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PLASMODIUM FALCIPARUM INFECTED ERYTHROCYTE CYTOADHERENCE TEST: ITS APPLICATION WITH BRAZILIAN ISOLATES AND INHIBITION BY HUMAN PLASMA

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In vivo *adherence of erythrocytes infected with trophozoites and schizonts of* P. falciparum *to capillary endothelium can be reproduced* in vitro *using C32 amelanotic melanoma cells. This adherence was shown to be inhibited by strain-specific antibody.*

In the present paper in vitro *adherehce of Brazilian strains and inhibition tests with acute malaria patients' plasma are reported.*

P. falciparum *infected erythrocytes obtained from patients were cultivated in RPMI 1640 medium. Tests were carried out whenever a 3% late trophozoite and/or early schizont infection was obtained. Monodispersed C32 amelanotic melanoma cells, seeded on coverslips, were overlayed with a 3% erythrocyte suspension, kept at 37ºC, 90 min., with gentle intermittent swirling. They were washed, stained with Giemsa and mounted. In inhibition studies washed infected erythrocytes were pre-incubated with plasma for 30 min., 37ºC, before adhesion tests, using normal serum preincubation as a control. Mean number of erythrocytes per cell, mean number per positive cell, level of significance of differences between test and control coverslips as calculated by Student t test, as well as percentage of inhibition are reported. Both partial binding and enhancement was obtained with several plasma samples, effect varying with the isolate tested.*

It has been known for many years that *P. falciparum* infected erythrocytes, after parasite maturation, adhere to intravenular and capillary endothelium (Miller, 1969), through knobs formed at the infected erythrocyte membrane (Luse & Miller, 1971). The phenomenon is believed to contribute to the pathogeny of cerebral malaria, through physical capillary obstruction, besides representing an escape mechanism of the parasite, by avoiding recirculation and phagocytosis and destruction of erythrocytes with membrane alterations.

Recently Udeinya et al. (1981) were able to reproduce the phenomenon *in vitro*, and the substitution of amelanotic melanoma C32 cell line for primary culture of endothelium as a target cell (Schmidt et al., 1982) was a simplification that allows the test to be made with more ease. Subsequently Udeinya et al. (1983a) showed that binding could be inhibited or reversed by strain-specific antibody. The reversal was also obtained *in vitro*, by injection of antibody into an infected *Aotus* monkey (David et al., 1983).

In view of the practical implications for chemotherapy, immunotherapy and immunoprophylaxis, several studies on the protein responsible for adhesion have been undertaken (Kilejian, 1979, 1980; David et al., 1983; Aley, Sherwood & Howard, 1984; Leech et al., 1984; Vernot-Hernandez & Heidrich, 1985). Recently, receptors on target cells implicated in binding have been identified (Barnwell, Ockenhouse & Knowles II, 1985; Roberts et al., 1985). It was also reported that among 12 sera from adults in the Gambia and Sudan, six inhibited binding of both a Brazilian and a Vietnam isolate. By absorption with one isolate, which inhibited binding of both, only the homologous binding inhibition was absorbed (Udeinya et al., 1983).

The growing incidence of malaria in Brazil, with a high percentage of *P. falciparum* in the Amazon region, as well as resistance to most chemotherapeutic agents, including mefloquine, have been emphasized by other authors at this Symposium, thus justifying the local introduction of new methodologies which may contribute to characterize strains and specific antibody response. Therefore, the exploration of the *in vitro* erythrocyte binding test and its inhibition by malaria patients' plasma seemed to be indicated. We have started by isolating several Brazilian strains of *P. falciparum*, setting up the cytoadherence test, and its inhibition with patients' plasma.

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MATERIALS AND METHODS

P. falciparum **isolates** – Parasite infected blood was obtained from patients through the courtesy of SUCEN – Superintendência de Controle de Endemias – laboratory of Dr. Marcos Boulos. Cultures were established by the Trager & Jensen method (1976), either in candle jars or with the addition of a 5% CO_2, 5% O_2, 90% N_2 gas mixture, at 37°C. Complete RPMI 1640 culture medium (CCM) was prepared with the addition of 25mM (5.94g/l) HEPES (N-2-Hydroxy-ethilpiperazine-N'-2-ethanesulfonic acid), 0.21% sodium bicarbonate, 25mg/l gentamycin and 10% heat-inactivated human type A serum. Patients' infected red blood cells (IHRBC) were washed, suspended in CCM at a 8% concentration. Culture medium was changed daily. Subcultures were made with the addition of fresh washed HRBC and IHRBC to fresh culture flasks. Serum and HRBC for cultures were obtained from donors at Hospital das Clínicas Blood Bank.

Amelanotic melanoma C32 – Cells were kept in tissue culture flasks, in CM with 10% fetal calf serum (CMFC). Confluent monolayers were trypsinised for subcultures. For adhesion tests, monolayers were trypsinised, cells were suspended in CMFC, suspensions adjusted to $3x10^4$ cells/ml. They were seeded on coverslips, incubated overnight at 37°C, either 18mm square coverslips, with 1.5ml suspension or 13mm diameter round coverslips with 0.5ml in Lux or Falcon multiwell culture trays. This results in monodispersed isolated cells, as described by Udeinya et al. (1983a).

Cytoadherence and inhibition test – Tests were carried out whenever parasite counts of cultures, as assessed in Giemsa stained smears, was at least 3%, consisting of mature trophozoites and/or early schizonts. Cultured IHRBC were washed three times in HBSS (pH 7.4), pelleted, and pellets suspended in an equal volume of plasma to be tested, incubated 30 min. 37°C. Subsequently CM (pH 7.4, adjusted with NaOH without bicarbonate), was added, to bring the final IHRBC concentration to 3%. Coverslips with melanoma cells were overlayed with either 0.25ml (round) or 1.0ml (square), and incubated at 37°C, 90 min., with gentle swirling every 15 min. They were gently washed in HBSS, fixed in methanol, stained with Giemsa, and counted. Most tests were carried out in duplicate, and for each experiment controls were made incubating IHRBC with normal serum. 100 to 200 cells were counted per coverslip, assessing number of cells with and without adherent IHRBC and individual cells with the corresponding number of IHRBR. For cytoadherence tests without inhibition, the preincubation with plasma was omitted. Only trophozoite and schizont IHRBC adhere in this test, while ring stages and non infected HRBC do not bind.

Patients' plasma – Plasma samples were obtained from patients during malaria attack, with either primary infection or after various infections, with a varying number of days since the current malaria attack started, as set out in Table IV. Material was either collected by ourselves at Belém (State of Pará), Paragominas (State of Pará), in São Paulo, (through the courtesy of SUCEN) or sent by Dr. C.E. Tosta (University of Brasília), collected at Ariquemes (State of Rondônia).

Statistical analysis – Counting of IHRBC per individual cell, allowed the calculation of mean number of adherent IHRBC, its standard deviation and standard error of mean for the total number of cells counted, and for positive cells only. By applying the Student t test, comparing controls and patients' plasma incubated samples, levels of significance (p) for the difference between control and experimental coverslips were obtained. The level of inhibition was also expressed as a percentage, where:

$$\%\ \text{inhibition} = \frac{\text{Nr adherent IHRBC control} - \text{Nr adherent IHRBC experimental}}{\text{Nr adherent IHRBC control}} \times 100$$

RESULTS

Table I lists a few isolates, and their cytoadherence. Binding intensity varied and was lost on sub-cultivation after a certain period of time.

TABLE I

Cytoadherence of erythrocytes infected with *P. falciparum* (IHRBC) to melanoma C32 targed cells (TC).

Strain	Origin	Days Kept in Culture	% TC with Adherent IHRBC	Mean NR IHRBC per TC	Mean NR IHRBC/ Positive TC
SP 14	Unknown	11	84%	4.30	5.00
		31	66%	1.40	2.20
		60	–	–	–
SP 15	Nova Olinda-Pará	8	78%	2.30	2.90
		37	–	–	–
		60	–	–	–
SP 18	200 Km off Porto Velho	28	40%	0.62	1.40
	–Rondônia (RO)	29	44%	0.75	1.60
SP 19	Ariquemes (RO)	18	–	–	–
SP 20	Mining At Morrinhos (RO)	23	30%	0.42	1.30
SP 22	Recrudescence of SP 18	7	–	–	–
SP 24	Mining At Prainha (RO)	12	73,5%	2.79	3.80
		16	76%	4.25	5.50
SP 28	Mining At Mutum (RO)	6	36%	0.60	1.68
SP 34	Mining At Mutum (RO)	6	27,27%	0.50	1.84
SP 35	Coliseu (MT)	4	20%	0.28	1.40
SP 37	Mining At Novo Mundo (MT)	5	35%	0.98	2.80

Table II gives a general idea of individual inhibition experiments. Both significant inhibition or enhancement of adhesion were observed, as well as practically no inhibition, when test coverslips were compared with controls. A better overview is obtained in Table III. Plasma SP 47 inhibited 4 out 6 strains against which it was tested; with other plasmas, both inhibition and enhancement were registered. On comparing individual strain interaction with several plasma samples, for instance, isolate SP 46 was inhibited by 2 in 4 plasmas and enhanced by 1; strain SP 48 was inhibited by 4 and enhanced by 1 in 6 plasmas; strain SP 50 was enhanced by 5 out of 6 plasmas; strain SP 51 was inhibited by 2 and enhanced by 2 out of 7 plasma samples.

For further information, Tables IV and V specify origin of patients' plasma and strains, respectively. Number of previous malaria attacks varied from none to 10, and duration of present attack from 2 to 13 days. However, no obvious differences due to these factors are evident, in the small number of samples tested.

TABLE II

Inhibition of cytoadherence by malaria patients' plasma

Experiment NR	Strain NR	Pre Incubation with Plasma NR	Mean NR IHRBC/TC	Inhibition P	Inhibition %	Mean NR IHRBC/ Positive TC	Inhibition P	Inhibition %
29	SP 46	N.S.	6.18±0.32			6.83±0.30		
		SP 47	3.91±0.28	P< 0.001 E	36.73	4.63±0.29	p< 0.001	32.21
31	SP 46	N.S.	2.58±0.17			3.10±0.18		
		SP42	2.24±0.19	–	13.18	3.33±0.59	–	–7.41
		B14	3.10±0.25	p< 0.001 E	–20.15	4.32±0.30	p< 0.02 E	–39.35
32	SP 46	N.S.	8.38±0.91			9.92±0.96		
		P5	5.21±0.65	p< 0.01	37.83	6.99±0.78	p< 0.02	29.36
		B14	8.18±0.67	–	2.39	9.50±0.72	–	4.23
33	SP 48	N.S.	11.67±0.51			12.11±0.50		
		SP 47	4.14±0.22	p< 0.001	64.52	4.73±0.22	p< 0.001	60.94
		B14	3.81±0.22	p<0.001	67.35	4.76±0.22	p< 0.001	60.69
		P4	7.60±0.33	p< 0.001	32.30	7.93±0.31	p< 0.001	34.51
		152	4.35±0.25	p< 0.001	62.72	4.35±0.25	p< 0.001	64.08
34	SP 45	N.S.	6.08±0.61			8.15±0.67		
		P4	1.90±0.28	p< 0.001	68.75	3.61±0.44	p<0.001	55.70
		SP47	3.76±0.58	p< 0.01	38.15	5.55±0.77	p<0.05	31.90
35	SP 48	N.S.	3.90±0.30			4.50±0.32		
		SP42	5.58±0.32	p< 0.001 E	–43.08	5.67±0.32	p< 0.01 E	–26.00
		123	4.62±0.27	–	–18.46	5.08±0.32	– E	–12.89
37	SP 50	N.S.	3.15±0.23			4.26±0.26		
		SP47	3.17±0.22	–	–0.63	4.09±0.23		4.16
		152	4.64±0.26	p< 0.001 E	–47.30	5.55±0.26	p< 0.001 E	–30.28
		SP50	6.21±0.37	p< 0.001 E	–97.14	7.07±0.37	p< 0,001 E	–65.96
		B14	6.05±0.23	p< 0.001 E	–92.06	6.62±0.23	p<0.001 E	–55.39
		P4	4.57±0.23	p< 0.001 E	–45.07	5.85±0.26	p<0.001 E	–37.32
		SP42	4.67±0.40	p< 0.001 E	–48.25	5.33±0.40	p< 0.04 E	–25.11
38	SP 51	N.S.	4.66±0.39			5.93±0.45		
		SP47	2.84±0.27	p< 0.001	39.05	3.89±0.33	p< 0.001	34.40
		SP50	3.42±0.24	p< 0.001	26.61	4.61±0.30	p< 0.02	22.65
		B14	4.07±0.34	–	12.66	5.55±.042	–	6.41
		P4	4.20±0.24	–	9.87	5.21±0.26	–	12.14
		152	9.27±0.65	p< 0.001 E	–98.92	10.30±0.68	p< 0.001 E	–73.69
		SP42	6.40±0.47	p< 0.001 E	–37.34	7.23±0.50	p = 0.05 E	–21.92
		150	4.38±0.30	–	0.06	5.96±0.36	–	0.50
39	SP 52	N.S.	1.06±0.32			1.86±0.21		
		SP47	0.75±0.04	p< 0.001	29.24	1.71±0.06	–	8.06
		152	0.88±0.06	p< 0.02	16.98	1.76±0.09	–	5.37
		B14	1.04±0.05	–	1.89	1.88±0.06	–	1.08
		B5	1.53±0.08	p<0.01 E	–44.33	2.31±0.09	p< 0.001 E	–24.20
40	SP 39	N.S.	4.48±0.27			5.19±0.29		
		123	4.56±0.18	–	–1.79	4.95±0.17	–	4.62
		SP50	4.64±0.26	–	–3.50	5.18±0.27	–	0.19
		155	3.74±0.20	p<0.04	16.51	4.23±0.20	p< 0.010	18.50
		SP42	3.67±0.22	p<0.02	18.08	4.38±0.24	p< 0.001	15.61

P. Student T test. Obtained by comparing adherence of controls (incubated with normal serum N.S.), with HRBC preincubated with patients plasma; ± = SEM

IHRBC Infected human red blood cells NS – Normal Serum E – Adherence Enhancement NR – Number

$$\% = \frac{\text{Mean NR. HRBC/TC Control} - \text{Mean NR. HRBC/TC with patients plasma}}{\text{Mean NR. HRBC/TC control}} \times 100$$

TABLE III

Cytoadherence inhibition. Comparison of inhibition for each strain and plasma

Plasma \ Strain	SP 47	P4	B14	152	SP 42	SP 50	P 5	123	B 5	150	155
SP 39					15,61(40) $p < 0,001$	0.19(40)		4.62(40)			18.50(40) $p < 0.01$
SP 45	31,90(34) $p < 0.05$	55,70(34) $p < 0.001$									
SP 46	32,21(29) $p < 0.001$		-39,35(31) $p < 0.02$ 4.23(32)		-7,41 (31)		29.36 (32) $p < 0.02$				
SP 48	60,94(33) $p < 0.001$	34,51(33) $p < 0.001$	60,69(33) $p < 0.001$	64,08(37) $p < 0.001$	-26,00(35) $p < 0.01$			-12,89 (35)			
SP 50	4,16(37)	-37,32(37) $p < 0.001$	-55,39(37) $p < 0.001$	-30,28(37) $p < 0.001$	-25,11(37) $p < 0.04$	-65,96(37) $p < 0.001$					
SP 51	34,40(38) $p < 0.001$	12,14(38)	6,40(38)	-73,69(38) $p < 0.001$	-21,92(38) $p = 0.05$	22,65(38) $p < 0,02$				0,5%(38)	
SP 52	8,06(39)		1,08 (39)	5,37 (39)					-24,20(39) $p < 0.001$.		

p Refers to significancy by Student t test, comparing mean numbers of erythrocytes per target cell with adherence of control with experimental coverslips
- Indicates enhancement
Experiment numbers in brackets (see Table II)

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Experiment numbers in brakets (see Table II)

TABLE IV

Origin of patients plasma used in cytoadherence inhibition tests.

Plasma Number	No. of Previous Malaria Attacks	Place of Probable Infection	Duration of Present Attack
B 5	"Several"	State of Rondônia	3 days
B 14	Two by *P. falciparum* Two by *P. vivax*	State of Rondônia	8 days
P 4	"Several"	State of Pará	2 days
P 5	One	State of Pará	2 days
SP 42	"Several"	State of Rondônia	3 days
SP 47	None	State of Rondônia	10 days
SP 50	None	State of Mato Grosso	7 days
123	Four	State of Rondônia	4 days
150	Five	State of Rondônia	3 days
152	Six	State of Rondônia	13 days
155	Ten	State of Rondônia	3 days

TABLE V

Strains tested in cytoadherence inhibition

Strain No.	Origin	Days in culture	Parasitemia on day of experiment	Experiment No.
Sp 39	Mato Grosso	4	4%	40
Sp 45	Rondônia	11	3%	34
Sp 46	Rondônia	4	7,3%	31
		1	3%	32
Sp 48	Rondônia	1	7%	33
		4	5%	35
Sp 50	Mato Grosso	2	8%	37
Sp 51	Mato Grosso	2	8,6%	38
Sp 52	Rondônia	8	5%	39

DISCUSSION

The loss of binding capacity of *P. falciparum* infected erythrocytes after some time in culture was reported by Udeinya et al. (1983), although one of their isolates maintained reasonable binding capacity up to 54 days. This is a big handicap in comparative studies between sera and strains. By the time cultures are sufficiently expanded to allow screening with numerous samples, binding capacity has generally been lost. In our first attempts at cytoadherence, we confirmed this loss of binding, as seen in Table I. In addition, isolates are not easily adapted to continuous culture. Therefore our inhibition experiments were carried out with recent isolates 1-11 days in culture (Table V).

In inhibition experiments, plasma from patients during active malaria disease, with a recent infection (symptoms between 2 and 13 days) were used. Most of them were workers in mining or agricultural projects, living constantly or intermittently in the endemic zone. Only two individuals with no previous malaria history, had a story of short stay in the Amazon region.

No correlation between duration of present disease, number of previous malarias, and effect on adhesion could be found. This is consistent with the small number of samples tested so far.

All our isolates originated from the State of Rondônia and Mato Grosso, which are, however, not a long distance apart. In the case of plasma samples, only the locality where the last infection was contracted is registered in Table IV. Some of the patients migrated to the area a few years or months ago, others relate internal migrations, within short distances, or from State to State. On others, no information is available. In a general way, internal migrations are widespread and this may result in interchange of parasite populations, the same applying to antibody response in infected individuals. This fact has to be kept in mind when comparing findings from different Brazilian geographic areas.

Udeinya et al. (1983a) reported binding inhibition by sera of individuals from Gambia and Sudan with isolates from Brazil and Vietnam. Six sera inhibited binding of both isolates, two had little effect on either isolate, and two inhibited binding of the Brazilian isolate, with little effect on the Vietnam isolate. Three sera inhibited binding of both isolates, and when absorbed with one, they had the inhibiting capacity for that isolate abolished or reduced, with little effect on the binding of the other isolate. Apparently Udeinya et al. worked with sera of healthly volunteers, living in the endemic area, while our samples came from acute malaria patients. This may explain the novel finding, that some samples, rather than inhibiting, actually enhance adherence.

On examining Table II, attention is called to the fact that in one experiment only enhancement was observed (exp. 37), and in another, only inhibition (exp. 33), and this could be due to some accidental experimental artifact. However, experiments including inhibition, enhancement, and lack of significant effect are also present (exps. 38, 39).

Table III gives a clearer idea, allowing to compare strains and plasma. Plasma SP 47 inhibited adhesion of 4 in 6 isolates; P 4 inhibited 2 and enhanced 1 in 4 isolates; B 14 enhanced 2 and inhibited 1 out of 6 strains; 152 enhanced 2 and inhibited 1 in 4 strains; SP 42 inhibited 1 and enhanced 3 in 5 isolates; SP 50 inhibited 1 and enhanced 1 in 3 strains.

If our findings of enhancement are confirmed, it may become possible to identify a factor in acute malaria patients' plasma, either of host or parasite origin, which contributes to the binding of infected erythrocytes to endothelium.

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