Ex vivo perfusion of human spleens maintains clearing and processing functions

Pierre A. Buffet, Geneviève Milon, Valentine Brousse, Jean-Michel Correas, Bertrand Dousset, Anne Couvelard, Reza Kianmanesh, Olivier Farges, Alain Sauvanet, François Paye, Marie-Noëlle Ungeheuer, Catherine Ottone, Huot Khun, Laurence Fiette, Ghislaine Guigon, Michel Huerré, Odile Mercereau-Puijalon, and Peter H. David

The spleen plays a central role in the pathophysiology of several potentially severe diseases such as inherited red cell membrane disorders, hemolytic anemias, and malaria. Research on these diseases is hampered by ethical constraints that limit human spleen tissue explorations. We identified a surgical situation—left splenopancreatectomy for benign pancreas tumors—allowing spleen retrieval at no risk for patients. Ex vivo perfusion of retrieved intact spleens for 4 to 6 hours maintained a preserved parenchymal structure, vascular flow, and metabolic activity. Function preservation was assessed by testing the ability of isolated-perfused spleens to retain Plasmodium falciparum–infected erythrocytes preexposed to the antimalarial drug artesunate (Art-iRBCs). More than 95% of Art-iRBCs were cleared from the perfusate in 2 hours. At each transit through isolated-perfused spleens, parasite remnants were removed from 0.2% to 0.23% of Art-iRBCs, a proportion consistent with the 0.02% to 1% pitting rate previously established in artesunate-treated patients. Histologic analysis showed that more than 90% of Art-iRBCs were retained and processed in the red pulp, providing the first direct evidence of a zone-dependent parasite clearance by the human spleen. Human-specific physiologic or pathophysiologic mechanisms involving clearing or processing functions of the spleen can now be experimentally explored in a human tissue context. (Blood. 2006;107: 3745-3752)

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Introduction

The spleen plays a key role in innate and adaptive immune responses, in selective clearance of aged or abnormal red blood cells (RBCs), and in removal of pathogens present in the blood. It is also central to the pathophysiology of several frequent and potentially severe diseases such as inherited red cell membrane disorders, hemolytic anemias, and malaria. Detailed studies on the mechanisms involved are hampered by 2 linked factors: the human spleen displays anatomic and physiologic features not observed in rodent models, and the human spleen is difficult to explore. The risk of life-threatening intraperitoneal bleeding makes spleen biopsy or needle-aspirate an unethical method for human research purposes. Histology studies from postmortem or splenectomy samples provide crucial information, but their scope is limited to late or complicated disease stages. So far, explorations of the human spleen have been restricted to “black box” approaches, in which intrasplenic tissue processing was inferred from the kinetics of elements circulating in the peripheral blood. Humanized mouse models provide an interesting research tool, but are limited by the rapid clearance of normal human RBCs and by the different spleen anatomy, vascularization/circulatory bed, and cellular subsets compared with humans.

The spleen is one of the few organs that may be removed while healthy. Left pancreas resection for tumor removal usually includes a splenectomy for vascular-related constraints. Retrieval of intact spleen is then possible without modifying the patient’s medical and surgical care, making experimental study of the human spleen clearing and processing functions during ex vivo perfusion theoretically feasible. However, unlike other isolated-perfused organs such as the heart, liver, or kidney, no simple macroscopic marker such as contractility, or bile/urine flow, is available to evaluate the preservation of spleen functions. Studies in splenectomized Plasmodium falciparum malaria patients have shown that most drug-exposed parasite clearance is spleen dependent. Furthermore, work in the last decades defined methods for accurate antimalarial drug efficacy assessment that provide tools for quantifying spleen-clearing function. Drug-exposed parasite clearance kinetics was established from clinical observations, in particular from artesunate-treated patients. Pitting, a phenomenon whereby erythrocytes containing inclusion bodies squeeze through the narrow interendothelial slits of the venous sinus wall, leaving a fraction of the cell containing the nondeformable inclusion bodies trailing behind on the reticulocyte meshwork side, was shown to account for a proportion of drug-exposed Plasmodium falciparum–infected RBC clearance.

In order to use clearance of artesunate-treated Plasmodium falciparum–infected red blood cells (Art-iRBCs) as a physiologic readout of spleen function, we measured clearance kinetics and pitting rate of Art-iRBCs in isolated-perfused spleens. We show here that ex vivo perfusion maintains these clearing functions at physiologic levels.

From the Biomedical Research Team, Medical Center, the Molecular Immunology of Parasites Unit, Centre National de la Recherche Scientifique, Unité de Recherche Associée (CNRS URA) 2581, Parasitology Department, the Immunophysiology and Intracellular Parasitism Unit, Parasitology Department, the Unité de Recherche et d’Expertise (URE) Histotechnology and Pathology, and the Public Health Platform, Institut Pasteur, Paris, France; and Assistance Publique–Hôpitaux de Paris, Paris, France.


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Reprints: Pierre A. Buffet, Biomedical Research Team, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France; e-mail: pabuffet@pasteur.fr.

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Patients, materials, and methods

Human spleen retrieval

Spleens were retrieved downstream of surgical interventions involving patients with left pancreas tumors, during which the spleen had to be removed for vascular-related constraints. Medical and surgical care was not modified and patient consent was obtained by the surgical team. Regulatory steps were according to the French law that guarantees patient safety, confidentiality, and protection from advertisement or economic incentive (art. L.1211-4, L.1211-3, L.1211-6 of the French Code of Public Health Regulations). The project was reviewed by the Necker Hospital investigational review board. The only captured clinical information was age, sex, ABO Rhesus blood group, and surgical indication. All documents were identified with a code number. Only the surgical team kept the list containing the spleen codes and patient names. At the end of each experiment, a detailed written macroscopic description of the spleen was provided to the referent pathologist along with photographs and more than 4 formaldehyde-fixed fragments from different parts of the spleen. Retrieved spleens were devoid of any macroscopic or microscopic alteration. Upon a 30- to 90-minute period of warm ischemia linked to the surgical procedure, the main splenic artery and vein were cannulated. The spleens were flushed with cold Krebs-albumin solution (25 mmol NaHCO3, 118 mmol NaCl, 4.7 mmol KCl, MgSO4, 1.2 mmol 7 H2O, 1.2 mmol NaH2PO4, CaCl2, 1.2 mmol 2 H2O, 7 mmol glucose, and 5 g human serum albumin [albumax], for 1 L sterile water) for transport to the laboratory. Once in the laboratory (cold ischemia time, 60-90 minutes), the spleen was connected to the perfusion device.

Ex vivo perfusion device

Preliminary establishment of the optimal perfusion conditions was performed with Dr Francis Paulmier and his team (INRA Nouzilly France) with 12 piglet spleens perfused with uninfected pig RBCs. Subsequently, 6 human spleens were retrieved, 3 of them perfused with human uninfected RBCS only, and 3 with human uninfected RBCs and Art-iRBCs, as explained in “Setting-up of model, assessment by metabolic markers, and vascular imaging.” Using a final perfusion device adapted from that described by Pabst et al (Figure 1). Major modifications of this device were a reduction of dead volume allowing the closed-circuit perfusion of a total volume of 150 mL, and the use of a manufactured glass warming chamber (Christian Roujoux, Institut Pasteur, Paris, France) allowing easy access to the spleen hilum while maintaining most of the capsule surface in a 37°C environment. A peristaltic pump (ISM 920 pump from Ismatec, Labortechniki, Glattbrugg-Zürich, Switzerland; and Masterflex 60419-17 tube from Tygon, Saint-Gobain, France) provided flow into sterile tubing (Transfusion kit; Transfumed, Sendal, Almaraz, Caceres, Spain). The perfusate was aspirated from a cylindric reservoir (Rubbermaid 300 mL; Curver, Luxembourg) through a bubble-trap (Transfusion kit; Transfused) then pushed through 2 m of silicone gas-permeable tubing (Tygon 3350 Silicon 1/8 × 3/16; Cole-Parmer Instrument Company, Vermont Hills, IL) around which a 3% CO2/100% O2 gas phase was maintained through a constant 1 L/min gas flow. Prior to entry into the spleen through a glass catheter, the perfusate is warmed using a 37.5°C-equilibrated heating coil. Cells, reagents, and samples for analysis are introduced into or removed from the reservoir. (B) Qualitative comparison of patient and IP-spleen parenchymal enhancement using contrast ultrasonography. Early and late parenchymal aspect after bolus injection of contrast agent, from an IP-spleen (Bi) and a representative human volunteer (Bii) showing the same progressive homogenous enhancement. For technical reasons, the ultrasonographic section is from anterior capsule to posterior capsule in IP-spleens, whereas it is from capsule to hilum in patients.

Figure 1. Description and vascular evaluation of the model. (A) Schematic diagram of the experimental set-up of the human IP-spleen. A peristaltic pump provides flow into polypropylene tubing. The perfusate is aspirated from the reservoir through a bubble-trap and then pushed into 2 m of silicone gas-permeable tubing around which a 3% CO2/100% O2 atmosphere is maintained through a constant 1 L/min gas flow. Prior to entry into the spleen through a glass catheter, the perfusate is warmed using a 37.5°C-equilibrated heating coil. Cells, reagents, and samples for analysis are introduced into or removed from the reservoir. (B) Qualitative comparison of patient and IP-spleen parenchymal enhancement using contrast ultrasonography. Early and late parenchymal aspect after bolus injection of contrast agent, from an IP-spleen (Bi) and a representative human volunteer (Bii) showing the same progressive homogenous enhancement. For technical reasons, the ultrasonographic section is from anterior capsule to posterior capsule in IP-spleens, whereas it is from capsule to hilum in patients.

medium flow from 1 mL/min to 100 to 150 mL/min over 40 to 60 minutes. Krebs-albumin medium was changed at least twice during this adaptation period (600-1000 mL total volume) until most of the patient’s RBCs were flushed from the spleen. At steady state, perfusate flow was 1 mL/g spleen/min, and temperature of the spleen capsule was maintained between 36.7°C and 37.2°C. In order to improve oxygen delivery to isolated-perfused spleens, O2 uninfected RBCs were added and allowed to circulate for 30 to 60 minutes before introducing Art-iRBCs (2.5%-6% parasitemia). Spleen aspect, gas flow through the artificial lung, perfusate aspect/color, as well as “arterial” pressure (ie, upstream of the spleen) were constantly monitored, and were recorded every 5 to 10 minutes. The arterial pressure was maintained between 60 to 120 mmHg. Five minutes before introducing Art-iRBCs, the spleen was rinsed again with 400 mL Krebs-albumin medium until the perfusate hematocrit level was less than .001 (0.1%)—in order to limit the influence of Art-iRBC dilution into uninfected RBCs on Art-iRBC concentration kinetics. After perfusion of Art-iRBCs, the initial hematocrit level was .016 (1.6%) or .04 (4%). Hematocrit, K+ and Na+ concentrations were recorded every 30 to 60 minutes. Glycemia was evaluated every 10 minutes using a

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Results

Setting-up of model, assessment by metabolic markers, and vascular imaging

The perfusion system was first established with spleens obtained from 12 anaesthetized piglets, as parameters such as spleen size and major vessel sections are similar to those of humans. The piglet spleens allowed for adjustment of the parameters to achieve appropriate physiologic metabolic and circulatory features (“Patients, materials, and methods”). The isolated human spleens were then set up in the experimental system shown Figure 1A. A typical experiment consisted of perfusing first Krebs-albumin solution, then red blood cells for 0.5 to 1 hour, and finally infected RBCs for 2 hours. The perfusion time for Krebs and RBCs was adjusted for each spleen until a steady state was reached, based on glucose consumption, stable flow, and arterial pressure. At the end of the experiment, the isolated-perfused human spleen was fixed and processed for histology. The physiologic and functional characteristics of the isolated-perfused spleen were assessed using several approaches. Ultrasonography at steady state showed a homogeneous echostructure of the parenchyma, devoid of hyperechogenic or hypoechochogenic structures such as air bubbles or large ischemic zones. Injection of the contrast agent (microbubbles) induced a progressive parenchymal enhancement in the isolated-perfused spleen (Figure 1Bi-ii), qualitatively similar to that observed in human volunteers (a representative example is shown Figure 1Bi-iv). Doppler analysis showed large permeable vessels, often localized in pairs, through which flows ran in opposite directions (data not shown). At steady state, the arterial pressure was maintained at 60 to 120 mmHg. During each experiment, the Na⁺ and K⁺ concentrations varied by less than 20%. There was significant glucose consumption, reflecting metabolic activity, so that glucose levels were monitored every 10 to 20 minutes, and extra glucose was added to maintain 1- to 2-g/L levels. Repeatedly measured arterial and venous O₂ and CO₂ partial pressures were within physiologic limits. From artery to vein, O₂ partial pressure decreased by more than 80 mmHg, whereas CO₂ partial pressure increased by more than 8 mmHg, showing O₂ consumption and CO₂ production, as expected to occur in an appropriately perfused and metabolically active organ. This points to a good spleen resistance to short periods of warm ischemia.

Clearance rate of infected red blood cells exposed to a lethal dose of artesunate (Art-iRBCs)

Perfusion of ring stage–infected RBCs previously exposed to a lethal dose of artesunate for 8 to 12 hours in vitro was started 10 to 30 minutes after a stable 1 mL/g per minute flow was reached. The concentration of artesunate used was in the range of the peak plasma level observed in patients treated intramuscularly with the drug.28 Typical morphology of Art-iRBCs containing dead parasite remnants with a shrunken, dense cytoplasm and pyknotic nucleus is shown in the Figure 2 (Aiii,iv).15 Sequential Giemsa-stained thin films of the circulating cells showed that Art-iRBC counts decreased with a half-life of 17 to 18 minutes, with an overall clearance time of approximately 120 minutes (2 experiments with differing initial parasitemia are shown in Figure 2). At that time, more than 95% of the parasite input could no longer be observed in the perfusate, indicating an efficient and rapid clearance. As a control, no change in parasitemia was observed when infected RBCs circulated through a device to which no spleen had been

dipstick glucometer (Accu-Chek and Glucotrend Premium; Roche Diagnostics, Mannheim, Germany). Glucose in water (150 g/L) was added as required to maintain glycemia between 1 and 2 g/L.

Ultrasonographic evaluation

The spleen was covered with transparent Saran wrap to prevent evaporation and ultrasonography gel leakage into the perfusate. At different time points, ultrasonography was performed (Philips HDI 5000; Philips, Bothell, WA). A 1-mL bolus of microbubbles (Sonovue; Bracco, Milan, Italy) was added into the reservoir and the parenchymal enhancement recorded using microvascular imaging analysis (MVI; Philips). In a separate prospective clinical study, human volunteers were administered a 6-mL bolus of microbubbles and the same ultrasonographic methodology was applied. The results of this study will be reported elsewhere (J.-M.C., manuscript in preparation). The qualitative characteristics of parenchymal enhancement of isolated-perfused spleens was compared with that observed in patient spleens.

Functional validation

P. falciparum parasites (strain FUP/CB) were cultured in RPMI medium supplemented with 5% albumax/5% AB human serum. Ring-stage parasites were incubated for 8 to 12 hours with artesunate (0.1 μg/mL) prior to centrifugation and introduction into the perfusate. At that time, the parasites (Art-iRBCs) were no longer viable as assessed by the absence of any reinvasion in culture. At different time points, 100 to 200 μL was collected from the perfusate and centrifuged, and the pellet was used for thin smears and erythrocyte membrane immunofluorescence (EMIF).

EMIF

EMIF was performed as described by Perlmann et al.27 Briefly, infected red blood cell monolayers were prepared from aliquots of the perfusate and fixed in 1% glutaraldehyde for 20 seconds (Sigma, St Louis, MO). Labeling of ring-injected erythrocyte surface antigen (RESA) was performed with sera from hyperimmune African adults (1:200 serum dilution in PBS/1% BSA) followed by Alexafluor 488–conjugated goat anti–human IgG (diluted 1:500; Molecular Probes, Eugene, OR). Parasite nuclei were stained with 10 μg/mL Hoechst 33342 (Molecular Probes). Slides were mounted with Vectashield medium (Vector laboratories, Burlingame, CA). Images were acquired on a Zeiss Axiovert 200 M microscope, using an Axiocam HRc camera controlled by Zeiss Axiovision software (all from Carl Zeiss, Heidelberg, Germany).

Histologic evaluation

At the end of the experiments, the spleen was gently perfused with 70 to 100 mL buffered 4% formaldehyde for 4 to 5 minutes (flow maintained below 20 mL/min, pressure below 15 cm H₂O) to allow the homogenous fixation of the tissue. Blocks (10 × 10 × 5 mm) were fixed in 4% buffered formaldehyde. Slides (3-μm thick) were stained with Giemsa for microscopic examination. Pictures were acquired on a Nikon E 800 microscope, using a Nikon digital still DXM 1200 camera controlled by an ACT-1 version 2 Nikon software (all from Nikon, Tokyo, Japan). Images were visualized with a Plan Apo 40 ×/0.95 DICm or a Plan Apo 100 ×/1.40 oil-immersion DiCM objective lens (Nikon). Giemsa-stained slides were photographed at × 400 magnification and read after the superimposition of a counting grid. Brown dots larger than 0.5 μm were counted and localized on 20 photographs for each spleen. Immunohistochemical analysis was performed essentially as described.6 Briefly, after appropriate preparation, sections were incubated with either anti-CD68 mouse monoclonal antibody (M0876, dilution 1:50 in PBS/BSA; Dako, Glostrup, Denmark) or a convalescent patient’s serum (dilution 1:75 in PBS/BSA). For detection of anti-CD68 and convalescent patient antibodies, rabbit anti–mouse IgG linked to peroxidase and a goat anti–human IgG linked to alkaline phosphatase were used, both at a 1:1000 dilution in PBS/BSA. The enzymatic complexes were revealed using aminobenzidine for peroxidase and fast blue for alkaline phosphatase.
Calculated pitting rate per spleen transit, % of iRBCs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Value (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.23 (0.02-1)</td>
</tr>
<tr>
<td>2</td>
<td>0.06 (90)</td>
</tr>
</tbody>
</table>

Pitting rate per body transit, % of iRBCs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Value (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>0.003 (0.001-0.05)</td>
</tr>
</tbody>
</table>

Estimated amount of Art-iRBCs pitted per spleen transit (DPRs) on Giemsa-stained smears from the perfusate.

- DPRs on Giemsa-stained smears
- Typical morphology of Art-iRBC containing dead parasite remnants
- Progressively increased in the perfusate, reflecting an active pitting process.
- O-iRBCs accounted for 66% to 80% of the RESA-positive RBCs after 90 minutes. The pitting rate per transit through isolated-perfused spleens (0.2%-0.23%) was similar to that deduced in human patients (0.02%-1%).

Total numbers of infected RBCs pitted per hour were also in the same range (Table 1).

On Giemsa-stained histologic sections, uninfected RBCs were observed as they squeezed to cross the sinus walls from the cords to the sinus lumen (Figure 3Bi-ii). Typical pictures of the pitting process were observed with Art-iRBCs (Figure 3Ci-ii), along with less conventional patterns such as stringlike formations linking O-iRBCs to their “just-pitted” dead parasite remnant (Figure 3Di) or “being-pitted” large dead parasite remnants with a bipolar cordal-luminal aspect (Figure 3Ciii,Di). In some instances, completely pitted infected RBCs were observed in the sinus lumen, with their “almost-pitted” dead parasite remnant still attached to the RBC membrane, sometimes in close contact with luminal cells (Figure 3Diii-iv). Pitting of Art-iRBCs was also observed by immunofluorescence using a human convalescent serum, with the immunoglobulins detected by alkaline-phosphatase–labeled anti-human Ig antibodies (Figure 3E). Overall, pitted infected RBCs represented 12% to 21% of the initial Art-iRBC loads (Table 1), indicating that other clearance mechanism(s) were involved.

Pitting of Art-iRBCs

Red blood cells leaving the reticular meshwork of the red pulp must cross the sinus wall to enter the venous system. During this process, erythrocytes containing inclusion bodies undergo pitting.

- Art-iRBCs containing dead parasite remnants
- Progressively increased in the perfusate, reflecting an active pitting process.
- O-iRBCs accounted for 66% to 80% of the RESA-positive RBCs after 90 minutes. The pitting rate per transit through isolated-perfused spleens (0.2%-0.23%) was similar to that deduced in human patients (0.02%-1%).

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Direct phagocytosis of Art-iRBCs in the cords

On Giemsa-stained sections of the spleen, dead parasite remnants appeared as brown dots either within intact Art-iRBCs (intraerythrocytic dead parasite remnants) or outside RBCs (extraerythrocytic positive EMIF reaction, but lack an intraerythrocytic dead parasite and hence are not stained with Hoechst 33342, a nuclear DNA marker. Figure 3Aiii-iv shows that the proportion of such O-iRBCs progressively increased in the perfusate, reflecting an active pitting process. O-iRBCs accounted for 66% to 80% of the RESA-positive RBCs after 90 minutes. The pitting rate per transit through isolated-perfused spleens (0.2%-0.23%) was similar to that deduced in human patients (0.02%-1%). Total numbers of infected RBCs pitted per hour were also in the same range (Table 1). On Giemsa-stained histologic sections, uninfected RBCs were observed as they squeezed to cross the sinus walls from the cords to the sinus lumen (Figure 3Bi-ii). Typical pictures of the pitting process were observed with Art-iRBCs (Figure 3Ci-ii), along with less conventional patterns such as stringlike formations linking O-iRBCs to their “just-pitted” dead parasite remnant (Figure 3Di) or “being-pitted” large dead parasite remnants with a bipolar cordal-luminal aspect (Figure 3Ciii,Di). In some instances, completely pitted infected RBCs were observed in the sinus lumen, with their “almost-pitted” dead parasite remnant still attached to the RBC membrane, sometimes in close contact with luminal cells (Figure 3Diii-iv). Pitting of Art-iRBCs was also observed by immunofluorescence using a human convalescent serum, with the immunoglobulins detected by alkaline-phosphatase–labeled anti-human Ig antibodies (Figure 3E). Overall, pitted infected RBCs represented 12% to 21% of the initial Art-iRBC loads (Table 1), indicating that other clearance mechanism(s) were involved.

**Table 1. Experimental pitting rate in isolated-perfused spleens compared with published data in artesunate-treated patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>DHA-treated patients data from Newton et al</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of red blood cells (RBCs) in IP-spleen perfusate or in patient blood, mL</td>
<td>13</td>
<td>10</td>
<td>1500-2000</td>
<td></td>
</tr>
<tr>
<td>iRBCs in IP-spleen perfusate or in patient blood</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial concentration, % of RBCs</td>
<td>2.6</td>
<td>5.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume, mL</td>
<td>0.338</td>
<td>0.563</td>
<td>&gt; 100</td>
<td></td>
</tr>
<tr>
<td>Total no.</td>
<td>3.38 × 10^9</td>
<td>5.63 × 10^9</td>
<td>&gt; 10^12</td>
<td></td>
</tr>
<tr>
<td>Parasite clearance time (corrected value), h</td>
<td>≲ 2 (≤ 40)</td>
<td>≲ 2 (≤ 40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak concentration of once-infected RBCs (O-iRBCs), % of RBCs‡§</td>
<td>0.31</td>
<td>1.19</td>
<td>1.8 (0.8-8.8)</td>
<td></td>
</tr>
<tr>
<td>Estimated global proportion of Art-iRBCs pitted during the experiment, %§</td>
<td>11.9</td>
<td>21.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated cumulative no. Art-iRBCs pitted</td>
<td>4.03 × 10^8</td>
<td>11.33 × 10^9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to peak of O-iRBCs (corrected value), h</td>
<td>2 (20)</td>
<td>1.5 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calculated no. of Art-iRBCs pitted per h.  × 10^6</td>
<td>4.03</td>
<td>7.55</td>
<td>21 (5-126)</td>
<td></td>
</tr>
<tr>
<td>Calculated amount of Art-iRBCs pitted per spleen transit, × 10^6</td>
<td>6.71</td>
<td>12.59</td>
<td>26 (6-177)</td>
<td></td>
</tr>
<tr>
<td>Pitting rate per body transit, % of iRBCs</td>
<td>0.2</td>
<td>0.23</td>
<td>0.06 (0.02-1)</td>
<td></td>
</tr>
</tbody>
</table>

NA indicates not assessed.

Assuming the whole RBC pool crossed IP-spleens once per minute.

*Number calculated assuming an RBC concentration of 10^10/mL (10^-3/L).

Observed parasite clearance time and time to peak of O-iRBCs were multiplied by 20 [corrected time] to take into account the fact that a given Art-iRBC crosses patient spleen every 20 minutes, but does so every minute through isolated perfused spleens.

The concentration of O-iRBCs at any time point was calculated using the following formula: concentration of O-iRBCs in the perfusate = % of iRBCs on Giemsa-stained smears: number of O-iRBCs/number of iRBCs on EMIF- and Hoechst-stained slides. The peak concentration was the highest observed value in each experiment. Because the peak concentration of O-iRBCs depends on parasitemia on Giemsa smears, it may not be perfectly synchronous with the peak proportion of O-iRBCs among EMIF-positive cells. This occurred during experiment 1.

The estimated pitting rate was calculated by dividing the peak concentration of O-iRBCs by the initial IRBC concentration, an acceptable assumption since the hematocrit level remained stable during experiments.

Indicates number of O-iRBCs produced. This minimal value was estimated under the hypothesis that no O-iRBC clearance occurred before the peak O-iRBC concentration was reached.

Assuming that the whole RBC pool crossed IP-spleens once per minute.

**Pitting rate per spleen transit = number of Art-iRBCs pitted during the experiment: number of spleen transits to reach the peak (ie, 60 for experiment 1 and 90 for experiment 2).**

Pitting rate was calculated per body transit in patients. It takes approximately 20 body transits for a given iRBC to cross the spleen once.
dead parasite remnants) (Figure 4A,Dii). Counting brown dots per × 400 field of Giemsa-stained sections (as seen in Figure 4A-B) showed that the number of brown dots in the spleen tissue at the end of experiments was related to the number of Art-iRBCs initially introduced in the system (one-way analysis of variance and multiple comparison tests, Bonferroni correction with 3 independent spleens and 20 sections/spleen, P < .05 for each paired comparison). In contrast, a low (1-5 dots per × 400 field) background was observed in the red pulp of an isolated-perfused spleen through which no Art-iRBCs had been perfused. This confirmed that the brown dots observed on sections of Art-iRBC-perfused spleens were attributable to dead parasite remnants. Extracellular dead parasite remnants accounted for 65.2% of dead parasite remnants (mean count for 3 experiments, 20 × 400 fields counted/experiment; range, 53%-79.5%) showing that, over 2 hours, isolated-perfused spleens had not only retained but also efficiently and rapidly processed the majority of Art-iRBCs. Intact Art-iRBCs were observed either free or colocalizing with cord cells (Figure 4A,Cia). Spleen sections double-stained with a serum from a convalescent *P falciparum* patient and anti-CD68 antibodies (Figure 4Cii) showed the same pattern, namely dead parasite remnants either inside intact Art-iRBCs or inside CD68+ macrophages, sometimes in close proximity with RBC fragments (Figure 4Ci-ic). A variety of morphologic patterns was observed, from fully intact Art-iRBCs in close contact with (Figure 4Ciia)—or apparently engulfed by (Figure 4Ciib)—macrophages to fragmented Art-iRBCs and dead parasite remnants within macrophages (Figure 4Ciic). Fragmentation of extracellular Art-iRBCs was very rarely observed. Taken together, these data strongly suggest that direct phagocytosis of intact Art-iRBCs by macrophages took place in isolated-perfused spleens, a process leading to intramacrophagic dead parasite remnants (Figure 2CiCd,CiiCd). Analysis of the localization of extracellular dead parasite remnants (see Figure 4Di for definitions) showed that 83.7% (range, 79%-87%) colocalized with cord cells. Only 4.4% (range, 1.9%-6.8%) of extracellular dead parasite remnants were free, whereas 11% (range, 9%-13%) colocalized with sinus wall cells.

**Figure 3. Kinetics and morphologic aspects of pitting.** EMIF-positive iRBCs (EMIF+–iRBC) (Ai) were distinguished from once-infected RBCs (O-iRBC, Aii arrow) by the presence of intraerythrocytic Hoechst-labeled dead parasite remnants (DPRs). The proportion of O-iRBCs in the perfusate increased during experiments (Aiii-iv). On Giemsa-stained histologic sections, uninfected RBCs were observed as they squeezed across the sinus walls (Bi-ii) from cords (co) to sinus lumens (sl). During pitting of Art-iRBCs, small DPRs were observed either free or colocalizing with cord cells (Di-ii)—a typical aspect of the pitting process (Ci-iii)—whereas large DPRs had a bispheval cordal-luminal aspect (Di). Some DPRs retained in the sinus wall were still linked to their once-host cell by a string-like formation (Dii) and incompletely pitted Art-iRBCs were observed in sinus lumens, with a membrane-bound DPR in contact with a luminal cell (Diii-iv). (E) Aspect of pitting as observed by immunohistochemistry using a human convalescent serum, the Ig being detected by alkaline-phosphatase labeled reagent.
the suitable quality of the perfusion of this peculiar zone in isolated-perfused spleens.

Discussion

Exploring the abdominal, prone-to-bleed, difficult-to-sample human spleen has often resembled studying a black box. The retrieval of healthy spleen after selected surgical interventions offered the opportunity to set up and validate a functional isolated-perfused human spleen system. The quantitative readout for the clearing function of the isolated-perfused human spleens provided results in close similarity with in vivo data obtained from malaria patients in terms of clearance kinetics, qualitative tissular processing, and pitting rate (Figure 2; Table 1). The pitting rate we are referring to in the present analysis is different from the rate of circulating pitted RBCs used as the gold standard for assessing spleen filtering function in patients with potential asplenia. "Classic" pitted RBCs result from macrophage-mediated removal of (nonmicrobial) intracellular remnants as evidenced by small surface depressions that can be visualized using differential interference microscopy (Nomarski optics). Because this pitting process removes part of the red cell membrane, pitted RBCs become less deformable and are cleared from the circulation by the spleen. This clearing function is impaired when the spleen is absent or not functional. It is commonly accepted by hematologists that a rate of circulating pitted RBCs more than 2% is an indication of a higher risk for overwhelming infection due to functional asplenia. By contrast, in the context of treated malaria patients, RBCs from which parasites have been removed (or pitted) are called once-infected red blood cells (O-iRBCs) and visualized as RESA-positive cells lacking a parasite remnant. Since O-iRBCs are not detected in the peripheral blood of asplenic malaria patients, their presence is a marker of spleen-clearing functions. Therefore the, "pitting rate" as we defined it in this paper (ie, spleen production of O-iRBCs) was an appropriate assessment of preserved function in isolated-perfused spleens.

Figure 4. Art-iRBC retention and processing. (A-B) Aspect and quantification of brown dots on Giemsa-stained sections showing that they correspond to dead parasite remnants. Brown dots colocalized with red blood cells (intracellular dead parasite remnants; Dii, α) or not (extracellular dead parasite remnants). Extracellular dead parasite remnants could be further classified as extracellular (Dii, β), intracellular in cord cells (Dii, γ) or intracellular in sinus wall cells (Dii, δ). Most extracellular dead parasite remnants colocalized with cord cells (A, Dii). (B) Mean number of brown dots per × 400 field on Giemsa-stained section as box plots for 4 different spleens in which different parasite loads had been introduced. (C-i) Aspect and localization of Art-iRBCs and extracellular dead parasite remnants at a cellular scale, analyzed either by Giemsa-staining (Ci) or by immunohistochemistry (Cii). Fragmented Art-iRBCs colocalized with cord cells/macrophages (Cii, row c). (D) Microcirculatory structures of the spleen were identified on Giemsa-stained sections (Di) allowing differential counting of intracellular and extracellular dead parasite remnants (Dii) on × 400 fields. To adjust for circulatory space on each field, dead parasite remnants numbers are expressed as mean (and standard error of the mean) for 100 RBCs in each zone (Diii). For statistical analysis, see “Art-iRBC retention and processing are zone dependent.”
opened for hypothesis-driven tissue investigations. The zone
dependence of Art-iRBC retention (Figure 4) is in keeping with the
classic paradigm that RBC quality control is more a function of the
slow microcirculation of the red pulp than of the fast microcircula-
tion of the perifolicular zone.6,11 We provide here the first
experimental confirmation of this paradigm in the context of
interactions between a human pathogen and the human spleen
tissue. The accumulation of Art-iRBCs in the red pulp might be
interpreted as a purely rheologic filtration similar to that observed
with carbonized plastic beads.33 This explanation is unlikely,
however, since in vitro exposure to artesunate results in infected
red blood cells with a normal or almost normal elongation index as
assessed by laser diffraction.14 Furthermore, pitting would have
hardly occurred with rigid infected RBCs. Taken together, our
clearing and processing data confirm that pitting of Art-iRBCs is an
intrasplenic process in humans, the remnant being removed as the
RBC crosses the wall of red pulp sinuses.14,15 Pitting accounted for
a fraction of parasite clearance, estimated as 12% to 21% of the
initial parasite load. The close correspondence between this
proportion of Art-iRBC cleared by pitting—as estimated from
kinetic analysis of the perfusate cells—on the one hand and the
proportion of extraerythrocytic dead parasite remnants deposited
into the sinus wall cells on the other hand (11%) is striking. It is
tempting to speculate that most extraerythrocytic dead parasite
remnants observed in or in close proximity to the sinus wall were
generated by pitting, while cordal dead parasite remnants were
created by phagocytosis of intact Art-iRBCs. This latter aspect is
reminiscent of the physiologic clearance of senescent RBCs, which
is handled through elimination of intact RBCs rather than lysed
debris.34 Hence, our results show that pitting is probably not the
only clearance mechanism of Art-iRBCs and that direct phagocyto-
sis of Art-iRBCs may account for a substantial part of parasite
clearance, a mechanism already observed in the spleen of a patient
who died from P. falciparum malaria.29 One complementary
hypothesis is that incomplete/abortive pitting triggers intrasinusal
phagocytosis, resulting in a 2-step mechanism of innate clearance.
Of importance, Art-iRBC clearance observed here—either through
phagocytosis or pitting—did not require any significant spleen
priming and was independent from most serum factors, since
perfusion was performed in Krebs–human albumin medium.

We used P. falciparum–infected RBCs as markers for spleen
filtering functions. In return, human isolated-perfused spleens may
dbow help explore the pathogenesis of P. falciparum malaria, the
most frequent and severe health problem in which the spleen plays
a central role.35 In malaria-endemic countries, splenectomy is
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Ex vivo perfusion of human spleens maintains clearing and processing functions

Pierre A. Buffet, Geneviève Milon, Valentine Brousse, Jean-Michel Correas, Bertrand Dousset, Anne Couvelard, Reza Kianmanesh, Olivier Farges, Alain Sauvanet, François Paye, Marie-Noëlle Ungeheuer, Catherine Ottone, Huot Khun, Laurence Fiette, Ghislaine Guigon, Michel Huerre, Odile Mercereau-Puijalon and Peter H. David