FRACTIONATION OF MOUSE MALARIOUS BLOOD ACCORDING TO PARASITE DEVELOPMENTAL STAGE, USING A PERCOLL-SORBITOL GRADIENT

H. GINSBURG*, I. LANDAU**, D. BACCAM**, D. MAZIER***

SUMMARY. Asexual intraerythrocytic malarial parasites permeabilize the membrane of their host cell to small monoelectrolytes and anions. Since permeabilization increases with parasite maturation, this property has been used previously to fractionate blood infected with Plasmodium falciparum and P. knowlesi according to the developmental stage of the parasite, using Percoll-sorbitol density gradients. We have extended this method to fractionate mouse blood infected with four species of rodent malaria: P. chabaudi, P. vinckei, P. yoelii and P. berghei. While the method works in principle in this case, the polyparasitism which characterizes these species prevented explicit separation according to developmental stage. Hence, erythrocytes harbouring several ring-stage parasites appeared in the same fraction which contained cells hosting a single trophozoite, and polyparasitized trophozoites were associated with singly-infected schizont. This observation implies that permeabilization of the host cell membrane results from the integrated metabolic activity of the parasite(s) and is not related to a specific phase of parasite development.


Fractionnement, à l'aide d'un gradient de Percoll-sorbitol, de sang de Souris paludéenne, en fonction du stade parasitaire.

RÉSUMÉ. Les stades érythrocytaires asexués des Plasmodium perméabilisent la membrane de leur cellule-hôte aux petites molécules non chargées et aux anions. Du fait de l'augmentation de cette perméabilisation au cours de la maturation du parasite, cette propriété a déjà été utilisée auparavant pour fractionner du sang infecté par Plasmodium falciparum et P. knowlesi, en utilisant un gradient de Percoll-sorbitol et ceci en fonction du stade de développement du parasite. Nous avons appliqué cette méthode au fractionnement de sang de Souris infectées par 4 espèces de Plasmodium de Rongeurs : P. chabaudi, P. vinckei, P. yoelii et P. berghei. Bien que la méthode s'applique en principe dans ce cas, le polyparasitisme des globules rouges, qui caractérise ces espèces, empêche une séparation stricte des parasites en fonction de leur âge. Ainsi, des érythrocytes hébergeant plusieurs anneaux se retrouvent dans la même fraction que des cellules contenant

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un trophozoïte unique et des érythrocytes contenant plusieurs trophozoïtes sont associés à des globules contenant un seul schizonte. Ces observations impliquent que la perméabilisation de la membrane de la cellule-hôte résulte de l’activité métabolique globale du ou des parasites et est sans rapport avec une phase évolutive spécifique.


Introduction

The intraerythrocytic development of malarial parasites is accompanied by substantial alterations in the permselectivity properties of the host cell membrane: The infected red blood cell becomes highly permeable to a variety of carbohydrates, amino acids and anions, which otherwise permeate into the cell at low pace through specialized transport systems, or by simple diffusion, if at all. This phenomenon has been described for different species of parasites and host cells: Plasmodium lophurae in duck red blood cells (Sherman and Tanigoshi, 1974, 1975), P. berghei in mouse (Homewood and Neame, 1974) and P. falciparum grown in human red blood cells in culture (Ginsburg et al., 1983, 1985; Kutner et al, 1983, Elford et al, 1985). It has been shown recently that the permeabilization of the host cell membrane progresses with parasite maturation, and on this basis, a technique has been devised for the isolation of P. falciparum-infected human erythrocytes according to the of developmental stage of the intracellular parasite (Kutner et al, 1985).

The extension of this technique to different species of murine malaria parasites developing in mice is presented in this report.

Materials and methods

Four different parasite species were tested: P. yoelii yoelii 265 BY, P. berghei berghei NK 65, P. chabaudi chabaudi AS and P. vinckei petteri 279 BY. Random bred Swiss albino mice, weighing 20-25 grams (obtained from IFFA-CREDO, France), were inoculated intraperitoneally with 10⁶-10⁷ parasites and the development of parasitemia was followed by microscopic inspection of Giemsa stained thin blood smears. When parasitemia was higher than 25-30 %, mice were bled into test tubes containing heparin. Blood was washed 3 times in 20 volumes of cold HEPES-buffered saline (HPS), plasma and buffy coat were removed by aspiration. Discontinuous Percoll-sorbitol gradients were prepared as described previously (Kutner et al, 1985) except that the Percoll concentration range was 10-40 %. Erythrocytes were suspended at 20 % hematocrit in HPS supplemented with sorbitol, 6 % w/v, and layered on top of the gradient. After 20 min centri-
fugation at 20,000 rpm in a fixed angle rotor, cell-containing layers were removed and washed successively in HPS containing decreasing concentrations of sorbitol. Thin blood smears were prepared from each layer, stained with Giemsa and inspected under the microscope.

Results

The distribution of cells obtained from mice infected with different parasite species in the Percoll-sorbitol gradient is depicted in figure 1. Distinct cell layers were observed throughout the gradients, although their position in the gradient varied somewhat with the species.

![Partitioning of cell fractions of parasitized blood in Percoll-sorbitol gradient. Percoll concentrations are shown on the left. Fractions were numbered from top to bottom, at their order of withdrawal from the gradient. See table I for morphological description.](image)

The morphological appearance of the parasites in the various layers of the gradient is described in table I and pictures of P. berghei-infected red blood cells in the different layers, are shown in figure 2.

Discussion

The intraerythrocytic development of malaria parasites involves dramatic changes in the structure and composition of the host cell membrane (see Sherman, 1985 for a recent review). One of the most obvious functional consequence of these changes is the permeabilization of the infected red blood cell to anions (Kutner
Table I. — Morphological description of erythrocytes infected with different species of parasites, as they appear in fractions isolated from the Percoll-sorbitol gradient. Numbering of layers goes from the top of the gradient to its bottom. Percentiles indicate % parasitemia.

<table>
<thead>
<tr>
<th>Species</th>
<th>Layer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. chabaudi</em></td>
<td>1</td>
<td>Swollen merozoites and debris of aged trophozoites.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Aged schizonts; Polyparasitized trophozoites; 100 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Aged and polyparasitized trophozoites; Schizonts, Male gametocytes; 100 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Polyparasitized trophozoites; 100 %</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Young polyparasitized trophozoites; Aged trophozoites; Female gametocytes;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Few uninfected reticulocytes; 100 %</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Few rings; Mostly aged and some young trophozoites; 20 %</td>
</tr>
<tr>
<td><em>P. vinckei</em></td>
<td>1</td>
<td>Cellular debris.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Free merozoites.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Mostly mature and some aged schizonts; Host cell membrane damaged; 70-80 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Few rings; Aged trophozoites and immature schizonts; Some reticulocytes; 80 %</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Mostly young and aged trophozoites; Polyparasitized younger stages; Some merozoites—probably immediately after invasion; 30 %</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Rings; 5-10 %</td>
</tr>
<tr>
<td><em>P. voelii</em></td>
<td>1</td>
<td>Debris of ghost and damaged parasites.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mature schizonts with abnormal morphology—no separation into merozoites; 90 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Highly polyparasitized trophozoites (&gt; 10/cell); Large schizonts; Young gametocytes; Many reticulocytes; 85 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Polyparasitized trophozoites (5-9/cell); Mature and aged schizonts; Many mature gametocytes; 90 %</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Aged trophozoites (2-4/cell); Many young schizonts; Many mature gametocytes; 90 %</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Young and aged trophozoites; Few young schizonts; 50 %</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>1</td>
<td>Free parasites and cellular debris.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Highly polyparasitized trophozoites (8-9/retic.); ~ 20 % mature schizonts in mature large and small erythrocytes (in small—8 nuclei/cell; in large—20); Trophozoites in reticulocytes are devoid of pigment; 95 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Polyparasitized reticulocytes (4-8/cell); Aged trophozoites and few young schizonts; 100 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Aged trophozoites; Polyparasitized reticulocytes (3-4/cell with younger trophs.); 95 %</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Aged trophozoites; Very few polyparasitized reticulocytes (2/cell); 80 %</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Rings and young trophozoites; 5-10 %</td>
</tr>
</tbody>
</table>

*et al, 1982, 1983* and to nonelectrolytes (Homewood and Neame, 1972; Sherman and Tanigoshi, 1974, 1975; Ginsburg *et al, 1983, 1985; Elford *et al, 1985*). Permeabilization results probably from structural defects induced in the host cell mem-
brane (Ginsburg and Stein, 1987) due to the insertion of parasite-derived poly-
peptides (Howard, 1982), and is seemingly vital to the normal development of
the parasite (Ginsburg, 1987), since blocking of the new transport pathways

The distribution of infected cells in the various layers of the Percoll-sorbitol
gradient has been shown to depend on the extent of permeabilization of the host
cell membrane (Kutner et al, 1985), which, in turn, depends on the stage of parasite
development. Although these phenomena were investigated in P. falciparum,
the present work indicates that they can be extended to murine species. In all
four species investigated, red blood cells containing the more mature parasites
were detected in the less dense layers of the gradient, suggesting that these cells
are more permeable to sorbitol. However, the general phenomenon of polypara-
sitism in murine malaria species (Garnham, 1970), resulted here in a distribution
which was apparently different from that observed in P. falciparum, where each
gradient layer contained only one distinct developmental stage. Thus, erythrocytes
harboring 2-3 parasites at the ring stage appeared in the same gradient layer with
cells containing one trophozoite and cells containing 2-3 trophozoites co-sedimented
with cells harboring one mature schizont, and so on. It is therefore suggested that
permeabilization of the host cell membrane resulted from the integral metabolic
activity of the intraerythrocytic parasites, i. e., that cells containing a larger
number of parasites were more permeabilized.

While this conclusion is true in its broadest sense, there were distinct differences
in the partitioning of cells infected with the various parasite species in the gra-
dient. Since this diversity may bear on the onset of permeabilization and on sys-
temic effects on non infected erythrocytes, it deserves special attention:

P. chabaudi: This species, unlike the others investigated in the present work,
develops synchronously and most parasites were at the trophozoite stage (mice
were bled in late morning). Since trophozoites were detected in the most dense
layer together with non infected cells, it is very probable that permeabilization
starts at or just after this stage. Partitioning of infected cells in the gradient depen-
ded almost exclusively on the number of parasites per host cell and on trophozoite
age. Most interestingly, layers 3 and 5 also contained gametocytes, the first mostly
female and the second mostly male gametocytes. Thus, this method could be
used for the differential investigation of these cells in the future.

P. vinckei: Enhanced permeability starts to develop at the ring stage but
cells polyparasitized with rings can be seen also in the less dense layers. In mature
stages, the host cell membrane was usually damaged, implying that it is more fragile
compared to cells infected with other species. Alternatively, these infected cells

Fig. 2. — Morphological appearance of P. berghei-infected cells in the different layers
of the Percoll-sorbitol gradient. Numbering of fractions refers to the designation in
figure 1.
may be substantially more permeable and thus became damaged during the post-gradient washings. Furthermore, the parasitemia in most layers was relatively low, implying that non infected cells were also permeabilized. It has been recently demonstrated that some of the polypeptides produced by the parasite are « secreted » extracellularly (Wilson, 1980; Jepsen and Andersen, 1981, Dasilva et al., 1983 for example). Such molecules should be sufficiently amphipatic to partition into the host cell membrane. As a result, they could cause structural imperfections with consequent permeabilization, both in the host cell and in non infected cells. Since this phenomenon was typical of P. vinckei, it is not unlikely that the nature of the polypeptide of this species is somewhat different from that of other species and deserves further experimentation.

P. yoelli: Permeabilization of the host cell membrane develops at the late trophozoite stage. Thereafter, it strongly depends on the number of parasites per cell, both at the trophozoite and the schizont stages. There is an indication for the presence of a sub-population of abnormal parasites which fail to develop and mature. Erythrocytes containing young gametocytes, i.e., where the host cytosol is still visible, are more swollen than those harboring mature gametocytes. This observation would be compatible with the decrease of the host cytoplasmic volume which occurs upon maturation of the gametocyte, or if the host cell membrane becomes totally permeabilized, e.g., also to cations.

P. berghei: Permeabilization occurs at the young trophozoite stage. It increases with parasite maturation, mostly from trophozoite to schizont. This observation points to a physiological difference between this species and P. yoelii, which is morphologically very similar (Garnham, 1970). There is a distinct change in the permeability of host reticulocytes as a function of parasite number. In fact, only reticulocytes were found to be polyparasitized. It seems as if parasite development accelerates the maturation of host reticulocytes inasmuch as polyparasitized cells harboring trophozoites are deeply stained while those hosting schizonts are as pale as normocytes.

In conclusion, the Percoll-sorbitol gradient which has been developed using cultured P. falciparum and seems applicable to P. knowlesi, P. cynomology and P. fragile (Aley et al., 1986), is much less effective for the fractionation of mouse blood infected with murine parasite species, probably due to their characteristic polyparasitism. However, since it is easier to isolate host cell membranes from erythrocytes infected with murine species (Heidrich et al., 1979), investigation of the composition of such membranes derived from different fractions obtained by this method, could elucidate the nature of the permeabilizing factors and their mode of action: The abundance of these factors in the host cell membrane should not depend on parasite age, but on the position of the infected cell in the Percoll-sorbitol gradient.

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REFERENCES


