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Malaria: Seroepidemiology and Serologic Diagnosis

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KAGAN, I. G. 1971. Malaria: Seroepidemiology and serologic diagnosis. *Experimental Parasitology*, **31**, 126–135. Recent advances on the application of serologic methods employing the indirect hemagglutination test with a *Plasmodium knowlesi* antigen for the study of malaria epidemiology are outlined. Work in progress on the stabilization of malaria antigens and the preparation of gluteraldehyde sensitized cells were reviewed. Fluorescent antibody studies in progress are discussed and work on the cross reactivity of *Babesia* antigens with malaria is mentioned.

INDEX DESCRIPTORS: Malaria; *Plasmodium knowlesi;* Indirect fluorescent antibody; Seroepidemiology; *Babesia argentina;* Indirect hemagglutination test; Serology; Epidemiology; Antigens; *Plasmodium vivax; Plasmodium falciparum; Plasmodium ovale;* Complement-fixation test.

In 1955 the Eighth World Health Assembly recommended that malaria be eradicated from the earth. By the end of 1969, malaria had been eradicated in areas affecting approximately 699 million people. An additional 703 million people are living in countries where eradication is in progress. Approximately 376 million people live in areas where malaria eradication has not been initiated (World Health Organization 1970).

The malaria eradication program has not been equally effective in all parts of the world. Years after eradication programs were conducted in Ceylon, outbreaks of malaria reached epidemic proportions. In Central America, because of changing economic conditions and mass population shifts, eradication programs have faltered. There may be more malaria in some parts of Central America today than before eradication programs were initiated (Bruce-Chwatt 1970a). Setbacks in eradication programs suggest a need for more efficient surveillance methods. Toward this end, serologic methods have been evaluated as epidemiologic tools.

Immunologic methods employed in malaria are indirect fluorescent antibody (IFA), agar gel diffusion, complement fixation, latex, and indirect hemagglutination (IHA) tests. Bruce-Chwatt (1970b) recently reviewed the seroepidemiology of malaria. In our laboratory, we have employed the IHA test for epidemiologic purposes and the IFA test for diagnosis (Kagan *et al.* 1969a).

INDIRECT HEMAGGLUTINATION TEST (IHA)

In 1968 we published a preliminary evaluation of the IHA test (Rogers *et al.* 1968). In 1969 we published a review of research being carried out in our laboratory on serologic methods as an epidemiologic tool in malaria (Kagan *et al.* 1969a). These studies showed that the IHA test is a sensitive and

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specific method for the detection of malaria antibody and that it can be used for epidemiologic purposes. The serologic results obtained with collections of sera from military recruits in four countries in the Western Hemisphere (United States, Brazil, Colombia, and Argentina) correlate well with the information on the prevalence of malaria in these countries obtained by classical malariometric methods (Kagan *et al.* 1969b). IHA tests of the U. S. collection gave no evidence of malaria. This result is consistent with the absence of malaria in the U. S. population. States in Brazil and Colombia, ranked according to serologic and malariometric endemicity, showed an 80% correlation. In Argentina, serologic reactors were found in states where active and passive surveillance indicated that malaria had been eradicated 10 years previously.

The studies in Argentina made it imperative to ascertain the duration of the antibody response once transmission had been interrupted. The persistence of malaria antibody in Tobago, West Indies, as measured by the IHA test following eradication in 1952–1955, was therefore evaluated (Mathews *et al.* 1970a). In this study, the significant finding was that malaria antibody could be detected in 10% of 40 residents who had high titers in 1955 and who were retested in 1969. Malaria antibody decays over a long period following the interruption of transmission in an endemic area. No antibody was detected in persons born after transmission ceased.

For the serologic method to be of value in the surveillance of malaria, the techniques used should be sensitive enough to detect the interruption of transmission when it occurs, to detect focal areas of malaria transmission within an endemic area, and to monitor the loss of antibody titer. To achieve these goals, we have devised an epidemiologic model that measures the geometric mean reciprocal titer (GMRT) and the percent positives in the collection. Since these two variables are interdependent, the relationship between the GMRT and the percent positives appears to be approximately linear. Plotting serologic results of over 20,000 sera from all parts of the world bears this out (Fig.1).

Since endemicity in a community can be measured or estimated by using either of these variables alone, the value of using the two together is not in estimating malaria endemicity, *per se*, but in measuring antibody decay and detecting focal areas of transmission. Studying 69 military veterans for 1 year following radical chemotherapeutic cure in the absence of reinfection, we noted that about 90% remained positive but that the GMRT fell (Fig. 2). Studies are in progress to evaluate entire communities before, during, and after eradication to ascertain whether the GMRT of the population living in an endemic area falls following eradication procedures.

A study of 20 communities on the islands of Leyte, Panay, and Mindanao in the Philippine Republic indicated that the immunologic method may detect focal areas of transmission. In two communities in the State of Cotabato, GMRT values were found to be elevated in all age groups. In communities surrounding these areas, the GMRT values were much lower. The presence of relatively high levels of antibody in young children (Mathews *et al.* 1970b) suggested that active transmission had occurred recently in the two areas with high GMRT values.

The distribution of antibody in different age groups in a population provides a number of clues to the history of malaria in a region. The presence of antibody in children under the age of 2 years indicates very recent transmission; however, the absence of antibody does not necessarily indicate the absence of transmission. In addition to antibody status, the following factors must be assessed to interpret accurately serologic

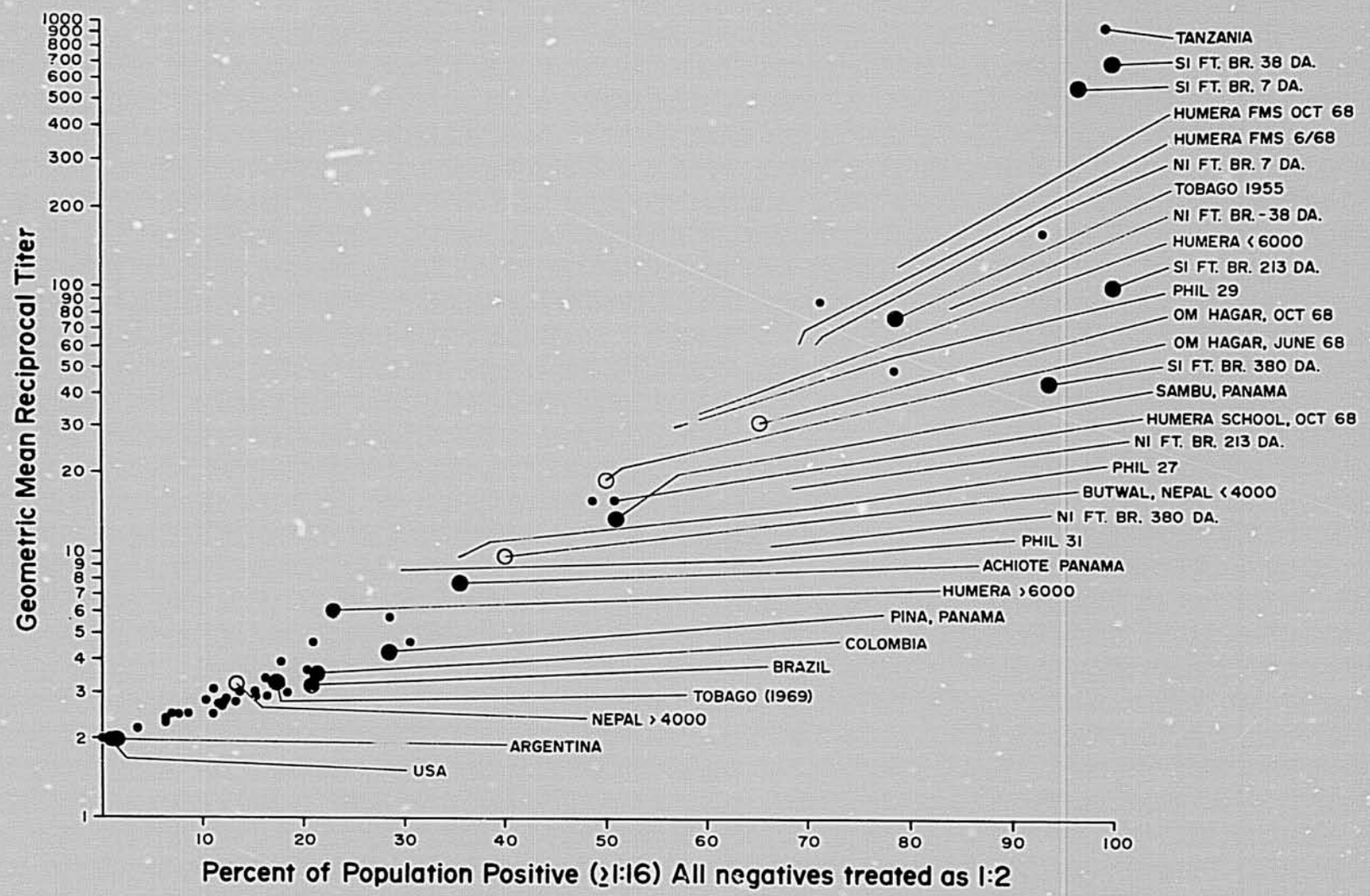

FIG. 1. Relation between the percent positive of the population sampled and the geometric mean reciprocal titer for malaria hemagglutination antibody in a number of surveys (Kagan, Mathews, and Sulzer 1969).

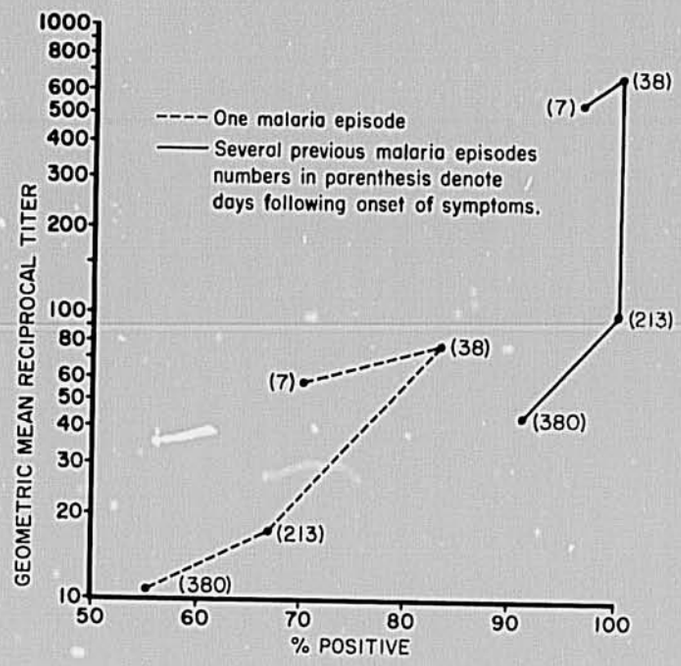

FIG. 2. The effect of previous malaria episodes on antibody response in U. S. military personnel.

data: the relative risk of each age group to exposure, travel experience, and access to malarial drugs. Antibody profiles for all age groups must be determined, and sampling rates must be sufficient to detect low levels of malaria antibody. In areas where transmission has recently been interrupted, antibody level could be expected to remain high in older age groups and to decline rapidly in the youngest age categories. Declining antibody levels in all age groups are indicative of interruption months or years previously. Although increasing antibody levels may be indicative of an increase in transmission, all changes in antibody profiles should be correlated with information available from classical, malariometric methods.

In a number of countries, work is in progress to evaluate the IHA test in conjunction with traditional malariometric programs. These studies are being carried out in areas where control programs have recently been initiated, where programs have been operational for a period of years without interruption of transmission, and where interruption has been effected.

To facilitate the collection of samples in an endemic areas and to avoid the necessity of collecting blood by veni-puncture, we studied blood absorbed to filter papers. After elution from ROPACO[2] filter paper (which has the consistency of a Whatman #3 paper) blood obtained by fingertip bleeding and dried yields results similar to those obtained with serum. The blood elements eluted from discs 10 mm in diameter punched from the papers in 0.2 ml of saline correspond approximately to a 1:8 dilution of the original serum.

Blood absorbed on filter papers, if kept dry and stored at −20 C, retains its immunologic properties for many months. The

[2] 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.

filter papers can be mailed from any place in the world to a central laboratory for titration.

The methods for preparing plasmodial antigens for use in IHA tests involve four major steps: freeing the parasite from the host cell, rupturing the parasite, fractionating the antigenic material, and storing the final product. Several methods have been used to accomplish each of these objectives.

The initial procedure in producing antigens for malaria IHA tests is removing the parasite from the host cell. This has been accomplished by saponin lysis (Spira and Zuckerman 1964; Schindler and Voller 1967; Mahoney *et al.* 1966; Cook *et al.* 1969); distilled water hypotonic lysis with or without additives such as Triton X-100 or CO_2 saturation (Stein and Desowitz 1964; Chavin 1966; Rogers *et al.* 1968); and pressure lysis (D'Antonio *et al.* 1966). Other methods, including immune lysis and sonication, are available, but these techniques have received little attention in the production of IHA antigens. In our hands treatment with distilled water containing 0.01% Triton X-100 produced the highest recovery of parasites. This method is rapid, is complete, and leaves little residual reagent to contaminate the antigen during subsequent processing.

Following the separation from the host cells, the parasites are ruptured to obtain soluble antigens. Several procedures have been employed: pressure disruption (Mahoney *et al.* 1966; D'Antonio *et al.* 1966; Rogers *et al.* 1968), freeze–thaw (Schindler and Voller 1967), tissue grinding (Stein and Desowitz 1964; Chavin 1966), or lyophilization followed by pressure (Spira and Zuckerman 1964), and sonication (our unpublished data). In our laboratory, three methods have been evaluated: pressure disruption with the French press, or more recently the Ribi press; freeze–thaw; and sonication. Of these methods, pressure disruption gave the best results. The Ribi press, operated at 17,000 psi, produced very uniform products with very little antigen denaturation. Other methods employed were not as satisfactory in terms of quality and quantity of antigen produced. Both freeze–thaw and sonication failed to achieve 100% disruption of the parasites, and the activity of the resulting antigens was much reduced.

The soluble parasite materials have been used directly in IHA tests as crude antigen (Mahoney *et al.* 1966; Stein and Desowitz 1964; Rogers *et al.* 1968; Spira and Zuckerman 1964; Schindler and Voller 1967) or subjected to fractionation to define better the active antigens or to improve their stability. Methods of fractionation include gel filtration (D'Antonio *et al.* 1966; Chavin 1966; Wellde *et al.* 1969; Turner and McGregor 1969), ion-exchange chromatography (Chavin 1966; Wellde *et al.* 1969), ammonium sulfate precipitation (Wellde *et al.* 1969), and rivanol precipitation (Chavin 1966). In our laboratory, rivanol precipitation has been the method of choice. This method has provided an antigen of increased stability without the great loss of activity found with other methods of fractionation. The optimal conditions for reproducible fractionation are being evaluated. Salt concentration and pH are important variables in this technique.

Some workers have used antigen prepared by rupturing red cells and parasites simultaneously either by a Hughes press (Turner and McGregor 1969) or by freeze–thawing the washed cells (Wellde *et al.* 1969). The supernatant is subsequently fractionated by "salting" or by chromatographic techniques. Other workers (McGregor *et al.* 1968) have used soluble antigens in the serum of infected patients in immunodiffusion tests. These additional sources of antigen are being explored as possible starting media for extracting stable, sensitive, and specific antigen for use in the IHA test. Rivanol fractionation of lysed infected red

blood cells has been shown (our unpublished data) to contain a stable antigen, but its extraction in large quantities has been difficult. Existing techniques are being modified to develop this procedure as a reliable source of antigen.

IHA antigens have been stored for long periods by freezing at −70 C or in limited cases by lyophilization. Incorporating highly purfied polyvinyl pyrollodone (PVP) has been found effective in stabilizing CF antigens for lyophilization (D'Antonio *et al.* 1966). In our hands PVP has given variable success in stabilizing IHA antigens. Research to develop more stable IHA antigens is continuing.

Another difficulty with IHA procedure is the fixation of antigen fractions to stabilized red cells. We have concentrated on the use of gluteraldehyde fixation of human O erythrocytes. The washed cells are fixed by a modification of the method of Weinbach (1969). In our procedure, the glucose concentration was reduced to 3.3% in the phosphate buffer to reduce the number of crenated cells. In addition, the cells were fixed for 30 min instead of 15, and were washed five times in distilled water instead of in saline. The variations in reactivity of the different batches of the red cells and changes in the reactivity of the same batch of cells due to aging and storage time were avoided by using gluteraldehyde fixed cells. With the development of lyophilized gluteraldehyde fixed cells, sensitized with stable plasmodial antigens, the usefulness of the IHA procedure as a field tool for the seroepidemiology of malaria will be greatly enhanced.

The antigen employed in our seroepidemiology studies is derived from *Plasmodium knowlesi*. This parasite produces a high parasitemia in the Rhesus monkey (*Macaca mulatta*). A goal for future work is the production of an efficient plasmodial antigen in a less expensive animal.

Indirect Fluorescent Antibody Test (IFA)

The IFA test is the test of choice in the serologic diagnosis of malaria (Bruce-Chwatt 1970b; Kagan *et al.* 1969a; Collins *et al.* 1968a; Wilson *et al.* 1971). The technique has been used for studies on the epidemiology of malaria by Collins and his associates (1967; 1968b) by Voller and Bruce-Chwatt (1968), and by McGregor and his associates (1965). In our initial studies on this technique in the fall of 1966, we found that smears of parasitized blood were inadequate as antigen. The paucity of parasites per field made it difficult to determine endpoints, and more important, we believed that the presence of soluble antibody in the thin smear might interfere with the test. Sulzer and Wilson (1967) reported the use of a thick smear antigen slide in the IFA test. With this technique, one can adjust the parasitemia so that 25–50 parasites field are visible. To eliminate soluble antibody components, the cells are washed before being used for antigen. By concentrating the parasites, one can obtain adequate numbers of schizonts and trophozoites in the field for evaluation. It is very important to have both types of parasite because we have found that the serum titer may vary and be much higher with the schizonts than with the trophozoites. In an evaluation of the thick smear antigen (Sulzer *et al.* 1969), slides of *Plasmodium vivax*, *P. falciparum*, and *P. brasilianum* were prepared. The lowest significant diagnostic titer was found to be 1:16. At this dilution, false-positive rates were less than 0.5% and false-negative rates were below 5%. Titers were reproducible within one 4-fold dilution.

The need for large amounts of antigen and the paucity of infected human patients with patent parasitemias made it important to look for other sources of antigen. In 1966 Young and his associates discovered that *Aotus trivirgatus* monkeys were susceptible

to *P. vivax* (Young *et al.* 1966). Since then, strains of *P. falciparum* and *P. malariae* were found to grow well in *Aotus*. We and many others have attempted to grow *P. ovale* in *Aotus* monkeys but with negative results. In continued efforts to obtain a simian source of *P. ovale*, we intend to reproduce the work of Bray (1957) who was able to obtain a parasitemia with human *P. ovale* in the chimpanzee.

In further evaluation of the malaria IFA test, the significance of the titer in the test was studied (Wilson *et al.* 1970). The level and the duration of the antibody response following treatment of experimental and natural infection have been studied by Collins and his associates (1968a) and by Lupascu and his coworkers (1969). A study to determine the pattern and persistence of malaria antibody in U. S. soldiers who returned from South Vietnam was devised to evaluate the parameters used in routine diagnosis of malaria by the IFA test. Two hundred thirty-one serum samples were collected at intervals up to 1 year from 69 men who had clinical attacks of *P. vivax* or *P. falciparum* infection in the United States after returning from Vietnam. These individuals received curative chemotherapy and were followed for a period of 12 months after the onset of symptoms. By 60 days after the onset of symptoms, antibody titers with homologous antigens ranged from negative to 1:4096, and 98.5% had positive reactions of 1:16 or above. Six months after cure, 53% of patients whose samples were tested had detectable antibodies and titers below 1:256. Of the individuals whose sera had titers of 1:256 to 1:4096, 98.7% had histories of recent infection. A titer of 1:16 or 1:64 is only suggestive of experience with malaria (Wilson *et al.* 1970).

Working with the same collection of sera, this laboratory showed that the antibody response was similar in patients with and without histories of previous malaria attacks, and, regardless of the number of malaria attacks the patient had experienced, the antibody response was in all instances the same. In the few cases in which a patient had a relapse and was re-treated, the titers before relapse were not significantly different from those in other patients following radical cure (Fisher *et al.* 1970).

In a study by Gleason and her associates (1971), agreement between slide diagnosis and IFA serology for 120 sera with slide-proven *P. vivax* and 39 sera with slide-proven *P. falciparum* was 76.7%; fourfold or greater differences between titers in tests with homologous and heterologous antigen were used as a basis for speciation. In 79 U. S. servicemen who had attacks of *P. vivax* or *P. falciparum* after returning home from Vietnam, correct classification was obtained in 81% of the cases during the 0–14-day period following the onset of symptoms and in 96.2%, from 15 to 60 days after the onset of symptoms. The IFA test was unreliable in speciating the type of malaria in sera tested 6 months after the onset of symptoms.

In a comparison of IFA and the IHA tests for malaria antibody (Wilson *et al.* 1971), titers of 226 sera drawn between the onset of symptoms and day 60 after onset were similar. The IHA test was found to maintain high titers for longer periods than the IFA test. The IHA test titers reflect enhancement of titer levels in patients with repeated malaria attacks, but the IFA test did not show this enhancement.

The literature contains some debate on whether the persistence of high antibody titers is evidence of current infection. Studies carried out on military returnees from southeast Asia definitely indicate that a titer of 1:256 or higher is suggestive of a very recent infection. If the subject has received no chemotherapy in the past 6 months, the infection is probably current. After successful chemotherapy, titers cannot be expected to decrease appreciably until at least 3 months have elapsed. If the titer persists for 6 months, however, a failure of chemotherapy should be suspected (Wilson *et al.* 1970). Whether this is the

case for people living in endemic areas is being investigated in a study of foreign students residing in the United States and found to have titers for malaria in the IFA test. In screening a large number of students from a number of schools in the eastern half of the country, 34 individuals were found with titers for malaria. These people are being followed for a short time to observe whether the titer is decreasing or fluctuating. After this stage, members of the group will be equally divided and half will be treated with curative doses of antimalarials as though they had an active infection with malaria. Both groups will be followed to see whether their titers decrease.

Supportive evidence that an elevated fluorescent antibody titer suggests an occult malarial infection is supplied by 10 cases of transfusion malaria we have studied (Sulzer and Wilson 1971). All 10 patients had multiple blood transfusions and subsequently developed malaria. One donor for each of 9 of the 10 cases was found to have malaria antibody in the IFA test. Three of the donors with positive antibody responses were confirmed by clinical evidence to be infected. In four cases, the infected donor was a native of an area with endemic malaria; the remaining five were military returnees from southeast Asia.

Babesiosis

In 1969, the fourth case of babesiosis in a human was diagnosed (Western *et al.* 1970). Of interest was the fact that in this case the serum was positive when tested with *P. falciparum* antigen in the malaria IFA test. The question of cross-reactions of malarial antisera in the IFA test with *Babesia argentina* was evaluated. Seven of 42 persons with either *P. falciparum* or *P. vivax*-induced malaria produced titers with the *B. argentina* antigen equal to titers obtained with the homologous antigen. Seven of eight cattle infected with *B. argentina* had a rise in titer of antibody to *P. falciparum* antigen. Three of 22 persons naturally infected with either *P. vivax*, *P. ovale*, or *P. falciparum* reacted with the *B. argentina* antigen.

In this study only one of many *Babesia* species was used as an antigen. The extent of cross-reactions with other *Babesia* species needs to be determined so that malarial antibody titers in areas hyperendemic for *Babesia* can be correctly interpreted.

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DISCUSSION

MR. E. H. FIFE: Dr. Kagan has touched on something that interests everyone involved with malaria surveys, diagnosis of malaria, and screening for malaria in Vietnam returnees. We need a good serologic tool to do survey work in endemic areas, and I'd like to add a bit of information of our own because we are engaged in serodiagnosis of malaria. This was some work that we did with sera from a group of Montagnards in the Northern Highlands of South Vietnam. We were trying to see whether we could incriminate or associate a curious idiopathic splenomegaly appearing in people in this particular geographic area with malaria, particularly with *P. malariae* infections. We did not find an association of *P. malariae* infection with the splenomegaly. However, we did find a curious thing in that among a group of some 50 individuals that were included in this survey, in less than 10% could we find or demonstrate malaria parasites of any stage on single thick blood films, whereas the incidence of high titers in the complement fixation test with *P. falciparum* antigen was over 90 per cent, and these sera gave complete fixation at a dilution of 1:256 or greater. In our CF test these titers were above the minimum considered to be of diagnostic significance. This means that if we went into an area with many semiimmune individuals and tried to use conventional hematological methods for establishing the incidence of infection, that we would misdiagnose or overlook many cases. I'm certain that a serologic test which would be easy to perform, such as Dr. Kagan described, will help us assess the incidence of malaria in endemic areas.

DR. I. G. KAGAN: Have you tested children with your CF test?

MR. FIFE: These were children because splenomegaly was occurring in seven to ten year olds. We did have a few adults; their titers were high, but the important thing is children's titers also were high. Furthermore, we were able to get as good reactions in *P. vivax* malaria using a purified *P. knowlesi* as we did using the homologous antigen. There's relatively little cross reaction with *P. falciparum* and/or *P. malariae*. Our *P. falciparum* antigen very seldom gives us even a titer of one with *P. vivax* or *P. malariae* antigen, and the *P. malariae* antigen does not cross react with either. So, our test has a relatively high level of specificity with regard to species, and there was considerable evidence from this particular group of sera that there was a number of individuals who had high titers with both the *P. falciparum* antigen and with the *P. knowlesi* antigen. This would indicate intercurrent infection with *P. vivax*.

DR. D. H. FERRIS: Does storage affect your serum titers?

DR. KAGAN: I showed you the results of tests on sera from Tobago. These sera were stored at −20 C for 15 years. I believe 90% were reactive after this interval of storage. Fortunately, we're dealing with an IgG antibody and not an IgM antibody. I believe IgM antibody becomes denatured on storage. We know that the serologic titer parallels the appearance of parasitemia, and one would expect when you have a positive slide that the serum should have a titer. We have demonstrated this in experimental infection in prison volunteers. We did find that some children with acute infections had serum specimens that lost titer on storage.