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Parasitology (1986), **93**, 389–399
With 4 figures in the text

Identification of non-cross-reacting antigens of *Onchocerca volvulus* with lymphatic filariasis serum pools

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(*Accepted* 21 *April* 1986)

SUMMARY

Onchocerca volvulus proteins labelled with ^{125}I were immunoprecipitated with onchocerciasis and lymphatic filariasis human serum pools in order to differentiate between cross-reacting and non-cross-reacting antigens. Analysis of the immunoprecipitates by two-dimensional gel electrophoresis revealed that all high molecular weight (M_r) *O. volvulus* antigens cross-reacted with the lymphatic filariasis serum pools. We observed, however, that at least 8 *O. volvulus* antigens were specifically immunoprecipitated only by the onchocerciasis serum pools, with M_r ranging from 20000 up to 43000 Daltons. These results suggest that the lower M_r *O. volvulus* antigens are more species specific than the other antigens. The significance of these findings for the immunodiagnosis of onchocerciasis is discussed.

INTRODUCTION

Onchocerca volvulus, a filarial nematode, is the causal organism of human onchocerciasis. This disease affects between 20 and 40 million people and is an important health problem causing high rates of blindness and skin pathology in the world today (World Health Organization, 1976).

Attempts to develop an immunodiagnosis for onchocerciasis have so far met with many problems. One is the lack of an experimental model for studying the humoral and cellular mechanisms operating during infection and their roles in the immunopathology of the disease. Another is the small amount of available filarial parasite, which can be obtained only from onchocerciasis patients undergoing nodulectomy (Nelson, 1981). The main drawback, however, is still the extensive cross-reactivity of antibodies induced by diverse species of filariae and even intestinal nematodes (Fujita, Tanaka, Sasa, Schichinone, Asai & Kurokawa, 1970; Tanaka, Fujita, Sasa, Tagawa, Naito & Kurokawa, 1970; Ambroise-Thomas, 1974).

The application of new serological methods to the study of filariasis resulted in diagnostic tests with high sensitivities, but no test developed so far has an acceptable level of specificity for a given species (Kagan & Norman, 1974; Voller & de Savigny, 1981).

Extensive antigenic homologies have been demonstrated between antigens derived from various *Onchocerca* spp. and lymphatic filariae like *Wuchereria bancrofti* and *Brugia malayi* (Forsyth, Copeman, Anders & Mitchell, 1981; Weiss, Gualzata, Wyss, Betschart, 1982; Ouassi, des Moutis, Cornette, Pierce & Capron, 1983; Phillip, Gomez-Priego, Parkhouse, Davies, Clark, Ogilvie & Beltrán-Hernández, 1984; Kaushal, Hussain & Ottessen, 1984).

Especially in West and Central Africa onchocerciasis and Bancroftian filariasis are co-endemic. The development of a reliable species-specific immunodiagnostic test for

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human onchocerciasis, in particular, the detection of pre-patent infection, has been recognized as a high priority (World Health Organization, 1984).

In the present study the extent of cross-reactivity and non-cross-reactivity of the *O. volvulus* antigens with lymphatic filariasis serum pools from India and Sri Lanka have been qualitatively analysed by two-dimensional gel electrophoresis of immunoprecipitated *O. volvulus* antigens. This represents a first step to define and isolate functionally important *O. volvulus* antigens for immunodiagnosis.

MATERIALS AND METHODS

Sera

All the individual sera were selected after demonstration of intensive cross-reaction with heterologous filarial antigen (*Dipetalonema viteae* in ELISA (Speiser, 1980)). For the 4 serum pools used, individual sera gave O.D. readings over 1·0. The onchocerciasis serum pool from Mali (O.V.–M) was prepared from 8 patients with parasitologically proven *O. volvulus* infections, living in a hyperendemic onchocerciasis area of Mali. At the time of bleeding no intestinal helminths could be detected. The onchocerciasis serum pool from Tanzania (O.V.–T) was prepared from sera of 10 adult patients also with parasitologically proven onchocerciasis. We have no information about concomitant helminth infections. The serum pool from India (F–I) was prepared from 7 adult males living in an endemic region for *Wuchereria bancrofti* in N.E. India, 5 patients had clinical signs of lymphatic filariasis (2 hydrocele, 3 lymphoedema) and 2 showed high blood eosinophilia (41 and 54%) at the time of bleeding. Blood examinations revealed no circulating microfilariae, stools were not examined. The serum pool from Sri Lanka immigrants consisted of 8 sera from adult males. Stool examinations revealed eggs of *Trichuris trichiura* (2), hook worm (3) and mixed infections (3). In all individuals no clinical signs of filariasis and no circulating microfilariae (in 1 ml of night blood) could be detected.

Antigens

O. volvulus worms, recovered from nodules excised at the Acha Hospital in Acha Tugi and at the Ad Lucem Hospital in Bafang (United Republic of Cameroon) were isolated according to the method of Schulz-Key, Albiez & Buettner (1977). The worms were carefully washed from host tissues, weighed out and frozen. A soluble antigen extract was prepared as described previously (Lobos & Weiss, 1985). Briefly, the worms were homogenized with 7 mM phosphate-buffered saline (PBS), pH 7·2 (18 mM NaCl, 1 mM PMSF). The homogenate was stirred overnight (o/n) at 4 °C, and then centrifuged at 800 ***g*** for 10 min, and the pellet was again homogenized in PBS containing 1 mM PMSF and extracted (o/n) at 4 °C. The fractions were pooled and centrifuged at 112000 ***g*** for 2 h at 4 °C. The supernatant fractions were collected and dialysed against PBS (o/n) at 4 °C, concentrated using XM10 membrane (Amicon), and the protein contents determined (Lowry, Rosebrough, Farr & Randall, 1951) and adjusted to a final concentration of 5·1 mg/ml. Antigens were stored in small aliquots at −70 °C.

Radio-iodination of antigens

O. volvulus antigens extracted with PBS buffer were radio-iodinated using the chloramine-T method (Greenwood, Hunter & Glover, 1963). Briefly, 125 μg of *O. volvulus* proteins in 25 μl of 0·5 M phosphate buffer, pH 7·5, were incubated with

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0·5 mCi of carrier-free $Na^{125}I$ (specific activity 13·5 Ci/mg, EIR, Switzerland) and 5 μl of chloramine-T (10 mg/ml in 0·1 M phosphate buffer) for 2 min. The reaction was stopped by the addition of 20 μl of sodium metabisulphite (10 mg/ml) and then diluted with 200 μl of TBS buffer, pH 7·5, containing 5 mM potassium iodide and 1 mg/ml BSA. Free iodine was separated from iodinated proteins using a Sephadex G-10 column pre-equilibrated with TBS buffer, pH 7·5, containing 1 mg/ml BSA. The first radioactive peak eluting after the void volume was collected and the bound radioactivity was determined by trichloroacetic acid (TCA) precipitation. TCA precipitable counts were always in the range 80–90%. The specific activity of the antigens was $7{\cdot}8 \times 10^5$ c.p.m./μg.

SDS–PAGE

PBS-extracted *O. volvulus* proteins, radio-isotope labelled proteins or immunoprecipitates were analysed according to Laemmli (1970) in 12·5% polyacrylamide gels. Samples were run under reducing conditions. The following M_r markers were run simultaneously: myosin (200 K), β-galactosidase (116 K), phosphorylase b (94 K), bovine serum albumin (67 K), ovalbumin (43 K), chymotrypsin (25 K). The slab gels were stained in Coomassie blue R-250 (Sigma). The gels were exposed to Kodak X-Omat film at −70°C.

Immunoprecipitation

Serum samples (2 μl) were incubated with 5 μl of radio-isotope labelled antigen (1·4 μg protein) in a total volume of 100 μl of TBS, pH 7·4, containing 0·5% Nonidet P-40. After incubating the suspension for 1 h at 0°C, 10 μl of rabbit anti-human IgG antiserum (Dakopatts, Glostrup, Denmark) was added and the suspension incubated overnight on ice. The amounts of human sera and anti-human IgG antiserum required to yield optimal immunoprecipitation of human IgG were previously determined.

The immunoprecipitates were washed with 1·1 ml of buffer containing 20 mM Tris HCl, pH 7·6, 150 mM NaCl, 1 mM EDTA, 2·5 M KCl and 0·5% Nonidet P-40 and pelleted by centrifugation at 10000 $\times g$ for 2 min. The washing was repeated twice. The pellets were then washed with 1·1 ml of a buffer containing 20 mM Tris HCl, pH 7·6, 150 mM NaCl, 1 mM EDTA, 0·5% Nonidet P-40, 0·5% sodium deoxycholate and 0·1% sodium dodecyl sulphate. Following centrifugation the immunoprecipitates were resuspended in sample buffer containing 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 0·01% (w/v) bromophenol blue, 10% (v/v) glycerol and 62·5 mM Tris HCl, pH 6·8, heated at 100°C for 3 min and analysed by SDS–PAGE. Immunoprecipitates analysed by two-dimensional gel electrophoresis were resuspended in lysis buffer containing 9·5 M urea, 2% (w/v) Nonidet P-40, 2% (v/v) ampholines, pH 9–11, and 2-mercaptoethanol for isoelectric focusing (IEF). Samples were incubated at 37°C for 10 min before loading.

Two-dimensional gel electrophoresis

Two-dimensional gels were run essentially as described by O'Farrell (1975) with the modifications described by Forchhammer (1979). The first dimension consisted of isolectric focusing (IEF) followed by SDS–PAGE in the second dimension.

IEF was performed in 14 × 0·35 cm tube gels containing 9·2 M urea (Serva, Heidelberg, West Germany), 2% Nonidet P-40, and 2% ampholine mixture (LKB, Bromma, Sweden, 0·18% pH 2·5–5, 0·54% pH 3·5–10, 1·09% pH 5–8, 0·18% pH 8–9·5). The gels were pre-run at 80 V for 15 min, followed by a stepwise increase in the voltage of 15 V

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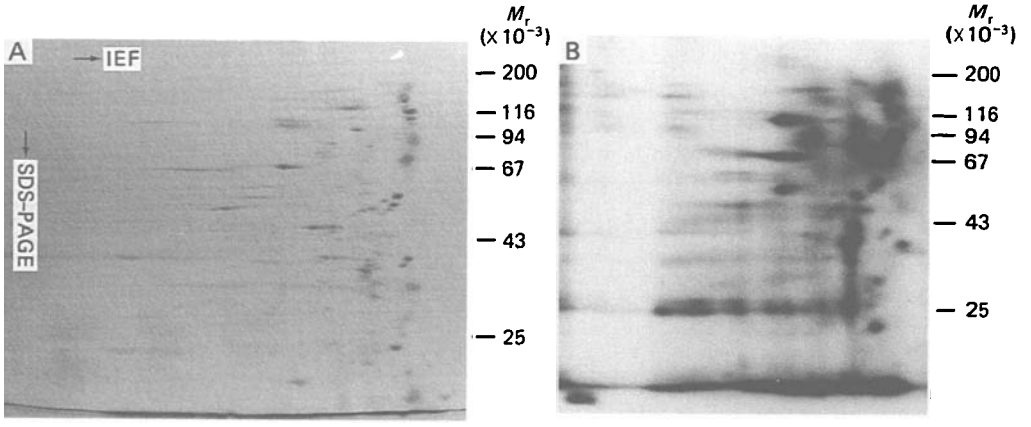

Fig. 1. (A) Coomassie blue-stained two-dimensional gel of *Onchocerca volvulus* proteins (356 μg). The extract was treated with 50 μg/ml DNase I and 50 μg/ml pancreatic RNase, lyophilized and made up to 9·5 M urea, 2% Nonidet P-40, 2% ampholines (pH 9–11), 5% 2-mercaptoethanol and analysed by IEF in the first dimension and SDS–PAGE in the second dimension. Acidic proteins are on the right. (B) Autoradiogram of ^{125}I-labelled *O. volvulus* proteins (5×10^5 c.p.m.) analysed by two-dimensional gels. The indicated molecular weights correspond to the positions of the following marker proteins: myosin heavy chain (200 K), beta-galactosidase (116 K), phosphorylase a (94 K), bovine serum albumin (67 K), ovalbumin (43 K) and chymotrypsin (25 K).

every 15 min up to 200 V. Samples were then loaded and run at a constant voltage of 300 V for 16 h, followed by 600 V for 1 h. Immediately after focusing, the pH gradient was measured after shaking 5 mm sections of the gel for 1 h in tightly capped vials containing 2 ml of 20 mM KCl made up in degassed distilled water. After equilibration in sodium dodecyl sulphate sample buffer for 30 min the IEF gels were loaded onto 12·5% acrylamide Laemmli discontinuous slab gels as described for SDS–PAGE. After staining and destaining (see above) the dried gels were exposed to Kodak X-Omat film in combination with intensifier screens (Kyokko, Japan) at −70°C.

RESULTS

O. volvulus proteins extracted with PBS were analysed using two-dimensional gel electrophoresis. The proteins were first resolved by isoelectric focusing in a pH gradient from 4·2 to 7·6 and separated by SDS–PAGE under reducing conditions. In general, the resolution was better when larger gels were used in the SDS–PAGE step as recommended by Johnson (1982). The Coomassie blue-stained polypeptides of *O. volvulus* are shown in Fig. 1. Up to 180 distinct polypeptides could be identified on the two-dimensional gels. The procedure resulted in resolution of many polypeptides that comigrate as single bands or doublets in SDS–PAGE. The majority of the proteins focused in the pH range 4·3–7·2. *O. volvulus* proteins labelled with ^{125}I are also shown in Fig. 1. Comparison with the Coomassie blue-stained pattern indicated that the majority of the *O. volvulus* polypeptides were labelled and approximately 200 distinct polypeptides could be identified.

O. volvulus antigens were immunoprecipitated with onchocerciasis and lymphatic filariasis serum pools, in order to differentiate between cross-reacting and non-cross-reacting antigens. Two onchocerciasis serum pools consisted of highly reactive

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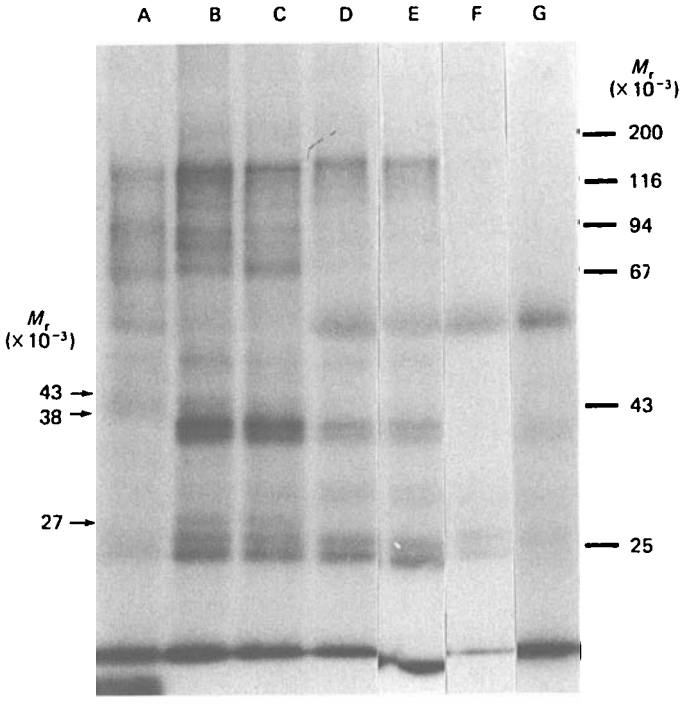

Fig. 2. Autoradiogram of ^{125}I-labelled *Onchocerca volvulus* proteins immunoprecipitated by onchocerciasis, lymphatic filariasis and normal human serum pools. Radio-isotope labelling, immunoprecipitation and analysis by SDS-PAGE in 12·5% polyacrylamide gels were as described in the Materials and Methods section. (A) ^{125}I-labelled *O. volvulus* proteins (3×10^3 c.p.m.); (B) immunoprecipitation with O.V.–T pool; (C) O.V.–M pool; (D) F–I pool; (E) F–S pool; (F) normal human serum (NHS) pool; (G) immunoprecipitation carried out only with rabbit anti-human IgG antiserum. Arrows at the left show antigens immunoprecipitated only by the onchocerciasis serum pools. Molecular weights corresponded to the positions of marker proteins as listed in Fig. 1.

individual sera from people with parasitologically proven onchocerciasis living in Mali (O.V.–M pool) or in Tanzania (O.V.–T pool). For the demonstration of cross-reactive antigens, we used two serum pools from individuals who had never been exposed to *O. volvulus*. The individual sera were selected after demonstration of intensive cross-reaction with heterologous filarial antigens (*Dipetalonema viteae* in ELISA). The sera chosen for the pool were all derived from amicrofilaraemic patients who showed clinical signs of lymphatic filariasis (F–I pool from India) or who were asymptomatic (F–S pool of immigrants from Sri Lanka).

The negative serum pool used as a control consisted of sera from healthy adult Swiss residents with negative parasitological and serological findings for gastrointestinal and tissue parasites and no clinical symptoms of any parasitic disease.

^{125}I-labelled *O. volvulus* antigens were immunoprecipitated with the serum pools and subjected to SDS–PAGE. The resulting autoradiography is shown in Fig. 2. The O.V.–M and O.V.–T serum pools gave the same immunoprecipitation patterns. Most of the *O. volvulus* antigens recognized by onchocerciasis serum pools were also immunoprecipitated by the F–I and F–S serum pools, with the exception of antigens migrating with M_r of 27 K and 39–43 K which were detected only by the onchocerciasis sera.

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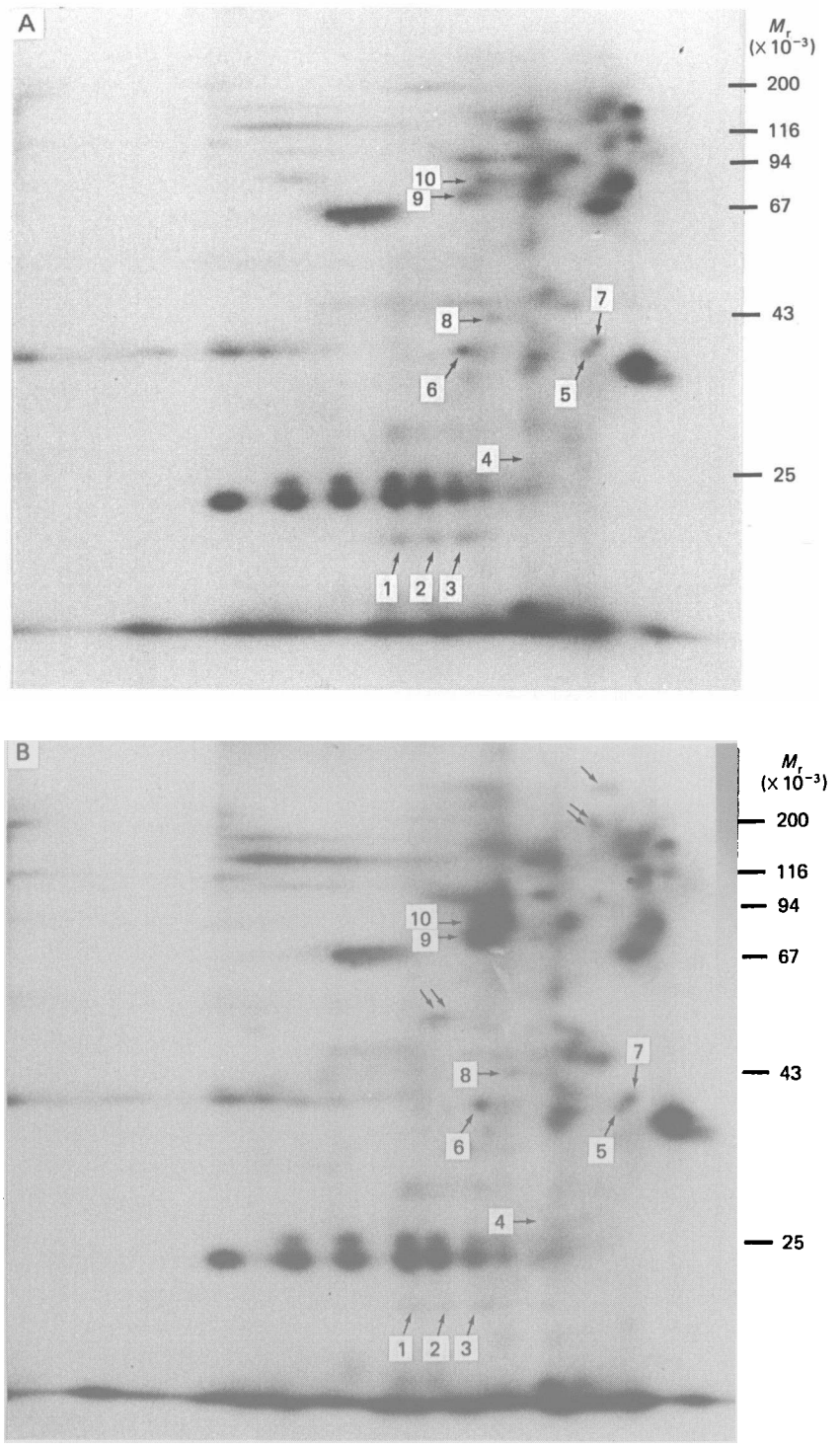

Fig. 3. For legend see opposite.

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Table 1. *The apparent molecular weights (M_r) and isoelectric points (pI) of the* Onchocerca volvulus *antigens recognized only by the onchocerciasis serum pools* (1–8)

Antigen	M_r ($\times 10^{-3}$)	pI
1	20	6·14
2	20	6·00
3	20	5·74
4	27	4·92
5	38	4·70
6	39	5·74
7	40	4·64
8	43	5·60
9	75	5·74
10	78	5·60

After a longer exposure a further antigen with M_r 20 K was seen in the immunoprecipitation patterns of both onchocerciasis sera.

Control experiments confirmed previous reports of the presence of human IgG in the parasite extract (Lobos & Weiss, 1985). Immunoprecipitation of labelled *O. volvulus* antigens by a second antibody (anti-human IgG antiserum) analysed by SDS–PAGE under reducing conditions demonstrated the presence of labelled IgG heavy and light chains in the parasite extract, migrating at 51 K and 25 K respectively as shown in Fig. 2, Lane G. The polypeptides with M_r 23, 36 and 37 K were probably non-specifically immunoprecipitated.

Two-dimensional electrophoresis improved the evaluation of the immunoprecipitation patterns. Up to 130 antigens were precipitated by the onchocerciasis serum pool from Tanzania (O.V.–T) and at least 125 antigens by the onchocerciasis serum pool from Mali (O.V.–M) (Fig. 3B and A respectively). The patterns were remarkably similar. The most obvious differences were in the polypeptides 9 and 10 (for molecular weights and approximate pI, see Table 1), which were strongly immunoprecipitated by the O.V.–T pool but only weakly recognized by the O.V.–M pool. In addition, 5 antigens were detected only by the O.V.–T and not by the O.V.–M pool (arrows in Fig. 3B).

The highly cross-reacting serum pools from Sri Lanka (F–S) and India (F–I) precipitated at least 84 and 85 *O. volvulus* antigens which were also detected by the homologous serum pools (see Fig. 4A and B respectively). Both serum pools showed the same precipitation patterns with the exception of one antigen migrating with M_r approximately 88 K and pI 4·82, which was immunoprecipitated only by the F–I pool (see arrow in Fig. 4B). This antigen is either present in small amounts or it is not stained by Coomassie blue since it could only be detected on silver-stained two-dimensional gels (data not shown).

Comparison between the immunoprecipitation patterns of the onchocerciasis and

Fig. 3. Autoradiograms of two-dimensional gels of ^{125}I-labelled *Onchocerca volvulus* antigens immunoprecipitated by onchocerciasis and lymphatic filariasis serum pools. Samples were prepared as described in the Materials and Methods section. First dimension electrofocusing was for 17 h. The second dimension SDS–PAGE was done in 12·5% polyacrylamide gels. (A) *O. volvulus* antigens immunoprecipitated by the O.V.–M pool. (B) Immunoprecipitation with the O.V.–T pool. The numbered arrows (1–8) in A and B show *O. volvulus* antigens immunoprecipitated only by the onchocerciasis serum pools. Acidic antigens are on the right. Numbers represent molecular weight standards as listed in Fig. 1.

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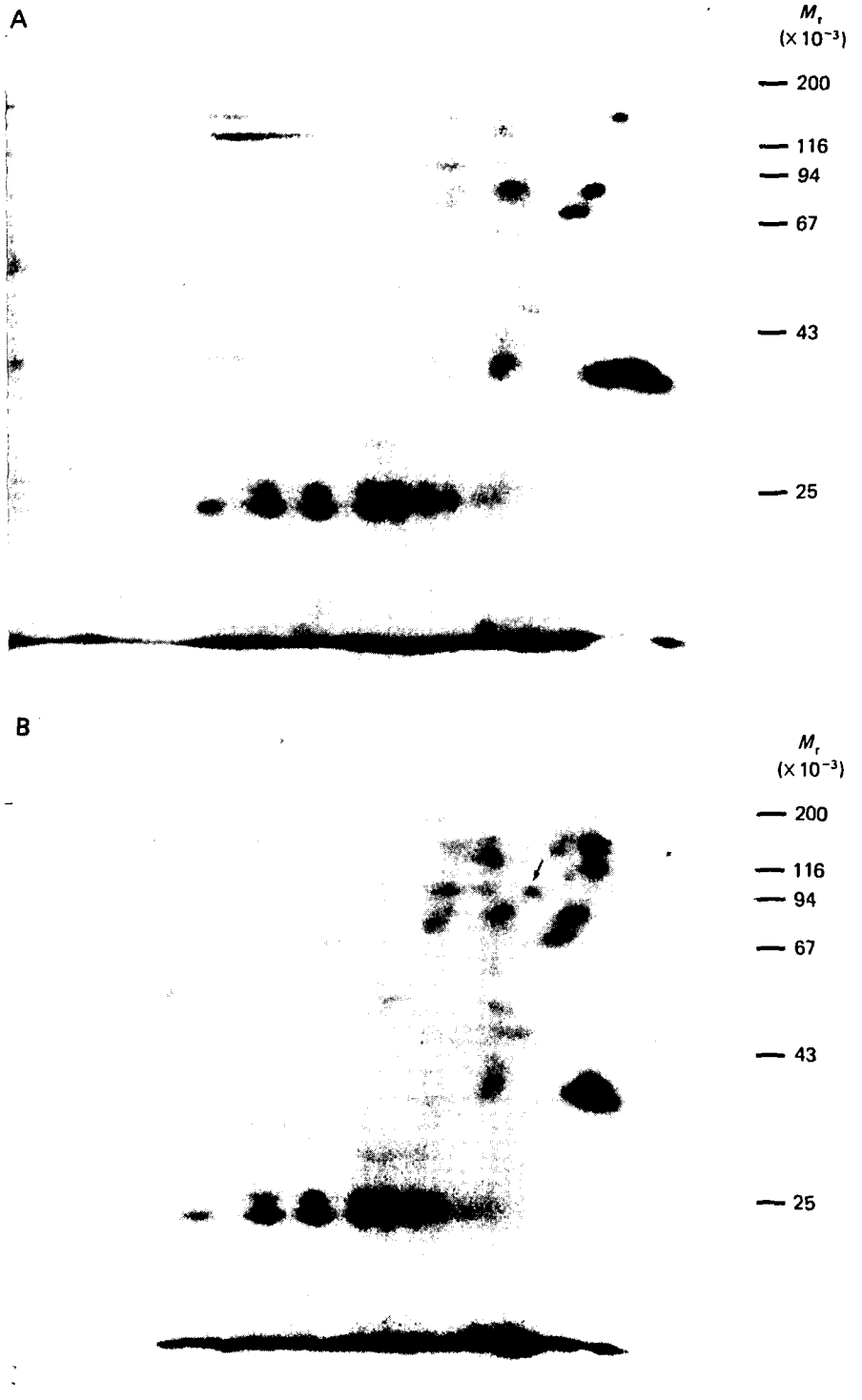

Fig. 4. For legend see opposite.

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lymphatic filariasis serum pools revealed striking differences. There were 8 *O. volvulus* antigens which were only recognized by the onchocerciasis serum pools. The apparent molecular weights and pI are given in Table 1, numbers 1–8. None of these antigens was detected even after extended exposure times in the gels of lymphatic filariasis serum pools.

DISCUSSION

In the present study we have qualitatively analysed the extent of cross-reactivity of *O. volvulus* antigens with lymphatic filariasis serum pools. Immunoprecipitations carried out with homologous onchocerciasis serum pools from two different geographic regions of Africa, Mali, (O.V.–M) and Tanzania (O.V.–T), and subsequently analysed by two-dimensional gels revealed striking similarities. We were able to detect up to 130 antigens with the O.V.–T serum pool and at least 125 antigens with the O.V.–M pool. Five antigens were detected only by the O.V.–T pool and variation in the intensity of two antigens was observed.

The complex pattern of antigens, identified by the anti-*O. volvulus* antibodies appeared similar to our previous results (Lobos & Weiss, 1985). In that study, however, unlabelled antigens were separated on two-dimensional gels, transferred electrophoretically to nitrocellulose membrane and then reacted with patient's serum and with ^{125}I-labelled protein A. The slight differences observed could be explained by differences in the techniques.

The immunoprecipitation data from Fig. 4A demonstrated that serum pools from humans infected with *Wuchereria bancrofti* contain antibodies against 85 radio-iodinated antigens of *O. volvulus*, providing evidence for extensive antigenic homologies between these filarial species. Previous reports have confirmed that the absence of circulating blood microfilariae is correlated to the presence of anti-microfilarial antibodies (Piessens, McGreevy, Ratiwayanto, McGreevy, Piessens, Koiman, Saroso & Dennis, 1980; McGreevy, Ratiwayanto, Sekar, McGreevy, Piessens, Koiman, Saroso & Dennis, 1980). The observed cross-reactivity of the *O. volvulus* antigens with the lymphatic filariasis serum pools may reflect the presence of *O. volvulus* microfilarial antigens and/or microfilarial epitopes present also in adult worms antigens.

The F–S serum pool also used as a control revealed the same cross-reactivity (with the exception of 1 antigen) with the labelled *O. volvulus* antigens (Fig. 4B).

The similarity in the profiles of cross-reacting antibodies in patients with clinical symptoms of bancroftian filariasis and asymptomatic 'endemic normals' was surprising. However, it has been reported previously that the so called 'endemic normals' show a marked immune responsiveness to filarial antigens even though they do not have any clinical or parasitological evidence of infection (Ottesen, Wekker & Heck, 1977; Ottesen, Weller, Lunde & Hussain, 1982). Moreover, individuals from this group were suffering from trichuris, hookworm or mixed infections and exposure to *Dirofilaria immitis* infective larvae cannot completely be excluded. This result suggests

Fig. 4. Autoradiograms of two-dimensional gels of ^{125}I-labelled *Onchocerca volvulus* antigens immunoprecipitated by lymphatic filariasis serum pools. Samples were prepared as described in the Materials and Methods section. First dimension electrofocusing was for 17 h. The second dimension SDS–PAGE was done in 12·5% polyacrylamide gels. (A) *O. volvulus* antigens immunoprecipitated by the lymphatic filariasis serum pool from Sri Lanka (F–S). (B) Immunoprecipitation with the lymphatic filariasis serum pool from India (F–I). Acidic antigens are on the right. Numbers represent molecular standards as listed in Fig. 1.

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that these helminths and *D. immitis* do not stimulate antibodies against the antigens detected only by individuals with onchocerciasis.

The most striking result was the detection and identification (see Table 1, Nos 1–8) of 8 antigens recognized only by onchocerciasis serum pools from East and West Africa but not by the F–S, F–I or NHS pools. These results suggest that they could be antigens bearing species-specific determinants. Such determinants could be further analysed by the production of monoclonal antibodies which could be extremely valuable reagents for use in diagnostic assays such as competitive immunoassays detecting specific host antibodies against *O. volvulus*.

We wish to thank Dr C. Thür (Ad Lucem Hospital, Bafang; United Republic of Cameroon) and Dr E. Herz (Acha Hospital, Acha Tugi; United Republic of Cameroon) for providing onchocerca nodules; Dr Marc Karam from the Onchocerciasis Control Programme for providing sera from Mali; Dr Marcel Tanner from the Swiss Tropical Institute Field Laboratory, Ifakara, Tanzania, for providing sera; Dr Gosh Dastidar from the School of Tropical Medicine Calcutta, India, for providing sera; Dr Benedikt Holzer, Inselspital, Berne, Switzerland, for providing sera from Sri Lanka immigrants; Dr Guadalupe Mengod, Biocenter, Basel, Switzerland, for helpful discussions and Mr Julio Olavarria for the photographic work. This investigation was suported by the Filariasis Component of UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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Printed in Great Britain

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