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APPLICATION OF THE INDIRECT HAEMAGGLUTINATION TEST FOR MALARIA

IV. OBSERVATIONS ON THE SPECIFICITY OF THE HAEMAGGLUTINATION
TEST WITH PLASMODIUM FALCIPARUM TEST CELLS. COMPARATIVE STUDIES
OF HAEMAGGLUTINATION AND FLUORESCENT ANTIBODY TESTS ON FIELD
MATERIAL FROM TANZANIA

by

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Introduction

There is a need for a reliable, inexpensive way for serological assessment of malaria. In this context we have implemented investigations of the possible use of the indirect haemagglutination test (IHA). Previously the weak point of the IHA test has been its reproducibility. The introduction of glutaraldehyde fixed cells greatly improved the reliability of the test. A field test should not be hampered by elaborate laboratory facilities; as a way of overcoming this difficulty we introduced the use of lyophilized test and control cells for use in the field (Meuwissen & Leeuwenberg, 1972) and described a simple field method for the absorption of heterophile antibodies in minute quantities of plasma (Meuwissen & Leeuwenberg, 1973).

In the present paper we report on the specificity of the IHA test with fixed sheep cells sensitized with P. falciparum (Palo Alto/Aotus) antigen and on the application of the IHA test to some 500 plasma samples collected from Africans of different age-groups. The IHA titres are compared with those obtained in indirect fluorescent antibody (IFA) tests with anti IgG and anti IgM conjugates.

Materials and methods

Sera. For assessment of the specificity of the IHA test with P. falciparum (Palo Alto/Aotus) test cells we used 545 sera from healthy Dutch blood donors and 189 samples from patients with non-malarious diseases. The latter sera were specified earlier (Meuwissen et al., 1972). From the collection of samples from malarious areas we have chosen those of Mto Wa Mbu. In this isolated settlement in the Arusha region of Tanzania medicated salt had been distributed since 1961 without much permanent success (Lelijveld, 1971; Draper & Voller, 1972). Five hundred and sixteen blood samples were collected in heparinized microcapillaries. After centrifugation the part with supernatant plasma was sealed and stored in deep-freeze until use.

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For dilution of the sample a volume of 20 μ l was taken from the capillary using a micro pipette with disposable tip (Eppendorf pipette). It was added to 180 μ l phosphate buffered saline, ph 7.2, for the preparation of a 1:10 dilution.

IHA test. For the indirect haemagglutination test we used the same procedures as described earlier (Meuwissen et al., 1972). All African sera were examined with the same batch of glutaraldehyde fixed sheep cells (sheep No. 22). The samples were absorbed with control cells following the method described by Meuwissen & Leeuwenberg (1973). As control cells we used fixed sheep cells sensitized with Aotus control antigen (Ao5). Sensitization of test cells was carried out with P. falciparum placental antigen. The sera were examined in permanent lucite "U" type plates.¹ From various brands and types of disposable plates only Biocult Linbro "U" type plates suited our purposes.² In addition some of the sera were also examined using cells sensitized with P. fieldi antigen and with another P. falciparum Aotus antigen (Palo Alto strain, Ao8). As parameters of the serological survey we used the percentage of sero-positives ($>1:40$) in the various age-groups and the mean titre index. The individual titre index is found by calculating the exponent to the basis of 2, after having divided the titre by 10. The titre of 1:20 ($=1:10 \times 2^1$) thus corresponds to a titre index of 1, while a titre of 1:40 ($1:10 \times 2^2$) corresponds to a titre index of 2. The mean titre index is calculated by dividing the sum of individual titre indices by the number of observations.

IFA test. All African samples were examined in the IFA test with P. falciparum antigen (Palo Alto strain) obtained from an owl monkey Ao2 and processed according to the method described by Voller (1971). The samples were tested with commercially available monospecific anti IgG and anti IgM conjugates.³ Although positive IFA titres occurred at a plasma dilution of 1:20, we had to use the same parameters for a valid comparison of IHA and IFA data. Therefore in this study a titre $>1:40$ was considered positive and for the calculation of the mean titre index all titres $\leq 1:40$ were given the individual index of 1.

Parasitological examination. The Giemsa stained thick blood films of the individuals of Mto Wa Mbu were examined at the laboratory in Amani. The parasite rate was determined for every age-group. The parasite density was estimated in each individual case. For each age-group it was expressed as the mean number of parasites per 200 leucocytes.

Results

A. Specificity of the IHA test

In Table 1 the results are given of the examination of 734 absorbed sera of patients and healthy blood donors in the IHA test with P. falciparum (Palo Alto/Aotus) sensitized test cells. Only 1 serum showed a positive titre. It was obtained from a boy who exceptionally showed only IgM immunoglobulins in his serum. All other sera were negative inclusive those of patients with diseases such as rheumatoid arthritis, syphilis, mononucleosis, disseminated lupus erythematoses, leukaemia, myelomatosis.

B. Examination of African sera

Parasitological and serological examination was done on 516 blood samples collected from people of various age-groups. The plasma samples were examined in the IHA test after absorption with the same cells. Thirteen samples (2.5%) showed persistent heterophile antibodies (see Table 2). These were not restricted to any particular age-group. These 13 samples were excluded from the comparison in IFA and IHA tests.

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³ Nordic Company, Tilburg, Netherlands.

Table 3 and Fig. 1 show the results of the examination of the remaining 503 samples which were all tested with cells sensitized with P. falciparum placental antigen. In the IFA test they were examined with P. falciparum antigen as well as anti IgG and anti IgM conjugate. At the time of blood collection a high percentage of the population had a patent parasitaemia.

There is a remarkably small difference in parasite rates between children and adults. Though not indicated in the table, the same applies to the parasite density. In successive age-groups the proportion of people with negative or low antibody titres shows a decrease, and with age there is an increase in the mean titre level. At the level of a serum dilution of 1:40 the percentage of sero-positives with the IHA test is higher than that obtained in the IFA test using IgM conjugates and lower than that observed in the IFA test using IgG conjugates. The mean titre indices suggest that in the younger age-groups IFA IgG antibody titres are higher than the IHA titres but in adults the opposite seems to be true. Moreover, the data suggest that the reactivity in the IHA test starts to increase in the age-group of 10-19 years; the titre level in the IFA test starts to rise earlier but this increase occurs more gradually. Table 4 and Fig. 1 show a further analysis of the results for people with positive or negative blood smears. These groups have a different serological reactivity. In most age-groups the mean titre observed with all three serological tests is higher in parasite carriers than in people without parasitaemia. Whereas there are among parasite carriers about four times as many sero-negatives in the IHA test (19.8%) as in the IFA IgG test (5.5%), there are only about one-and-a-half times as many (35.4% compared with 20.6%) in persons without patent parasitaemia.

For further exploration of this point the relationship was studied between trophozoite density at three levels of IHA titres in various age-groups. Table 5 indicates that the geometric mean trophozoite density was generally lowest in adult trophozoite carriers with IHA titres of >1:160.

Part of the samples was also examined in the IHA test with cells sensitized with P. falciparum (Aotus) antigen and with P. fieldi antigen. The results confirmed the earlier observation that P. falciparum (Aotus) test cells were more reactive than the cells sensitized with our batch of P. falciparum placental antigen, whereas the P. fieldi test cells were less sensitive (Meuwissen & Leeuwenberg, 1973).

The results of IHA tests using the placental falciparum antigen and the P. falciparum Palo Alto strain (Aotus) antigen are compared in Table 6, separately for persons with or without patent parasitaemia and for three levels of reactivity in the IHA test. It is evident that the number of negative or weak positive reactions among parasite carriers is considerably lower when using the P. falciparum (Aotus) antigen as compared to the placental antigen.

Discussion

Information on the specificity of the test used for serological survey operations is of great importance. For assessment of the effect of malaria control, the method should be sufficiently specific even when malaria infections become rare. When the serological test is not 100% specific and the incidence of malaria infections in a population decreases, there will be an increase in the percentage of the positive tests that have to be ascribed to unspecific reaction. For testing the specificity of the test we used cells sensitized with the most reactive antigen available, P. falciparum (Aotus) antigen. After absorption of the sera with control cells we saw only one positive reaction in a group of more than 700 samples. It is our impression that the specificity of the IHA test is sufficiently established.

The results of the examination of the African sera indicate that differences exist between IFA and IHA tests. The IHA test with P. falciparum placental antigen shows twice as many non-reactors at a dilution of 1:40 of the serum as the IFA IgG test (29.7% compared with 15.1%) (Table 3). In comparison with the IFA test, and assuming that its specificity is restricted to malaria, it is striking that the IHA test with test cells sensitized with

placental antigen is only half as sensitive in the group of parasite carriers as it is in people without a parasitaemia. In the literature data on comparative studies of IHA and IFA tests are extremely scarce. Sadun et al. (1969) compared the results of IHA tests with P. falciparum sensitized test cells and those of the Soluble Antigen Fluorescent Antibody (SAFA) test with P. falciparum antigen. In volunteers they followed the serological response in nine P. falciparum infections and 10 P. vivax infections. The patients had intermittent parasitaemias for several months. It is reported that the time-course development of antibodies followed almost parallel lines in both tests and that antibodies were detected at approximately the same time after the onset of parasitaemia. Somewhat at variance with their comments, the paper is accompanied by eight graphs, which illustrate that in four P. falciparum cases and in three out of four vivax cases early during the parasitaemia IHA titres are lower than SAFA titres. In the P. falciparum cases IHA titres were higher than SAFA titres after about three months.

Wilson et al. (1971) compared titres in the IHA test using P. knowlesi test cells with those obtained through the IFA test with P. vivax antigen in samples of Viet-Nam veterans with P. vivax infections. They conclude that the IFA test detected antibodies slightly more efficiently than the IHA test during the initial two weeks after the onset of symptoms. However, from the third week onwards, and clearly after seven months, the IHA test detected antibodies somewhat more efficiently than the IFA test. The IHA test detected significantly higher malarial antibody levels in patients who had previous experience with malaria. This difference was not observed in the IFA test. The authors suggest that possible explanations might be the use of heterologous antigens, the occurrence of non-precipitating antibodies detected by the IFA test only, the inhibition of the IHA test by non-precipitating antibodies, or the enhancement of IHA titres by auto-antibodies present in people with previous attacks. In the present study we have considered the following possibilities to explain the different reactivity observed in IHA and IFA tests:

1. The influence of the use of P. falciparum (Palo Alto/Aotus) antigen in the IFA test, on one hand and that of P. falciparum placental antigen in the IHA test on the other. Table 6 indicates that in IHA tests with both falciparum antigens, the higher reactivity is achieved with the P. falciparum Palo Alto/Aotus antigen.
2. During parasitaemia free circulating, soluble antigens in the serum of the patient could inhibit the IHA test more than the IFA test. Soluble antigens can be demonstrated in P. falciparum infections and the presence of S antigens is directly correlated to the degree of parasitaemia at the time of examination (McGregor, 1972). The data presented in Table 5 do not rule out the possibility that soluble antigens could inhibit the IHA titre level. In most age-groups the mean number of trophozoites was smallest in trophozoite carriers with the highest titre level. This could be explained as an absence of IHA inhibition through soluble antigen; on the other hand this could just as well be a feature of concomitant protective immunity. Detailed longitudinal observations in experimental models are needed for the investigation of this point.
3. After a malaria infection the IHA test could revert to negative sooner than the IFA test does, but we have no evidence that this does happen.
4. In a primary infection it may very well take longer for the IHA test to become positive than the IFA test. A low degree of affinity of malaria antibodies could remain inapparent in the IFA test but show in the IHA test. We now have preliminary evidence from observations in owl monkeys that in primary infections the IHA test can remain negative, at a 1:40 dilution of the sera, for a period of several weeks, while the IFA test is already clearly positive. It is well known that the affinity of the circulating antibodies increases in general during the process of immunization. Hypothetically it could be presumed that early in the malaria infection the affinity of antimalarial antibodies is sufficient for demonstration in the IFA test but insufficient to form stable antibody bridges between adjacent sensitized cells. Later in the primary infection and also during re-infections

antibodies would be reactive in both the IHA test and in the IFA test. Therefore it is indicated to implement longitudinal observations on the course of the IHA test during primary infections and re-infections.

5. Antimalarial drug administration could have a greater influence on the IHA antibody titre level than on that of the IFA. This should be further studied as Desowitz et al. (1966) have observed a significant drop in the IHA titres as a result of antimalarial treatment. Such a sudden drop has not been reported in IFA titre levels. As our sera were collected in an area where chloroquinized salt has been irregularly distributed since more than 10 years, we must therefore consider the possibility of an influence on the IHA test results.

SUMMARY

The specificity of the indirect haemagglutination (IHA) test with Plasmodium falciparum placental antigen sensitized test cells was examined using sera of healthy donors and various patients without malaria infections. Only one non-specific antibody reaction was seen in more than 700 tests.

IHA titres and indirect fluorescent antibody (IFA) titres obtained with IgG and IgM conjugates were compared in 503 human sera from a locality in Tanzania. In successive age groups an increasing number of sero-positive reactors was seen both in IFA and IHA tests. The increase in reactivity and in mean titre levels started in the IFA test earlier than in the IHA test. The increase was more gradual in the IFA test. Parasite carriers had higher antibody levels than people without apparent parasitaemia. The IHA test seemed to be less reactive than the IFA test with anti IgG conjugate, particularly in parasite carriers.

When conducting the IHA test with P. falciparum Palo Alto/Aotus antigen the reactivity was higher than with the P. falciparum placental antigen and thus closer to that of the IFA test with IgG conjugates.

RESUME

Sur des sérums provenant de donneurs de sang bien portants et de malades atteints d'affections non paludéennes, les auteurs ont étudié la spécificité de l'épreuve d'hémagglutination indirecte (IHA) réalisée avec des cellules sensibilisées par l'antigène placentaire de Plasmodium falciparum. Sur plus de 700 épreuves, une seule s'est révélée non spécifique.

D'autre part, les titres IHA et les titres IFA (immunofluorescence indirecte) obtenus avec des conjugués anti-IgG et anti-IgM ont été comparés sur 503 échantillons de sérum humain prélevés dans une localité de Tanzanie. Aussi bien dans les épreuves IFA que dans les épreuves IHA, il est apparu que le nombre des réactions positives augmentait avec l'âge. L'âge auquel commence l'augmentation de la réactivité et du titre moyen d'anticorps est moins élevé avec l'épreuve IFA qu'avec l'épreuve IHA. L'augmentation en fonction de l'âge est plus graduelle dans l'épreuve IFA. Chez les porteurs de parasites, les titres d'anticorps étaient plus élevés que chez les sujets ne présentant pas de parasitémie apparente. Dans l'épreuve IHA, la réactivité semble être moins grande que dans l'épreuve IFA pratiquée avec un conjugué anti-IgG, en particulier chez les porteurs de parasites.

Lorsque l'épreuve IHA est faite avec l'antigène de P. falciparum Palo Alto/Aotus, la réactivité est plus forte qu'avec l'antigène placentaire de P. falciparum et se rapproche de celle obtenue dans l'épreuve IFA pratiquée avec un conjugué anti-IgG.

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TABLE 1. SPECIFICITY OF THE IHA TEST FOR MALARIA

Examination of non-malarious sera with test cells sensitized with P. falciparum - Palo Alto strain-Aotus antigen (Ao8) after absorption with control cells sensitized with Aotus control antigen (Ao5)

Sera of patients ^a Healthy blood donors' age	No. examined	IHA titre <1:40	Aspecifically positive IHA titre (≥1:140)
	189	188	1
15-20 yrs	25	25	0
21-25 "	123	123	0
26-30 "	97	97	0
31-40 "	133	133	0
41-50 "	102	102	0
51-60 "	50	50	0
61-70 "	15	15	0
Total	734	733	1

^a For specification of diagnosis see Meuwissen et al. (1972).

TABLE 2. PERSISTENT HETEROPHILE ANTIBODIES AFTER ABSORPTION WITH CONTROL CELLS IN IHA TEST WITH CONTROL CELLS (Ao5 CONTROL ANTIGEN)

Age	Months	Years						Total
		2-4	5-9	10-19	20-39	40-59	60	
No. examined	53	85	132	107	80	45	14	516
Titre 1:40	0	1	4	0	3	0	0	8
" 1:80	0	0	0	0	1	0	1	2
" 1:160	0	0	0	2	0	1	0	3

TABLE 3. COMPARISON OF IFA IGM, IFA IGG AND IHA ANTIBODY TITRES IN ABSORBED AFRICAN SERA (IFA IGM AND IFA IGG TEST WITH P. FALCIPARUM PALO ALTO STRAIN/AOTUS ANTIGEN, IHA TEST WITH P. FALCIPARUM PLACENTAL ANTIGEN)

Age	No. examined	Parasite rate	Percentage with titre \leq 1:40			Mean titre index		
			IFA IGM	IFA IGG	IHA	IFA IGM	IFA IGG	IHA
< 3 months	4	(25)	(75)	(50)	(50)	(1.5)	(2.5)	(2.0)
3-5 "	5	(40)	(100)	(20)	(40)	(1.0)	(2.6)	(2.2)
6-11 "	27	30	74	44	59	1.4	1.8	2.0
12-23 "	17	47	47	29	41	2.4	3.0	2.4
2-4 years	84	33	63	23	42	1.7	3.4	2.6
5-9 "	128	45	50	16	37	2.0	3.8	2.8
10-19 "	105	43	40	10	23	2.2	4.1	3.5
20-39 "	76	26	29	9	12	2.6	4.3	5.1
40-59 "	44	23	30	0	11	2.8	5.1	6.0
> 60	13	23	31	0	8	2.9	4.8	6.2
Total	503	36.3	46.5	15.1	29.7	1.9	3.9	3.5

N.B. Figures in parenthesis indicate data from \leq 5 people.

TABLE 4. COMPARISON OF IFA IGG, IFA IGM AND IHA ANTIBODY TITRES IN ABSORBED SERA OF PEOPLE WITHOUT AND WITH (IFA IGG AND IFA IGM TEST WITH P. FALCIPARUM PALO ALTO STRAIN/AOTUS ANTIGEN, IHA TEST WITH P. FALCIPARUM PLACENTAL ANTIGEN)

Patent parasitaemia	No. examined		Percentage with titre \leq 1:40				Mean titre index						
	-	+	IFA IGM	IFA IGG	IHA	IFA IGM	IFA IGG	IHA	IFA IGM	IFA IGG	IHA		
\leq 3 months	3	1	(100)	(0)	(67)	(0)	(67)	(0)	(0)	(3)	(2.7)	(2.0)	(2.0)
3-5 "	3	2	(100)	(100)	(33)	(0)	(33)	(50)	(1.0)	(1.0)	(2.0)	(3.5)	(2.7)
6-11 "	19	8	84	50	63	13	63	50	1.4	1.6	1.9	3.0	1.7
12-23 "	9	8	67	25	67	13	67	13	1.9	3.0	2.6	3.5	2.0
2-4 years	56	28	61	67	50	11	50	25	1.7	1.8	3.2	3.8	2.3
5-9 "	70	58	60	38	44	5	44	29	1.7	2.3	3.4	4.4	2.4
10-19 "	60	45	47	31	33	2	33	9	1.9	2.5	3.8	4.4	2.9
20-39 "	56	20	34	15	14	5	14	5	2.4	3.2	4.1	5.0	4.9
40-59 "	34	10	33	20	12	0	12	10	2.7	3.2	5.1	5.0	5.8
60 "	10	3	20	(67)	10	(0)	10	(0)	3.3	(1.7)	5.1	(4.0)	6.5
Total	320	183	50.4	37.9	35.4	5.5	35.4	19.8	2.0	2.4	3.7	4.3	3.4

N.B. Percentages of \leq 5 people in parenthesis.

TABLE 5. COMPARISON IN VARIOUS AGE-GROUPS OF THE GEOMETRIC MEAN NUMBER OF TROPHOZOITES PER 200 LEUCOCYTES IN ALL PEOPLE EXAMINED (A) AND IN TROPHOZOITE CARRIERS ONLY (B) AT DIFFERENT LEVELS OF IHA TITRES (IHA TEST WITH P. FALCIPARUM PLACENTAL ANTIGEN)

Age-group	No. examined	No. without trophozoites	A		No. of trophozoite carriers	B	
			Mean trophozoite density (geometr. mean) according to IHA titres \leftarrow 1:40 1:80 \rightarrow 1:160 1:160	Mean trophozoite density (geometr. mean) in trophozoite carriers, according to IHA titres \leftarrow 1:40 1:80 \rightarrow 1:160 1:160			
<3 months	4	4	-	-	0	-	-
3-5 "	5	4	(3)	-	1	(268)	-
6-11 "	27	19	3	16 (4)	8	(81)	(20)
12-23 "	17	9	2	15 (8)	8	(21)	(64)
2-4 years	84	60	2	4	24	73	40
5-9 "	128	77	3	6	51	36	56
10-19 "	105	63	3	3	42	75	34
20-39 "	76	57	2	2	19	(71)	13
40-59 "	44	36	(2)	0	8	(18)	5
60 "	13	10	(0)	(0)	3	-	(24)
Total	503	339	2.4	3.9	164	52.5	27.0

N.B. Mean densities of \leftarrow 5 people in parenthesis.

TABLE 6. DISTRIBUTION OF PERSONS WITHOUT AND WITH APPARENT PARASITAEMIA ACCORDING TO IHA TITRE LEVELS.
A. IHA TESTS WITH P. FALCIPARUM PLACENTAL ANTIGEN. B. IHA TESTS WITH P. FALCIPARUM (PALO ALTO/AOTUS ANTIGEN)

Age-group	No. examined	No. without parasites	Persons without apparent parasitaemia. Distribution (%) according to IHA titres ↙ 1:40 1:80 ↘ 1:160	No. of parasite carriers	Persons with parasitaemia. Distribution (%) according to IHA titres ↙ 1:40 1:80 ↘ 1:160
<u>A</u> IHA placental antigen ↙ 3 months	4	3	(67) (33) -	1	(100) -
3-5 "	5	3	(67) - (33)	2	(100) -
6-11 "	27	19	79 16 5	8	50 37 13
12-23 "	17	9	78 11 11	8	25 62 13
2-4 years	84	56	61 28 11	28	39 32 29
5-9 "	128	70	69 20 11	58	36 31 33
10-19 "	105	60	47 33 20	45	18 29 53
20-39 "	76	56	20 25 55	20	10 30 60
40-59 "	44	34	12 21 67	10	10 0 90
60 "	13	10	10 10 80	3	- (100)
Total	503	320	47.5 24.1 28.4	183	28.4 29.5 42.1
<u>B</u> IHA <u>P. falciparum</u> Ao. antigen ↙ 3 months	3	2	(100) -	1	- (100)
3-5 "	3	1	(100) -	2	(100) -
6-11 "	11	10	70 30 -	1	(100) -
12-23 "	8	5	(100) -	3	(67) -
2-4 years	35	29	73 17 10	6	- 50 50
5-9 "	65	42	53 33 14	23	13 30 57
10-19 "	49	32	44 12 44	17	18 18 64
20-39 "	7	4	- (25) (75)	3	- (67) (33)
40-59 "	11	9	33 22 44	2	- (100)
60 "	2	2	- (100)	-	-
Total	194	136	55 21 24	58	17 31 52

N.B. Percentages of ↙ 5 people in parenthesis.

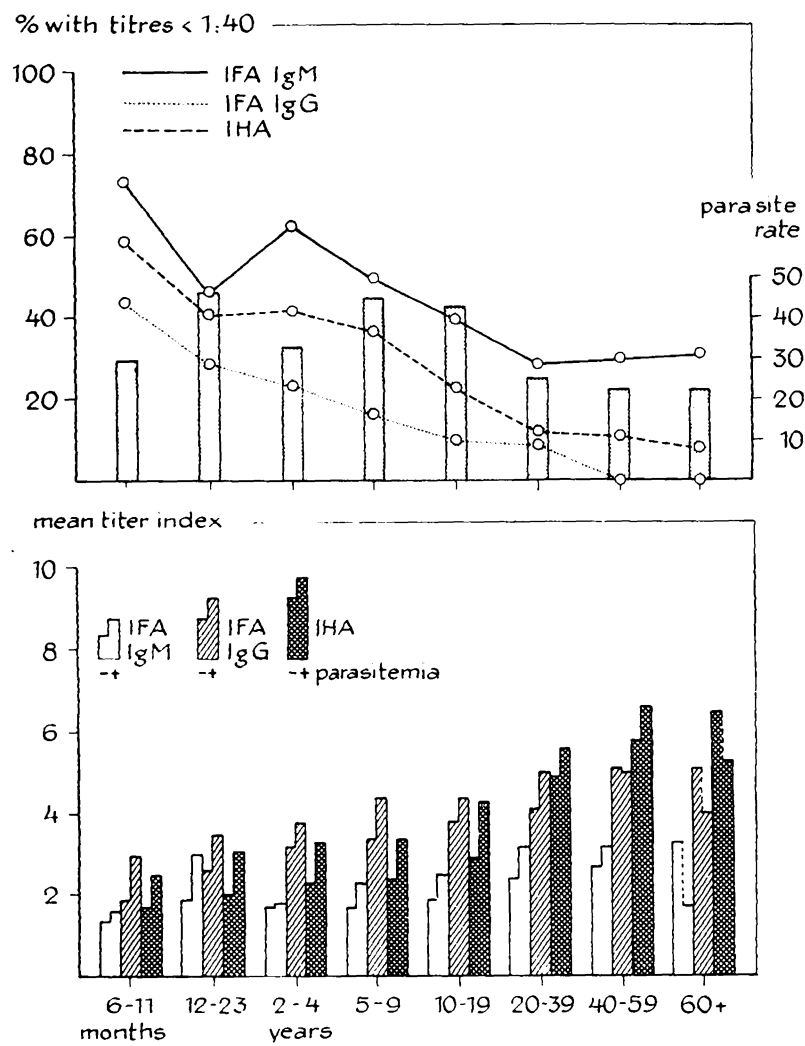


Fig. 1 Results of immunofluorescent antibody tests, using IgG and IgM conjugates, and of the haemagglutination test in relation to age and parasitaemia

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