THE SEROLOGY OF MALARIA: RECENT APPLICATIONS*

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THE serology of malaria has a long and varied history. Only in the last decade have new serologic techniques, such as the indirect fluorescent antibody and hemagglutination tests, been explored for use in solving problems associated with epidemiology, speciation, and diagnosis. In our laboratory, studies on the serology of malaria have been devoted to the evaluation of the indirect hemagglutination (IHA) test as an epidemiologic tool. We believe that malariometric techniques currently employed for the assessment and eradication of malaria are not adequate and that new methods require evaluation. This is especially true in the undeveloped areas of the world where trained manpower to conduct various aspects of case finding, fever and spleen surveys, and slide examination are lacking. We are also studying the indirect fluorescent antibody technique (IFA) with emphasis on its evaluation as a diagnostic method. I shall discuss some of the accomplishments of this program.

INDIRECT HEMAGGLUTINATION STUDIES

Desowitz and Stein[1] and Stein and Desowitz[2] described an IHA test utilizing formalin and sheep red cells treated with tannic acid and sensitized with antigens from *Plasmodium cynomolgi* and *P. coatneyi.* This test was later used in a field study of malaria immunity in Australian New Guinea.[3, 4] Bray and El-Nahal[5, 6] reported difficulties with this test system and recommended using fresh sheep red cells treated with tannic acid. Mahoney et al.[7] fractionated antigens from the plasmodia of *P. knowlesi* following disruption of the parasites in a French press.

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Since the IHA test has proved to be useful in seroepidemiologic studies on toxoplasmosis[8, 9] and other parasitic diseases,[10] we attempted to standardize and evaluate the test for malaria.[11] Antigen is prepared from lysing mature schizonts from splenectomized rhesus monkeys infected with the *Anopheles hackeri* strain of *P. knowlesi*.* The infected cells are washed and lysed by adding at least 10 volumes of distilled water. The freed plasmodia were washed by centrifugation and stored at −70° C. Blood from a 3-km. monkey could be processed in four hours to yield 5 to 20 ml. of plasmodial sediment. The plasmodia are disrupted by using a cooled French pressure cell,† operated at 20,000 pounds per square inch. The antigen is quite labile and cannot be stored for long periods. Methods for stabilizing the antigen are currently under study.

The IHA test is carried out with human group O erythrocytes that have been tanned with 1:20,000 tannic acid solution and sensitized with malaria antigen. A microtitration method employing 0.05-ml. dilutions of serum is used for antibody titration.

To facilitate the collection of blood, samples obtained on filter paper by finger stick were compared with sera collected by venipuncture. These studies indicate that a finger-stick filter-paper technique yields comparable results and thus can be used to collect specimens in the field. The filter paper ROPACO 1023.038,‡ which met most of the requirements, was cut in 1 × 3-inch rectangles and imprinted with two circles, each 14 mm. in diameter. For field studies the paper rectangles were returned to the diagnostic laboratory in plastic bags with glassine interleaves and a dessicant.

In the laboratory a 13/32-inch disc was punched from within the filled circle. The disc was immersed in 0.2 ml. of phosphate buffered saline solution (PBSS) and agitated twice during the 30-minute soaking period. The disc was removed with a rod, which was rolled over it to express some of the eluate. Approximately 0.13 ml. of eluate could be obtained in this manner. The amount is approximately a 1:16 dilution of the original serum sample.

To evaluate the test for specificity, sera from 61 residents of St. Lawrence Island, Alaska, 11 chronic tuberculosis patients from Atlanta,

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Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U. S. Department of Health, Education, and Welfare.

TABLE I. TESTS ON THE SERA OF INDIVIDUALS HAVING NO KNOWN HISTORY OF MALARIA

	Titer						
Source	*0*	*2*	*4*	*8*	*16*	*32+*	*Total*
Normal Alaskans	54	3	4				61
Syphilis, primary and secondary	28	2	8	4	1		43
Chronic tuberculosis	5	4	2				11
Parasitology battery	105	13	19	19	6*	4*	166

*Includes three cases of schistosomiasis and four cases of filariasis.

TABLE II. TESTS OF THE SERA OF INDIVIDUALS WITH SLIDE-PROVED MALARIA

	Titer						
Species	*0*	*2*	*4*	*8*	*16*	*32+*	*Total*
P. vivax Honduras	1	0	0	0	2	127	130
P. falciparum U.S.	0	1	0	0	1	15	17
Mixed species U.S.	2	0	0	3	3	77	85
Mixed species U.S., Vietnam	5	0	0	1	2	127	135

Ga., and 43 syphilis patients from throughout the United States were assumed to be from malaria-free areas. A parasitology diagnostic battery of 166 sera were also tested. The sera contained 12 subgroups of specimens known to be serologically positive for echinococcosis, filariasis, schistosomiasis, trichinosis, or other nonmalarious diseases, or negative for all these diseases. Titers of 1:16 and greater were uncommon (0.9%) in sera assumed to be free of antibody against malaria (Table I). In the parasitology diagnostic battery, sera with titers of 1:16 or greater (6%) may represent true positives because they were collected from individuals living in areas where malaria may be endemic.

To evaluate the test for sensitivity, specimens from patients with proved infections were evaluated. Sera from 17 cases of *P. falciparum* infection primarily represented citizens of the United States who con-

Table III. MALARIA ANTIBODY TITERS OBTAINED WITH MILITARY RECRUIT SERA

Country	*Total*	*Titers*																	
		0	*2*	*3*	*4*	*8*	*9*	*16*	*27*	*32*	*64*	*81*	*128*	*243*	*256*	*512*	*1,024*	*2,048*	*4,096*
United States	2,237	2,203	7		7	13		7											
Brazil	2,681	1,979	76		68	153		134		129	59		34		27	12	8	2	
Colombia	2,961	2,162	62		108	134		166		146	93		46		31	8	2	1	2
Argentina	3,077	2,877		58			100		30			10		2					

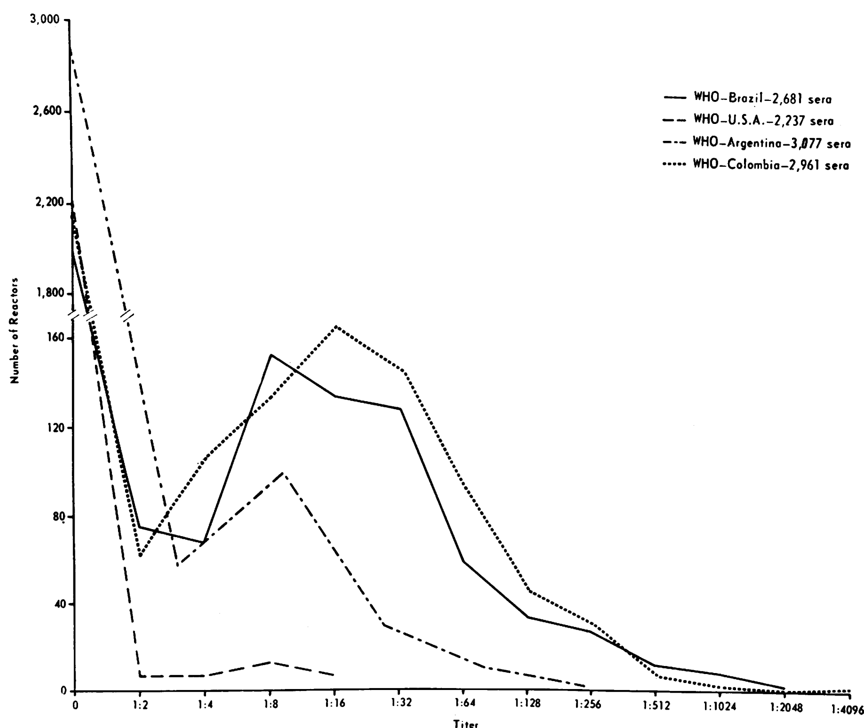

Fig. 1. The frequency distribution of indirect hemagglutination titers for malaria obtained with sera of military recruits.

tracted malaria in a country with known endemic malaria. The sera from 130 persons with *P. vivax* infection were collected in a hyperendemic focus of malaria in Honduras.

Testing sera from persons with malaria diagnosed by blood-smear examination revealed a sensitivity of 96% when a titer of 1:16 or greater was considered a positive reaction (Table II). Sera were collected and stored at −20° C. over a 12-month period without a change of titer.

To evaluate the test for seroepidemiologic studies, 10,956 serum specimens representing four collections of military-recruit sera were tested. The subjects were males varying in age from 18-22 years. The collections consisted of 2,237 sera from the United States, 2,681 from Brazil, 2,961 from Colombia, and 3,077 from Argentina. Table III lists the titers obtained with the military recruit sera. For epidemiologic purposes, a titer of 1:8 or greater was considered positive. This deci-

TABLE IV. BRAZIL: COMPARISON OF SEROLOGIC AND BLOOD-SLIDE RESULTS FOR MALARIA FROM STATES FROM WHICH BOTH DATA WERE AVAILABLE. STATES ARE RANKED FROM HIGHEST TO LOWEST VALUE.

	Serology			*Slide examination*		
Rank	*State code*	*Name of state*	*Per cent positive*	*State code*	*Name of state*	*Per cent positive*
1	3	Roraima	100	3	Roraima	16.8
2	4	Amazonas	39.8	25	Goias	15.7
3	5	Para	39.3	7	Maranhão	15.5
4	2	Acre	37.5	5	Para	14.1
5	12	Pernambuco	33.6	16	Bahia	8.7
6	7	Maranhão	27.6	8	Piaui	6.6
7	25	Goias	25.6	4	Amazonas	5.5
8	24	Mato Grosso	25.4	2	Acre	4.7
9	13	Alagôas	25.0	18	Espirito Santo	4.6
10	8	Piaui	23.3	24	Mato Grosso	3.5
11	17	Minas Gerais	18.6	17	Minas Gerais	3.4
12	16	Bahia	17.3	22	Santa Catarina	3.3
13	10	Rio Grande do Norte	15.8	9	Ceará	2.3
14	22	Santa Catarina	12.5	21	Paraná	2.0
15	11	Paraiba	8.8	12	Pernambuco	1.3
16	19	Rio de Janeiro	8.3	19	Rio de Janeiro	0.6
17	9	Ceará	4.6	11	Paraiba	0.4
18	21	Paraná	4.3	27	Guanabara	0.3
19	15	Sergipe	0.0	15	Sergipe	0.1
20	18	Espirito Santo	0.0	13	Alagôas	0.0
21	27	Guanabara	0.0	10	Rio Grande do Norte	0.0

sion was based on the frequency distribution of the titers in each collection as shown in Figure 1. On this basis, the following prevalence of positive reactors were obtained: United States, 20 (1%) specimens positive; Brazil, 558 (21%) specimens positive; Colombia, 629 (21%) specimens positive; and Argentina, 142 (4.6%) specimens positive. The similar frequency distribution of titers obtained in Brazil and Colombia, where malaria is widely endemic, and the lower frequency distribution in Argentina, where malaria endemicity occurs at a low level in limited parts of the country, suggest that the test is measuring specific malaria antibody.

The sera positivity rate differs markedly from the rate of malaria positivity obtained by blood-smear examination in countries where malaria is endemic. In the United States collection, 99% of the sera examined were negative. This finding attests to the high specificity of the test in confirming the absence of malaria in this country. In Brazil and Colombia the serologic prevalence rate was 21% but the slide pos-

itivity rates for these countries varied from 3 to 5%. The discrepancy in the two methods is probably due to the fact that the IHA test can detect antibody in the blood of an individual many years after infection, whereas the slide method detects only parasites present at the moment the blood is taken for examination. Comparison of serologic and blood-slide results from states in Brazil from which both data were available (Table IV) indicates that 8 of the 10 states with the highest serologic prevalence rates were also among the 10 states with the highest slide-positive rates. The same correlation was found in Colombia. The high correlation suggests that both methods are measuring the prevalence of malaria.

The persistence of the hemagglutination antibody can best be studied in areas where malaria has been eradicated or in individuals who have received radical curative treatment and do not live in endemic areas. A study in an area where malaria has been eradicated was made in Tobago. The last case of autochthonous malaria was reported in Tobago in 1953 and, except for a small introduced outbreak of *P. malariae* in 1966, the island has remained free of malaria. Through the courtesy of Wilbur Downs 84 sera collected in 1955 in Tobago were titrated for malaria antibody. In 1969 a second collection was made, and 40 of the individuals whose sera were collected in 1955 were bled again. Twenty-three sera were obtained by venipuncture and 13 by the filter-paper method. The prevalence for the 40 individuals fell from 84% to 10% and the geometric mean reciprocal titer from 52.5 to 3.3. Because the minimal detectable titer indicated with filter paper is 1:16, all negative sera are considered 1:2 dilutions for calculating the geometric mean reciprocal titer. The four positive individuals were the oldest people in the sample, and their titers were *1:128*, *1:64*, *1:16*, and *1:16*. The prevalence of malaria antibody in the 943 individuals sampled from five areas in Tobago was 1.52% and the geometric mean reciprocal titer was *2:1*. These studies indicate that malaria has indeed been eradicated in the island and that antibodies can persist in some individuals for at least 14 years.

A potential use of the serologic method is to delineate the extent of malaria transmission. The question of whether malaria is being transmitted in Nepal above an elevation of 4,000 feet was studied. In a collection of 163 individuals living in villages above this altitude, 22 (13.5%) were positive. Of the 22 positive, 19 were males with a history

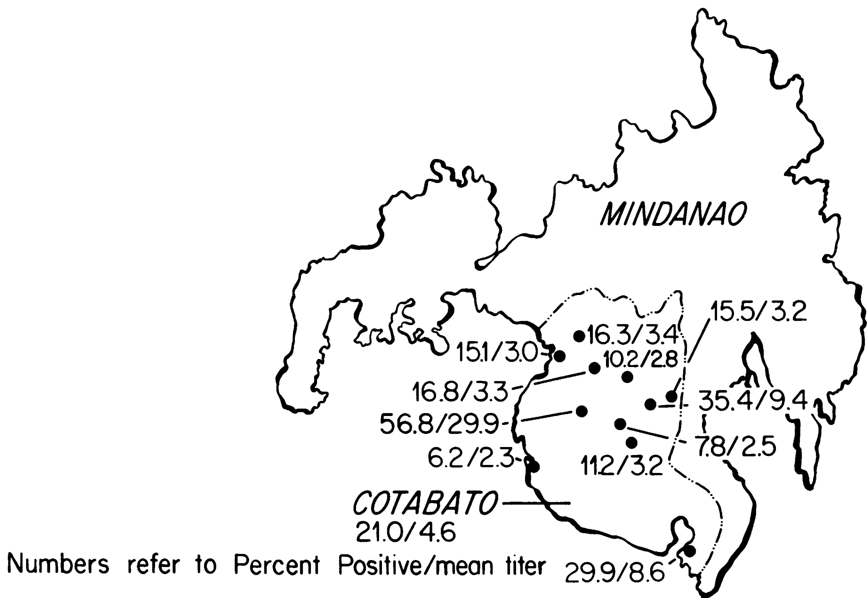

Fig. 2. Distribution of malaria antibodies and geometric mean reciprocal titers for a number of villages in the province of Cotabato on the island of Mindanao, Philippine Republic.

of travel to areas below 4,000 feet. This contrasted with a prevalence of 40% for 502 samples in an endemic area below 4,000 feet. In Ethiopia only 23% of 92 sera collected from individuals living above 6,000 feet were positive compared to 58% of 122 samples collected below 6,000 feet.

The IHA test may also be useful in detecting focal outbreaks of malaria in an endemic area. In a Philippine study done in the province of Cotabato, the prevalence of malarial IHA antibody ranged from 10 to 56%. Two villages with positive serologic rates of 30 and 56% and high geometric mean reciprocal titers were found in a geographical cluster of villages with rates ranging from 10 to 15% and low mean titers (Figure 2). These data suggest that in the two villages with high serologic prevalence, active transmission may be taking place. These malaria "hot spots" can be readily detected with such a survey method.

In Ethiopia serum samples taken in two locations in both wet and dry seasons showed that the positivity rate and the mean titer increased

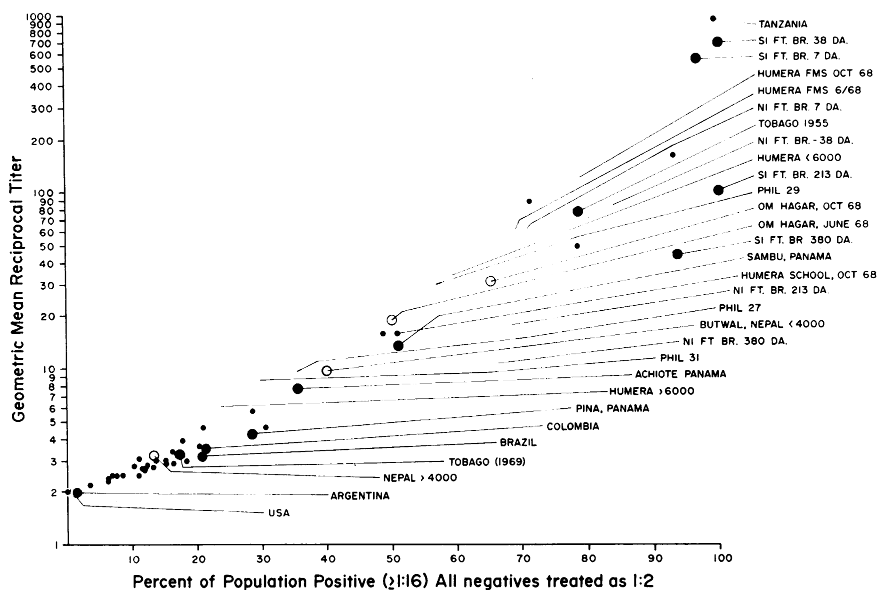

Fig. 3. Relation between the per cent of the population sampled and the geometric mean reciprocal titer for malaria hemagglutination antibody in a number of surveys.

TABLE V. ETHIOPIA: SEASONAL FLUCTUATION OF MALARIA ANTIBODY LEVELS

Location	*June 1968 (dry)* Per cent positive	Mean titer	*October 1968 (wet)* Per cent positive	Mean titer
Om Hagar School	50.0	18.8	65.5	30.5
Humera Farms	68.4	56.1	78.2	113.0

markedly in the wet season, the time of peak transmission. This finding indicates the potential use of the IHA test in monitoring seasonal changes in malaria transmission (Table V).

A correlation between the mean geometric reciprocal titer for a survey population and the endemicity of malaria is apparent. To date approximately 20,000 sera, representing collections from the Western Hemisphere, Africa, and Asia have been titrated. These were all "grab samples" and represented many types of populations and epidemiologic situations. The number is large, however, and many of the biases may

have been canceled out. This point, however, cannot be assumed and matched populations will be studied. When one plots, on a logarithmic scale, the geometric mean titer versus the per cent positive in the collection, a straight-line relation appears (Figure 3). Serial specimens drawn from a single area but under differing epidemiologic situations move along this line in a predictable manner. This suggests that if a large number of samples are collected from an area, one may readily characterize the endemicity of malaria as hypoendemic, endemic, or holoendemic. Specimens collected at various intervals will make it possible to assess changes in malaria incidence in an area. This assessment can be made rapidly because a collection of several thousand sera can be titrated in a few days by the microtitration method.

In summary: the IHA test may have specialized applications in assessing the prevalence of malaria. Because of the long duration of malaria antibody in a person who has been infected, recent outbreaks of malaria can be detected only by testing young children or by noting a rise in the geometric mean titer over a given period. Since the mean geometric titer and per cent positive of a carefully selected cross section of the population reflects the endemicity of malaria, small surveys in selected areas will readily detect focal outbreaks or a change in the epidemiology of the infection. The technique can be used to delineate the extent of malaria in a country. Other more practical applications, such as monitoring the effect of eradication, or a chemoprophylaxis program in endemic areas, are under study.

Fluorescent Antibody Studies

The fluorescent antibody (FA) test for malaria was initially introduced by Tobie and Coatney[12] and Voller and Bray.[13] In the last few years a number of workers have made important contributions with this method.[14-24] The FA test is the serologic technique most widely used for the diagnosis of malaria at the present time. Reports by McGregor et al.[25, 26] in Gambia, West Africa, on the use of the fluorescent antibody technique to measure the status of immunity in a population residing in an endemic area have shown that serologic methods may be used to good advantage in the epidemiologic assessment of malaria endemicity.

The FA test, because of technical problems, does not lend itself to mass-screening methods. Nonetheless epidemiologic surveys on relative-

TABLE VI. NUMBER AND PER CENT OF REACTORS TO THREE PLASMODIUM ANTIGENS AMONG 184 SERA FROM INDIVIDUALS NOT EXPOSED TO MALARIA AND 49 SERA FROM INDIVIDUALS WITH A POSITIVE SLIDE DIAGNOSIS*

		Washed-cell thick-smear antigens					
		P. falciparum		P. vivax		P. brasilianum	
Sera group	*Titers*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Donors not exposed to malaria	Negative	157	85.3	180	97.8	183	99.5
	1:4	26	14.2	4	2.2	1	0.5
	1:16	1	0.5	0	0.0	0	0.0
Donors with positive slide diag.†	Negative	6	12.2	4	8.2	9	18.4
	1:4	10	20.4	4	8.2	3	6.1
	1:16	33	67.4	41	83.6	37	75.5

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†All sera in this group had previously had titers of at least 1:16 with one of the three antigens. On this second titration, two of these sera had negative reactions with all three antigens.

ly large groups of individuals have been made in Nigeria by Collins et al.[23] and Voller and Bruce-Chwatt.[24]

Use of a soluble antigen fluorescent antibody test with *P. falciparum* and chimpanzee erythrocyte lysates from experimental infections in chimpanzees as antigens adsorbed to cellulose acetate discs may be a more rapid means of performing the fluorescent antibody technique.[27] A stable antigen fractionated by sequential elution with chromatography from a DEAE Sephadex A 25 column gave fractions that were very active in this test. Approximately 50,000 tests can be performed with the amount of antigen normally collected from one infected chimpanzee. This technique is very promising and merits further study because fluorescence is read by a fluorometer, and objective criteria of positive and negative reactions are possible.

We have used a washed-cell thick-smear antigen in all our IFA studies.[28] Washing the parasitized cells removes soluble serum components, especially gamma globulin that may contain malaria antibody and thus may interfere in the test. When such a washed antigen is prepared as a thick smear, the number of plasmodia per field can be controlled. This greatly facilitates the reading of the test results.[29]

The sensitivity and specificity of the test are very high.[29] These parameters were evaluated with a battery of 232 sera, of which 184 were from persons never exposed to malaria and 49 from persons with patent plasmodial infections. All sera were randomized, coded, and

TABLE VII. DISTRIBUTION OF TITERS FOR TWO ANTISERA WITH FOUR ANTIGENS IN THE INDIRECT FLUORESCENT ANTIBODY (IFA) TEST FOR MALARIA*

Reciprocal Titer	*Plasmodium Antigens* P. vivax *E.T.*†	*MOR.*‡	P. falciparum *E.T.*	*MOR.*	P. brasilianum§ *E.T.*	*MOR.*	P. fieldi *E.T.*	*MOR.*
Negative				6		4		2
4				14		9	2	3
16	16	9	22	27		25	10	8
64	29	30	28	2				
256		1			6			
1,024					34			
4,096					2			
Total replicates	45	40	50	49	42	38	12	13

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†*Plasmodium malariae* demonstrated in circulating blood. Mother of an infant with congenital malaria.

‡District medical officer, Sepik, New Guinea. Reported *Plasmodium vivax* infection with treatment one year prior to sampling.

§*P. brasilianum* antigen was used as an equivalent to *P. malariae*.

tested with antigens prepared from *Plasmodium vivax*, *P. falciparum*, and *P. brasilianum*, the latter used as a substitute for *P. malariae*. As seen in Table VI only one of the sera from the nonexposed group had a positive reaction at 1:16 with the *P. falciparum* antigen. Specificity is, therefore, greater than 99%. The false positive rates with the *P. brasilianum* and *P. vivax* antigens at the 1:4 dilution were less than 3%, but 14% of the sera reacted with the *P. falciparum* antigen. For this reason a positive reaction at 1:4 with any antigen species is regarded as a questionable positive, and the lowest acceptable diagnostic titer for malaria is 1:16.

The 49 positive sera included in this battery had been tested previously and found to have low antibody concentration. Sera of low titer were chosen so that the most rigorous test of sensitivity might be made. The inclusion of many sera of high antibody titers, which are readily detected by serologic tests, would give a sensitivity rate that might be misleading. As noted in the footnote to Table VI, two of the sera retested gave no reaction at the 1:16 dilution with any of the antigens. This constituted a false negative rate of less than 5%. All other sera were positive at 1:16 with at least one antigen. Some sera reacted

only with their homologous antigen. For a diagnostic procedure with the highest sensitivity homologous antigens of the human species should be employed. For this reason we maintain *P. vivax* and *P. falciparum* in Aotus sp. and Ateles sp. monkeys respectively. If homologous human plasmodial antigens are not available, however, a *P. vivax* antigen may be used. With this antigen, the specificity would be better than 95%; the sensitivity with sera of low titer about 92%.

Reproducibility of titers with the thick-smear antigen on a test-to-test basis is excellent (Table VII). Two positive sera were tested with each of four antigen species many times. All titers were replicated within plus or minus one fourfold dilution except for the MOR. serum tested with the *P. falciparum* antigen.

The IFA test can be used to determine the infecting plasmodium species when one cannot make a determination with stained blood slides.[30] Etiologic diagnosis may be difficult because the parasites may have been distorted by the effect of drugs, the parasitemia too scanty to reveal diagnostic forms, or the species identification in doubt because the slides were improperly prepared. The method may be used to detect responsible donors in transfusion malaria.

An evaluation of species identification was made with 206 sera from 93 military personnel who, after returning from Southeast Asia, relapsed with malaria infections. Both acute and convalescent sera were tested. In each case, the infecting plasmodium species was determined by stained blood slides. Since only *P. vivax* and *P. falciparum* infections were involved, antigens of these two species alone were employed in the evaluation.

Comparisons were made on the basis of fourfold titer differences with the two antigens. The species representing the antigen that gave the highest titer was designated as the infecting species. When matched with the slide results, the correct species was identified serologically for 89% of the specimens. Six per cent gave the same titer for both antigens; one case (0.5%) was misdiagnosed, and 5% were negative. Species determinations have become routine in our laboratory and have proved very useful in transfusion malaria and in correcting some misdiagnoses based on improperly stained blood films.

The rise and fall of antibody titer in returning military personnel may be useful diagnostically in that high antibody titer may indicate recent or current infection, especially if there is no history of recent

treatment.[31] Sera from 69 individuals reporting to a military hospital in the United States with clinical cases of malaria were studied. In the first two weeks after onset of symptoms the titers were relatively high, with a geometric mean of 1:184. After six months of treatment the titers fell to a mean of 1:9. In this group only one serum had a titer of 1:256. These data clearly suggest that a titer of 1:256 in a returning soldier indicates recent malaria infection. If there is no history of treatment and if parasitemia is not detectable an antibody titer of 1:256 may be regarded as serologic evidence of current malaria infection.

In summary: when a washed-cell thick-smear antigen is used, the IFA test for malaria gives good reproducibility, sensitivity, and specificity. The infecting species of malaria can be identified serologically. When malaria is suspected but definitive diagnosis cannot be made by stained blood film, IFA test results may be useful. For maximum diagnostic sensitivity, homologous plasmodial species antigen should be used. Human plasmodial species from infections in Aotus and Ateles monkeys make excellent antigen. *P. brasilianum* appears to give serological results identical with those of *P. malariae* antigen.

Summary

Studies in progress on the application of the indirect hemagglutination test for epidemiologic purposes of malaria are outlined. The test is both sensitive and specific, and antibody can be titrated from plasma eluted from filter paper. The technique may be used to determine serologic positive rates in a population, to determine the extent of malaria transmission, and to characterize the endemicity of malaria.

The indirect fluorescent antibody test is also under evaluation for diagnostic purposes. Evaluation of sensitivity and specificity using a washed-cell thick-smear antigen indicates a procedure of high sensitivity and specificity. Species indentification by using homologous malarial antigen is possible. The significance and duration of antibody in individuals who have undergone radical cure by chemotherapy shows a fall in titer to levels below 1:256 in six months. Human plasmodial species in monkeys may be used as antigen.

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