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THE MEASUREMENT OF ANTIBODY IN HUMAN MALARIA
INFECTIONS BY A FORMOLIZED TANNED SHEEP CELL
HAEMAGGLUTINATION TEST¹

by

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It is well known that an immune response occurs in malaria infections of man and animals. However, there have been few methods available to detect and measure humoral antibodies elaborated against the parasite Eaton (1938) described an agglutination test using homologous antigen and the sera of monkeys chronically infected or recovered from Plasmodium knowlesi. Eaton & Coggeshall (1939) demonstrated complement fixing antibodies in monkeys immunized by injections of killed Plasmodium knowlesi antigen. They came to the conclusion that such antibodies were group-, not strain-specific and did not confer protection against further infection. Sutliff et al. (1950) found the complement fixation test with P. knowlesi valuable in detecting cases where the clinical picture and history indicates malaria but parasitaemia is not demonstrable. More recently the fluorescent antibody technique has been applied by Tobie & Coatney (1961), Tobie, Kuvin, Coatney & Evans (1962), and Voller & Bray (1962).

The tanned cell haemagglutination test has proved to be a remarkably sensitive test requiring small amounts of antibody and antigen and has been employed to detect antibody developed against a number of parasites such as Toxoplasma (Lunde & Jacob, 1959), and Schistosoma (Kagan, 1955). Desowitz & Stein (1962) described the application of the tanned cell haemagglutination method using antibody developed against P. berghei and homologous antigen. The present report concerns the modification of that test, employing formolized tanned sheep cells to determine the reactions of various Plasmodia antigens and sera of human patients with malaria.

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MATERIALS AND METHODS

Preparation of antigens

Aqueous extracts of P. berghei, P. cynomolgi and P. vivax were used as antigen in these experiments. The sources of P. berghei were either young infected four to six-week-old rats showing 30-40 parasites per oil immersion field or splenectomized infected adult rats. Ten days after splenectomy these rats were given at three to four-day intervals, four intraperitoneal injections of 0.5 ml heavily-infected young rat's blood and then bled by cardiac puncture when 30-40 parasites per oil immersion field in a thin film were seen. P. cynomolgi antigen was prepared from blood of a heavily-infected Rhesus monkey.¹ P. vivax antigen was obtained from the blood of malaria patients in the Singapore General Hospital.

Preparation of antigen was the same for all species. The heparinized, infected blood was centrifuged at 4000 r.p.m. for 10 minutes and the plasma decanted. This was followed by two washings of the red cells with physiological saline. The sedimented cells were haemolysed in five volumes of distilled water and then centrifuged at 4000 r.p.m. for 10 minutes. The supernatant was discarded and the sediment haemolysed again, centrifuged and washed twice in phosphate-buffered saline (PBS) at pH 7.2. The final deposit was resuspended in 1 ml PBS and thoroughly ground in an all-glass tissue homogenizer. The homogenate was centrifuged at 1000 r.p.m. for five minutes and the supernatant used as antigen. When not needed immediately the antigen was stored at -20°C. Control antigen was prepared from normal, uninfected red cells in the same manner as above.

Preparation of formolized, tanned sheep red blood cells

Sheep blood was collected in an equal amount of Alsevers' solution and stored in the refrigerator for several days before further treatment. The cells were formolized by a modification of Csizmas' (1960) method. One hundred ml of blood were washed by centrifugation seven times in physiological saline and the washed cells resuspended in nine volumes of PBS. Two hundred ml of the suspension were placed in an Erlenmeie

¹ This has been kindly supplied by Drs Eyles and Warren of the United States Public Health Service Far East Research Project at the Institute for Medical Research in Kuala Lumpur, Federation of Malaya.

flask into which a cellophane bag containing 50 ml of 20% formalin (formol) was dipped. The flask was shaken for two hours after which time the bag was punctured and formalin allowed to pour into the cell suspension. The cells and formalin were then mixed with a magnetic stirrer for another 18 hours. The mass of clotted material found after this period was removed, the suspension filtered through gauze and finally washed seven times in 10 volumes of PBS.

The formolized cells were tanned by mixing an equal volume of 1:20 000 tannic acid in PBS with a 2.5% cell suspension also in PBS and the mixture incubated at 37°C for 15 minutes. After incubation the cells were washed once and resuspended in PBS to a 2.5% dilution.

Sensitization of cells with antigen and method for carrying out the test

The performance of the tests depends upon determining the optimum concentration of antigen for sensitizing the formolized tanned cells. Concentrations higher than the optimum produced false positive reactions and therefore it was necessary first to test each lot of antigen with a solution of 0.125% w/v bovine crystalline albumin in PBS (BCAS). Sensitization of the cells was carried out by mixing equal volumes of the 2.5% tanned cell suspension with an antigen dilution and four volumes PBS. A series of antigen dilutions were used ranging from 1:10 to 1:100. The mixture was kept for 15 minutes at room temperature, centrifuged at 1000-1500 r.p.m. for five minutes and finally resuspended in two volumes of BCAS. The suspension was centrifuged again at 1000 r.p.m. for five minutes and finally resuspended in one volume of BCAS, restoring it to the original dilution of 2.5%. The test was carried out in 75 x 12 mm tubes which had been thoroughly cleaned. To 0.5 ml (one drop) of BCAS was added one drop of the formolized tanned cell suspension which had been sensitized with the serial dilutions of antigen. The highest concentration which produced a negative pattern after 24 hours was chosen. Usually this proved to be an antigen diluted to approximately 1:50.

The serum to be tested was inactivated at 56° for 30 minutes. The inactivated serum was absorbed overnight in the refrigerator with an equal volume of packed sheep cells which had been washed three times with PBS. Two-fold dilutions of the inactivated, absorbed serum from 1:100 to 1:25 600 were made with BCAS. To 0.5 ml

of the serum dilution was added one drop of the sensitized cell suspension, the tube shaken and read at four hours after which time it was again thoroughly shaken and read after 24 hours. Readings were made according to the description of haemagglutination patterns given by Stavitsky (1954) and Boyden (1951).

RESULTS

The results are summarized in Table 1 and Figure 1. Of the 27 patients whose sera were tested, 25 were infected with P. vivax, one mixed infection of P. vivax and P. falciparum, and one with P. falciparum. Thirty-eight control sera were obtained from the General Hospital Blood Bank, and were taken from adults apparently free of malaria. However, while there is no malaria in Singapore it is endemic in the Federation of Malaya and the surrounding Indonesian islands and it is therefore difficult to ascertain whether the donors of the control sera had not contracted malaria sometime during their lives. Thus a certain proportion of sera of normal subjects reacted, although generally at much lower titres than the infected patients. The average titre of the sera from infected patients as compared with that of the controls was five times higher with P. berghei antigen, eight times higher with P. cynomolgi antigen and 12 times higher with P. vivax antigen. Although the average titre of the controls was highest with P. vivax antigen the titre of sera of the infected patients also increased proportionately to 1:9170. Maximum titre of sera from infected subjects using P. berghei antigen was 1:3200, with P. cynomolgi antigen 1:6400 and with P. vivax antigen 1:25 600. It is of interest to note that the serum of the patient infected with P. falciparum and that of a child six years old with P. vivax were negative with P. berghei antigen but gave titres of 1:400 and 1:800 respectively with P. vivax antigen. Some typical haemagglutination tests are shown in Table 2.

Control antigen prepared from uninfected blood did not produce any positive haemagglutination reactions.

DISCUSSION

A major obstacle in devising a practical serological test for malaria immunity is the difficulty in obtaining sufficient antigen from the small, intracellular parasite. The formalized, tanned red cell haemagglutination test overcomes this in that only minute, probably microgram, quantities of antigen protein are required and less than one ml of patient's serum is sufficient. The method seems to be highly sensitive, titres as high as 1:25 600 being detected with homologous antigen. The ability to obtain positive results with P. berghei antigen indicates that a source of antigen is readily available for routine experimental determination of the presence of antibody, at least in the case of P. vivax infections. Admittedly, the titres with heterologous antigen are lower than with homologous antigen but a titre above 1:400 with P. berghei antigen seems indicative of infection with P. vivax. Work is now in progress to follow the immune reaction as evidenced by haemagglutination titres during the course of malaria infections in an attempt to correlate the state of infection and level of immune protection with the haemagglutination titre.

Another possible application of the technique is the determination of the antigenic relationship between different species of Plasmodia. Although positive results are obtained with heterologous antigen it is to be noted that higher titres are generally given with P. cynomolgi antigen than with P. berghei antigen when tested against P. vivax antisera indicating a closer relationship of the former to P. vivax. The single sample of serum from a P. falciparum case failed to cross react with either P. berghei or P. cynomolgi antigen while with P. vivax antigen a titre of 1:400 was obtained. Sera from patients infected with P. falciparum are now being collected for additional serological trials. It will also be of interest to follow the course of haemagglutination titres after chemotherapy to assess whether the technique can indicate the achievement of radical cure.

The reason for the positive reactions with the "normal" control sera also requires further investigation. It is virtually impossible to obtain sera from indigenous residents of Singapore who have had no known contact with malaria. The average titre of the normal sera is higher with P. vivax antigen than with P. berghei

or P. cynomolgi antigens and is suggestive that some of the positive reactions with "normal" sera may be due to the presence of antibody. An attempt is being made to obtain sera from individuals who have never been in a malarious area in order to ascertain whether the positive reactions in control sera were due to low concentrations of antibody or a normal non-specific factor in some sera.

SUMMARY

A formalized, tanned sheep cell haemagglutination test has been employed to measure antibody developed in human malaria infections. The preparation of antigen from infected, haemolysed red blood cells and the technique for carrying out the test are described. Antigen was derived from P. berghei, P. cynomolgi, and P. vivax. The majority of the sera was obtained from patients suffering from P. vivax infections. Sera of these patients gave highest titres (up to 1:25 600) with P. cynomolgi and P. vivax antigens. Positive haemagglutination reactions were noted with P. berghei antigens but in lower titres.

A proportion of the "normal" control sera obtained from the Singapore General Hospital Blood Bank produced positive reactions but generally at titres considerably lower than with patients' sera. The possible causes of these positive control reactions are discussed.

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TABLE 1. RESULTS OF HAEMAGGLUTINATION TESTS USING SERA FROM INFECTED AND NON-INFECTED PERSONS AND THREE PLASMODIAL ANTIGENS

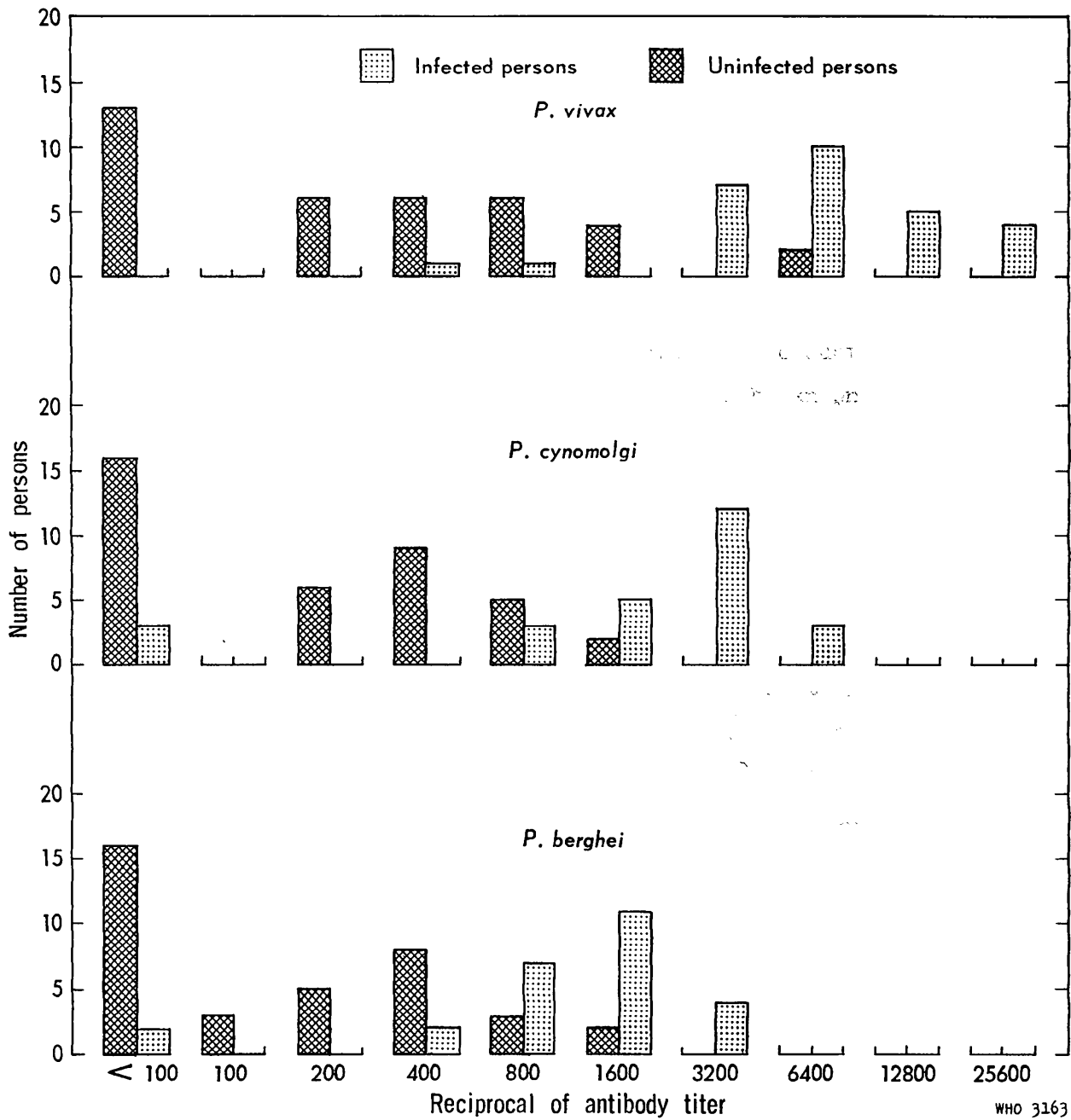
	Titres of antibody (reciprocal)									
	100	100	200	400	800	1 600	3 200	6 400	12 800	25 600
<u>P. vivax</u> antigen										
Infected subjects	-	-	-	1	1	-	7	10	5	4
Non-infected subjects	13	-	6	6	6	4	-	2	-	-
<u>P. cynomolgi</u> antigen										
Infected subjects	16	-	6	9	5	2	-	-	-	-
Non-infected subjects	3	-	-	-	3	5	12	3	-	-
<u>P. berghei</u> antigens										
Infected subjects	16	3	5	8	3	2	-	-	-	-
Non-infected subjects	2	-	-	2	7	11	4	-	-	-

TABLE 2. SOME TYPICAL HAEMAGGLUTINATION REACTIONS WITH P. BERGHEI
P. CYNOMOLGI AND P. VIVAX ANTIGENS

Patient No.	Age (years)	Infection	Titre with <u>P. berghei</u>	Titre with <u>P. cynomolgi</u>	Titre with <u>P. vivax</u>
1	6	<u>P. vivax</u>	0	1:800	1:800
3	28	<u>P. falciparum</u>	0	0	1:400
4	25	<u>P. vivax</u>	1:800	1:1 600	1:3 200
7	29	<u>P. vivax</u>	1:800	1:1 600	1:3 200
11	13	<u>P. vivax</u>	1:1 600	1:1 600	1:6 400
12	25	<u>P. vivax</u>	1:400	1:3 200	1:3 200
13	10	<u>P. vivax</u>	1:800	1:1 600	1:6 400
15	11	<u>P. vivax</u>	1:1 600	1:6 400	1:6 400
16	25	<u>P. vivax</u> + <u>P. falciparum</u>	1:800	1:3 200	1:12 800
17	30	<u>P. vivax</u>	1:1 600	1:1 600	1:3 200
19	19	<u>P. vivax</u>	1:1 600	1:3 200	1:6 400
20	30	<u>P. vivax</u>	1:1 600	1:6 400	1:12 800
21	18	<u>P. vivax</u>	1:1 600	1:3 200	1:6 400
23	31	<u>P. vivax</u>	1:1 600	1:6 400	1:12 800

FIG. 1

DISTRIBUTION OF HEMAGGLUTINATION TITRES, USING VARIOUS PLASMODIAL ANTIGENS, AMONGST A GROUP OF NORMAL AND MALARIA INFECTED PERSONS



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