotman, T. Huima, and A. M. Prince. 1991. Characterization of an *Onchocerca volvulus* cDNA clone encoding a genus-specific antigen present in infective larvae and adult worms. *Mol. Biochem. Parasitol.* 45:65-76.
 - 15a. Ogunrinade, A. F., R. Chandrashekar, and G. J. Weil. Unpublished data.
 16. Ogunrinade, A. F., R. Chandrashekar, G. J. Weil, and O. O. Kale. 1992. Use of a recombinant antigen (OC 3.6 cDNA) for the serological diagnosis of onchocerciasis in exposed Nigerian children. *J. Trop. Pediatr. (Oxford)* 38:103-105.
 17. Ogunrinade, A. F., O. O. Kale, R. Chandrashekar, and G. J. Weil. 1992. Field evaluation of IgG₄ serology for the diagnosis of onchocerciasis in children. *Trop. Med. Parasitol.* 43:59-61.
 18. Ottesen, E. A., F. Skaravil, S. P. Tripathy, R. W. Poindexter, and R. Hussain. 1985. Prominence of IgG₄ in the IgG antibody response to human filariasis. *J. Immunol.* 134:2707-2712.
 19. Prost, A. 1980. Latence parasitaire dans l'onchocercose. *Bull. W.H.O.* 58:923-925.
 20. Ramachandran, C. P. 1993. Improved immunodiagnostic tests to monitor onchocerciasis control programmes—a multicenter effort. *Parasitol. Today* 9:76-79.
 21. Reimer, C. B., D. J. Phillips, C. H. Aloisio, D. D. Moore, C. G. Galland, T. W. Wells, C. M. Black, and J. S. McDougal. 1984. Evaluation of thirty one mouse monoclonal antibodies to human IgG epitopes. *Hybridoma* 3:263-275.
 22. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of poly-peptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31-40.
 23. Smith, D. B., M. R. Rubira, R. J. Simpson, K. M. Davern, W. U. Tiu, P. G. Board, and G. F. Mitchell. 1988. Expression of an enzymatically active parasite molecule in *Escherichia coli*: *Schistosoma japonicum* glutathione S-transferase. *Mol. Biochem. Parasitol.* 27:249-256.
 24. Southgate, B. A. 1984. Recent advances in the epidemiology and control of filarial infections including entomological aspects of transmission. *Trans. R. Soc. Trop. Med. Hyg.* 78(Suppl.):19-28.
 25. Tada, I., M. Korenaga, K. Shiwaku, E. O. Ogunba, G. O. Ufomadu, and E. W. B. Nwoke. 1987. Specific serodiagnosis with adult *Onchocerca volvulus* antigen in ELISA. *Am. J. Trop. Med. Hyg.* 36:383-392.
 26. Taylor, H. R. 1985. Report of a workshop: research priorities for immunologic aspects of onchocerciasis. *J. Infect. Dis.* 152:389-392.
 27. Taylor, H. R., E. Keyvan-Larijani, H. S. Newland, A. T. White, and B. M. Greene. 1987. Sensitivity of skin snips in the diagnosis of onchocerciasis. *Trop. Med. Parasitol.* 38:145-147.
 28. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
 29. Weil, G. J., A. F. Ogunrinade, R. Chandrashekar, and O. O. Kale. 1990. IgG₄ subclass antibody serology for onchocerciasis. *J. Infect. Dis.* 161:549-554.
 30. Weiss, N., and M. Karam. 1987. Humoral immune responses in human onchocerciasis: detection of serum antibodies in early infections. *CIBA Found. Symp.* 127:180-188.
 31. Weiss, N., and M. Karam. 1989. Evaluation of a specific enzyme immunoassay for onchocerciasis using a low molecular weight antigen fraction of *Onchocerca volvulus*. *Am. J. Trop. Med. Hyg.* 40:261-267.
 32. World Health Organization Expert Committee on Epidemiology of Onchocerciasis. 1976. Epidemiology of onchocerciasis. Report of a WHO expert committee. WHO Tech. Rep. Ser. 597:1-193.