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9. ABSTRACT

An indirect hemagglutination (IHA) test for malaria was evaluated. Antigen was prepared from mature schizonts of Plasmodium knowlesi harvested from the blood of the rhesus monkey. The plasmodia were freed from red blood cells with distilled water and then disrupted in a French pressure cell. A 2% NaCl extract of the disrupted plasmodia was used to sensitize human, group O tanned erythrocytes. This IHA test detected antibody titers of 1:16 or greater in 98% of slide-proved cases of malaria and in less than 1% of serum from persons without a history of malaria. The major problems in this technique included harvesting the correct stage of plasmodia for antigen, using the French pressure cell, and storing the labile disrupted preparation. Control of these variables has made possible a test that can detect antibody against a spectrum of plasmodia that infect primates, with a minimum expenditure of antigen.

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A MODIFIED, INDIRECT MICROHEMAGGLUTINATION TEST FOR MALARIA*

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During the past 50 years, many attempts have been made to develop serologic tests for malaria with precipitin, agglutination, indirect hemagglutination (IHA), complement-fixation (CF) and indirect fluorescent-antibody tests (IFA).¹ The major problems with these tests have been related to specificity and to difficulty in obtaining sufficient antigen. The serologic procedure employed by many today both in diagnosis and in research is the IFA test. High cost and lack of standardization of the procedure have prevented its use both in extensive routine diagnostic work and in epidemiologic studies.

Stein and Desowitz² described an IHA test with formalin- and tannic acid-treated sheep red cells sensitized with antigens from *Plasmodium cynomolgi* and *P. coatneyi*; this test was subsequently used in a field study of immunity to malaria in Australian New Guinea.³ Bray and El-Nahal reported difficulties with this test system and recommended a technique with fresh sheep red cells treated with tannic acid.⁴

Mahoney *et al.*⁵ fractionated antigens prepared from the plasmodia of *P. knowlesi* after disruption of the parasites in a French press. They found antigens active in IHA tests in both the supernatant fluid and the residue after centrifugation at 125,000 G for 90 minutes. With tanned sheep erythrocytes as carriers, these antigens detected antibody in all 16 serum samples from patients with *P. vivax* infections, but in only six of 32 samples from volunteers infected with *P. falciparum*. *P. vivax* serum titers ranged from 1:100 to 1:6,400, and the *P. falciparum* titers ranged from 1:40 to 1:80. Normal monkey and normal human serum samples gave negative results at a 1:40 dilution. Serum from two of 27 patients with syphilis was positive, with titers of 1:320 and 1:80.

Since the IHA test has proved useful in sero-epidemiologic studies on toxoplasmosis^{6,7} and other parasitic diseases,⁸ an attempt was made to standardize and evaluate an IHA test for malaria.

* The use of trade names is for identification only and does not constitute endorsement by the Public Health Service nor by the U. S. Department of Health, Education, and Welfare.

Such a test might be employed as a supplement to the IFA test for diagnostic purposes and may be useful in studying the epidemiology of malaria.

MATERIALS AND METHODS

Antigen Preparation

Splenectomized rhesus monkeys were infected with the *Anopheles hacketti* strain of *P. knowlesi*† by blood transfer. The animals were anesthetized and exsanguinated when 10% or more of their red blood cells contained mature schizonts. Either heparin or sodium citrate was used as an anticoagulant. All further procedures were carried out at 4°C.

The blood cells were washed three times in 10 volumes of 0.85% saline solution by centrifugation at 1,100 G for 5 minutes, and lysed by the addition of at least 10 volumes of distilled water. After centrifugation at 3,000 G for 5 minutes, the sediment consisted of plasmodia, leukocyte nuclei, and some red-cell stroma. Washing the sediment three times with saline solution facilitated removal of most of the fluffy white stroma. The final sediment was stored at -70°C before further processing. Blood from a monkey weighing 3 kg could be processed in 4 hours to yield 5 to 20 ml of plasmodial sediment.

For further preparation, the plasmodia were thawed in tepid water, placed in a cooled French pressure cell,‡ and two volumes of antigen diluent (equal volumes of 4% NaCl and M/15 phosphate buffer at pH 6.4) were added. The cell was operated at 20,000 pounds per square inch. Antigen diluent used for rinsing the cell brought the final volume to four times the volume of plasmodial sediment. The disrupted material was centrifuged at 8,000 G for 15 minutes. The supernatant fluid was tan to pink in color and opalescent; the residue was black and appeared to consist of malaria pigment and amorphous debris. The supernatant fluid was considered as undiluted antigen and was used as such, or

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‡ American Instrument Company, Inc., Silver Spring, Maryland.

diluted with antigen diluent, when titration tests showed that dilution was compatible with suitable cell sensitivity. The undiluted antigen was stored at -70°C .

Test Procedure

Human, group O blood, either fresh or stored for less than 21 days in acid citrate dextrose (ACD) at 4°C , was washed three times with phosphate-buffered saline solution (PBS: equal volumes of 0.85% NaCl and M/15 phosphate buffer at pH 7.2). The buffy coat was discarded. The erythrocytes were suspended in 40 volumes of PBS, and an equal volume of 1:20,000 (w/v) tannic acid in PBS was added. The mixture was incubated for 15 minutes in an ice-bath. After being washed twice with PBS, the cells were ready for sensitizing.

The packed, tanned cells were sensitized by adding five volumes of antigen and by mixing and incubating for 15 minutes at 37°C . To decrease nonspecific agglutination, an equal volume of PBS with 5% normal rabbit serum (NRS) was added, and this mixture incubated an additional 5 minutes. After one wash in PBS at pH 7.2 containing 1% NRS, the cells were suspended in 50 volumes of this solution and used immediately. Cells with 1:10,000 Merthiolate were useful for one week if stored at 4°C . Stored cells were resuspended in fresh PBS with 1% NRS before use.

Plastic plates* with an 8×12 array of wells were used. Each well first received 0.05 ml of PBS with 1% NRS. A 0.05-ml diluting loop filled with test serum was placed in the first well in each row and the serum serially diluted in a doubling stepwise manner, beginning with a 1:2 dilution.

During agitation, 0.025 ml of the 2% sensitized-cell suspension was dropped into each test well. Positive and negative control serum samples were titrated each day. The completed test was read 45 to 90 minutes later. A smooth mat of agglutinated cells, a 2+ reading according to Stavitsky,⁹ was considered to be a positive reaction. In order to detect nonspecific agglutination reactions, positive serum samples were tested with a 2% suspension of tanned red blood cells sensitized with 5% NRS.

*Cooke Engineering Company, Alexandria, Virginia.

Serum Samples Titrated

Serum from 61 residents of St. Lawrence Island, Alaska, 11 patients with chronic tuberculosis from Atlanta, Georgia, and 43 patients with syphilis from throughout the United States of America were assumed to be from malaria-free areas. A parasitologic diagnostic battery of 166 serum samples containing 12 subgroups of specimens known to be serologically positive for echinococcosis, filariasis, schistosomiasis, trichinosis, or other nonmalarious diseases, or negative for all these diseases, was also tested.

Serum samples assumed to contain antibody to malaria were from patients with infections proved by blood film. Serum from 17 cases of *P. falciparum* infection primarily represented U. S. citizens who contracted malaria in a country with known endemic malaria. The serum from 130 cases of *P. vivax* infection were collected in a hyperendemic focus of malaria in Honduras.

RESULTS

Factors Influencing the Test

Preliminary studies were carried out to characterize those variables that influenced the stability, sensitivity, and specificity of the IHA test. Conditions having an insignificant effect on the sensitivity of the test were splenectomy of the monkey, contamination of the antigen with host components, pressure of the French press, centrifuge speed, PBS osmolarity, tannic-acid concentration, temperature, and pH during sensitization of the cells.

Collins *et al.* have previously reported that rhesus monkeys received in the United States of America for medical research may have had prior experience with malaria.¹⁰ They found that 65% of the rhesus monkeys received from New Delhi had serum reactive in the IFA test when tested with several different simian-malaria antigens. We have found over 50% of our monkeys from the same source to have serum reactive by IHA, with *P. knowlesi* antigens. This prior experience and, at times, partial immunity, may interfere with experimentally induced malaria. The use of splenectomized animals has made the course of infection more predictable. All monkeys were splenectomized prior to infection for antigen production.

The plasmodial preparation before disruption in the French press contained white-blood-cell

nuclei and some red-cell stroma, as well as unknown quantities of adsorbed host elements. These antigenic materials have not appeared to affect the specificity of the test, but further delineation of their influence awaits the production of a purer preparation.

Antigen extraction from the whole plasmodia was carried out in a cooled French press at 20,000 pounds per square inch. We found the antigen to be useful in the IHA test when produced at lower pressures, but believed that the best yield was obtained at the maximum pressure. The disrupted material was centrifuged with forces varying from 3,000 to 48,000 G without affecting the activity of the final product.

The PBS used as buffer had an osmolarity of 228 milliosmols. Another formula of PBS with 308 milliosmols of solute did not greatly alter the sensitivity of the test. As did Shioiri,¹¹ we found that the concentration of tannic acid could be widely varied, but that 1:20,000 as used above was the most effective. Our antigen preparation was effective in sensitizing tanned red cells when incubated at 37°, 22°, or 4°C within the pH range 5.6 to 7.2.

Several variables were found to be essential for standardization of this technique. These included control of the developmental stage of the parasite prepared for antigen, physical characteristics of antigen storage, species of origin of the red blood cells, temperature of tanning the red cells, and osmolarity of the antigen diluent.

The activity of antigens from *P. knowlesi* varied according to the stage at which they were harvested. Antigens that were reactive with serum from animals immune to *P. knowlesi* could be extracted from several stages of the erythrocytic cycle.

Antigens having the greatest degree of cross-reactivity with serum from other primate malaria infections were present in increasing concentrations as the parasite became more mature. These antigens have detected antibodies against the following species of *Plasmodium* that infect primates: *P. inui*, *P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. knowlesi*, *P. brasilianum*, *P. malariae*, *P. ovale*, *P. vivax*, and *P. falciparum*.

The best preparations were made from blood containing an equal number of mature schizonts and young ring forms. These preparations could be diluted 20 times and still maximally sensitize tanned cells. Preparations of ring stages were not as active. The *P. knowlesi* infections generally

developed synchronously and required harvesting within a critical 2-hour period to yield this quality of preparation.

The active antigens of *P. knowlesi* were rather labile. Plasmodia stored at -70°C showed a gradual loss in antigen activity over a 3-month period. Disrupted plasmodia stored in antigen diluent at -70°C lost activity in 2 weeks. When stored at 4°C, the disrupted preparation was inactivated in 3 days. Once adsorbed onto red cells and washed free of other plasmodial constituents, the antigens appeared to remain stable until contamination occurred or the cells spontaneously agglutinated.

The use of human, group O or rhesus-monkey erythrocytes improved the reliability of sensitization. Serum samples were tested with red cells from the same species. Rabbit and sheep cells have occasionally been sensitized to an equal degree as human cells, but have been less reliable. Human, group O cells from five different donors have been of equal quality. Those drawn while the donor was fasting, and stored in ACD, have been least fragile, confirming the report of Indeglia *et al.*¹²

A water-bath at 4°C was necessary for adequate tanning of cells. A 37°C water-bath did not promote red-blood-cell changes that allowed sufficient adsorption of the malaria antigens.

The antigen diluent found effective for obtaining maximum sensitivity of human O cells, tanned at 4°C, was 2% NaCl with phosphate buffer. Use of this diluent was not essential during disruption of the plasmodia. The sensitization of red cells with a weak antigen was enhanced when salt solution approaching 2% concentration was used (Table 1). Concentrated solutions of antigen have adequately sensitized cells with an antigen diluent with 0.5% NaCl.

Test Reproducibility

In an evaluation of titer reproducibility, 30 serum samples were titrated as blind duplicates six times (Table 2). As expected, tests performed on the same day with the same lot of cells were least variable. One determination differed from its duplicate by one dilution, or less, 90% of the time and by two dilutions, or less, 95% of the time. Similar reproducibility within two serum dilutions could be obtained when different lots of cells were used on different days if the titers were adjusted by observing the reaction of the positive, control serum samples. Similar results

TABLE 1
Effect of salt concentration on the sensitizing of human red blood cells

	Titers			
	Normal human	<i>P. malariae</i> human	<i>P. vivax</i> human	<i>P. knowlesi</i> monkey
Human O cells, tanned at 4°				
Dilute antigen in 0.7% NaCl.....	Neg.	1:40	1:40	1:40
Dilute antigen in 0.8% NaCl.....	Neg.	1:320	1:160	1:1,280
Dilute antigen in 1.0% NaCl.....	Neg.	1:640	1:640	1:1,280
Dilute antigen in 1.3% NaCl.....	Neg.	1:5,120	1:5,120	1:5,120

were also obtained with different technicians and different test readers.

Test Specificity and Sensitivity

To evaluate the significance of titer, we tested various groups of serum (Table 3). Titers of 1:16 and greater were uncommon (0.9%) in serum samples assumed to be free of antibody against malaria. In the parasitologic diagnostic battery, serum samples with a titer of 1:16 or greater (6%) may represent true positive results as they were predominantly from areas where malaria may be endemic. The testing of serum from persons with malaria diagnosed by blood-smear examination revealed a sensitivity of 98% when a titer of 1:16 or greater was considered a positive reaction.

Serum samples were collected and stored at -20°C without a change of titer over a 12-month period. Preliminary results on serum collected by filter-paper absorption of capillary blood and compared with that collected by venipuncture have indicated that a filter-paper technique will yield comparable results and thus aid the collection of specimens.

DISCUSSION

The tests devised to detect malaria antibody often differ in their specificity of reaction and final results. The source of antigen and its method of preparation partially limit the antibodies that will be detected. Different test results may also be obtained because the test system employed may have a tendency to detect preferentially the immunoglobulins of different classes.

The agglutination of parasitized cells, as reported by Eaton,¹³ appears to be species-specific. Brown observed that parasitized cells agglutinated when exposed to antibody formed

TABLE 2
Agreement of duplicate titrations in an evaluation of 30 serum samples

Test conditions	Number of duplicate determinations	Percent of duplicate determinations differing by	
		1 dilution or less	2 dilutions or less
Tests made on the same day with the same lot of cells	330	90%	97%
Tests made on different days with the same lot of cells	180	80%	95%
Tests made on different days with different lots of cells	360	69%	86%
Tests made on different days with different lots of cells, but adjusted according to control-serum results	360	69%	93%

against that specific relapse stabilate.¹⁴ Cross-reactions were infrequent.

Previously described IHA tests have demonstrated a moderate degree of cross-reactivity between plasmodia infecting man and simians. Stein and Desowitz found that antigens from *P. cynomolgi* gave a high titer with *P. vivax* serum, but a low titer with *P. falciparum* serum.² Antigens from *P. coatneyi* have high titers with *P. falciparum* serum, but low titers with *P. vivax* serum. Mahoney *et al.*⁵ used an extract of *P. knowlesi* in an IHA test that gave high titered reactions with all *P. vivax* serum. Titers of 1:40 or greater were found in only 19% of the *P. falciparum* serum.

When the IFA test is used, cross-reactions among malaria infections of simians and man

TABLE 3
Distribution of IHA titers among serum samples from selected groups of persons

Source of specimens	Titers							Total specimens
	Negative	1:2	1:4	1:8	1:16	1:32	1:64+	
Normal Alaskans	54	3	4	0	0	0	0	61
Patients with tuberculosis	5	4	2	0	0	0	0	11
Patients with syphilis	28	2	8	4	1	0	0	43
Parasitologic diagnostic battery	105	13	19	19	6*	2*	2*	166
Patients infected with <i>P. falciparum</i>	0	1†	0	0	1	1	14	17
Patients infected with <i>P. vivax</i>	1‡	0	0	0	2	5	122	130

* These 10 serum samples included four from persons with filariasis, three with schistosomiasis, and one each with syphilis, trichinosis, and visceral larva migrans.

† Serum drawn early in experimental infection; IFA titer of 1:4 not considered significant.

‡ One-year-old male, normal serum proteins by electrophoresis, IFA test negative with *P. vivax* antigen.

have been common.¹⁶⁻¹⁷ The highest titer obtained with a given serum was usually obtained with the homologous antigen, but strain differences have not been demonstrated serologically.

Antigens extracted from *P. knowlesi* by Kligler¹⁸ and by Eaton¹⁹ have shown broad generic reactivity when used in the CF test. Antigens prepared from the serum of *P. knowlesi*-infected monkeys gave less interspecies reactivity than a plasmodial antigen preparation.²⁰

Todorovic has described the preparation of a serum antigen from acute infections with *P. gallinaceum* in chickens.²¹ In the tube latex-agglutination (TLA) test, the antigen has shown generic cross-reactivity. Antigens from *P. gallinaceum* infections reacted with antiserum to *P. gallinaceum*, *P. berghei*, *P. knowlesi*, *P. malariae*, *P. falciparum*, and *P. vivax*.

The use of soluble antigens in the CF, IHA, and TLA tests has been associated with more cross-reactions than have been found with parasitized cells in agglutination tests. The dry smear of parasitized blood as used in the IFA test has provided an intermediate degree of cross-reactivity. Close attention to the stage of the developing parasite has allowed us to obtain antigens that are common to many species of *Plasmodium*. These antigens may be structural elements, metabolites or secretions of parasitic origin, or altered host elements. The antigens are apparently released into the plasma when the merozoites are liberated. The activity in young ring stages is greatly reduced.

The thermostability of malaria antigens has not often been commented upon. Most preparations have apparently been rather stable. Todorovic's antigen is stable for several months at 4°C. IFA test antigens are generally stored as dry smears at -70°C and remain usable for 1 year or more. Our crude extract of *P. knowlesi* plasmodia has been very labile; storage at -70°C has been essential. This lability suggests that reactions continue in the undiluted preparation and result in alteration of the effective antigens. The development of effective preservatives is necessary.

The IHA test offers certain advantages in the serologic diagnosis of malaria. It requires little time per test: a technician working with sensitized cells can titrate over 400 serum samples per day. This may be further improved by the use of automated techniques. The antigen requirement of IHA is certainly much higher than that of the IFA test, but it utilizes plasmodia that infect simians. Each monkey can provide sufficient antigen for 3,800 complete titrations, or more than 10,000 screening tests. These characteristics make possible the use of the IHA test in studies of the seroepidemiology of malaria.

SUMMARY

An indirect hemagglutination (IHA) test for malaria was evaluated. Antigen was prepared from mature schizonts of *Plasmodium knowlesi* harvested from the blood of the rhesus monkey. The plasmodia were freed from red blood cells with distilled water and then disrupted in a

French pressure cell. A 2% NaCl extract of the disrupted plasmodia was used to sensitize human, group O tanned erythrocytes.

This HIA test detected antibody titers of 1:16 or greater in 98% of slide-proved cases of malaria and in less than 1% of serum from persons without a history of malaria. The major problems in this technique included harvesting the correct stage of plasmodia for antigen, using the French pressure cell, and storing the labile disrupted preparation. Control of these variables has made possible a test that can detect antibody against a spectrum of plasmodia that infect primates, with a minimum expenditure of antigen.

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