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Studies on various aspects of the indirect haemagglutination test for malaria*

J. H. E. T. MEUWISSEN, ANNA D. E. M. LEEUWENBERG, & G. E. MOLENKAMP

This paper gives the results of studies on various technical aspects of the indirect haemagglutination (IHA) test for malaria, on the similarity of the results obtained in the IHA test and in the indirect fluorescent antibody test, on the use of various plasmodial extracts as sensitizing antigens in the IHA test, and on the influence of heterophile antibodies on the titres obtained in the IHA test. Some longitudinal observations on induced malaria infections of man and monkey showed that the infection can induce the production of heterophile antibodies: their appearance, however, remains unpredictable. In some infections agglutinins against host erythrocyte components are also produced. Absorption of sera with tanned sheep cells sensitized with noninfected host red blood cell antigens is advocated as a control on the IHA titre for specific agglutinins.

The indirect haemagglutination (IHA) test, so successfully applied in various fields of microbiology and immunology, still awaits wide acceptance as a routine method for the seroepidemiological study of malaria despite the fact that it was introduced for that purpose in 1962 by Desowitz & Stein (1962). It is obvious that a simple reproducible serological test for epidemiological studies in the field would be of great practical importance. Such a test could be applied to the assessment of malaria endemicity, to the study of fluctuations in the intensity of malaria transmission, and to the evaluation of malaria control and eradication measures. For some years we have from time to time used the IHA technique in the course of experimental studies of malaria. This paper presents a preliminary report on our experience with the IHA test with particular attention to attempts to assess the influence on the test of the development of heterophile antibodies. It is hoped that the paper may stimulate other workers to explore ways of further improving the test.

MATERIALS AND METHODS

Sera

Human serum samples collected from 276 people and sent to the laboratory between 1965 and 1970 for examination by the indirect fluorescent antibody (IFA) test for malaria were also used for this study on haemagglutinins. Most of the sera were from Caucasians with symptoms of fever or splenomegaly, who had attended the outpatient department of a tropical diseases institute in the Netherlands, or from those having a routine medical examination following a stay in the tropics. Serum samples from four neurosyphilitic patients, blood-inoculated with *Plasmodium ovale* (Meuwissen, 1966), were also examined by the IHA test.[1] Sera collected at regular intervals from rhesus monkeys inoculated between 1968 and 1970 with various species and strains of plasmodia were also used. All sera had been stored at −20°C without preservatives.

Parasites

The following strains and species of malaria parasites were used: *Plasmodium cynomolgi bastianellii*, *P. c. langur*, *P. c. ceylonensis*, *P. inui*, *P. knowlesi*, *P. knowlesi* Guy strain, *P. fieldi*, and *P. vinckei*. *P. fieldi* was obtained from Dr W. Collins (Chamblee, Ga., USA) and *P. vinckei* from the late Professor Vincke (Antwerp, Belgium); the other species of plasmodia were received from Dr A. Voller (London, England).

* From the Department of Medical Microbiology, University of Nijmegen, Nijmegen, The Netherlands.

[1] Some samples in the longitudinal follow-up study had been used in other experiments and were no longer available for examination by the IHA test.

Preparation of sensitive antigen

Splenectomized rhesus monkeys were infected by intravenous inoculation with the primate plasmodia. The animals were bled at the moment of maximum parasite density, when most of the parasites were schizonts. The blood was defibrinated with glass beads and the cells were washed in phosphate buffered saline (PBS), pH 7.2, and resuspended in the original volume of PBS. To eliminate the white blood cells the erythrocytes were twice aggregated by addition of an equal volume of 3% dextran in PBS. To concentrate the parasitized cells, the erythrocytes were suspended in at least 3 volumes of PBS and centrifugated for 15 min at 1 100 *g* at 4°C. The lighter parasitized cells tended to form a darker band on top of the other erythrocytes. This band was carefully removed with a bent pipette. Following repeated separation of the bands through washing and centrifugation the remaining cells were resuspended in PBS, the suspension was centrifuged, and the darker bands were combined. In this way a suspension containing mostly parasitized cells was prepared. These packed parasitized erythrocytes were stored at −70°C in volumes of 0.1 ml. On the day of the actual test, 0.9 ml of PBS, pH 5.5, was added to the cells and the suspension was subjected to ultrasonic treatment for exactly 10 seconds, with a Branson Sonifier, model J-17V, equipped with a microtip and with the activity level control indicator at position 10 on the scale. After the ultrasonic treatment the suspension was centrifuged at 8 500 *g* for 15 min at 4°C. The supernatant was the undiluted sensitizing antigen.

P. vinckei was inoculated intraperitoneally into Swiss mice. Heavily infected blood from these animals was collected in acid citrate dextrose solution and the sensitizing antigen was prepared as described above.

Preparation of sensitized cells

Sheep erythrocytes collected in Alsever solution and stored at 4°C were used within 8 days. As required 0.3-ml quantities of packed thrice-washed cells were resuspended in 9.7 ml of 0.0025% tannic acid solution in PBS, pH 7.2, incubated at 4°C for 15 min, and washed several times, with a final washing in PBS, pH 5.5.

For antigen titrations, volumes of 0.06 ml of tanned cells were suspended in PBS, pH 5.5, up to a final volume of 1 ml after the addition of increasing quantities of antigen—namely, 0.06, 0.1, 0.2, and 0.3 ml—in different tubes. In a sixth tube, control cells were prepared without the addition of antigen. After incubation at 37°C for 30 min the cells were washed twice in a diluent, i.e. 1% inactivated rabbit serum in PBS, pH 7.2. Suspensions of 1.3% sensitized cells and nonsensitized control cells were prepared: 0.025 ml of these suspensions were added to equal volumes of sera, inactivated at 56°C for 30 min, and diluted in the microtitre system (Sever, 1962) in a diluent, starting at a dilution of 1 : 40. The microtitre plates were shaken mechanically for 2 min. The test was read after incubation for 1 h at room temperature and after storage overnight in a refrigerator. For the optimum quantity of sensitizing antigen, we used the smallest quantity that showed the highest haemagglutinin titre with a set of positive reference serum samples, in the absence of haemagglutination with sensitizied cells combined with a negative reference serum, and with nonsensitized cells combined with the positive references. Grades of agglutination were read according to Stavitsky (1954). A grade ± was considered positive.

Indirect haemagglutination test (IHA test)

For the actual test, the optimum quantity of sensitizing antigen was added to tanned cells as determined in the antigen titration test. In some tests, tanned cells were sensitized with the constituents of noninfected erythrocytes prepared from noninfected blood. This " control antigen " was prepared from noninfected blood in the same way as were the specific antigens from parasitized erythrocytes. All serological samples were inactivated at 56°C for 30 min. As a routine, they were tested with specifically sensitized cells and with nonsensitized tanned sheep cells as a control on the presence of heterophile antibodies, i.e., antibodies reacting with antigenic determinants of noninfected heterologous erythrocytes.

Absorption procedure.

In some tests, serum samples were examined after absorption. Inactivated serum, diluted 1 : 5, was mixed with 4 volumes of a 25% suspension of erythrocytes. In most experiments fresh sheep cells were used, but in others tanned sheep cells sensitized with " control antigen " were employed. After incubation at room temperature for 1 h the 1 : 20 diluted supernatant serum sample was used in the IHA test.

Table 1. IHA titres obtained with *P. vinckei* as sensitizing antigen. Influence of the duration of ultrasonic treatment on *P. vinckei* antigen (lot no. 96.02.14) tested in duplicate with mouse serum (A 11 F 6)

	IHA titres after ultrasonic treatment for the following no. of seconds:							
	10	20	40	60	80	100	120	140
first test	320	320	320	320	160	80	80	40
second test	640	320	80	160	160	160	80	40

RESULTS

Technical factors that influenced the reproducibility of the test

The preparation of antigen. The duration of the ultrasonic treatment appeared to be critical for the disruption of the parasitized cells. Treatment at the indicated level of energy output for less than 10 seconds did not result in complete disruption of all cells: more than 10 seconds however, reduced the antigenicity of the crude antigen (see Table 1). Repeated freezing and thawing also had a detrimental effect on the antigenicity. Crude antigen stored at −70°C did not lose its antigenicity within 20 weeks of storage but at −20°C the antigen was much less stable.

The pH during sensitization of the tanned cells. In our experience, the cells spontaneously agglutinate when the tanned cells are sensitized at a pH that approaches neutrality.

The concentration of sensitized cells. One source of variability in the test appeared to be the concentration of the sensitized cells added to the diluted serum samples. At low concentrations the agglutination patterns are difficult to read and at higher concentrations the titres tend to decrease.

Source of cells. Table 2 shows the results of tests on different days with 1 negative and 4 positive sera that were absorbed with fresh erythrocytes. In these tests 6 different sheep were used as sources of cells. The cells were sensitized with the same batch of antigen. The table demonstrates that good reproducibility can be achieved when the same source of cells is used and that greater variability results from the use of erythrocytes from different sheep.

Source of antigen. The results of tests with 8 human sera that were examined with cells of 1 sheep sensitized with 3 batches of *P. fieldi* antigen showed that different batches of antigen can be a source of variability in the test.

Comparison of titres obtained with IHA test and the IFA test

Comparison in tests of human sera. Table 3 shows the relationship between the IHA titres obtained with tanned cells sensitized with *P. c. bastianellii* antigen, and the titres obtained in the IFA test with *P. fieldi* antigen.

Of the 276 sera tested, 233 had an IHA titre $\leqslant 1:40$; 215 of these 233 also had an IFA titre $\leqslant 1:40$, indicating that in the low range of antibody titres both tests gave similar results in 92% of the tests. At the upper end of the scale the antibody titres were similar in about 70% of cases, 30 sera with IHA titres $\geqslant 1:160$ having IFA titres $\geqslant 1:80$, although the IFA titres tended to be somewhat lower than the IHA titres. However, 18 (44%) of 44 sera with an IFA titre $\geqslant 1:80$ had an IHA titre $\leqslant 1:40$ and 17 (40%) of 43 sera with an IHA titre $\geqslant 1:80$ had an IFA titre $\leqslant 1:40$. Seven of the latter 17 sera, however, contained heterophile haemagglutinins: after they had been absorbed, the IHA titres became $\leqslant 1:40$.

Comparison in a longitudinal study of monkey malaria. For a better comparison of the IHA and IFA tests a longitudinal study was made of five malaria infections in monkeys: one sporozoite-induced infection (D24) and four blood-induced infections with *P. cynomolgi bastianellii*, *P. c. langur*, *P. knowlesi*, and *P. inui*. In these five monkeys the heterophile antibody titre remained $< 1:40$ during the whole observation period of 80 days after the first day of parasitaemia. In these sera the development of the IHA titres and of the IFA titres closely paralleled each other.

The IHA antibody titres were higher in most animals than the IFA titres. However, this difference

Table 2. Indirect haemagglutination test for malaria with one batch of *P. c. bastianellii* antigen. Titres obtained on different days with four positive and one negative test serum, absorbed with fresh sheep cells, in tests with sensitized (Sens.) and nonsensitized (Non) tanned cells: six different sheep cell donors were used

Serum	Sheep no. 18 Sens.	18 Non	12 Sens.	12 Non	9 Sens.	9 Non	1 Sens.	1 Non	13 Sens.	13 Non	16 Sens.	16 Non	Frequency of observations of various titres
190	1 280	<40	1 280	<40	5 120	(SP)	640	<40	640	<40			2 at 5 120
	1 280	<40	640	<40	5 120	(SP)							6 at 1 280
			1 280	<40	1 280	<40							6 at 640
			640	<40	1 280	<40							
			640	<40									
105	1 280	<40	640	<40							320	<40	1 at 1 280
			640	<40									2 at 640
													1 at 320
244	160	<40	640	<40	640	<40	320	<40	160	<40			5 at 640
	640	<20	640	<40	640	<40	160	<40					4 at 320
			320	<40									3 at 160
			320	<40									
			320	<40									
276	320	<40	320	<40	640	<40	320	<40					3 at 640
	640	<40	320	<40	640	<40							7 at 320
			320	<40	320	<40							1 at 160
			160	<40									
			320	<40									
negative serum	<40	<40	<40	<40	SP	<40	<40	<40	<40	<40			3 SP
	<40	<40	<40	<40	SP	<40	<40	<40					11 at <40
			SP	<40	<40	<40							
			<40	<40	<40	<40							
			<40	<40									

could have resulted partly from the fact that *P. cynomolgi* antigen was used in the IHA test whereas *P. fieldi* antigen was used in the IFA test. This is in agreement with the observation that the difference between IHA and IFA titre values tended to be greater in two animals that received a homologous inoculation with *P. cynomolgi bastianellii*.

IHA tests with various sensitizing antigens

In order to demonstrate differences in reactivity, antigens prepared from *P. cynomolgi bastianellii*, *P. c. langur*, *P. ceylonensis*, *P. inui*, *P. fieldi*, *P. knowlesi*, and *P. knowlesi* Guy strain were compared in IHA tests with sera from persons with proved malaria parasitaemia. None of the sera used in this experiment agglutinated nonsensitized tanned sheep cells. A control antigen prepared from noninfected rhesus monkey erythrocytes was added to the series of plasmodial antigens. Table 4 shows the mean antibody titres of 10 sera from persons with a *P. falciparum* infection, of 14 sera from persons with *P. vivax* infections, of 3 sera from persons with *P. malariae*

Table 3. Distribution of antibody titres in the IHA test of 276 human sera examined with tanned cells sensitized with *P. c. bastianellii* antigen according to the antibody titre in the IFA test with *P. fieldi* antigen

IHA titre	IFA titre								
	<20	20	40	80	160	320	640	1 280	Total
<40	101	56	39	12	1				209
40	13	3	3	4	1				24
80	4	3	4	3	2	1			17
160		1	3	5	1				10
320				4	0	0	1	1	6
640	1			1	1	1			4
1 280					3	0	1		4
2 561	0	0	0	0	0	0	0	0	0
⩾5 120			1	0	0	1			2
total	119	63	50	29	9	3	2	1	276

Table 4. Comparison of the mean titres obtained with seven sensitizing plasmodial antigens in the IHA test for malaria with sera negative for heterophile antibodies, and obtained from subjects with a natural malaria infection

Infection	No. of sera	Antigen							
		P. c. bastianellii	*P. c. langur*	*P. c. ceylonensis*	*P. inui*	*P. fieldi*	*P. knowlesi*	*P. knowlesi* Guy strain	*Control* antigen
P. falciparum	10	$10 \times 2^{4.1}$	$10 \times 2^{3.1}$	$10 \times 2^{3.0}$	$10 \times 2^{4.7}$	$10 \times 2^{2.8}$	$10 \times 2^{2.2}$	$10 \times 2^{2.5}$	$10 \times 2^{1.6}$
P. vivax	14	$10 \times 2^{5.1}$	$10 \times 2^{3.6}$	$10 \times 2^{3.5}$	$10 \times 2^{4.9}$	$10 \times 2^{3.4}$	$10 \times 2^{3.1}$	$10 \times 2^{2.4}$	$10 \times 2^{1.2}$
P. malariae	3	$10 \times 2^{6.0}$	$10 \times 2^{4.3}$	$10 \times 2^{4.3}$	$10 \times 2^{5.3}$	$10 \times 2^{4.7}$	$10 \times 2^{4.7}$	$10 \times 2^{4.0}$	$10 \times 2^{1.3}$
P. ovale	2	$10 \times 2^{5.0}$	$10 \times 2^{3.5}$	$10 \times 2^{3.5}$	$10 \times 2^{4.5}$	$10 \times 2^{2.5}$	$10 \times 2^{2.5}$	$10 \times 2^{2.5}$	$10 \times 2^{1.5}$

infections, and of 2 sera from persons with *P. ovale* infections. Comparisons should be made only between the titres of the sera against the different monkey malaria antigens and not between the results of the different sera. It is apparent that, whatever the source of the malaria serum samples, the IHA titres were highest in tests with *P. cynomolgi* and *P. inui* as sensitizing antigen and were lowest in those with *P. knowlesi* antigen. No category of sera had a particularly greater reactivity with any one of the sensitizing antigens. The sera examined in this experiment,with the exception of two sera from the *P. falciparum* group, did not show at a dilution of 1 : 40 haemagglutination with tanned cells sensitized with control antigen.

Studies on the frequency of aspecific positive reactions in the IHA test for malaria

Examination of nonmalarious human sera. In this experiment we studied sera that could have been positive for aspecific reactions in the IHA test for malaria. For these tests tanned cells sensitized with *P. c. bastianellii* antigen were used. As indicated in Table 5, 19 (10%) of 189 sera had a titre ⩾1 : 40 in the IHA test. After absorption with fresh sheep cells, however, only 5 (3%) were still reactive in this test.

Examination of bovine sera from animals infected with Anaplasma marginale. Sera from Nigerian cattle infected with *Anaplasma marginale* were tested using

Table 5. IHA antibody titres of nonmalarious human sera before and after absorption with fresh sheep cells in sera with a titre ⩾ 1 : 40 for tanned cells sensitized with *P. c. bastianellii* antigen

Sera from people with:	IHA titre before absorption		IHA titre after absorption		
	<1 : 40	⩾1 : 40	<1 : 40	1 : 40	⩾1 : 80
clinically suspected rheumatoid arthritis: titre in the Rose-Waaler test					
⩾1 : 64	32	4	3	1	
⩽1 : 32	98	4	3	1	
positive Wassermann test	2	1	1		
Reiter's disease	0	1		1	
mononucleosis	0	4	4		
periarteritis nodosa	1	0			
disseminated lupus erythematosus	1	3	3		
hepatotenticular degeneration (Morbus Wilson)	1	0			
carcinosis	1	0			
dysglobulinaemia	2	0			
pancytopenia	1	0			
idiopathic haemolytic anaemia	1	0			
IgG paraproteinaemia	5	0			
increased IgM level of unknown origin	0	2		1	1
leukaemia	13	0			
myelomatosis	12	0			
total	170	19	14	4	1

tanned cells sensitized with *P. c. bastianellii* antigen. Without absorption with fresh sheep cells, 6 of 8 sera had positive titres between 1 : 80 and 1 : 1280. After absorption all titres were <1 : 40.

Development of heterophile antibodies during malaria infections and their influence on the IHA test

During some parasitaemias in man and animals we observed the development of antibodies against unsensitized tanned sheep cells.

P. ovale infections. The development of heterophile antibodies in a blood-induced *P. ovale* infection in patient D may be used as an example (Meuwissen, 1966). Prior to the inoculation with *P. ovale*, this patient had received several inoculations with *P. vivax*. From the sixth day of parasitaemia onwards antibodies against nonsensitized tanned sheep cells were present. Haemagglutinins against tanned sheep cells sensitized with *P. c. bastianellii* antigen appeared before this. The development of heterophile antibodies was observed in the sera of 2 of the 4 patients with *P. ovale*; in both of them one or more induced infections with *P. vivax* had preceded the inoculation with *P. ovale*.

Monkey malaria infections. In 5 of 10 non-splenectomized rhesus monkeys, one sporozoite-induced and four blood-induced malaria infections were accompanied by the development of haemagglutinins against sensitized tanned sheep cells and of heterophile antibodies. In three monkeys low heterophile antibody titres were present before the inoculation. In 3 of the animals the highest titre

Table 6. Distribution of IHA antibody titres of 276 sera from individuals tested with tanned cells sensitized with *P. c. bastianellii* antigen according to the IHA titre with nonsensitized cells

IHA titre with sensitized tanned cells	IHA titre with nonsensitized tanned cells				
	<40	40	80	⩾160	Total
<40	208	1	0	0	209
40	13	11	0	0	24
80	9	2	6	0	17
160	9	0	0	1	10
320	5	1	0	0	6
640	3	0	1	0	4
1 280	3	0	0	1	4
2 560	0	0	0	0	0
5 120	1	0	0	1	2
total	251	15	7	3	276

of heterophile antibodies was seen after a second rise in parasite density. In all 5 animals the haemagglutinin titres for sensitized cells started to rise within a few days of the appearance of circulating parasites.

Heterophile antibodies in human sera. The development of heterophile antibodies in natural malaria infections was further studied in an examination of the 276 human sera (see earlier section). Table 6 shows that 13 (30%) of 43 sera with an IHA titre ⩾1 : 80 with cells sensitized with *P. bastianellii* antigen contained heterophile antibodies with titres ⩾1 : 40.

Further analysis of circulating haemagglutinins

Since the origin and specificity of the antigenic determinants reacting in the IHA test with circulating haemagglutinins is unknown, a further analysis was carried out with three types of tanned cell: (1) cells sensitized with *P. c. bastianellii* antigen, (2) nonsensitized tanned cells, and (3) cells sensitized with control antigen

Absorption experiments with fresh sheep cells. Of the 276 human sera, the 50 with IHA titres ⩾1 : 80 in tests with cells sensitized with *P. bastianellii* antigen were tested again before and after absorption with fresh sheep cells. Before absorption, 32 (64%) of the sera reacted only with the specifically sensitized cells. Two sera that reacted with specifically sensitized cells and with nonsensitized cells lost all reactivity during the absorption. Five sera that reacted with specifically sensitized cells and with cells sensitized with control antigen lost not only their heterophile reactivity but also a great part of their haemagglutinin reactivity with specifically sensitized cells. Of the 11 sera with reactivity for all three types of cells, only 3 retained specific haemagglutinin reactivity but in one of them the absorption was not completely effective.

Absorption with tanned sheep cells sensitized with control antigen. Of a similar series of 34 human and primate sera with IHA titres ⩾1 : 80 in tests with specifically sensitized cells, 14 also reacted with nonsensitized cells, with cells sensitized with control antigen, or with both. These 14 sera can be arranged in four groups according to the results of tests after absorption with fresh sheep cells or with tanned cells sensitized with control antigen. Table 7 shows that the 5 sera in group A reacted with all 3 types of cell. Their heterophile antibody activity could be absorbed as well by the tanned cells sensitized with control antigen as by the fresh sheep cells, without a complete loss of haemagglutinins for specifically sensitized cells in 4 of the 5 sera. In the 4 sera of group B and the 2 of group C, fresh sheep cells failed to absorb heterophile antibodies. However, they could be absorbed by tanned cells sensitized with

Table 7. IHA titres of human and primate malarious sera in tests with tanned cells sensitized with *P. c. bastianellii* antigen (1), with nonsensitized tanned cells (2), and with tanned cells sensitized with control antigen (3). The tests were carried out before and after absorption either with fresh sheep cells or with tanned cells sensitized with control antigen

Serum no.	IHA antibody titre								
	Before absorption			After absorption			After absorption with tanned cells sensitized with control antigen		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
group A									
SM 1	160	40	40	80	<40	<40	80	<40	<40
SM 27/2	2 560	40	80	1 280	<40	<40	1 280	<40	<40
F 33/2	80	40	40	80	<40	<40	80	<40	<40
E 40/12	80	40	<40	40	<40	<40	<40	<40	<40
SM 194	160	⩾160	160	<80	<80	<80	40	<40	<40
group B									
SM D6	160	80	<40	80	80	<40	160	<40	<40
SM D7	320	40	<40	320	40	<40	320	<40	<40
E 78/4	1 280	80	80	1 280	80	80	1 280	<40	<40
C 10/19	80	80	40	80	80	<40	<40	<40	<40
group C									
SM 85	40	40	160	80	<80	160	80	<40	<40
SM 229	160	160	160	<40	<40	80	<40	<40	<40
group D									
SM 39-2	20 480	<40	⩾640	40 960	<80	640	20 480	<40	320
SM 39/3	20 480	<40	>640	40 960	<80	⩾1 280	20 480	<40	640

control antigen. Four of these 6 sera did not show an appreciable loss in IHA titre for specifically sensitized cells. It was remarkable that in the group B sera, fresh sheep cells failed to absorb heterophiles that reacted with nonsensitized tanned sheep cells. For the group D sera the results of absorption experiments carried out on two serum samples of a Moroccan patient with a falciparum parasitaemia are shown. The heterophile antibody activity of these sera could not be absorbed either with fresh sheep cells or with the cells sensitized with control antigen.

DISCUSSION

Technical aspects

Following the introduction of the IHA test for serological studies of malaria (Desowitz & Stein, 1962; Stein & Desowitz, 1964) four other centres have reported on their results with this technique (Bray, 1965; Bray & El-Nahal, 1966; Mahoney et al., 1966; Rogers et al., 1968; Wellde et al., 1969). The techniques applied in the previous studies differ from each other in several respects. The details of the various techniques used in the other five studies as well as our own are summarized in Table 8. It will be seen that our technique combined points from the other methods. In the present study blood was collected in a flask with glass beads for defibrination and for the elimination of thrombocytes and most of the polymorphonuclear leucocytes; the lymphocytes and monocytes were then separated from the red blood cells by agglutination of the latter with dextran solution. The object of this procedure was to concentrate the parasitized erythrocytes. No attempt was made to isolate the parasite from its host erythrocyte, the maximum amount of antigenic

Table 8. Comparison of techniques used in six studies of the indirect haemagglutination test for malaria [a]

Techniques	Study no.					
	1	2	3	4	5	6
Purification of antigen						
elimination of thrombocytes	—	—	—	—	—	+
elimination of polynuclear leucocytes	—	+	—	—	—	+
elimination of lymphocytes/ monocytes with dextran	—	+	—	—	—	+
Isolation of parasites from infected red cells	+	+	+	+	—	—
applied with distilled water	+	—	—	+	—	—
applied by treatment with saponin	—	+	+	—	—	—
Homogenization of parasitized material by freezing and thawing	—	+	—	+	+	—
treatment in French pressure cell	—	—	+	+	—	—
	—	—	—	—	—	+
tissue grinder	+	+	—	—	—	—
Chromatographic separation	—	—	—	—	+	—
Carrier:						
formalinised red cell	+	—	—	—	—	—
tanned red cell	+	+	+	+	+	+
Temperature during treatment with tannic acid	37°C	37°C	?	4°C	4°C	4°C
Strength of tannic acid solution	1/20 000	1/20 000	?	1/20 000	1/10 000	1/40 000
Source of carrier cell	sheep	sheep	sheep	human[c]	sheep	sheep
Temperature during sensitization	room temp.	40°C	37°C	37°C	room temp.	37°C
Period of sensitization	15 min	15 min	15 min	15 min	30 min	15 min
pH during sensitization	7.2	7.2	5.6	7.2	5.6	5.5
Serum inactivated	+	+	?	?	+	+
Absorbed with fresh sheep cells	+	+	?	—	+	(+)
Diluent [b]	0.125 % BSA	1 % RS	0.4 % RS	1 % RS	1 % RS	1 % RS

[a] 1. Study by Stein & Desowitz (1964)
2. Studies by Bray (1965) and Bray & El Nahal (1966)
3. Study by Mahoney et al. (1966)
4. Study by Rogers et al. (1968)
5. Study by Wellde et al. (1965)
6. Present study.

[b] BSA = bovine serum albumin
RS = rabbit serum.

[c] Group O blood.

material was desired. A simple ultrasonic treatment was suitable for homogenization of the parasitized cells.

In our tests sheep cells were used after tests with tanned chicken cells and a few tests using human blood group O erythrocytes had given poor results (unpublished data). The main drawback of the chicken cells was that the chickens varied considerably in their suitability as cell donors. Even an individual chicken showed day-to-day variation in this respect. Moreover the quantity of blood that could be obtained from an individual chicken was rather small.

Our studies confirm that antigenic material is present in the supernatant of disrupted parasitized cells and that it is thermolabile. Storage at temperatures above −70°C, and long ultrasonic treatment, lessened the antigenicity of the cell extract. As reported by Mahoney et al (1965), we found it better to sensitize the tanned cells at pH 5.5 instead of at pH 7.2. With regard to the sensitivity of the test it appears that the concentration of the sensitized cell suspension used in the microtitre plates had some influence on the outcome of the test, a high concentration tending to decrease the average titre. The reproducibility of the test depended partly on differences in carrier cells from different donor sheep.

Since little is known about the nature of the sensitizing antigen, the malaria antigen content of a parasitized cell extract cannot be assessed. As was to be expected, different batches of one species of antigen showed differences in reactivity with the same lot of sera. The sensitizing antigen may be either purely of parasite origin or it may be material composed of host and parasite components or even host cell material that has been modified by the presence of the parasite. In view of this uncertainty it appears probable that difficulties with the preparation of the sensitizing antigen will remain as long as there is no reliable measure of the specific antigen as content of the mixture of parasitized cell material. The other possibility has already been indicated by Wellde et al. (1969) in studies on chromatographic separation of the specific antigens; their observations should be confirmed and their experimental approach should be used more extensively by others.

Comparison of the IHA and IFA tests

The comparison of results obtained by applying the IHA and IFA techniques to the same sera confirms in general the observations of Sadun et al. (1969) and Wilson et al. (1971). In their studies it was found that both tests provided a specific and sensitive method of following the course of antibody development in malaria infections. In the present study, 276 sera were tested with both methods. These sera were arbitrarily selected from individuals who had complained of symptoms that could be related to a possible malaria infection. In most cases parasitaemia had not been demonstrated and only 43% of these sera showed an IFA titre $\geqslant 1:20$, indicating that only a minority of the subjects tested had experienced a recent malaria attack. The data show that sera with low antibody contents give comparable titres with both methods and that in human sera with seropositive reactions, the titres in the IHA tests with *P. c. bastianellii* antigen tend to be higher in general than those in the IFA test with *P. fieldi* antigen. The longitudinal studies of monkey malaria seem to confirm these observations. They also show that in IHA reactions with homologous antigen the differences between titres in this test with *P. cynomolgi* antigen and the IFA titres obtained with *P. fieldi* antigen tend to be greater than those obtained with heterologous antigens in both serological tests

Comparison of different sensitizing antigens

In accordance with current theories (WHO Scientific Group on the Immunology of Malaria 1968), our observations with the use of 7 different sensitizing antigens indicate that the IHA test for malaria demonstrates group-specific antibodies. In the study of human sera, only heterologous antigens were used. *P. c. bastianellii* and *P. inui* antigens proved to be the best sensitizing antigens for all tests, perhaps because of the developmental stage of the circulating parasites that were used for the preparation of the antigen. It seems that schizonts provide better antigens than young trophozoites. This could also be a possible explanation for the relatively poor antigenicity of the antigens prepared from *P. knowlesi*, the primate plasmodium with a unique 24-hour cycle. It is probable that with this species the antigen was not prepared within the short period when the greatest number of schizonts were circulating.

Specificity of the test and influence of heterophile antibodies

As was shown in Table 5, 3% of the 170 non-malarious human sera, specially selected from patients with other disease entities that would give specific positive titres in the IHA test for malaria, had an IHA titre $\geqslant 1:40$ and only about 0.5% of them had an IHA titre $\geqslant 1:80$ with specifically sensitized cells. This suggests that the IHA test is specific for malaria. It seems unlikely that the sera react with some erythrocyte antigenic determinants freed from the host cells during the process of haemolysis. Otherwise the bovine sera from infections with *Anaplasma marginale* and the serum from a patient with an idiopathic haemolytic anaemia, after absorption with fresh sheep cells, should both have shown positive titres with the specifically sensitized cells. Moreover, the observations on the malaria infections in rhesus monkeys indicate that the host erythrocyte determinants are not the main part of the sensitizing antigens. These antigens are prepared from para-

sitized blood of rhesus monkeys. Nevertheless the monkey serum samples after absorption with cells sensitized with control antigen prepared from noninfected rhesus monkey red cell material still showed reactivity with the specifically sensitized cells.

The IHA test for malaria seems to be affected more by the influence of heterophile antibodies than by nonspecificity. Adeniyi-Jones (1967) found a high incidence of agglutinins against tanned sheep cell erythrocytes in Nigerian sera. In contrast to the present observation, she reported that agglutination was not affected by absorption with normal sheep cells and that the absorption with tanned cells met with varied success. Kano et al. (1968) found agglutinins to guineapig erythrocytes and to rat erythrocytes in Gambian sera. In both these papers the authors suggest a possible relationship with malaria. This view was opposed by Greenwood (1970) in his study on heterophile antibodies in Nigerian sera. Others have reported on the possible relation between malaria infections and other unusual host responses. Curtain et al. (1965) demonstrated a high incidence of cold agglutination, Houba & Allison (1966) an increased incidence of rheumatoid factor-like antibodies, and Shaper et al. (1968) circulating autoantibodies to heart, thyroid, and gastric parietal cells. In the present study, the relationship in time between malaria parasitaemia and the development of agglutinins against nonsensitized tanned sheep cells was demonstrated in a *P. ovale* infection and in both blood-induced and sporozoite-induced primate malaria infections in rhesus monkeys. The development of these heterophile antibodies occurred in an unpredictable way. Some patients and animals did not show a rise in heterophile antibody titre. Moreover, some animals produced these antibodies early during the primate parasitaemia while others did so many weeks after the commencement of the patent parasitaemia. The rise in heterophile antibody titres occurred some time after the increase in the titre of haemagglutinins for specifically sensitized cells. About 30% of 276 human sera with an IHA titre $\geqslant 1:80$ for specifically sensitized cells also showed haemagglutinin activity for non-sensitized tanned cells.

The significance of the production of these heterophile antibodies became apparent after absorption with fresh sheep cells. Some of the sera lost not only their haemagglutinating capacity for non-sensitized cells but also that for specifically sensitized cells. In one serum the absorption was only partly successful.

In later experiments, it was shown that certain sera also appeared to have some haemagglutinins that reacted with determinants of the control antigen prepared from rhesus monkey red cell material. Some sera that did not react with nonsensitized tanned sheep cells reacted with cells sensitized with this control antigen. This second type of heterophile antibody could in most cases be absorbed with fresh sheep cells but, as is shown in Table 7, not always. Exceptional cases were encountered that had haemagglutinins for tanned cells sensitized with control antigen; these heterophile antibodies could not be absorbed.

In view of the possibility that different types of haemagglutinin with different specificities develop during infections with plasmodia, it is necessary to determine to what extent all these haemagglutinins form an antibody family with broad cross-reactivity and with definite affinities, which differ from host to host, for erythrocytes of various species.

So far we have not had the opportunity of studying the incidence of heterophile antibodies in the field. Nevertheless it appears from our preliminary studies that both specifically sensitized tanned sheep cells and tanned cells sensitized with control antigen should be used in the IHA test. All sera with haemagglutinins for specifically sensitized cells should be absorbed preferably with cells sensitized with host erythrocytic control antigen and then tested again. Otherwise the interpretation of the IHA titres of malaria sera is likely to be incorrect.

In the introduction to this paper a plea was made for a simple serological field test for malaria. The necessity to include a routine absorption procedure in the serological titration involved in the IHA, test detracts from that ideal. It is clear that further studies on the nature of the specific sensitizing antigens and on methods for their isolation are necessary.

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RÉSUMÉ

ÉTUDES SUR DIVERS ASPECTS DE L'ÉPREUVE D'HÉMAGGLUTINATION INDIRECTE DANS LE PALUDISME

L'épreuve d'hémagglutination indirecte (HAI), appliquée avec tant de succès à divers domaines de la microbiologie et de l'immunologie, en est encore à attendre d'être admise comme méthode de routine dans l'étude séroépidémiologique du paludisme. Depuis quelques années, les auteurs utilisent de temps à autre cette technique au cours de recherches expérimentales sur le paludisme. Cet article constitue un rapport préliminaire sur leurs travaux, particulièrement sur ceux qui visent à perfectionner la méthode et à apprécier l'influence qu'exerce sur l'épreuve la formation d'anticorps hétérophiles.

Les souches et espèces de parasites étaient: *Plasmodium cynomolgi bastianellii, P. c. langur, P. c. ceylonensis, P. inui, P. knowlesi, P. knowlesi* Guy, *P. fieldi* et *P. vinckei.* L'antigène sensibilisant a été obtenu en injectant par voie intraveineuse à des singes rhésus splénectomisés les plasmodiums appropriés. On procédait à la saignée au moment où la densité parasitaire était à son maximum, essentiellement sous forme de schizontes. Les cellules sensibilisées étaient des globules rouges de mouton traités à l'acide tannique. En pratique, l'antigène sensibilisant était ajouté aux cellules tannées dans la proportion optimale déterminée par titration de l'antigène.

En comparant les résultats obtenus par l'HAI et l'IFI (épreuve d'immunofluorescence indirecte) appliquées aux 276 mêmes sérums, on a constaté que les deux méthodes fournissent un moyen sensible et spécifique de suivre la formation des anticorps au cours du paludisme. Les sérums avaient été choisis arbitrairement parmi des sujets se plaignant de symptômes en relation possible avec cette maladie. Le plus souvent, il n'a pas été possible de mettre en évidence la présence de parasites dans le sang. Il ressort des résultats que, lorsque la quantité d'anticorps est faible, les titres obtenus par l'un ou l'autre procédé sont très voisins. De plus, dans les sérums humains à réaction positive, les titres d'HAI à l'antigène *P. cynomolgi bastianellii* ont tendance à être généralement plus forts que ceux trouvés par IFI avec l'antigène *P. fieldi.* L'emploi de sept antigènes différents indique que l'HAI démontre la présence d'anticorps possédant une spécificité de groupe. Les antigènes *P. cynomolgi bastianellii* et *P. inui* se sont révélés les plus sensibilisants dans chaque épreuve. L'HAI semble être spécifique pour le paludisme. En d'autres termes, c'est plutôt l'intervention d'anticorps hétérophiles que le défaut de spécificité qui est gênante pour cet examen. Certaines infections comportent également des agglutinines actives sur les composants des hématies de l'hôte. Comme témoin du titrage des agglutinines spécifiques par l'HAI, on préconise l'absorption des sérums à l'aide de cellules de mouton tannées sensibilisées par des antigènes d'hématies d'un sujet non infecté. La nécessité d'adjoindre systématiquement une technique d'absorption au titrage sérologique par l'HAI complique l'épreuve.

Il reste bien sûr nécessaire de poursuivre les recherches sur la nature des antigènes sensibilisants spécifiques et sur les méthodes propres à les isoler. De ces travaux dépend le perfectionnement de cette technique sérologique d'étude du paludisme.

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