AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY

1. SUBJECT CLASSIFICATION

A. PRIMARY: Public Health

B. SECONDARY: Malaria

2. TITLE AND SUBTITLE

Indirect flourescent-antibody tests for parasitic diseases: V. An evaluation of a thick-smear antigen in the IFA test for malaria antibodies

3. AUTHOR(S)

Sulzer, A.J.; Wilson, Marianna; Hall, E.C.

4. DOCUMENT DATE	5. NUMBER OF PAGES	6. ARC NUMBER
1969	7 p.	ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS

Public Health Service, Center for Disease Control, Parasitology Branch, Atlanta, Georgia 30333

8. SUPPLEMENTARY NOTES *(Sponsoring Organization, Publishers, Availability)*

(In American Journal of Tropical Medicine and Hygiene, v. 18, no. 2, p. 199-205)

9. ABSTRACT

This study was performed to evaluate a washed-cell, thick-smear antigen for use in the indirect flourescent-antibody test for malaria. Thick-smear antigens of Plasmodium vivax, P. falciparum, and P. brasilianum were prepared from parasitized erythrocytes washed free of soluble serum components by several changes of phosphate-buffered saline solution, pH 7.2. Serum samples both positive and negative for malaria antibody were tested with blind and double-blind techniques. The lowest significant diagnostic titer was found to be 1:16. At this dilution, false positive rates were less than 0.5%, false negative rates were below 5%, and the range of titer reproducibility was plus or minus one fourfold dilution. Use of the modified antigen had certain advantages over the conventional whole-blood, thin-smear antigen. Binding of the antigen donor's antibody to his plasmodia is avoided. This effect, previously attributed to an in vivo reaction, seems to occur in vitro. Reading is facilitated by the large number of plasmodia per field.

10. CONTROL NUMBER	11. PRICE OF DOCUMENT
PN-AAC-635	
12. DESCRIPTORS	13. PROJECT NUMBER
	14. CONTRACT NUMBER: PASA RA(HA)-5-68 Res.
	15. TYPE OF DOCUMENT

AID 590-1 (4-74)

614.532
S954a

Printed in United States of America
Reprinted from THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE
Vol. 18, No. 2, March 1969
p. 199-205
Copyright © 1969 by The American Society of Tropical Medicine and Hygiene

INDIRECT FLUORESCENT-ANTIBODY TESTS FOR PARASITIC DISEASES

V. AN EVALUATION OF A THICK-SMEAR ANTIGEN IN THE IFA TEST FOR MALARIA ANTIBODIES

levandi
A. J. SULZER, MARIANNA WILSON, AND ELMER C. HALL
U. S. Public Health Service, Health Services and Mental Health Administration, National Communicable Disease Center, Atlanta, Georgia 30333

ABSTRACT: This study was performed to evaluate a washed-cell, thick-smear antigen for use in the indirect fluorescent-antibody test for malaria. Thick-smear antigens of *Plasmodium vivax*, *P. falciparum*, and *P. brasilianum* were prepared from parasitized erythrocytes washed free of soluble serum components by several changes of phosphate-buffered saline solution, pH 7.2. Serum samples both positive and negative for malaria antibody were tested with blind and double-blind techniques. The lowest significant diagnostic titer was found to be 1:16. At this dilution, false positive rates were less than 0.5%, false negative rates were below 5%, and the range of titer reproducibility was plus or minus one fourfold dilution. Use of the modified antigen had certain advantages over the conventional whole-blood, thin-smear antigen. Binding of the antigen donor's antibody to his plasmodia is avoided. This effect, previously attributed to an *in vivo* reaction, seems to occur *in vitro*. Reading is facilitated by the large number of plasmodia per field.

Antigen preparation and techniques for performing the indirect fluorescent-antibody (IFA) test for malaria antibodies have varied little since Kuvin *et al.* first described the test in 1962.[1] Various problems related to the antigen have been noticed: the *in vivo* binding of antibody,[2,3] the difficulty in standardizing antigen, and the need for mature plasmodia to detect the highest possible antibody levels.[4] With thin-smear antigens, extended searches are often necessary because of sparseness of plasmodia.

Certain reactions characteristic of the malaria IFA test have never been delineated in detail, perhaps in part because of the problems cited. The lowest titers indicative of clinical malaria have been given as 1:80[5] and 1:20.[6] Reproducibility of titers has been reported to be plus or minus one threefold dilution on the basis of 19 replicate tests of only one serum sample tested with only one antigen.[4] Sensitivity of the test has not been explored definitively.

In an attempt to overcome some of these problems, we prepared and evaluated a thick-smear antigen for use in the malaria IFA test. Thick-smear antigen prepared from whole blood was useless, however, because of high levels of nonspecific fluorescence. Extraneous gamma globulin has been found to be an interfering factor in the sensitivity and specificity of other IFA tests.[8] It was thought that the donor's soluble serum components, especially any portion consisting of specific malaria antibody, might interfere in the malaria IFA test when antigen was prepared from whole blood. We therefore washed the parasitized cells to remove these components before preparing a thick-smear antigen.[9]

A thick-smear antigen prepared from washed parasitized erythrocytes overcame all the problems encountered earlier. Washing the blood had additional benefits.[9] We report an evaluation of the sensitivity and specificity, and a preliminary study of the reproducibility of test results with the washed-cell, thick-smear antigen.

MATERIALS AND METHODS

Preparation of the Antigen

Heparinized blood containing plasmodia was centrifuged, and the plasma was removed. The cells were resuspended in 10 times their volume of phosphate-buffered physiological saline solution (PBS, pH 7.2), centrifuged, and the PBS was discarded. Resuspension and centrifugation were repeated five times. The cells were resuspended in PBS to their original volume. One drop of the suspension was dispensed on each of several slides with a hypodermic syringe equipped with a 26-gauge needle. From the drops, thick smears were prepared, stained, and the number of plasmodia per high-power field was determined. When too

A. . . D.
Reference Center
Room 1656 NS

many were present, the cell suspension was diluted by the addition of PBS. When plasmodia were too few, the suspension was concentrated by centrifugation and removal of excess PBS. Storage of washed cells overnight or 48 hours at 4° C did not reduce their suitability for antigen. In one instance, washed cells were stored at 4° C for 10 days with no adverse effect.

When antigen slides were to be prepared the same day the blood was drawn, an alternative method for controlling the number of plasmodia per field was used. Thin smears, prepared from the donor's blood, were fixed and stained. The average number of parasites per high-power field was determined. This number was multiplied by 15, the average concentration factor of thick over thin films for malaria parasites.[10] The cells were then washed as already described and suspended in PBS. The volume of the suspended cells was determined by the desired number of plasmodia per field in the finished antigen mounts. For example, if the thin-smear count averaged two plasmodia per field and the desired number per field in the finished antigen was 15, the washed cells were suspended in PBS to twice the volume of the original blood specimen.

Antigen slides were prepared by dispensing one drop of the suspended cells in a 1.5-cm circle scribed on a 2- by 3-inch microscope slide. The drop was spread to make a thick smear within the circle. Care was taken that the cells did not settle out of suspension in the syringe. The mounts were allowed to air-dry at room temperature for about ½ hour. The dried slides were wrapped in tissue paper and stored at −70° C until needed. Antigen slides have been stored for as long as 12 months with no detectable change in reaction. Three antigens—*Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium brasilianum* (simian equivalent to *Plasmodium malariae*[5, 11])—were employed throughout the tests. In addition, *Plasmodium fieldi* antigen was used in one phase of the study.

Performance of the Test

Antigen slides were removed from storage, placed in staining racks, and washed for 10 minutes in distilled water on a slowly moving slide rotator. After the slides were washed, they were removed from the bath; excess wash fluid was removed by careful blotting.

The antigen slides were labeled and placed in moist chambers prepared by lining shallow pans with wet filter paper. Serum samples to be tested were diluted with PBS (pH 7.2) in fourfold steps starting at a dilution of 1:4. About 0.05 ml of each dilution was dispensed on a labeled antigen mount. The chamber was covered and incubated at 37° C for 30 minutes.

After incubation, excess serum was removed from each slide with a gentle stream of PBS. The slides were placed in a staining rack in PBS on a slowly moving slide rotator for 15 minutes. The slides were then removed from the wash and replaced in a moist chamber. The appropriate dilution of anti-human gamma globulin conjugate, Cohn's Fraction II (0.05 ml), containing Evans blue counterstain (0.2%) was distributed over all antigen mounts.[12] The slides were covered and incubated again at 37° C for 30 minutes. Excess conjugate was removed by a gentle saline stream followed by immersion in PBS and washing on the rotator for 15 minutes.

Excess wash fluid was removed from the slides by careful blotting, and the slides were distributed on trays. A drop of buffered glycerol (pH 8.0) was placed on each mount and a coverslip superimposed. Two positive control serum samples, one negative control serum, and a saline-solution control were included with each test. Labels on all slides, including the controls, were covered with tape; the slides were then randomized and coded in order that all readings would be made "blind."

All tests were evaluated on a Leitz Ortholux fluorescence microscope equipped with BG-12 and UG-2 exciter filters and a Leitz 470 μ ocular filter. Plasmodia can be located readily by the pigment's refraction of blue light under the BG-12 filter. Specific fluorescence can then be evaluated under the UG-2 filter. Fluorescence was graded from negative to 4+. A reading of 2+ was considered positive.

Antiserum

Ninety-two serum samples submitted for routine testing for malarial antibodies were used in this study. Blood smears accompanying the serum were examined at the National Malaria Repository, NCDC, where one or more species of malaria was identified in all 92 instances.

For an initial check of nonspecific reactions,

especially those due to syphilis, 26 serum samples were obtained from American teen-agers who had never been outside the United States. These donors all had primary or secondary syphilis.

Another group of 233 serum samples was assembled to evaluate further the sensitivity and specificity of the thick-smear antigen. Untested serum from 184 persons with no history of exposure to malaria was included. Fifty-six of the donors were Eskimos from Alaska; 128 were American Indian schoolchildren with an ascariasis rate of 49%, a trichuriasis rate of 39%, and an amebic-prevalence rate of 72%. The other 49 persons had malaria diagnosed by slide and low IFA titers (1:16–1:64) with at least one antigen on previous tests. These were included to avoid any presumptive bias in testing the "negative" groups. There were 29 cases of *P. vivax*, 13 of *P. falciparum*, three of *P. vivax* and *P. falciparum*, three of *P. malariae*, and one of *Plasmodium ovale*. The 233 serum samples were coded and randomized to assure objectivity in the performance and reading of the IFA test.

Two positive serum samples were used as control. One, "Mor," was from a male donor with a history of *P. vivax* infection. The other, "ET," was from a female donor who had a patent parasitemia of *P. malariae*.[6] Negative serum and saline-solution (conjugate) controls were also included with each test.

RESULTS

Several investigators have reported the binding of donor's antibody by plasmodia, an effect attributed to an *in vivo* reaction.[2-5] We thought this might be due to an *in vitro* reaction of the antibody that was incorporated with whole-blood antigens. Malaria antibody has been titrated from dried blood specimens on filter paper,[12] and we thought that whole-blood smears on glass slides were similar. Any malaria antibody that was in the donor's blood would be present in the dried blood film, and it could react with the plasmodia at any time.

To investigate this possibility, we drew blood from a monkey parasitized by *P. brasilianum*. A portion of this blood was washed as already described and a portion left unwashed. Both portions were stored at 4° C, and antigen slides were prepared daily from each. After three days' storage, antigen prepared from the unwashed cells showed positive reactions in saline-solution and negative serum controls. Antigen prepared from the washed portion was satisfactory even after the cell suspension had been stored for 10 days.

P. vivax reacted similarly to *P. brasilianum* but within a shorter time. Antigens prepared from cells of four donors with *P. vivax* malaria washed within 30 minutes of removal from the donors were satisfactory, although the donors' plasma had malaria-antibody titers of 1:1,024 in three cases and 1:64 in the other. Blood samples drawn from 10 different persons with *P. vivax* malaria were stored at 4° C for about 12 hours and then washed. All lots of antigen prepared from these patients gave positive reactions in the saline-solution and negative serum controls.

Among 92 slide-positive specimens submitted for routine testing for malaria antibody, 89 were positive by the IFA test at a titer of 1:16 or above with one or more antigens, and three were negative. The highest titer obtained was 1:4,096.

TABLE 1

Reactions to three plasmodium antigens of serum from 184 persons not exposed to malaria and 49 with a positive slide diagnosis

Serum group	Titers	Washed-cell thick-smear antigens					
		P. falciparum		*P. vivax*		*P. brasilianum*	
		(*No.*)	(%)	(*No.*)	(%)	(*No.*)	(%)
Donors not exposed to malaria	Negative	157	[illegible]	180	97.8	183	99.5
	1:4	26	[illegible]	4	2.2	1	0.5
	1:16	1	[illegible]	0	0.0	0	0.0
Donors with positive slide diagnosis*	Negative	6	[illegible]	4	8.2	9	18.4
	1:4	10	[illegible]	4	8.2	3	6.1
	1:16	33	67.4	41	83.6	37	75.5

* All samples in this group had previously had titers of at least 1:[illegible] with one of the three antigens. On this second titration, two of these serum samples had negative reactions with all three antigens.

TABLE 2

Day-to-day reproducibility of IFA titers based on two titrations of 49 slide-positive serum samples with three antigens

Reproducibility* (*No. of serum samples*)	Antigen			Total
	P. falciparum	*P. vivax*	*P. brasilianum*	
With exact agreement on both titers	19 (39%)	23 (47%)	16 (33%)	58 (40%)
With a 4-fold difference between titers	22 (45%)	23 (47%)	21 (43%)	66 (45%)
With a 16-fold difference between titers	6 (12%)	3 (6%)	9 (18%)	18 (12%)
With a 64-fold difference between titers	2 (4%)		3 (6%)	5 (3%)
Total samples tested	49	49	49	147(100%)

* Differences in reproducibility among the three antigens are not statistically significant by the chi-square test.

One of the three negative serum samples was from a *P. ovale* infection to which the homologous antigen was not available. Four serum samples from *P. falciparum* and five from *P. vivax* cases reacted only with their homologous antigens. For IFA diagnosis of malaria in man, therefore, the homologous antigens appear essential to avoid an occasional false negative reaction.

Among the 26 serum samples from persons with syphilis, only one was reactive in the test. This serum gave identical results when retested with the *P. falciparum* antigen at a dilution of 1:4.

Among the 233 serum samples tested with three malaria antigens, only one serum from a person not exposed to malaria reacted at a dilution of 1:16 (Table 1). This titer was obtained with the *P. falciparum* antigen giving a false positive rate of 0.5% at the 1:16 dilution. At the 1:4 dilution, false positive rates were 14.7, 2.2, and 0.5% for *P. falciparum*, *P. vivax*, and *P. brasilianum* antigens.

Among the 49 serum samples from persons with slide-diagnosed malaria, negative reactions were obtained on 12.2, 8.2, and 18.4% with the three antigens. In addition, titers of 1:4 were obtained on 20.4, 8.2, and 6.1% of the samples. However, only two of the 49 samples gave negative reactions at the 1:16 dilution level with all three antigens on the second titration. This reflects the influence of day-to-day reproducibility of titers on the sensitivity of the IFA test. Over-all, a titer of 1:16 appears to provide the most favorable balance of sensitivity and specificity.

Reproducibility of titers on a day-to-day basis is indicated by a comparison of the two titers obtained on the 49 slide-positive serum samples. Table 2 shows the number of samples with the same titers and with fourfold, 16-fold, and 64-fold differences in titers on the two determinations. Differences in reproducibility among the three antigens are not statistically significant by the chi-square test; hence, day-to-day reproducibility can be estimated from the combined data (Table 2). For example, titers obtained on different days are either the same or one fourfold dilution apart 85% (40 + 45) of the time. Conversely, differences exceed one fourfold dilution 15% of the time; however, only 3% of the differences are as much as 64-fold (three dilutions) apart. These figures correspond to 97% reproducibility within ± one fourfold dilution (16-fold range).

Replicate testing of the two positive control serum samples, "Mor" and "ET," also provides information on day-to-day reproducibility. Multi-

TABLE 3

Distribution of titers for two antiserum samples with four antigens in the IFA test for malaria

Reciprocal titer	Plasmodium antigens							
	P. vivax		*P. falciparum*		*P. brasilianum*‡		*P. fieldi*	
	ET*	Mor†	ET	Mor	ET	Mor	ET	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

* *P. malariae* was demonstrated in the circulating blood of this mother of an infant with congenital malaria.[9]

† A district medical officer, Sepik, New Guinea, he had reported *P. vivax* infection with treatment 1 year before sampling.

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

TABLE 4

Comparison of infecting species of Plasmodium *with difference in antibody titer obtained with homologous and heterologous antigen in vivax and falciparum malaria*

	No. of serum samples*	No. vivax	No. falciparum
No difference in titer	9	7	2
Homologous titer greater:			
4-fold	12	7	5
16-fold	11	8	3
64-fold	12	8	4
256-fold	9	9	0
1,024-fold	4	4	0
Heterologous titer greater:			
4-fold	3	2	1
16-fold	0	0	0
64-fold	1	1	0
Total	61	46	15

* All donors were military men returned from Vietnam. Many gave a history of recurrent attacks of malaria; this was true in the case with a heterologous titer 64-fold greater than that with the infecting *Plasmodium* at the time the specimen tested was drawn.

ple titers on each antiserum were obtained in routine IFA testing with four antigens, two of simian and two of human origin. Objectivity was assured by randomizing and blind-testing of the control serum samples within each run. Table 3 shows the distribution of titers for each antigen-antiserum combination. With the exception of two replicates with "Mor" serum and the *P. falciparum* antigen, replicate titers on each antiserum were reproducible on a day-to-day basis within limits of ± one fourfold dilution (16-fold range). This corresponds to an over-all reproducibility of 99.3% (287 of 289 replicates). However, titers on the "ET" antiserum were reproducible within a fourfold range (two serially adjacent titers) on 98.7% of the replicates.

As shown in Table 3, the highest titers for each serum were obtained with the antigens most similar to the infecting species. Serologic speciation would be possible if significantly greater titers were obtained with homologous antigens than with heterologous antigens. Table 4 shows the differences in titers of 61 antiserum samples, 46 from slide-positive *P. vivax* cases and 15 from slide-positive *P. falciparum* cases tested with *P. vivax* and *P. falciparum* antigens. Considering 16-fold or greater differences in titer with homologous and heterologous antigens significant (as seems reasonable from Tables 2 and 3), 59% of the infecting species were identified serologically. Only one antiserum titrated significantly higher with the heterologous antigen. In this instance, there was a history of recent falciparum infection, although *P. vivax* was identified on the blood smear at the time the serum was drawn.

DISCUSSION

Several authors have reported an *in vivo* reaction of the antigen donor's antibody and parasites.[2,5] Our results indicate that this reaction is probably not *in vivo*, but *in vitro*. If the parasitized cells are washed free of the donor's serum within 30 minutes of the specimen's being drawn, antigen prepared from the cells is always satisfactory. This is true even when the donor's antibody level is 1:1,024. On the other hand, if the whole blood is allowed to stand several hours before washing, plasmodia in antigen preparations may react brilliantly in the saline-solution and negative controls. *P. vivax* is especially sensitive; parasitized cells that remain in serum for only 12 hours are unfit for antigen. Other species may be more stable.

Antigens prepared from whole blood include any antibody that has developed in the donor. Although this antibody may not have been taken up by the plasmodia at the time of antigen preparation, it may react with them at any time during storage or during the testing procedure. That this should occur, especially in persons with high antibody titers, is not surprising, for it has been demonstrated that antibody can be titrated from dried blood on filter paper.[12,13] Because the reaction of the donor's antibody with his plasmodia seems to occur *in vitro* and not *in vivo*, this interfering factor can be avoided. Use of washed-cell antigens removes the necessity of selecting antigen donors before the development of specific malaria antibody as previously suggested.[4,5]

The washed-cell thick-smear antigen has several other advantages. The number of plasmodia from mount to mount and from lot to lot of cells can be kept constant. With 15 to 30 organisms per field, the scanning of two to three fields at random is ample to evaluate the reaction. The large number of parasites per field will usually include several schizonts, which give higher titers than

trophozoites in the same field with some antiserum.[11] Particularly with *P. brasilianum* antigens, the individual merozoites within the mature schizonts are often delineated in an intense green pattern, whereas young trophozoites in the same field may have no specific fluorescence.

Titers do not seem to be affected by wide fluctuations in numbers of plasmodia, contrary to previous report.[11] We found that titers obtained with two antiserum samples did not vary significantly in repeated tests with antigen mounts that had 25 to 50 or 100 to 150 plasmodia per field.

Pretreatment of the antigen slides with 0.1% HCl to lyse the erythrocytes, as reported,[1,8,5,7,11,12] is not advisable with the thick-smear antigen. We have found this treatment to reduce titers as much as 64-fold. This effect could be due to removal of an acid-sensitive antigen component from the plasmodia.

Results of the initial evaluations, with serum from 92 persons with slide-positive cases of malaria and from 26 American teen-agers with syphilis, were encouraging. Failure to detect one of the 92 slide-positive cases could be attributed to lack of the homologous antigen, *P. ovale*. Detection of four *P. falciparum* and five *P. vivax* cases with only their homologous antigens emphasized the need for homologous antigens for all antiserum samples tested. Since the only reaction of the syphilitic serum was at a 1:4 dilution with the *P. falciparum* antigen, positive reactions at dilution levels of 1:16 or greater may be considered diagnostic of malaria. Our results with the syphilitic serum confirmed previous findings that antiserum to *Treponema pallidum* does not cross-react in the malaria IFA test.[1,12]

Intestinal parasitic infections rarely lead to false positive reactions in the malaria IFA test. Of the 184 serum samples from persons never exposed to malaria, 128 were from persons with a high prevalence of intestinal parasites. Only one of these samples was positive at the 1:16 dilution level.

Nonspecific reactions at the 1:4 dilution level (Table 1) indicate that 1:16 is the lowest reliable diagnostic titer for the test. This agrees well with the 1:20 level designated by Collins *et al.*[7] and is fivefold less than the 1:80 level designated by McQuay *et al.*[10] We also confirmed the findings of Diggs and Sadun[12] that *P. falciparum* antigen gives a higher percentage of nonspecific reactions than do other *Plasmodium* species.

Sensitivity of the washed-cell antigen, *i.e.*, the detection rate for malarial antibodies, appears to be excellent even at the lowest diagnostic level. However, due to variables inherent in all test procedures, fluctuation of titers of serum samples with low antibody content between positive at the 1:16 level and negative in repeated tests is to be expected.

Comparison of duplicate titrations on the 49 positive serum samples and replicates of the control serum (Table 2) indicates that titers are at least 97% reproducible within plus or minus one fourfold dilution. Reproducibility was not antigen-dependent. This study was limited to day-to-day variations in the IFA test. A more detailed study with multiple tests of each serum will be required to determine within-day reproducibility.

P. vivax and *P. falciparum* infections may be occasionally differentiated by the IFA test, especially when the homologous titers are at least 16-fold greater than the heterologous titers (Table 4). Thorough appraisals of significance of titer differences with antigens of all *Plasmodium* species infecting man must be made before serologic speciation can be applied with confidence to malaria infections in man.

SUMMARY

For use in the malaria IFA test, thick-smear antigens were prepared from washed, parasitized blood cells. Removal of the donor's antibodies and other soluble serum components by washing prevented their interference in the reaction. The number of plasmodia per field in the finished antigen could be controlled. The false positive rate with this antigen was less than 1% at the 1:16 dilution level. The detection rate of known malaria infections was 95% with serum samples of low titer. Reproducibility is not dependent on serum-antigen relations and is within a plus or minus one fourfold dilution range. *P. vivax* and *P. brasilianum* antigens were more specific in reaction than *P. falciparum* antigen. In a comparison of serum from persons with vivax and falciparum malaria tested with antigens of the same species, the titer was significantly greater with the homologous antigen in 59% of the instances.

REFERENCES

1. Kuvin, S. F., Tobie, J. E., Evans, C. B., Coatney, G. R., and Contacos, P. G., 1962. Fluorescent antibody studies on the course of antibody production and serum gamma globulin levels in normal volunteers infected with human and simian malaria. *Am. J. Trop. Med. & Hyg., 11:* 429–436.
2. Kreier, J. P., and Ristic, Miodrag, 1964. Detection of a *Plasmodium berghei*-antibody complex formed *in vivo*. *Am. J. Trop. Med. & Hyg., 13:* 6–10.
3. Sodeman, W. A., Jr., and Jeffery, G. M., 1965. Immunofluorescent studies of *Plasmodium berghei*: A "natural" antibody in white mice. *Am. J. Trop. Med. & Hyg., 14:* 187–190.
4. Sodeman, W. A., Jr., and Jeffery, G. M., 1966. Indirect fluorescent antibody test for malaria antibody. *Pub. Health Rep., 81:* 1037–1041.
5. Ciuca, M., Bona, C., Ciplea, A. G., Ianco, L., Negulici, E. B., and Constantinesco, P., 1964. Les mécanismes de l'immunité acquise dans l'infection à *P. vivax*. *Arch. Roum. Path. Exper. Microbiol., 23:* 749–762.
6. McQuay, R. M., Silberman, Simone, Mudrik, Pola, and Keith, L. E., 1967. Congenital malaria in Chicago, a case report and a review of published reports (U.S.A.). *Am. J. Trop. Med. & Hyg., 16:* 258–266.
7. Collins, W. E., Jeffery, G. M., Guinn, Elizabeth, and Skinner, J. C., 1966. Fluorescent antibody studies in human malaria. IV. Cross-reactions between human and simian malaria. *Am. J. Trop. Med. & Hyg., 15:* 11–15.
8. Sulzer, A. J., and Kagan, I. G., 1967. Indirect fluorescent antibody tests for parasitic diseases. III. Conjugate-antigen relationships in the tests for trichinosis and schistosomiasis. *Am. J. Med. Techn., 33:* 192–199.
9. Sulzer, A. J., and Wilson, Marianna, 1967. The use of thick-smear antigen slides in the malaria indirect fluorescent antibody test. *J. Parasitol., 53:* 1110–1111.
10. Field, J. W., Sandosham, A. A., and Fong, Y. L., 1963. *The Microscopical Diagnosis of Human Malaria*. Economy Printers, Ltd., Kuala Lumpur.
11. Collins, W. E., Skinner, J. C., and Coitman, R. E., 1967. Fluorescent antibody studies in human malaria. V. Response of sera from Nigerians to five *Plasmodium* antigens. *Am. J. Trop. Med. & Hyg., 16:* 568–571.
12. Diggs, C. L., and Sadun, E. H., 1965. Serological cross reactivity between *Plasmodium vivax* and *Plasmodium falciparum* as determined by a modified fluorescent antibody test. *Exper. Parasitol., 16:* 217–223.