ACCELERATED SENESCENCE OF HUMAN ERYTHROCYTES CULTURED WITH PLASMODIUM FALCIPARUM

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ABSTRACT

Red blood cells infected with *Plasmodium falciparum* (IRBCs) undergo changes primarily in their membrane composition which contribute to malaria pathogenesis. However, all manifestations (e.g. anemia) cannot be accounted for by IRBCs alone. Uninfected erythrocytes (URBCs) may play a role, but have been under-researched. We wanted to document changes in the erythrocyte membrane that could contribute to URBC reduced lifespan and malaria-associated anemia. Human erythrocytes were cultured with *P. falciparum*, washed at the trophozoite stage, and IRBCs and URBCs were separated by centrifugation on Percoll density gradient, thus obtaining erythrocyte fractions of different densities/ages. IRBC and URBC purified membranes were analyzed and compared to control normal erythrocytes (NRBCs) of the same age, from the same donor, kept in the same conditions.

*P. falciparum* accelerated aging of both IRBCs and URBCs, causing a significant shift in the cell population towards the more dense (old) fraction. Protein, phospholipid and cholesterol content were reduced in IRBCs and young URBCs. Compared to NRBCs, the young and medium uninfected fractions had higher levels of lipid peroxidation and phospholipid saturation (due to the loss of polyunsaturated fatty acids, PUFA) and lower phosphatidylserine. In IRBCs, thiobarbituric reactive substances (TBARS) were higher and PUFA and phosphatidylserine lower than NRBCs and URBCs. In comparison, trophozoite membranes had lower phospholipid (particularly sphingomyelin and phosphatidylserine) and cholesterol content, and a higher degree of saturation. Parasite-induced peroxidative damage might account for these modifications.

In summary, we demonstrated that *P. falciparum* induces membrane changes associated with accelerated senescence in both infected and uninfected erythrocytes. URBCs will then likely contribute to malaria anemia (they are recognized as senescent cells and are removed) and to organ damage (they aggravate congestion in small vessels).

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INTRODUCTION

Clinical malaria is a consequence of Plasmodium invading and developing inside red blood cells (RBCs). Infection is accompanied by profound changes in the host cell that are well documented for *P. falciparum*, the parasite which causes the life threatening form of malaria. *P. falciparum* particularly affects lipid composition of the infected red blood cell (IRBC) (cholesterol level, phospholipid pattern, organization and degree of saturation)\(^1,2\), but also its deformability and antigenic, osmotic and transport properties\(^3,4\). Known consequences are sequestration and stiffness (involved in organ damage, e.g. cerebral malaria) and removal. If these changes are well researched for IRBCs, little is known as to the possible alterations in the membrane composition and structure of uninfected RBC (URBC) from malaria patients or *P. falciparum* in vitro cultures\(^1,5\).

Knowing whether URBCs are affected as well and how, is important because some of the clinical manifestations of falciparum malaria cannot be accounted for by IRBCs alone. For instance, falciparum malaria causes different degrees of anemia\(^6\) which is only partly due to direct destruction of IRBCs by the growing parasite or their removal by the spleen. Severe anemia, which occurs particularly in young children and pregnant woman living in malaria endemic areas, is a case-defining condition for severe and complicated malaria. URBCs in malaria patients have a shorter life span, but the reasons for this are not completely understood.\(^7\) The presence of circulating monocytes containing URBCs suggests that during malaria infection uninfected erythrocytes develop membrane modifications that activate monocytes to phagocytize them\(^8\). One possibility is that URBCs are seen as senescent cells by the organism and thus removed.

In order to assess if premature removal of RBCs may be related to changes in membrane structure and/or composition consistent with an accelerated aging we undertook a systematic comparison of the membrane composition of normal RBCs (NRBC) vs. URBCs and IRBCs from in vitro cultured *P. falciparum*. Since RBC aging is accompanied by increased cell density\(^9\), we fractionated RBCs on a...
Percoll/sorbitol gradient in groups of different densities, corresponding to different ages. Each URBC and IRBC fraction was analyzed for membrane lipid composition, stage of lipid peroxidation and sensitivity to oxidative stress and compared to the corresponding fraction of NRBCs.

Parasite membranes from trophozoite-stage *P. falciparum* were studied in parallel. We reasoned that a better knowledge of the lipid composition of trophozoites membranes would help solving the paradox of parasites, which grow in a iron-porphyrin rich environment, accumulating hemozoin that is toxic for host membranes \(^{10,11}\) and yet survive.
MATERIALS AND METHODS

Reagents. RPMI 1640 medium was purchased from Gibco BRL, human A-positive red blood cells and plasma were kindly provided by the Blood Bank of the National Cancer Institute, Milano, Italy.

Standard phospholipids (PL), 1,1,3,3-tetraethoxypropane, acetyl thiocholine, NADP$^+$ and thiobarbituric acid were purchased from Sigma; silica gel, acid washed, from BDH; silica gel plates (Kieselgel 60, HPTLC) from Merck; standard fatty acid methyl esters from Alltech.

Parasite cultures

Freshly collected complete human blood with CPD (citrate/phosphate/dextrose) as anticoagulant was banked at 4° C for less than 15 days and used for parasite cultures. P. falciparum cultures were carried out according to Trager and Jensen’s 12 with slight modifications. Briefly, a CQ-sensitive (D10) was maintained at 5% hematocrit at 37°C in complete culture medium (RPMI 1640 supplemented with NaHCO₃ 24 mM, 10% heat-inactivated A-positive human plasma, 20mM Hepes and 2mM glutamine).

All cultures were maintained in a standard gas mixture consisting of 1% O₂, 5% CO₂, 94% N₂. When parasitemia exceeded 5%, subcultures were taken; the culture medium was changed every second day.

After 3-4 days of culture at the trophozoite stage, cells were washed twice with serum-free culture medium, resuspended to 25% hematocrit and fractionated on to a Percoll/4% sorbitol (w/v) gradient 13. This procedure allowed the separation of IRBCs at the top of the gradient (d=1.078) and URBCs in three bands of different density and age: young (d=1.091), medium (d=1.104) and old (d=1.117). As control, NRBCs were cultured for 3-4 days in the same conditions and density separated in four discrete bands: very young (d=1.078), young (d=1.091), medium (d=1.104) and old (d=1.117). The cell number in each fraction was counted with an hemocytometer, whereas the mean corpuscular volume (MCV) was determined by using an automated Coulter.

Preparation and analysis of erythrocyte ghosts. NRBC and URBC ghosts were prepared by hypotonic lysis and extensive washing in 20 volumes of 5mM NaHPO₄ buffer pH 8.0. IRBC ghosts were purified from parasite by lysis with 0.07% saponin in PBS at 37°C for 10 min and washed according to Hsiao 5.

Final washing of RBC ghosts was performed with 10 mM Tris HCl buffer pH 7.4. Parasite released from
saponin lysis was washed twice with PBS, lysed with 5 mM phosphate buffer, pH 8.0 and centrifuged at 100,000 g for 30 min. to pellet trophozoite membranes.

RBC and trophozoite membranes were subjected to total lipid extraction and partitioning in accordance to Folch et al.\textsuperscript{14}. Total lipid extract was used for lipid analysis: phospholipid (PL) phosphorus was determined according to Bartlett\textsuperscript{15}, whereas cholesterol (Cho) was quantified by densitometric analysis after separation by high performance thin layer chromatography (HPTLC) in hexane/diethyl ether/acetic acid (90:10:1, by vol.) and visualization with a solution of p-anisaldehyde/acetic acid/sulphuric acid (1:100:2, by vol.). An aliquot of the total lipid extract was fractionated in the different lipid components by silicic acid column chromatography\textsuperscript{16}. Compositional analysis of PL was performed by HPTLC separation in chloroform/methanol/acetic acid/water (60:40:4:2, by vol.) and quantification by densitometric analysis after spraying with a specific phosphate ester reagent\textsuperscript{17}. PL fatty acid composition was determined by gas liquid chromatography and stage of lipid peroxidation and sensitivity to oxidative stress of ghosts as TBARS production as previously reported\textsuperscript{11}.

Other analyses: Protein content and acetyl cholinesterase (AChE) activity were determined in the ghost pellet accordingly to Peterson\textsuperscript{18} and Vander Jagt et al\textsuperscript{19}, respectively. An aliquot of the different RBC fractions was lysed with 0.1% Triton X-100 and the hemolysate used for the determination of hemoglobin by its Soret band absorption at 412 nm and glucose 6 phosphate dehydrogenase (G6PDH) activity\textsuperscript{20}. An aliquot of RBC ghosts and parasite homogenate was ruptured by sonication at 40 watts (3 times for 5 sec each) with cooling, and assayed for glutamate dehydrogenase (GDH)\textsuperscript{19} and parasite lactate dehydrogenase (pLDH) using 3-acetyl pyridine NAD (APAD) as a coenzyme in the reaction leading to the formation of APADH (εM= 9.1) and pyruvate from lactate. To exclude possible interferences of saponin in the enzymatic assays an aliquot of NRBC lysed with 0.07 % saponin was subjected to the different enzyme determinations.

Statistical analysis. For the assessment of changes occurring after exposure to \textit{P.f.} URBCs or IRBCs were compared to populations of NRBCs by using the Student’s paired \textit{t} test. The two-sample independent-group \textit{t} test was used for comparison of medium or old RBCs to young RBCs of the same
type. For the assessment of the percentage distribution in the four density fractions the data were analyzed by the two-way analysis of variance, with one factor for repeated measurements (URBCs vs. NRBCs) and one factor for independent measurements (days).
RESULTS

Density fractionation of RBC control and cultured with P. falciparum. NRBCs banked for different times at 4°C were cultured for 72 hr at 37°C and then separated by Percoll gradient. In agreement with published data, a progressive shift toward medium and older fractions was seen with aging (data not shown).

The rate at which such changes occur varied from donor to donor. Therefore, we selected for further study RBCs stored for ≤ 15 days and always compared with RBCs from the same donor to avoid intersubject variability. As shown in Figure 1, RBCs co-cultured with P.falciparum (URBCs) showed modifications in density that resulted in a significant increase in the number of cells sedimenting in the old fraction, with a corresponding decrease of the young fraction. The modifications induced by P.f. were not significantly different among RBC banked for different days.
Figure 1. Density fractionation of control and uninfected erythrocytes.

Cells were banked at 4°C for the indicated time and then maintained at 37°C for 72 hr in the presence or not of *P. falciparum* parasite before fractionation on Percoll/sorbitol gradient. Data were analyzed by two-way analysis of variance

- ■: Control RBC (NRBC); □: Uninfected RBC (URBC)
- Young URBC vs young NRBC: ° p < 0.01
- Old URBC vs old NRBC: * p < 0.003
In parallel, the increase in density was associated with a decrease of the MCV and of AChE, an erythrocyte membrane-bound enzyme, and G6PDH, two specific markers of RBC aging (Table 1).

| Table 1. Markers of RBC aging in different RBC populations |
|----------------|----------------|----------------|----------------|
|                | NRBC           | URBC           | IRBC           |
|                | very young     | young medium   | old            | young medium   | old            | young medium   | old            | 98.2 ± 0.3#   |
| MCV            | 98.2 ± 0.5     | 92.5±0.7       | 84.5 ± 0.1     | 81.2 ±0.5      | 90.7 ±0.4#     | 84.7±0.3#      | 80.5 ±0.5#     | 98.2± 0.3#   |
| AChE (U/mg protein) | 5.6 ±0.9      | 4.9 ±1.2       | 4.4 ±0.5       | 3.1 ±0.9       | 4.6 ±1.5#     | 4.3 ±1#        | 3.2 ±0.5#      | 3.9 ±1*     |
| G6PDH (U/grHb) | 5.5 ±1         | 4.4±0.8        | 3.6 ±1         | 2.5± 0.6       | N.T.          | N.T.           | N.T.          | N.T.       |

Specific activities of AChE (U/mg membrane protein), G6PDH (U/g Hb). AChE was assayed in RBC membranes, G6PDH in RBC hemolysate. Results are the mean (+ S.D.) of 6-8 determinations. N.T.= not tested. Data analyzed by paired t test: URBCs vs. NRBCs of corresponding density; IRBCs vs very young NRBCs

* p < 0.01
# Not significantly different

MCV and AChE showed a similar age-related decrease in URBCs. In IRBCs, collected from the top of the gradient, AChE showed a significantly lower activity compared to that of the corresponding very young NRBC fraction, whereas the MCV was not significantly different.

To assess if parasite contamination had occurred in IRBC and URBC membranes, we measured the activities of two parasite marker enzymes, pLDH (the parasite-specific form of lactic dehydrogenase) and GDH (a mitochondrial enzyme present only in the parasite). In the fraction containing *P. falciparum* trophozoites the pLDH value was 4.1 ± 0.2 U/mg protein and GDH was 83.3 ± 4.0 mU/mg protein. Conversely, pLDH was detectable only in trace amounts in IRBC ghosts (0.2 ± 0.1 U/mg protein) whereas GDH was not detectable in either URBCs or IRBCs.
Protein and lipid content in density fractionated RBC. Figure 2 shows the protein, PL and Cho content in the various RBC fractions.

![Bar chart showing protein, PL, and Cho content in different RBC fractions](image)

**Figure 2. Effect of aging and *P. falciparum* on the protein and lipid content of the different erythrocyte populations.**

Control (NRBC) and uninfected RBCs (URBC) were density separated by Percoll/sorbitol gradient and ghosts from the various fractions were obtained by lysis and extensive washing with 5 mM NaHPO₄ buffer, pH 8.0; ghosts from infected RBCs (IRBC) were obtained by lysis with 0.07% saponin (w/v). Lipids were extracted and PL quantified by phosphorus determination and cholesterol by thin layer chromatography and densitometric analysis as described in “Material and methods”. Protein content is expressed as µg/10⁷ cells, PL and Cho as µmol/10¹⁰ cells. Data shown are the mean ± S.D. of 5 experiments (paired t test for comparison of IRBCs vs. very young NRBCs, and young URBCs vs. young NRBCs)

* p < 0.01

Protein and lipid content progressively decreased as RBCs became older. In contrast, mean cell hemoglobin content did not change with age (25-30 pg/cell, data not shown). A loss of all the membrane components was found in the IRBC fraction when compared to the control fraction of corresponding density (very young NRBC) and in the young URBC fraction compared to young NRBC. Medium and old URBCs were not significantly different from NRBC of the same density.
PL and Cho decreased similarly, with the result that the Cho/PL molar ratio was unchanged. These differences could not be seen when NRBCs and URBCs were compared before density fractionation (data not shown).

**PL distribution and fatty acid pattern.** Data on the PL composition of ghosts from NRBCs and URBCs after density fractionation are summarized in Table 2.

| Table 2. Phospholipid distribution of control and uninfected human erythrocyte ghosts |
|-----------------------------------------------|-----------------------------------------------|
| **NRBC**                                      | **URBC**                                      |
| young  | medium  | old  | Young | medium  | old  |
| PE     | 29.1 ± 0.5 | 30.0 ± 0.3 | 28.2 ± 0.6 | 29.2 ± 0.9 | 30.0 ± 0.7 | 30.0 ± 1.3 |
| PS+PI  | 14.0 ± 0.2 | 11.4 ± 0.5°° | 10.1 ± 0.3°° | 11.1 ± 0.3** | 9.7 ± 0.2°°** | 7.0 ± 0.4°°** |
| PC     | 31.4 ± 0.8 | 32.9 ± 0.5° | 34.4 ± 1.2°° | 30.6 ± 1.0 | 32.9 ± 1.3 | 34.2 ± 0.5° |
| SM     | 25.5 ± 0.3 | 25.7 ± 0.8 | 27.3 ± 0.4° | 29.1 ± 0.4* | 27.4 ± 0.9 | 28.8 ± 0.6 |

Lipids were extracted from erythrocyte ghosts and PL purified from other lipid components as described in “Material and methods”. Individual PL species were separated by HPTLC and quantified by densitometric analysis. PE= phosphatidylethanolamine, PS=phosphatidylserine, PI= phosphatidylinositol, PC= phosphatidylcholine, SM=sphingomyelin.

Results represent the percentage of total PL and are the mean (+ S.D.) of 3-6 determinations made in duplicate.

Data were compared by using the two-sample independent *t* test for within group comparisons (vs. young RBCs), and the paired *t* test for between-group comparisons (cells of the same age)

° *p* < 0.05 and °° *p* < 0.01 vs young RBC of the same group.

* *p* < 0.05 and ** *p* < 0.01 vs NRBC of the same age.

Phosphatidylinositol (PI), quantified with phosphatidylserine (PS), was present in all fractions in a very low percentage (< 1%). In NRBCs, aging caused a decrease in PS and an increase in phosphatidylcholine (PC) and sphingomyelin (SM) content. A similar trend was present in URBCs, although in each fraction the percentage of PS was significantly lower compared to the NRBC fraction.
of the same age. SM was higher in all URBC fractions, although significantly different only in the young age. When IRBC were tested, very low levels of PS and PC were found, while PE was higher than in NRBCs (Figure 3).

![Figure 3. Phospholipid distribution of infected erythrocyte (IRBC) ghosts and *P.falciparum* trophozoite membranes compared to control erythrocytes (NRBC). PL were extracted, purified and analyzed by HPTLC separation and densitometric analysis as described in “Materials and methods”. Results represent the percentage of total PL and are the mean (+ S.D.) of three determinations made in duplicate. IRBCs were compared to trophozoites (unpaired *t* test, *p* < 0.01) and young NRBCs (paired *t* test, °p < 0.01).]

*P.falciparum* trophozoites too had very low levels of PS, but differently from IRBC, PC was one of the most represented PL species, and SM was low.

The degree of PL unsaturation, as shown by the percentage of polyunsaturated fatty acids (PUFA) and the Double Bond Index (DBI, defined as the total number of unsaturated methylenes), decreased with age in NRBCs and URBCs and was comparatively lower in URBCs than in NRBCs (Table 3).
Table 3. Phospholipid fatty acid composition of control and uninfected erythrocyte ghosts

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>NRBC young</th>
<th>medium</th>
<th>Old</th>
<th>URBC young</th>
<th>medium</th>
<th>old</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>21.9 ± 0.6</td>
<td>24.9 ± 0.8°</td>
<td>25.8 ± 1.5°</td>
<td>25.1 ± 1.4**</td>
<td>26.1 ± 0.5</td>
<td>26.0 ± 0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>19.4 ± 2.2</td>
<td>19.1 ± 1.2</td>
<td>18.2 ± 1</td>
<td>18.6 ± 0.1</td>
<td>18.6 ± 0.5</td>
<td>18.6 ± 0.8</td>
</tr>
<tr>
<td>18:1, n-9</td>
<td>19.6 ± 2.0</td>
<td>18.0 ± 1.5</td>
<td>20.7 ± 2.1</td>
<td>20.4 ± 1</td>
<td>19.8 ± 0.8</td>
<td>21.1 ± 2.1</td>
</tr>
<tr>
<td>18:2, n-6</td>
<td>11.6 ± 0.1</td>
<td>12.7 ± 0.3</td>
<td>12.5 ± 0.2</td>
<td>11.1 ± 0.1</td>
<td>12.0 ± 0.3</td>
<td>11.8 ± 1.4</td>
</tr>
<tr>
<td>20:4, n-6</td>
<td>18.5 ± 0.5</td>
<td>17.1 ± 0.5°</td>
<td>15.5 ± 0.3°°</td>
<td>17.5 ± 0.2*</td>
<td>15.5 ± 0.2**</td>
<td>15.8 ± 0.2</td>
</tr>
<tr>
<td>22:5, n-3</td>
<td>2.7 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>2.0 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>22:6, n-3</td>
<td>5.9 ± 0.3</td>
<td>5.4 ± 0.5</td>
<td>4.8 ± 0.2</td>
<td>4.8 ± 0.8*</td>
<td>5.1 ± 0.6</td>
<td>4.4 ± 0.6</td>
</tr>
</tbody>
</table>

PUFA (%)  
NRBC: 38.7  
URBC: 35.8

DBI  
NRBC: 165.7  
URBC: 152.4

PL fatty acid composition was determined by gas liquid chromatography. DBI= Double Bond Index (obtained by multiplying the percentage of each fatty acid by the number of double bonds in that acid). Results are the mean (+ S.D.) of 4-8 determinations

(Paired t test for comparison of URBCs vs NRBCs of the same age, * p < 0.05 and ** p < 0.01; unpaired t test for comparison of medium and old NRBC vs young NRBCs, ° p < 0.05 and °° p < 0.01)

In NRBCs, palmitic acid (C16:0) increased with age, while arachidonic acid (C20: 4n-6) and docosahexaenoic acid (C22: 6 n-3) decreased, leading to a decrease of DBI (Table 3). In URBCs, C16:0 was significantly higher in young cells, while C20: 4n-6 was significantly lower in both young and medium cells as compared to the corresponding fractions of NRBCs; PUFA and DBI also decreased with age and were comparatively lower than NRBC controls (Table 3). In IRBCs, PUFA and DBI were lower than NRBCs and URBCs (Table 4). Saturated fatty acids (C16:0 and C18:0) were significantly higher, while all the unsaturated (C18:1n-9, C18:2n-6, C20:4n-6, C22:6n-3) were significantly lower than NRBCs and URBCs. C22: 5n-3 was not found. P. falciparum trophozoites had low, yet measurable levels of C14:0 which was undetectable in RBCs, very low levels of C20:4n-6 and C22:6n-3, and no
C22:5n-3. Parasites showed a DBI close to IRBCs, although C18:0 and C18:2n-6 were not statistically different from NRBCs and URBCs.

Table 4. Phospholipid fatty acid composition of infected erythrocyte ghosts and Plasmodium falciparum plasma membranes

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>IRBC</th>
<th>P.falciparum trophozoite</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0 n.d</td>
<td></td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>16:0</td>
<td>32.1 ± 0.5**</td>
<td>37.2 ± 1.5**</td>
</tr>
<tr>
<td>18:0</td>
<td>30.5 ± 1.0**</td>
<td>18.8 ± 0.8**</td>
</tr>
<tr>
<td>18:1,n-9</td>
<td>16.5 ± 0.7*</td>
<td>22.2 ± 1.3**</td>
</tr>
<tr>
<td>18:2,n-6</td>
<td>7.1 ± 0.8**</td>
<td>11.9 ± 1.4*</td>
</tr>
<tr>
<td>20:4,n-6</td>
<td>8.3 ± 0.3**</td>
<td>6.6 ± 0.6*</td>
</tr>
<tr>
<td>22:5,n-3</td>
<td>n.d</td>
<td>n.d.</td>
</tr>
<tr>
<td>22:6,n-3</td>
<td>4.6 ± 0.6♦</td>
<td>2.2 ± 0.5**</td>
</tr>
<tr>
<td>PUFA (%)</td>
<td>20</td>
<td>20.7</td>
</tr>
<tr>
<td>DBI</td>
<td>91.5</td>
<td>85.6</td>
</tr>
</tbody>
</table>

PL fatty acid composition was determined and DBI calculated as described in Table 3. Results are the mean (± SD) of 3 determinations. N.d.= not detectable

Paired t test for comparison of IRBC vs young NRBC and young URBC (see table 3):
• p < 0.05 and ** p < 0.01, ♦ p < 0.05 only vs young NRBC

Unpaired t-test for comparison of trophozoites vs IRBC membranes:
• p < 0.05 and ♦ ♦ p < 0.01

Stage of lipid peroxidation and sensitivity to oxidative stress. To investigate whether the modified fatty acid pattern could be related to different levels of lipid peroxidation, we examined the production of TBARS in basal conditions or after stimulation with the prooxidant system iron/ascorbate (Figure 4). Figure 4A shows higher basal levels of TBARS in young and medium URBC ghosts compared to NRBCs of the same age. Old URBCs were not significantly different from old NRBCs. A three fold increase in the level of basal peroxidation was found in IRBCs compared to NRBCs and URBCs. When challenged by iron-ascorbate, IRBC ghosts had the highest values of peroxidation, whereas, among
URBCs, only ghosts from young URBCs appeared more sensitive than their age-matched controls (Figure 4B).

Figure 4. Lipid peroxidation of ghosts from control, uninfected and infected erythrocytes
RBC ghosts were assayed for thiobarbituric reactive substances (TBARS) before or after induction for 3 hr at 37°C with the prooxidant system iron-ascorbate. Results (mean ± S.D.) are expressed as nmol TBARS/µmol red cell phospholipids
° p < 0.01 vs NRBC of the same age
* p < 0.01 vs NRBC and URBC
A: TBARS at basal conditions
B: TBARS after iron/ascorbate challenge
DISCUSSION

The present study shows that *P. falciparum* induces biochemical modifications in the membranes of both infected and uninfected erythrocytes mimicking the physiological cell’s aging process. These effects might be produced through *P. falciparum*-induced oxidative stress.

Cell populations obtained from the same donor which were either cultured but non-exposed to (NRBC, control RBC), co-cultured but not infected (URBC), or infected with *P. falciparum* (IRBC) were compared. A novel protocol was used (cell storage for different times at 4°C and culture at 37°C for 72 hr, the *P. f.* intraerythrocytic cycle) before Percoll fractionation, and the recovery in each fraction was quantified and compared. The implications of the methodology used in explaining our results vis-à-vis discrepancies in the literature are addressed further below.

We confirmed and quantified in NRBCs the changes occurring during RBC aging 21-26: (i) decreased AChE and G6PDH activities; (ii) decreased protein, PL and Cho; (iii) decreased PS; (iv) decreased PUFA and DBI. Some of these changes were more marked in cells infected with, or exposed to (notably young URBC) *P. falciparum*, as compared to NRBCs of the corresponding density (age). The AchE activity of young URBCs which was not different from that of homologous NRBCs when expressed per mg of protein, was actually lower, due to the lower content of protein per cell of this URBC fraction.

RBCs which are not infected (URBCs) but share the same environment with *P. falciparum* infected cells, become older prematurely, as suggested by the increase in the number of cells sedimenting in the medium and most dense fractions and the relative decrease of the young fraction, compared to NRBCs. These findings were reproducible and independent of the donor and storage time at 4°C.

Changes induced by *P. falciparum* to the properties of IRBCs are well documented. PL content increases due to the high biosynthetic activity 27 necessary for membrane biogenesis and essential to parasite’s survival and its composition is modified 1,28.

It is important to draw attention at this point on the paucity and conflicting nature of data reported in the literature when whole infected RBCs (reflecting the separate contribution of the erythrocyte and the
intracellular parasite itself) are studied and when total RBC populations of infected and uninfected cells are compared with no prior fractionation \textsuperscript{1,2,5,29,30}. This variability has been attributed to different parasite species, level of parasitemia, developmental stages of the parasite, or purity of cell membranes. Based on our results, additional factors, which can be controlled by standardizing the experimental methodology, contribute to these discrepancies. RBCs from different donors can be very different in their lipid composition, and, within each donor, they vary with age. Aging (in vivo and/or during storage at 4°C) is accompanied by physical and biochemical modifications including enzymatic activities, lipid composition and peroxidative damage of membrane PL \textsuperscript{21-26,31}. Therefore, one should compare infected and uninfected RBC only with control RBCs from the same donor, banked for the same number of days and maintained under the same culture conditions; RBC fractions (separated on a density gradient) should be used instead of whole unFractionated cell population; infected and uninfected RBC fractions should be compared to control fractions of the same density (age). It is also worth noting that \textit{P.falciparum} shows a preference for invading young RBCs \textsuperscript{32}. We ourselves did not see any difference between whole control and URBCs before density fractionation. In the whole RBC population, the decrease in lipid and protein content due to the loss of the younger RBC and to the lower protein and lipid content of IRBC and young URBC is masked by the relative increase in the old fraction. This helps explaining why previous studies which used unFractionated RBC populations could not see differences between normal and uninfected RBC \textsuperscript{5,29}.

Our results are not influenced by contaminants. The purity of RBCs derived from \textit{P.falciparum} cultures was verified by measuring the activities of pLDH (the parasite-specific form of LDH assayed by the NAD analogue APAD), and GDH (a mitochondrial enzyme present only in the parasite) \textsuperscript{19,33}. Other features too (see below) support the purity of IRBCs and URBCs from parasites.

Several data indicate that oxidative stress increases with age in RBCs, and intensifies with \textit{P.falciparum} infection. In agreement with published data, we too found increased endogenous TBARS in older RBCs, suggesting a higher level of lipid peroxidation in aged erythrocytes\textsuperscript{25,34}. Hydroperoxides have been shown to exert an inhibitory effect on the PL reacylating reactions\textsuperscript{35}, and could contribute to the
impairment of fatty acid esterification found in aged erythrocytes\textsuperscript{26,36} as well as to the loss of PUFA and PL observed with RBC aging in the present and previous works. On the other hand, endogenous TBARS levels were higher in IRBCs and also in young and medium URBCs compared to NRBCs of the same densities. This finding supports the view that peroxidative processes increase in RBCs during \textit{P.falciparum} infection\textsuperscript{37}. Membrane damage is enhanced in IRBCs, as shown by the lower unsaturation index (DBI) and the modified PL pattern, whereby PS and PC are markedly decreased. The lower DBI of IRBC ghosts, (about 50\% that of NRBC), is accounted for by the loss of PUFA and the increase of both the saturated species palmitic and stearic acid. Compared to \textit{P.falciparum} trophozoites, IRBCs had very similar DBI and PUFA values, but a much higher content in stearic acid and a lower content in oleic and linoleic acid, as well as higher proportions of SM and PS (as molar percentage and absolute value/mg protein). Low percentages of SM and PS in trophozoite membranes and in the whole infected RBCs were reported also by others\textsuperscript{5,29}. These differences, along with the lower content of PL and Cho of trophozoite (0.6 \(\mu\text{mol/mg protein}\) and 0.12 \(\mu\text{mol/mg protein}\), respectively) are a further indication of the negligible contamination of parasite membrane by RBC membranes in our specimens. Also the lower AChE activity of IRBCs, compared to the NRBCs of the same density (very young fraction), could be ascribed to \textit{P.falciparum}-induced oxidative stress and/or the reduced content of PUFA\textsuperscript{38-40}. \textit{P.falciparum} accelerated also the age-related modifications of URBCs, particularly the young fraction. We found a loss in arachidonic and docosahexaenoic acid but, differently from IRBCs, the increase in saturated species was limited to palmitic acid. On a percentage molar basis, lower PS levels were found in URBCs compared with NRBCs of the same density, which were apparently compensated by increased SM. However, since young URBCs have a lower PL content compared to the control fractions, all the PL species were reduced in absolute terms, albeit to a lower extent compared to PS. The greater loss in PS could be partly ascribed to its richness in unsaturated fatty acids, which are most vulnerable to peroxidative breakdown, but also to its increased exposure on the outer surface of the cell membrane. Lipid asymmetry of the membrane, maintained by an aminophospholipid translocase activity, is reduced in aged erythrocytes\textsuperscript{41} or erythrocytes under oxidative stress\textsuperscript{42}. Altered membrane
PL organization, particularly a greater PS exposure on the outer surface of the membrane, has been reported in both IRBCs $^{2,43,44}$ and URBCs $^{45,46}$, though not confirmed by others $^{29,47}$. Oxidative stress induced by *P. falciparum* might contribute to the loss of PS by changing the transbilayer organization of the membrane phospholipids and enhancing PS exposure on the outer surface.

Iron-ascorbate induction was studied to assess whether *P. falciparum* infection could induce changes in RBC susceptibility to lipid peroxidation. Membranes obtained from IRBCs and young URBCs are more sensitive to peroxidation than those from NRBCs, as shown by the levels of TBARS production, despite their lower degree of unsaturation. This apparent incongruity could be related to the different lipid distribution in the membrane, promoting the presence of defect points, which facilitate radical attack. A lower content of vitamin E has been found in intact parasitized erythrocytes$^{48}$; therefore, a depletion in endogenous antioxidant protection, in particular vitamin E, can not be excluded and deserves further investigation.

Alterations in the organization of PL has been shown to compromise membrane integrity and lead to early hemolysis or reticuloendothelial clearance$^{49}$ or phagocytosis$^{41,50}$. Therefore the observed abnormalities in the organization of RBC PL may have pathophysiological implications and possibly shorten cells’ life span.

The nature of the modifications observed in IRBC and URBC plasma membranes suggests that *P. falciparum* can exert a strong oxidative stress in RBC culture.

Differently from erythrocyte membranes, parasite membranes appear to be quite resistant to oxidative stress. They are very poor in cholesterol and have a lower content of PL, characterized by a higher level of fatty acid saturation than RBCs (see also Hsiao et al.$^5$). These features will very likely contribute to parasite resistance to the oxidant environment where they live and critical processes taking place in the parasite itself. The release of hemoglobin-derived heme and its oxidation and crystallization to hemozoin (malaria pigment) in the parasite food vacuole generates toxic radicals. Even hemozoin that is considered a non toxic storage of heme for the parasites, has been shown to catalyze peroxidative processes in cell culture and cell free systems$^{10,11,51}$. Parasites seem to avail of different tools to cope
with such an environment: (i) they are rich of antioxidant enzymes either imported from host or newly synthesized; (ii) as shown here, the composition of their membranes is quite resistant to oxidative damage; and (iii) to minimize contact with oxygen, they have evolved a way to survive in microaerophylic conditions. The food vacuole, the organelle where the digestion of hemoglobin occurs, is likely to be an anaerobic compartment, as we recently suggested.

In conclusion, the results of this study show that the growth of *P.falciparum* affects primarily the host RBC but modifies also the surrounding uninfected RBCs, while preserving the integrity of the microorganism. These findings have implications for both a better understanding of pathological manifestations of malaria, leading to its severe complications. The changes in URBCs can shorten the cell’s life span through premature removal, thus contributing to chronic and severe anemia observed in areas of intense malaria transmission, where particularly young children and pregnant women live with large numbers of parasites in their blood. In addition, these changes may be a factor of increased RBC membrane rigidity, thus contributing to cells jamming in small vessels and hence to organ damage of severe malaria. For the parasite, under the circumstances, approaches using membrane oxidants are unlikely to be effective.

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references


Accelerated senescence of human erythrocytes cultured with Plasmodium falciparum

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