Immune complexes in *Wuchereria bancrofti* infection in man

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The levels of immune complexes in the sera of patients with *Wuchereria bancrofti* infection were determined by enzyme-linked immunosorbent assay, using a rabbit antibody to the adult *Setaria digitata* antigens SD2-4, and by the Clq-binding assay. Approximately 3–7% of microfilaraemic subjects and 30–40% of amicrofilaraemic symptomatic patients had levels of immune complexes that were significantly higher than the levels observed in non-filarial control subjects. The antigen in the polyethylene glycol-precipitated immune complexes was isolated. This *W. bancrofti* antigen was found to be similar to the *S. digitata* antigen SD2-4. Both antigens had the properties of an acidic glycoprotein of isoelectric point around pH 3.

Serum antibody determination is considered satisfactory for general diagnosis in the majority of cases of Bancroftian filariasis; however, there is no correlation between the serum antibody level and the clinical status or the intensity of infection (1, 2). Furthermore, because of wide antigenic cross-reactivities, antibody determination does not permit the identification of the infecting species. It is not uncommon to see patients who present with symptoms and signs suggestive of filarial infection, but who are serum antibody-negative. This may be due to the poor sensitivity of the assay system or to masking of antibodies by antigens in immune complexes. Therefore, the current emphasis is on determination of circulating antigens. It is very likely that circulating antigens, if present, would be complexed because most individuals in the endemic areas would have serum antibodies as a result of repeated exposure to infection.

*Wuchereria bancrofti* infections in man are characterized by a wide spectrum of host immune responses. In endemic areas, although the majority of people are exposed to the infection, only a small proportion develop clinical disease. This may present as one of the classical lymphatic forms, ranging from acute filarial fever with lymphangitis and lymphadenitis to the more chronic lymphoedema, hydrocele, and elephantiasis, or as a hypersensitivity state such as tropical pulmonary eosinophilia, or as an atypical form such as filarial arthritis. The immunological phenomena responsible for these different manifestations of the disease are poorly understood, but it is possible that immune complexes play an important role.

Antigenic cross-reactivity is probably the most serious limitation in filarial serology based on antibody determination. The same limitations will apply to antigen and immune-complex determinations unless the molecular basis of antigenic cross-reactivity is defined and taken into account in the development of new techniques.

A further prerequisite for the determination of circulating antigens and immune complexes is information on the number of different antigens involved, their origin, and correlation with disease status. Unless the molecular and antigenic characteristics of the adult, microfilarial, and larval antigens are known, their quantitative determination will be impossible. In the case of *W. bancrofti*, such characterization is extremely difficult as the parasite is not available for antigen extraction. Immune complexes isolated from patients’ sera may, however, be a good source of biologically active antigen.

Circulating immune complexes have been demonstrated in filarial infections (3–6), but the isolation and characterization of the antigen in the immune complex was not attempted in any of these studies. Also, the diverse techniques and the different types of patients and control populations studied make direct comparison of the results almost impossible. For the same reasons, the diagnostic value of circulating antigens and immune complexes cannot be evaluated.

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The purpose of the present investigation was to detect circulating specific adult antigen immune complexes in *W. bancrofti* infection, and, if possible, to isolate and characterize the antigen(s) in these complexes.

**MATERIALS AND METHODS**

**Collection of sera**

Blood samples were collected from patients attending an antifilarial clinic in Colombo, Sri Lanka, and from microfilaraemic individuals detected during night blood surveys. In the clinic, 58 microfilaraemic subjects were selected, and serum antibody positive by immunofluorescence against *W. bancrofti* microfilarial surface antigens (7) and by enzyme-linked immunosorbent assay (ELISA) against adult *Setaria digitata* antigen SD2-4 (8). All 42 microfilaraemic subjects detected during night blood surveys were asymptomatic. Microfilariae in night blood were detected by the nucleopore membrane filtration technique. A further 48 non-microfilarial control subjects were selected from an area free of filariasis. These subjects did not have any serological, parasitological, or clinical evidence of filarial infection.

Informed consent was obtained in all cases. Samples of 5 ml of venous blood were collected, the serum was separated and stored in aliquots of 0.5 ml at −20 °C until used.

**Preparation of rabbit anti-SD2-4 antiserum**

*S. digitata* antigen SD2-4 was prepared as described previously (8), and 1 mg of antigen in 0.5 ml of Freund’s complete adjuvant was given to each rabbit, intramuscularly, in the hind leg. This was followed by 4 booster injections of antigen in Freund’s incomplete adjuvant at 10-day intervals. Two weeks after the last injection, the animals were “test bled”. The animals that produced good antisera (as assessed by Ouchterlony analysis) were bled, and the sera collected and pooled.

The antigenic specificity of the rabbit anti-SD2-4 antiserum was tested by Ouchterlony analysis and by inhibition of ELISA, in which the reaction between antibodies in sera from *W. bancrofti*-infected patients and the insoluble antigen SD2-4 was inhibited with the rabbit anti-SD2-4 antiserum. Polystyrene tubes were coated with antigen SD2-4, and various quantities of anti-SD2-4 antiserum, mixed with 25 μl of patients’ sera, were added. As a control, normal rabbit serum was used in place of anti-SD2-4 antiserum. The amount of human antibody bound was determined by ELISA using a goat antibody specific to human Ig (absorbed against rabbit Ig) and coupled to horseradish peroxidase. The reduction in the ELISA titre upon the addition of the rabbit antiserum to antigen SD2-4 was calculated.

**Determination of filarial antigen-specific immune complexes**

The ELISA was used to determine the immune complexes in patients’ sera binding to insoluble anti-SD2-4 antibody through free antigen valencies expressed on the complex.

The IgG fraction of the anti-SD2-4 antiserum was prepared by DEAE-cellulose chromatography (9). Polystyrene tubes were coated with this antibody preparation at a predetermined concentration of 2 μg of antibody/ml per tube, the tubes were washed, and 25 μl of the test human serum was added in 1 ml of phosphate-buffered saline containing 0.1 ml of Tween 20 per litre. The tubes were incubated at 37 °C for 3 h and at 4 °C for 1 h. The amount of bound human immunoglobulin was determined by ELISA using a peroxidase-conjugated goat antibody specific to human Ig. The ELISA reading was assumed to be a measure of the immune complexes in the test serum. The mean value of the ELISA reading for the non-filarial control group was taken as the cut-off point and ELISA readings greater than this mean + 2S.D. were considered as significantly positive.

**Determination of serum immune complexes by Clq-radioimmunoassay (Clq-RIA)**

Clq-RIA was performed as described by Hay et al. (10) with certain modifications. Aliquots of 1 ml of human Clq in phosphate-buffered saline (PBS, pH 7.4) were incubated in polystyrene tubes for 3 days at 4 °C. After 3 washes with PBS, the tubes were filled with a 0.1 g/litre solution of gelatin in PBS and incubated at room temperature for 2 h. After 3 more washes, the tubes were used in the Clq assay.

Aliquots of 50 μl of the test serum were mixed with 100 μl of sodium ethylenediaminetetraacetic acid (EDTA, adjusted to pH 7.5 with NaOH) and incubated for 30 min at 37 °C. The mixture was then transferred to an ice bath. Duplicate 50-μl samples were placed in the Clq-coated tubes together with 950 μl of PBS–Tweens 20. Coated tubes containing 1 ml of PBS were used as controls. The tubes were incubated for 1 h at 37 °C and for 30 min at 4 °C.

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C1q-RIA was performed by S. Dissanayake at the Department of Immunology, Middlesex Hospital Medical School, London W1, England.

Provided by Dr F. C. Hay and Dr Lynn J. Nineham, Department of Immunology, Middlesex Hospital Medical School, London.
Fig. 1. Antibody activity of the rabbit antiserum to SD2-4, as assessed by Ouchterlony analysis. Peripheral small wells (1), sera from W. bancrofti-infected subjects; peripheral large wells (2), rabbit anti-SD2-4 antiserum; centre well (3), antigen SD2-4.

Fig. 4. Rocket immunoelectrophoresis of antigen isolated from PEG-precipitated immune complexes by preparative isoelectric focusing.

Fig. 5. Isoelectric focusing in thin-layer polyacrylamide gel.

2, 3: antigen SD2-4 (2 = 100 μg, 3 = 25 μg)
4, 5: low molecular-weight material from Sephacryl G-150 fractionation of the PEG precipitate
1, 6, 7, 8: high-molecular-weight material from the Sephacryl G-150 column (1, 6, 8 = 100 μg; 7 = 25μg).
Unbound proteins were then removed by washing 3 times with PBS-Tween. Immune complexes bound to the Clq coating were detected by incubating the tubes with 1 μg of affinity chromatography-purified, 125I-labelled, rabbit antibodies to human immunoglobulin in 1 ml of PBS-Tween. Unbound labelled protein was removed by washing and the tubes were counted for radioactivity, the amount of bound radioactivity being a measure of immune complexes in the patients' serum, expressed as ng of bound 125I-labelled antibody.

**Isolation of immune complexes**

Immune complexes were isolated by precipitation with polyethylene glycol (PEG, relative molecular mass 6000). Sera containing high levels of immune complexes (as determined by the ELISA) were pooled and diluted 1:1 with barbital buffer (pH 7.4, containing 0.02 mol/litre EDTA). A solution of PEG in barbital buffer (pH 7.4) was added to give a final PEG concentration of 50 g/litre. The mixture was stirred at 37 °C for 3 h and left to stand at 4 °C overnight. The precipitate was collected by centrifugation at 10 000 g for 30 min, suspended in 10 ml of dilute acetic acid (pH 3) containing 0.1 mol/litre NaCl, and dialysed against barbital buffer. The soluble material was collected, concentrated, and fractionated by isoelectric focusing.

**Isoelectric focusing in polyacrylamide gel (IEF-PAG)**

IEF-PAG was performed in thin layers of 5% polyacrylamide gel with a pH gradient of 3–10 (2% ampholyte). The method used was as described in the instruction manual supplied with the LKB Multiphor electrophoretic apparatus. The protein concentration in the samples was adjusted to 10 g/litre and up to 50 μl of the sample was applied to slots made in the gel near the cathode. Focusing was performed in the LKB Multiphor apparatus using a constant wattage power supply, set for 300 V/80 mA initially and 950 V/2 mA finally. After focusing, the gel was washed in 125 ml/litre trichloroacetic acid for 12 h and stained for glycoproteins (using periodic acid-Schiff stain) followed by protein staining with Coomassie brilliant blue (11).

**Preparative isoelectric focusing in sucrose density gradients**

The extracted immune complexes were also fractionated by preparative isoelectric focusing in a pH gradient of 3–10. Focusing was performed in an LKB 110-ml preparative isoelectric focusing column. The dilute acetic acid extract was dialysed against acetate buffer, pH 6, and focused at 900 V for 20 h. After focusing, 5-ml fractions were collected, and the pH was determined; they were then dialysed against PBS and the protein concentration was calculated from the optical density at 280 nm. These fractions were then used in the ELISA and in rocket immunoelectrophoresis.

**ELISA with isoelectrically focused fractions**

The fractions obtained from the preparative focusing column were coated onto polystyrene tubes at a protein concentration of 2 μg/ml per tube. To the washed tubes were then added 50 μl of a pooled antibody-positive human filarial serum in 1 ml of PBS-Tween. For each fraction, a negative serum served as a control. Bound human antibodies were then determined by ELISA using a peroxidase-conjugated antibody specific to human Ig. To eliminate the high background noise from immunoglobulin in some focused fractions (mainly in the pH range, 6–10), the reading obtained with the negative serum was subtracted from that obtained with the positive serum.

**Rocket immunoelectrophoresis in agarose gels coupled to IEF-PAG**

The material extracted from the PEG precipitate of immune-complex sera was fractionated by IEF-PAG. The polyacrylamide gel was then cut into narrow strips and overlaid onto an agarose gel containing the rabbit anti-SD2-4 antiserum and electrophoresed in the second direction until precipitation appeared. The polyacrylamide gel was then removed, the agarose gel was washed in PBS, dried, and stained with Coomassie brilliant blue.

**RESULTS**

**Antibody activity of rabbit anti-SD2-4 antiserum**

Results of Ouchterlony analysis (Fig. 1) and inhibition of ELISA (Fig. 2) showed the presence of antibodies of similar antigenic specificity in the rabbit antiserum and in sera of patients infected with *W. bancrofti*. However, Ouchterlony analysis showed only partial identity and the maximum ELISA inhibition obtained was about 50%.

**Levels of antibodies and immune complexes**

As has been shown previously (1), the microfilaraemic subjects did not have serum antibodies reacting with surface antigens of *W. bancrofti* micro-

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filariae, but had high levels of antibodies reacting with the adult antigen SD2-4. The symptomatic amicrofilaraemic patients had antibodies reacting with both antigens (Table 1).

The levels of immune complexes obtained for the non-filarial control group by the Clq method were relatively high (mean, 135 ± 60 ng of labelled antibody), but the percentages of filarial patients with significantly high levels of immune complexes (more than 2 standard deviations greater than the mean for the control group), as determined by Clq assay and the ELISA, were comparable (Table 1). In general, approximately 30–40% of the amicrofilaraemic patients and 3–7% of the microfilaraemic subjects had significantly higher levels of immune complexes than the non-filarial control group.

Isolation and partial characterization of antigens in immune complexes

The presence of filarial antigen(s) in the PEG-precipitated immune complexes was shown by the reactivity of the isolated material against W. bancrofti sera in the ELISA (Fig. 3) and by immunoprecipitation in agarose gels containing the rabbit antiserum to SD2-4 (Fig. 4). These results demonstrated at least partial identity between the W. bancrofti antigens in patients’ sera and S. digitata antigen SD2-4. The isoelectric focusing experiments showed that the antigen in the immune complexes, in common with SD2-4, is anodic in nature (isoelectric point around pH 3). Furthermore, the W. bancrofti antigen and the SD2-4 focused at the same position in thin layer IEF–PAG (Fig. 5). Both antigens were found to stain with glycoprotein (periodic acid–Schiff) and protein (Coomassie brilliant blue) stains, but better results were obtained with a combination of the two staining procedures. It was therefore concluded that the antigens were glycoprotein in nature. In addition, when the isolated immune-complex material was fractionated on Sephadex G-150 under acidic conditions, two peaks were obtained (high-molecular-weight-void and low-molecular-weight-included fractions) and the antigen was detected in both fractions (unpublished data). Heat treatment of the antigens at 56 °C for 30 min did not destroy the antigenic activity as tested by ELISA (unpublished data).

Table 1. Levels of antibodies and immune complexes in filarial subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of subjects</th>
<th>Antibody to W. bancrofti microfilarial surface antigen by IFA (at 1:16 dilution)</th>
<th>Antibody to SD2-4 by ELISA (OD at 495 nm)</th>
<th>Immune-complexes by Clq-RIA</th>
<th>Immune-complexes by ELISA (OD at 495 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-filarial controls</td>
<td>48</td>
<td>all negative</td>
<td>0.14 ± 0.07</td>
<td>135 ± 60</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Microfilaraemic asymptomatic subjects</td>
<td>42</td>
<td>all negative</td>
<td>1.1 ± 0.45 (91%)</td>
<td>169 ± 95 (7%)</td>
<td>0.09 ± 0.04 (3%)</td>
</tr>
<tr>
<td>Amicrofilaraemic symptomatic subjects</td>
<td>58</td>
<td>all positive</td>
<td>0.48 ± 0.3 (69%)</td>
<td>234 ± 135 (30%)</td>
<td>0.18 ± 0.11 (40%)</td>
</tr>
</tbody>
</table>

* Mean ± SD. Figures in parentheses give the percentage of results more than 2 SD greater than the mean of the control group.

b Expressed as ng of bound 125I-labelled antibody (mean ± SD). Figures in parentheses give the percentage of results more than 2 SD greater than the mean of the control group.

c Significantly different from controls (Student’s t-test, P < 0.01).
DISCUSSION

Although circulating immune complexes and antigens have been demonstrated in *W. bancrofti* infections (6, 12, 13), the characteristics of the antigen(s) are not known. The present report is perhaps the first on the determination of immune complexes containing adult antigen, with emphasis on isolation and characterization of the antigen.

The presence of more than one antigen is shown by the occurrence of serum antibodies reacting with adult, microfilarial, and larval antigens (1, 2, 14-17). Our data and conclusions are applicable only to *W. bancrofti* antigens (in immune complexes) cross-reacting with the heterologous *S. digitata* antigen SD2-4. The SD2-4 antigen is probably similar to the *W. bancrofti* adult antigen; this conclusion is based on our previous observation that the SD2-4 antigen is suitable for serodiagnosis of *W. bancrofti* infection by antibody determination (1) and also on the physicochemical characteristics reported in this paper.

The detection of relatively high levels of immune complexes by the Clq-binding assay in the non-filarial controls was probably caused by the non-specificity of the method. In tropical areas, people are repeatedly exposed to a wide range of parasitic diseases and it is possible that the non-filarial control sera contained unrelated immune complexes. It should be noted, however, that even aggregated immunoglobulins are Clq-reactive and these may have been present in the test sera as a result of its prior storage at $-20 \degree C$.

The ELISA immune-complex determination was more antigen-specific, but suffered from two major limitations. Firstly, only the immune complexes with free antigen valencies (antigen excess) were reactive, resulting in possible underestimation of the total. Also, the presence of uncomplexed free antigen in the serum may have blocked the binding sites on the insolubilized antibody, causing an error in the same direction. The second limitation, and perhaps the more important, was that antiglobulins may have reacted with the insoluble rabbit Ig, causing a false positive reading, or overestimation of the immune complexes. In this context, it would have been

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Footnote: See footnote a, page 919.
possible to carry out prior absorption of test sera with insoluble rabbit Ig, but the effect of such treatment on the subsequent ELISA result is not known. Even the prior screening of test sera for antiglobulins would not solve the problem as the competitive binding of antiglobulins is determined by their relative binding affinities and concentrations, which are difficult to quantify. Au et al. (6) determined the antiglobulin levels, together with immune complexes, but the antiglobulin data were of little help in the interpretation of their immune-complex data. We are therefore of the opinion that determination of serum antigens and immune complexes by conventional immunoassay is less sensitive and less specific than the presently available antibody determinations, unless Fab or F(ab')2 fragments of highly specific antibodies (preferably monoclonal) are employed as the insoluble phase in radiometric saturation analysis (18). Highly purified, characterized antigens are indispensable for such assays.

In spite of these methodological differences and limitations, both the C1q assay and the ELISA gave comparable results for immune complexes. Significantly high levels were seen in 30–40% of the microfilaraemic patients, compared with only 3–7% of the microfilaraemic subjects. Therefore, the sensitivity of the immune-complex determination is much lower than that of the antibody determinations (Table 1).

The presence of immune complexes containing adult antigen in a much higher percentage of microfilaraemic subjects than of microfilaraemic subjects compares well with the observed antibody levels in these patients. The apparently lower levels of antibodies in amicrofilaraemic subjects were probably a result of masking of antibodies by antigens (in immune complexes). In contrast to the asymptomatic microfilaraemic subjects, the amicrofilaraemic patients had received treatment for various lengths of time and this would have accelerated the release of adult antigens (19).

Our findings on immune complexes in amicro-

filaraemic symptomatic patients are in agreement with those of Ottesen,6 who found high levels of circulating C1q-binding immune complexes in patients with clinical filariasis. Au et al. (6) report high levels of circulating adult antigens in Malayan and Bancroftian microfilaraemic sera, but their data are not directly comparable with ours because the assay systems were different. The crude antigen preparation of Au et al. may have contained multiple antigens and therefore different antigens may have been detected in different populations of patients.

The presence of W. bancrofti antigen in the PEG-precipitated immune complexes was shown by their ELISA reactivity against the patients' sera (Fig. 3) and by immunoprecipitation (Fig. 4). The isoelectric point of the reactive component was around pH 3 (anodic). Spencer et al. (20) have reported similar anodic migration in preparative isoelectric focusing of adult antigens of Brugia malayi and B. pahangi.

Both the W. bancrofti and the SD2-4 antigens stained with glycoprotein stain (periodic acid–Schiff) and protein stain (Coomassie brilliant blue). In SDS-polyacrylamide gel electrophoresis, both antigens stained better with the glycoprotein stain (unpublished data). Furthermore, both antigens were stable in 0.1 mol/litre perchloric acid, a characteristic of carbohydrate-rich substances. It is highly probable that the antigens in adult S. digitata and in circulating immune complexes in W. bancrofti infections are glycoproteins. When attempts were made to fractionate crude extracts of S. digitata and acid-dissociated PEG precipitates, antigenic activity was seen in both high molecular weight and low molecular weight material obtained from Sephadex columns (unpublished data). It is possible that the antigen exists in an aggregated form. The antigen in the W. bancrofti immune complexes was very similar to that in SD2-4, and showed identical mobility in thin-layer isoelectric focusing (Fig. 5).

ACKNOWLEDGEMENTS

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RÉSUMÉ

LES IMMUN-COMPLEXES DANS LES INFECTIONS HUMAINES À WUCHERERIA BANCROFTI

On a déterminé, chez des malades porteurs d'une infection à Wuchereria bancrofti, les taux sériques d'immun-complexes par la méthode immuno-enzymatique ELISA en utilisant un anticorps de lapin dirigé contre l'anti-

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6 See footnote a, page 919.
corps SD2-4 de Setaria digitata adulte, ainsi que par fixation à la fraction Clq du complément. Environ 3 à 7% des malades microfilarémiques et 30 à 40% des malades symptomatices non microfilarémiques présentaient des taux sériques d'immun-complexes sensiblement supérieurs à ceux observés chez des témoins indemnes de filariose. On a procédé à l'isolement de l'antigène présent dans les immun-complexes précipités au polypehtylène-glycol. Cet antigène de W. bancrofti s'est révélé analogue à l'antigène SD2-4 de S. digitata. Ces deux antigènes ont les propriétés d'une glycoprotéine acide dont le point isoelectrique se situe aux alentours de pH3.

REFERENCES


