Notes

Fluorescent Antibody Studies on Malaria Parasites *

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Although immunofluorescent staining has been extensively applied to studies of bacteria since Coons et al.[a] developed this technique, its first use in malariology was when Brooke et al.[b] showed that *Plasmodium berghei* could be stained by fluorescent antibody. Using this method Ingram et al.[c] demonstrated that *Plasmodium cynomolgi bastianellii* could be differentiated from *Plasmodium gallinaceum*. More recently Tobie & Coatney [d] were able to stain *Plasmodium vivax* by this method.

This note deals with preliminary work using the fluorescent antibody technique in an attempt to differentiate species and strains of *Plasmodium*.

Materials and methods

Conjugation. The standard method of conjugation used [e] is as follows.

A 10-ml quantity of serum was diluted with 10 ml of normal saline cooled to 4°C in a beaker in an ice-bath. Mechanical stirring was arranged, care being taken to avoid frothing. Then 20 ml of saturated ammonium sulfate, cooled to 4°C, were added slowly over a period of 20 minutes. Stirring was continued for 90 minutes. The suspension was then centrifuged at 10 000 *g* for 10 minutes and the precipitate washed into a container with 20 ml of half-saturated ammonium sulfate. Centrifugation was repeated and the precipitate of globulin was dissolved in distilled water.

This globulin solution was dialysed overnight against normal saline buffered to pH 7.0 with 0.01 M phosphate buffer (PBS). The solution was then passed down a Sephadex G-25 [f] column. The protein fraction was collected and tested with Nessler's solution to confirm that all the ammonium sulfate had been removed. The protein content was then determined by reading the absorption at 280 mμ in the spectrophotometer.

The globulin was then diluted with saline and 0.5 M bicarbonate carbonate buffer at pH 9.0 to give a solution in which the final concentration of globulin was 10 mg/ml and 25% of the total volume was buffer. The buffered globulin was stirred at 4°C and fluorescein isothiocyanate powder, 1 mg per 20 mg of protein, was added slowly to the surface of the conjugation mixture, and stirring was continued for 18 hours. Free dye was removed from the conjugate by passing it through a Sephadex column. The conjugate was then stored in bijou bottles at -30°C.

Before use, the conjugates were absorbed twice with acetone-dried pigeon liver powder, 100 mg per ml of conjugate. They were then shaken at room temperature for 30 minutes and then centrifuged at 25 000 *g* for 30 minutes. Sodium azide (0.3%) was added to each conjugate, which was then stored at 4°C.

Preparation of blood smears. Thin blood smears were made, air-dried, and fixed in acetone for one minute at room temperature followed by rapid air drying. Slides prepared in this manner could be kept over calcium chloride at 4°C for two weeks without deterioration. Storage of slides at room temperature resulted in a rapid loss of their ability to react. Immediately before staining the smears were immersed in PBS for 20 seconds.

For *direct staining*, labelled antibody was applied to the smears for 30 minutes in a damp chamber at room temperature, followed by washing and mounting in PBS. Controls were set up in which labelled non-immune sera were tested against smears from both infected animals and non-infected animals. In the *indirect method*, unlabelled immune

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[a] Coons, A. H., Creech, H. J., Jones, R. N. & Berliner, E. (1942) *J. Immunol.*, **45**, 159.

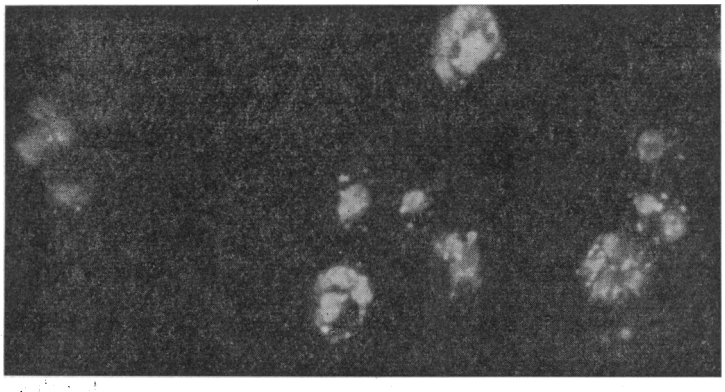
[b] Brooke, M. M., Melvin, M. & Healey, G. R. (1959) *Staining* Plasmodium berghei *with fluorescein-labelled antibodies* (Paper presented at the Sixth Congresses on Tropical Medicine and Malaria, Lisbon, 1958).

[c] Ingram, R. L., Otken, L. B. & Jumper, J. R. (1961) *Proc. Soc. exp. Biol. (N.Y.)*, **106**, 52.

[d] Tobie, J. E. & Coatney, G. R. (1961) *Exp. Parasit.*, **11**, 128.

[e] Coons, A. H. & Kaplan, M. H. (1950) *J. exp. Med.*, **91**,1.

[f] AB Pharmacia, Sweden.



×1000.

FIG. 1

FLUORESCENT *P. BERGHEI* PARASITES AND FLUORESCENT STIPPLING OF THE INFECTED HOST CELLS IN A SMEAR OF INFECTED RAT BLOOD EXPOSED TO HOMOLOGOUS FLUORESCEIN-LABELLED ANTISERUM

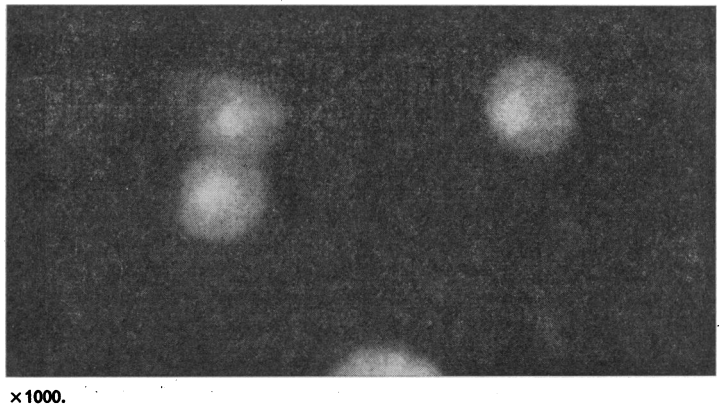


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FIG. 2

FLUORESCENT *P. BASTIANELLII* PARASITES AND FLUORESCENT STIPPLING OF INFECTED HOST CELLS IN A SMEAR OF INFECTED MONKEY BLOOD WHICH HAD BEEN EXPOSED TO UNLABELLED HOMOLOGOUS ANTISERUM FOLLOWED BY LABELLED ANTI-HUMAN GAMMA-GLOBULIN PREPARED IN A RABBIT

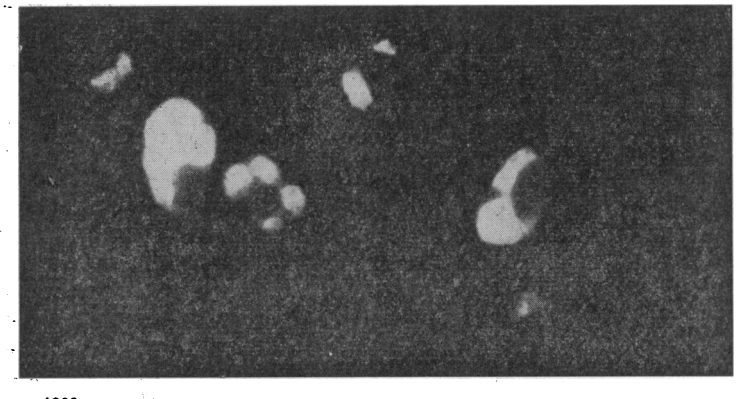

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FIG. 3

FLUORESCENT *P. GALLINACEUM* PARASITES IN A SMEAR OF INFECTED CHICKEN BLOOD EXPOSED TO HOMOLOGOUS FLUORESCEIN-LABELLED ANTISERUM

Note absence of stippling of the infected cells

TABLE 1

PRODUCTION OF ANTISERA TO SPECIES OF *PLASMODIUM*

Species of *Plasmodium*	Host	Method of infection
P. berghei	Rats 240-260	Blood passage; repeatedly challenged
P. gallinaceum 8B	Chicken 96	Blood passage
P. juxtanucleare 14C	Chicken 97	Blood passage
P. bastianellii	Monkey 200	Infected by sporozoites; challenged with infected blood
P. bastianellii	Monkey 227	Infected by sporozoites; challenged with infected blood
P. bastianellii	Human 1	Infected by sporozoites
P. vivax	Human 1 (European)	Infected by mosquito bite; challenged with sporozoites
P. vivax	Human 4[a] (African)	Bitten by infected mosquitos; challenged with infected blood
P. ovale	Human 6 (European)	Infected by mosquito bite

[a] No parasites were recorded in blood smears from human 4

serum of globulin was applied to the smears for 30 minutes. After washing, the preparations were overlayered with conjugated antibody prepared against the globulin of the first serum. As controls, unlabelled non-immune sera or saline were used as the first stage in the indirect method.

Control sera were obtained from clinically normal non-infected animals. Antisera were produced as shown in Table 1. Smears were made from heavily infected blood: *P. berghei* and *P. vinckei* from rats and mice, *P. gallinaceum* strains 8A and 8B and *P. juxtanucleare* strains 14C and 14A from chickens *P. bastianellii*, *P. gonderi* and *P. osmaniae* from monkeys, and *P. vivax* from humans.

Results

The results of immunofluorescent staining using control sera and homologous or heterologous antisera are given in Tables 2-6. In general the direct method gave the best results since the background staining was higher when the indirect method was used.

When *P. berghei* was stained with the homologous serum (Fig. 1) the cytoplasm of the parasite fluoresced whereas the nucleus did not.[b] In the present work fluorescent stippling of the infected host cells could be clearly seen. The stipples were larger, but

TABLE 2

STAINING RESULTS[a] (DIRECT METHOD) AFTER EXPOSURE OF BLOOD SMEARS TO FLUORESCEIN-LABELLED SERUM FROM RATS, EITHER INFECTED WITH *P. BERGHEI* OR NON-INFECTED CONTROLS

Smear		Sera	
Parasite	Host	*P. berghei* immune	Control
P. berghei	Rat	+++	—
P. berghei	Mouse	+++	—
P. vinckei	Rat	±	—
P. vinckei	Mouse	±	—
P. gallinaceum 8B	Chicken	—	—
P. juxtanucleare 14C	Chicken	—	—
P. bastianellii	Monkey	—	—
P. gonderi	Monkey	—	—
P. osmaniae	Monkey	—	—
P. vivax	Human	—	—

[a] The intensity of fluorescence was estimated visually, +++ indicating the brightest, — being negative, while ++, + and ± represent successively decreasing intermediate intensities. NT = not tested.

TABLE 3

STAINING RESULTS[a] (INDIRECT METHOD) AFTER EXPOSURE OF BLOOD SMEARS TO ANTISERUM FROM RATS, EITHER INFECTED WITH *P. BERGHEI* OR NON-INFECTED CONTROLS, FOLLOWED BY FLUORESCEIN-LABELLED RABBIT ANTIRAT GLOBULIN

Smear		Sera	
Parasite	Host	*P. berghei* immune	Control
P. berghei	Rat	+++	±
P. vinckei	Rat	+	±
P. gallinaceum 8B	Chicken	+	±
P. juxtanucleare 14C	Chicken	—	—
P. bastianellii	Monkey	—	±
P. gonderi	Monkey	+	±
P. osmaniae	Monkey	+	±

[a] For explanation of symbols see Table 2.

TABLE 4

STAINING RESULTS[a] (DIRECT METHOD) AFTER EXPOSURE OF BLOOD SMEARS TO FLUORESCEIN-LABELLED ANTISERUM FROM CHICKEN, EITHER INFECTED WITH *P. GALLINACEUM* 8B OR *P. JUXTANUCLEARE* 14C OR NON-INFECTED CONTROLS

Smear		Sera		
Parasite	Host	*P. gallinaceum* immune	*P. juxtanucleare* immune	Control
P. gallinaceum 8B	Chicken	+++	—	—
P. gallinaceum 8A	Chicken	+++	—	—
P. berghei	Rat	—	—	—
P. vinckei	Rat	—	—	—
P. bastianellii	Monkey	—	—	—
P. gonderi	Monkey	—	—	—
P. juxtanucleare 14A	Chicken	—	+++	—
P. juxtanucleare 14C	Chicken	—	+++	—

[a] For explanation of symbols see Table 2.

Discussion

The strong cross-reactions between the primate malarias *P. vivax*, *P. bastianellii*, *P. gonderi* and *P. osmaniae* (Table 5) would suggest that these share common antigens. This is rather surprising in view of the results of cross-immunity tests. Garnham[g] reported no cross-immunity even between the subspecies *P. cynomologi cynomologi* and *P. bastianellii*. The absence of any staining of the above species tested with the *P. ovale* immune serum cannot be regarded as significant until this serum has been used against the homologous parasite. This work agrees with that of Tobie & Coatney,[d] who found a strong cross-reaction between *P. vivax* and *P. bastianellii* and a weaker cross-reaction between those species and *P. berghei*.

The weaker reaction with serum from human 4, an African, is interesting in that racial innate immunity might explain why the reaction was less strong than with the serum from human 1, a European (Table 5). Both patients were equally immune to reinfection with *P. vivax*.

TABLE 5

STAINING RESULTS[a] (INDIRECT METHOD) AFTER EXPOSURE OF BLOOD SMEARS TO ANTISERUM FROM HUMANS, EITHER INFECTED WITH *P. VIVAX*, *P. BASTIANELLII* OR *P. OVALE* OR CONTROL NON-INFECTED, FOLLOWED BY FLUORESCEIN-LABELLED RABBIT ANTIHUMAN GAMMA-GLOBULIN

Smear		Sera				
Parasite	Host	*P. vivax* immune 1	*P. vivax* immune 4	*P. bastianellii* immune 2	*P. ovale* immune 6	Control 3
P. bastianellii	Monkey	+++	++	+++	±	—
P. vivax	Human	+++	++	+++	NT	—
P. osmaniae	Monkey	+++	+	+++	±	—
P. gonderi	Monkey	+++	+	+++	±	—
P. berghei	Rat	+	NT	+	NT	±
P. gallinaceum 8B	Chicken	—	NT	—	NT	—

[a] For explanation of symbols see Table 2.

fewer, than those observed in smears of the primate malarias stained with antisera against *P. bastianellii* (Fig. 2) or *P. vivax*. The stippling was especially evident in smears of *P. bastianellii* and *P. osmaniae*.

The labelled avian sera gave strong staining of the parasite with very low background fluorescence when tested against the homologous species. No antigenic fluorescent stipples, as seen in the mammalian malarias, were observed (Fig. 3).

The labelled avian sera from chickens infected with *P. gallinaceum* or *P. juxtanucleare* (Table 4) were species- but not strain-specific. This would indicate that these species have no common antigens nor do they possess any of the antigens of the mammalian species of *Plasmodium*. Ingram et al.[c] found that antisera produced in rabbits by the injec-

[g] Garnham, P. C. C. (1959) *Riv. Parasssit.*, **20**, 273.

TABLE 6

STAINING RESULTS [a] (INDIRECT METHOD) AFTER EXPOSURE OF BLOOD SMEARS TO WHOLE ANTISERUM OR GAMMA-GLOBULIN FROM MONKEYS, EITHER INFECTED WITH *P. BASTIANELLII* OR NON-INFECTED CONTROLS, FOLLOWED BY FLUORESCEIN-LABELLED RABBIT ANTIHUMAN GAMMA-GLOBULIN

Smear		Sera			
Parasite	Host	*P. bastianellii* immune serum 227	Control serum	*P. bastianellii* immune γ-globulin 200	Control gamma-globulin
P. bastianellii	Monkey	+++	–	+++	–
P. vivax	Human	+++	–	+++	–

[a] For explanation of symbols see Table 2.

tion of *P. gallinaceum* sporozoites, when labelled, stained the erythrocytic stages of *P. gallinaceum* but not those of *P. bastianellii*.

Fluorescent stippling of the infected red blood cell was a feature common to the mammalian malarias (Fig. 1 and 2) but was not observed in the avian infections (Fig. 3). Tobie & Coatney [d] suggested that the stippling, which they observed in infections of *P. vivax* and *P. bastianellii*, represented Schuffner's dots. In the present work larger fluorescent granules were found in *P. berghei* infections, in which Schuffner's dots have not been shown by Giemsa staining. It is clear that the stippling marks the position of antigenic material in or on the infected host cells.

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Classification of Antimalarial Drugs in Relation to Different Stages in the Life-cycle of the Parasite: Commentary on a Diagram

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One of the ways of classifying antimalarial drugs is according to their action on particular stages of the life-cycle of the parasite. The teaching of the principles of chemotherapy of malaria can be greatly facilitated by the use of a diagram indicating clearly the rationale of the use of different antimalarials for different purposes, both in the prevention and cure of individual infections and in the two principal phases (attack and consolidation) of malaria eradication programmes.

Such a diagram is shown here, illustrating the classification of the most common antimalarials and accompanied by some comments on these. A number of less known or not generally used compounds have not been included.

All the data concerning the rationale of chemotherapy of malaria, the description of individual compounds in common use, guidance as to dosage and so on will be found in the monograph *Chemotherapy of malaria.* [a] A large amount of recent information on the use of chemotherapy in malaria eradication and on the trends of research in this field

[a] Covell, J., Coatney, G. R., Field, J. W. & Singh J. (1955) *Chemotherapy of malaria*, Geneva (*World Health Organization: Monograph Series*, No. 27).