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THE FLUORESCENT ANTIBODY TECHNIQUE AS A MEASURE  
OF ANTIBODY TO MALARIA PARASITES

by

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The fluorescent antibody technique has been used successfully to stain malaria parasites (Brooke et al., 1958; Ingram et al., 1961; Tobie & Coatney, 1961; Voller, 1962) and the indirect method has been successfully applied to the measure of circulating antibody to malaria parasites (Kuvin et al., 1962).

Independent attempts to adapt the indirect method in Liberia to measure the distribution of antibody concentration throughout a semi-immune population have also been successful and are described below.

Methods, materials and results

1. Fixation of the Laverania falcipara blood smear for antigen: acetone fixation for five minutes followed by quick drying in a relative humidity of less than 40 per cent. or similar acetone fixation followed by treatment with 0.1 per cent. hydrochloric acid (1 ml concentrated HCl to 1000 ml water) for five minutes gave good preservation of the parasites and erythrocytes, caused staining of the parasite cytoplasm but not the parasite nuclei, gave false positive results with up to 1/100 dilution of non-immune serum and gave results up to 1/6400 with immune serum.

Fixation with methanol gave good preservation but a very messy blood film after staining with a great deal of deposit between erythrocytes, and caused staining of the parasite nuclei only.

Treatment with 0.1 per cent. hydrochloric acid (or 0.2 per cent. acetic acid, both diluted in water or saline) of unfixed slides for five minutes gave rather poor fixation

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and disrupted the integrity of both erythrocyte and parasite to some degree. Disruption of integrity became less and less as storage of the blood film was prolonged beyond twenty-four hours. This treatment gave good staining, no false positives with non-immune serum and results up to a dilution of 1/6400 of immune serum.

2. The blood smear containing erythrocytic forms of L. falcipara as antigen was best when used directly and no advantage was gained from washing the erythrocytes, removing white cells and resuspending the erythrocytes in non-immune serum or goat serum prior to making the smear. The parasites used can be taken from children of any age, preferably having parasitaemias above 25 000/mm<sup>3</sup>. These smears are best used after more than twenty-four hours of drying in a desiccator.

3. The anti-human globulin serum tagged with fluorescein isothiocyanate. This was purchased from Microbiological Associates Inc. and the first batch obtained was undoubtedly poor, probably because it had taken four months to reach the Institute (by air!). A second batch has given consistently good results. No difference was found between goat and rabbit antiserum. The antiserum can be heated to 56°C for half an hour without destroying its activity (as can test serum). The commercial product can be diluted to 1/20 without impairing activity. Thus five ml of antiserum can be diluted to 100 ml and, as one drop only is used, nearly 2000 slides can be processed using one five ml bottle of antiserum, which costs \$ 5.00.

4. The measurement test for antibody was performed by treating a smear of parasites for five minutes with 0.1 per cent. HCl, 10 minutes washing in phosphate buffered saline pH 7.2 (PBS), half-hour layering with one standard drop of serum or serum dilution under test, 15 minutes washing in PBS, half-hour layering with tagged antiserum, 15 minutes washing in PBS, mounting in PBS and examining by U/V microscope. A Zeiss binocular microscope has been used with an Osram HBO 200 mercury vapour burner. Exciter filters were mounted on the lamp-housing and barrier filters between the objective and eye-piece lenses.

5. Parasite staining was awarded the following code marks: ++ for brilliant staining, + for definite staining,  $\frac{+}{-}$  for faint staining where usually larger parasites were just visible but small parasites could not be seen.  $\frac{+}{-}$  represented the end-point reaction. The test was applied to various age-groups in a local population living under conditions

of endemic malaria risk. For results, see Tables 1, 2, 3 and 5.

6. The length of time that dried unfixed smears can be left at room temperature in a desiccator before use was found to be up to 12 days. At 14 days some deterioration occurred.

7. The processing was performed at 4°C, 20°C and 37.5°C without essentially altering the results.

8. Milk from immune mothers was tested and found to contain none or only very little antibody in a few cases. The milk was tested before and after removing fat.

9. Attempts were made to shorten the time of the test by reducing all layering and washing times to 10 minutes and using a mechanical shaker. The results were the same but there was some tendency for the smear to wash off. No real advantage was gained as only about 40 minutes were gained in a two-and-a-half-hour process.

10. The tagged antiserum, absorbed on liver powder, diluted 1/20 and stored at -20°C kept for four to six weeks without change. Some false positive results were obtained after this time, probably due to the appearance of free fluorescein.

11. Serum from Liberian adults living in Kpain in the centre of a DDT residual spray malaria eradication pilot project was tested. The spraying had continued for three years and had resulted in the apparent disappearance of A. gambiae and A. funestus. Titres as high as 1/1600 were found and little diminution of circulating antibody was apparent (see Table 3).

12. No evidence was found of bound antibody to circulating parasites.

13. All circulating parasites in the blood, including gametocytes, have been stained and all portions of the parasite except the pigment have been stained.

14. While the erythrocyte parasitized by the older parasites has been found to stain weakly and is presumed therefore to be weakly antigenic, the erythrocytes parasitized by younger parasites stain hardly at all. There may be some support here for the thesis that the parasitized erythrocyte is not importantly antigenic while the parasite is relatively young. Thus malarial immunity (and take-up by phagocytes) may be excited only by the older parasites in erythrocytes and, more particularly, by the merozoites

and other products of the final schizogony process. On the other hand, the lesions in the erythrocytes stain strongly at all times and are apparently strongly antigenic.

15. Drops of blood from finger-pricks have been taken up on to four cm<sup>2</sup> of filter-paper, dried and stored in a desiccator. As late as two weeks later, these have been inserted into Tygon plastic tubes blocked at one end. Into the tubes 0.3 ml of PBS was run and the tubes rolled and squeezed periodically over half an hour. The liquid was then expressed and used as test serum. The end concentration of serum is about 1/10. The results are shown in Table 4.

#### REFERENCES

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TABLE 1. THE END-POINT REACTION FOR SERUM DILUTIONS USING  
L. FALCIPARA SLIDE ANTIGEN, ACETONE FIXED, AND A POOR BATCH  
OF RABBIT AND GOAT ANTI-HUMAN GLOBULIN SERUM CONJUGATED WITH  
FLUORESCHEIN ISOTHIOCYANATE

Serum from	Reciprocal of serum dilution	Corrected reciprocal serum dilution*
European non-immunes	25, 100, 100, 50, 50, 100, 100, 100	1
Liberian (immune) cord blood	200, 1600, 3200, 800, 800, 400	2, 16, 32, 8, 8, 8
Liberian 6-12 months	200, 200	4, 4
Liberian 12-30 months	400, 1600	8
Liberian 30-48 months	200, 1600	2
Liberian 4-10 years	1600, 800	
Liberian adults	1600, 1600, 3200, 1600, 1600, 800, 1600, 800, 3200, 6400, 6400, 3200, 3200	64, 16, 32, 16, 32, 16, 32, 8, 32, 32, 32
Liberian adults on three years chemo-prophylaxis	1600, 800, 100	64, 8, 1

\* Corrected by bringing the non-immune control to one where a batch of staining included a control.

TABLE 2. THE END-POINT REACTION FOR SERUM DILUTIONS USING L. FALCIPARA SLIDE ANTIGEN, HYDROCHLORIC ACID TREATED, AND A POOR BATCH OF RABBIT OR GOAT ANTI-HUMAN GLOBULIN SERUM CONJUGATED WITH FLUORESCEIN ISOTHIOCYANATE

Serum from	Reciprocal of serum dilution
European non-immune	1, 1, 1, 1, 1, 1, 1, 1
Liberian (immune) cord blood	50, 50, 100
Liberian 6-12 months	25, 1
Liberian 12-30 months	10, 1
Liberian 38-48 months	50
Liberian 4-10 years	100, 50, 25
Liberian adults	200, 100, 25, 50, 25, 50, 50, 50, 100, 100
Liberian adults on three years chemo-prophylaxis	25, 100

TABLE 3. THE END-POINT REACTION FOR SERUM DILUTIONS USING L. FALCIPARA SLIDE ANTIGEN, HYDROCHLORIC ACID TREATED, AND A GOOD BATCH OF RABBIT ANTI-HUMAN GLOBULIN SERUM CONJUGATED WITH FLUORESCCEIN ISOTHIOCYANATE

Serum from	Reciprocal of serum dilution
European non-immunes	0, 0, 0, 0, 0, 0, 0
European cord-blood	2, 0
Liberian (immune) cord blood	100, 100, 100, 400, 800, 400, 800, 6400, 800, 400, 1600
Liberian 0-2 weeks	5, 25, 25, 400, 1600, 400, 50, 1600, 1600, 400, 800
Liberian 2 weeks-6 months	2, 2, 2, 5, 25, 25, 50, 400, 50, 400, 25, 5, 25, 50, 50, 100, 100, 10, 10, 10, 1600, 50
Liberian 6-12 months	0, 25, 2, 5, 25, 100, 100, 50, 100
Liberian 12-30 months	5, 10, 25, 25, 10, 25, 200, 200, 10, 25, 5
Liberian 30-48 months	50, 100, 50, 100, 50, 50, 25, 200, 400, 400
Liberian 4-10 years	100, 200, 200, 400, 25, 200, 800, 200
Liberian adults under 40 years	50, 800, 400, 800, 400, 400, 1600, 100, 3200
Liberian adults over 40 years	3200, 1600, 3200
Liberian adults after clinical malaria	3200, 3200, 800, 200, 1600, 3200, 6400
Liberian adults in area protected for 3 years by DDT spraying	400, 100, 200, 200, 200, 1600, 200, 50, 200
Adults from Nigeria and Cameroons	100, 100, 200, 1600
Liberian mothers' milk	0, 0, 5, 0, 2, 5

Results are in chronological order and the tendency towards rise in titre is probably due to move in time into the wet season when malaria transmission is considerably increased and clinical malaria equally increased.

TABLE 4. STAIN REACTION USING BLOOD ELUTED FROM  
 FILTER PAPERS USING L. FALCIPARA SLIDE ANTIGEN AS IN TABLE 3

Blood from	Number	Results
European non-immunes	10	<sup>+</sup> , 1 ; -, 9
Liberian adults	50	++, 48 ; +, 2
Liberian 4-10 years	25	++, 22 ; +, 3
Liberian 24-48 months	10	++, 9 ; +, 1
Liberian 12-24 months	10	++, 8 ; +, 2
Liberian 1-12 months	10	++, 5 ; +, 4; <sup>+</sup> , 1 -, 1
Liberian under 3 years protected 3 years by DDT spraying	10	+, 1 ; <sup>+</sup> , 2 ; -, 7
Liberian under 3 years unprotected	6	++, 2 ; +, 4

Note: Slide antigen - L. falcipara second serum layer - rabbit anti-human globulin serum conjugated with fluorescein isothiocyanate. Stain reactions: ++ brilliant, + easily visible, <sup>+</sup> not all parasites visible, - no reaction.

TABLE 5. END-POINT REACTION FOR SERUM DILUTIONS USING L. FALCIPARA  
AVERAGE TITRE COMBINING TABLES 2 AND 3

Serum from	Average reciprocal of dilution
Liberian cord blood	864
Liberian 0-2 weeks	628
Liberian 2 weeks-6 months	131
Liberian 6-12 months	39
Liberian 12-30 months	42
Liberian 30-48 months	134
Liberian 4-10 years	209
Liberian adults under 40 years	467
Liberian adults over 40 years	2333
Liberian adults after clinical malaria	2657
Liberian adults protected for three years by DDT spraying	350

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