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Sulzer, A.J.; Wilson, Marianna

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9. ABSTRACT

In one decade, the indirect fluorescent antibody (IFA) test for malaria has developed from its first experimental stages into a widely used tool for studying the incidence of malaria. Although other serological tests, such as complement fixation, hemagglutination, and gel precipitation, are used to detect malarial antibody, the IFA test is the method of choice for both individual diagnosis and epidemiological studies. It has been applied most extensively in Africa. Comparatively few studies have been performed in other areas, but the IFA test should be a most useful method for assessing the results of malaria eradication projects in the Western Hemisphere. It would be beneficial to standardize the test procedure for accurate comparisons of studies performed in different laboratories. Although the basic test technique is similar worldwide, every group has its own modifications and thus introduces unnecessary variables. Results can be generally compared now, but some differences in results may be due to test procedures which might be resolved by standardization of methods. Technical developments can be made to improve test efficiency. The present test is tedious, time-consuming, and requires skill and a well-equipped laboratory. The introduction of multiple-place antigen slides has reduced the procedure time. A multispecies antigen, consisting of all four human malaria species in the same amount, would greatly reduce the time and effort now required for large-scale surveys. Automation of the test procedure and determination of results would not only improve test efficiency but be valuable in standardization by eliminating subjective judgments of fluorescence.

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THE FLUORESCENT ANTIBODY TEST FOR MALARIA

Authors: Alexander J. Sulzer A.J.
Marianna Wilson
Parasitology Section
Center for Disease Control
Atlanta, Georgia

Referee: Elvio Sadun
Dept. of Medical Zoology
Walter Reed Army Institute of Research
Washington, D.C.

INTRODUCTION

The fluorescent antibody (FA) technique, as developed by Coons, Creech, and Jones,[1] depends on immune serum globulin labelled with fluorescent dye to detect homologous antigen. This procedure, with several modifications, has been widely used with many diseases. Brooke, Healy, and Melvin[2] were the first to use the technique to detect malaria parasites in blood films. Since that time, the FA test for malaria has evolved into a sophisticated tool used for both research and diagnostic purposes. There are presently two types of FA tests used in malaria studies; the direct test, a continuation of the original work of Brooke et al., and the indirect test, a procedure used to detect specific malarial antibody rather than parasites or antigen.

DIRECT FLUORESCENT ANTIBODY TEST

The direct test is used to detect parasites, elucidate antigenic relationships, and to study pathologic states associated with malarial infection. Because the indirect method evolved from the direct method, a review of what has been accomplished with the latter serves as an introduction to the indirect test.

In 1961, Ingram, Otken, and Jumper[3] demonstrated antigenic differences between *Plasmodium gallinaceum* and *P. cynomolgi* using the direct FA test. In the same year, Tobie and Coatney[4] found cross-reactions between antisera and antigens of simian, human, and rodent malarias. Antigenic dissimilarities between the rodent malaria, *P. berghei*, and several primate malarias were reported by Voller in 1962.[5] He also found an absence of cross-reactions between two avian malarias, *P. gallinaceum* and *P. juxtanucleare*, as well as between these two and several primate malarias.

The first use of direct fluorescent antibody staining of exoerythrocytic stages of malaria was reported in two studies in 1963. Ingram and Carver[6] stained tissue forms of *P. gallinaceum* with conjugated *P. gallinaceum* antiserum. They also reported lack of specificity in staining some species but recognized the value of the method in studying the malarial cycle in the host. Voller and Taffs[7] found no fluorescence in tissue sections

containing *P. gallinaceum* schizonts treated with *P. juxtanucleare* antiserum; however, the exoerythrocytic stages stained well with homologous antisera. Ward and Conran[8] reported that conjugated antiserum to *P. cynomolgi* was highly specific for the tissue stages of *P. cynomolgi.*

Application of direct fluorescent antibody staining to the sporozite stage of malaria was first attempted by Coradetti et al.[9] in 1964. Using serum of rabbits immunized with sporozoites of *P. gallinaceum,* they found that the tagged antiserum specifically stained sporozoites of *P. gallinaceum* but not those of *P. giovannolai.* Lack of cross-reactions between avian *P. gallinaceum* sporozoites and antisera to *P. vivax, P. falciparum,* and *P. cynomolgi* was demonstrated by Sodeman and Jeffrey.[10] They obtained strong reactions with tagged homologous antisera and avian sporozoites, but no reactions occurred with fluorescent tagged primate malarial antisera. They also reported the use of Evans blue counterstain to control non-specific staining of background material.

The use of direct fluorescent antibody staining in the study of pathology associated with human malaria has been especially useful when applied to the nephrotic syndrome. Ward and Kibukamusoke[11] used conjugates specific to the corresponding immunoglobulins to demonstrate deposits of IgM, IgG, and IgA immunoglobulins in kidney biopsy sections from patients with the nephrotic syndrome. In 3 of 13 cases, they also found *P. malariae* antigens associated with these deposits. Their failure to demonstrate malarial antigens in all cases may have been, in addition to the reasons they give, because they used only anti-*P. malariae* conjugate. Conjugates specific for other *Plasmodia* might have been useful if the antigen present in the complex was other than *P. malariae.* Allison et al.[12] also showed deposits of immune complexes in renal biopsy specimens from children with the nephrotic syndrome and active infection with *P. malariae.* Ward and Conran,[13] using a *P. cynomolgi*-rhesus monkey model, demonstrated that other species of malaria might produce the same effect. They also extended the evidence that the human nephrotic syndrome associated with malaria is, in fact, due to deposits of antibody-antigen complexes in the kidney.

Adeniyi, Hendrickse, and Houba[14] found that 20% of patients exhibiting the malarial nephrotic syndrome had a selective proteinuria and that one third of this group responded well to prednisolone therapy. The observed reduction in proteinuria was accompanied by disappearance of glomerular deposits of immunoglobulins. Direct fluorescent antibody staining of renal biopsy sections is the method of choice to evaluate effectiveness of therapy in such cases.

INDIRECT FLUORESCENT ANTIBODY TEST

The necessity of tagging every serum with fluorescein renders the direct fluorescent antibody test impractical as a routine diagnostic method for detection of serum antibody. The indirect method that uses a fluorescein conjugated anti-globulin serum is better adapted for this purpose. Kuvin et al.[15] were the first to use indirect fluorescent antibody (IFA) techniques as a practical means of malarial antibody detection.

IFA Test Reagents

Antigen

Antigen for the IFA test consists of thin or thick smears of infected blood. Initially[15] sera were tested with malaria organisms obtained from individuals other than the serum source. The reason for this is obscure, but presumably it was thought to give more dependable results than when the antiserum from a patient was tested with his own parasites. Later it was recognized that the antigen donor developed malarial antibody which might interfere in the test. Kreier and Ristic[16] described an in vivo reaction with *P. berghei* of the mouse donor's antibody with its parasites. They made thin smears of parasitized whole mouse blood and treated these smears with conjugated anti-mouse globulin. Positive fluorescence of parasites was seen in blood slides made three to eight days after onset of parasitemia. To avoid this in vivo reaction, Ciuca[17] and Sodeman and Jeffery[18] recommended that parasitized erythrocytes for antigen be drawn before the infected donor had an opportunity to produce significant amounts of antibody.

Subsequently, Sulzer, Wilson, and Hall[19] demonstrated that if parasitized erythrocytes are washed free of plasma soon after they are taken from the donor, the donor's antibody status is immaterial. Even if the donor has an antibody titer of 1:1024 or greater, antigen prepared from his erythrocytes by prompt washing was completely

satisfactory. This indicates that the in vivo reaction of malaria antibody can be ascribed to the presence of antibody in the whole blood thin films. This also accounts for the lack of success of initial attempts to use thick blood films as antigen, which would contain relatively large amounts of immunoglobulins. The in vivo reaction described was, in reality, an in vitro reaction of antibody incorporated with the whole blood smears.

Acidified water containing 0.5 to 1.0 percent hydrocloric acid was used for dehemaglobulinizing whole blood thin smear antigens. This water may have been a factor in the usefulness of this type of antigen. Solutions of low pH are known to cause disassociation of antigen-antibody complexes.[20] If a small amount of the donor's antibody is present in the whole blood antigens, it may be removed by the acid lysis step. It has been reported, however, that acid lysis of thick smear washed cell antigens reduces the titer by as much as 64-fold with some antisera.[19] Whether this reduction is due to removal of an acid soluble moiety from the plasmodial antigen or to some other reason, it may account for the lower titers reported by those using acid lysis as compared with those using only distilled water for this step.

For the greatest sensitivity and reactivity, test antigens must contain the schizont stage of development. Although schizonts are mentioned in early reports[15] as being present in antigens, no stress was placed on the necessity to include them. Since many of the initial studies were performed with parasites from human sources, *P. falciparum* antigens probably contained no schizonts since they are rarely seen in the peripheral blood of human patients. Sodeman and Jeffery[18] mentioned that "more mature forms" seemed to give better reactions. Sulzer et al.[19] mentioned that schizonts seemed to have greater reactivity than trophozoites. Targett[21] reported in detail on the greater reactivity of the schizont antigen. He ascribed this property to a quantitative difference in the antigenic response of schizonts as compared with the response of trophozoites; but there were no firm data to support this assumption. To whatever cause this difference may be due, it is plain that to compare reactions in the malaria IFA test, the stage of parasites used for antigens must be specified. If one antigen consists only of trophozoite stages and the other contains schizonts, differences or similarities of reactions may be more apparent than real.

Sources of antigenic material have been a major problem in the IFA test procedure since its inception. For human malarias, homologous antigens were obtained from experimentally infected human volunteers when available; alternatively, simian malarial species were employed but these lacked specificity and sensitivity. When schizonts were found to give the greatest reactivity, human sources were rendered more unsatisfactory, since it is not always possible to obtain material containing high percentages of schizonts.

The susceptibility of the South American night monkey, *Aotus*, to infection with three human malaria species has been of great value. Young, Porter, and Johnson[22] reported that *P. vivax* would grow readily in splenectomized *Aotus*. Geiman and Meagher[23] infected *Aotus* with *P. falciparum* and produced very high parasitemias. Many life cycle stages, especially schizonts, that are rarely seen in man appear in the peripheral circulation of the monkey. Geiman and Siddiqui[24] reported adaptation of *P. malariae* to the *Aotus*. *P. brasilianum*, a quartan parasite of New World primates, morphologically resembles *P. malariae*. For the IFA test it can be used as an antigenic substitute for *P. malariae*[25] and is easily maintained in *Ateles* monkeys. The availability of these parasites in experimental animals has made homologous antigens of three of the human malarias readily available to laboratories that perform IFA tests. The other human species, *P. ovale*, can only be maintained in chimpanzees or man.[26] Fortunately, this is the least common and least virulent of the human malarial parasites.

Conjugate

The term "conjugate" in fluorescent antibody work usually refers to an anti-gamma globulin serum which has been tagged or labelled with fluorescein isothiocyanate (FITC). Production of this reagent has been the subject of extensive research; the interested reader is referred to either Cherry, Hebert, and Pittman[27] or Nairn.[28]

A prime requirement of the conjugate is that it react with the immunoglobulin containing the specific antibody to be detected. As noted below, the IgG portion of the immunoglobulins usually accounts for the major portion of malarial antibody; therefore, conjugates used in the IFA test for malaria should at least have activity toward this constituent. If all antibody is to be detected,

activity to all immunoglobulins should be present. For routine diagnostic work, this is preferred.

In certain instances, it is desirable to detect antibodies in a particular immunoglobulin class in the presence of antibodies in the other immunoglobulin classes.[29] The IFA test is very convenient for this purpose. Class specific immunoglobulin conjugates are now commercially available; methods for their production are described in the sources cited above.

A satisfactory conjugate should have little or no activity to the antigen or, if it does, this activity should be easily eliminated by dilution. Working dilutions or titers of commercial conjugates for use in parasitic IFA tests have, until recently, rarely exceeded 1:20. However, conjugates which can be diluted 1:200 to 1:400 for use in the test have been produced commercially. This is a most important development for it appears that such conjugates will increase the reactivity of the test. Whether this increase in reactivity, coupled with a similar effect of schizont antigens, will influence specificity adversely remains to be seen. Conjugates with working titers of more than 1:100 are usually diluted far beyond the level at which nonspecific staining occurs, a distinct advantage in IFA tests.

Test Procedure

In whatever laboratory performed, test procedures are essentially variations of the same technique. Microscope slides of parasitized blood films are prepared and stored at -70° until needed. If desired, they can be treated with acetone for fixation and then dehemaglobulinized in water which can be acidified. Serum dilutions are applied and allowed to react, either at room temperature or at 37°C for 20 to 30 minutes. One or more washing steps follow to remove unreacted serum components. Anti-species conjugate, diluted to an optimal level and sometimes in combination with Evans blue counterstain, is then applied and the slide is incubated a second time for 20 to 30 minutes after which it is washed again. Buffered glycerol is then placed on each reaction site, a coverslip is superimposed, and the reaction determined using a fluorescence microscope. Illumination is usually by a mercury arc light through a darkfield condenser. Appropriate exciter and barrier filters are employed; these vary according to the worker.

Test Parameters

Definitions

Accurate knowledge of five parameters – sensitivity, specificity, minimal significant dilution, reactivity, and reproducibility – is essential for interpretation of serologic test results. The test is calibrated by determining these parameters. Precision of test interpretation cannot exceed the precision with which these basic determinations are made.

Sensitivity is a measure of the efficiency of antibody detection. This is especially important when antibody concentration is low, as it is in the beginning of the infection. It is influenced not only by antibody concentration but also by antigen quality and test conditions. It is usually stated in percent of known positive sera that are detected.

Specificity can be described as either the false positive rate or as the percentage of positive reactions that represent specific antibody. Other organisms may share a common antigen with the agent in question, and exposure to it may stimulate cross-reacting antibodies. Although this antibody is specific for the shared antigen, its presence will reduce specificity of the test by contributing to the false positive rate. Non-specific factors in sera as well as antigen quality and test conditions may also contribute to the false positive rate.

The minimal significant titer is determined by the conflict between requirements for sensitivity and specificity; conditions that increase one of these parameters very often decrease the other. The lowest dilution of known positive sera for which sensitivity and specificity are in optimal balance is designated as the minimal titer significant for specific antibody.

Reactivity (the endpoint titer) may be defined as the relative degree to which an antiserum may be diluted before it ceases to give a positive reaction. In the IFA test this is influenced greatly by the antigen and the quality of the conjugate. Flexibility and usefulness of the test are greatly enhanced when reactivity is high. When considerations of sensitivity and specificity compel setting a relatively high minimal significant titer, reactivity must at least exceed this level – indeed, this is the major determining factor in sensitivity. When antibody levels are used to determine current status of infection, the reactivity should be great enough for there to be a marked difference between antibody titers during and after infection. Determination of

species by serology, as well as determination of phylogenetic relationships, depends on titer differences; in such instances, reactivity at relatively high levels is a prime requirement. The same criteria apply to the study of antigenic specificities of different life cycle stages employed as antigen.

Reproducibility is usually applied to titer determination. Test conditions are controlled so that the titer of a given serum, when tested two or more times, will not vary more than two dilution factors in a majority (usually 95%) of titrations.

The five parameters defined above are interdependent and a change in any one factor in the test procedure may significantly alter one or all of them. The malaria IFA test has never been standardized to any extent; each laboratory has its own variation. For this reason, results from any given laboratory must be interpreted with parameter determinations made in that laboratory. Comparisons between laboratories, therefore, can be made only on a relatively broad basis due to the number of variables involved.

Another factor to be carefully considered when evaluating experimental results is the extent to which the experimental design reduces the effect of bias.[30] This factor is too often neglected in practice. If no mention is made of bias-excluding design in reports, one must assume that it was not introduced. Design against experimental bias is especially important in estimating test parameters as well as in comparing or correlating titers.

Published Studies on Test Parameters

There has been a gradual change in numerical values of test parameters in the malaria IFA test. Collins, Jeffery, and Skinner in 1964[31] implied that a positive reaction in undiluted serum is specific and a reaction of 2 plus is positive and reproducible. In 1965, Collins et al.[32] working with simian malarial antisera, reported titers of 1:40 or above as positive, but in 1966[33] with human antisera, they reported 1:20 as the minimal significant dilution. In 1968[34] Collins, Jeffery, and Skinner again reported 1:20 as the lowest positive dilution indicative of exposure to malaria. McQuay et al.[35] reported 1:80 as the lowest significant titer with 1:40 as only suspicious. Voller reported minimum titers of 1:25 in 1964[36] and 1:20 in 1968;[37] Meuwissen[38,39] also used 1:20. In 1969 El-Nahal[40] used an initial dilution of 1:10, while Dranga, Marinov, and Miha[41], in the same year preferred 1:20. In all these studies, the dilution series is in twofold increments, which indicates that reproducibility is within plus or minus one twofold dilution. The literature contains no data from studies designed to support the lowest significant titer or twofold dilutions.

Sodeman and Jeffery,[18] using three antisera and one antigen, studied reproducibility of titers. They concluded that titers could be reproduced within plus or minus one threefold dilution. They also noted that counterstain with Evans blue, applied according to Diggs and Sadun,[42] improves visualization of the test.

Sulzer et al.[19] published an evaluation of the sensitivity, specificity, and reproducibility of washed-cell thick-smear antigen. They limited the study to three antigens: *Plasmodium falciparum*, *P. vivax*, and *P. brasilianum*; the latter was considered by them to be equivalent to *P. malariae*. The majority of their 141 positive sera were from *P. vivax* cases. Included in the battery were 210 sera from persons never exposed to malaria. Sulzer et al.[19] found that positive reactions at 1:16 or greater were indicative of the presence of malarial antibody. Specificity at this level was better than 99%, sensitivity 95%, and reproducibility plus or minus one fourfold dilution. This study confirmed the work of Diggs and Sadun,[42] that homologous antigens must be used to detect the greatest percentage of cases because some sera did not react with other than their homologous antigens. Results of this study are inconclusive when applied to antisera other than *P. vivax* because of the lack of sufficient numbers of antisera from other human malaria species.

Cross-Reactions Between Malarial Species

One of the first concerns with the IFA test for malaria, as with any serological test, was a source of antigen. Human patients with a given *Plasmodium* infection and with a parasitemia of the required level were not always available. If simian malarial antigens could be proved to be suitable for testing human malarial antisera, a more convenient source of antigen could be maintained. In addition, phylogenetic relationships would be revealed by serological cross-reactions of various *Plasmodium species*. Therefore, the serological relationships of various human and simian malarias was one of the first subjects of intensive study by IFA methods.

Primate Malarias

Tobie et al.[43] found that antisera to *P. vivax* and *P. cynomolgi* cross-reacted with their respective antigens, and the titer was always greater with the homologous parasite; there were, however, two strains of *P. vivax* which could not be separated by titer differences. Voller[5] found cross-reactions between primate malarias. On the basis of similar titers obtained with human antisera titrated against *P. falciparum* and *P. cynomolgi bastianellii*, Kuvin and Voller[44] suggested that the latter species might be used as antigen in serologic investigations of human malaria. Tobie et al.[45] suggested that the lower titers obtained in heterologous as compared to homologous systems in *P. cynomolgi* antigen-antibody studies were due to a specific antigen confined to *P. cynomolgi*. This would explain the equal titers found when *P. vivax* antisera were tested with both *P. vivax* and *P. cynomolgi* antigens. If the *P. cynomolgi* antigen contained schizonts and the *P. vivax* antigen did not, the difference might also be explained.

Collins et al.[32] studied sera from monkeys with undetermined malaria infections by testing with known simian malarial antigens. They suggested that heterologous reactions may be greater than homologous reactions. This effect, again, might be due to differences in the life cycle stages found in a given species of antigen. The differences in titer in this study are very small; with each antiserum the titers fall with only one exception within fourfold limits of one another.

This study was followed by two others in which sera from known infections of human and simian malaria as well as antigens of the same species were tested.[33,46] A large amount of cross-reactivity was found, and homologous reactions were usually greater. However, antisera to *P. inui* and *P. coatneyi* reacted at higher titers with *P. fieldi* than with their homologous antigens. In 1967, sera from Nigerians were tested with three human and two simian malaria species antigens.[25] The most efficient antigen was *P. falciparum*, which was closely followed by *P. brasilianum* and *P. fieldi*. Again, the importance of cross-reactions as measured by titer differences is hard to assess, since neither stage of parasite antigen nor reproducibility of titers is given. Either one, or a combination of both, could have markedly influenced the results. It is clear from these studies, however, that *P. fieldi* may be a good simian substitute antigen for testing *P. falciparum* antisera and that *P. brasilianum* may be good for testing *P. malariae* antisera.

The French group of Coudert and associates[47-49] tested antisera from cases of *P. falciparum* and *P. vivax* infections, and used *P. vivax*, *P. falciparum*, and *P. cynomolgi bastianellii* as antigens. Using the simian antigen they detected both human species almost as well as when they used homologous antigens. Whether schizonts were present or not in any of the antigens was not mentioned.

Meuwissen[39] compared simian antigens for possible use in detecting human malaria cases, and he agreed with Collins that *P. fieldi* was a good substitute for human antigens and better than *P. cynomolgi*. With heterologous antigens, some titers were lower than with homologous antigens. With antisera to *P. falciparum*, titers of 1:1280 were not uncommon. Meuwissen does not mention the stage of parasite he used as antigen.

In an elegant study of cross-reactions, Collins et al.[50] demonstrated serological differences in 14 strains of *P. inui*. They employed a carefully considered experimental design which included coding and blind testing, statistical comparisons, and other factors to insure that bias would be reduced to a minimum and that the results would be valid. With similar methods, it may be possible to greatly increase our knowledge of phylogenetic relationships between the various species of malarias. It is regrettable that such care in experimental design is so rare in scientific publications.

The nonprimate malarias have served as convenient laboratory models for the study of the IFA reaction. Avian and rodent models are much less expensive than primates; the cost of maintaining primates prohibits their use in many laboratories. The difficulty in assembling appropriate batteries of antisera and antigens of the human malarias confines their use to the relatively few laboratories to which such materials are available.

Rodent and Avian Malarias

In 1962 Voller[5] reported few cross-reactions between rodent and avian antigens and human and simian malarial antisera. In 1965[51] he found some cross-reaction between *P. berghei* and *P. vinckei* in rats, but the heterologous reactions were much lower than the homologous reactions. There were no cross-reactions between *P. berghei* and the avian malarias *P. gallinaceum* and *P. juxtanucleare*. El-Nahal[52] illustrated the serological similarities

and differences between two strains of *P. berghei* and between *P. berghei* and *P. chabaudi* and *P. vinckei*. There were no differences between the two strains of *P. berghei* or between the other two species, but they could be readily differentiated one from the other.

In the study of cross-reactions of malaria antisera, the work of El-Nahal[53] with exoerythrocytic schizonts as antigen in the IFA test is most interesting. The antigens were *P. berghei yoelii* from the rat, *P. malariae* from humans, two strains of *P. cynomolgi*, and the avian *P. gallinaceum*. Antisera to four rodent malarias reacted only with *P. berghei* antigen. Antisera to three stains of *P. cynomolgi* reacted to *P. cynomolgi*, but antisera to *P. inui*, *P. shortti*, and *P. malariae* failed to react. Only *P. malariae* antisera reacted with *P. malariae* antigen; *P. falciparum* antisera did not react. The only reactor to *P. gallinaceum* antigen was the *P. gallinaceum* anti-serum; *P. juxtanucleare* antiserum failed to react. El-Nahal suggests that exoerythrocyte schizonts may prove to be species-specific antigens in IFA methods. Future studies should include a greater number of species and antisera to confirm this finding. Phylogenetic studies would be greatly enhanced by a very specific antigen in the IFA test. Another of his interesting observations was the occurrence of a "prozone" with *P. cynomolgi* antisera. This effect is probably due to some blocking agent in the system and not to the mechanism usually assumed to account for the classic prozone phenomenon encountered in agglutination and precipitin tests.

Of the nonprimate malarias used as antigen to detect human malarial antibody, *P. gallinaceum* has been reported to be satisfactory.[54,55] Kielmann et al.[56] reported that the avian parasite was as reactive to human malarial sera as *P. falciparum* and exceeded that of *P. cynomolgi*. These workers mentioned that they did not find an in vivo reaction of antibody with the *P. gallinaceum*. None of the chickens used as an antigenic source had malarial antibody when tested with anti-chicken conjugate. The findings of Keilmann and his co-workers should be carefully confirmed since the availability of an antigen as easily maintained as *P. gallinaceum* would benefit all laboratories performing diagnostic tests for malarial antibodies.

In many laboratories simian malarial antigen has become the basis for tests in both diagnostic and epidemiological studies of malaria in human populations. As convenient as simian or avian antigens are, if greatest sensitivity is to be achieved, the homologous antigens to all species suspected are essential. Since the *Aotus* monkey can be easily infected with human malarial species, it is now possible for all laboratories to obtain the homologous antigens.

Cross-reactions between malaria and babesia

In 1968, Cox and Milar[57] demonstrated that prior infection with certain malaria species effectively protected rodents against subsequent challenge with *Babesia*. Suspecting that this cross-protection was due to common antigens shared by both genera, Cox, Milar, and Patterson[58] detected serological cross-reactions between *Plasmodium* and *Babesia* by gel precipitation and bentonite flocculation. They then used the IFA test to study the antibody response in mice to *Babesia* infection.[59] Very weak cross-reactions were observed between the morphologically similar *B. microti* and *B. rodhaini*. In 1970[60] Cox and Turner used the IFA test to determine antigenic relationships between malaria and babesia. *P. vinckei*, *P. chabaudi*, *P. berghei berghei*, *P. berghei yoelii*, *B. rodhaini*, and *B. microti* antisera and antigens were produced and cross-matched. The greatest titers were always obtained with the homologous system, but there were considerable cross-reactions between all six species. Morphological similarities of the *Plasmodium* species were matched by serological results, but the two morphologically similar *Babesia* species were found to be serologically dissimilar.

Until 1970, only three cases of human babesiasis had been recognized, all in splenectomized individuals. Western et al.[61] reported *Babesia microti* infection in a fourth human with a functional spleen. The initial diagnosis by stained blood films was *P. falciparum*: this is not surprising because of the close morphological resemblance of *P. falciparum*; and *B. microti* trophozoites. Sera from the patient reacted in the IFA test to both *B. microti* and *P. falciparum* antigens.

The serological relationships of *Babesia* and *Plasmodium* warrant more extensive studies with the IFA test. Although very few cases of babesiasis have been diagnosed in man, subclinical infections may be more common than previously thought. Man and the animal hosts of *Babesia* live in close proximity in many areas of the world, some of which are endemic for malaria. Inapparent (sub-

clinical) infections of *Babesia* may confuse the interpretation of IFA test results for malaria if cross-reactions occur to any extent. In endemic malarial regions, a stained blood slide containing only *Babesia* could be easily misdiagnosed as malaria. It is to be hoped that many more reports on this subject will appear in the future.

Antibody Patterns

Antibody Production

After development of the IFA test for malaria, several groups immediately began to study the production of antibody in experimental infections. Kuvin et al.[15,62,63] and Tobie et al.[43,45,64] found that, in *P. vivax* and *P. cynomolgi* infections, patent parasitemia preceded antibody reactions by a few days to a week or more. Maximal antibody reactions were detected one to one and one half months later. Antibody titer dropped after treatment but usually did not reach negative levels during the experimental periods. Homologous antigen-antibody combinations exhibited the greatest reactivity. In contrast with the results of Kuvin and Tobie and their co-workers, Coudert et al.,[65] using *P. cynomolgi* as antigen, reported that positive antibody responses to *P. vivax* infections preceded patent parasitemias. They suggested that this result may have been due to the methods of infection.

To study this relationship of parasitemia and antibody response, Lunn et al.[66] infected volunteers with *P. vivax* and placed five on suppressive chloroquine therapy. Four of these individuals did not develop antibodies until 38 to 77 days after chloroquine was discontinued, and 2 to 6 days after onset of patent parasitemia. The other did produce antibodies while on suppressive chloroquine and before a patent parasitemia was observed; antibody increased fourfold after onset of patent parasitemia. The authors attributed this patient's early production of antibody to subpatent parasitemia that occurred because of the low serum chloroquine levels.

Antibody production to infection with *P. falciparum* and *P. malariae* has also been extensively studied. In a series of three papers on experimental infections, Collins et al.[31,67,68] reported weak cross-reactions between the two species. Antibody to *P. falciparum* could persist up to twenty months with only slight diminutions of titer. Antibody titer in non-immune (not previously exposed) patients fluctuated more widely than did titers in semi-immune (previously exposed) *P. falciparum* patients. The authors suggested that parasites must be present to maintain high antibody levels. They reported that semi-immune patients had higher antibody levels than non-immune patients. However, they also mentioned that they used a different lot of conjugate and new fluorescence equipment when testing the semi-immunes. This could easily account for the higher reactions. Lunn et al.[69] found parasitemias preceding positive antibody reactions by three to nine days, and globulin increases were correlated with recrudescence of parasitemias in patients with *P. falciparum* infections. Lupascu et al.[70] found in experimental *P. malariae* infections that parasitemias preceded antibody reactions by four to six days. They hypothesized that antibodies detected by IFA are involved with the protective process; they based their ideas on the supposition of an in vivo antigen-antibody reaction with intracellular parasites and the observation of high titers with low parasitemias and low titers with increased parasitemias.

Very little work has been done with experimental *P. ovale* infections. Meuwissen[38] found that antibody titers appeared two to six days after onset of patent parasitemias; he could not relate parasite density and antibody level.

Except for several casual references,[15,62,69,70] no particular attention was directed toward malarial antibody decay until 1968. In that year, Collins et al.[34] studied the antibody persistence in induced malarial infections. Of 14 patients infected with *P. falciparum*, one maintained a low positive antibody response up to 8 years after the termination of the infection. Initial negative responses were seen as early as six months to three years after termination. With *P. malariae*, negative reactions were observed three to six years after treatment, with low positive results as late as ten to thirteen years. Patients with *P. vivax* were tested three years after termination of infection, and all were negative. Multiple infections increased the duration of positive antibody responses. Six years or more after parasitemia, *P. malariae* patients not given curative treatment more consistently had positive results than patients given treatment. Lupascu et al.[71,72] extended the results with *P. malariae* infections. They reported that, with induced infections, titers were generally negative six months to one and one

half years after radical treatment. Blood donors, considered index cases of transfusion-induced malaria, generally became negative one month to one year after treatment.

Antibody persistence has been reported in nonimmune populations with natural infections. Luby, Collins, and Kaiser[73] found low levels of antibody remaining in U. S. citizens who had been infected 13 years before with *P. vivax*. Wilson, Sulzer, and Runcik[74] followed the antibody response after treatment of U. S. servicemen who experienced clinical attacks of *P. vivax* or *P. falciparum* after returning from Vietnam. They reported to detectable antibody in 36 of 68 patients 6 months after radical treatment with the majority of positive reactions at 1:16. The patients were followed for only one year after treatment.

Immunoglobulin Response to Induced Infection

The immunoglobulin response to malaria infection and its relationship to the antibody detected by IFA are of much interest. Kuvin et al.[62] noted in experimental infections that the increase in total gamma globulin was correlated with the rise in malarial antibody titer, but that the excess gamma globulin did not consist entirely of malarial antibody. Ciuca[17] agreed with this result. Abele et al.[75] extended the work to show that increases in $beta_2$-M macroglobulins closely coincided with the appearance of malaria antibody detected by IFA. Lunn et al.[69] also found that the initial increase in gamma globulins closely coincided with the appearance of antibodies. They reported a transient rise in the gamma globulins and $alpha_2$ globulins, a transient decrease in the albumin and $alpha_2$ globulins, and no consistent change in the beta globulins during each episode of parasitemia. Tobie et al.[76] reported that IgM globulin production was greatly increased during experimental infections of *P. vivax* and *P. cynomolgi*. Increases in IgG were also large but not as striking as the IgM levels. Increases in antibody levels were correlated with increases in immunoglobulin. In the same year, Tobie et al.[77] published findings that suggested dead plasmodia elicited slight antibody responses in *P. vivax* infections. They concluded that the antibody response was probably due to asexual forms and not to sporozoites or exoerythrocytic parasites. They agreed with McGregor[78] that the late asexual stages provided the antigenic stimulus for the production of specific antibody. This is not surprising, because the rupture of the schizont is the point in the life cycle when metabolic products as well as merozoites are discharged into the circulatory system. Cohen and Butcher[79] mention several studies which suggest that protective antibody acts against mature schizonts or extra-cellular merozoites.

Anti-IgM, IgG, and IgA immunoglobulin conjugates have recently been used to study the IgM, IgG, and IgA response to malarial infection as detected by IFA. Cox, Crandall, and Turner[80] have used the malarial parasites of mice as models for these studies. In 1969, mice infected with *P. vinckei* and *P. chabaudi* were examined. During the early stages of infection, IgM antibody levels were higher than those of IgG, but the IgG levels soon rose to higher levels than the IgM. There was no apparent decline in IgM 39 days after infection nor increase in antibody levels after challenge. In 1970, Cox[81] showed that titers were either the same as, or one dilution less when obtained with an anti-IgG conjugate than when obtained with a conjugate that detected both IgM and IgG. Titers with the anti-IgM conjugate were much lower. He concluded that the relative levels of IgM and IgG could not be used to indicate how recently the infection had been acquired, because the IgM antibody levels remained elevated during convalescence.

A somewhat different situation exists in *P. berghei yoelii* infections.[82] The pattern of antibody production during initial infection was similar to those seen with *P. vinckei* and *P. chabaudi*. However, mice infected with *P. berghei yoelii* showed marked increases in antibody titers after challenge. The authors suggested that this result might be due to the low parasitemia produced by *P. berghei yoelii* infection; this low parasitemia might provide only a small initial antigenic stimulus compared with the other rodent malarias.

Collins et al.[83] used specific immunoglobulin conjugates to study antibody patterns in experimental human infections. In those infected with *P. vivax*, the IgG response rose and peaked slightly earlier than the IgM response. In *P. falciparum* infections, both peaked at approximately the same time. Regardless of whether or not the patients were treated, the IgM and IgA responses returned to low levels, while the IgG levels remained elevated. They suggested that these responses could be of practical use in distinguishing between

recent infection and past experience with malaria.

Non-immune Populations

In areas where there is no natural transmission of malaria, imported and induced infections often present diagnostic problems. In the United States, the number of imported malaria cases has increased dramatically since 1966.[84] Transfusion-induced malaria is a direct result of importation. The person who receives blood from several donors and experiences a malaria attack obviously acquired the malaria from a donor with occult infection. Blood film examinations of the donors are generally useless because of the low level of parasitemia. Donor history of infection or travel in malarious areas may not always be completely reliable. Many authors[71,85-88] have used the IFA test very successfully to detect infected donors.

People with occult infections who reside in a non-immune population represent a threat to continued freedom from malaria. Dranga et al.[41] tested a Rumanian population from an area that was formerly hyperendemic for malaria, but that had been under malaria control for 20 years. Nineteen persons (8.8%), all of whom were born before 1950, had positive titers ranging from 1:20 to 1:5120 to *P. malariae*. *P. malariae* was subsequently isolated from one of these people. In a nonmalarious village, only two people (0.7%) reacted with *P. malariae*. This finding suggested that high IFA titers may indicate occult malaria infections which might account for transfusion malaria years after eradication. In countries under malaria control where surveillance methods are not very effective, natural transmission from occult carriers could create an epidemic situation. In the last five years, several instances of natural transmission in the United States have been reported.[84,89-91] The IFA test can be an asset in the epidemiological investigations of such cases.

Induced malaria among heroin users is also a direct result of imported malaria. Two clusters involving six cases of induced malaria among drug users were reported in 1970.[84] In both clusters, the infected individuals had shared injection equipment with Vietnam veterans who had a history of malaria. With heroin addiction on the increase in the United States, this type of induced malaria will probably become more common. Here again, the IFA test can be most useful in detecting asymptomatic malaria infections to maintain malaria control.

Congenital malaria is very rarely seen in the United States. The reports on two recent cases have included IFA results.[29,35] One mother had not been in a malarious region for seven years and the other, for three; *P. malariae* was diagnosed in both of the women after the children were found to be infected. Harvey et al.[29] used anti-IgM conjugate to demonstrate malarial IgM antibody in both the mother and child.

Very few studies of malaria infection have been made in non-immune populations. Meuwissen[39] tested sera from 18 patients naturally infected. As in experimental infections, maximum antibody response developed within two to four weeks after the onset of parasitemia, then decreased gradually after treatment. Leibovitz et al.[92] tested sera from 17 military returnees with malaria. 183 returnees exposed in Vietnam, but with no history of malaria, and 50 persons with no known exposure to malaria. IFA titers were positive in all 17 infected cases; no reaction was detected in the 50 non-exposed individuals, but 49 of the other 183 returnees (26%) had positive reactions. Based on their results, the authors suggested that IFA test as a screening tool to detect infected individuals with no history of clinical malaria. If the positive reactors had been given antimalarial treatment and antibody titers had been followed, this study would have been of more value.

Studies on Immune Populations

The role that antibody plays in malarial immunity has been extensively studied in endemic areas. Classically two methods have been used to determine malarial endemicity; the spleen rate, or the percentage of young children with enlarged spleens, and the parasite rate, or the percentage of persons found with malarial parasites in peripheral blood. However, spleen rates are known to be influenced by many other diseases, and malarial parasite rates detected by blood film examination are generally low in adults. Circulating parasites in the peripheral blood cannot be detected in blood films in a high percentage of the older population. If these adults have occult infections, serological methods might be useful in determining true endemicity. For this purpose, the IFA test for malaria has been found to be a valuable asset.

Voller and Bray[93] were the first to use the IFA test to determine antibody levels in a population from a malaria endemic area. Sera of Liberian children with heavy patent parasitemias of *P.*

falciparum, *P. malariae*, or *P. ovale* were tested. High titers were demonstrated in cord bloods; antibody titers declined after birth, then rose to high levels at four years of age, paralleling the known pattern of gamma globulin levels. Kuvin and Voller[44] found that West Africans living in Britain for three months to seven years had relatively low antibody titers. Since the same patients had high gamma globulin levels, they felt that malarial antibody represented very little of the excess globulin. They suggested that antibody production is decreased after the patient leaves the malarious area because of lack of antigenic stimulation by repeated infection. Voller and Wilson[36] treated a group of Gambian mothers and their newborn children with anti-malarial drugs. After seven months the mothers' antibody levels were much lower than those of mothers in an untreated group. After nine months of therapy, the children had no detectable antibody, while in an untreated group the reactions were mostly positive. As Voller and Wilson observed, this finding indicates that repeated infections are necessary to maintain high antibody levels. They imply that positive IFA titers might be indicative of current or recent infection with malaria, whether or not *Plasmodia* can be found in the peripheral blood.

Several attempts have been made to determine how much of the total gamma globulin was actually due to malarial antibody. Curtain et al.,[94] using column chromatography, found that only 6 to 11% of the total 7S globulin in people from holoendemic malarious areas was specific malarial antibody. Cohen and Butcher,[95] allowing for nonspecific absorption by the parasite preparations, reported 5.4% of total IgG in Gambian sera was specific malarial antibody. Curtain, Gorman, and Kidson[96] tried to correlate malarial antibody titers with gamma globulin levels in Melanesian children. Malarial antibody titers were lower in children protected by chemotherapy, but gamma globulin levels did not differ from those of unprotected children with relatively high antibody titers. The authors suggested that the high gamma globulin levels could be due not only to malaria, but also to many other infectious disease.

Turner and Voller[97] compared IgD, IgA, IgG, and IgM immunoglobulin levels of Nigerian adults with those of British adults. IgD and IgA levels were similar in both groups, but IgG and IgM levels were significantly higher among Nigerians than British. No correlation of immunoglobulin levels with malarial antibody titers was found. Turner and Voller were quite aware that the elevated immunoglobulin levels could be due to diseases other than malaria. In the second part of this study, McFarlane and Voller[98] found that IgA, IgG, and IgM levels were not significantly different in persons with or without peripheral parasitemia, although the IgA and IgM levels were higher and the IgG levels lower in the group with detectable parasitemia. The IFA titers were greater in those persons with positive blood films.

McFarlane et al.[99] measured IFA responses and immunoglobulin levels of a Nigerian population. The development of IFA titers paralleled the development of IgG. Serum IgG was lowest when the children were between four weeks and four months of age and reached adult levels at about five years. Adult levels of serum IgM were reached at one year. They suggest that the IgM detected at birth may be indicative of infection.

Initial studies indicated that the IFA test could be useful in determining malarial endemicity. McGregor et al.[100] made a cross-sectional study of malaria in Gambia by comparing ages, parasite counts, and IFA test results. Antibody levels were high in newborn infants, fell rapidly during the first year, and then rose throughout childhood; this is the classic pattern of maternal transfer of antibody. As IFA titers rose, parasitemias dropped, indicating some correlation between titer and immunity. McGregor and his co-workers considered the sum of duration and density of parasitemia as the factor determining antibody titer. They suggested the IFA test as an excellent method of determining malarial endemicity. However, since a well equipped laboratory is essential for performance of the test, it was not considered suitable for field work.

Coudert et al.[101] tested sera from North Africa and found the IFA test to be of great value in regions endemic for malaria. Titers in general were proportional to the endemicity and increased with age. For diagnostic purposes in such populations, a negative result indicated no infection and a positive result only indicated infection at some unknown time. Collins et al.[25] tested Nigerian sera with five *Plasmodium* antigens. Highest titers were seen with *P. falciparum* antigen; the authors also confirmed that antibody titer increased with age. Rey, McGregor, and Mattern[102] evaluated the usefulness of the IFA test in hypo- and hyperendemic areas in West Africa. Using *P. falciparum*

as antigen, they concluded that the test is excellent for obtaining epidemiological information, but in endemic zones it is of little diagnostic value in determining current infection.

More sophisticated studies of malarial endemicity have been reported since 1967. Collins et al.[103] tested sera from three areas of Malaysia: hyperendemic, hypoendemic, and an area where malaria control measures were in progress. *P. falciparum* and *P. fieldi* antigens were found to have the greatest reactivity in all three areas. However, *P. vivax* was the most prevalent species in blood films from the hypoendemic and the area under malaria control. The hyperendemic region had high parasitemia rates and high IFA titers. The hypoendemic area had low parasitemia rates and low IFA titers. Where control measures had been taken, parasitemia rates were low but the IFA titers high.

A study of the effect of malaria control measures was performed by Harverson, Wilson, and Hall.[104] Sera from children in Bathurst, where mosquito spraying is practiced, were compared with sera of children from a region in Gambia that is hyperendemic for malaria. Spleen rates were greater, hemoglobin levels lower, and the percentage of children with peripheral malaria parasites was much greater in the hyperendemic area than in Bathurst. Children in Bathurst had low antibody titers, while those from the hyperendemic area had high titers. The authors considered the IFA test to be a very good indicator of the effectiveness of control measures. Another confirmation of the value of the IFA test for epidemiological purposes was published by Voller and Bruce-Chwatt[37] who found that 853 of 914 sera (92%) collected in Nigeria were positive in the IFA test. They stress the necessity for caution in interpreting serological results and the importance of thorough knowledge of the local epidemiology of malaria.

Voller, Lelijveld, and Matoba[105] surveyed the IgG, IgM, and malarial IFA levels of several groups in northeastern Tanzania. They studied populations at three different altitudes: 1) below 750 feet, an area of intense transmission; 2) 1800-3000 feet, an area of sporadic transmission; and 3) above 4500 feet, an area of little or no transmission. Malarial antibody was detectable in almost everyone tested in Area 1, and mean titers increased with age. In Area 2, only half of the children under 2 years old had detectable antibody; however, the older age groups were virtually all positive at levels lower than those in Area 1. In Area 3, very few of those under 2 years old had malarial antibody; only one-third of the older persons were positive at very low levels. The titers found in Area 3 might be due to the mobility of the population traveling to malarious areas, or they may be false positives (see discussion on *Babesia*). The immunoglobulin studies agreed with those of other workers. The number of positive IFA reactions was always greater than the number of positive blood slides. Because of the increasing IFA titers and decreasing parasite rates with age, Voller et al. suggested that the antibody levels reflect the development of functional immunity. *P. fieldi* was used as antigen; the authors stated that if *P. falciparum* (the most prevalent species) antigen had been used, titers would have been higher but would have occupied the same relative positions.

A serological malarial survey in the highlands of Ethiopia was reported by Collins, Warren, and Skinner.[106] Malaria eradication activities had not been initiated in the study area. All individuals were examined for malaria parasites, and the IFA test was performed with all four human malaria species. The IFA results indicated very little malaria transmission over 6300 feet. At elevations below 6000 feet, positive responses were detected in 36.7% of the people. *P. falciparum* antigen gave the highest number of positive responses, followed by *P. ovale*, *P. malariae*, and *P. vivax*. The higher titers with *P. ovale* as compared with those of *P. vivax* suggest that *P. ovale* is more prevalent in this area than previously thought.

The relationship between malaria and pregnancy in Nigeria was the subject of a report by Gilles et al.[107] Malarial antibody titers were determined before and during pregnancy. Although many of the women developed peripheral parasitemias during pregnancy, the IFA titers did not show consistent changes. There was a progressive fall in both IgG and IgM levels during pregnancy. This was a clear demonstration that reduction of effective immunity during pregnancy was not reflected in IFA titer levels. At least in this study, it is plain that IFA titers may not be an indication of the immune state of the individual to malaria. Williams and McFarlane[108] reported on malarial antibody in maternal and cord sera of Nigerian mothers and their infants. Titers in the IFA test ranged from 1:1280 to 1:5120 in the

mothers and from 1:640 to 1:2560 in the cord bloods. All of the malarial antibody was found in the IgG fraction, although the presence of IgM was demonstrated by immunoelectrophoresis in both maternal and cord serum. The authors concluded that malarial immunity of the newborn Nigerian is due to passively acquired maternal IgG antibody.

Tropical splenomegaly has been investigated by both the direct and indirect FA tests. Gebbif et al.[109] found that splenomegaly in Ugandan children was associated with sinusoidal infiltration of the liver and elevated malaria IFA titers. This appears to be the first instance in which malarial antibody was associated with a particular type of pathology. Marsden et al.,[110] investigating tropical splenomegaly in Uganda, considered malaria infection among other possible causes. The presence of malarial organisms in some patients and elevated malarial IFA titers were only suggestive of a connection between splenomegaly and malarial infection. Vanier, Hutt, and Cook[111] found elevated IFA titers in children with gross splenomegaly in Uganda and concluded that malaria infection was the most common cause of this condition.

An unusual, but possibly very useful method of obtaining malarial antibody was described by Kibukamusoke and Wilks.[112] Urinary proteins from patients with the nephrotic syndrome were concentrated by gel filtration with Sephadex G-25 and found to contain much globulin. Malaria IFA tests were performed on both serum and the concentrated urinary proteins. Though kidney pathology and antibody titer in the urinary proteins could not be correlated, serum antibody titers and urinary protein titers were related. Although the authors maintain that their data support the conclusion that most of the elevated gamma globulin found in Ugandans is due to malarial infection, the connection seems tenuous at best. However, the method may be very useful in areas where cultural factors pose a problem in collecting blood samples. The presence of urinary antibody to malaria should be studied for its correlation with active infection, since this might furnish a useful tool for epidemiological studies.

SUMMATION

In one decade, the IFA test has developed from its first experimental stages into a widely used tool for studying the incidence of malaria. Although other serological tests, such as complement fixation, hemagglutination, and gel precipitation, are used to detect malarial antibody, the IFA test is the method of choice for both individual diagnosis and epidemiological studies. It has been applied most extensively in Africa. Comparatively few studies have been performed in other areas of the world, particularly the Western Hemisphere. Projects for malaria eradication are being actively pursued in these areas; the IFA test should be a most useful method for assessing the results.

Standardization of the test procedure would be beneficial for accurate comparisons of studies performed in different laboratories. Although the basic test technique is similar worldwide, every group has its own modifications and thus introduces unnecessary variables. General comparison of results can be made at this time, but some differences in results may be due to test procedures which might be resolved by standardization of methods.

Technical developments can be made which will improve test efficiency. The test is presently tedious, time-consuming, and requires skill and a well-equipped laboratory. The introduction of multiple-place antigen slides has reduced the procedure time. A multispecies antigen, consisting of all four human malaria species in the same mount, would greatly reduce the time and effort presently required for large-scale surveys. Automation of the test procedure and determination of results would not only improve test efficiency but would also be valuable in standardization by eliminating subjective judgments of fluorescence.

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