Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 81, Suppl. II :95-109, 1986. *Intern. Symp. on Malaria*

THE ALTERED MEMBRANE OF MALARIA-INFECTED ERYTHROCYTES: VARIABLE EPITOPES AND IMMUNE EVASION / CONSERVED EPITOPES AND CYTOADHERENCE

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The morphological, antigenic and functional properties of the altered erythrocyte membrane of P. falciparum *and* P. knowlesi *infected cells are compared. These malaria species share the expression of antigenically diverse epitopes on high molecular weight malarial proteins inserted into the host cell membrane. This antigenic diversity promotes evasion of isolate-specific immune responses. Infected cells from both species also share an acquired functional property of cytoadherence to endothelial cells lining blood vessels, although the specific properties of cytoadherence differ in their patterns of organ distribution.* P. falciparum*-infected cells of diverse origin can also be shown to cytoadhere specifically to pure thrombospondin coated on plastic. Since this molecule is synthesized and released from endothelial cells it is a likely candidate as the host cell receptor for infected erythrocytes. Functional properties such as cytoadherence imply conservation of structure, at least at the secondary level of protein folding. It is predicted that infected erythrocytes will also express antigenically conserved epitopes related to this function. Serological evidence for antigenically conserved epitopes on diverse natural* P. falciparum *isolates has now been obtained. Immunoelectronmicroscopy has identified the knobs of* P. falciparum*-infected cells, which serve as the points of cytoadherence, as the sites for expression of both antigenically diverse and conserved epitopes. It is probable that the diverse epitopes involved in immune evasion and the conserved epitopes involved in cytoadherence are on the same cell-surface molecule.*

As the asexual malaria parasite matures within the host erythrocyte there are several profound alterations in the properties of the erythrocyte membrane. These diverse alterations generally appear together at the mid-trophozoite stage and reach maximum expression at the late-trophozoite/early-schizont stage. This review will focus on morphological, antigenic and functional alterations involved in immune evasion and cytoadherence. Other important properties of the infected erythrocyte membrane, such as changes in lipids, lectin receptors or permeability to anions, cations and metabolites are reviewed elsewhere e.g. (Howard, 1982; Ginsburg et al., 1983; Kutner, Ginsburg & Cabantchik, 1983; Elford et al., 1985).

Sections A-C below review the key methodological approaches used in this work and the properties of the biological reagents. The subsequent sections deal with the experimental observations, their interpretation and the current models for antigenic variation and cytoadherence.

A. Malarial Parasites — Two malaria species have provided most of the relevant experimental information: *P. knowlesi*, a simian malaria which can infect man, and *P. falciparum*, the most lethal of the four human malarias.

P. knowlesi has been studied in the rhesus monkey *(M. mulatta)* wherein the phenomenon of antigenic variation in malaria was first established (Eaton, 1938; Brown & Brown, 1965; Brown, 1973). The natural host of *P. knowlesi* is the Kra monkey *(M. fascicularis)*. *P. knowlesi* is readily cloned by micromanipulation and can be expanded to high parasitemias ($\geqslant$ 30%) in the rhesus monkey. Cryopreserved ring-stage parasites can be cultured for 16-20hrs. to obtain a synchronous population of late-trophozoite-infected erythrocytes (the asexual cycle of *P. knowlesi* is complete in 24hrs.). Methods for purification of *P. knowlesi*-infected cells and the surface membrane have been developed (Aley et al., 1984).

P. falciparum has been studied in the squirrel monkey *(Saimiri sciureus)* (Hommel, David & Oligino, 1983) and by our laboratory, in the owl monkey *(Aotus trivirgatus)* (Geiman & Meagher, 1967). These laboratory hosts offer similar properties as the *P. knowlesi* / rhesus system, except for significantly lower parasite yields. Cryopreserved blood of 10-30% ring-stages can be thawed and in this case cultured 24-30hrs. to obtain trophozoite-infected cells (the asexual cycle of *P. falciparum* is complete in 48hrs.) (Barnwell et al., 1983). *P. falciparum* infected cells of $\geqslant$ 80% purity can be prepared using Percoll gradients containing sorbitol (Aley, Sherwood & Howard, 1984; Aley et al., 1986).

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We have also studied *P. falciparum* isolates collected directly from infected patients at the Medical Research Council Laboratories, Fajara, The Gambia (Marsh & Howard, 1986). *P. falciparum* infections are characterized by the appearance of immature, but not mature, parasitized erythrocytes in the peripheral circulation. The ring-infected blood was collected from patients and chloroquine administered. Serum was collected at this time ('acute' serum). The blood was washed once and cryopreserved in liquid nitrogen. Three weeks after cure of the acute attack, a second serum sample was collected ('convalescent' serum). Infected blood could then be thawed and cultured 24-30hrs. to obtain mature parasites in the original cells from the patient. We could then assay the immuno-reactivity of acute and convalescent sera with the surface of infected cells.

The *P. falciparum* parasites taken directly from *Aotus* or man offer an important advantage over parasites derived from continuous *in vitro* culture in human erythrocytes. Adaptation of Gambian isolates to *in vitro* culture led to rapid loss of infected cell agglutinability by adult 'immune' sera and complete loss of the capacity to cytoadhere *in vitro* (Marsh & Howard, 1986). This change in phenotype at the infected cells surface was not accompanied by any morphological changes in the expression of knobs. A comparative study of several geographically diverse *P. falciparum* isolates also showed that continuous *in vitro* culture often leads to partial or complete loss of the *in vitro* cytoadherence property without loss of knob expression (Udeinya et al., 1983). Earlier experiments had also shown that parasites which express knobs *in vitro* (ie K^+) can switch to a knobless phenotype (ie K^-) (Langreth et al., 1979). Thus, *in vitro* derived parasites selected for properties of growth in an environment substantially different to that *in vivo* generally lack the complete expression of the surface membrane alterations important for antigenic variation and cytoadherence.

B. Identification of Cell-surface Antigens on Infected Erythrocytes – The proteins on malaria-infected erythrocytes have been radiolabeled by several methods in an attempt to specifically label cell-surface molecules (Howard, Barnwell & Kao, 1982, 1983; Howard et al., 1982, 1986). To date, the most useful method has been ^{125}I-label surface proteins by the lactoperoxidase-H_2O_2-$Na^{125}I$ system (Howard, Barnwell & Kao, 1983; Leech et al., 1984). This method labels several host erythrocyte surface proteins very efficiently, however, the malarial proteins described here can be readily identified since they are of comparatively high M_r on SDS-PAGE (M_r 200,000-300,000) compared to host molecules and they exhibit size diversity in different isolates. Several criteria have been proposed for assessment of the degree of surface versus intracellular labeling of infected cells (Howard, 1982; Howard et al., 1982). In view of the fragility of malaria-infected erythrocytes and the increased permeability of the altered host membrane additional criteria are useful to more definitively establish that a ^{125}I-labeled protein unique to the infected cells is in fact on the cell surface. One useful method has been to study the effects of exogenous proteases on the ^{125}I-labeled proteins with intact labeled cells. The new ^{125}I-labeled proteins of *P. knowlesi* (Howard, Kao & Barnwell, 1983) and *P. falciparum*-infected cells (Leech et al., 1984) were both shown to be cleaved by exogenous trypsin. In the latter case an additional point in favor of a cell-surface location for the ^{125}I-labeled protein was the correspondence between sensitivity of this molecule to trypsin and sensitivity of the *in vitro* cytoadherence phenomenon to this enzyme over a range of concentrations. Both properties were sensitive to trypsin at a very low level (0.1 μg/ml, 5 min, 23°C).

A more telling argument for expression of ^{125}I-labeled proteins on the surface of infected cells has come from the correspondence between reactivity (or not) of antibodies with the cell surface and capacity of the same antibody panel to specifically immunoprecipitate the labeled protein. The capacity of rhesus monkey or rabbit antisera to agglutinate *P. knowlesi*-infected cells of different variant phenotype matched exactly their capacity to immunoprecipitate the M_r 180,000-230,000 variant antigen (Howard, Barnwell & Kao, 1983; Howard, Kao & Barnwell, 1983). The capacity of *Aotus* antisera to block or reverse *in vitro* cytoadherence of different *P. falciparum* isolates matched their capacity to immunoprecipitate the M_r ~300,000 antigen of this species (Leech et al., 1984).

Since antibody is unable to penetrate the cell-surface membrane, it can be used as a probe for cell-surface antigens. Antibodies of defined cell surface immunoreactivity (agglutination/blockade of cytoadherence) have been added to ^{125}I-labeled infected cells, unbound antibody removed by washing and immune complexes purified on Protein A Sepharose after mild detergent extraction (Howard, Kao & Barnwell, 1983; Leech et al., 1984).

Three methods for demonstrating binding of antibodies to the surface of intact infected cells have been instrumental in this work. It is important to note that these methods have allowed us to measure cell-surface reactive antibodies in polyclonal, polyspecific monkey or human sera containing many other anibodies against intracellular malarial antigens.

1) Bound antibodies can be detected by indirect immunofluorescence using fluorescein-conjugated tertiary antibody (Howard et al., 1982; Hommel, David & Oligino, 1983; Marsh, Sherwood, Howard, 1986). This method, shown in Fig. 1, has the advantage, when coupled with ethidium bromide staining of parasite DNA, of allowing one to directly measure the proportion of infected cells that are reactive and the amount of antibody bound to each cell.

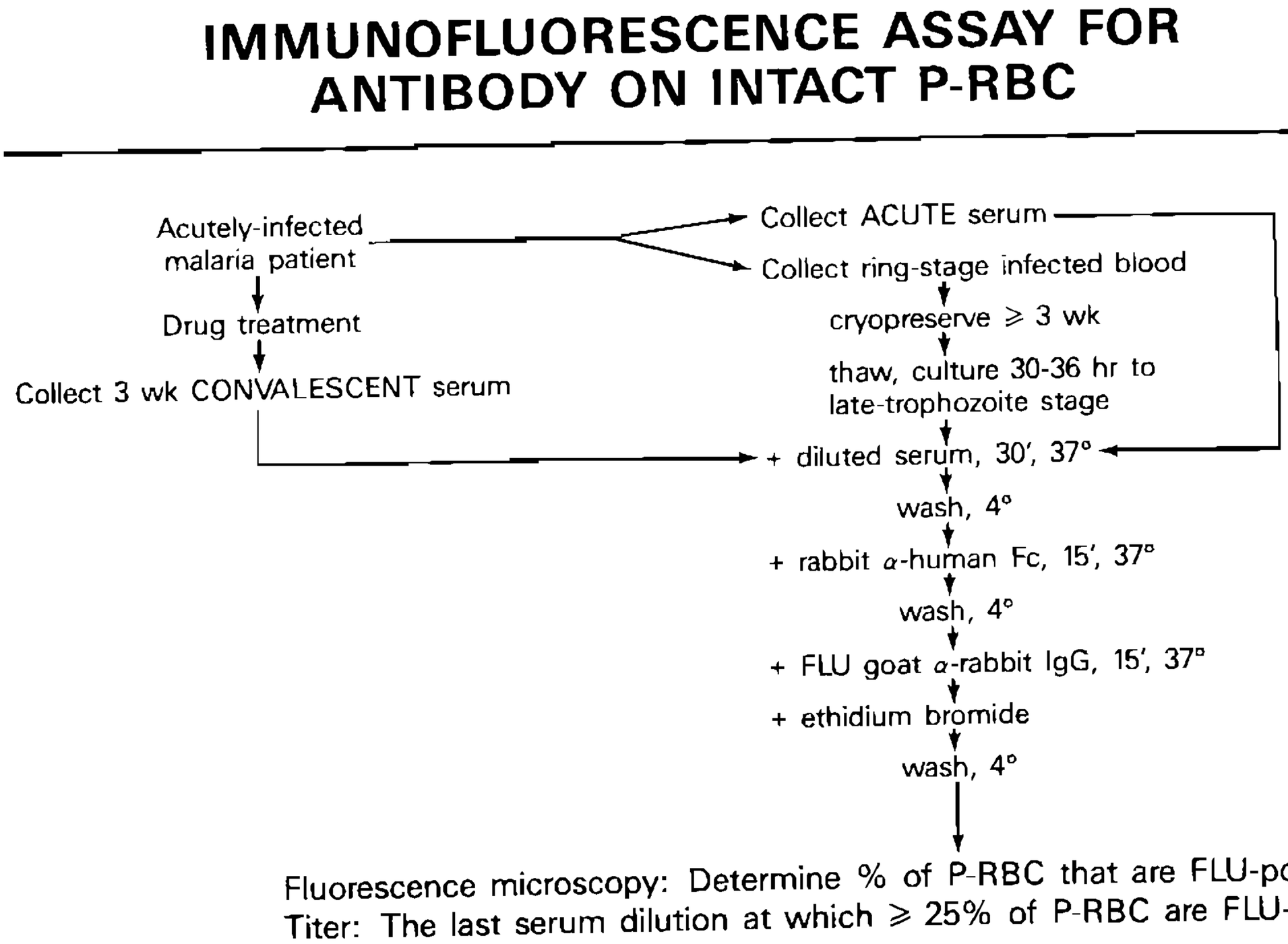

Fig. 1: The immunofluorescence assay for detection of binding of antibodies from human sera to the surface of *P. falciparum*-infected erythrocytes from patients (from Marsh, Sherwood & Howard, 1986). Cryopreserved ring-stage infected cells are thawed and cultured until the mid-to late-trophozoite stage one for this assay.

2) Cell surface binding of antibodies has also been detected by agglutination. Experiments with *P. knowlesi* infected cells utilized serum induced macroagglutination method with purified infected cells (Eaton, 1938; Brown & Brown, 1965; Barnwell et al., 1983). *P. falciparum* infected *Aotus* erythrocytes are also agglutinated by immune serum (Sherwood et al., 1985). For studies on isolates from *P. falciparum*-infected children we applied the micro-agglutination method which employs ethidium bromide to stain parasite nuclei (Sherwood et al., 1985; Marsh, Sherwood & Howard, 1986). Clumps of antibody-agglutinated cells can then be visualized by fluorescence microscopy without the necessity for purification of the infected cells. Agglutinated cells can also be detected by phase-contrast or polarized-light microscopy (Marsh, Sherwood & Howard, 1986) but under these conditions quantitation of the number of agglutinates is more difficult. The micro-agglutination method requires only the addition of primary antibody and does not require cell washing. It is therefore more simple to perform with many samples than the indirect-immunofluorescence technique. However, one cannot assess whether infected cells that are not in agglutinates are non-reactive with the particular antiserum (i.e. a negative subpopulation) or whether they are simply non-agglutinated due to mechanical or kinetic constraints.

3) The third method for detection of antibody interaction with the surface of infected cells is the capacity of sera to block or reverse cytoadherence. *In vitro* attachment of *P. falciparum*-infected erythrocytes to endothelial cells or the C32 line of amelanotic melanoma cells can be blocked by *Aotus* or human sera from immunized hosts (Udeinya et al., 1983). This presumably reflects binding of antibodies to cell-surface antigens in close proximity to the molecule(s) involved in cytoadherence. *In vitro* cytoadherence could also be reversed if antibody was added to bound cells (Udeinya et al., 1983). Importantly, passive transfer of anti-malarial *Aotus* serum to an infected animal reversed cytoadherence as well, since a pulse of mature infected erythrocytes

were flushed from their sites of vascular sequestration into the peripheral blood (David et al., 1983). These results suggest that the interaction between the ligand/receptor complex of infected cell/endothelial cell is of relatively low affinity.

Cell surface reactivity of antisera has also been studied by immunoelectronmicroscopy. Early studies demonstrated binding of antibodies to knobs on the surface of *P. falciparum* infected cells from *Aotus* monkeys or *in vitro* cultures (Kilejian, Abati & Trager, 1977; Langreth & Reese, 1979). Antibodies did not bind to the surface membrane of infected cells between knobs. In contrast, rhesus monkey antisera which reacted with *P. knowlesi* infected cells were shown by immunoelectronmicroscopy to bind evenly to the entire surface membrane (Hommel & David, 1981). Recently we have continued this approach using *P. falciparum*-infected cells cultured directly from a Gambian malaria patient and acute/convalescent homologous sera or heterologous West African sera from adults (Howard, Aikawa & Marsh, in preparation). Bound antibody was detected using either gold conjugated-goat anti-human IgG or gold conjugated-Protein A. These results are summarized below.

C. *In vitro* assays for cytoadherence – *In vitro* attachment assays have been developed to study the cytoadherence property of *P. falciparum*-infected erythrocytes.

1) Human endothelial cells were obtained from umbilical cords by collagenase treatment and cultured as an attached monolayer on plastic (Udeinya et al., 1981). When *P. falciparum*-infected blood from *Aotus* monkeys or infected human blood from culture was overlayed onto the endothelial cells then gently washed, adherent cells could be examined by Giemsa staining and light microscopy. Greater than 90% of the adherent cells were trophozoite-infected erythrocytes, whereas the initial blood contained < 5% parasitized cells (Udeinya et al., 1981). The specificity of this interaction was further demonstrated as follows: knobless (K^-) parasites that do not sequester *in vivo* also failed to attach *in vitro*; uninfected erythrocytes from normal or infected *Aotus* monkeys/humans failed to attach; infected cells and uninfected erythrocytes did not attach to human dermal fibroblasts (Udeinya et al., 1981).

2) Several cultured human cell lines were screened for their capacity to attach *P. falciparum*-infected erythrocytes (Schmidt et al., 1982). Binding was observed to a human amelanotic melanoma cell line (C32 cells), amnion epithelial cells and human aortic endothelial cells. The C32 cells were chosen as a convenient cell line for routine *in vitro* assays of cytoadherence. Attachment of infected cells to C32 melanoma cells paralled attachment to endothelial cells in several respects: adhesion was parasite stage-specific since only mature asexual stages of infected cells bound; *P. vivax*-infected erythrocytes which do not sequester *in vivo* failed to attach to either cell type *in vitro*; K^- *P. falciparum* parasites also failed to bind to the C32 melanoma cells; finally, transmission electron microscopy showed that attachment to C32 cells was mediated by the knob protusions on the infected cells (Schmidt et al., 1982).

3) Most recently, an alternative *in vitro* test for cytoadherence has been developed utilizing the human platelet-derived glycoprotein thrombospondin (Roberts et al., 1985). Since thrombospondin immobilized on plastic will specifically attach K^+ *P. falciparum*-infected erythrocytes, it has been proposed that this host protein may mediate the cytoadherence of infected cells to venular endothelium (Roberts et al., 1985). It should be noted that although this study employed thrombospondin purified from thrombin-activated human platelets, thrombospondin has been shown to be synthesized and secreted from cultured endothelial cells (McPherson, Sage & Bornstein, 1981; Mosher, Doyle & Jaffe, 1982). A schematic drawing of the structure of thrombospondin is shown in Fig. 2.

Thrombospondin was purified from human platelets and attached to bacteriological plastic dishes as 10μl droplets containing 10-200μg/ml of protein (Roberts et al., 1985). The protein is efficiently adsorbed to the plastic and can be assayed by immunoreactivity with monoclonal antibodies or specific rabbit antisera. The specificity of attachment of K^+ *P. falciparum*-infected cells was parallel to the earlier studies with endothelial cells and the C32 melanoma cell line: uninfected erythrocytes did not stick to immobilized thrombospondin; K^- infected cells did not attach nor did immature infected cells (rings) of K^+ parasites. In the thrombospondin study additional controls were performed using other purified mouse or human proteins often involved in cell-cell adhesion. *P. falciparum*-infected erythrocytes did not attach to immobilize mouse laminin, human Factor VIII, human plasma vitronectin or fibronectin (Roberts et al., 1985).

In view of the difficulties in obtaining human umbilical cord endothelial cells and considerable variability in the adhesiveness of individual fresh preparations, the C32 melanoma cells provide an easier cellular assay for cytoadherence of *P. falciparum*-infected erythrocytes. Adherence to thrombospondin is also an easy and rapid method, the most time-consuming aspect of these methods being the light microscopy analysis of bound infected cells.

HUMAN PLATELET THROMBOSPONDIN

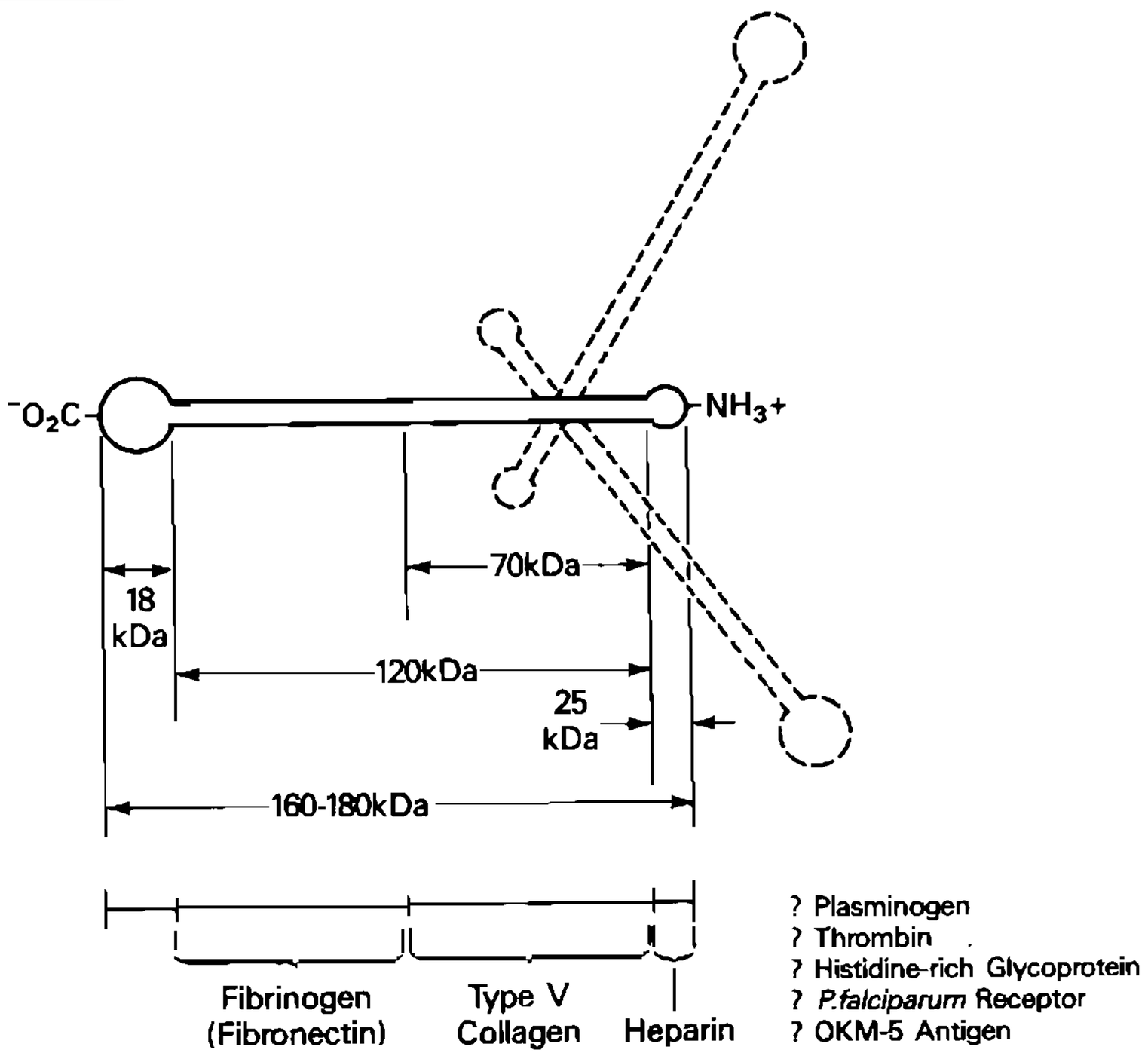

Fig. 2: A schematic drawing of human platelet thrombospondin (kindly provided by Dr. D. Roberts) which summarises some of the properties of this protein. The native molecule is a trimer of three peptide claims each with a denatured $M_r \sim 180{,}000$. The disulphide linkages which link the three chains are not shown here. The arrangement of each chain with globular regions at each terminus has been proposed by Lawler et al., 1985, on the basis of law angle rotary shadowing electron microscopy of individual molecules, coupled with structural mapping of the molecule by controlled proteolysis and reactivity with monoclonal antibodies: The NH_2-terminal globular structures are very close together and include the region of each peptide capable of binding heparin. Other domains of the molecule which bind Type V collagen and fibrinogen are also indicated. Several additional host molecules have been shown to bind to thrombospondin (eg. plasminogen, thrombin, histidine-rich glycoprotein) but their binding sites are unknown.

D. Variant Antigens on Infected Erythrocytes – Cloned asexual *P. knowlesi* parasites were shown to undergo antigenic variation at the infected cell surface by both agglutination and immunofluorescence assays (Barnwell et al., 1983). Thus, infected cells of clone Pk1(A^+) were agglutinated by sera from monkeys infected with this parasite, but were not agglutinated by sera from animals infected with the clone Pk1(B^+)1^+ which was derived from Pk1(A^+) under immune pressure *in vivo*. Conversely, sera from monkeys infected only with Pk1(B^+)1^+ agglutinated cells of this phenotype, but not the Pk1(A^+) cells. These experiments (reviewed by Howard, 1984) demonstrated that a cloned malaria parasite has the genetic capacity to express multiple and antigenically non-cross-reactive epitopes of a cell-surface molecule. Immunochemical studies showed that the surface variant antigen was a malarial protein of M_r 180,000-230,000 in different clones. The antigen was specifically immunoprecipitated from a given parasite only by antisera capable of agglutinating infected erythrocytes of that phenotype (Howard, Barnwell & Kao, 1983; Howard, Kao & Barnwell, 1983).

Similar studies were performed with *P. falciparum* infections in squirrel monkeys. During multiple repeated infections of monkeys with non-cloned *P. falciparum* isolates, peaks of parasitemia could sometimes be recovered which expressed a different cell surface phenotype as defined by the immunofluorescence assay (Hommel, David & Oligino, 1983). A difference in cell surface phenotype was also demonstrated when cloned *P. falciparum* parasites were passaged between intact and splenoctomized squirrel monkeys (Hommel, David & Oligino, 1983). Although it was

not clear whether *P. falciparum* clones could undergo antigenic variation *in vivo* as in the *P. knowlesi* model, it was clear that antigenic diversity of cell surface phenotype could also contribute to evasion of variant-specific immune responses in *P. falciparum*. Extensive studies on the *P. knowlesi*/rhesus monkey system had demonstrated that the capacity of this parasite to express different infected cell surface phenotypes allowed it to evade immune responses against earlier variants in a chronic or rechallenge infection (Brown & Brown, 1965; Brown, 1973; Barnwell et al., 1983).

Antigenic diversity at the surface of *P. falciparum*-infected erythrocytes was also evident from studies on the blockade or reversal of *in vitro* cytoadherence with *Aotus* monkey antisera. Antisera from monkeys infected or immunized only one or two times with uncloned *P. falciparum* isolates were shown to specifically block the cytoadherence of infected cells from the same isolate when preincubated with the infected cells (Udeinya et al., 1983). Such antisera did not block attachment of other laboratory isolates. In contrast, sera from repeatedly immunized animals would block cytoadherence of several different isolates. Similar isolate-specific interaction of *Aotus* antibodies with the surface of infected cells was obtained by addition of sera after the cells had attached (Udeinya et al., 1983). In this case cytoadherence was reversed with sera from animals infected with the same isolate. These results with antibodies suggest that an isolate-specific epitope(s) is at or near the surface molecule(s) involved in cytoadherence. Due to the relatively large size of antibody, steric block of the presumed erythrocyte receptor for the host cell need not imply direct interaction of the blocking or reversing antibody with the receptor itself.

Once a panel of *Aotus* sera had been characterized in this fashion for their capacity to block/reverse cytoadherence, it was used to probe the molecular identify of the infected cell surface antigen bearing isolate specific determinants. We identified a malarial protein of very large size ($M_r \sim 300{,}000$) which displayed size heterogenity with different isolates and, most importantly, antibody binding correlated with the activity of these sera in cytoadherence blockade/reversal (Leech et al., 1984). Thus, those sera which blocked attachment also immunoprecipitated the ^{125}I-surface labeled molecule whereas those without blocking activity did not. The protein could also be labeled by biosynthetic uptake of ^{35}S-methionine or ^{3}H-amino acids and shown to interact with blocking/reversing antibody at the surface of intact infected cells (Leech et al., 1984).

Other studies correlated the expression of this protein with expression of knobs on the infected erythrocyte membrane (Aley, Sherwood & Howard, 1984). ^{125}I-surface labeling of K^+ *Aotus*-infected erythrocytes identified a size heterogenous $M_r \sim 300{,}000$ protein that was absent from the surface of K^- *Aotus*-infected cells or uninfected erythrocytes. We could also identify an analogous surface protein on trophozoite-infected erythrocytes cultured directly from blood of Gambian malaria patients (Aley, Sherwood & Howard, 1986). With different patients the size of this protein varied ($M_r \sim 250{,}000$-$350{,}000$) and in some cases multiple bands were observed.

Most recently we have investigated the antigenic diversity of the surface antigen(s) on *P. falciparum*-infected erythrocytes from Gambian malaria patients using acute and convalescent sera from the same patients (Marsh & Howard, 1986). The serological reactivity of this serum panel with the surface of infected erythrocytes was tested by the micro-agglutination and indirect immunofluorescence assays described earlier. Fig. 3 shows that in each of 10 cases the acute serum reacted weakly, or generally not at all, with the infected cells from the same patient (i.e. homologous reactions). In *all* cases the homologous convalescent serum reacted strongly with the surface of infected cells from the same patient but not with uninfected cells. Often, neither the acute nor convalescent sera would react with the surface of infected erythrocytes from the other patients (i.e. heterologous reactions). Hence, with this particular panel of sera and isolates the reactions of convalescent sera with the homologous parasites was generally isolate-specific. In other cases (eg. 188, 189) both the acute and convalescent sera reacted with infected erythrocytes from some but not all heterologous infections. Importantly, the acute and convalescent sera were of identical titer with the heterologous infected cells when this occured. We concluded that in these examples an isolate-specific homologous response developed during convalescence and that these individuals had been immunized previously with parasites which shared identical or cross-reactive surface phenotypes with some members of the panel of parasites.

The isolate-specific human antibodies were IgG, since they were reactive with anti-Fc secondary antibodies in the immunofluorescence assay (and not with anti-μ antibodies) and were depleted by passage of serum through Protein A Sepharose (Marsh & Howard, 1986). This study demonstrated that there is extensive antigenic diversity at the infected cell's surface in the natural population of human *P. falciparum* parasites: from 10 patients presenting to the MRC clinic over a two week period 10 different serological phenotypes were defined by the matrix of acute/convalescent serum reactivities (see Fig. 3). It was also remarkable that in each patient an isolate-specific antibody against the infected cell's surface developed during the three week convalescent

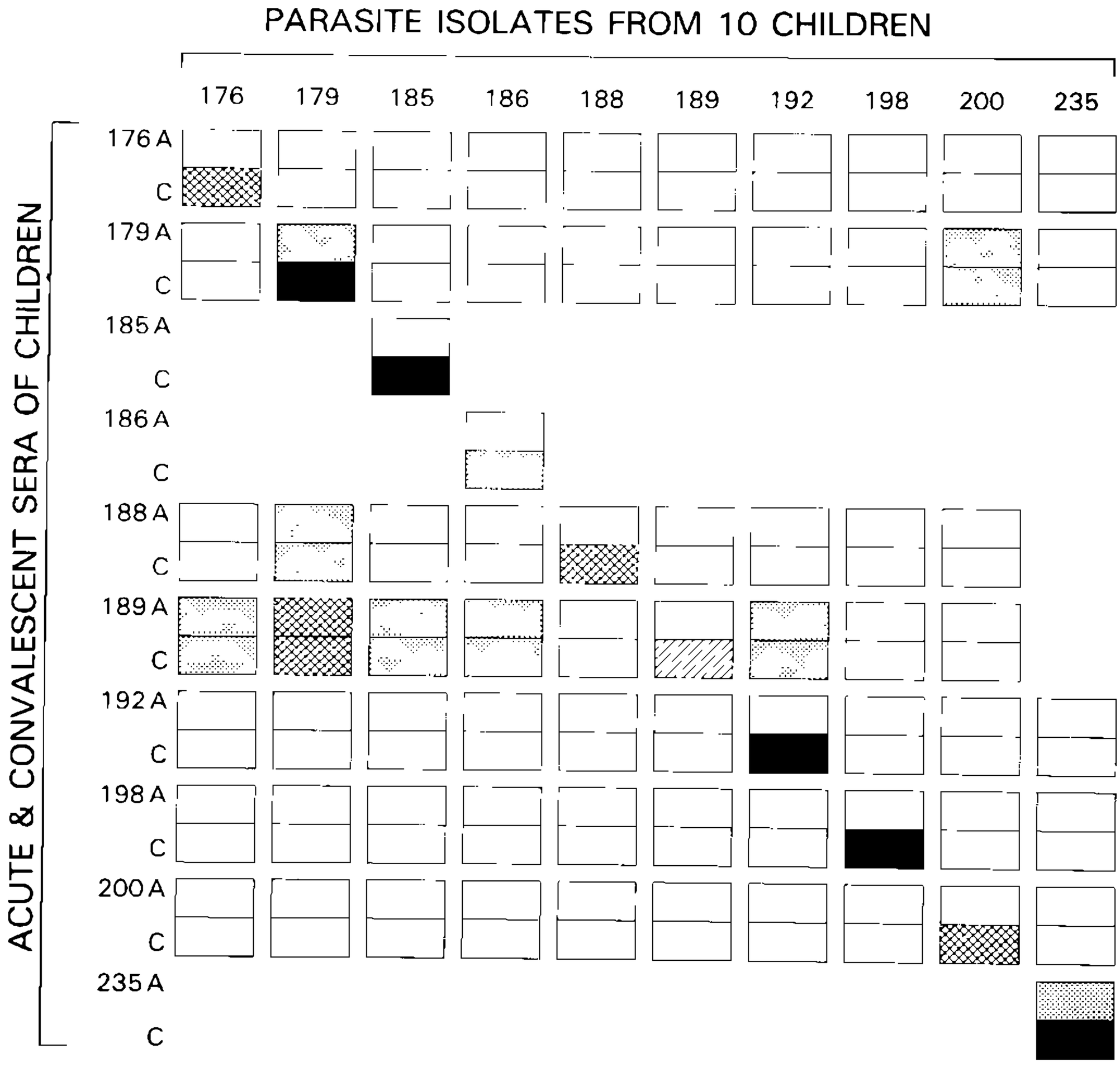

Fig. 3: Interaction of serum antibodies from Gambian malaria patients with the surface of *P. falciparum*-infected erythrocytes from the same patients. Antibody binding measured semi-quantitatively by titration of sera in the micro-agglutination test (from Marsh & Howard, 1986).

period after chloroquine treatment. The isolate-specific epitope(s) at the cell surface appear to be highly immunogenic.

The subcellular localization of the isolate-specific antigen has been revealed using immuno-electronmicroscopy with rabbit anti-Fc antibody and Protein A gold (Howard, Aikawa & Marsh, in preparation). Fig. 4 shows schematically that all of the Protein A-gold was deposited at the surface of knobs but not at the intervening areas of erythrocyte membrane. The variant-specific epitope(s) recognized by homologous convalescent human serum are localized at knobs.

Immunoprecipitation and cell surface labeling studies are underway with the panel of human sera and parasites from the same patients to attempt to identify the *P. falciparum* surface molecule responsible for this antigenic diversity. By analogy with our studies on *Aotus* infected erythrocytes (Leech et al., 1984), we anticipate that the size heterogeneous, $M_r \sim 300{,}000$ surface protein on these infected human erythrocytes (Aley, Sherwood & Howard, 1986) will be specifically immunoprecipitated only by those sera which contain surface-reactive antibodies. We note that there are several similarities in properties of the *P. knowlesi* variant antigen as defined with cloned parasites and the *P. falciparum* surface antigen identified with *Aotus* and human infections: both molecules are relatively large ($M_r \sim 200{,}000$ *versus* $M_r \sim 300{,}000$) and size heterogeneous with different parasites; both molecules are extracted from infected erythrocytes by agents which disrupt the membrane cytoskeleton (eg. SDS and NaOH) but are not extracted by neutral detergents; both molecules (as ^{125}I-labeled protein) are sensitive to exogenous trypsin with intact cells; both molecules exhibit antigenic heterogeneity, within the progeny of clones for *P. knowlesi* and with non-cloned isolates of *P. falciparum*. It is therefore likely that the antigenic diversity of

LOCALIZATION OF *P.falciparum* ANTIGENS ON INFECTED HUMAN ERYTHROCYTES BY IMMUNOELECTRONMICROSCOPY

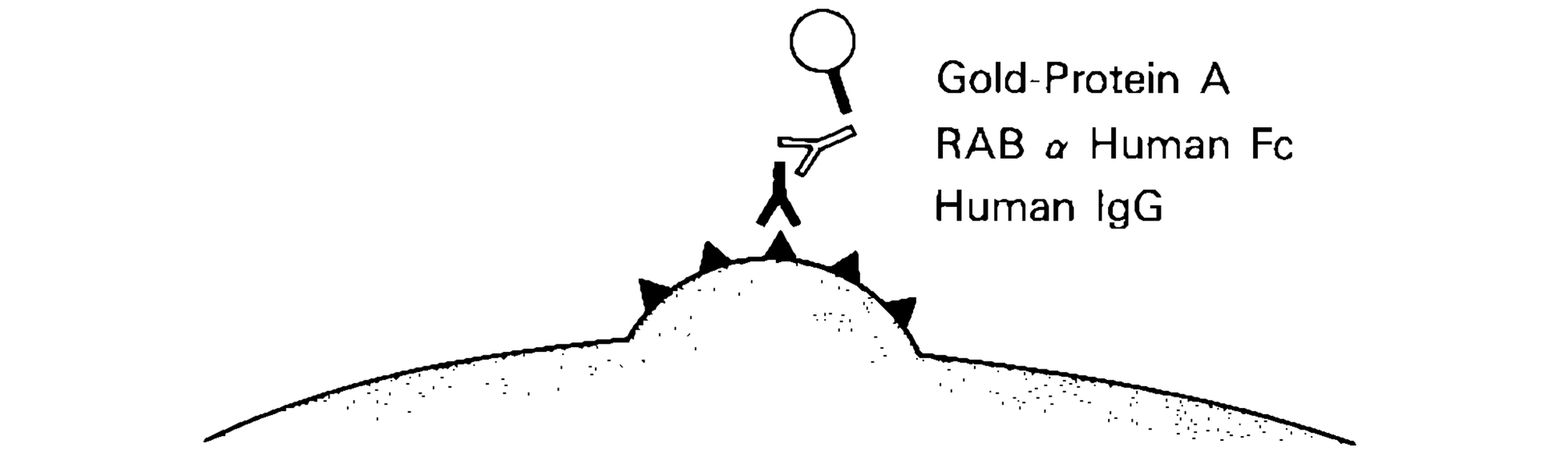

Fig. 4: A schematic drawing of the localization of isolate-specific and conserved surface antigens on infected erythrocytes using Protein A-gold and immunoelectronmicroscopy. Protein A-gold was localized at the surface of knobs but not on the infected cell surface membrane between knobs, nor on uninfected cells (Howard, Aikawa & Marsh, in preparation).

natural *P. falciparum* infections at the infected erythrocyte membrane reflects the same process for evasion of variant-specific host immune responses as with *P. knowlesi*. Thus, the immune system of the host responds rapidly to a particular infection by production of variant-specific antibodies which recognize the surface of infected erythrocytes. These variant-specific responses are evaded by the parasite's capacity to express an alternative antigenic form of the surface antigen. It is also possible that individual asexual parasites of *P. falciparum* do not all have the capacity to switch to another phenotype. We emphasize that studies on non-cloned populations do not allow one to exclude the possibility that parasites of different phenotypes are present in the original inoculumn and that these organisms are independently derived genetically. Even were this the case, the demonstration that each of 10 different isolates from Gambian children were phenotypically distinct (Marsh & Howard, 1986) suggests that isolate-specific immune responses against previous infections would generally be ineffective against subsequent inocula.

While the demonstration of antigenic variation and antigenic diversity at the infected erythrocyte membrane indicates a parasite mechanism for evasion of specific immune responses, it does not explain why the parasite evolved the capacity to express a cell surface antigen in the first place. Why does the asexual malaria parasite express a highly immunogenic malarial protein on infected cells which elicits immune responses that it must then evade? This problem is discussed next.

E. Conserved Antigens on Infected Erythrocytes – Functional changes in the surface membrane properties of malaria-infected erythrocytes would imply the expression of molecular structures that should be conserved in order to retain the particular functional property. If such functional structures were sterically accessible to antibody when expressed on intact infected cells, they would represent antigenic epitopes that would be antigenically conserved in all parasites, regardless of phenotypic diversity and variation in other properties. Thus, expression of malarial enzymes, ligand-receptor molecules or transport proteins on infected erythrocytes would be expected to lead to the expression of antigenically conserved surface antigens. Given that the malaria parasite undergoes a complex series of differentiation steps and asexual multiplication roughly 30-fold within the infected erythrocyte, it is anticipated that multiple functional changes

must occur in the properties of the erythrocyte surface membrane. It would be surprising if an erythrocyte membrane, designed for exchange of gases, anions and cations, lactate and glucose could support the metabolic traffic required of an intracellular malaria parasite. In support of this argument, there is now considerable evidence for marked changes in the permeability properties of the infected erythrocyte membrane, including increased permeability to many low-molecular weight anions, neutral and zwitterionic compounds (Ginsburg et al., 1983; Kutner, Ginsburg & Cabantchik, 1983), and amino acids such as glutamine that are not taken up at all by uninfected erythrocytes (Elford et al., 1985). Although biophysical data has been collected on these altered membrane properties, there is no information on the molecular structure of the components involved and no information on their antigenicity.

With some malaria species, infected erythrocytes containing mature asexual parasites also aquire another functional property lacking from uninfected erythrocytes, namely the capacity to cytoadhere to endothelial cells lining blood vessels. This cytoadherence property implys the expression of a receptor/ligand on infected erythrocytes, hence the expression of a functional structure that might be antigenically conserved. This cytoadherence property is expressed, for example, by infected erythrocytes of *P. falciparum, P. knowlesi, P. fragile* and *P. coatneyi* (the latter two are simian malarias analogous to *P. falciparum*), but is not expressed by *P. malariae* or *P. vivax*. Cytoadherence leads to the sequestration of mature infected cells (but not ring-stages) from the peripheral circulation. Several ideas have been put forward to explain why some malaria species sequester: attachment to venous capillaries may place the parasite in an environment of relatively low O_2 concentration in which *in vitro* parasite growth is known to be favored (Trager & Jensen, 1976); attachment may prevent passage of mature parasitized erythrocytes through organs such as spleen and liver where a variety of localized specific and non-specific immune responses could destroy mature infected cells; the blood concentrations of essential metabolites differ in various tissues and may be optimal for parasite growth in the organs were sequestration occurs. As yet there is no simple unifying explanation for the fact that different species which do sequester may attach to different tissues in the same host (eg. *P. knowlesi versus P. fragile* in the rhesus monkey). Nor do we know why other species fail to sequester in hosts in which another parasite does sequester (eg. *P. vivax* and *P. malariae versus P. falciparum* in human infections). The importance of cytoadherence for *P. falciparum* specifically is clear from the fact that laboratory-derived K^- variants in *Aotus* monkeys lacking expression of the functional property of cytoadherence are avirulent (Lanners & Trager, 1984; Green et al., 1985; Langreth & Peterson, 1985). There is now serological evidence for expression of an antigenically conserved epitope on the surface of *P. falciparum* infected erythrocytes from Gambian malaria patients and indirect evidence which links this conserved epitope to the cytoadherence property. The same panel of *P. falciparum*-infected erythrocytes that was shown to be serologically diverse in surface antigen phenotype using sera from the acutely-infected patients (see Fig. 3), was also shown to express a conserved surface antigen using sera from Gambian adults (Marsh & Howard, 1986) Fig. 5. The majority of Gambian adults are clinically immune since they do not suffer acute symptoms even though they may carry low level asexual infections. Sera from a panel of adults were tested in the micro-agglutination test for cell-surface reactivity with the infected cells (Fig. 5). Most of the sera contained antibodies which recognized all of these isolates. Interestingly, serum from one adult who lived in an urban area and suffered an acute *P. falciparum* attack (number 22) was negative for surface-reactive antibodies. We then tested whether this broad sero-reactivity represented the accumulation of a collection of isolate-specific antibodies some of which happen to cross-react with the panel of isolates, or whether the immune adults posses a pan-specific antibody (Marsh & Howard, 1986). Antibodies from a high titer cross-reactive adult serum were adsorbed onto purified infected erythrocytes from one child and after washing to remove unbound antibodies, bound antibodies eluted with isotonic acid solution. The eluted antibodies reacted in the micro-agglutination test with the surface of infected cells from several other isolates, indicating that the antibodies are pan-specific. Furthermore, exhaustive absorption of this adult serum with the purified infected erythrocytes not only totally depleted sero-reactivity with cells of the same isolate, but also abolished reactivity with other isolates. Thus, sera of adult 'immune' Gambians contains an antibody capable of binding to the cell surface of infected erythrocytes from different patients. More recently we have shown that 'immune' adult sera from Nigeria, The Gambia and Ghana all recognize a cell surface antigen on the surface of infected erythrocytes from The Gambia or Ghana (Howard, Rock & Marsh, in preparation).

The link between expression of the antigenically conserved epitope(s) and cytoadherence is as follows. When Gambian isolates collected directly as rings are cultured for one cycle of growth in the original erythrocytes, the mature asexual stages which develop express the capacity to cytoadhere *in vitro*. They also express the surface antigen(s) recognized by isolate-specific antibodies

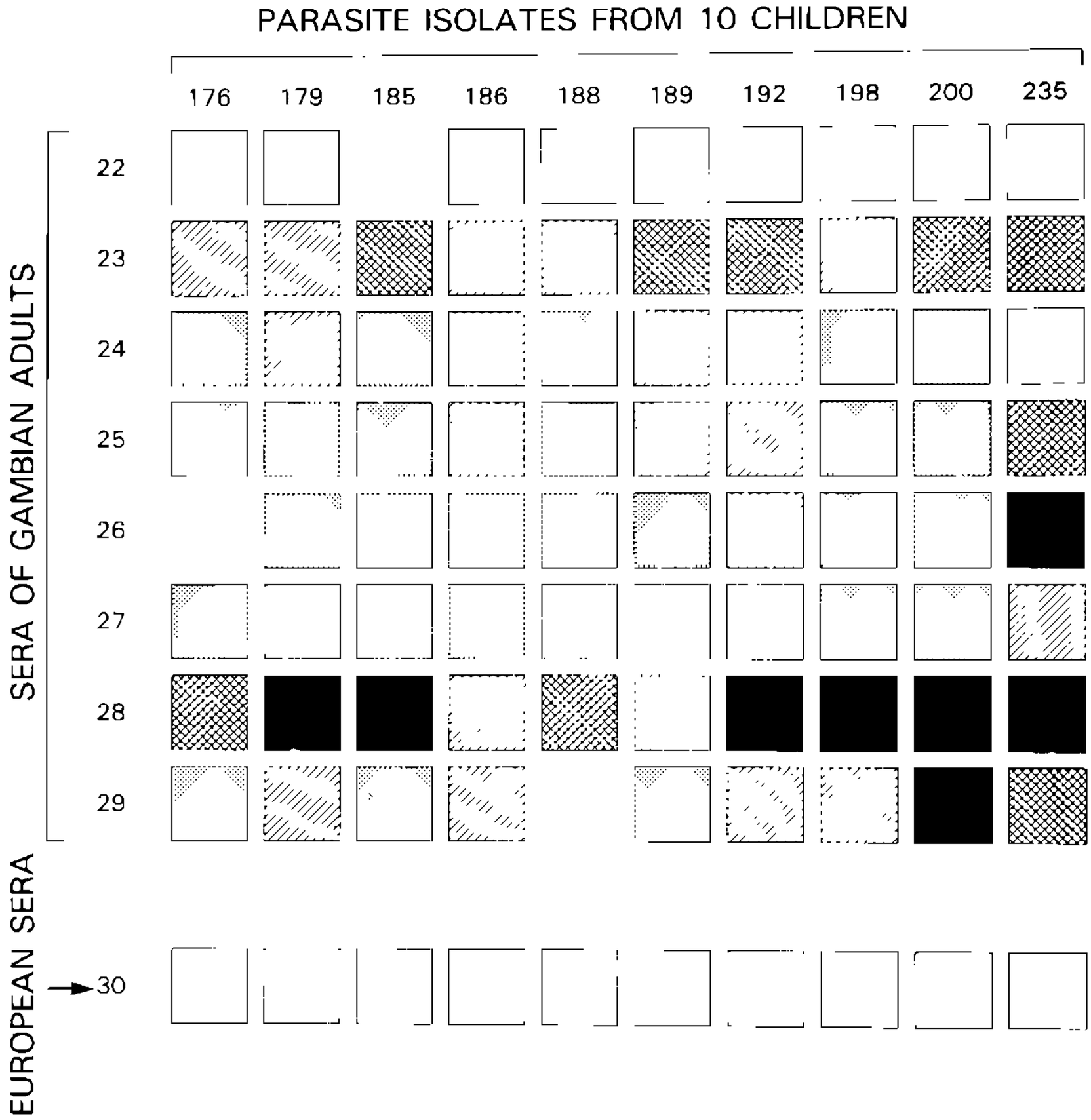

Fig. 5: Interaction of serum antibodies from adult Gambians with the surface of *P. falciparum*-infected erythrocytes from the same panel of isolates described in Fig. 4. Antibody binging measured semiquantitatively by titration of sera in the micro-agglutination test (from Marsh & Howard, 1986).

from homologous convalescent sera and the conserved antigen recognized by adult sera. However, after one or two cycles of reinvasion (ie $\leqslant 4$ days) these parasites no longer express the capacity to cytoadhere, nor do they express the diverse or conserved surface antigen(s). No morphological differences are apparent in the surface membrane knobs expressed by the original cytoadherent parasites or *in vitro* derived non-adherent organisms (Marsh & Howard, 1986). Since as discussed above the functional property of cytoadherence is unlikely to be mediated by an antigenically diverse structure, we hypothesize that the concomitant loss of the antigenically conserved surface antigen and cytoadherence property reflect the same molecular change in the cell membrane.

F. The Host Cell Receptor for Cytoadherence of *P. falciparum*-Infected Erythrocytes – Two independent studies have recently described host cell molecules with properties expected for the endothelial cell surface receptor recognized by *P. falciparum*-infected erythrocytes (Barnwell, Ockenhouse & Knowles, 1985; Roberts et al., 1985).

Our own work identified pure thrombospondin as a candidate host receptor based on the specific attachment of infected erythrocytes to thrombospondin-coated plastic (see Section 3 above). Fig. 6 shows the critical results which established a correlation between attachment to C32 melanoma cells and attachment to thrombospondin. K^+ *P. falciparum* parasites from *Aotus* monkeys which sequester *in vivo* attached to C32 cells and thrombospondin. K^- parasites which do not sequester *in vivo* did not attach in either case. With a variety of K^+ parasites adapted to

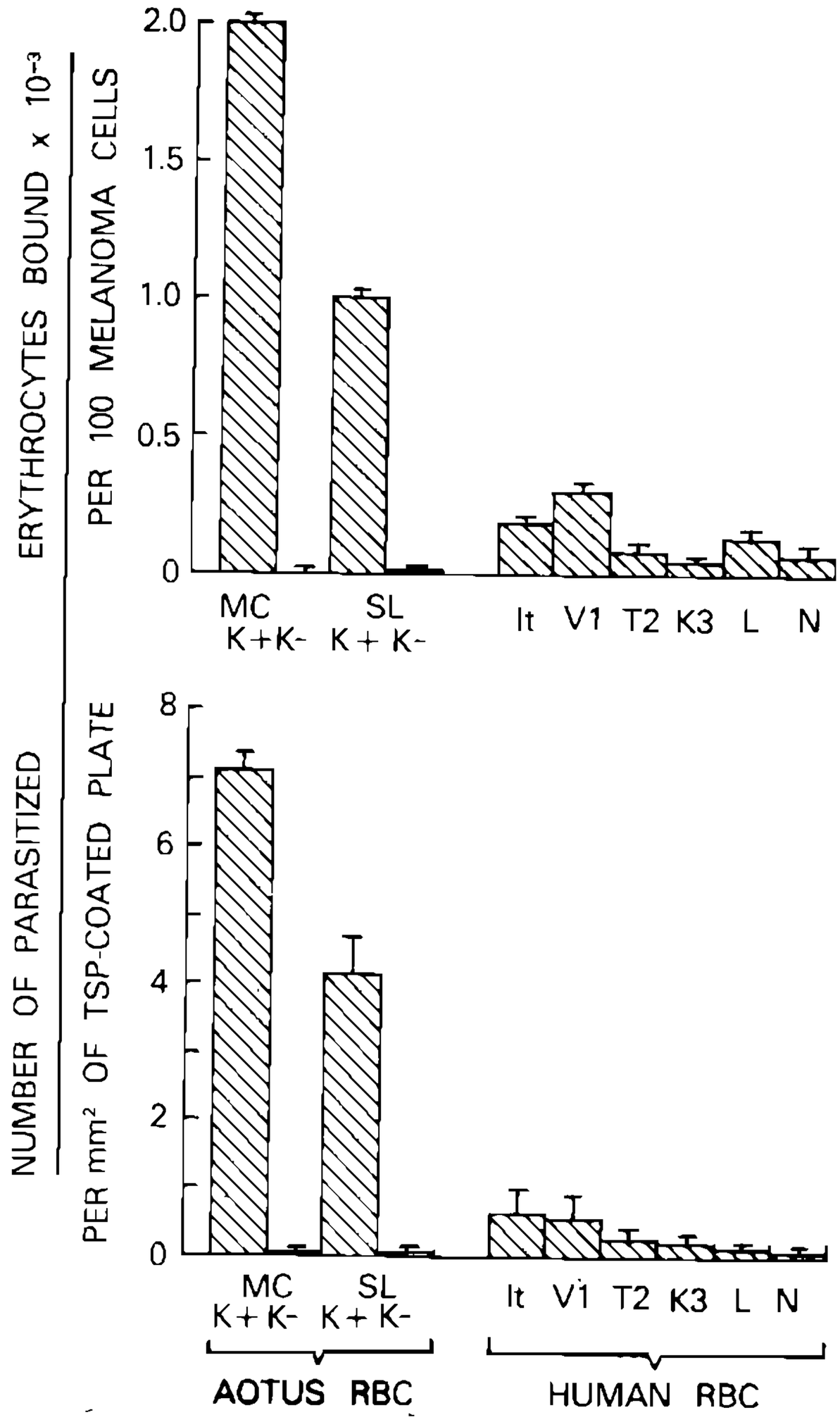

Fig. 6: Binding of *P. falciparum* parasites in *Aotus* or human erythrocytes to immobilized thrombospondin (TSP) and C32 melanoma cells. MC, Malayan Camp strain; SL, St. Lucia strain; It, clone ItG_2F6 from Brazilian strain It; V1, Vietnam/Cambodja strain; T2, Thailand strain; K3, Kenya strain; L & N, Liberia and Nigeria strains. Binding of unparasitised cells to thrombospondin-coated plates was 86 ± 40 cells mm^{-2} (modified from Roberts et al., 1985).

continuous growth in human erythrocytes *in vitro*, low levels of attachment were observed to C32 cells and immobilized thrombospondin. None of these parasites attached to laminin, Factor VIII, vitronectin or fibronectin (Roberts et al., 1985).

Additional evidence for the specificity of this attachment of infected cells to thrombospondin was obtained by competition experiments summarized in Fig. 7. Addition of soluble thrombospondin inhibited the binding of infected cells to C32 melanoma cells and immobilized thrombospondin in a dose-dependent manner. Similarly, addition of increasing levels of rabbit antibody to thrombospondin also inhibited the degree of attachment. Purified rabbit IgG from control animals had no inhibitory effect. A monoclonal antibody specific for thrombospondin also inhibited attachment (Fig. 7).

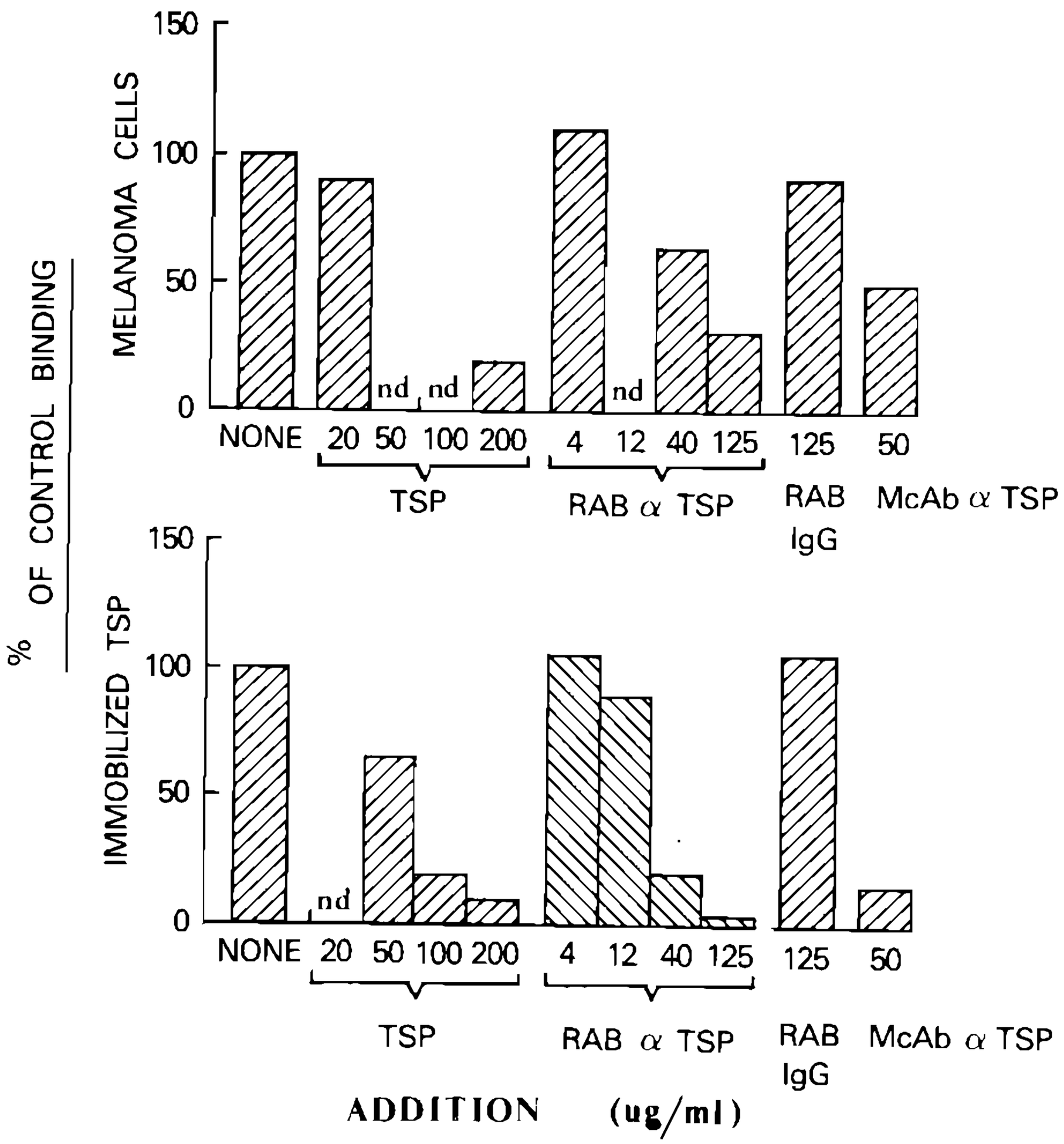

Fig. 7: Inhibition of attachment of *P. falciparum*-infected erythrocytes (Malayan Camp K^+ strain in *Aotus* erythrocytes) to immobilized thrombospondin (TSP) and melanoma cells using soluble thrombospondin and anti-thrombospondin antibodies. Infected cells were preincubated with various levels of soluble thrombospondin, rabbit antibody to thrombospondin, control rabbit IgG or a mouse monoclonal antibody (McAb) to thrombospondin, then added to C32 cells or thrombospondin attached to plastic. nd: not determined (modified from Roberts et al., 1985).

Thrombospondin is a major glycoprotein of platelet α-granules and has been much studied for its role in thrombin-mediated platelet aggregation. Thrombin activates secretion of this protein, after which it appears to be involved in cross-linking platelets through binding the membrane associated receptor(s) (Lawler, Chao & Fang, 1977; Phillips, Jennings & Prasanna, 1980).

Thrombospondin is also synthesized and secreted by a variety of cell types including cultured human umbilical vein and bovine aortic endothelial cells (McPherson, Sage & Bornstein, 1981; Mosher, Doyle & Jaffe, 1982), fibroblasts from several tissues (Raugi et al., 1982; Jaffe et al., 1985), monocytes and macrophages (Jaffe, Ruggiero & Falcone, 1985). In several of these examples thrombospondin is secreted as a soluble protein which then is incorporated into the extracellular matrix. Thus, we suggested that attachment of *P. falciparum*-infected erythrocytes to endothelial cells could be mediated by thrombospondin, but would also require another membrane-associated protein to anchor the soluble thrombospondin (Roberts et al., 1985). Support for this suggestion has come from the independent studies of Barnwell and collegues (Barnwell, Ockenhouse & Knowles, 1985; Asch et al., 1986) on the nature of the host receptor for infected erythrocytes.

The distribution of cell surface reactivity for a monoclonal antibody (OKM 5 from Ortho Diagnostic Systems Inc.) with various cell types has been shown to parallel their capacity to cytoadhere *P. falciparum*-infected erythrocytes (Barnwell et al., 1985). OKM 5 antibody reacts with the surface of monocytes, human endothelial cells and platelets, and importantly in this connection, with the surface of the cytoadherent C32 melanoma cell line but not with the surface of the G361 non cytoadherent melanoma cell line. Monocytes have also been shown to cytoadhere *P. falciparum*-infected erythrocytes (Ockenhouse, Schulman & Shear, 1984). The surface ^{125}I-labeled protein on C32 cells that was specifically immunoprecipitated by OKM 5 antibody had the same M_r (~ 88,000) as that identified previously with endothelial cells. Pretreatment of adherent cells with monoclonal antibody OKM 5 inhibited attachment of infected erythrocytes (> 92% inhibition with 2μg antibody/ml). Other monoclonal antibodies against surface antigens on melanoma cells and endothelial cells had no inhibitory effect. Furthermore, attachment of *P. falciparum*-infected erythrocytes to C32 melanoma cells, endothelial cells and monocytes was reversed by > 83% with OKM 5 but not with other monoclonal antibodies. This study also showed that soluble 1M KCl extracts of C32 melanoma cells could inhibit cytoadherence of infected erythrocytes to both melanoma cells and monocytes. This inhibitory capacity was totally reversed when OKM 5 antibody, but not other antibodies, was added to the soluble extract (Barnwell, Ockenhouse & Knowles, 1985).

More recently, Asch et al., 1986 have shown that thrombospondin attaches specifically to the OKM 5 antigen. ^{125}I-thrombospondin binding to various cell surfaces was inhibitable with OKM 5 antibody. Immunoaffinity purification of the OKM 5 antigen yielded a single M_r 88,000 glycoprotein band which bound ^{125}I-thrombospondin after transfer to nitrocellulose (Asch et al., 1986).

From the combined data on thrombospondin and the OKM 5 antigen several hypotheses, none necessarily exclusive, can be derived for the host cell component of cytoadherence. Does thrombospondin or the OKM 5 antigen each separately bind the infected erythrocyte surface receptor? Do these molecules share a common region recognized by infected cells? Must thrombospondin be coupled to the OKM 5 antigen for it to play a role in cytoadherence? Does the cell surface distribution and affinity of these putative receptors for infected erythrocytes alter on endothelial cells from different tissues in different individuals? Perhaps the special symptoms of cerebral malaria originate from peturbation of the number of one or other of these host cell ligands in brain capillaries. Another potential advantage from these studies on the host cell ligand(s) for cytoadherence may be the possibility of affinity purification of the functional, cytoadherent portion of the infected cell surface receptor.

G. Variant and Conserved Antigens on the same Molecule? – Our understanding of the surface protein expressed on *P. falciparum*-infected erythrocytes is summarized in schematic form in Fig. 8. This model is hypothetical and serves only as a convenient pictorial summary of the following essential points:

i) To date we have identified only one new malarial protein on the surface of K^+-infected cells that is absent from the surface of K^--infected cells. The protein of $M_r \sim 300{,}000$.

ii) From its detergent solubility properties it appears to transverse the lipid bilayer and attach to submembrane cytoskeleton. This portion of the molecule would probably be antigenically and structurally conserved since it mediates an attachment function (protein-protein interaction).

iii) The trypsin sensitivity of the external portion of the protein (which can be ^{125}I-labeled by cell-surface radiolabeling) correlates with the trypsin sensitivity of the cytoadherence property.

iv) The capacity of antisera to block or reverse cytoadherence *in vitro* correlated with their capacity to react with the biosynthetically labeled protein at the cell surface.

v) From points (i), (ii) and (iii) we concluded that this protein bears a domain that mediates cytoadherence to endothelial cells. Such a functional domain should be antigenically conserved. Recently we identified epitope(s) on *P. falciparum*-infected erythrocytes from Gambian children that are antigenically conserved in all isolates examined. These epitopes may correlate with the functional portion of the molecule shown here attached to thrombospondin. This domain appears to be poorly immunogenic since children who suffer acute *P. falciparum* infections do not make antibodies (IgG or IgM) to the conserved epitope(s). In contrast, sera from immune adults who have been repeatedly infected do contain such antibodies (Marsh & Howard, 1986).

vi) The molecule also contains an antigenically variable domain that can be defined as serologically distinct with convalescent antisera from infected children or antisera from infected *Aotus* monkeys. *In vitro* studies show that antisera against this variable domain can block cytoadherence, probably through a steric inhibitory effect on the functional, cytoadherent part of the molecule. We do not know whether cloned *P. falciparum* parasites can express alternative antigenic forms of this domain, as with *P. knowlesi*. Even so, the antigenic diversity of this part of the molecule is great in natural infections (Marsh & Howard, 1986). This antigenically diverse part is highly immunogenic, since three week convalescent sera from infected children invariably contained IgG antibody that was serologically specific for the homologous isolates.

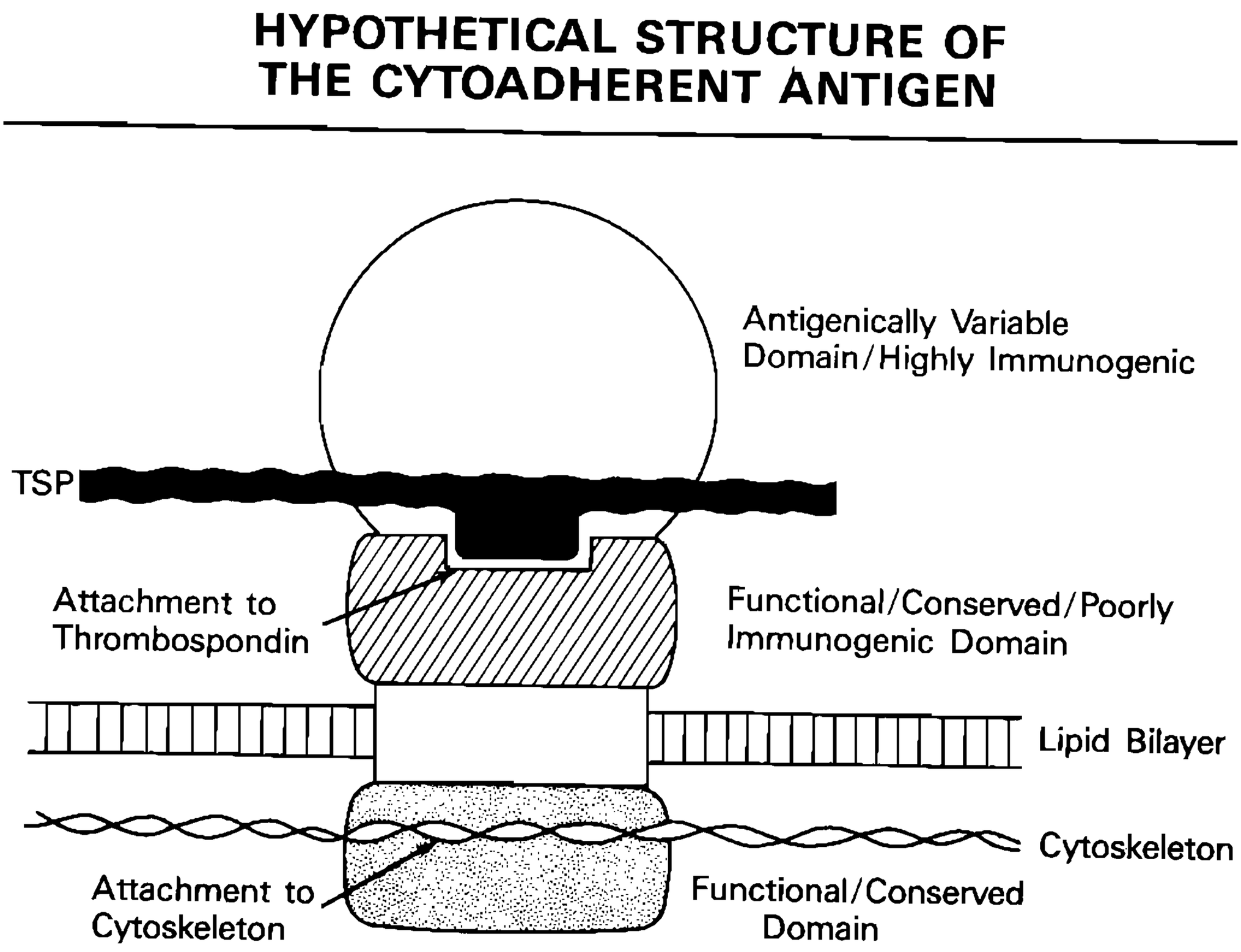

Fig. 8: A schematic drawing of different domains of the $M_r \sim 300{,}000$ *P. falciparum* antigen expressed on infected erythrocytes. Four types of domain are indicated: a submembrane region attached to cytoskeleton; a hydrophobic membrane-spanning region (the protein could traverse the membrane several times); a functional domain which binds thrombospondin (TSP); an antigenically variable domain.

Detailed structural information on this molecule is needed to confirm these ideas. The successful cloning and sequencing of the gene for this malarial protein would not only provide information on the protein arrangement in the host cell membrane (a M_r 300,000 protein could transverse the lipid bilayer several times) but lead to identification of the functional, cytoadherent structure. From such knowledge it may be possible to design a vaccine strategy based on *in vivo* blockade or reversal of cytoadherence.

H. Questions for the Future – Aside from the obvious paucity of structural information on the $M_r \sim 300{,}000$ protein on infected erythrocytes, there are several areas of further research involving interesting biological questions. For example, why is the antigenically conserved epitope

expressed on *P. falciparum*-infected erythrocytes from different children not recognized by the infected individuals? Perhaps the adults, who do have serum antibodies against this conserved epitope(s), have overcome specific immunosuppression of antibody responses against this structure.

Epidemiological studies should be performed in different malarious areas of the world to determine if there is a correlation between immune responses against the antigenically conserved surface antigen on infected erythrocytes and protective anti-malarial immunity. It would also be interesting to determine the serological diversity of the *P. falciparum* surface antigen in the same individual with different acute malaria attacks (we would predict a different antigen with each attack), and, the serological diversity of infections in different individuals at the same time and location.

Finally, studies on the host cell ligands involved in cytoadherence may shed light on the pathogenesis of cerebral malaria. Inflammatory and/or immune responses during acute infection in particular individuals may alter the cell-surface phenotype of capillary endothelial cells in brain and promote cytoadherence in this tissue.