INTRODUCTION

Various immuno-diagnostic methods have been studied using closely related filarial species from animals as the antigen. A comprehensive review of such work has been made by KAGAN (1963). All these methods have failed to supply a satisfactory immuno-diagnostic test for the detection of human filariasis owing to their poor specificity.

The present studies were designed to determine whether a fluorescent antibody (FA) technique for bancroftian filariasis could be developed by using micro-fragments of microfilariae and/or infective stage larvae of *Wuchereria bancrofti* as antigens. In this technique economy of worm materials must be an important consideration as they have to be obtained directly from infected persons or laboratory infected mosquitoes.

MATERIALS AND METHODS

All the filarial materials such as microfilariae, the infective stage larvae, elephantiasis sera etc., used in this study came from various districts of Fiji where filariasis is caused by the non-periodic form of *Wuchereria bancrofti var. pacifica* (IYENGAR, 1954).

Sources of antigens

Microfilariae of *W. bancrofti* were isolated and concentrated from blood of adult Fijian males having microfilaraemia. The method used was modified from that described by FRANKS and STOLL (1945). Blood samples were drawn from patients with a microfilaria count of 20–40 per 20 c.mm. blood into a flask containing dextrose citrate in normal physiological saline (sodium citrate 7 g. and dextrose 1.7 g. in 70 ml. saline) until the citrate solution and the blood were in the ratio of 1 in 4. Dextran 0.6% was then added. The red cells formed rouleaux and settled rapidly. The supernate was withdrawn and centrifuged at 2,000 rpm for 10 minutes to sediment the microfilariae and white blood cells. Saponin 1% was added to the sediment to lyse any contaminating red cells. The WBC-microfilariae sediment was washed 3 times in physiological saline to remove the saponin and the final deposit was frozen in saline before being flown over to the author's laboratory. Throughout the procedure observations were kept on the viability of the microfilariae.

Infective stage larvae were dissected from experimentally infected mosquitoes, *Aedes polynesiensis* (Marks), as described by ROZEBOOM and CABRERA (1965). The larvae were picked up by a fine tip Pasteur pipette and put in physiological saline, frozen and flown to the author's laboratory.

Financial assistance for this work came from a grant provided by the South Pacific Medical Research Committee of the Medical Research Council of New Zealand. This was carried out in association with the studies undertaken by Associate Professor F. N. Macnamara. I would like to express my gratitude to him and to the staff of Wellcome Virus Laboratory, Suva, Fiji for collecting the filarial material, and to Dr. R. H. J. Jones for supplying the sera from New Zealand citizens.

Special acknowledgement is due to Dr. T. Maguire for his help and advice in the immunofluorescent technique, Mr. B. A. Smirk and Mr. B. P. Connor for the production of the illustrations, Professor J. A. R. Miles for the allocation of facilities and Dr. S. Thompson who encouraged me to publish this work.
Preparation of purified antigen

Back in the author's laboratory, the worm material (microfilariae or infective stage larvae) was washed once with physiological saline and then transferred into a clean 3 cm. diameter petri dish. Each worm, together with a little saline, was picked up under the microscope using a micro-capillary pipette.

A well, about 5 mm. in diameter and 2 mm. in depth was made with a ring of molten Noyer cement (G. T. Gurr Ltd., London) forming a cup within a 10 cm. diameter glass petri dish. After the cement had dried, the bottom of the well was covered with a very thin layer of distilled water and the petri dish, with the well, was then cooled to --20°C. in a Pearse's Type "H" cryostat (SLEE Co. Ltd., London). The worms were transferred directly into this well after having been picked with the micro-capillary pipette. When the well was full, its wall was then removed from the frozen mass of worm material by cutting with a scalpel. The frozen mass was then separated from the petri dish by friction heat, transferred onto a sectioning block and sections, 8 \( \mu \) thick, were cut at --20°C. with the cryostat. The sections were put on clean glass microscopic slides, air dried at room temperature, treated with acetone for 10 minutes and then dried again at 37°C. for 15 minutes before being used in the FA test. The fixed sections when not used immediately were stored at --70°C. in a moisture-free container.

Sera used

All human sera were stored at --20°C. after being collected and were used undiluted and without further treatment after thawing. There were 3 batches:—

(a) sera from 60 elephantiasis patients
(b) 208 sera from inhabitants of Lau Islands, Fiji, where filariasis is endemic
(c) 106 sera from inhabitants of New Zealand where filariasis does not occur.

Immunofluorescence technique

The indirect fluorescent antibody technique using fluorescein isothiocyanate-labelled rabbit anti-human globulin was employed. The anti-human globulin was prepared by inoculating 6 months old New Zealand bred albino rabbits intramuscularly with 1 ml. of pooled human sera (from 5 healthy persons) mixed with 1 ml. of Freund's complete adjuvant. Three weeks later the rabbits were inoculated subcutaneously with 1 ml. of pooled human sera without adjuvant. Subcutaneous inoculations were continued at weekly intervals until a precipitation antibody titre of at least 1 in 100 was obtained. Separation and purification of the rabbit anti-human serum globulin was carried out according to the method of D'Alessio et al. (1970), and labelled anti-human globulin was prepared as described by Marshall et al. (1958).

FA staining was carried out as follows. The fixed microfilarial (or infective larval) fragments were flooded with the test human serum and incubated for 30 minutes in a humidified chamber at room temperature. The specimens were then run through 5 one-minute washes in phosphate buffered saline (PBS, pH 7-6) and then finally treated with the fluorescein-labelled anti-human globulins for another 30 minutes in a humidified chamber at room temperature. The specimens were again subjected to 5 one-minute washes in PBS and then mounted in buffered glycerol (pH 8-9).

In each batch of sera tested, the following controls were included:

(i) filarial fragments treated with the fluorescein conjugate
(ii) filarial fragments treated with the test serum but not with the fluorescein conjugate.
(iii) filarial fragments treated with normal human serum from a healthy New Zealander, who had no history of ever being in contact with filariasis, and fluorescein conjugate.
(iv) filarial fragments treated neither with serum nor fluorescein conjugate.

Fluorescein microscopy was accomplished by use of a binocular Reichert "Zetopan" microscope equipped with a Wotan HBO 200W light source, dark field condenser, Leitz Wetzler 45/0-65 objective, BG 12/6 mm. exciter filter, clear barrier filter and wide field 8 + eyepieces.
INDIRECT FLUORESCENT ANTIBODY TECHNIQUE

Results

Microfilarial fragments as antigen

In the control samples, the fragments in most cases were just barely visible (Fig. 1A). In those cases where no microfilarial fragments were visible under UV light microscopy, the fragments were then re-stained with haematoxylin for ordinary light microscopy to confirm the presence of such fragments. In all the samples treated with elephantiasis sera a brightly fluorescent area was visible at the cut ends of the microfilariae. This fluorescence extended slightly into the body of the embryonic worm (Fig. 1B, C and D). When the microfilaria was cut only at the proximal or distal segment, the fluorescence was visible only in the cut area, and the remainder of the uncut head or tail was non-fluorescent (Fig. 1C). Furthermore, in those microfilarial fragments where cellular material spilt from their cut ends before fixation, the whole mass of this material fluoresced brightly (Fig. 1D). All microfilarial fragments in one section when treated with the same elephantiasis serum did not fluoresce with the same intensity; nevertheless the difference between a positive serum treated fragment and a negative serum treated one could still easily be distinguished.

The results obtained from the testing of the sera from Lau Islands are summarized in Table I. In a few instances where very weak fluorescence was obtained as compared to that observed when elephantiasis sera were used and it was difficult to decide between a positive and negative reaction, the serum concerned was retested. If similar weak fluorescence was obtained the serum was then classified as negative. The age distributions of infected individuals among the male and female are also shown in Table I. The 106 sera obtained from New Zealanders gave completely negative results with the indirect FA test.

TABLE I. Age-sex distribution of persons of the Lau Islands (Fiji) with antibodies against Wuchereria bancrofti demonstrable in the indirect Fluorescent Antibody test.

<table>
<thead>
<tr>
<th>Sex</th>
<th>5-9 Ex %+</th>
<th>10-14 Ex %+</th>
<th>15-19 Ex %+</th>
<th>20-29 Ex %+</th>
<th>30-39 Ex %+</th>
<th>40-49 Ex %+</th>
<th>50+ Ex %+</th>
<th>All ages Ex %+</th>
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<tbody>
<tr>
<td>Male</td>
<td>8/14 57</td>
<td>13/26 50</td>
<td>6/15 40</td>
<td>4/7 57</td>
<td>3/10 30</td>
<td>4/8 50</td>
<td>3/9 33</td>
<td>41/89 45</td>
</tr>
<tr>
<td>Female</td>
<td>7/18 39</td>
<td>9/22 41</td>
<td>7/14 50</td>
<td>7/17 41</td>
<td>8/19 42</td>
<td>7/18 39</td>
<td>3/11 27</td>
<td>48/119 40</td>
</tr>
<tr>
<td>Both sexes</td>
<td>15/32 47</td>
<td>22/48 46</td>
<td>13/29 45</td>
<td>10/24 42</td>
<td>11/29 38</td>
<td>11/26 42</td>
<td>8/20 30</td>
<td>88/208 42</td>
</tr>
</tbody>
</table>

Ex = Number of sera showing fluorescence/number of sera examined.
%+ = Percentage of sera showing fluorescence.

Infective larval fragments as antigen

Similar results were obtained with this antigen as when microfilarial fragments were used. In the negative control samples, the fragments were barely visible though they were much easier to identify under the UV light microscope than the microfilarial fragments because of their larger size (Fig. 2A). In samples treated with elephantiasis serum, the infective larval fragments fluoresced at the cut ends. The fluorescence appeared to be more extensive than that observed when microfilariae were used (Fig. 2B).

Owing to the lack of infective larval antigen only 57 sera from the Lau Islands were again tested. Out of this number of sera again tested, only 4 gave contradictory results to those obtained when microfilariae were the antigens. The particulars of the 4 sera and the results obtained with the 2 antigens are reported in Table II.
Fig. 1. Micro-fragments of microfilaria of *Wuchereria bancrofti* used as antigen in the indirect fluorescent antibody test.

A.—Fragments treated with normal human serum obtained from a person who has never been exposed to filariasis. The fragment does not fluoresce (arrow). \( \times 600 \).

B, C and D.—Fragments treated with elephantiasis serum. Specific fluorescence appears at the cut ends of the fragments. B: \( \times 600 \). C and D: \( \times 850 \).

Fig. 2. Micro-fragments of infective stage larva of *Wuchereria bancrofti* used as antigen in the indirect fluorescent antibody test.

A.—Fragment treated with normal human serum obtained from a person who has never been exposed to filariasis. The fragment does not fluoresce (arrow). \( \times 850 \).

B.—Fragments treated with elephantiasis serum. Cut ends of the larva fluoresce more brightly. \( \times 850 \).
TABLE II. Results obtained when microfilarial and infective stage larval fragments were used as antigens.

<table>
<thead>
<tr>
<th>Serum Number*</th>
<th>Sex</th>
<th>Age (Years)</th>
<th>Fluorescent antibody test</th>
</tr>
</thead>
<tbody>
<tr>
<td>L7110</td>
<td>Male</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>L7004</td>
<td>Female</td>
<td>17</td>
<td>+</td>
</tr>
<tr>
<td>L1191</td>
<td>Male</td>
<td>24</td>
<td>+</td>
</tr>
<tr>
<td>L1164</td>
<td>Male</td>
<td>15</td>
<td>-</td>
</tr>
</tbody>
</table>

*Sera from Lau Islanders.

Discussion and conclusion

With regard to work done by previous authors (CHOWDHURY and SCHILLER, 1962; MANTOVANI and SULZER, 1967; ISHII et al., 1969) this study confirms their observations that the internal tissue of both the microfilaria and infective stage larva is the site of antigen-antibody combination in the FA test for filarial infection.

Not only did different serum samples result in different fluorescent intensities with the same antigen but also the two different antigenic preparations gave different intensities with the same serum. Nevertheless, the interpretation of results obtained with the sera from Lau Islands, tested separately against the microfilariae and infective stage larva, were almost identical; with the exception of 4 sera (see Table II). No adequate reason could be given by the author to explain this apparent contradictory finding. Hence, a final choice between microfilariae and infective stage larvae as antigen cannot be made until their antigenic differences have been further studied and understood.

With a 100% positive antibody reaction for the elephantiasis sera, the findings in this investigation agreed with those reported by WONG and GUEST (1969).

A 42% positive antibody reaction out of the 208 Lau islanders’ sera examined was slightly higher than the filariasis incidence rate (about 30%) previously reported in epidemiological surveys (BURNETT, 1960; SYMES, 1960; IYENGAR, 1965; MATAIKA et al., 1971). However, this result might be explained by the fact that in all these surveys the method used for registering a positive case was microscopic examination for the presence of microfilariae in 20 c.mm. blood smears. Such a test would not register those who were infected but did not have circulating microfilariae (BEAVER, 1970). The indirect immunofluorescence test would pick out these cases. Another factor which might contribute to this higher percentage of prevalence was that in Fiji, where *Dirofilaria immitis* of the dog is common, (80%, J. U. MATAIKA, pers. comm., 1971) man could act as a paratenic host for this filarial worm (NOBLE and NOBLE, 1964; SPRENT, 1969) with possible cross-serological reactions (GARCIA et al., 1968; TANAKA et al., 1970; FUJITA et al., 1970).

No statistical significance in the differences of prevalence of filarial antibody in age and sex groups was obtained. However, with regard to Table I, the early peak of antibody prevalence may indicate that children are sensitized to filarial antigen at an early age, but the infective larvae fail to grow to maturity, or having grown to adult worms fail to reproduce microfilariae owing possibly to a single sex infection at this very early age of the children. The asymptote of prevalence of about 50% suggests that a state of dynamic equilibrium between the decline of humoral antibody and the induction of
further production of antibody from reinfection is reached in a fairly early age group. This equilibrium is maintained probably throughout the whole life-span of many people, or at least for 45 years for all people, in an endemic population. The slight drop in the percentage of older people (50 years and above) who have filarial serum antibody may be due to some older people, after having been subjected to numerous bites by infective mosquitoes through the years, evolving an entirely different immune response which cannot be detected by the humoral type of antigen-antibody reaction.

Although the degree of specificity of this FA test has not been fully ascertained, on the whole this technique warrants further investigation. In the foreseeable future it may even become the answer to the need for a more reliable and sensitive test for detecting not only new filariasis cases but also people who are still harbouring adult filarial worms although temporarily without microfilaraemia after chemotherapeutic treatment with diethylcarbamazine (Hawking, 1962; WHO, 1962 and 1967) and hence are potentially still sources of infection (Kessel et al., 1970; Mahoney and Kessel, 1971).

Summary

The indirect fluorescent antibody technique, using the actual causal agent as antigen, was evaluated as an immuno-diagnostic test for detecting patients who are sensitive to bancroftian filarial antigen. To achieve the economy in the use of the antigenic material micro-fragments, cut by means of a cryostat, of microfilariae and infective stage larvae of *Wuchereria bancrofti* were used.

The usefulness of this technique was assessed with 3 batches of human sera:—60 sera from elephantiasis patients, 208 sera collected in an endemic area of filariasis, and 105 sera from healthy citizens of New Zealand where filariasis is rare and found only in immigrants from the Pacific Islands. All elephantiasis sera gave good bright fluorescence with the internal tissue of both the microfilaria and infective stage larva, whereas all sera from New Zealanders failed to produce fluorescence. 42% of the sera obtained from the filariasis endemic area gave fluorescence of an intensity comparable to that observed from the elephantiasis sera. This percentage of the prevalence of filarial antibody obtained was compared with the microfilaraemia rates reported from previous epidemiological surveys. This difference and the pattern of the prevalence of filarial antibody was discussed and may be regarded as an indication of the sensitivity of this indirect fluorescent antibody technique in detecting also those persons who are sensitized to filarial antigen but do not possess detectable microfilariae in their peripheral blood.

REFERENCES