

Antigenic characterization of adult *Wuchereria bancrofti* filarial nematodes

T. M. MORGAN,¹ INGE SUTANTO,² PURNOMO,² SUKARTONO,²
F. PARTONO² and R. M. MAIZELS^{1*}

¹ Department of Pure and Applied Biology, Imperial College of Science and Technology,
Prince Consort Road, London SW7 2BB, UK

² Department of Parasitology, Faculty of Medicine, University of Jakarta, Salemba
Raya 6, Jakarta, Indonesia

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SUMMARY

Adult *Wuchereria bancrofti* were recovered from infected *Presbytis cristatus* monkeys and radio-isotope labelled extrinsically with ¹²⁵I and *in vitro* with [³⁵S]methionine. ¹²⁵I labelling of the surface of adult *W. bancrofti* permitted a comparison between the major surface antigens of this species and those from the related lymphatic filariae, *Brugia malayi* and *B. pahangi*. All species bear a prominent M_r 29000 surface antigen but among the differences observed were the strongly labelled molecules with M_r 58000 and 67000 in *W. bancrofti* which are extremely faint in the *Brugia* species. The [³⁵S]methionine label was effectively incorporated into somatic parasite proteins *in vitro* although it was not possible to identify any secreted proteins in this way. The antigenicity of these products was investigated using a variety of sera from homologous and heterologous infections and the immunoprecipitation patterns highlighted particular differences between somatic proteins of male and female worms. One secreted antigen was detected, however, by virtue of its phosphorylcholine epitopes, in the culture medium of mixed adult worms; medium from male *W. bancrofti* adults was negative although homogenates of either sex of adult *W. bancrofti* were strongly positive in the same system.

INTRODUCTION

Wuchereria bancrofti is the most widespread of the causative agents of lymphatic filariasis (Sasa, 1976) producing a broad spectrum of pathological manifestations in infected people (Ottesen, 1984). Differential diagnosis of the infection is, at present, based on minor differences in the morphology of the microfilarial stage of the life-cycle or by the presence of clinical pathology. Immunological techniques utilizing the presence of humoral antibody to a particular pathogen have proved of value in the diagnosis of many infectious diseases but, in infections such as filariasis in which antibody may exist well beyond parasite clearance, unambiguous diagnosis of the presence of the parasite is not currently possible. A more thorough understanding of the full antigenic profile of the human lymphatic filarial parasites, however, may help identify suitable diagnostic target antigens.

Larval stages of *W. bancrofti*, although difficult to obtain in quantity, have been examined by a number of techniques; thus microfilarial surface antigens have been analysed by means of immunofluorescence (Ridley & Hedge, 1977; Dissanayake & Ismail, 1980), surface radio-iodination (Maizels, Philipp, Dasgupta & Partono, 1984; Maizels, Burke, Sutanto, Purnomo & Partono, 1986) and *in vitro* cultivation to study

* To whom correspondence and reprint requests should be addressed.

secreted IVR (*in vitro* released) products (Kharat, Kaliraj, Ghirkinar & Harinath, 1981; Reddy, Piessens & Harinath, 1984; Maizels *et al.* 1986). Antigens of *W. bancrofti* L3 have also been studied, by radio-iodination of major surface antigens (Maizels *et al.* 1986) and the collection of IVR products (Malhotra & Harinath, 1984; Maizels *et al.* 1986).

The characterization of adult worm antigens has been severely restricted due to the high degree of host specificity of *W. bancrofti*. In 1982, Palmieri, Connor, Purnomo, Dennis & Marwoto established experimental infections of *W. bancrofti* in the leaf monkey *Presbytis cristatus*. Using this model small numbers of adult *W. bancrofti* may be obtained, and by utilizing the techniques of surface radio-iodination (Bolton & Hunter, 1973; Markwell & Fox, 1978; Parkhouse, Phillip & Ogilvie, 1981) and *in vitro* [³⁵S]methionine incorporation (Parkhouse & Clark, 1983) we have been able to examine the antigenic profile of adult worms. These techniques have been previously used in the study of other human filarial nematodes including *Brugia malayi* (Kaushal, Hussain, Nash & Ottesen, 1982; Maizels, Partono, Oemijati, Denham & Ogilvie, 1983*a*), *B. timori* (Maizels, Partono, Oemijati & Ogilvie, 1983*b*) and *Onchocerca volvulus* (Philipp, Gomez-Priego, Parkhouse, Davies, Clark, Ogilvie & Beltran-Hernandez, 1984; Taylor, Goddard & McMahon, 1986). By utilizing these techniques we have been able to analyse the adult stage of *W. bancrofti* and we report here the identification of a number of surface and somatic antigens of this parasite.

MATERIALS AND METHODS

Parasites

Infective larvae (L3) of *Wuchereria bancrofti* were obtained by dissection of *Aedes togoi* mosquitoes 16 days after feeding on microfilaraemic human blood. Approximately 700 larvae were then inoculated subcutaneously into the groin region of adult *Presbytis cristatus* monkeys (Palmieri *et al.* 1982). Between 40 and 42 weeks later the monkeys were sacrificed and adult worms recovered by total body dissection.

Radio-iodination

The methods of Iodogen (Markwell & Fox, 1978) and Bolton-Hunter (Bolton & Hunter, 1973) mediated radio-iodination were followed as for adult *Brugia* worms (Kaushal *et al.* 1982; Maizels *et al.* 1983; Sutanto, Maizels & Denham, 1985). After iodination a modified solubilization procedure was used in which the labelled adults were washed in phosphate-buffered saline (PBS) and then incubated for 16 h at 4 °C in 1.5% *n*-octylglucoside (nOG; Sigma O-8001) in PBS containing protease inhibitors (PBS-I). The detergent solution containing solubilized surface-labelled antigens was removed and the worms washed 3 times in PBS inhibitors before homogenization in nOG PBS-I in a ground-glass tissue homogenizer (Jencons H103/32/94). The homogenates were subsequently centrifuged at 10000 *g* for 30 min and the supernatant fluid decanted. Protease inhibitors included in the buffer were: 25 µg/ml α -*N*-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK; Sigma T-7254), 1 mM phenylmethylsulphonyl-fluoride (PMSF; Sigma P-7626), 5 µM pepstatin (Sigma P-4265) and 1 mM ethylenediaminetetra acetic acid (EDTA; Sigma ED-2SS).

In vitro radio-isotope labelling

Parasites were incubated with 500 μCi of [^{35}S]methionine (Amersham SJ.204) for 48 h in methionine-free MEM (made as for Gibco formulation cat. no. 041-1095) containing 1% glucose, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco 043-5070). After culture for 48 h the supernatant fraction was recovered and the parasites washed 3 times in PBS-I and incubated for 16 h in nOG PBS-I at 4 °C. After recovery of this supernatant fraction, worms were further washed in PBS-I and homogenized in the detergent buffer. The homogenate was then centrifuged at 10000 g for 30 min and the supernatant fluid decanted. Somatic proteins not soluble in 1.5% nOG were further extracted by boiling in 5% SDS, 5% fresh 2-mercaptoethanol in 8 M urea, 10 mM phosphate buffer, pH 7.4. Material solubilized by this harsh extraction procedure was recovered by centrifugation at 10000 g for 30 min.

SDS-PAGE and immunoprecipitation analysis

Radio-isotope labelled antigens were analysed for relative molecular weight and immunoreactivity on 5–25% polyacrylamide gradient gels as described previously (Sutanto *et al.* 1985). Prior to electrophoresis the samples were mixed in loading buffer and reduced in 5% 2-mercaptoethanol. Polyacrylamide gels containing ^{35}S -labelled antigens were immersed in Amplify (Amersham International) after electrophoresis for fluorography. Gels were dried and then exposed to Fuji X-ray film at -80 °C. Relative molecular weights were calculated by reference to standard marker proteins (Pharmacia) of range 14400–94000 Daltons.

Antigens, labelled by the above methods, were incubated in PBS with 5 μl of human serum overnight at 4 °C. Antigen–antibody complexes were precipitated by reaction with 50 μl of 10% suspension of formalin-killed *Staphylococcus aureus* bacteria (BRL Immunoprecipitin, 9321SA). The precipitates were washed 3 times in PBS–0.1% Triton X-100 (Sigma) and resolubilized in loading buffer. Serum samples were collected from Indonesian patients infected with *B. malayi* from Tanjungpinang and Buru Island, Indonesia, and *W. bancrofti* from Jakarta. Further samples from *W. bancrofti* patients from Bihar, India, were kindly provided by Dr A. Dasgupta, All India Institute of Medical Sciences, New Delhi. Serum from patients infected with *Onchocerca volvulus*, *Loa loa*, and *Strongyloides stercoralis* were provided by Dr J. Lillywhite, Hospital for Tropical Disease, London, and hookworm serum from Dr G. Schad of the University of Pennsylvania.

Immunoradiometric assay (IRMA)

A mouse IgM monoclonal antibody Bp-1, which was raised against a detergent-released fraction of *B. pahangi* (Sutanto *et al.* 1985) with a specificity for the hapten phosphorylcholine (Maizels, Denham & Sutanto, 1985a) was used in an immunoradiometric assay (IRMA) for PC-bearing filarial antigens (Forsyth, Mitchell & Copeman, 1984; Maizels *et al.* 1985a). Titertek immunoassay plates (Flow 77-176-05) were coated with 50 $\mu\text{l}/\text{well}$ of a 1 $\mu\text{g}/\text{ml}$ solution of Bp-1 in PBS overnight at room temperature. Unreacted sites were blocked with a 0.5% solution of bovine serum albumin (BSA) for 30 min at room temperature. After washing in PBS–0.1% Tween 20 (Sigma P-1379) for 3 \times 3 min the plates were incubated with parasite preparations for 6 h at 37 °C. The plates were then washed as above and to each well approximately 50000 c.p.m. of ^{125}I -labelled BP-1 were added in PBS with 5% normal cat serum–0.1% Tween 20 and

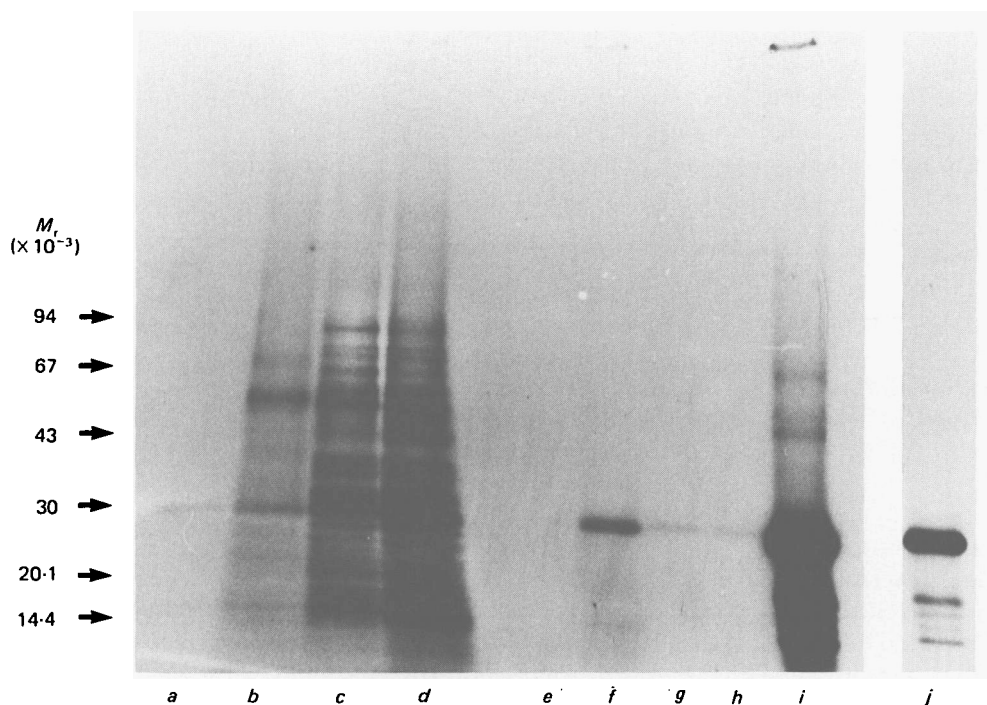


Fig. 1. Analysis of surface-labelled antigens of adult *Wuchereria bancrofti* by means of nOG solubilization and electrophoresis on SDS-PAGE. (a), (b) Iodogen-labelled molecules non-disruptively solubilized in nOG (2 replicate experiments); (c), (d) Bolton-Hunter-labelled molecules nOG solubilized (replicate experiments); (e); (f) homogenates of Iodogen-labelled adult worms after nOG solubilization; (g), (h) homogenates of Bolton-Hunter labelled worms after nOG solubilization; (i) DOC-solubilized Iodogen-labelled *Brugia pahangi*; (j), same track as (i) but less exposed.

incubated overnight at room temperature. Excess radio-isotope labelled antibody was removed by 3 further washing steps and each well counted in an LKB gamma counter.

RESULTS AND DISCUSSION

Parasite recovery

From 6 *Presbytis* monkeys used as laboratory hosts for *W. bancrofti*, each infected with approximately 700 infective larvae (L3), a total of 22 adult worms was recovered (0.5% recovery), 7 males and 15 females; 2 monkeys which were microfilaria positive (mf+ve) yielded no adult *W. bancrofti* on sacrifice. As had been anticipated, the restricted number of adults recovered from the *Presbytis* model made radio-isotope labelling techniques particularly appropriate for antigenic analysis of small quantities of material. A reflection of the difficulty in obtaining such parasite material is that the results reported in this paper are the first analyses of the antigens of adult *W. bancrofti* filaria, parasites which currently infect between 100–200 million people.

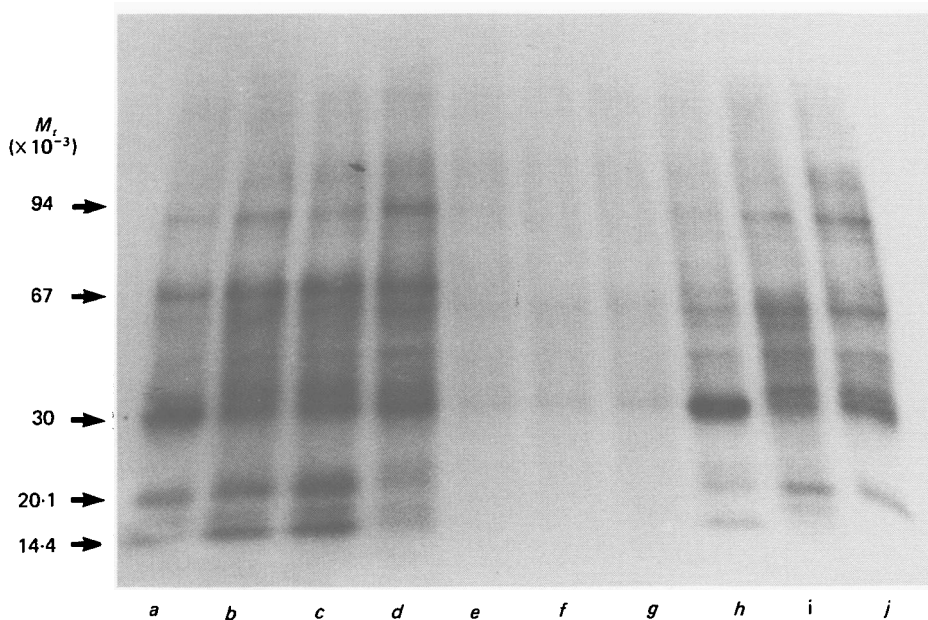


Fig. 2. Immunoprecipitation of Bolton-Hunter-labelled surface antigens of adult *Wuchereria bancrofti*. (a) Jakartan *W. bancrofti* infection serum pool; (b) and (c) pools of Indian *W. bancrofti* infection sera; (d) *Brugia malayi* infection serum pool; (e) normal human serum; (f) *Toxocara canis* infection serum; (g) Hookworm infection serum; (h) *Loa loa* infection serum; (i) *Strongyloides stercoralis* infection serum; (j) *Onchocerca volvulus* infection serum.

Surface radio-iodination

One direct approach to identifying surface antigens of parasitic nematodes is that of radio-iodination of intact viable parasites (Parkhouse *et al.* 1981). A comparison was made of two techniques for the iodination of adult *W. bancrofti* using the Iodogen (Markwell & Fox, 1978) and Bolton-Hunter (Bolton & Hunter, 1973) procedures. Each experiment was performed twice and following iodination labelled parasites were incubated for 16 h in *n*-octylglucoside. The supernatant fraction containing detergent-released molecules was recovered before homogenization of the adult worm material and analysed by PAGE. The results are presented in Fig. 1 and show that Iodogen mediates labelling of molecules of M_r 15 000, 20 000, 29 000, 51 000, 58 000 and 67 000 (15–67 K) which are released by nOG (Fig. 1 *a, b*). One of the replicate experiments with Iodogen gave a very low yield (Fig. 1 *a*) but the two patterns were consistent. The M_r 15, 20 and 29 K bands shown here resemble the pattern obtained by Iodogen labelling of both *B. pahangi* (Sutanto *et al.* 1985) and *B. malayi* (Maizels *et al.* 1985*a*). The higher molecular weight proteins, major bands at M_r 58 and 67 K and a minor band at 51 K do not correspond closely to those seen in *Brugia* parasites. Comparison of nOG-solubilized *W. bancrofti* and deoxycholate (DOC)-solubilized *B. pahangi* molecules revealed that although proteins of these sizes are present in *B. pahangi* they are very weakly labelled (Fig. 1 *i, j*) and apparent only when the gel track is over-exposed.

Bolton-Hunter radio-iodination labelled a large number of molecules in addition to the major proteins labelled by the Iodogen method (Fig. 1 *c, d*) as has been previously found for *Brugia* species (Maizels *et al.* 1983; Sutanto *et al.* 1985). At least 12 extra molecules were observed in these preparations between M_r 16 and 94 K, in addition to

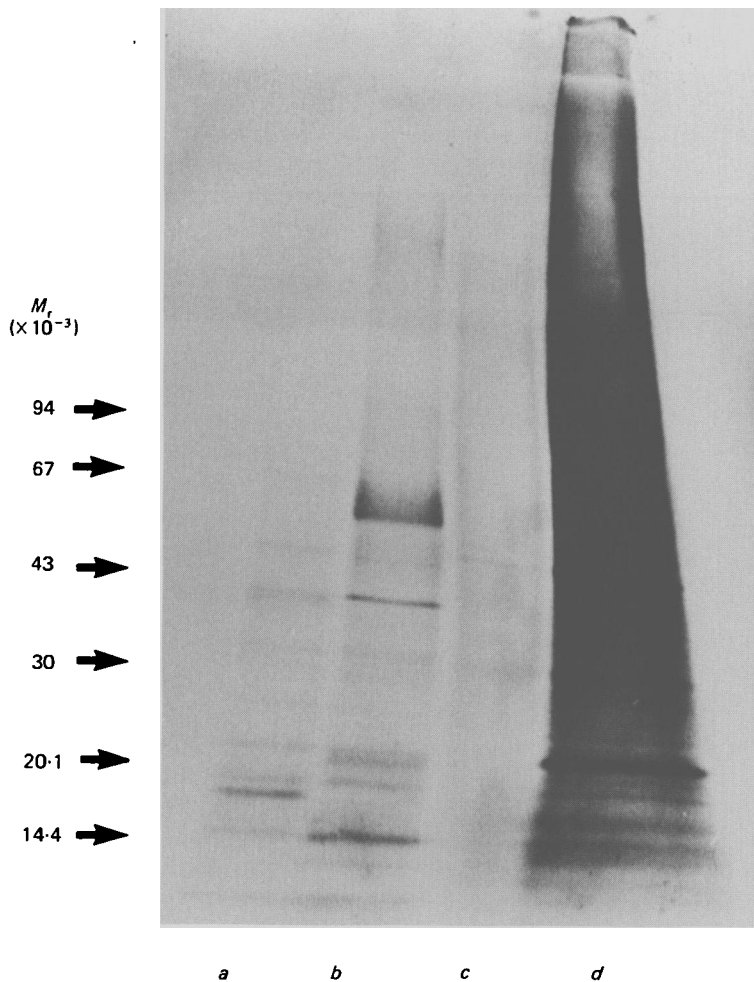


Fig. 3. *In vitro* [^{35}S]methionine-labelled antigens of adult *Wuchereria bancrofti*. (a) Male homogenate in nOG; (b) mixed adult (7 female/1 male) homogenate in nOG; (c) and (d) SDS-urea-2ME extraction of male and mixed adult material respectively.

a molecule of M_r approximately 140 K. The Bolton-Hunter reagent, however, has been shown to penetrate into the soma of the worm, labelling proteins which are not located on the surface (Marshall & Howells, 1985).

Homogenization of labelled worms after the initial non-disruptive incubation in nOG produced a single band of M_r 29 K, one of the major surface molecules of the nematode, indicating that the first detergent solubilization does not remove all of the labelled protein from the worm surface. This was evident on both Iodogen (Fig. 1 *e, f*) and both Bolton-Hunter (Fig. 1 *g, h*) labellings. Further extraction in a combination of SDS, urea and 2-mercaptoethanol (Sutanto *et al.* 1985) was carried out but this material did not resolve into discrete bands on SDS-PAGE.

To assess the antigenicity of surface-labelled proteins immunoprecipitation was performed using both Iodogen and Bolton-Hunter reagent-labelled worm preparations. Due to low levels of ^{125}I incorporation achieved using the Iodogen method no

immunoprecipitated proteins could be visualized. Using the Bolton–Hunter-labelled worm material, however, the antigenicity of several proteins was demonstrated by immunoprecipitation with a variety of infection sera, both from filarial and other nematode infections (Fig. 2). Compared to normal human serum control (Track *e*) molecules of apparent M_r of 15, 20, 29, 51, 58 and 85 K were recognized by each of the *W. bancrofti* infection serum pools used in this study (*a–c*). One *B. malayi* serum pool recognized the antigens of M_r 29–85 K but only weakly precipitated the smaller proteins of 15 and 20 K (*d*); this result contrasts with that obtained with adult *B. pahangi* surface antigens (Maizels, Sutanto, Gomez-Priego, Lillywhite & Denham, 1985*b*) in which the *Brugia* M_r 15 and 20 K bands are equally precipitated by sera from *Brugia* or *Wuchereria* infections. Of the other nematode infection sera tested neither *Toxocara canis* (*f*) nor hookworm (*g*) produced immunoprecipitation above the normal human serum background (*e*).

Three other sera tested showed interesting precipitation patterns, which may be compared to those reported with Iodogen-labelled *Brugia* adult antigens (Maizels *et al.* 1985*b*). *Loa* infection serum (from an individual with unknown prior clinical history) reacted particularly strongly with the M_r 29 K antigen of *W. bancrofti* in a similar pattern to that which it precipitated from labelled *Brugia* material. Secondly, sera from Onchocerciasis patients consistently recognize the 20 K antigen of *Brugia* with intermittent precipitation of other antigens (Maizels *et al.* 1986). In the case of *W. bancrofti* antigens, the M_r 20 K antigen was precipitated by the *O. volvulus* serum (*i*) and, in addition, antigens of M_r 29, 32, 51 and 85 K but not those at 15 and 67 K. Finally, when a *Strongyloides* serum was tested (*j*), a similar pattern of precipitation was seen to that found with *Onchocerca*. Cross-reactivity of *Strongyloides* sera was not observed with Iodogen-labelled *Brugia* surface antigens (Maizels *et al.* 1985*b*) but such sera have been reported to be more cross-reactive with somatic filarial antigens than sera from other non-filarial infections (Kaushal, Hussain & Ottesen, 1984).

Metabolic labelling with [³⁵S]methionine

From the parasites available, *in vitro* [³⁵S]methionine labelling was performed on one group of 2 adult males and a second group of 7 females and 1 male. Although the quantity of labelled proteins released *in vitro* was very low, by using large volumes of the culture supernatants some IVR (*in vitro* released) molecules were visualized by SDS–PAGE at M_r 52, 67, 77 and 91 K but only in the culture containing female worms (data not shown). Upon examination of the ³⁵S-labelled somatic worm material recovered by homogenization, in addition to common antigens shared by each sex, specific differences were observed (Fig. 3) with a male-specific molecule of M_r 17.5 K, (Fig. 3*a*) and female-specific molecules of M_r 21, 52, 62 K and a high M_r band of over 200 K (Fig. 3*b*). Material insoluble in mild detergent was extracted with SDS, urea and 2-mercaptoethanol (Fig. 3*c, d*). The differences between the preparations were highlighted by the immunoprecipitation patterns of a variety of sera with these somatic antigens (Fig. 4).

In these immunoprecipitation experiments, lymphatic filarial infection sera each precipitated the female M_r 62 K antigen together with a number of other antigens (Fig. 4*a–c*), although precipitation by the *B. malayi* serum pool (*a*) was relatively weak. Again this contrasts with the strong reaction of *W. bancrofti* infection sera towards surface antigens from adult *Brugia* (Maizels *et al.* 1985*b*). The Jakartan and Indian serum pools used showed minor differences in the precipitation patterns of the

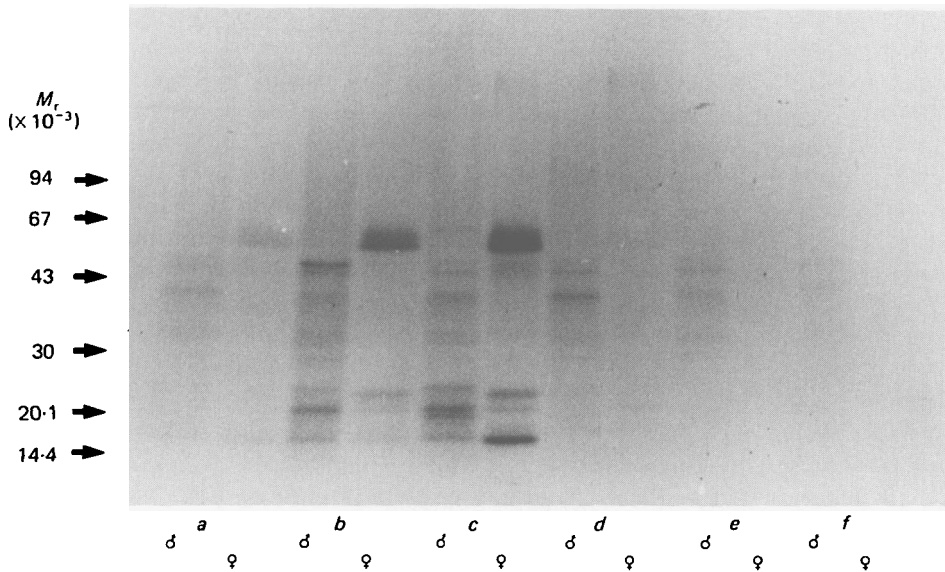


Fig. 4. Immunoprecipitation of [^{35}S]methionine-labelled somatic antigens of adult *Wuchereria bancrofti*. (a) *Brugia malayi* infection serum pool; (b) Jakartan *W. bancrofti* infection serum pool; (c) Indian *W. bancrofti* infection serum pool; (d) *O. volvulus* infection serum; (e) *Toxocara canis* infection serum; (f) normal human serum. In each case (♂) refers to antigen from adult males and (♀) to antigen from predominantly female parasites.

sex-specific antigens; antigens from the male *in vitro* cultured worms of M_r 17.5 and 71 K were only visible in the Indian pool precipitate (c) and 54 K antigen was preferentially recognized by the Jakartan sera (b). Of the female antigens the Indian serum pool precipitated both the M_r 14.5 and 52 K antigens more strongly than the Jakartan sera.

The precipitation pattern of the *O. volvulus* (d) and *Toxocara* (e) sera closely resembled that obtained with normal human serum (f). The onchocerciasis serum sample, however, did precipitate the M_r 200 K antigen which was faintly represented in the lymphatic filarial sera precipitates.

Phosphorylcholine-bearing molecules

Several species of helminth parasite have been reported to contain the hapten phosphorylcholine (PC), including *Ascaris suum*, *Nippostrongylus brasiliensis* and *Trichinella spiralis* (Pery, Petit, Poulain & Luffau, 1974; Sugane & Oshima, 1983). Recently, the presence of phosphorylcholine-bearing molecules has been examined in filarial infections (Forsyth *et al.* 1984; Forsyth, Spark, Kazura, Brown, Peters, Heywood, Dissanayake & Mitchell, 1985; Gualzata, Weiss & Heuser, 1986). Such antigens have been reported both in parasite material and in the sera of infected patients, and a number of monoclonal antibodies raised against filarial antigens have been found to recognize PC as their target epitope (Forsyth *et al.* 1985; Maizels *et al.* 1985a). Analysis of the *in vitro* culture fluids and extracts of male and female adult *W. bancrofti* for phosphorylcholine-bearing molecules was performed in an immunoradiometric assay (IRMA) (Forsyth *et al.* 1984) using Bp-1 as described previously (Maizels *et al.* 1985a).

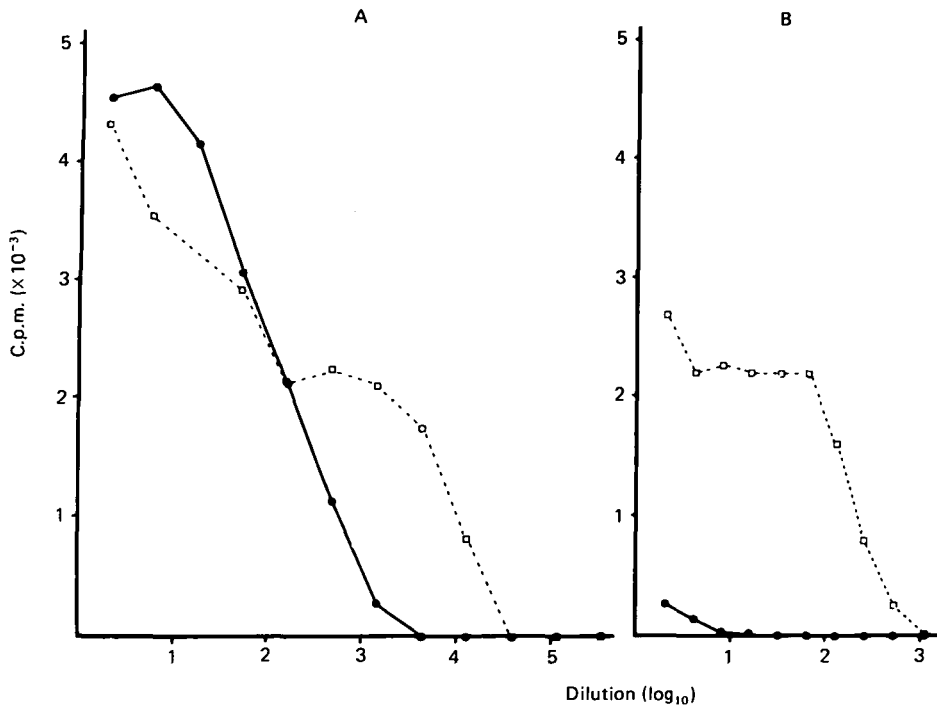


Fig. 5. Detection of filarial antigen in IVR and somatic material of adult male and female lymphatic filariae by immunoradiometric assay with monoclonal antibody Bp-1. (A) Somatic material of female (□) and male (●) *Wuchereria bancrofti*; (B) IVR of female (□) and male (●) adult worms.

PC-bearing molecules were readily detectable in somatic extracts of the *W. bancrofti* adults (Fig. 5A). Although the male extract did not titrate as far as the female, it must be noted that the overall concentration of the male preparation was significantly lower than that of the female indicating that the level of PC-positive molecules in the male would be relatively higher. The male extract also showed a far steeper titration slope, perhaps indicating the presence of different PC-conjugated molecules, or a different number of PC haptenic groups/molecule in each sex.

In vitro released PC-bearing molecules were not found in the male culture supernatant (Fig. 5B) although significant quantities were detected from the culture containing female worms. The incorporation of [³⁵S]methionine attested to the viability of the male worms *in vitro*, but in view of their number it was possible that the level of PC released may have been below the threshold of detection. We are currently investigating whether male *Brugia* parasites release PC-containing molecules *in vitro* and whether the non-secretion of PC by *W. bancrofti* adult males reflects an interspecific difference in addition to the observed difference between the sexes.

We have provided here a first analysis of the antigenic profile of adult *W. bancrofti* parasites, and compared the pattern of surface proteins with those known for the related filariae *B. malayi* and *B. pahangi*. Although adult surface antigens from the two *Brugia* species cannot be distinguished (Selkirk, Denham, Partono, Sutanto & Maizels, 1986), *W. bancrofti* surface antigens appear to be somewhat distinct, particularly in the *M_r* 51–67 K range. Further differences between the genera may be inferred from the

differential immunoprecipitation of labelled somatic proteins. Whether such differences are related to the different clinical manifestations of these filarial species (Partono, 1984) is open to speculation.

These results presented here were made possible by applying radio-isotopic techniques to scarce quantities of parasite material, and such approaches may continue to be necessary as long as the laboratory production of *W. bancrofti* is so difficult.

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