# Fluorescent Antibody Staining as a Measure of Malarial Antibody. (27687)

A. VOLLER* AND R. S. BRAY (Introduced by R. J. C. Hoeppli)
*London School of Hygiene and Tropical Medicine, and the Liberian Institute, American Foundation for Tropical Medicine†*

The fluorescent antibody technic has been employed to stain malaria parasites(1, 2,3) using both the direct and indirect method. The indirect method offers the advantage of greater brilliance and the opportunity to use serial dilutions of a number of test sera without the time-consuming necessity of conjugating each test serum with dye. This report concerns our preliminary experience in using the indirect method and serial dilutions of test sera as a measure of circulating antibody to malaria.

*Materials and methods.* Air-dried thin blood films of *Laverania falcipara* (= *Plasmodium falciparum*), *Plasmodium malariae* and *P. ovale* were taken from children aged 6 months to 7 years showing heavy blood infections. All smears were collected in the area immediately surrounding the Liberian Institute at Harbel or its field station at Voinjama, Western Province (240 miles by road from Harbel).

The thin smears were treated as follows to determine the best method of fixation: fixation in dry acetone for 5 minutes and quick drying in an atmosphere of below 50% R.H.; in dry methyl alcohol for 10 seconds; in Lillie's formol for 5 minutes; treatment in 0.1% hydrochloric acid in water or physiological saline for 5 minutes then transfer to phosphate-buffered physiological saline at pH 7.2 (PBS).

Air-dried thin smears of sporozoites of *L. falcipara* from the salivary glands of *Anopheles gambiae* were fixed in dry acetone. Whole guts of *Anopheles gambiae* infected with oocysts of *L. falcipara* were used fixed for 10 minutes in dry acetone or unfixed and kept in PBS.

Blood was collected sterilely from adults and older children from the cubital vein; from young children and infants during circumcision or from the jugular vein, and from the cord at delivery, and the serum separated. Sodium azide was used where necessary to maintain sterility. Goat or rabbit anti-serum to human globulin conjugated with fluorescein isothiocyanate was obtained commercially (Microbiological Associates, Inc.) and absorbed once with mouse-liver powder before use.

The treated smear was washed with PBS and a selected portion was covered with one drop of the diluted test serum for 30 minutes at room temperature (23°C). The smear was washed thoroughly for 15 minutes in PBS and covered with one drop of the anti-globulin serum at a dilution of 1 in 20 for 30 minutes at room temperature. The smear was washed again for 15 minutes in PBS, mounted in PBS and examined by means of a Zeiss fluorescent microscope. Variants of this technic included washing parasitised red cells and resuspending them in goat serum prior to smearing and fixation, layering with goat serum prior to layering with test serum, absorption of the test serum with mouse-liver for 30 minutes.

The staining reaction was awarded 3 values: ++ = brilliant, + = dimmer but well defined and ± = visible but indistinct or visible only in some cases. The values were awarded to the parasitised erythrocyte envelope, the lesions in the parasitised erythrocyte, the parasite cytoplasm and the parasite nucleus.

*Results.* Fixation in Lillie's formol gave no staining reaction. Fixation with methyl alcohol gave poor results. All other treatments of the blood smear allowed staining. Both fixation in acetone and treatment with 0.1% hydrochloric acid gave consistent results. Fixation with acetone gave the best fixation and the most satisfactory staining but also gave false positive results. Treat-

* This work was done at the Liberian Institute during the senior author's tenure of a World Health Organisation fellowship.

† This work was assisted by a grant from World Health Organisation to the Liberian Institute.

ment with 0.1% hydrochloric acid frequently destroyed the integrity of the erythrocytes and distorted the parasite but did not give false positive results.

The technic failed to stain sporozoites or whole oocysts; blood parasites in the absence of test serum; the nucleus of blood parasites when acetone-fixed; the cytoplasm of blood parasites and the lesions in the parasitised erythrocyte when methyl alcohol-fixed. With acetone fixation erythrocytic parasite cytoplasm, erythrocyte lesions and the parasitised erythrocyte envelope all stained brilliantly, when undiluted or low dilutions of immune serum were used. Prior layering with goat serum and inactivation of test serum at 56°C did not alter the staining reaction. The cytoplasm of erythrocytic asexual forms of *L. falcipara*, *P. malariae* and *P. ovale* and the cytoplasm of erythrocytic sexual forms of *L. falcipara* and *P. malariae* were successfully stained when acetone-fixed and employing immune serum to a dilution of 1 in 6400. The same forms were also stained when using non-immune serum to a dilution of 1 in 100. Washing the parasitised erythrocytes and resuspending in goat serum gave a slight improvement but did not materially reduce the false positive results when non-immune serum was employed.

When treated with 0.1% hydrochloric acid the cytoplasm of the parasite, occasionally the nucleus of the parasite, the erythrocyte lesions and the parasitised erythrocyte envelope all stained brilliantly using immune serum to a dilution of 1 in 200. Only undiluted non-immune serum gave similar results.

Table I shows results of using dilutions of test serum and *L. falcipara* blood smears. The end-point of the acetone-fixed material is the ± value for parasite cytoplasm. The end-point of the 0.1% hydrochloric acid treated material is the last ++ value for parasite cytoplasm as there is a sharp drop to a + or ± or negative value at this point probably depending on the efficiency of absorption of free fluorescein in the conjugated anti-serum. When using 0.1% hydrochloric acid treatment an even better end-point is the staining of the parasitised erythrocyte envelope which drops from a + or ++ value to a complete lack of staining at the same dilution as the above drop in parasite staining.

It will be seen that false positive results seriously mitigate the value of the technic

TABLE I.

| Test serum from | End points, acetone-fixed smears of *L. falcipara* | | End points, 0.1% HCl-treated smears of *L. falcipara* |
|---|---|---|---|
| | Dilution X | Corrected dilution* where controlled | Dilution X |
| Foreign non-immunes | 25, 100, 100, 50, 50, 100, 100, 100 | 1 | 1, 1, 1, 1, 1, 1, 1, 1, 0‡, 0‡ |
| Local cord blood | 200, 1600, 3200, 800, 800, 400 | 2, 16, 32, 8, 8, 8 | 50, 50, 100, 100‡ |
| Local 6-12 mo | 200, 200 | 4, 4 | 25, 1, 0‡ |
| " 12-30 " | 400, 1600 | 8 | 10, 1†, 5‡, 10‡, 25‡ |
| " 30 mo - 4 yr | 200, 1600 | 2 | 50, 50‡, 100‡, 50‡ |
| " 4-10 yr | 1600, 800 | — | 100, 50, 25 |
| Local 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 | 200, 100, 25, 50, 25, 50, 50, 50, 100, 100 |
| Local adults under chemoprophylaxis for 2-5 yr | 1600, 800, 100 | 64, 8, 1 | 25, 100 |

* This is corrected by reducing the dilutions so that the non-immune control is 1 where the series of slides used contained a non-immune control.

† Child living in a DDT-sprayed zone.

‡ New batch of fluorescein-conjugated anti-globulin serum.

when using acetone-fixation. Positive results were obtained using non-immune serum up to a dilution of 1 in 100. However positive results using immune serum were obtained up to a dilution of 1 in 6400. Treatment with 0.1% hydrochloric acid gave better and seemingly more reliable results especially if the conjugated anti-globulin serum is fresh and well absorbed. On the other hand acetone-fixed material when kept dry remained unchanged for weeks whereas treatment with 0.1% hydrochloric acid required immediate use of the smear and the dried unfixed smear could be kept only a few days before treatment as it soon commenced to lose its antigenic properties.

Several individual results are worthy of note. The gametocytes of *L. falcipara* and *P. malariae* stained brilliantly. Two local adults under chemoprophylaxis (300 mg chloroquine base or 400 mg amodiaquine base weekly) for 3 and 5 years showed high titres in their sera. Cord blood sera from local mothers showed titres as high as some immune adults and considerably higher than non-immune. A non-immune under chemoprophylaxis (25 mg pyrimethamine weekly) inoculated with sporozoites of *L. falcipara* by allowing infected *A. gambiae* to bite on 6 out of 8 consecutive days showed no subsequent rise in titre. Two local adults with titres of 1 in 100 and 1 in 50 were infected with sporozoites of *L. falcipara;* one (1 in 100) showed parasites and clinical symptoms and the other (1 in 50) showed neither parasites nor clinical symptoms. There was no difference between the staining reactions obtained using parasites and sera from Voinjama and Harbel when all combinations were employed.

Lesions in the erythrocytes parasitised by *L. falcipara* and *P. ovale* were seen at an earlier stage of development than can be demonstrated by Romanowski stains. In the case of *L. falcipara* about 20 tiny scattered dots appeared in the erythrocyte when the parasite was still very small. As the parasite grew the dots grew larger in size and eventually appeared to coalesce to form several large lesions. These large lesions were the well known Maurer's clefts as was demonstrated by Giemsa staining of slides of the same series. In the case of *P. ovale* a series of small scattered dots could also be seen in erythrocytes containing very small parasites. The dots grew slightly in size and were seen to be Schüffner's dots. Zieman's stippling of the host erythrocyte to *P. malariae* was also seen but individual dots could not be detected.

*Discussion.* The technic proved to have some value in measuring circulating antibody to malaria. The measurements made on individuals among a population at constant risk follow broadly the classic concepts of immunity to stable malaria(4). It proved possible to demonstrate high titres of antibody in foetal blood at birth; a fact long suspected in human malaria and shown to be the case in murine malaria(5). The few sera of children showed an initial decline in antibody titre with age, followed by a rise to a high level by the age of 4 years, which is in accord with epidemiological knowledge. Equally the results found here tend to mirror the pattern of gamma globulin levels among African populations at risk to malaria(6). We have no way of knowing if the antibody being measured is effectively antiparasitic though one result tends to show otherwise. The difference in titre between subjects has been disappointingly small though in line with previous experience with immunological tests in malaria.

The brilliance of the gametocyte staining would seem to show that gametocytes are effectively antigenic despite the knowledge that the gametocyte of *L. falcipara* is able to circulate in the host for weeks without impairment of activity. The technic was useful in demonstrating lesions in parasitised erythrocytes and for the first time the young ring of *L. falcipara* was seen to have caused lesions in the host erythrocyte. Maurer's clefts appear to arise from the coalescence of a large number of small lesions which appear earlier.

It would seem that the sporozoites of *L. falcipara* are not effectively antigenic when inoculated into a susceptible host. They are known to circulate for only 30-40 minutes before invading liver cells. It is possible that this is too short a time to stimulate measurable antibody response.

The technic as a measure of antibody

has not proved entirely consistent in our hands. Variation of the slide antigen and of aliquots of anti-globulin sera have given variation in the results using the same test serum. The use of the hydrochloric acid treatment of smears has tended to minimise this. It is hoped that concentration on standardising the antigen by using parasites of similar age from infants only (below 12 months) and on absorbing and purifying the conjugated anti-globulin serum will remove the inconsistencies.

*Summary.* The erythrocytic forms of 3 species of human malaria parasites but not the sporozoites and oocysts of *L. falciparа* have been stained by the indirect method of the fluorescent antibody technic. The technic has been used to measure the circulating antibody to malaria with some success and the results so far confirm classic concepts of malarial immunity. Antibody to malaria has been demonstrated in cord blood from immune mothers.

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Received May 21, 1962. P.S.E.B.M., 1962, v110.