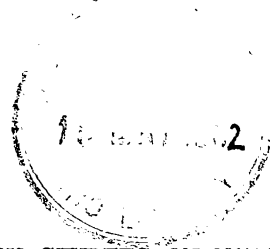


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WHO/Ma.1/344 ✓
27 April 1962

ORIGINAL: ENGLISH



FLUORESCENT ANTIBODY STUDIES ON MALARIA PARASITES¹

by

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Although immunofluorescent staining has been extensively applied to studies of bacteria since Coons (1942) developed this technique, its first use in malariology was when Brooke (1961) showed that Plasmodium berghei could be stained by fluorescent antibody. Using this method Ingram et al. (1961) demonstrated that P. cynomolgi bastianellii could be differentiated from P. gallinaceum. More recently Tobie & Coatney (1961) were able to stain P. vivax by this method.

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

Materials and methods

The standard method of conjugation used (Coons & Kaplan, 1950) is as follows:

Ten ml of serum were 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. Twenty ml of saturated ammonium sulfate, cooled to 4°C, was 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.

¹ Advance report on an investigation carried out with the financial assistance of the World Health Organization. The full report together with photomicrographs will be published at a later date.

This globulin solution was dialyzed 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 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 m μ 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/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 bijoux bottles at -20°C. Before use the conjugates were absorbed twice with acetone dried pigeon liver powder, 100 mg/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 serum of globulin was applied to the smears for 30 minutes. After washing the preparations were overlaid with conjugated antibody prepared against the globulin of the first serum. As controls unlabelled non-immune serum 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 according to Table 1. Smears were made from heavily infected blood, P. berghei from rats and mice, P. vinckei from rats and mice, P. gallinaceum strains 8A and 8B from chicken, P. juxtannucleare strains 14C and 14A from chicken, P. bastianellii, P. gonderi, P. osmaniae from monkeys, and P. vivax from humans.

Results

The results of immunofluorescent staining using control sera, homologous or heterologous antisera, are given in Tables 2 to 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 the cytoplasm of the parasite fluoresced whereas the nucleus did not (Brooke, 1961). In the present work fluorescent stippling of the infected host cells could be clearly seen. The stipples were larger, but fewer, than those observed in smears of the primate malarias stained with antisera against P. bastianellii 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.

TABLE 1. PRODUCTION OF ANTISERA TO SPECIES OF PLASMODIUM

Species of Plasmodium	Host	Method of Infection
<u>P. berghei</u>	rats	Blood passage. Repeatedly challenged.
<u>P. gallinaceum</u> 8B	chicken	Blood passage.
<u>P. juxtannucleare</u> 14C	chicken	Blood passage.
<u>P. bastianellii</u>	monkey	Infected by sporozoites. Challenged with infected blood.
"	monkey	Infected by sporozoites. Challenged with infected blood.
"	human 1	Infected by sporozoites.
<u>P. vivax</u>	human 1 (European)	Infected by mosquito bite. Challenged with sporozoites.
"	*human 4 (African)	Bitten by infected mosquitos. Challenged with infected blood.
<u>P. ovale</u>	human 6 (European)	Infected by mosquito bite.

* No parasites were recorded in blood smears from human. 4.

TABLE 2. STAINING RESULTS AFTER EXPOSURE OF BLOOD SMEARS TO FLUORESCHEIN LABELLED SERUM FROM RATS, EITHER INFECTED WITH P. BERGHEI OR NON-INFECTED CONTROLS. DIRECT METHOD.

Parasite	Host	Sera	
		<u>P. berghei</u> immune	Control
<u>P. berghei</u>	rat	+++	-
<u>P. berghei</u>	mouse	+++	-
<u>P. vinckei</u>	rat	+	-
<u>P. vinckei</u>	mouse	+	-
<u>P. gallinaceum 8B</u>	chicken	-	-
<u>P. juxtannucleare 14C</u>	chicken	-	-
<u>P. bastianellii</u>	monkey	-	-
<u>P. gonderi</u>	monkey	-	-
<u>P. osmani</u>	monkey	-	-
<u>P. vivax</u>	human	-	-

The intensity of fluorescence was estimated visually, +++ indicating the brightest, - being negative, while ++, +, +, represent successively decreasing intermediate intensities. N.T. indicates that no test was carried out.

TABLE 3. STAINING RESULTS AFTER EXPOSURE OF BLOOD SMEARS TO ANTISERUM FROM RATS, EITHER INFECTED WITH P. BERGHEI OR NON-INFECTED CONTROLS, FOLLOWED BY FLUORESCCEIN LABELLED RABBIT ANTI-RAT GLOBULIN. INDIRECT METHOD.

Smear		Sera	
Parasite	Host	<u>P. berghei</u> immune	Control
<u>P. berghei</u>	rat	+++	<u>+</u>
<u>P. vinckei</u>	rat	+	<u>+</u>
<u>P. gallinaceum 8B</u>	chicken	+	<u>+</u>
<u>P. juxtannucleare 14C</u>	chicken	-	-
<u>P. bastianellii</u>	monkey	+	<u>+</u>
<u>P. gonderi</u>	monkey	+	<u>+</u>
<u>P. osmaniae</u>	monkey	+	<u>+</u>

TABLE 4. STAINING RESULTS AFTER EXPOSURE OF BLOOD SMEARS TO FLUORESCEIN LABELLED ANTISERUM FROM CHICKEN, EITHER INFECTED WITH P. GALLINACEUM 8B, P. JUXTANUCLEARE 14C, OR NON-INFECTED CONTROLS. DIRECT METHOD.

Smear		Sera		
Parasite	Host	<u>P. gallinaceum</u> immune	<u>P. juxtannucleare</u> immune	Control
<u>P. gallinaceum 8B</u>	chicken	+++	-	-
<u>P. gallinaceum 8A</u>	chicken	+++	-	-
<u>P. berghei</u>	rat	-	-	-
<u>P. vinckei</u>	rat	-	-	-
<u>P. bastianellii</u>	monkey	-	-	-
<u>P. gonderi</u>	monkey	-	-	-
<u>P. juxtannucleare 14A</u>	chicken	-	+++	-
<u>P. juxtannucleare 14C</u>	chicken	-	+++	-

TABLE 5. STAINING RESULTS AFTER EXPOSURE OF BLOOD SMEARS TO ANTISERUM FROM HUMANS, EITHER INFECTED WITH P. VIVAX, P. BASTIANELLII, P. OVALE, OR CONTROL NON-INFECTED, FOLLOWED BY FLUORESCENIN LABELLED RABBIT ANTI-HUMAN γ GLOBULIN. INDIRECT METHOD.

Smear		Sera					
Parasite	Host	P. vivax immune (1)	P. vivax immune (4)	P. bastianellii immune (2)	P. ovale immune (6)	Control (3)	
<u>P. bastianellii</u>	monkey	+++	++	+++	±	-	
<u>P. vivax</u>	human	+++	++	+++	N.T.	-	
<u>P. osmaniae</u>	monkey	+++	+	+++	±	-	
<u>P. gonderi</u>	monkey	+++	+	+++	±	-	
<u>P. berghei</u>	rat	+	N.T.	+	N.T.	+	
<u>P. gallinaceum</u> 8B	chicken	-	N.T.	-	N.T.	-	

TABLE 6.. STAINING RESULTS AFTER EXPOSURE OF BLOOD SMEARS TO WHOLE ANTISERUM
OR THE γ GLOBULIN FROM MONKEYS, EITHER INFECTED WITH P. BASTIANELLII, OR
NON-INFECTED CONTROLS, FOLLOWED BY FLUORESCEN LABELLED RABBIT
ANTI-HUMAN γ GLOBULIN. INDIRECT METHOD.

Smear		Sera			
Parasite	Host	<u>P. bastianellii</u> immune (227) serum	Control serum	<u>P. bastianellii</u> immune (200) γ globulin	Control γ globulin
<u>P. bastianellii</u>	monkey	+++	-	+++	-
<u>P. vivax</u>	human	+++	-	+++	-

Discussion

The strong cross reactions between the primate malarial parasites 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 (1959) reported no cross immunity even between the sub-species P. cynomolgi cynomolgi 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 (1961) 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 genetic 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.

The labelled avian sera from chicken infected with P. gallinaceum or P. juxtannucleare, (Table 4), were species specific but not strain specific. This would indicate that these species have no common antigens nor possess any of the antigens of the mammalian species of Plasmodium. Ingram et al. (1961) found that antisera produced in rabbits by the injection 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 malarial parasites but was not observed in the avian infections. Tobie & Coatney (1961) 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.

Summary

Several species of malaria parasites were stained with convalescent sera by immunofluorescent techniques. Strong cross reactions were found between the primate malarias: P. vivax, P. bastianellii, P. gonderi and P. osmaniae. These species cross reacted less strongly with P. berghei. The avian malarias P. gallinaceum and P. juxtannucleare were stained with homologous sera, the reactions being species but not strain specific. Fluorescent stippling of an immune nature was observed in the infected host cells in the mammalian malaria.

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ACKNOWLEDGEMENTS

I wish to thank Professor P. C. C. Garnham for his encouragement and advice, Dr Cohen for separating the monkey γ globulin, and Mr P. G. Shute for supplying the human sera.

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