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ALTERATIONS IN MEMBRANE PERMEABILITY OF MALARIA-INFECTED HUMAN ERYTHROCYTES ARE RELATED TO THE GROWTH STAGE OF THE PARASITE *

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During the intraerythrocytic growth of *Plasmodium falciparum* in culture, marked changes are observed in the permeability properties of the host cell membrane. Anionic substances otherwise impermeant to normal cells, become highly permeant to infected cells. These changes in permeability become apparent as rings mature into trophozoites and remain throughout schizogony. The permeability changes to anionic substances are not manifested as degradation of band 3, the purported erythrocyte anion transporter. They probably reflect alterations of a more general nature.

Studies performed with animal models of malaria have indicated that the membrane of red blood cells infected with plasmodia undergoes parasite-dependent perturbation in structure [1–12] and function [13–16]. The causal relationship between functional and structural alterations is not well understood. A possible means to shed some light on the aetiology of the alleged alterations is to quantitatively follow defined red cell membrane properties through the various developmental stages of intraerythrocytic parasite [5]. Using synchronized in vitro cultures of *Plasmodium falciparum* infected human erythrocytes and anionic surface probes impermeant to normal cells [13–19], we found marked abnormalities in the host cell membrane permeability barrier as parasites develop into trophozoites. The abnormalities which are not a result of invasion per se and are not manifested as chemical degradation of band 3, the erythrocyte anion transporter [19], most probably reflect subtle perturbations of the membrane matrix caused by factors of parasitic origin.

We have chosen the amino-reactive reagent 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonic acid ($^3H_2$DIDS) to monitor alterations of membrane properties because in normal erythrocytes it is demonstrably impermeant [17,18] and because it labels specifically and stoichiometrically band 3 membrane polypeptides [18,20] known to be involved in the physiologically important Cl/HCO_3^- exchange [19]. This enabled us to follow both reorganization of the host cell membrane throughout the various stages of malarial infection as well as the fate of band 3 polypeptides, the predominant integral membrane component of erythrocytes [19]. Moreover, prelimimary work indicated that H_2DIDS modification of infected cells which have reached the ring stage, did not block their further development into trophozoites and schizonts.

Parasitized cells were grown in vitro [21] and were synchronized using a modified version of a previously published method [22]. At the various

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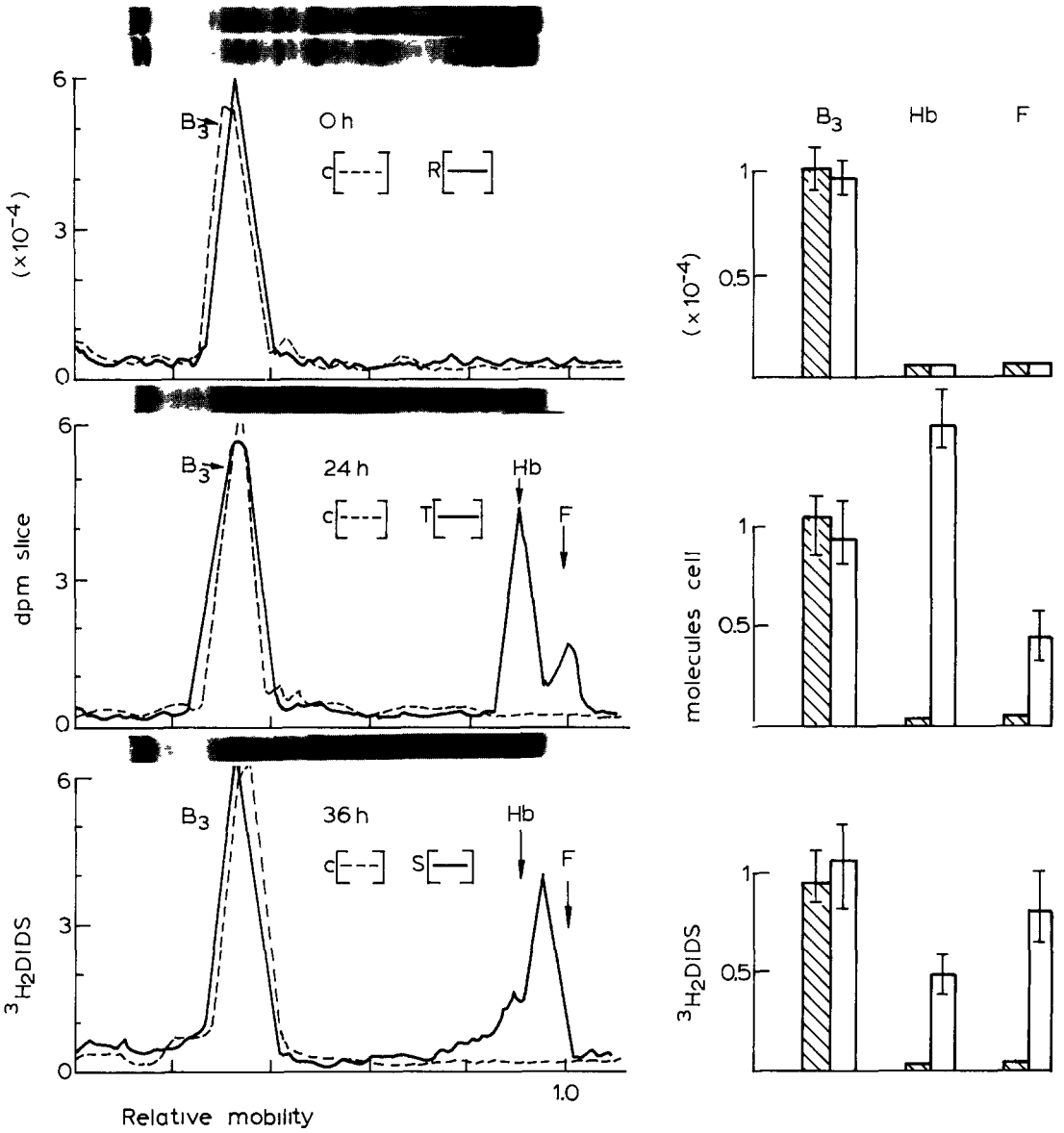

Fig 1 SDS-polyacrylamide gel electrophoresis of intact cells from infected cultures grown synchronously and labeled with $^3H_2$DIDS Red blood cells of type O^+ were infected with *Plasmodium falciparum* and grown as previously described [21] Synchronized growth was accomplished by a modification of an established method [22] as follows One volume of packed cells from infected cultures was resuspended in 20 vol of 5% mannitol incubated at 25°C for 15 min, centrifuged and washed with RPMI-1640 medium After centrifugation, the pellet containing rings and uninfected cells was resuspended in growth medium and grown for an additional 38 to 40 h The mannitol treatment was repeated to yield 10% parasitemia of only ring (R) forms (Giemsa method) The rings were either labeled or further grown, either for 24 h to yield trophozoites (T) or 36 h to yield schizonts (S)

Labeling of cells washed with phosphate buffered medium (150 mM NaCl, 10 mM phosphate, pH 7 4) was performed either at the R, T, or S stage, or with uninfected cells (C) 'grown' in the same conditions as previously described [21] Briefly, 200 μl packed cells were resuspended with 2 0 ml phosphate-buffered saline containing 10 μM $^3H_2$DIDS (4 10^9 dpm/μmol) and incubated for 30 min at 37°C The cells were washed twice with the above medium containing bovine serum albumin (0 1%), twice with medium alone, and finally resuspended in the same medium. S and T were separated and enriched from C and R by the Ficoll method [23] and finally washed with the same medium The radioactivity, number of cells and parasitemia (P) were determined, the latter using the Giemsa method An aliquot of cells was dissolved in boiling SDS and $2 \cdot 10^7$ cells were electrophoresed in SDS-polyacrylamide gel

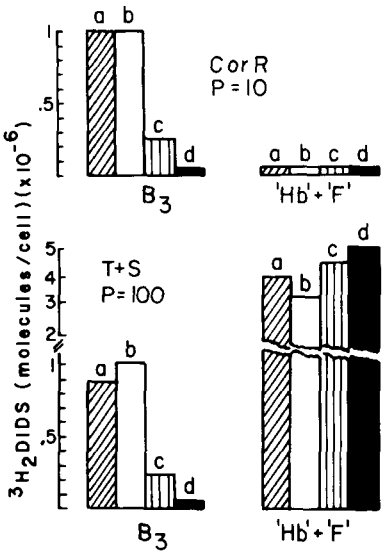

Fig 2 The effect of anion transport blocking on the H_2DIDS-labeling properties of malaria infected cells Uninfected cells (C) or infected cells (P)=20, containing all parasitic stages (R=10, T=5, S=5), were first incubated with either buffer or unlabeled H_2DIDS (20 μM) and washed as described in Fig 1 The buffer treated cells were subsequently labeled with $^3H_2$DIDS (20 μM) either in the absence (a) or in the presence (b) of 0 2 mM phloretin, or in the presence of 1 mM DNDS (c) Similarly, the H_2DIDS-treated cells were labeled with $^3H_2$DIDS (d) All labeled cells were processed through a Ficoll cushion, counted for radioactivity and cell number, and electrophoresed as shown in Fig 1 The labeling in B3 fraction and in the combined Hb+F fraction are given in terms of $^3H_2$DIDS molecules associated with the respective fraction per cell Data for T+S cells were normalized from the observed 70–100 P after verifying that uninfected cells from infected cultures behaved towards $^3H_2$DIDS labeling just as control cells

stages of growth the cells were labeled with $^3H_2$DIDS and analyzed for labeling yield and radioelectroporetic profiles in SDS-polyacrylamide electrophoresis gels (Fig. 1, left). Normal cells and cells from infected cultures which reached the ring stage, have virtually all the label restricted to the band 3 area. However, in trophozoites and schizonts, in addition to a similarly quantitative labeling of band 3, we found a marked labeling in the hemoglobin (Hb) and lipid areas. When normalized to 100% infected cells, the labeling of these two components, as described in Fig. 1, was calculated to be as high or even higher than that found in band 3 (Fig. 1, right) and certainly much higher if increasing concentrations of $^3H_2$DIDS were used (see Fig. 2). The labeled Hb or Hb-degradation products of $^3H_2$DIDS-treated trophozoites and schizonts could be quantitatively released by osmotic lysis using isoosmotic mannitol [22]. This indicates that labeled Hb originated from intact host cells which retained their permselectivity towards cations and other large solutes relative to water (i.e., osmotic barrier) and not from a sub-population of highly permeabilized, namely, totally leaky cells. While the labeling of cytoplasmic hemoglobin (s) clearly indicated the permeabilization of host cell membranes, the labeling of lipids could also result, as recently suggested [12], from the externalization of endofacial lipid domains otherwise inaccessible to disulphonic stilbenes [18].

In order to ascertain whether $^3H_2$DIDS access into the cytoplasm of infected cells was gained by a modification of band 3 which provides the primary route of anion traffic in red cell membranes, a suspension of red cells containing all stages of parasite growth was first incubated with either unlabeled H_2DIDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) or phloretin to fully block all 'specific' anion transport sites [19], and subsequently the cells were labeled with $^3H_2$DIDS and separated into fractions of rings and uninfected cells and of trophozoites and schizonts (Fig. 2). While band 3 labeling at any developmental stage was markedly reduced by the various chemical pretreatments, no protection either to hemoglobin or lipid labeling of trophozoites and schizonts was afforded. An essentially similar picture was ob-

electrophoresis tubes as described elsewhere Gels were sliced immediately after electrophoresis, dried over night at 60°C, dissolved with 0.5 ml 30% H_2O_2 for 6 h, and counted for radioactivity in Packard's Insta-Gel with PRIAS instrument (Packard) (left panel) Coomassie blue staining on parallel gels was performed as described before [24] (top panel) B3, Hb, and F denote band 3 (95 kDa), hemoglobin(s) (16.5 kDa) and the SDS front (probably lipids), respectively The number of H_2DIDS molecules per cell, as distributed among B3, Hb and F, are given in the right panel These numbers were calculated from the left panel and corrected to P=100 Slashed columns, control cells, ring cells or uninfected cells from infected cultures (bottom layer of Ficoll) Blank columns. R (bottom layer of Ficoll), S and T (upper layer of Ficoll) corrected to P=100%

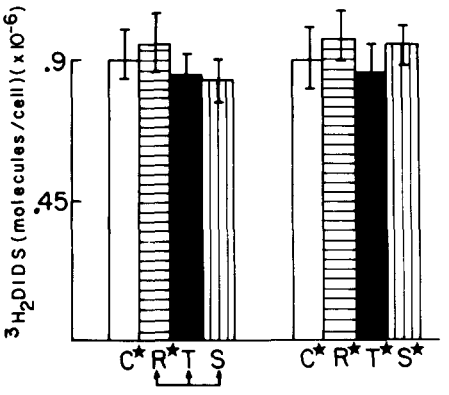

Fig 3 B3 in the different developmental stages of *Plasmodium falciparum* Left panel. Control cells or infected cells at the R stage were first labeled with 10 μM $^3H_2$DIDS as shown in Fig 1 and analyzed by SDS-polyacrylamide gel electrophoresis either at 0, 24 or 36 h of growth Right panel control cells or infected cells at the R, T or S stage were labeled with $^3H_2$DIDS as described above (data taken from Fig 1) Data are given as $^3H_2$DIDS labeled B3 per cell after normalization of labeling profiles to P = 100 Control cells, rings and uninfected cells from cultures after 0, 24 and 36 h of growth gave essentially the same results

tained by prelabeling synchronized rings with unlabeled H_2DIDS and labeling the resulting trophozoites and schizonts with $^3H_2$DIDS (not shown).

These results indicate that the abnormal labeling profiles of cells and, in particular, the impaired membrane barrier properties are probably not directly associated with band 3 anion transport capacity, inasmuch as the chemical integrity of the proteins is fully retained throughout all stages of intraerythrocytic parasite growth. This is also corroborated by the fact that infected cells labeled at the ring stage, quantitatively retain the label in band 3 as they develop into trophozoites and schizonts (Fig. 3). This property was shown to be independent of the degree of labeling of band 3 in the range $(0.4–1.1) \cdot 10^6$ B3 polypeptides per cell. However, these results do not exclude the possibility that band 3 has been structurally modified in a manner which escapes present detection techniques.

The recent suggestion that some degradation of band 3 occurred in *Plasmodium berghei* infected mouse erythrocytes [7] could not be corroborated with *Plasmodium berghei* infected rat erythrocytes using the present labeling techniques (not shown). Furthermore, no labeling or cytoplasmic components and of lipids was observed at any stage of parasite growth in rat cells (to be published elsewhere). These observations underscore the fact that alterations in host cell membrane properties might differ among different animal cells infected with either the same or with different plasmodia.

Finally, the abnormally high permeability to bulky anionic substances found here with *Plasmodium falciparum* infected erythrocytes, might also serve to rationalize the selective osmotic lysis of trophozoites and schizonts obaintained with isoosmotic solutions of polyols such as sorbitol and mannitol [22] as well as of a variety of other sugar analogs, all of which permeate slowly across membranes of uninfected cells. These abnormal membrane properties are not necessarily a sign of a general permeabilization of the host membrane, but seem to be alterations of a discrete nature, inasmuch as they are found only with distinct classes of substances (Kutner et al., to be published elsewhere) and only at specific times after the invasion of cells (i.e., from trophozoite stage and on). We therefore assume that these alterations resulted from membrane perturbations caused by factors associated with the intraerythrocytic growth of the parasite and not with the initial invasion of the parasite into host cels. We also believe that a reduction in the permselectivity of parasitized red cells could be exploited for the design of new drugs to affect selectively parasite growth and propagation.

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