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# OPTIMIZATION AND INHIBITION OF THE ADHERENT ABILITY OF *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES

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*The vast majority of the 1-2 million malaria associated deaths that occur each year are due to anemia and cerebral malaria (the attachment of erythrocytes containing mature forms of* Plasmodium falciparum *to the endothelial cells that line the vascular beds of the brain). A "model system" for the study of cerebral malaria employs amelanotic melanoma cells as the "target" cells in an* in vitro *cytoadherence assay. Using this model system we determined that the optimum pH for adherence is 6.6 to 6.8, that high concentrations of $Ca^{2+}$ (50mM) result in increased levels of binding, and that the type of buffer used influences adherence (Bis Tris> MOPS> HEPES> PIPES). We also observed that the ability of infected erythrocytes to cytoadhere varied from (erythrocyte) donor to donor. We have produced murine monoclonal antibodies against* P. falciparum*-infected red cells which recognize modified forms of human band 3; these inhibit the adherence of infected erythrocytes to melanoma cells in a dose-responsive fashion. Antimalarials (chloroquine, quinacrine, mefloquine, artemisinin), on the other hand, affected adherence in an indirect fashion i.e. since cytoadherence is due, in part, to the presence of knobs on the surface of the infected erythrocyte, and knob formation is dependent on intracellular parasite growth, when plasmodial development is inhibited so is knob production, and consequently adherence is ablated.*

Key words: cytoadherence – malaria – *Plasmodium falciparum*

More than 100 years ago the hallmark of *Plasmodium falciparum* infections was identified: only erythrocytes containing ring-stage parasites and gametocytes are found in the peripheral circulation (Bignami & Bastianelli, 1890). Sequestration, the attachment of erythrocytes infected with mature stages parasites to endothelial cells lining the post-capillary venules, is responsible for the absence of these stages from the peripheral blood. The principal organs in which sequestration takes place are the heart, lung, kidney, and liver (Aikawa et al., 1990; Pongponratn et al., 1991). Sequestration in the brain microvessels – a special pathology of *P. falciparum* infections called cerebral malaria – may totally occlude blood flow, and result in confusion, lethargy, and deep coma (MacPherson et al., 1985; Warrell, 1987; Howard & Gilladoga, 1989).

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In 1981 Udeinya et al. reported the development of an *in vitro* model for sequestration. Using cultured human umbilical vein endothelial cells (HUVECs) overlain by a suspension of *P. falciparum*-infected red cells, followed by microscopic examination of the HUVECs, they were able to show a specific adherence of infected red cells (iRBCs) to the monolayer of target cells. They also noted that not all HUVECs bound iRBCs. (The basis for this still remains to be described). The lack of availability of a continuous HUVEC line, and the high degree of variability in HUVEC adhesiveness – presumably due to differences in individual donors as well as changes associated with the number of passages in culture – prompted a search for other cytoadherent target cells. Schmidt et al. (1982) screened 18 cell types and found the C32 amelanotic melanoma cell (ATCC CRL1585), a continuous cell line, to have a greater cytoadherent capacity than HUVECs. Stage specificity for *in vitro* adherence to melanoma cells and HUVECs matched that observed *in vivo:* red cells bearing mature trophozoites (24+hr) and early schizonts (36hr) were the most adherent (Schmidt et al., 1982;

Marsh et al., 1988). Uninfected red cells and erythrocytes containing ring-stage parasites were non-adhesive. The cytoadherence assay described by Udeinya et al. (1981) has since been modified by several workers (Sherman & Valdez, 1989; Udeinya et al., 1985; Wright et al., 1990; David et al., 1983; Marsh et al., 1988) and used in the hope of better defining the causes of cerebral malaria. However, with this *in vitro* assay considerable variation in the degree of binding was observed both in laboratory cultures and with blood obtained from malaria patients; this variability was neither related to the initial parasitemia nor adaptation to culture conditions. In a study carried out in the Gambia using amelanotic melanoma cells as the targets no correlation was found between the binding capacity of infected cells from subjects with cerebral malaria and those with uncomplicated malaria (Marsh et al., 1989). A second study with Thai patients (Ho et al., 1991) also found no correlation between the degree of adherence and cerebral malaria, although cytoadherence was greater for isolates from individuals with severe malaria. The present work reports on attempts to optimize the cytoadherence assay and to elucidate some of the environmental and cellular factors that may contribute to sequestration and to the development of cerebral malaria.

## MATERIALS METHODS

*Chemicals and cell culture* – The antimalarials chloroquine diphosphate and quinacrine hydrochloride and the buffers MOPS, MES, PIPES, and Bis Tris were obtained from Sigma (St. Louis, Mo.), HEPES was purchased from JR Scientific (Woodland, Ca.). Calcium chloride and calcium lactate were from Matheson, Colemen and Bell (Cincinnati, Ohio). Mefloquine hydrochloride was obtained through the courtesy of Dr Wilbur Milhous, Walter Reed Army Institute for Research (Washington, D.C.). Artemisinin was obtained from Polysciences (Warington, Pa.) and was prepared as a $10^{-2}$M stock in dimethylsulfoxide (DMSO).

Human erythrocytes were obtained by venipuncture of human donors. Blood was collected into Vacutainer tubes and stored at 4 °C until needed. Before being used in cultures the red cells were washed 3X in RPMI 1640 medium and then were stored at 4 °C. All red cells were used within 3 days of washing, and samples of different donors to be used in a single experiment were washed at the same time. All *P. falciparum* lines (ItG2, Ituxi, from Brazil; FCR-3 from the Gambia; and B6B6+ (Biggs et al., 1990), derived from ItG2) were cultured in $O^+$ human red cells with 10% (v/v) human serum, and HEPES buffered RPMI 1640 (Trager & Jensen, 1976) supplemented with 50μM hypoxanthine. The knobby status of the various lines was maintained by subjecting iRBCs to gelatin flotation (Pasvol et al., 1978); the "floating" fraction was subcultured in the case of knobby ($K^+$) lines FCR-3 and ItG2, whereas the pellet was subcultured in the case of the knobless ($K^o$) line B8B6+. The C32 amelanotic melanoma cell line was grown according to established procedures (Sherman & Valdez, 1989).

*Cytoadherence assay* – Melanoma cells were seeded at 1 X $10^4$ cells/ml onto each well of a sterile 12 well microscope slide (Celline, Newfield, New Jersey) until the fluid formed a bead (approximately 20μl). After seeding, the slide was incubated in a humidified chamber at 37 °C for 24 h. Fixation of melanoma cells was accomplished by decanting the fluid from the slide before being placed in 3-5 day old formaldehyde fixative (1% paraformadehyde (w/v) (Sigma), 4% (w/v) sucrose in 0.1M sodium cacodylate (Polyscience), pH 7.0). The cytoadherence assay consisted of a HEPES/RPMI rinsed slide bearing the fixed melanoma cells being placed in a plastic box with 6 (3.5cm X 10.5cm) fluidtight compartments (Vlchek Plastics, Ohio). Remaining rinse liquid was aspirated off before the addition of 5ml of liquid containing 400 μl of human red blood cells infected with *P. falciparum*.The parasitemia was 5-10%, and within each experiment a common pool of infected blood was used. Slides were incubated in moist air at ambient temperature (usually 27 °C) for 90 min, and the blood cells were kept in suspension by agitation on a rocking table (Bellco). After incubation, the slides were retrieved, and nonadherent cells removed by dipping the slides in a series of three beakers containing 100-200 ml of HEPES/RPMI. The slides were fixed in 0.5% (v/v) glutaraldehyde, 4% (w/v) sucrose in 0.1M sodium cacodylate (pH 7.0) for 15-30 minutes at room temperature. After fixation the slides were stained overnight with Giemsa stain (10%). The number of infected ertythrocytes and melanoma cells was determined by counting the number of iRBCs and melanoma cells in several fields distributed across the slide. Counting of cells

was continued until either 300 melanoma cells or 900 iRBCs had been observed. If uninfected erythrocytes made up more than 5% of the bound erythrocyte population the entire experiment was rejected. Buffers of indicated pH (see figure legends) were prepared by adjusting each buffer solution to the lowest pH shown and then removing aliquots as the pH was adjusted upward with 0.1N NaOH.

*Immunizations and monoclonal antibody production* – Monoclonal hybridoma antibodies were produced according to Zola & Brooks (1982). Briefly, 1-2 x $10^7$ iRBCs (60% parasitemia) with mature forms (trophozoites and schizonts) of the FCR3-3 strain were injected intraperitoneally into BALB/c mice every week for a total or three injections. A final boost, with the same number of parasites, was given intravenously 72 h before the mice were sacrificed. Spleen cells were fused with the mouse P3-X63-Ag8.653 cell line and selection of hybridomas with hypoxanthine-aminopterin-thymidine medium was as previously described (Kohler & Milstein, 1975). Growing colonies were screened for the production of antibodies to iRBC (FCR-3 and ItG2) membranes by an indirect immunofluorescent assay (Winograd & Sherman, 1989; Crandall & Sherman, 1991). All colonies were subcloned twice by limiting dilution, and the immunoglobulin class and subclass were determined by using a kit from Sangstat Medical (Menlo Park, Ca.). Ascites fluid was produced by injecting 4 x $10^6$ hybridoma cells into the peritoneal cavity of Pristane (2,6,10,14-tetramethylpentadecane) primed BALB/c mice.

*Inhibition of cytoadherence* — Infected red cells (FCR-3) were exposed to antimalarials: antimalarials were added to cultures containing 8 h old ring-stage parasites, and incubated for 8-12 h; antimalarials were also incubated for up to 6 h with red cells containing trophozoites. In addition, antimalarials were added during the time period when schizont or trophozoite-infected cells were in contact with melanoma cells, that is, during the time of the binding assay.

The monoclonal antibodies 4A3 and 1C4 (Winograd & Sherman, 1989; Crandall & Sherman, 1991) were added directly to the adhesion buffer, and allowed to interact for the 90 min assay period.

## RESULTS

*Influence of buffer, pH, and calcium levels on cytoadherence* – Maximum binding for the knobby FCR-3 line was observed at pH 6.9, and the number of cytoadherent cells dropped off abruptly when the pH of the binding buffer was increased to 7.0 and above (Fig. 1). This is in agreement with the results of Marsh et al. (1988) using melanoma cells, but is different from the findings of Udeinya et al. (1989) with HUVECs. In the present work when the pH was decreased below 6.8, the degree of binding diminished, but this decline was less dramatic

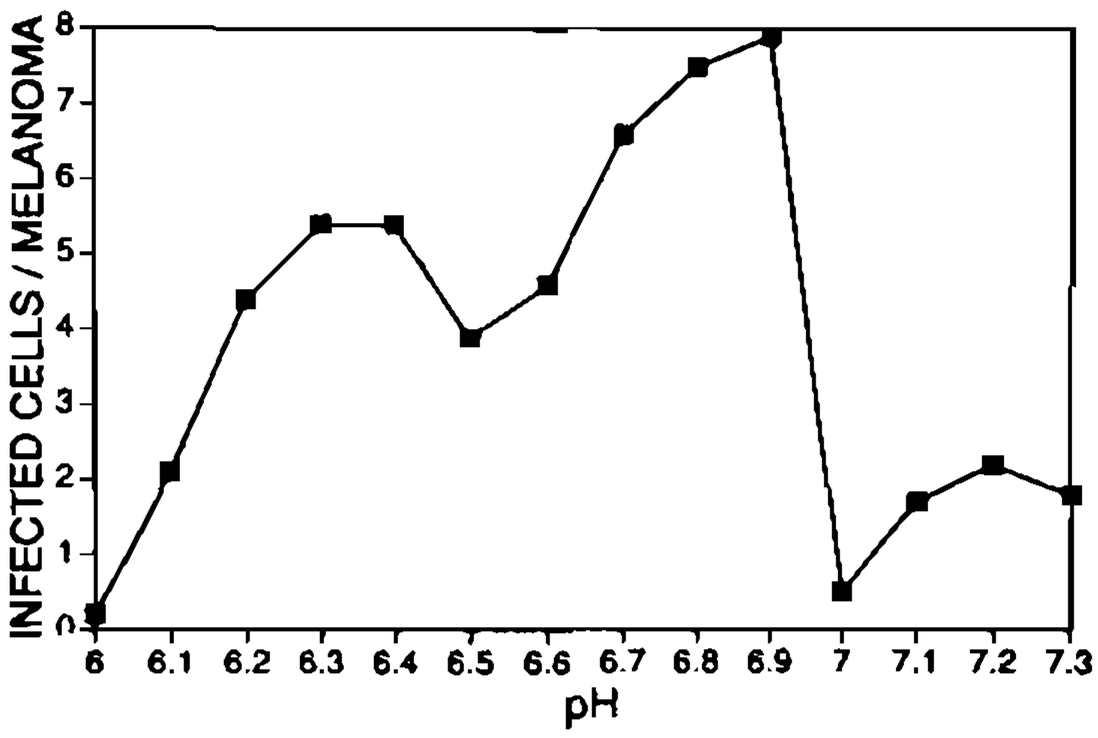


Fig. 1: adherence vs. pH: human blood containing mature forms of the *Plasmodium falciparum* strain FCR-3 was suspended in 5ml of 25mM Bis Tris /155mM NaCl and was incubated with formalin-fixed melanoma cells. The ratio of infected erythrocytes to melanoma cells is indicated on the y axis and the pH of the incubation solution is indicated on the x axis.

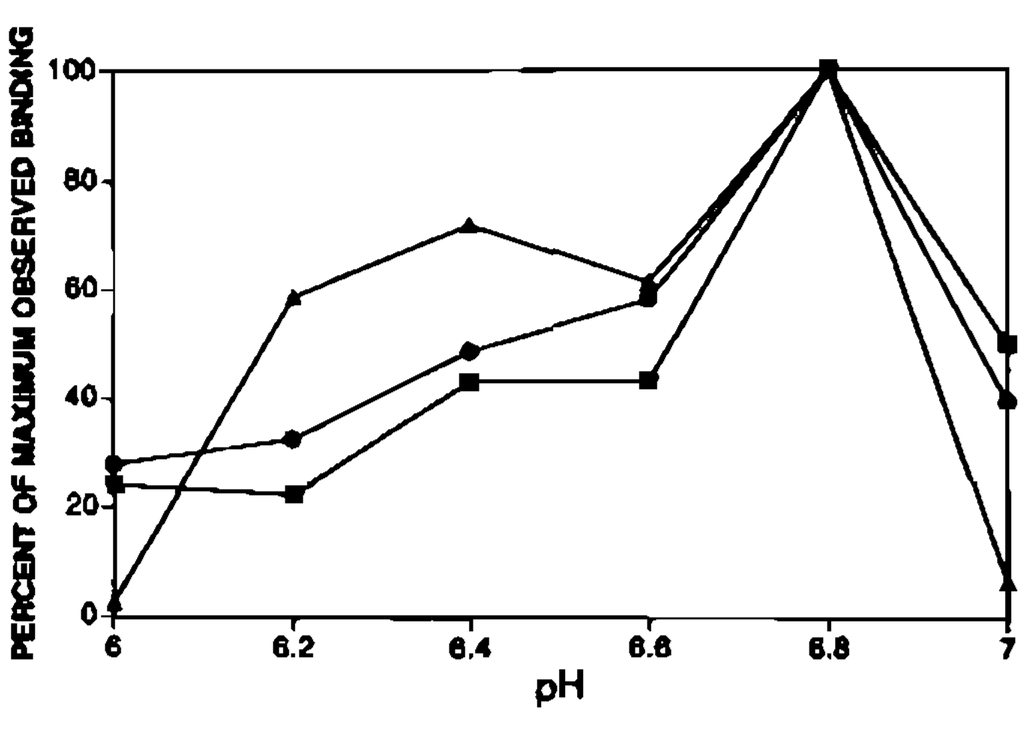


Fig. 2: the effect of varying pH of the medium on the ability of three strains to adhere: human erythrocytes infected with the strains FCR-3 (▲), B8B6+ (■), and ItG2 (●), were allowed to adhere to formalin-fixed melanoma cells. The erythrocytes were suspended in 25mM Bis Tris /155mM NaCl that was adjusted to the pH indicated on the x axis.

than when the pH was greater than 7.0. Below pH 6.0 and above pH 7.2, we observed a small number of bound iRBCs, consistent with random (nonspecific) background binding. The pH optimum for binding was similar for the FCR-3 line, the ItG2 (a knobby, cytoadherent) line and the B8B6+ (a knobless, cytoadherent) line (Fig. 2). The optimum pH range for cytoadherence (6.2-6.8) was lower than the optimum pH range (7.0-7.4)) for *in vitro* parasite growth. The parasite growth medium (HEPES/RPMI 1640) contains 25mM HEPES buffer to prevent changes in pH due to the production of lactic acid by the parasites. Although the pKa of HEPES, 7.5, is suitable for tissue culture work, in the pH range at which maximal cytoadherence was observed to take place HEPES is a poor buffer (Good & Izawa, 1968). Therefore the ability of iRBCs to cytoadhere in various biological buffers was examined to determine whether the type of buffer affected cytoadherence. Binding was best in Bis Tris, whereas other buffers (including HEPES) were distinctly inferior (Fig. 3). Addition of serum (up to 15%) did not affect binding in Bis Tris (data not shown). The effect of varying the $Ca^{2+}$ concentration in the binding buffer was also determined. Maximal cytoadherence was observed when the concentration of $Ca^{2+}$ was ~ 50mM; higher concentrations of $Ca^{2+}$ (in the form of $CaCl_2$) resulted in decreased cytoadherence (Fig. 4, top). The lines B8B6+ and ItG2 responded to $Ca^{2+}$ levels in the same way as FCR-3 (Fig. 4, bottom) ($Ca^{2+}$ in the form of calcium lactate also increased adhesion, but unlike $CaCl_2$ did not inhibit binding at concentrations > 50mM). Varying the amount of the divalent ion $Mg^{2+}$ (1mM to 70mM), or the monovalent ion $K^+$ (10mM to 160mM) in the medium had no effect on binding. In order to construct a buffer that would be optimal for cytoadherence, we repeated the pH studies (see Fig. 1). We observed that the addition of 50mM $Ca^{2+}$ to the binding buffer narrowed the pH range in which cytoadherence occurred, and in the presence of $Ca^{2+}$ the pH optimum for cytoadherence was decreased by 0.3 pH units (Fig. 5).

*Influence of red cell donors on cytoadherence* – We observed that the degree of adhesion under standard laboratory conditions varied in a cyclical fashion (Fig. 6). The period of the cycle (approximately 14 days) appeared to be related to the frequency of individual donors donating blood as well as our practice of using a pool of red cells from individual donors for the cultures. Therefore we attempted to determine whether individual donors promoted or inhibited adherence. The effect of erythrocyte source on adhesion of iRBCs was determined in two separate experiments. Two donors (PA and AH) produced significantly higher adhesion ratios despite the fact that the stages of parasite development and parasitemia

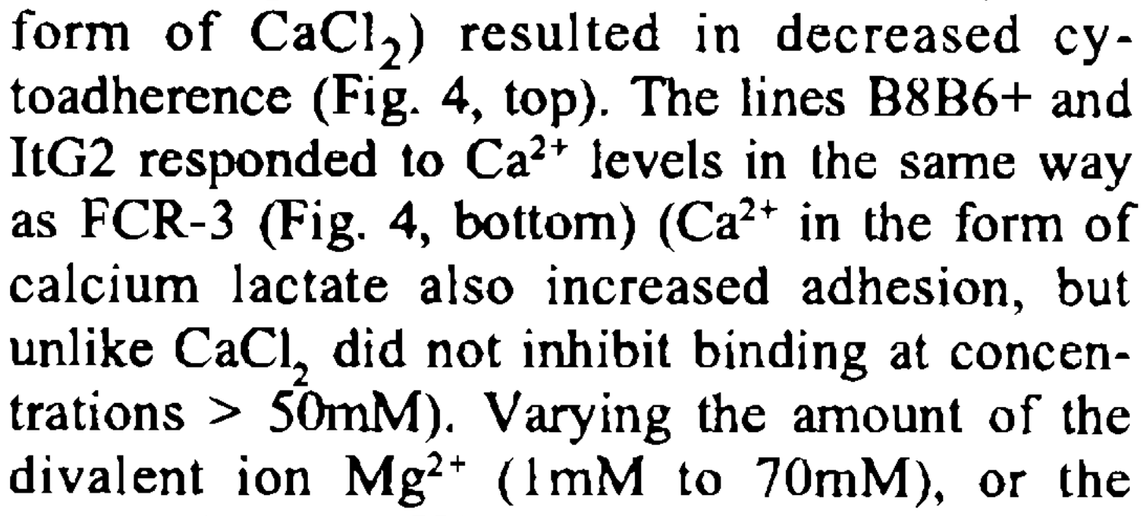


Fig. 3: the effect of various buffers on adherence: human erythrocytes infected with the strain FCR-3 were suspended in a solution of 25mM buffer (as marked on the x axis)/155mM Na Cl (pH 6.8) and allowed to adhere to formalin-fixed melanoma cells.

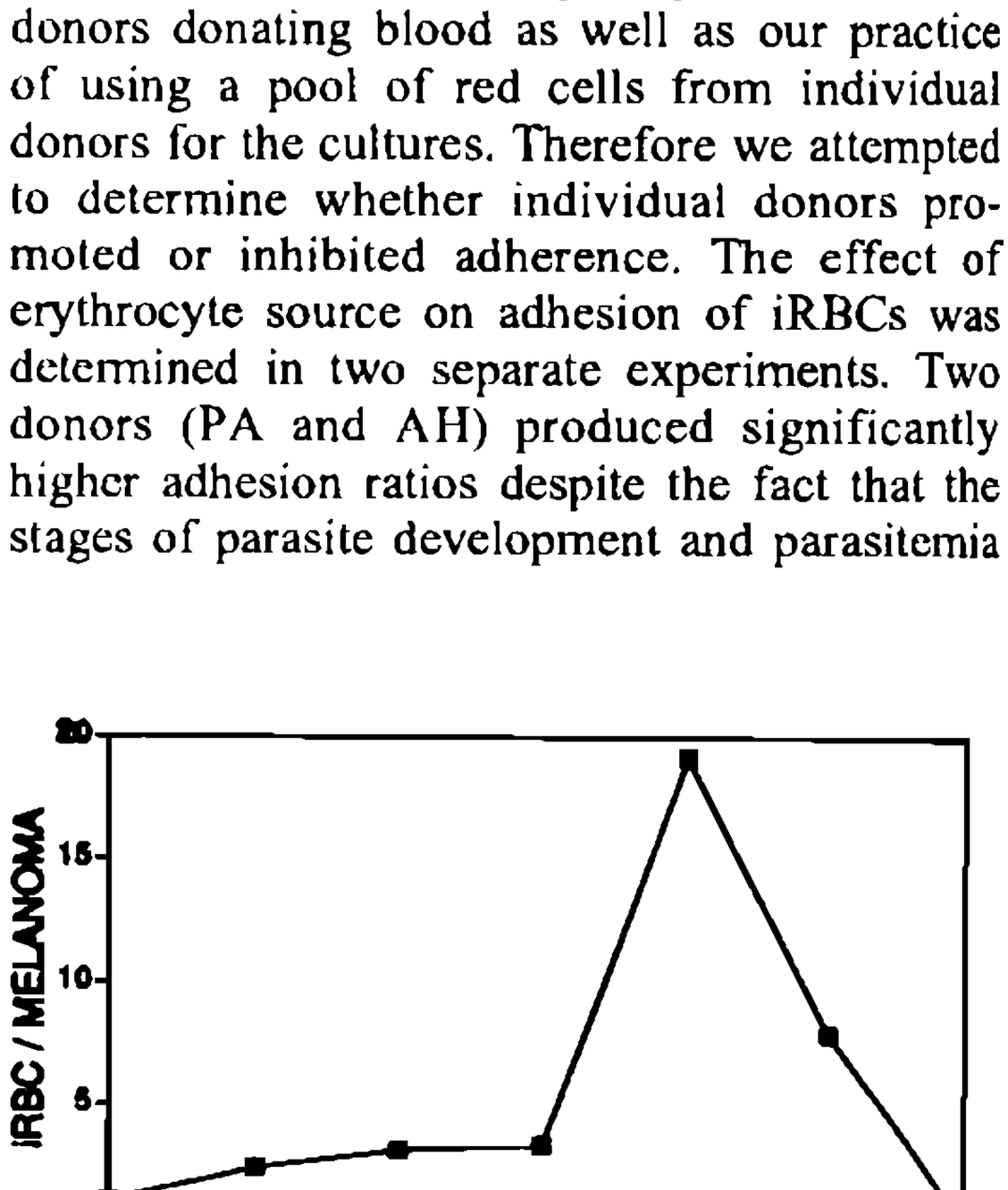


Fig. 4: the effect of $Ca^{2+}$ levels in the incubation buffer: solutions with a known $Ca^{2+}$ concentration were made by combining 25mM is Tris /155mM Na Cl (pH 6.8) and 25mM Bis Tris /55mM Na Cl/100mM $CaCl_2$ (pH 6.8). Human erythrocytes infected with the strain FCR-3 (top), and B8B6+ (bottom, ●) and ItG2 (bottom, ■) were then allowed to adhere to formalin-fixed melanoma cells.

TABLE I

Relationship of donor, parasitemia and developmental stage of parasites to cytoadherence

Experiment 1:

| Donor | Parasitemia | Stage of development (%) | | | | iRBC/Melanoma |
|---|---|---|---|---|---|---|
| | | R | T | 2N | S | |
| PA | 4.6 | 0 | 34 | 42 | 23 | 19.7 |
| AH | 5.2 | 0 | 46 | 50 | 4 | 19.1 |
| EP | 5.5 | 0 | 67 | 16 | 16 | 11.5 |
| JJ | 4.4 | 0 | 69 | 21 | 9 | 11.5 |
| JR | 5.7 | 0 | 60 | 26 | 12 | 10.0 |
| DP | 4.1 | 0 | 72 | 14 | 14 | 10.6 |

Experiment 2:

| Donor | Parasitemia | Stage of development (%) | | | | iRBC/Melanoma |
|---|---|---|---|---|---|---|
| | | R | T | 2N | S | |
| PA | 4.9 | 0 | 89 | 12 | 0 | 29.8 |
| AH | 4.4 | 0 | 89 | 11 | 0 | 59.8 |
| EP | 6.5 | 0 | 94 | 6 | 0 | 32.4 |
| BS | 6.4 | 0 | 95 | 5 | 0 | 17.9 |
| GE | 5.1 | 0 | 88 | 12 | 0 | 29.1 |
| HM | 5.8 | 0 | 80 | 18 | 2 | 27.3 |

R: ring; T: trophozoite; 2N: schizont, 2 nuclei; S: schizont, multinucleate.

TABLE II

The effect of antimalarials on the cytoadherence of *Plasmodium falciparum* (FCR-3 strain)-infected cells (IRBC) to amelanotic melanoma cells

| | Adhesion index | IRBC/melanoma cell |
|---|---|---|
| Chloroquine diphosphate | | |
| Control | 1.0 | 9.3 |
| $10^{-5}$ M | 0.87 | 8.1 |
| $10^{-4}$ M | 0.76 | 7.1 |
| $10^{-3}$ M | 0.69 | 6.4 |
| Artemisinin | | |
| Control | 1.0 | 5.0 |
| $10^{-8}$ M | 1.0 | 5.0 |
| $10^{-7}$ M | 0.96 | 4.8 |
| $10^{-6}$ M | 0.88 | 4.4 |
| Mefloquine hydrochloride | | |
| Control | 1.0 | 11.9 |
| $10^{-7}$ M | 1.1 | 12.8 |
| $10^{-6}$ M | 1.3 | 15.0 |
| $10^{-5}$ M | 1.3 | 15.0 |
| $10^{-4}$ M | 0.8 | 10.0 |
| Quinacrine hydrochloride | | |
| Control | 1.0 | 3.0 |
| $10^{-5}$ M | 0.9 | 3.5 |
| $10^{-4}$ M | 1.2 | 3.6 |
| $10^{-3}$ M | 1.0 | 3.1 |

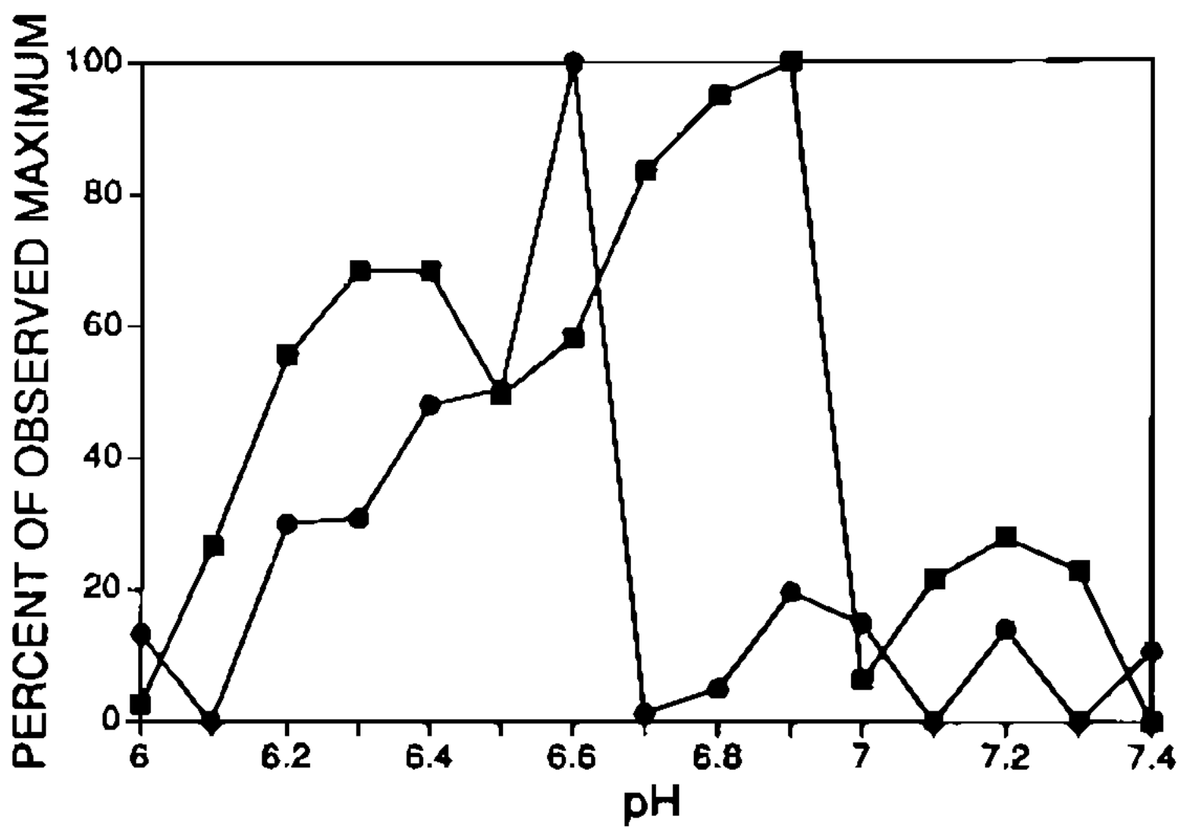


Fig. 5: adherence vs pH in the presence of 50mM $Ca^{2+}$: human blood containing mature forms of the *Plasmodium falciparum* strain FCR-3 was suspended in 5ml of 25mM Bis Tris /155mM NaCl (■) or 25mM Bis Tris / 55mM NaCl /100mM $CaCl_2$ (●) adjusted to the pH indicated on the x axis and allowed to adhere to fixed melanoma cells. The maximum number of infected erythrocytes per melanoma observed for the unsupplemented samples was 5, while the maximum observed for the samples containing 50mM $Ca^{2+}$ was 35.

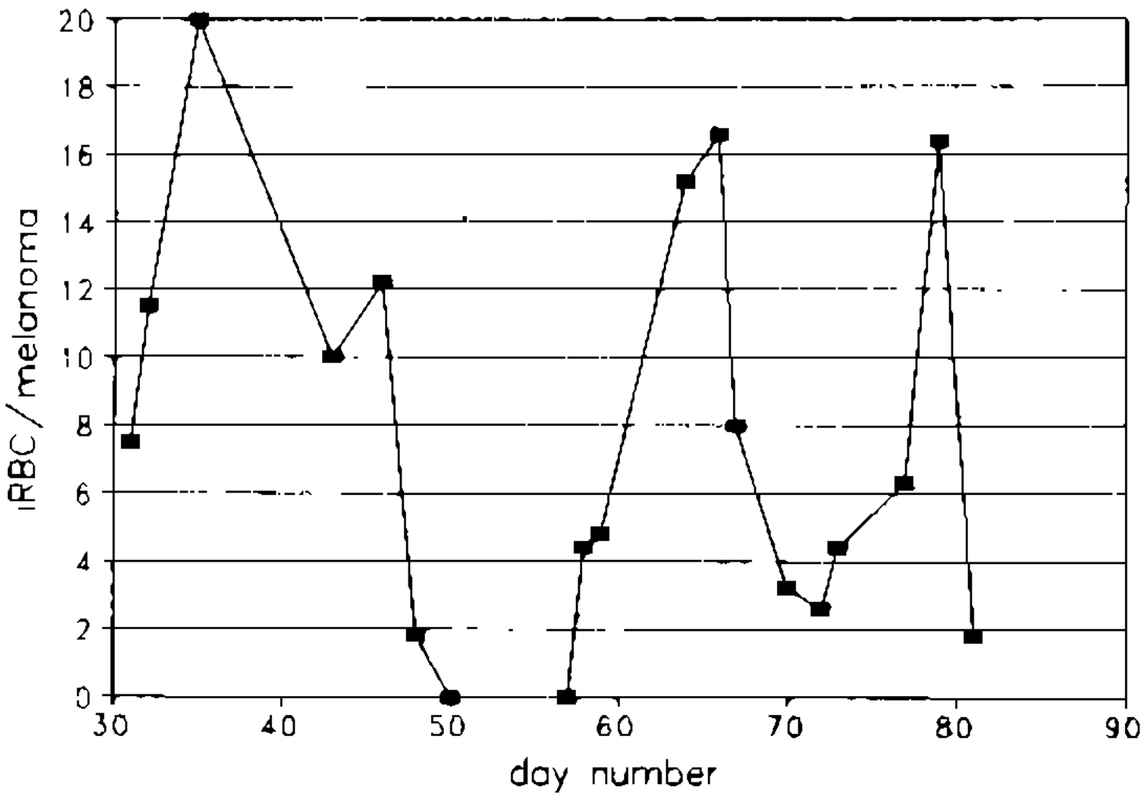


Fig. 6: variation in the degree of adhrence of red cells to amelanotic melanoma cells during a period of 60 days. The adhesion ratio of the control assays (no additions, Bis Tris buffer with $Ca^{2+}$ only) from experiments conducted over 60 days were plotted against the day number (January 1st = day 1). The period from day 50 to 55 resulted in adhesion ratios which were exceptionally low, and therefore were considered to be doubtful. This may have been due to a pool of donors with red cells unsuitable for adherence or the lack of expression of suitable ligands on the malanoma cells.

in all samples were approximately equal (Fig. 7 and Table I). In the second experiment blood was obtained from a different group of donors (some donors appeared in both groups, but the blood used was donated on different dates). The results of the adherence assay for the second experiment are shown in Fig. 8 and Table I. In agreement with the previous experiment, the donor AH produced significantly higher levels of adhesion, however, the donor PA produced a level of adhesion which was average. One donor, BS, produced a level of adhesion which was lower than that seen in the five other samples.

*Effects of antimalarials and anti-band 3 antibody* – When iRBCs bearing ring-stage parasites or parasites at the trophozoite/schizont stage were incubated in the presence of antimalarials for periods of up 12 h there was a cessation of parasite growth, the appearance of degenerative forms, and a lack of expression of knobs; consequently such drug-treated cells did not bind to melanoma cells. There was no significant inhibition of adherence when iRBCs were incubated with antimalarials during the time (90 min) of the assay (Table II).

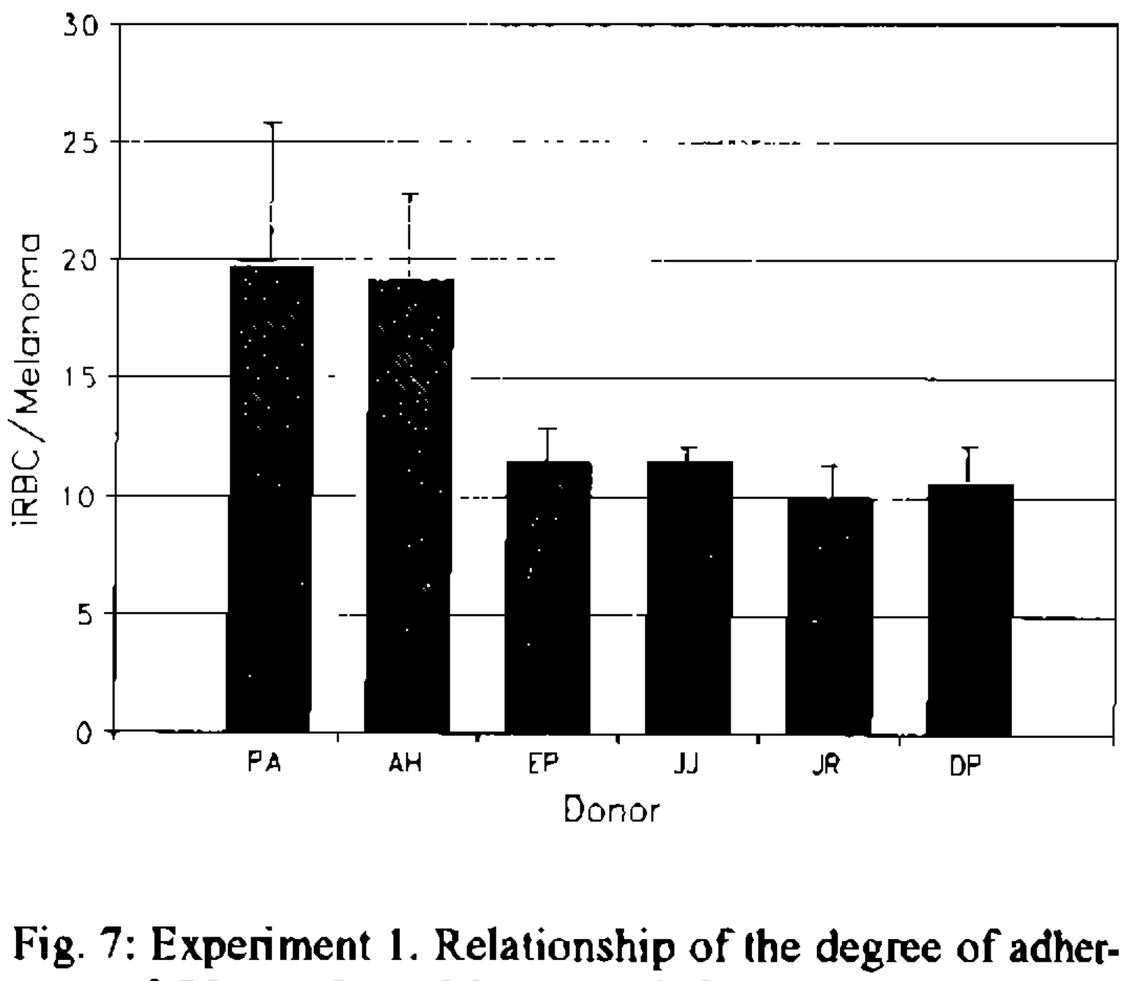


Fig. 7: Experiment 1. Relationship of the degree of adherence of *Plasmodium falciparum*-infected red cells (FCR-3) to individual red cells donors. Values represent the mean +/- standard deviation.

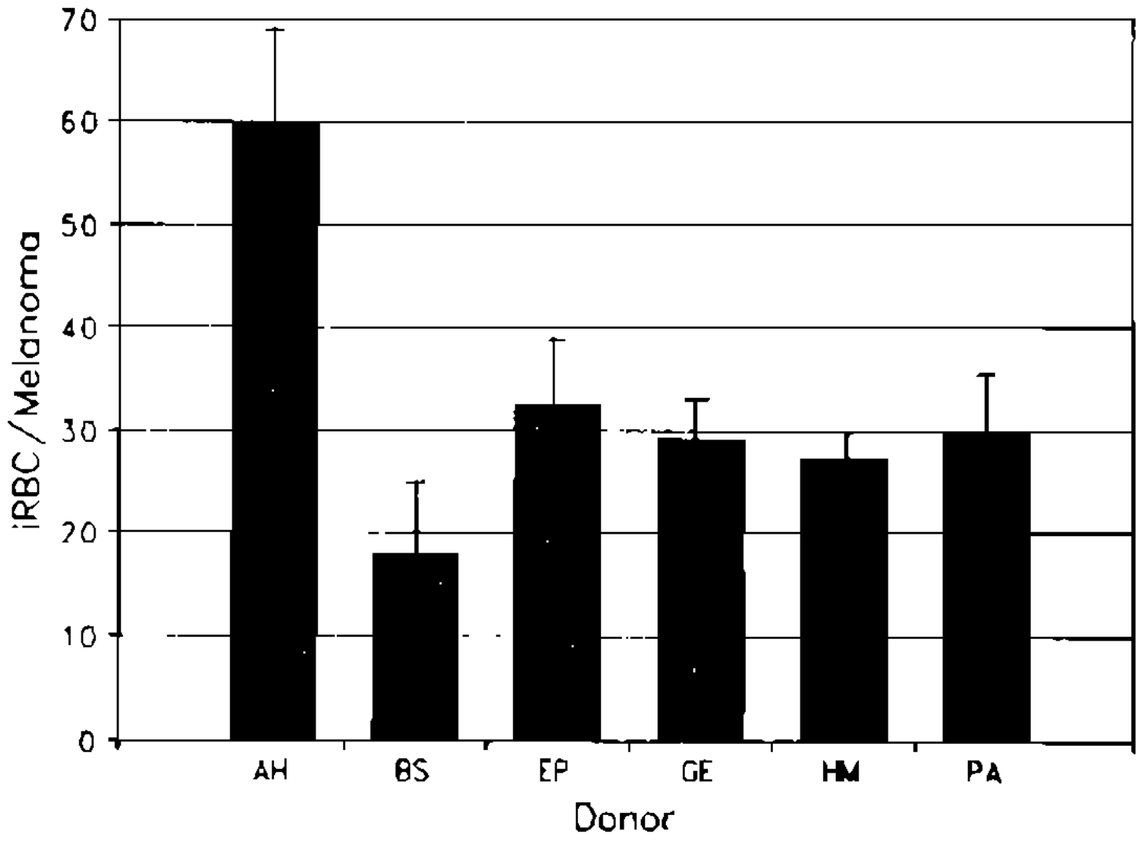


Fig. 8: Experiment 2. Relationship of the degree of adherence of malaria-infected red cells (FCR-3) to the individual red cell donor. Values represent the mean +/- standard deviation.

The effect of adding ascites fluid from the antibody lines 1C4 and 4A3 is shown in Fig. 9. The antibody lines 1C4 and 4A3 were found to inhibit adhesion of iRBCs in a dose-responsive fashion. Inhibition of adherence with both antibodies was found not to be additive and adding amounts of 1C4 beyond those shown on the graph did not increase the inhibition beyond 80% (data not shown).

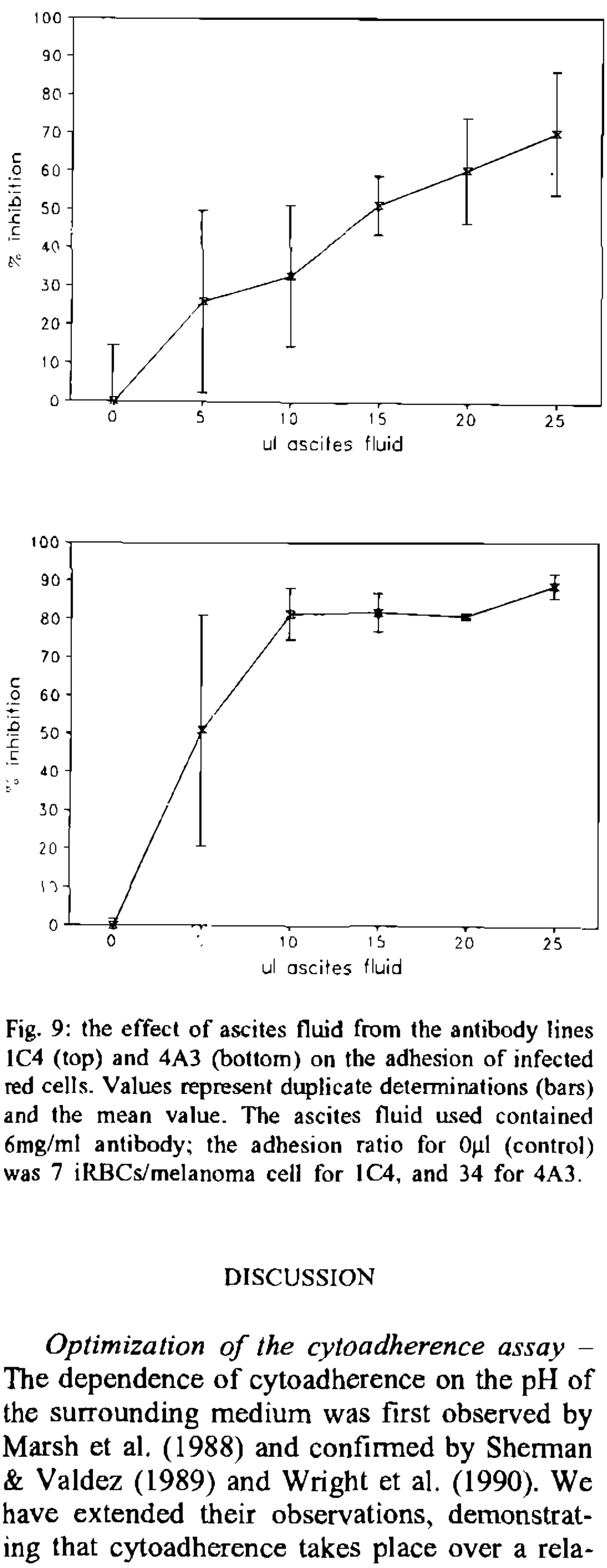


Fig. 9: the effect of ascites fluid from the antibody lines 1C4 (top) and 4A3 (bottom) on the adhesion of infected red cells. Values represent duplicate determinations (bars) and the mean value. The ascites fluid used contained 6mg/ml antibody; the adhesion ratio for 0µl (control) was 7 iRBCs/melanoma cell for 1C4, and 34 for 4A3.

## DISCUSSION

*Optimization of the cytoadherence assay* – The dependence of cytoadherence on the pH of the surrounding medium was first observed by Marsh et al. (1988) and confirmed by Sherman & Valdez (1989) and Wright et al. (1990). We have extended their observations, demonstrating that cytoadherence takes place over a relatively narrow pH range (6.0-7.0), and that the optimal pH range for cytoadherence can be very narrow (0.2 pH units). The optimal pH for cytoadherence is below that normally maintained in mammalian cell cultures. Indeed, the buffer HEPES is frequently added to cell culture media, because its pKa prevents rapid acidification of the medium by the metabolic products of the growing cells. Although HEPES is also suitable for growing malaria parasites, it would appear that a buffer with a lower pKa would be preferable for cytoadherence experiments. In a healthy human the pH of the blood plasma is rigidly maintained at pH 7.4 (Ganong, 1977), a value distinctly higher than the pH optimum for adherence; however, individuals infected with *P. falciparum* frequently have abnormally low levels of blood pH (Fisher, 1983) as well as high lactate levels (White et al., 1983, 1985; Taylor et al., 1988; Molyneux et al., 1989). In the postcapillary venules, especially those containing sequestered cells, the pH may be depressed further, and $CO_2$ levels elevated. Indeed, we observed that when infected erythrocytes suspended in HEPES/RPMI (pH 7.4) were exposed to high (up to 100%) levels of $CO_2$ they became highly cytoadherent (results not shown). Although high $CO_2$ levels may be significant *in vivo* we found that more consistent patterns of adhesion were obtained when the pH was controlled (by using a buffer with a suitable pKa, adjusting the pH, and performing the experiments in ambient air), instead of attempting to control the level of $CO_2$ in the medium.

Calcium affected cytoadherence (Fig. 4) at a much higher concentration (50mM instead of 1mM) than has been observed in other $Ca^{2+}$ dependent cell-cell adhesion system (Mohandas et al., 1985; reviewed by Takeichi, 1988; Heimark et al., 1990). The presence of high (50mM) level of $Ca^{2+}$ in the medium may enhance binding by reducing the surface charge on the target cell (i.e. high levels of $Ca^{2+}$ reduce the surface charge of HUVECs (Vargas et al., 1989) thereby permitting cells to come closer to one another). Since the addition of calcium to the binding buffer had the effect of lowering the optimal binding pH it suggests that calcium and pH do not act independently of one another in affecting cytoadherence. It is interesting to note that the effects of pH and $Ca^{2+}$ on the ability of several diverse lines to bind to amelanotic melanoma cells was the same. This suggests that all of the lines tested (two knobby and one knobless) use a similar or

identical mechanism to cytoadhere. Although the effects of pH and $Ca^{2+}$ were common to all the lines tested, the individual lines did not respond to the same extent. The highest ratio of iRBCs to melanoma cells was observed when the FCR-3 line was used, whereas the knobless B8B6+ line usually showed the fewest number of iRBCs per melanoma cell. This suggest that although the different lines probably use a common mechanism to adhere, the number of cytoadherence sites may vary from line to line. Preliminary experiments (Smith, unpublished) have indicated that the buffer composition that results in optimal binding of *P. falciparum*-infected erythrocytes to amelanotic melanoma cells is also the same as that which results in maximal binding of iRBCs to freshly isolated HUVECs (ATCC CRL 1730). This suggests that the cytoadherence mechanisms for endothelial and amelanotic melanoma cells are similar. Finally, it is of interest to note that the environmental conditions which promote cytoadherence to melanoma cells described in this report are similar to those that increased rosetting (Carlson et al., 1990).

The changes in the medium that promoted cytoadherence were specific for the *falciparum* infected red cell i.e. the same environmental conditions did not increase the number of adherent uninfected erythrocytes. This indicates that the conditions that promote erythrocyte adhesion are not present in uninfected cells, and that we are observing a parasite induced adhesion phenomenon.

Alterations in the composition of the binding buffer resulted in an increased number of iRBCs that bound to an individual melanoma cell, however, altering the composition of the binding buffer itself did not change the percentage of melanoma cells that were competent to bind iRBCs. Thus, the cytoadherence property (i.e. the capacity to bind iRBCs) of an individual melanoma cell remained independent of the composition of the medium.

The cytoadherence study involving blood donors was centered on a single parasite line (FCR-3) and involved several sources of red cells. The data obtained make it clear that some of the variation in binding of iRBCs is attributable to the individual red blood cell donor (Figs. 7 and 8 and Table I). One of the donors, PA, displayed better than average binding in the first experiment, but only average binding in the second, therefore, it appears that an increase in adherent ability may be affected by the erythrocyte's environment. (However, the possibility that PA's infected erythrocytes remained constant, and that the melanoma targets expressed different amounts of ligand cannot be completely excluded). The donor AH consistently produced above average adherence, and was intentionally included in the experiment because of a coincidental rise in the adherence ratio observed in the cytoadherence assays and the known inclusion of AH blood in the pool of blood used to culture the parasite. The adherence ratio shown for AH in the second experiment may in fact be lower than was potentially possible since in our experience the melanoma cells are completely covered with iRBCs above adherence ratios of 60 (that is, the system saturates at about 60 iRBCs per melanoma cell). It is interesting to note that in both adherence experiments the majority of the donors assayed produced mean adherence values which were (within each experiment) not significantly different from one another. It would be of considerable interest to extend these studies to a larger number of red cell donors using a single cloned parasite line and then to determine the adherent ability of these iRBCs. Were a highly cytoadherent subpopulation to be identified it would suggest that an individual's red cells, as well as parasite strain, influence adherence. Were such a subpopulation to be found it might contain individuals with a greater susceptibility to develop cerebral malaria.

*Inhibition of cytoadherence* – None of the antimalarials tested directly blocked adhesion. Indeed, what the studies with antimalarials show is that if knob expression is suppressed adherence is ablated. Consequently, any factor that inhibits parasite growth and reduces knob formation adversely affects cytoadherence. Therefore, we presume the *in vivo* effect of an antimalarial on adherence would be indirect and result from inhibition of parasite maturation rather than blocking adhesion *per se*.

The antibodies 1C4 and 4A3 were able to inhibit adherence of iRBCs is a dose responsive fashion. 4A3 was the better inhibitory agent; this may be due to its epitope being closer to the adhesin on the red cell surface, or alternatively may be due to 4A3 being a relatively bulky IgM, whereas 1C4 is a smaller $IgG_{2a}$. 1C4 and 4A3 both recognize modified forms of human band 3 (Winograd & Sherman, 1989; Crandall & Sherman, 1991), therefore it was not entirely unexpected that adding both of the antibodies did not result in complete inhibition

of adherence. Cytoadherence should be abolished once all of the surface adhesins are saturated, but complete inhibition of adherence was not obtained by adding increased amounts of either antibody. Since 1C4 and 4A3 block adhesion by interacting with parasite-induced modified forms of band 3 molecules, and inhibition of binding with these monoclonal antibodies was always less than 100%, it suggests that adhesive molecules unrelated to band 3 are also involved in cytoadherence.

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