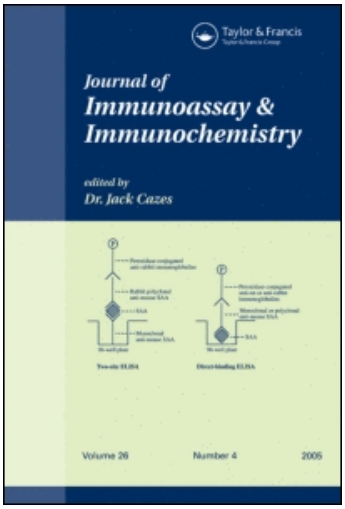

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ANALYSIS OF PAIRED SERUM, URINE AND FILTER PAPER BLOOD SPECIMENS FOR PRESENCE OF FILARIAL ANTIGEN BY IMMUNORADIOMETRIC ASSAY

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ABSTRACT

Paired serum, urine, and finger-prick whole blood dried on filter paper were analyzed by immunoradiometric assay (IRMA) for filarial antigen using Brugia malayi-specific rabbit antibody. Nine sera and 6 urines from the 10 paired serum-urine samples obtained from individuals with microfilaremia contained IRMA detectable filarial antigen. In contrast, all serum and urine specimens from patients with chronic infections, endemic and non-endemic controls were negative. Whole blood eluted from filter paper spots contained IRMA detectable material; their degree of positivity agreed well with IRMA binding levels obtained with paired urines. Reduced recovery of antigen dried on filter paper was observed at antigen levels <10 ng/ml equivalents, presumably due to irreversible absorption onto the filter paper. Urine and finger-prick filter paper blood specimens can be used in the diagnosis of microfilaremic infections that have been associated with circulating antigen in the blood.

(KEY WORDS: Filariasis, immunoradiometric assay, filarial antigens, filter paper blood, urine, diagnosis)

INTRODUCTION

Filariasis is a major health problem in tropical countries. Accurate diagnosis of this mosquito-borne nematode infection is not always possible with currently employed parasitological tests due to their limited sensitivity which is insufficient to detect low microfilaremia and occult filariasis (1,2). Detection of parasite material in the blood or urine of a patient would provide more accurate definition of a current infection. Detection of circulating filarial antigen in the serum of filarial patients has been reported by several workers. Hamilton *et al.* have used antibodies specific for *Brugia malayi* (*Bm*) adult worms to detect crossreactive filarial antigens in *Wuchereria bancrofti* (*Wb*)-infected patients with an IRMA (3). Forsyth *et al.* have employed monoclonal antibody specific for *Onchocerca gibsoni* to detect *Wb* parasite-derived antigens in infected patients (4). In an attempt to simplify specimen collection, Malhotra *et al.* have shown that human antibody specific for filarial antigens may be detected in blood spotted on filter paper using an ELISA (5). Immunoreactive material of filarial origin (antigen) has also been reported in the urine of filarial patients and its presence is suggestive of a current, active infection (6,7).

The present study had two main objectives. First, serum and urine specimens from the same patients were analyzed to examine the relationship between levels of IRMA-detectable antigen in the blood and urine. Second, filarial antigen in finger-prick whole blood dried on filter paper was compared to urine specimens obtained from

the same patients. Recovery studies were performed to examine the efficiency of filarial antigen elution from filter paper disks.

MATERIALS AND METHODS

Serum and urine samples:

A total of 33 paired human serum and urine samples were collected from four groups of individuals: Viz., non-infected, non-endemic normal (USA) volunteers (n=4); clinically normal, microscopically *microfilariae (mf) negative, endemic normal* individuals (n=9); patients with microfilariae in their peripheral blood (microfilaremics) without any clinical symptoms (n=10); and patients with clinical manifestations of filariasis without circulating microfilariae in their blood (chronic filarial patients) (n=10). The presence of microfilariae in peripheral blood was confirmed by a night wet blood smear test (8). Filarial and endemic normal blood and urine samples were collected from Sevagram and surrounding villages (India). *The urine samples were centrifuged* (1000 x g, 15 min. 4°C) to remove sediment. Serum was separated from cells immediately after collection and all sera were stored at -20°C with 0.1% sodium azide until used.

Filter paper whole blood specimens:

Filter paper blood samples were collected on Whatman 3 filter paper as previously described (5). Finger-prick whole blood was

collected in such a manner that it covered a circle with a 0.9 cm diameter (equivalent to 20 microliters of blood). Blood on the filter paper was dried at room temperature and stored for up to 6 months at 4°C in sealed plastic bags until used. Filter paper specimens tested for specific antibody have shown good stability in Sevagram for periods up to 12 months when stored at 4°C sealed in a polyethylene bags.

The filter paper with dried blood was cut into small pieces, mixed with 0.5 ml of phosphate buffered saline (PBS), pH 7.5 containing 0.5% Tween-20 and 0.2% bovine serum albumin and agitated 2 hrs at room temperature. The eluates were centrifuged (600 x g, 15 min, at 4°C) and the supernatants were analyzed immediately. Urine specimens (24 hr) were collected from the same patients at the time of blood collection and aliquots were frozen at -20°C until used.

Immunoassay reagents:

The B. malayi antigen (BmA) added to non-endemic human sera for a reference and positive control in the recovery study was prepared by Dr. Rabia Hussain (9) from adult male/female worms isolated from jirds, Meriones unguiculatus. The polyclonal anti-BmA antibody was prepared by Dr. Hussain by hyperimmunization of rabbits with BmA. The specific rabbit anti-BmA was purified by affinity chromatography of an DEAE-52 IgG fraction on Sepharose 4B-BmA. Chromatographically-purified rabbit IgG (Cooper Biomedical, Malvern, PA), BmA and affinity-purifed rabbit anti-BmA were each separately

coupled to Sepharose 4B using the procedure of Axen et al. (10). Affinity-purified rabbit IgG anti-BmA (100 ug) was radioiodinated with 2 mCi of ^{125}I-NaI (Amersham, Arlington Heights, IL) using the chloramine T method (10). Immunoreactivity of the labeled anti-BmA was 77% as determined by an overnight incubation of 120,000 cpm at 23°C with excess BmA-Sepharose followed by buffer washes to remove unbound labelled antibody.

Immunoradiometric Assay (IRMA) for Antigen:

B. malayi crossreactive antigen in serum, whole blood filter paper eluate and urine specimens was measured by an solid phase radioimmunoassay as has been previously described (3). Serum, urine or filter paper extracts (0.02 to 0.05 ml; undiluted except for filter paper extracts) were added to a 12 x 75 mm test tubes containing 0.2 to 0.5 ml of a 1% v/v concentration of either rabbit anti-BmA-Sepharose or normal rabbit IgG-Sepharose. Following a 16-18 hr rotation at 23°C, unbound serum proteins were removed with three 2.5 ml buffer washes using PBS, pH 7.5, 0.5% Tween 20 and 0.2% bovine serum albumin. ^{125}I-rabbit anti-BmA was then added (3-6 ng in 0.5 ml/tube) and the tubes were rotated 16-18 hrs at 23°C. Following a second buffer wash, bound radioactivity was quantitated in a 16 well gamma counter (CAP-RIA 16: Capintec, Ramsey, NJ) Results were expressed as a % B_{max}, where B_{max} represents the immunoreactive cpm added as determined by incubation of the ^{125}I-anti-BmA with excess BmA-Sepharose.

Binding levels to the normal rabbit IgG-Sepharose were used to define the positive binding threshold and to control for autoantibody interference (e.g. rheumatoid factor). Dose response curves were generated by analyzing non-endemic human serum containing added quantities of BmA (1 to 100 ng/ml). This reference serum was analyzed in each assay to monitor sensitivity and to provide a reference for heterologous interpolation of relative antigen levels in ng/ml equivalents (12).

Filter Paper Recovery Studies.

The efficiency of elution of filarial antigen in whole blood dried on filter paper was examined using serum or whole blood from non-endemic controls to which known amounts of BmA (0 to 100 ng/ml) were added. Whole blood or serum (0.02 ml) containing 0 to 20 ng/ml of B. malayi antigen was spotted on individual Whatman 3 filter paper disks and air dried. Elution of antigen from whole blood or serum on the filter paper was studied by analysis of the eluates in the IRMA. Recovery was determined by comparing the IRMA binding levels obtained with eluted whole blood or serum containing antigen versus serum with no added antigen.

RESULTS

Paired Urine and Serum Samples.

Paired urine and serum specimens from the same patients were analyzed concurrently in the IRMA for BmA crossreactive compon-

ents. The binding levels of multiple sera and urine specimens obtained in the IRMA are presented in Figure 1. All but one serum from the 10 microfilaremic patients were positive for BmA using the mean binding levels + 2 SD of the endemic control sera as a criterion for positivity (bottom panel, Figure 1). In contrast, only 6 paired urine samples from the same patients showed positive binding levels (top panel, Figure 1). No detectable binding above North American and endemic control levels was observed in patients with advanced chronic pathology who also had no detectable Mf in their blood.

Elution Efficiency of Filarial Antigen from Filter Paper.

The efficiency of antigen elution from the dried filter paper spot (0.02 ml of whole blood volume) was examined by comparing IRMA binding levels produced by an eluate from a given filter paper blood spot with IRMA results obtained by concurrently analyzing the same specimen not dried on paper (Figure 2). There was no detectable reduction in antigen detection by the IRMA at the 10 ng/ml level of BmA. A progressive loss in detectable antigen was observed at BmA concentrations < 10 ng/ml equiv. when whole blood was spotted on the filter paper, dried and eluted with buffer containing BSA carrier protein. The 20% reduction at 4 ng/ml of BmA and 90% at 2 ng/ml of BmA was attributed to irreversible adsorption of filarial antigen to the Whatman paper.

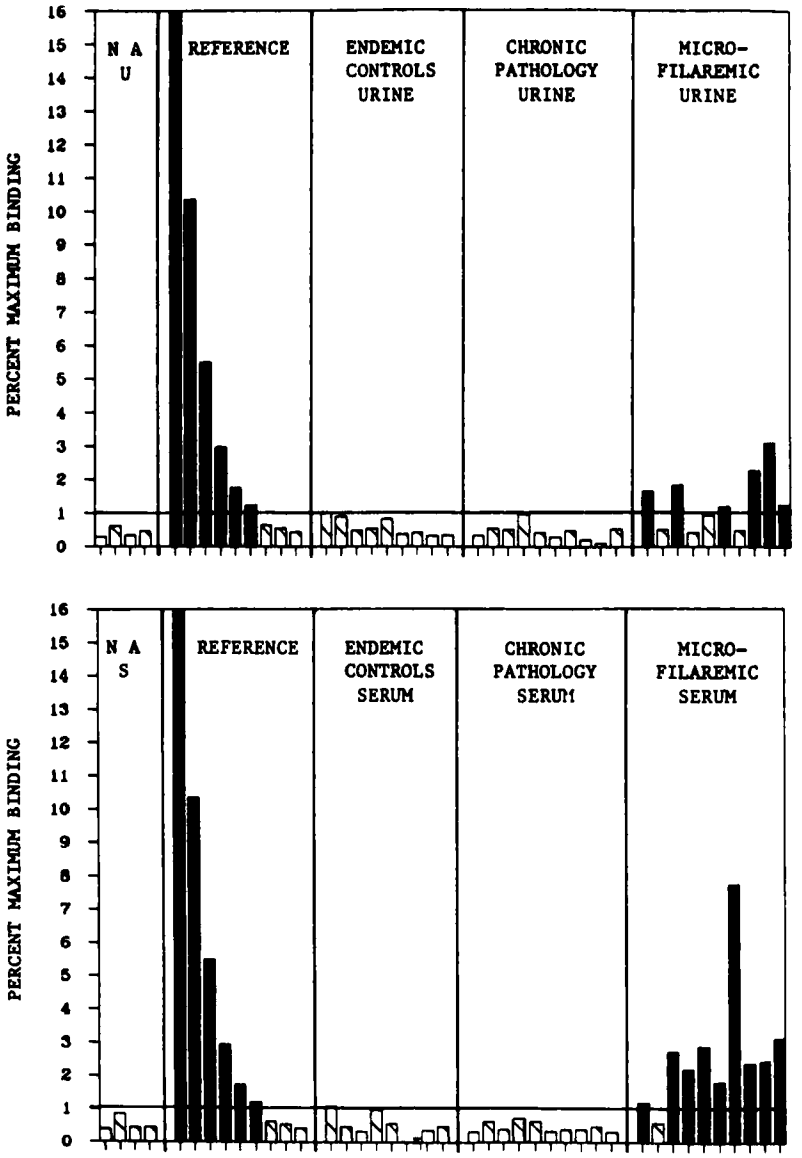

FIGURE 1.
B. malayi antigen immunoreactivity analysis of paired urine (U) (top panel) and serum (S) (bottom panel) collected from 4 groups of individuals: North American (NA) non-endemic controls, amicrofilaremic endemic controls and filarial patients with chronic pathology or microfilaremia. Bm-crossreactive antigen was detected only in the unconcentrated urine and serum of patients with microfilaremia. Binding levels were compared to reference serum containing no antibody and defined amounts of Bm-antigen.

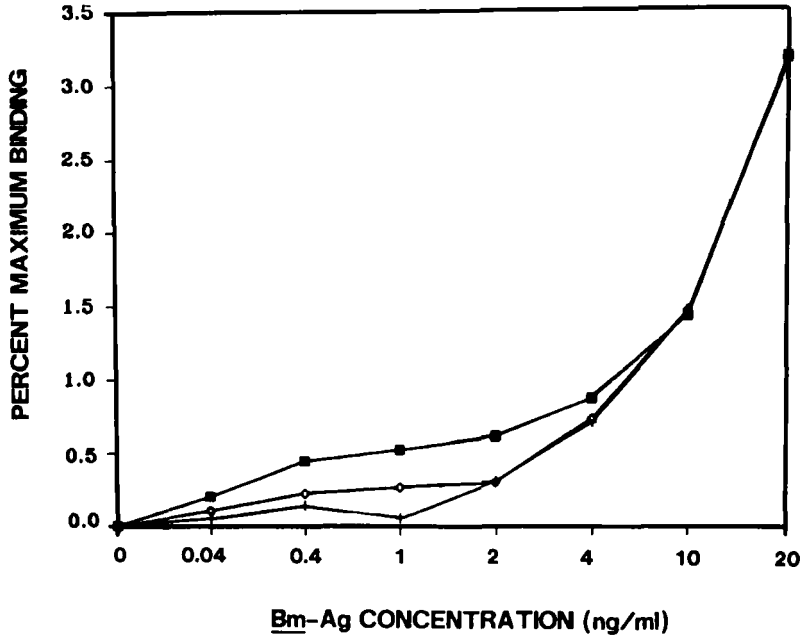

FIGURE 2.
Recovery of Bm antigen eluted from filter paper spotted with 20 ul of serum (◇) or whole blood (✚) containing BmA (0-100 ng/ml). IRMA binding levels obtained with eluates were compared to those obtained by direct analysis of the same serum (■). The limit of sensitivity of the IRMA under conditions of this experiment was 1 ng/ml (0.5% Bmax). Levels of BmA in serum or whole blood were reduced by 20% at 4 ng/ml equiv. and 90% at 2 ng/ml equiv. of BmA when whole blood and serum were spotted on filter paper, dried and eluted with buffer. Above 10 ng/ml, no detectable loss in antigen was observed due to filter paper adsorption.

Paired Urine and Filter Paper Blood Samples.

Whole blood specimens spotted on filter paper and a paired urine specimen collected from W. bancrofti microfilaremic patients were analyzed concurrently in the IRMA for BmA immunoreactive components (Figure 3). In all but one case, binding levels > 10 ng/ml equiv-

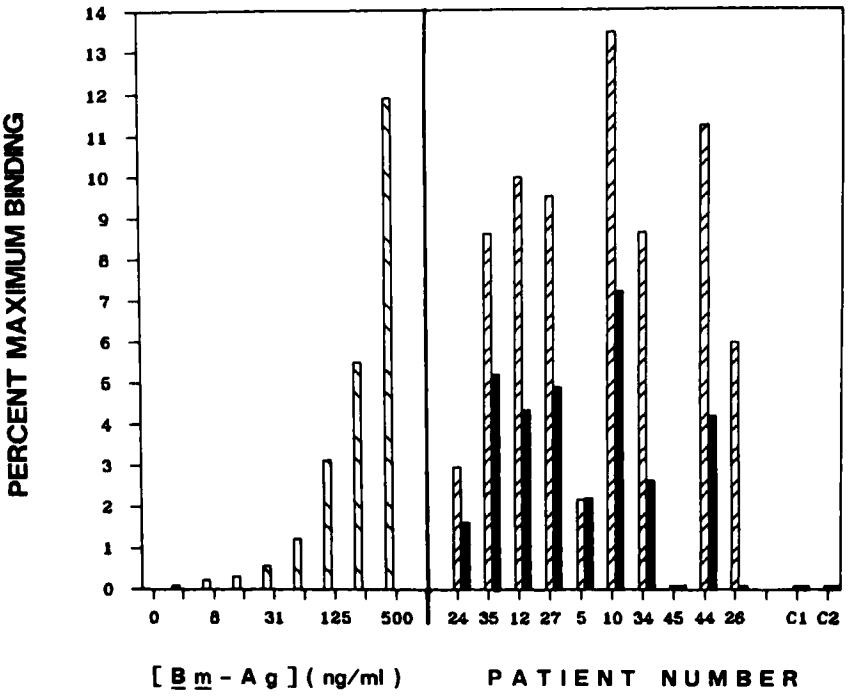

FIGURE 3.
B. malayi-antigen immunoreactivity analysis of paired urine (black bars) and whole blood filter paper specimens (hatched bars) collected at the same time from 10 filarial patients with microfilaremia and 2 endemic controls (right panel) . Binding levels were compared to reference serum containing no antibody and added amounts of Bm-antigen (500 to 1.5 ug/ml) (left panel). Immunoreactive material was detected in all but one eluted whole blood filter paper specimen and 8 of 10 urine specimens.

alents were detected in filter paper whole blood samples. In two cases, the binding levels obtained with urines were below the limit of detectability of the assay.

Table 1 displays the microfilariae count in the 10 microfilaremic patients as detected by a wet whole blood smear and the relative filarial antigen levels detected in the immunoassay. There is

TABLE 1

Patient Number	Mf Count in whole blood (# per 20 mm^3)	Relative Antigen Level in Serum (% Bmax) •
26	2	5.98
24	3	2.96
45	6	0.02
5	10	2.17
12	10	10.00
34	10	8.66
35	17	8.63
44	22	11.24
27	31	9.53
10	34	13.43

• Percent maximum binding in solid phase radioimmunoassay

a suggestive trend of higher levels of immunoassay detectable filarial antigen in the serum of patients with the higher Mf counts.

DISCUSSION

Recent isotopic and non-isotopic immunoassays have facilitated the diagnosis of human filariasis through more accurate detection of host antibody and parasite antigen in human blood (1,2). Despite these advances in laboratory methodology, patient specimen collection still remains a major problem in serological diagnosis. Individuals living in tropical areas where filariasis is endemic are reluctant to give blood by venipuncture. Thus, there has been a need to examine the usefulness of finger-prick whole blood

samples and urine as possible alternative test specimens. Earlier studies by our group have shown that the IRMA can detect B. malayi crossreactive components in undiluted urine samples from microfilaremic patients. This study was unable, however, to examine the concominant presence of filarial antigen in the blood and urine of infected patients due to the unavailability of paired specimens. The present study compares binding levels obtained with paired urine and serum samples from the same infected individuals and it examines the utility of filter paper finger-prick blood specimens.

The frequency of positivity in the BmA IRMA was 90% with 0.05 ml of undiluted serum and 60% with 0.1 ml of undiluted urine from the same microfilaremic patients (Figure 1). IRMA binding levels obtained with serum were generally 2 to 10 times higher than those observed in the paired urine sample. There was no direct correlation between the relative level of antigen circulating in the blood and that detected in the urine. One urine sample was negative in the IRMA, despite a high level of IRMA binding with the paired serum. This lack of correlation presumably reflects differential loss in kidney function between patients as a result of chronic filariasis infections.

Blood samples collected on filter paper have been previously used in an ELISA to detect host antibody specific for filarial antigens (5). This study expands these observations and shows that filter paper eluates can be employed in the IRMA as a substitute for undiluted serum samples. There can be significant losses of antigen on the filter paper, especially when the level of antigen in the blood is low. Filter paper eluates of non-endemic whole

blood should be used in parallel in each assay to control for non-specific binding and to establish a positive threshold. Because the IRMA employs both a solid phase and labeled antibody, there was concern that rheumatoid factor (anti-immunoglobulin antibodies) in the patients sera may cause false positive binding results. False positive IRMA binding levels resulting from either rheumatoid factor, hypergammaglobulinemia or other factors in the blood were monitored using polyclonal rabbit IgG in place of rabbit anti-BmA on a solid phase. False positive results were not observed with the sera tested in this study.

One reported difficulty with the filarial antigen detection in human serum is the presence of specific host antibody that either complexes antigen and clears it from circulation or interfers in the immunoassay (2). Host antibody can reduce the sensitivity of the antigen IRMA by masking antigen determinants which otherwise would be bound by solid phase or labeled antibody. In addition to its ease of collection, urine may selectively filter antigen while impeding larger antibody molecules. This theoretical advantage, however, is overshadowed by the fact that the kidney must be damaged sufficiently to allow these proteins to pass. This problem is reflected in this study as a lower frequency of IRMA positivity observed with urine than was obtained with the serum specimens.

An important group of filariasis-infected individuals that may be identified by a serological test are those who have an early infection which is prepatent (e.g. the level of microfilariae in the blood is below the limit of detection of microscopic assays).

Individuals with chronic filarial infections can be generally diagnosed by characteristic pathology which in its advanced stages may include elephantiasis and scrotal swelling. While the presence of host antibody can be an interfering factor, immunoassays remain the most sensitive methods available for the diagnosis of patients with early infections. This study has demonstrated that urine and filter paper whole blood specimens can be used in conjunction with the immunoassay for the diagnosis of human filariasis. Restrictions in the amount of antigen that may be detected in urine or eluted from whole blood filter paper specimens indicate that the greatest application of these specimens may be in the diagnosis of human microfilaremic infections.

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