

Detection of Antigens and Antibodies in the Urine of Humans with *Plasmodium falciparum* Malaria

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Humans infected with *Plasmodium falciparum* frequently have elevated levels of proteins in their urine, but it is unclear if any of these proteins are parasite antigens or antimalarial antibodies. To resolve this question, urine samples from malaria patients and controls living in Thailand and Ghana were evaluated. Urine samples from 85% of the patients had elevated protein levels and contained proteins with M_r s ranging from <29,000 to >224,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antisera were produced against urine from infected and control subjects. Antisera raised against infected, but not control, urine were positive by indirect immunofluorescence on *P. falciparum* parasites and immunoprecipitated approximately 12 unique bands from extracts of parasites metabolically labeled with ³⁵S-methionine. These data suggest that a variety of *P. falciparum* antigens are released into urine during acute infection. It is also likely that anti-*P. falciparum* antibodies are present in the urine of malaria patients because samples from these patients, but not controls, were positive in indirect immunofluorescence assays and immunoprecipitated at least 19 *P. falciparum* antigens from extracts of metabolically labeled parasites. The detection of malarial antigens and antibodies in urine may lead to a new approach for the diagnosis of malaria.

Individuals with fever commonly have elevated levels of protein in their urine (8, 10, 16, 25). It is therefore not surprising that proteinuria has been reported in patients infected with *Plasmodium falciparum*, a disease characterized by cyclical fevers (3, 7, 17, 19). Recently, Ehrlich and Horstmann examined urine samples from seven malaria patients by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and found that *P. falciparum* patients excreted a variety of proteins (5). It is unknown, however, if any of these proteins are parasite antigens or potentially antimalarial antibodies.

Antigens and/or antibodies to a variety of pathogens including *Leishmania donovani* (14), *Trypanosoma cruzi* (12), *Mycobacterium leprae* (11), and human immunodeficiency virus type 1 (HIV-1) (4) have been found in urine. The identification of malarial antigens in urine would have practical benefits. First, there may be a correlation between the number and molecular weights of malarial antigens released into urine and the level of renal pathology present in the host. Such a relationship would be of clinical value. Secondly, the detection of malarial antigens or antibodies in urine could provide new approaches for the diagnosis of malaria.

In this study, we sought to determine if *P. falciparum* antigens and/or antibodies were excreted into human urine. To identify malarial antigens, mice were immunized with urine from infected and normal human subjects. The resulting antisera were used in immunoblotting, immunoprecipitation, and immunofluorescence assays for antigen characterization. At least 12 proteins of parasite origin were detected in the urine of infected patients. Immunofluorescence and immunoprecipitation studies also demonstrated the presence of antimalarial antibodies to a variety of malarial antigens in the urine of patients with acute *P. falciparum* malaria.

MATERIALS AND METHODS

Urine samples. Urine samples were collected in the Chantaburi region of Thailand from adults with *P. falciparum* infections. Clinical histories were taken by the attending physicians, and parasitemias were confirmed by blood smears. Clean-catch urine samples were then obtained from the patients before the administration of antimalarial chemotherapy. Urine samples were transferred to sterile test tubes, immediately frozen at -20°C, and shipped to Georgetown University on dry ice. A total of 30 urine samples from 10 female and 20 male patients were obtained. The patients had a mean age of 32 years (standard deviation, 15 years) and a mean parasitemia of 1.1%, with counts ranging from 0.014 to 5.4%. Many of the subjects reported having had malaria multiple times. For comparison, 19 negative control urine samples were obtained from Thai adults residing in Bangkok where *P. falciparum* is not transmitted. Samples and clinical data were obtained in 1988 by H. Wilde through the DiaTech Program, Program for Appropriate Technology and Health (P.A.T.H.), Seattle, Wash.

Urine samples were also collected from 16 children residing in Accra, Ghana, who were infected with *P. falciparum*. Midstream urine samples were obtained whenever possible from 10 females and 6 males, with a mean age of 5 years (standard deviation, 2.5 years) and a mean parasitemia of 2.4% with counts ranging from 0.3 to 7.3%. After being collected, urine samples were centrifuged, the fluid phase was collected, and 0.1% sodium azide was added prior to freezing. All samples were kept frozen during transport and maintained at -20°C until used. In addition, midstream urine samples were collected from adolescents who had *Schistosoma haematobium* infections but were negative by blood smear for malaria. Urine from patients with *S. haematobium* was selected as a negative control because these patients have proteinuria due to a parasitic infection. Ghanaian samples were collected in 1983 as part of the Coordinated Research Programme on Nuclear Techniques for the Detec-

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Several additional negative control urine samples were studied. These included 10 clean-catch urine samples from healthy Americans and a 24-h sample from an American with multiple myeloma containing Bence-Jones proteins. These urine samples were frozen and treated in the same manner as those from Thai adults.

Pooled urine samples were used in many of the studies. To prepare these, equal volumes of urine from the 30 Thai patients were combined; the pool is referred to as pooled (+) Thai urine. A second pool was prepared by combining urine samples from 16 Ghanaian malaria patients and is referred to as pooled (+) Ghanaian urine. A similar pool was prepared from uninfected Thai, Ghanaian, and American controls and is referred to as pooled control urine.

Urinalysis. Individual urine samples were thawed, and urinalysis was performed by using Multistix 10 SG reagent strips (Miles Diagnostics, Elkhart, Ind.). The analysis included measurements of glucose, bilirubin, ketones, specific gravity, blood, pH, protein, urobilinogen, nitrites, and leukocytes.

Preparation of antisera. Female BALB/c mice, 6 to 10 weeks old (Harlan Sprague-Dawley, Frederick, Md.) were immunized three times with (i) pooled (+) Thai urine, (ii) pooled (+) Ghanaian urine, or (iii) pooled control urine. In the first immunization, mice were injected with 200 μ l of urine emulsified in Freund complete adjuvant (Calbiochem, La Jolla, Calif.) in a single subcutaneous site. Approximately 14 days later, they were injected intraperitoneally with 200 μ l of urine in Freund incomplete adjuvant. The third boost consisted of 200 μ l of urine (without adjuvant) injected intraperitoneally. Fourteen to 30 days later, the mice were anesthetized with sodium pentobarbital and blood was collected directly from the heart. Sera were stored at -20°C until used.

IFA assay. Sera from immunized mice and unconcentrated urine samples from patients with *P. falciparum* were tested for the presence of antimalarial antibodies by indirect immunofluorescence antibody (IFA) analysis. Routine in vitro cultures of *P. falciparum* parasites of the NF54 (Netherlands Airport strain) and Malayan Camp (MC) strains were maintained by using the method described by Trager and Jensen (22). Blood smears of cultured parasites were prepared, air dried, and fixed in acetone. The IFA method of Voller was followed (24). In brief, serum samples were diluted in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) and applied to the parasite smears for 20 min at room temperature. Smears were then washed in three changes of 0.1 M PBS (pH 7.4), and a 1:40 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, Mo.) was applied for 20 min at room temperature. Smears were washed in three changes of 0.1 M PBS (pH 7.4), mounted in barbital-glycerol buffer (0.1 M sodium barbital, 50% glycerol [pH 8.6]) and examined by use of a Laborlux epifluorescence microscope (Leitz, Wetzlar, Germany) equipped with a band pass filter (450 to 490 nm).

SDS-PAGE. Proteins in pooled urine samples were separated under nonreducing conditions by SDS-PAGE by using the method described by Laemmli (15). In brief, urine samples were diluted 1:1 in nonreducing sample buffer, incubated at 95°C for 4 min, and then separated on an SDS-10% polyacrylamide slab gel by using a Mini-protein II Electrophoresis Cell (Bio-Rad Laboratories, Richmond, Calif.). Proteins were stained by using the silver stain method of Merrill et al. (18). Prestained molecular-weight

standards were purchased from Bethesda Research Laboratories (Bethesda, Md.).

Immunoblot analysis. The transfer of proteins was performed by using the method of Towbin et al. (23). In brief, proteins were transferred onto nitrocellulose sheets (0.45- μm pore size; Bio-Rad Laboratories) by using a mini Trans-blot Electrophoresis Transfer Cell (Bio-Rad) and following the directions of the manufacturer. After transfer, nitrocellulose papers were soaked in blocking solution (0.3% bovine serum albumin and 0.3% Tween-20 in 0.1 M PBS [pH 7.4]) overnight. The blots were then incubated with a 1:500 dilution of sera from immunized mice in blocking solution diluted 1:100 with 0.1 M PBS (pH 7.4) for 4 h. Blots were then washed three times with 0.05% Tween-20 in 0.1 M PBS (pH 7.4) and incubated for 2 h with a 1:500 dilution of horseradish peroxidase-labeled goat anti-mouse polyvalent immunoglobulin (Sigma) in blocking solution. After three additional washes, the blots were developed by using the 4-Cl-1-naphthol substrate (60 mg of horseradish peroxidase color development reagent [Bio-Rad] in 20 ml of methanol combined with 100 ml of 0.015% H_2O_2 in 0.1 M PBS [pH 7.4]). All incubations and washes were performed at 4°C , with samples being agitated at the lowest speed possible on a clinical rotator (Thomas Scientific, Swedesboro, N.J.).

Metabolic labeling of parasites. Asynchronous cultures of *P. falciparum* NF54 parasites were maintained in vitro as described by Trager and Jensen (22). For metabolic labeling studies, erythrocytes which had been kept in the refrigerator for 3 weeks were used to ensure that leukocytes were no longer viable. Since mature erythrocytes do not incorporate amino acids, [^{35}S]methionine ([^{35}S]Met)-labeled proteins in this study were synthesized by the parasite.

For metabolic-labeling studies, parasites were cultured in media deficient in methionine by using Selectamine RPMI-1640 (Gibco Laboratories, Grand Island, N.Y.). Medium was supplemented with 10% normal human A^+ serum, 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.27% sodium bicarbonate, and 10 μg of hypoxanthine (Sigma) per liter. [^{35}S]Met (185 MBq) (ICN Biomedicals Inc., Irvine, Calif.) was added at a concentration of 100 $\mu\text{Ci}/\text{ml}$. Cultures were gassed with 3% O_2 -6% CO_2 -91% N_2 and maintained in 75- cm^2 flasks at a 5% hematocrit. The cells were cultured with the radioactive amino acid for 12 h. Final parasitemias were between 7 and 10%.

After metabolic labeling, infected erythrocytes were lysed with buffer containing 1% Triton X-100 in 0.1 M PBS (pH 7.4)-1 mM iodoacetamide (Sigma)-1 mM phenylmethylsulfonyl fluoride (Sigma) for 15 min at 4°C and then centrifuged at $1,125 \times g$ for 10 min. The Triton X-100-soluble material was used in the immune precipitations studies described below.

Immune precipitation. Immune precipitations were performed by use of the method suggested by Kessler (13) with modifications. To prevent nonspecific binding of malarial proteins to protein A, 1 ml of the Triton X-100-soluble extract was incubated with 0.5 ml of a 50% slurry of protein A-Sepharose (Pharmacia, Piscataway, N.J.) for 30 min at room temperature. Aliquots (50 μl) of the preadsorbed extract were then incubated with (i) 10 μl of sera from mice immunized with control human urine, (ii) 10 μl of sera from mice immunized with pooled (+) Ghanaian urine, (iii) 50 μl of pooled control human urine, or (iv) 50 μl of pooled (+) Ghanaian urine for 16 h at 4°C . The aliquots were then incubated with 75 μl of a 50% slurry of protein A-Sepharose

TABLE 1. Summary of urinalysis results

Subjects	No. of subjects with:												
	Protein ^a					Bilirubin ^b				Blood ^b			
	-	tr	30-100 mg/dl	100-300 mg/dl	300-2,000 mg/dl	-	+	++	+++	-	+-	+	++
<i>P. falciparum</i> infected													
Thailand (n = 30)	0	4	12	13	1	18	8	3	1	22	3	2	3
Ghana (n = 16)	1	2	6	5	2	14	1	1	0	14	1	0	1
Uninfected													
Thailand (n = 19)	14	5	0	0	0	19	0	0	0	19	0	0	0
United States (n = 10)	6	4	0	0	0	10	0	0	0	10	0	0	0
Proteinuria controls													
<i>S. haematobium</i> ^c (n = 8)	0	2	3	2	1	8	0	0	0	7	1	0	0
Myeloma ^d (n = 1)	0	0	0	1	0	1	0	0	0	1	0	0	0

^a -, negative. The lowest level detectable is 15 to 30 mg of albumin per dl.

^b Qualitative estimates expressed as negative (-), trace (+-), positive (+), high (++), and very high (+++). The lowest levels detectable are as follows: bilirubin, 0.4 to 0.8 mg/dl; blood, 0.015 to 0.062 mg of hemoglobin per dl.

^c Patients with *S. haematobium* from Ghana.

^d American patient with multiple myeloma.

for 1 h at room temperature. The samples were washed once with 1% bovine serum albumin (Sigma) in NETT buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris base [pH 7.4], 3 mM Na₂S₂O₃, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride), twice with NETT buffer, once with 650 mM NaCl in NETT buffer, and a finally again in NETT buffer. Immunoprecipitated samples were combined with 25 μ l of reducing sample buffer, heated at 95°C for 5 min, and separated on an SDS-10% polyacrylamide gel. Gels were incubated in Resolution (E.M. Corp., Chestnut Hill, Mass.), dried, and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) for 1 to 5 days at -65°C.

RESULTS

Summary of urinalysis. Elevated levels of proteins (≥ 30 mg/dl) were found in the urine of 26 of 30 (87%) of the adult patients from Thailand and 13 of 16 (81%) of the infected children from Ghana (Table 1). Protein levels varied among the patients, with the majority of specimens containing 30 to 300 mg/dl. Proteinuria was not observed in the normal American control subjects but was recorded for individuals with *S. haematobium* infections and the adult with multiple myeloma (100 to 300 mg/dl). Increased levels of hemoglobin (≥ 0.045 mg/dl) were detected in the urine of only 5 of 30 and 1 of 16 malaria patients from Thailand and Ghana, respectively (Table 1). Bilirubin, which is common in *P. falciparum* patients with severe renal complications (20), was observed in 12 of 30 (40%) Thai and 2 of 16 (13%) Ghanaian patients.

All urine samples from control and infected individuals had values within the normal range for specific gravity, pH, urobilinogen, and glucose. Bacteriuria was measured by the detection of nitrites, and approximately 12% of the urine samples from infected patients were found to be positive (i.e., ≥ 0.1 mg/dl). Urine samples from the control group were negative for bilirubin, hemoglobin, nitrites, and leukocytes.

SDS-PAGE analysis of urine samples. In initial studies, 5 μ l of (i) pooled (+) Thai, (ii) pooled (+) Ghanaian, and (iii) control urine samples were separated under nonreducing conditions by SDS-PAGE and visualized by silver staining (Fig. 1). Results clearly show that the concentration of protein in infected urine samples was considerably greater than in uninfected urine samples, thus supporting the above

urological finding of proteinuria. There were numerous protein bands present in urine samples of malaria patients from Thailand and Ghana that were not seen in the urine of control subjects (Fig. 1, lanes 1 and 2 versus lanes 3 to 6). The total number (approximately 15) and the M_r s of these bands (ranging from $<29,000$ to $>224,000$) were similar in pooled (+) urine samples from both Thai adults and Ghanaian children. Urine from uninfected, normal Thais (lane 3) and Americans (lane 6) showed only a few weak bands. Urine from proteinuria-control patients showed a clear band of Bence-Jones proteins (dimer of light chains) in the individual with multiple myeloma (lane 4) and a single distinct band at an M_r of 224,000 in subjects with *S. haematobium*

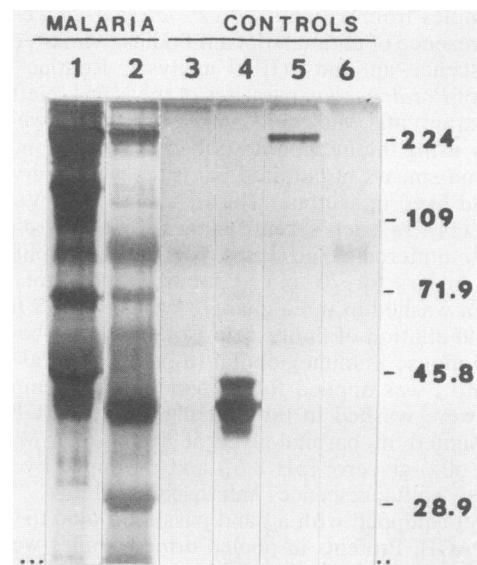


FIG. 1. SDS-PAGE analysis of urine samples. Pooled urine samples were diluted 1:1 in nonreducing sample buffer, separated on an SDS-10% polyacrylamide gel, and visualized by using silver stain. Each lane contains 5 μ l of urine. Lanes: 1, pooled (+) Ghanaian urine; 2, pooled (+) Thai urine; 3, pooled control urine from Thailand; 4, urine from a patient with multiple myeloma; 5, pooled control urine from Ghanaian patients with schistosomiasis; and 6, pooled control urine from normal Americans.

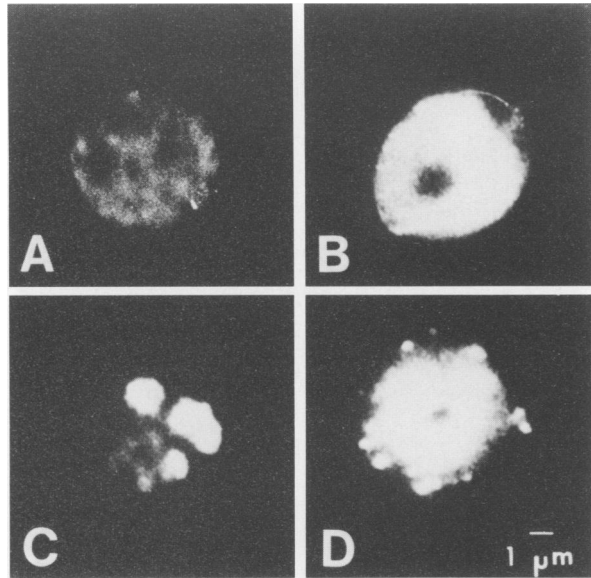


FIG. 2. Representative patterns of IFA produced by sera from mice immunized with urine from patients with *P. falciparum* malaria. Acetone-fixed *P. falciparum* blood smears were treated with sera from mice immunized with pooled (+) Ghanaian urine (antibody titer, 1:500). Similar patterns were produced by using sera from mice immunized with pooled (+) Thai urine. Sera from mice immunized with pooled control urine were routinely negative at 1:50 dilution.

infections (lane 5). Thus, SDS-PAGE results demonstrate both a high concentration of protein and unique protein bands in pooled urine samples from malaria-infected individuals.

Detection of malarial antigens in urine. Antisera from mice immunized with pooled (+) Thai and pooled (+) Ghanaian urine were tested by IFA analysis (Fig. 2). Results showed that sera from these mice, but not from mice immunized with normal urine, reacted specifically with *P. falciparum* parasites (Fig. 2). Thus, mice produced antibodies to malarial antigens present in pooled (+) Thai and pooled (+) Ghanaian urine samples.

In general, the antisera produced several different patterns of fluorescence depending on the developmental stage of the asexual parasite (Fig. 2). Staining was seen as diffuse fluorescence in infected but not normal erythrocytes (Fig. 2A), associated with the parasite itself (Fig. 2B and C), in packets in the cytosol of infected erythrocytes (Fig. 2D), and surrounding freed merozoites (data not shown). IFA patterns were similar when sera from mice immunized with pooled (+) Thai or (+) Ghanaian urine were used and when the two different strains of *P. falciparum* parasites were used. Sera from mice immunized with control human urine were routinely negative for parasite reactivity.

When the above-described antisera were used, unique antigens were detected by immunoblot analysis in the urine of patients with malaria from Thailand and Ghana. As can be seen in Fig. 3, lanes 1 and 3, approximately 9 to 12 bands, with M_r s ranging from 20,000 to 200,000, were observed in the urine from infected patients, many of which were not present in the matched control urine samples (lanes 2 and 4). Similar results were obtained when antisera raised against pooled (+) Thai and pooled (+) Ghanaian urine were utilized, indicating the presence of similar proteins in the urine

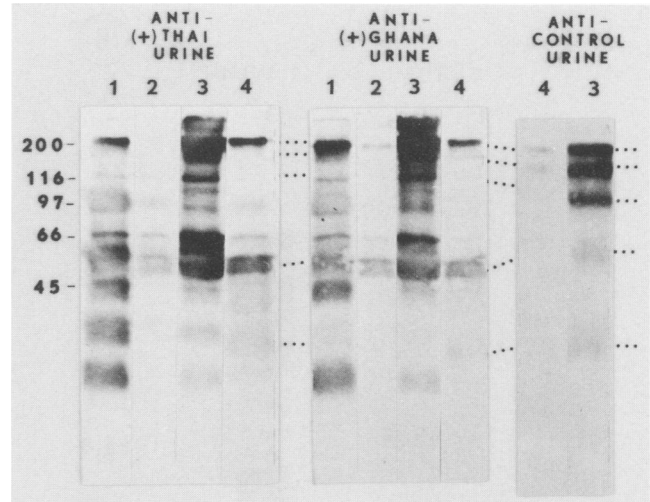


FIG. 3. Immunoblot analysis of pooled urine samples. Urine samples were separated by SDS-PAGE and transferred to nitrocellulose. Urine samples were from Thai patients (lane 1), uninfected Thais (lane 2), Ghanaian patients (lane 3), and Ghanaians who were malaria negative but had schistosomiasis (lane 4). Blots were treated with sera from mice immunized with pooled (+) Thai urine, pooled (+) Ghanaian urine, or pooled control urine. Dotted lines denote proteins normally found in all urine. Some of the other bands may be of parasitic origin.

of both groups of patients. Sera from mice immunized with control human urine recognized approximately five bands in urine samples from patients with malaria and schistosomiasis (represented by dotted lines). On the basis of the M_r s, some of these bands most likely are albumin, transferrin, and immunoglobulin.

To determine if the unique bands seen above were detected in the urine of individual malarial patients, urine samples from 21 infected and 7 control Thai adults were assessed by immunoblotting. The results are summarized in Table 2. In total, 15 bands were observed in the 21 patients and 4 bands were observed in the 7 controls. For simplicity,

TABLE 2. Detection of antigens in individual urine samples

Antigen band designation	Approx M_r (10^3)	% of urine samples from ^a :	
		<i>P. falciparum</i> -infected patients (n = 21)	Noninfected controls (n = 7)
A	250	100	18
B	224	44	0
C	210	78	0
D	175	62	0
E	155	80	0
F	112	48	0
G	103	100	0
H	90	48	0
I	83	100	96
J	70	10	0
K	67	100	100
L	59	81	0
M	40	71	50
N	35	38	0
O	32	100	0

^a Percentages of the urine samples in which the antigen was detected by immunoblotting.

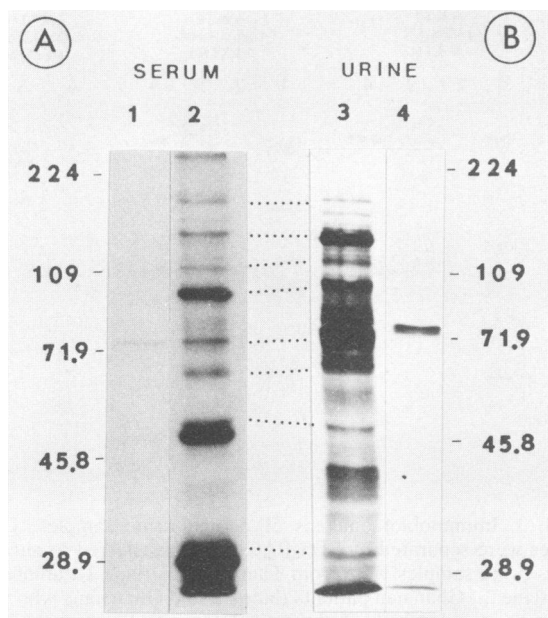


FIG. 4. Immune precipitation of proteins from *P. falciparum* parasites metabolically labeled with [35 S]Met. (A) Parasite antigens were immunoprecipitated by using sera from mice immunized with pooled control urine (lane 1) and pooled (+) Ghanaian urine (lane 2). (B) Parasite antigens were immunoprecipitated by using pooled (+) urine from Ghanaian patients (lane 3) and pooled control urine (lane 4). Dotted lines designate antigens that may appear in urine in the form of immune complexes.

the bands have been assigned letters (Table 2). Bands A, I, K, and M appear to represent normal proteins commonly found in urine, whereas the remaining 11 bands were detected only in samples from malaria patients. Table 2 shows that many of the unique bands were frequently found in urine samples (i.e., bands B, C, D, E, L, M, and O). The results suggest that some of the unique bands may be of parasitic origin and that they are routinely excreted during acute *P. falciparum* infection.

Identification of parasite proteins. To determine whether any of the unique bands detected were of parasite origin, antisera to pooled (+) Ghanaian and control urine were used to immunoprecipitate antigens from extracts of *P. falciparum* parasites metabolically labeled with [35 S]Met. The results are shown in Fig. 4A. At least 12 unique proteins were precipitated by the antisera of mice immunized with urine from malaria patients (lane 2). The antigens had approximate M_r s of 215,000, 187,000, 179,000, 139,000, 103,000, 62,000, 55,000, 52,000, 40,000 (faint), 34,000, 30,000 and 25,000. Only the band of M_r 74,000 was precipitated from the extracts by sera from mice immunized with control urine (lane 1).

Detection of antimalarial antibodies in urine and identification of their corresponding malarial antigens. To determine if antimalarial antibodies were present in the urine of patients with *P. falciparum* malaria, urine samples from 20 Thai and 6 Ghanaian patients and from 3 controls were tested by IFA. Antibodies were detected in all 26 patients; the 3 controls were negative. Three different patterns of fluorescence were observed: (i) fluorescence restricted to the parasite itself (Fig. 5A to C), (ii) diffuse fluorescence associated with infected but not normal erythrocytes (Fig. 5D), and (iii) as packets of fluorescence (Fig. 5E and F). Antibodies that

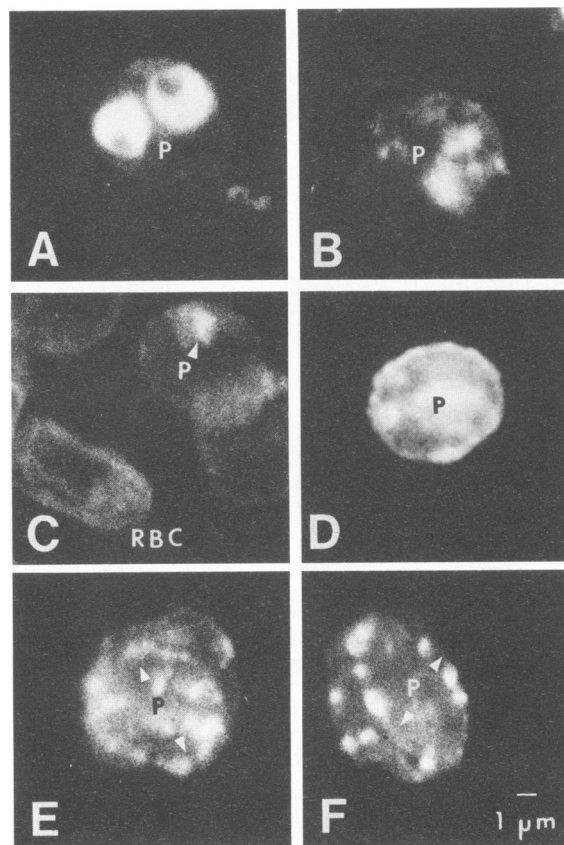


FIG. 5. Indirect immunofluorescence patterns produced by urine from *P. falciparum* patients. Acetone-fixed *P. falciparum* blood smears were treated with 5- μ l urine samples from malaria patients.

reacted with normal (uninfected) erythrocytes were detected in only 3 of 26 patients (Fig. 5C).

To determine the M_r of the parasite antigens recognized by antibodies in urine, immune precipitation studies were performed by using pooled (+) Ghanaian urine as a source of antibodies. The results are shown in Fig. 4B. Approximately 20 [35 S]Met-labeled protein bands, with M_r s ranging from <25,000 to 160,000, were identified (lane 3). Urine from negative controls precipitated only one band, which had an M_r of 74,000. Thus, it appears that antibodies that reacted with at least 19 different malarial polypeptides could be detected in human urine. A comparison of the immunoprecipitation results shown in Fig. 4A (lane 2) and 4B (lane 3) suggests that some antigens (indicated by dotted lines) and their corresponding antibodies may both be excreted into urine during acute malaria infections.

DISCUSSION

Data from previous studies strongly suggested that malarial antigens and possibly antibodies might be released into urine during acute infection. Immunohistological studies of renal tissue from patients with *P. falciparum* had revealed the presence of immune complexes, including C3, antigen, and antibody lining the glomeruli (2, 6, 20) and intact parasitized erythrocytes sequestered in the nephrons (21). Occasionally a few intact erythrocytes were seen in the urine of patients (1, 7, 20). These results, along with the documented reports of proteinuria (3, 7, 17, 19), made it likely

that parasite antigens and antibodies would be excreted into urine.

Results from the current study support this conclusion. Elevated levels of proteins (>30 mg/dl) were present in the urine of 84% of the patients studied (Table 1). Protein levels in these patients were approximately equivalent to those observed in proteinuria controls, namely individuals with chronic *S. haematobium* infections and multiple myeloma. When mice were immunized with pooled urine from malaria patients, they produced antibodies that reacted with *P. falciparum* parasites in the IFA assay. Mice immunized with pooled urine from control subjects failed to produce antibodies that reacted with malarial parasites. These data suggest that malarial antigens are present in the urine of *P. falciparum* patients.

Additional studies showed that antisera from mice immunized with pooled (+) urine immunoprecipitated a set of [³⁵S]Met-labeled *P. falciparum* proteins. Since only malarial proteins are labeled in *in vitro* cultures, the bands shown in Fig. 4 (lane 2) represent proteins synthesized by *P. falciparum* parasites. A single malarial protein was immunoprecipitated by using antisera from mice immunized with a pool of control urine (Fig. 2, lane 1). This band may be the result of nonspecific binding or may represent a cross-reactive epitope between malarial parasites and an altered host protein present in urine. On the basis of these results, it seems logical to conclude that malarial antigens are released into urine during acute infection and that most of the unique bands depicted in Fig. 4 (lane 2) represent the molecular weights of these antigens within the parasite itself. Undoubtedly, the numbers and molecular weights of these antigens could differ in urine.

Clearly, the antisera used in immunoblotting studies react with both malaria- and host-derived antigens (Fig. 2 and Table 2). The antisera to control urine detected a set of bands in the urine of both malaria and control subjects. Conversely, anti-pooled (+) Thai and anti-pooled (+) Ghanaian antisera reacted with proteins in control and infected urine. The common reactivities are shown by dotted lines in Fig. 3. However when anti-pooled (+) urine antiserum was used, additional reactivities were observed in the urine of malaria patients that are absent in the controls. Because proteins could be diluted out in a "pool," individual urine samples were assessed (Table 2). As expected, several of the antigens were found in most urine samples, but others were detected only in the urine of malaria patients (Table 2). Some of the unique proteins were found in the majority of malaria patients tested. Although it is possible that some of the unique bands in Table 2 represent reactivities to elevated levels of host proteins (e.g., acute-phase proteins), data from IFA and immunoprecipitation studies strongly suggest that many are parasite proteins.

It is also likely that antimalarial antibodies of multiple antigenic specificities are excreted during acute *P. falciparum* infection. Urine from Ghanaian children with acute malaria, but not from adolescents with chronic schistosomiasis, contained antibodies that reacted with *P. falciparum* parasites by IFA and specifically immunoprecipitated [³⁵S]Met-labeled parasite antigens. The finding of antibody in urine is not surprising since SDS-PAGE analysis of urine from malaria patients showed that they contained large amounts of high-molecular-weight proteins in the size range of immunoglobulin (Fig. 1).

During *P. falciparum* infection, both mild and severe renal pathological changes have been demonstrated (1, 2, 3, 6, 9, 19, 20). Histologically, parasitized erythrocytes and malaria

pigment-laden macrophages have been found in the glomerular capillary loop during uncomplicated malaria (20). These changes are not associated with severe renal failure and are thought to be responsible for the mild proteinuria observed during acute infection (3, 7, 17, 19). One therefore wondered if antigens or antibodies would be released when renal pathology was minimal. In the patients included in this study, parasitemias ranged from 0.014 to 5.4% in Thai adults and from 0.3 to 7.3% in Ghanaian children. Serum blood urea nitrogen and creatinine data demonstrated that severe kidney complications were not present in these patients (data not shown). The finding of both high- (>200,000) and low-molecular-weight proteins in urine by SDS-PAGE demonstrates glomerulotubular involvement (5) but does not necessarily indicate severe renal pathology. Thus, it appears that renal dysfunction was not present in the majority of patients studied and that it is not a prerequisite for the release of *P. falciparum* antigens or antibodies into urine. In another infection where polyclonal activation is also dominant, antibody to HIV-1 has been demonstrated in the urine of patients with HIV-1 infections in the absence of demonstrable proteinuria (4).

Identification of parasite proteins and antimalarial antibodies in urine suggests that a urine-based assay for diagnosis of malaria may be feasible. An assay that detects antigen or antibody in urine would be noninvasive and thus would prevent direct exposure of health-care workers to blood. It is not known how long malarial antigens or antibodies are excreted into urine, but the observation that proteinuria is transient and rapidly returns to normal following chemotherapy (5) suggests that there maybe a direct correlation between the presence of malarial antigens or antibodies and acute infection. Recently, Kohanteb et al. (14) reported finding antigens and antibodies in concentrated urine samples of patients with *L. donovani*. In this case, antileishmania antibodies persisted longer in urine than parasite antigens following chemotherapy. Similar studies are needed in *P. falciparum* patients. An additional important consideration in a urine-based diagnostic approach is the quantity of antigen or antibody present. Parasite antigens have been detected in the urine of patients with *T. cruzi* (12) and *M. leprae* (11), but the urine was concentrated 25 and 100 times, respectively. In the current study, straight (unconcentrated) urine was used throughout. Thus, the concentration of malarial antigens or antibodies present in freshly collected urine samples should be sufficient for direct use in a diagnostic assay. These considerations along with the finding of similar antigens in the urine of children in Africa and adults in Southeast Asia (Fig. 3) suggest that a diagnostic assay based on the detection of specific antigens or antibodies should be considered.

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