

## Interaction of Plasmodium falciparum and Convalescent Serum in vitro

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**OBJECTIVE:** The studies of Cohen et al<sup>1</sup>, and subsequently, other workers, demonstrate clearly an important role for antibody in immunity to Plasmodium falciparum infections. Exploitation of these findings requires extensive study of numerous aspects of the protective effect including its mechanism and specificity. Study of the problem in the intact experimental animal or patient poses many problems which could be circumvented by the availability of an assay of the protective antibody in vitro. Recent development in Cohen's laboratory in London indicate that such an assay might be feasible for P. knowlesi.<sup>2</sup> His data suggest that inhibition of plasmodial development occurs through reaction of antibody with the extracellular merozoites. If this is the case, the development of an in vitro assay could be approached by achieving repenetration in vitro and then studying the inhibition of the phenomenon by patient serum. Implicit in these requirements is the ability to detect the repenetration event with certainty. The present study was designed to (a) explore the possibility that in vitro erythrocyte penetration by P. falciparum can be detected by a marked recipient cell technique and (b) study, if feasible, the effect of serum from patients with a history of recurrent malaria on penetration in vitro.

**DESCRIPTION:** The system for detection of penetration in vitro involves the inclusion of fetal erythrocytes in the culture; these cells can later be distinguished from adult cells by differential elution of hemoglobin. The presence of a parasite within a fetal cell is taken as evidence of penetration in vitro.

Washed erythrocytes from heparinized cord blood and from patients with acute falciparum malaria were suspended in tissue culture medium 199 modified by the addition of NaHCO<sub>3</sub> (1.7 mg/ml), Penicillin G (400 units/ml), and streptomycin (400 µg/ml) and equilibrated with 5% CO<sub>2</sub> in air. Additional glucose was added in various amounts as described below. The pH of the medium immediately after gassing was 6.7–6.9. Mixtures of the cell suspensions were prepared so that the final total erythrocyte concentration was 1X10<sup>8</sup>/ml with 1% of the cells parasitized. In most experiments, equal numbers of adult and fetal cells were employed; if necessary, erythrocytes from a normal adult donor were used to reduce the final parasitized cell concentration. Compatible normal serum was included in each culture. The culture mixtures were incubated without agitation at 37°C in disposable plastic serological microtitration trays sealed with cellophane tape. The trays were sterilized prior to use by ultraviolet irradiation. After an appropriate interval, thin blood films were prepared and fixed with ethanol. The hemoglobin of the adult erythrocytes was eluted with citrate buffer (pH 3.4 ± 0.05); on treatment of the slides with eosin, only the fetal cells were stained<sup>4</sup>. The preparations were counterstained with Giemsa's stain for visualization of the parasites. Sera were collected from patients with a history of recurrent malaria. All sera and all parasites were obtained in Cholburi Province. Globulin fractions of sera were prepared by precipitation in 1/3 saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in the cold; the redissolved preparations were dialyzed exhaustively against saline.

**PROGRESS:** Experiments have been performed with 28 isolates of P. falciparum. In 24 cases, progressive development with infection of fetal cells was observed. Penetration was detected as early as 24 hours and was usually maximal at 48 hours. However, since there was considerable variation in the rate of development, preliminary observations on control preparations were used to decide on the optimal time for

harvest; on occasion this was as late as 60 hours after initiation of culture. All intermediate stages of asexual development were observed in adult (donor) cells. On one occasion parasites identified as immature gametocytes were seen after 24 hours of culture. Parasites in fetal cells were usually small rings, although on a few occasions somewhat larger trophozoites were observed.

The percentage of fetal erythrocytes parasitized was variable but values as high as eight per cent were recorded. Thin blood films prepared from adult erythrocytes alone and from fetal erythrocytes alone served as controls on the specificity of differential hemoglobin elution. Adult cells were essentially devoid of hemoglobin after elution, whereas fetal cells showed uniform uptake of eosin. Control slides of cell mixtures prepared prior to incubation at 37°C showed no parasitized hemoglobin containing cells.

Thirteen sera from patients with histories of repeated attacks of malaria were studied for an effect on penetration of fetal cell by P. falciparum in vitro. In these experiments, the medium consisted of equal parts of modified 199 and the test serum. Supplemental glucose (100 mg% final concentration) was added to that already present in the culture medium. Eight replicate cultures were employed for each serum sample. The mean percentage of fetal erythrocytes parasitized was determined for each serum and compared with the value for normal serum used in a control set of cultures. Experiments were performed on two P. falciparum isolates. It is apparent that all of the test sera allowed less fetal cell penetration than the controls.

Since it was recognized that inhibition of penetration could be caused by a variety of serum components (or deficiencies of serum components) efforts were made to adapt the system for testing of serum fractions. Experiments were performed to test the effect of varying proportions of serum, culture medium and normal globulin on fetal cell penetration. Again eight-fold replicate cultures were employed. The supplemental glucose in the culture medium prior to admixture with serum and globulin was 200 mg%. The results are summarized in Table II. The data indicates that the globulin preparations cannot substitute for serum; however, when serum is present at a concentration of 0.25 ml per ml of mixture (25% serum) penetration is comparable in extent to that obtained with 50% serum. On this basis further experiments were conducted with 25% serum, 50% globulin, and 25% 199.

The results of two experiments on the effect of varying amounts of added glucose are summarized in Table III. With the exception of one mean value, both experiments indicate that approximately 200 mg% of added glucose is optimal. Greater or lesser amounts seem to be inhibitory. The similarity in the extent of penetration obtained in the two experiments is probably fortuitous.

Studies of the effect of globulin prepared from the serum of patients with a history of recurrent malaria are now in progress.

**SUMMARY:** (1) An experimental system which allows detection of invasion of erythrocytes by P. falciparum in vitro has been devised. Human fetal erythrocytes are employed as the recipient cells. The lower solubility of fetal hemoglobin in acid is exploited as a cell marker by which the fetal cells can be distinguished in artificial admixture with adult erythrocytes. Parasites in the previously uninfected fetal cells serve as evidence of invasion in vitro.

(2) Fewer fetal cells were penetrated in the presence of thirteen sera from patients with a history of recurrent malaria than in a normal control serum. With one of these sera no fetal cell penetration was observed.

(3) It has been shown feasible to perform these culture experiments in the presence of a globulin fraction of serum; experiments testing the effect of globulin from patients with a history of recurrent malaria are in progress.

- REFERENCES:** (1) Cohen, S., McGregor, I.A., and Carrington, S.P., *Nature* 192: 733, 1961.  
(2) Cohen, S., Butcher, G.A., and Crandall, R.B., *Nature* 223: 368, 1969.  
(3) Trigg, P.I., *Parasitology*, 59: 925, 1969.  
(4) Betke, von K., and Kleihauer, E., *Blut*, 4:241, 1958.

Table I. Inhibition of penetration of fetal erythrocytes by P. falciparum in vitro by serum from patients with a history of recurrent malaria

Serum No.	Mean percentage of fetal cells parasitized	
	Exp. 1	Exp. 2
1	.20	3.5
2	.86	5.6
3	.53	4.0
4	.60	3.6
5	.40	—
6	.27	—
7	0	—
8	.43	—
9	.54	—
10	—	4.9
11	—	4.5
12	—	3.35
13	—	6.8
Control	1.6	8.8

Table II. Effect of varying proportions (by volume) of culture medium 199, normal serum and normal serum globulin on the percentage of fetal erythrocytes parasitized in vitro

% Globulin	% 199	% Serum							
		0		10		25		50	
0	25	NS	NS*	0.3	0.2	0.4	0.1	1.0	0.3
	50	0	0	0	0.02	0.5	0.03	1.8	0.5
25	25	0.1	0	0.1	0.2	0.7	0.3	1.6	1.2
	50	0	0	0	0.05	0.4	0.6		
50	25	0	0	0.1	0.2	1.6	1.1		
	50	0	0						

\* NS—slides not satisfactory.

Units are mean per cent fetal erythrocytes parasitized. The two values in each block are from two experiments with different P. falciparum isolates. Values greater than 0.1% are rounded to the nearest 0.1%.

Table III.

Added glucose final concentration mg%	Average % fetal erythrocytes parasitized	
	Experiment No. 1	Experiment No. 2
50	0.10	—
100	0.17	—
150	0.32	—
200	0.05	0.40
250	0.38	—
400	—	0.30
600	—	0.15
800	—	0.15
1000	—	0.20