# VARIATION IN THE CYTOADHERENCE CHARACTERISTICS OF MALARIA PARASITES: IS THIS A TRUE VIRULENCE FACTOR?

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*The sequestration of* Plasmodium *falciparum-infected erythrocytes to the endothelial cells of brain capillaries is believed to represent one of the determining factors in the pathogenesis of cerebral malaria. In vitro studies of cytoadherence provide an experimental approach to underetand the mechanism of sequestration and the respective roles played by parasite and host components in this interaction.*

*This paper critically reviews current studies on cytoadherence, with particular emphasis on the nature of the information provided by such studies and their limitations. The paper also describes how·cytoadherence studies using the patient's own monocytes can provide original information on the level of receptor up-regulation in the course of malarial infection.*

Key words: malaria – cytoadherence – thrombospondin – ICAM-1 – CD36 – monocytes – sequestration – cerebral malaria – *Plasmodium falciparum*

Sequestration is a process by which late asexual stages of malaria parasites are retained in the capillary system and, thus, disappear from the peripheral bloodstream. The crucial question is whether the cytoadherence to endothelial cells, which is responsible for sequestration, affects the "virulence" of the parasite and can, thereby, be responsible for the more severe forms of the disease.

This review paper discusses the value of looking at *in vitro* cytoadherence characteristics as a means for investigating the pathophysiology of severe disease. Using experimental results from studies performed with material collected in a study of cerebral malaria in African children, it shows that (i) different parasites may bind with different affinity to various cell types (inc. melanoma cells, monocytes and U937), going from complete absence of binding to strong binding; (ii) patient's own monocytes may be used to investigate cytoadherence and give an indication on the level of receptor

"up-regulation" during infection; and (iii) that, while there is no substantial correlation between cytoadherence assays and disease severity, some of the features of cytoadherence may be altered in severe forms of malaria.

## FEATURES OF SEQUESTRATION

Sequestration is not a general feature of malaria parasites and, while it occurs in *Plasmodium falciparum, P. fragile, P. coatneyi* and *P. chabaudi*, it is absent in most other species, including the other three human malaria parasites, *P. vivax, P. ovale* and *P. malariae*. For a given parasite, the degree and location of sequestration is, to a large extent, dependent upon the nature of the host. For example, the heart, liver and lung are major sites for the sequestration of *P. falciparum* in the owl monkey (*Aotus trivirgatus*), while the spleen is the dominant site for the same parasite in the squirrel monkey (*Saimiri sciureus*) (Fremount & Rossan, 1974). In the owl monkey, there are substantial differences in the intensity of cytoadherence, depending on the karyotype of the animal. The cytoadherence to capillary endothelium in the brain is a feature of human infection and does not occur in any of the primate models.

Sequestration is stage-specific and occurs only when the parasite transforms from the ring – to the mature trophozoite stage (i.e. only during the second half of the 48 hour life cycle of P. falciparum). In infected patients, sequestration is usually extremely efficient and the finding of late asexual stages in the peripheral blood is a relatively rare event, even in endemic areas or situations where very heavy infections are observed. The notable exception is the case of splenectomized patients, where high level infections are observed with all developmental stages of the parasite present in the peripheral blood (Israeli et al., 1987). In experimental hosts where the spleen represents a major site for sequestration, the absence of a spleen has been shown to facilitate the selection of non-cytoadherent parasite populations (David et al., 1983). The demonstration that a parasite population can switch from a cytoadherent to non-cytoadherent phenotype (a process which occurs very fast in the splenectomized squirrel monkey) was the first tangible evidence that substantial differences may exist between different parasite populations with regard to their ability to stick to host cells.

Ultrastructural studies of P. falciparum-infected cells shows the presence of electron-dense protrusions of the erythrocyte membrane (or "Knobs"). In human pathology specimens, the close contact observed between knobs and endothelial cells has been interpreted as evidence that knobs were responsible for cytoadherence (MacPherson et al., 1985). This correlation is, however, far from clear-cut. Among the data which contradict this hypothesis are the facts that: P. malariae/P. brasilianum have knobs but do not cytoadhere (Smith & Theakston, 1970; Sterling et al., 1972); P. chabaudi has no knobs but cytoadheres (Cox et al., 1987); "knobless" isolates of P. falciparum may cytoadhere (McCallum-Deighton, 1988; Udomsangpetch et al., 1989; Biggs et al., 1989), and "knobby" isolates of P. falciparum taken from splenectomised hosts do not (David et al., 1983).

SEQUESTRATION AND DISEASE

Plasmodium falciparum is the most lethal of human malaria parasites, particularly because of the severity of some of its clinical forms, including cerebral malaria. While the pathophysiology of cerebral malaria has not yet been satisfactorily elucidated, sequestration is cur-

rently believed to represent an important aetiological factor (Warrell, 1987). In a situation where large numbers of highly metabolically active stages of malaria parasites are attached to the surface of capillary endothelial cells, it is conceivable that these parasites may be responsible for a dramatic alteration of the metabolic balance within the confined space of a cerebral capillary and, thus, induce coma either through a depletion of oxygen, glucose and other nutrients, or through the direct effect of toxic malaria metabolites or through an indirect effect involving host factors (e.g. cytokines) produced in response to malaria antigens. The features of coma in cerebral malaria are indeed compatible with such a hypothesis.

The evidence that cytokines may be a severity factor in malaria is essentially circumstantial (i.e. IL-1, TNF and IL-6 are more frequently increased in severe than mild forms — Grau et al., 1989; Kern et al., 1989; Grau et., 1990). This feature may be of importance in the understanding of the role of sequestration because one of the effects of these cytokines is to regulate the expression of the endothelial cell surface antigens which may act as "receptors" for cytoadherence.

The fact that only a small proportion of individuals infected with P. falciparum develop cerebral malaria is one of the unexplained features of the disease. This may be due either to a variable virulence of different parasites isolates, to different host characteristics or to epidemiological features of the infection. In support of the role of genetic factors in the control of susceptibility is the observation, recently made in the Gambia, that certain HLA Class II haplotypes (e.g. DRw13-DQw5) were less frequently associated with severe disease and were commonest in parts of Africa where falciparum malaria is endemic (Hill et al., 1991). In support of an epidemiological explanation of the phenomenon, is the observation that severe disease occurs only with low frequency in areas of greatest endemicity and is particularly common in areas of low endemicity in proximity to a high transmission zone (as described in a detailed study of malaria incidence in the township of Brazzaville – Trape et al., 1987). The possible implication of this observation being that the early development of immunity in high endemicity zones may protect against severe disease.

## IN VITRO ASSAYS FOR CYTOADHERENCE

The various *in vitro* assays currently used to examine the cytoadherence of *P. falciparum*-infected erythrocytes have been extensively reviewed elsewhere (Howard, 1988; Barnwell, 1989; Hommel, 1990).

Because of their endothelial nature, assays with primary cultures of human umbilical vein entodhelial cells (HUVEC) were the first to be used for cytoadherence studies (Udeinya et al., 1981). The finding that an established cell line of human amelanotic melanoma cells (ATCC C32), had adherence features comparable to that of HUVEC's (Schmidt et al., 1982) was considered a convenient alternative and the melanoma cells binding assay became, for some time, "the standard model for the assessment of cytoadherent capability" (Warrell et al., 1990). The assay was rendered quantitative either by direct counting of the infected erythrocytes adhering to melanoma cells or by prior radiolabelling of infected erythrocytes with either $^{51}$-Chromium (David et al., 1983) or $^{3}$H-hypoxanthine (Wright et al., 1990). Apart from monocyte cell lines (see below), none of the other cell lines tested by various authors, has so far been shown to be of use for *in vitro* cytoadherence studies. One important feature of these studies is the fact that cytoadherence appears to be host cell specific inasmuch as human or primate infected etythrocytes bind only to human or primate cells, while mouse infected erythrocytes bind only to murine cells (Cox et al., 1987). "Rosetting" is a form of cytoadherence, where infected erythrocytes bind to normal erythrocytes *in vitro* (Wahlgren et al., 1989); this assay has been used to investigate the comparative cytoadherence of different *P. falciparum* strains (Carlson et al., 1990; Hasler et al., 1990). The precise correlation between rosetting and other assays has not yet been clearly established, but as isolates which are capable of binding melanoma cells but are not rosetting and isolates which are rosetting but do not bind melanoma cells, have both been identified (Udomsangpetch et al., 1989), this suggests that the two assays may measure different adhesins.

### DIVERSITY OF PARASITE CYTOADHERENCE

Whatever the *in vitro* assay used, different malaria isolates have been shown to exhibit a considerable degree of diversity in their cytoadherent ability. The results obtained in our study of Malawian isolates using the melanoma cell binding assay (Goldring et al., 1992) (Fig. 1) are comparable to previous reports from Brazil (Kloetzel et al., 1986), the Gambia (Marsh et al., 1988), Papua New Guinea (Southwell et al., 1989), and Thailand (Ho et al., 1991). It is noteworthy that, while a percentage of non-cytoadherent isolates was found in all the reported studies, the overall number of non-cytoadherent isolates appears to be substantially higher in the Malawian sample. When rosetting is used to compare isolates (as was done in two independent studies from The Gambia (Carlson et al., 1990; Hasler et al., 1990), a wide spread from a high level of cytoadherence to a complete absence of cytoadherence was also observed. No convincing correlation between the degree of cytoadherence of a given isolate and the severity of the disease induced by this isolate could be found in either group of studies, particularly when an overall view is taken.
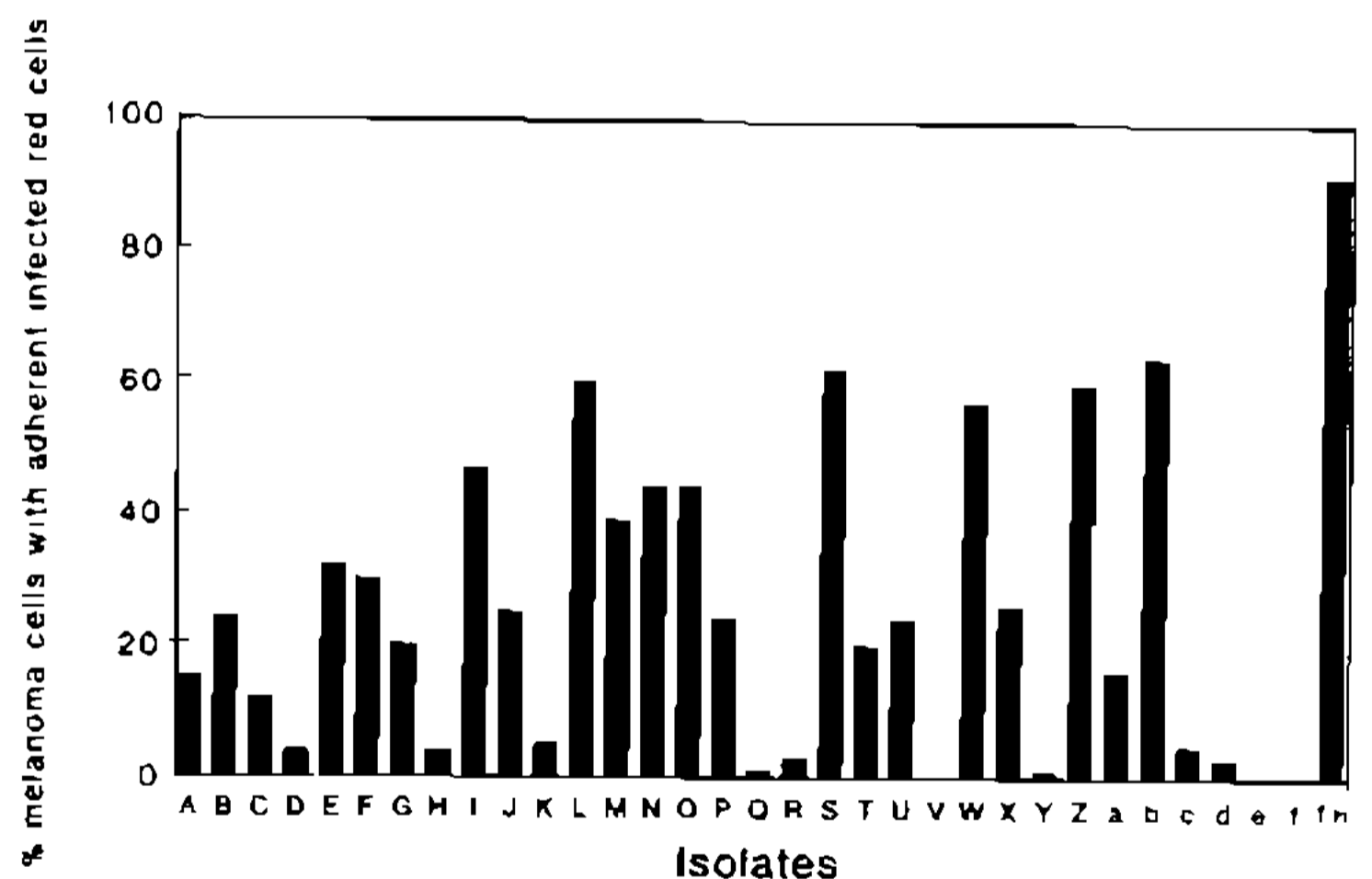


Fig. 1: cytoadherence of *Plasmodium falciparum* infected erythrocytes from 32 African (A-f) patients and one Thai (Th) patient to melanoma cells.

## MOLECULES INVOLVED IN CYTOADHERENCE

The cytoadherence of malaria-infected erythrocytes to "high endothelial" cells involves the existence of specific receptor molecules on the surface of the red cell (malaria "adhesins"), capable of binding their counterpart on the surface of endothelial cells.

The identification of PfEMP-1 (Leech et al., 1984) as the most likely candidate malaria adhesin is based on a combination of different circumstantial evidence, i.e. the presence of neoantigens on the surface of erythrocytes, the extreme antigenic diversity of these molecules and the inhibition of cytoadherence either by antibodies which recognize strain-specific

PfEMP-1 molecules or by trypsin-treatment which removes critical PfEMP-1 epitopes (see reviews by Hommel & Semoff, 1988; Berendt et al., 1990). Neither PfEMP-1 nor any other malarial antigen has, as yet, been shown capable of binding to endothelial receptors.

In the absence of an easy experimental access to human high entodhelial cells, most studies of cytoadherence receptors have concentrated on either melanoma cells or HUVECs and three potential candidate cytoadherence receptors have been identified so far: thrombospondin (Roberts et al., 1985), CD 36 (Panton et al., 1987; Ockenhouse et al., 1989) and ICAM-1 (Berendt et al., 1989). The isolate-specific diversity of cytoadherence observed in in vitro assays and the presence of a percentage of noncytoadherence isolates in all the samples tested suggests that more than one cytoadherence receptor is involved (a variable depending on the type of parasite involved). This also suggests that the current list of candidate receptors is still incomplete (especially if one considers that all the "in vitro noncytoadherent" parasites were sequestering in the patient from whom they have been isolated). The use of transfected simian COS-cells expressing either ICAM-1 of CD36 genes has been a useful method for identifying isolates having specific adhesins to one of these two receptors (Berendt et al., 1989; Oquendo et al., 1989).

REGULATION OF CYTOADHERENCE

The cell-cell interaction responsible for cytoadherence may be altered by either parasite – or host – induced changes in the cell surfaces. It has been shown, for instance, that neuraminidase-treatment of infected erythrocytes increased cytoadherence to melanoma cells in vitro (Sherman & Valdez, 1989) and, as malaria infection can also induce the loss of sialic acid in red cells (Howard et al., 1988), it is conceivable that this may, at least in certain circumstances, affect the intensity of sequestration. It is equally possible that erythrocytes from some patients may be more or less susceptible to parasite-induced changes or may express malaria adhesins more effectively on their surface; this is, for example, the case for thalassemic red cells, which express malarial neoantigens more effectively than normal cells (Luzzi et al., 1991).

The role of host factors in the regulation of endothelial surface receptors (e.g. integrins), is of even greater importance. It has been shown,

for example, that the expression of ICAM-1 in endothelial cells may be enhanced by IFN-γ, IL-1 and TNF (Rothlein et al., 1986). Malaria infection itself is capable of inducing the release of cytokines by host cells and extremely high levels of IL-1, TNF and IL-6 have been reported in various experimental models (Clark, 1978), in severe P. falciparum infections (Grau et al, 1989; Kwiatkowski et al., 1990) and in less severe P. vivax infections (Mendis et al., 1990). The malaria antigens which induce this increased cytokine production are believed to be molecules (perhaps lipids), having an "endotoxin-like" activity (Playfair et al., 1990; Taverne et al., 1990).

The up-regulation of surface receptors may enhance cytoadherence, as shown to be the case in HUVEC's treated in vitro with IL-1 or TNF (Berendt et al., 1989). It is conceivable that it is "enhanced" cytoadherence, rather than "baseline" cytoadherence, which is responsible for the severe forms of the disease. Such a working hypothesis would be attractive for two reasons: (i) in view of the fact that endothelial cells are phenotypically distinct in different organs, enhancement would be expected to occur preferentially in certain sites (e.g. cerebral endothelium) and this would account for the observation that, while the expression of receptors, such as CD36, is poor on normal cerebral endothelium it may be up-regulated during infection; and (ii) enhancement may be genetically-controlled, either at the level of the host's ability to respond to malarial endotoxin or at the level of a variable susceptibility of endothelial cells to regulatory molecules, such as cytokines. This may explain the various features of falciparum malaria, particularly the relative rarity of cerebral malaria in a situation where parasite are always sequestrated, regardless of disease severity.

Antimalarial antibodies may directly affect sequestration of infected cells, by inhibition or reversal of cytoadherence. This effect was demonstrated both in vitro using the melanoma cell binding assay and in vivo by the passive transfer of immune serum to P. falciparum-infected squirrel monkeys (David et al., 1983; Singh et al., 1988). This effect of antibodies, which is highly isolate-specific, indicates a close relationship between malaria adhesins and the neoantigens expressed on the surface of malaria-infected erythrocytes (Hommel et al., 1983). In our hands, the cytoadherence of all Malawian isolates capable of binding melanoma

cells was inhibited by means of a serum pool prepared from over 700 immune adult blood donors. The serum pool had, however, no inhibitory effect on the cytoadherence to human monocytes or the U937 cell line and, when used for immunotherapy in malaria infected children, it failed to produce any detectable reversal of sequestration (Taylor et al., 1992).

In endemic areas, severe malaria infection no longer occurs in children over 4-5 years of age and this is believed to be due to an "antitoxic immunity", which precceds by a number of years the onset of an anti-parasite immunity. It is conceivable that anti-toxic immunity may work by interference with the production of cytokines in response to "malarial endotoxins" and thus (among other effects) avoid the induction of an enhanced cytoadherence which may be necessary for the pathogenic effects; this would explain why severe disease no longer occurs in school-age children, despite the heavy parasitaemia which may be observed.

## MONOCYTES AS SUBSTITUTES FOR "HIGH ENDOTHELIAL CELLS"

The "ideal assay" for measuring cytoadherence should closely resemble what happens *in vivo* and include the three components of host-parasite relationship: the relevant endothelial cells, malaria-infected cells and human plasma. Such an assay would measure the host's ability to bind parasites, the relative parasite "stickiness" and some of the regulatory factors that may be produced by the host in response to infection (Fig. 2 summarizes the features which an ideal cytoadherence assay would be expected to measure). While plasma and infected red cells are easily accessible from pa-

tient blood, the same is not true for "high endothelial" cells from sites of parasite sequestration. The only accessible cell, with properties similar to the endothelial cells is the peripheral monocyte. In this section we will outline why monocytes may be a substitute for endothelial cells and introduce a model which closely resembles *in vivo* sequestration.

The plasma membrane of monocytes has a number of surface proteins which are also found on the surface of endothelial cells, including not only glycoproteins, receptors, and MHC class I and II antigens (see Table), but also receptors for cytokines such as IL-1, IL-6, IL-8 and TNF, which stimulate both types of cells. Endothelial cells and monocytes also share common functions, such as antigen presentation with an associated increase in Class II MHC expression, the induction of T cell proliferation and IL-2 production and the ability to enable T cells to assist the secretion of immunoglobulins by B cells (Shore et al., 1986). Both cells may, therefore, contribute to the plasma levels of lymphokines in malaria by secreting TNF and IL-6 in response to IL-1 and TNF (Sironi et al., 1989; Navarro et al., 1989). Monocytes and endothelial cells are also alike in their responses to pharmacological agents and the effects these agents have on their metabolism (e.g. the negative regulatory effect of glucocorticoids on the cytokine system) (Waage et al., 1990).

While the ability of monocytes and monocytic cell lines (U937, HL60) to bind schizont-infected erythrocytes *in vitro* had previously been demonstrated (David et al., 1983; Barnwell et al., 1985), we have extended this work by looking at the behaviour of monocytes from different individuals, as well as parasite isolates from different patients. This study (Goldring et al., 1992) has shown that, as in the case of melanoma cell binding (Fig. 1) or rosetting, there are substantial differences between isolates, but in addition monocytes from different donors are also variable in their ability to bind malaria-infected cells (Fig. 3). In contrast to melanoma cell binding and rosetting (Singh et al., 1988; Wahlgren et al., 1989), cytoadherence to monocytes can neither be inhibited nor reversed by antibodies; this feature is similar to that observed when hyperimmune serum is passively transferred to infected children (Taylor et al., 1992), but different from what had been observed in the squirrel monkey model (David et al., 1983).
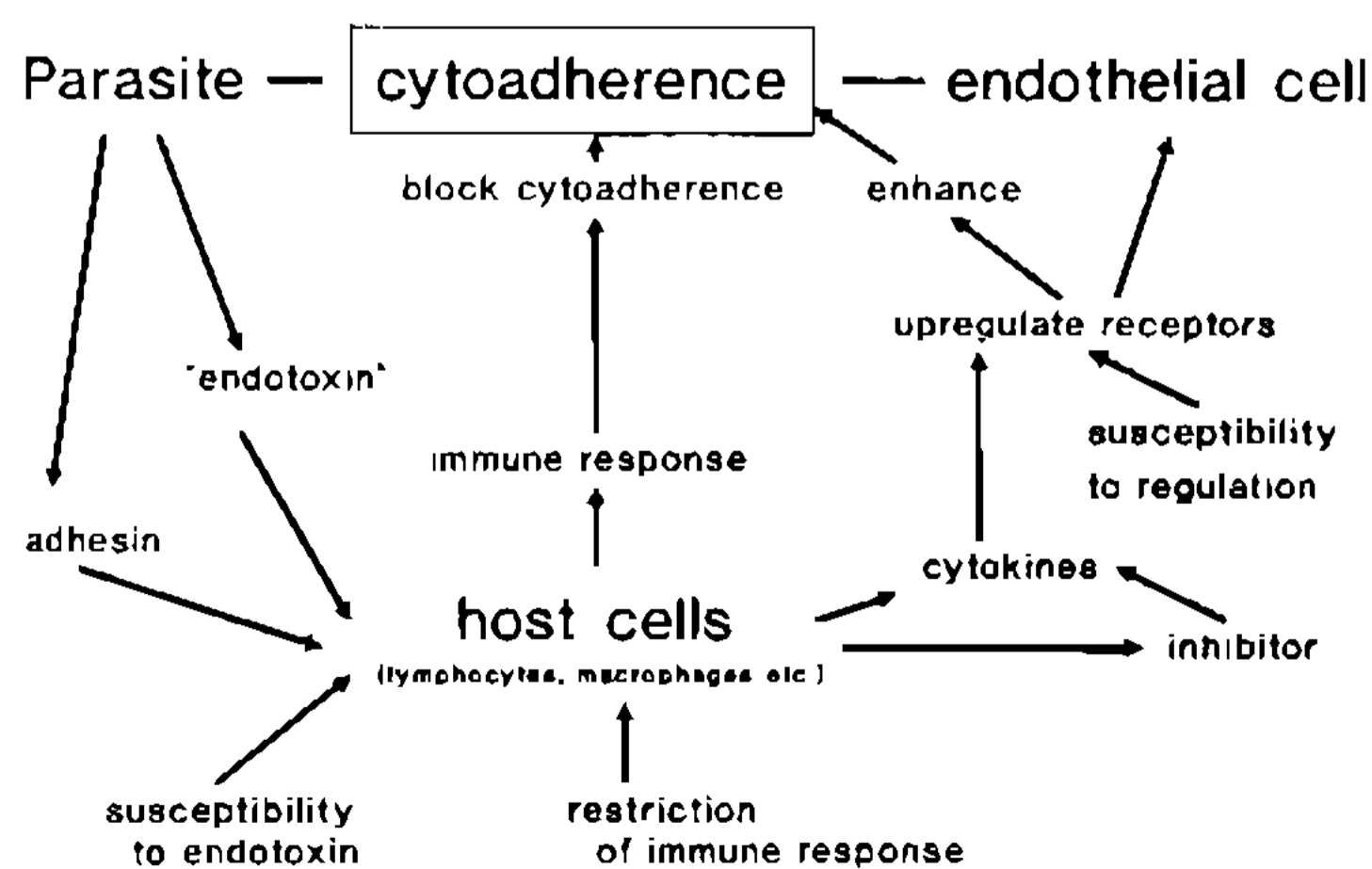


Fig. 2: the features to be considered in an "ideal" cytoadherence assay.

TABLE

Cell-surface molecules expressed by both monocytes and endothelial cells

| Molecules on cell surface | Monocytes | Endothelial cell | References |
|---|---|---|---|
| CD36 (gp VI) | + | + | Barnwell (1985) |
| CD11a (LFA-1) | + | + | Simmons et al. (1988) |
| CD54 (ICAM-1) | + | + | Warwyk et al. (1989) |
| Class 1 MHC | + | + | Keilke (1989) |
| Class 11 MHC | + | + | Beilke (1989) |
| factor VIII | + | + | Jaffe (1992) |
| C3 receptor | + | + | Friedman (1984) |
| Fc receptor | + | + | Friedman (1984) |
| G-CSF receptor | + | + | Bussolino et al. (1989) |
| GM-CSF receptor | + | + | Bussolino et al. (1989) |
| CD9 CD13 CD29 | + | + | Knapp et al. (1989) |
| CD31 CDW32 CD42a | + | + | Knapp et al. (1989) |
| CD42b CD43 CD47 | + | + | Knapp et al. (1989) |
| CDW49b CDW49d CD51 | + | + | Knapp et al. (1989) |
| CD61 CD62 CD63 | + | + | Knapp et al. (1989) |
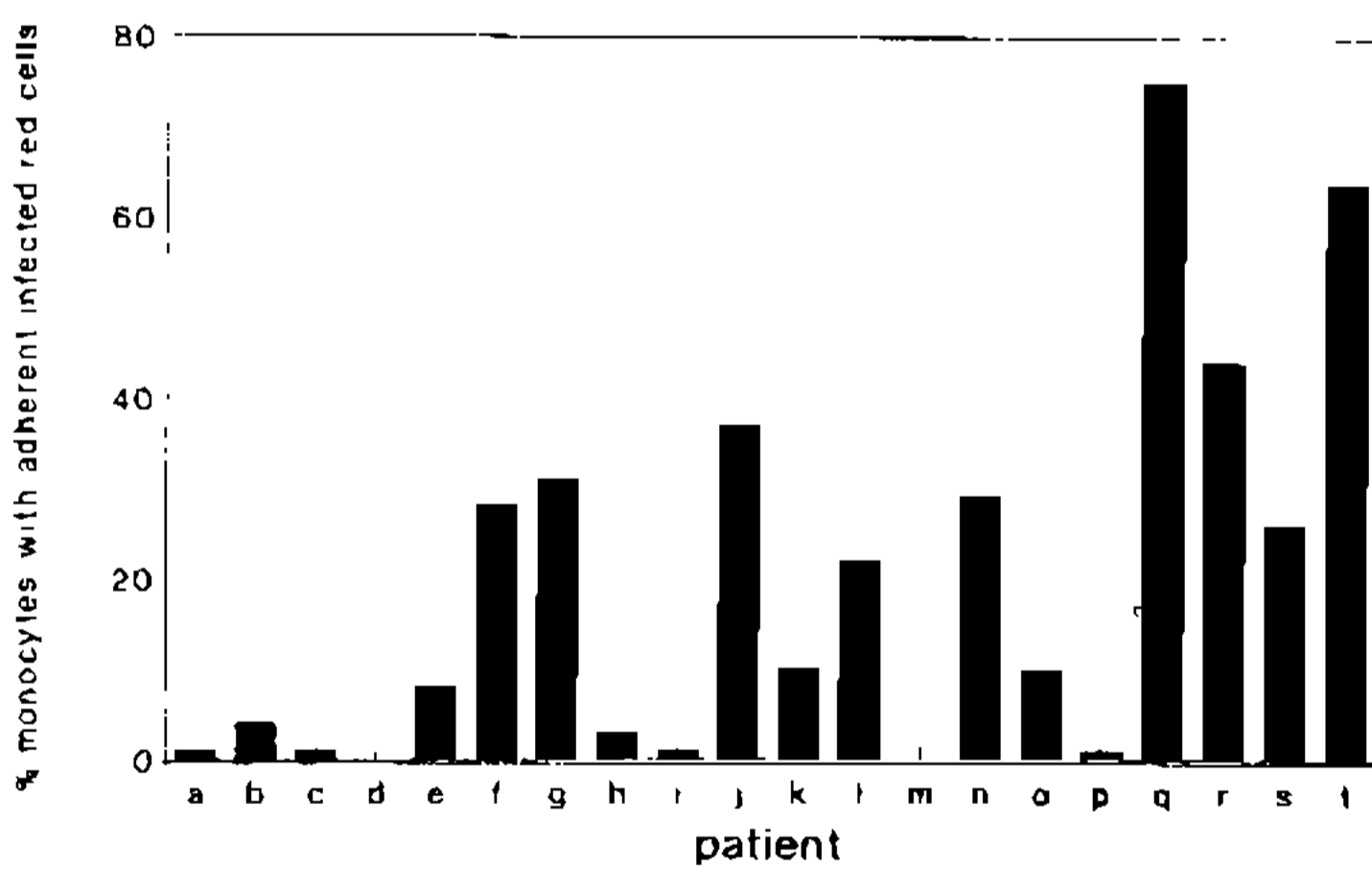


Fig. 3: cytoadherence of *Plasmodium falciparum* infected erythrocytes to monocytes from the same patient. Each of the 20 (a-t) isolate/monocytes was obtained from patients during infection.

It is conceivable that, during malarial infection both monocytes and endothelial cells may be exposed to the same array of cytokines and hence common responses may be measured by looking at monocytes taken during infection (since endothelial cells are not as easily accessible). Both cells respond to IFN-γ, TNF and lymphotoxin by upregulating Class 1 MHC antigens (Cotran, 1987) and ICAM-1 expres-

sion Pober et al., 1986; Wawryk et al., 1989); by the production of colony stimulating factors (Broudy et al., 1987; Clarke & Kamen, 1987) and by the production of IL-1 and IL-6 (Navarro et al., 1989; Sironi et al., 1989).

In malaria, the stimulation of monocytes has already been documented, both in terms of the secretion of TNF, neopterin and IFN-γ (Kwiatkowski et al., 1990; Brown et al., 1990) and in terms of an increased metabolism (Allison, 1988). Of particular interest is the fact that monocytes from patients with malaria appear to be able to secrete greater quantities of TNF than convalescent monocytes from the same patient (Kwiatkowski et al., 1990). Monocytes during malaria infections have also been shown to be associated with parasitized erythrocytes in sites of sequestration such as the bone marrow and the placenta (Wickramasinghe et al., 1987).

Using monocytes taken from children with different clinical forms of malaria (ranging from mild to severe) and at different stages of infection (acute infection or after recovery), we have

examined the possibility of measuring "enhanced cytoadherence" for the homologous malaria parasite, i.e. parasite isolate taken from the same patient during acute infection and cryopreserved until use. Preliminary results are presented on Fig. 4, which shows: (i) that it is possible to examine the cytoadherence of such monocyte/parasite combinations *in vitro;* (ii) that combinations of monocytes/parasites can be found in both mild and severe groups, where no cytoadherence is observed; and (iii) that, in the severe group, there is a significant decrease in cytoadherence, when comparing the acute and convalescent monocytes, a difference which does not exist in the mild group. While no definitive conclusions can be drawn from these preliminary results, in view of the small number of combinations tested, this pattern is nevertheless of interest, since it is suggestive of a correlation between enhanced cytoadherence and severe disease.
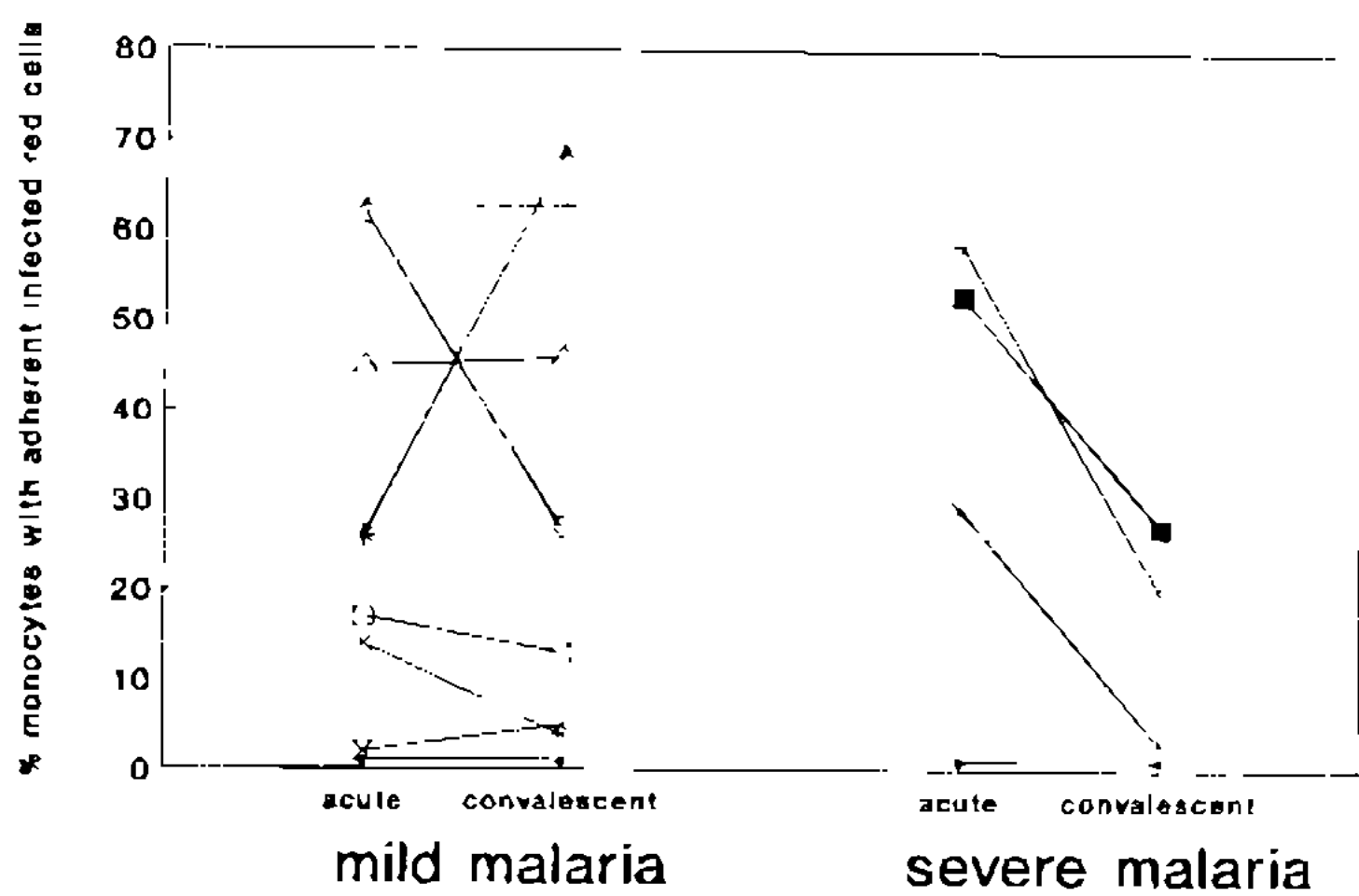


Fig. 4: cytoadherence of *Plasmodium falciparum* infected erythrocytes to monocytes from the same patient. Monocytes were obtained at the acute and convalescent stages of infection from patients with mild or severe malaria. Cryopreserved parasites were cultured for the assay with 'convalescent monocytes'.

CONCLUSIONS

(i) Whatever cytoadherence assay is used (melanoma cell, HUVEC, monocyte or transfected COS-cell binding or rosetting), the correlation between *in vitro* cytoadherence and *in vivo* sequestration is poor. For each assay, a group of parasites are found which exhibit no cytoadherence, despite sequestration *in vivo*. The correlation between this type of *in vitro* assay and disease severity cannot, under the circumstances, be expected to be very good (and it is not).

(ii) The diversity of cytoadherence from one isolate to another is considerable, ranging from

high cytoadherence to no cytoadherence. This diversity is both quantitative and qualitative (i.e. a parasite isolate may be a "high binder" for monocyte and not bind melanoma cells). This diversity is due both to the existence of different malarial adhesins on the infected erythrocyte and to the existence of different cytoadherence receptors on host cells.

(iii) The complexity of the cell-cell interactions involved in cytoadherence *in vivo* are difficult to evaluate with the simplistic assays currently in use. There is a need to develop evaluation techniques which take account not only of the diversity in the intrinsic cytoadherence characteristics of parasites and host cells, but also to their ability to respond to regulatory mechanisms which may enhance or reduce cytoadherence. It is only when such assays become available that a rational study of the correlation between cytoadherence and disease severity may be undertaken.

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