RAPID COMMUNICATION

Merocyanine 540-Sensitized Photoinactivation of Human Erythrocytes Parasitized by Plasmodium falciparum

By Orla M. Smith, Stephen A. Dolan, James A. Dvorak, Thomas E. Wellems, and Fritz Sieber

The purpose of this study was to evaluate the photosensitizing dye merocyanine 540 (MC540) as a means for ex vivo purging of Plasmodium falciparum-infected erythrocytes from human blood. Parasitized red blood cells bound more dye than nonparasitized cells, and exposure to MC540 and light under conditions that are relatively well tolerated by normal erythrocytes and normal pluripotent hematopoietic stem cells reduced the concentration of parasitized cells by as much as 1,000-fold. Cells parasitized by the chloroquine-sensitive HB3 clone and the chloroquine-resistant Dd2 clone of P falciparum were equally susceptible to MC540-sensitized photolysis. These data suggest the potential usefulness of MC540 in the purging of P falciparum-infected blood.

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MERO CYANINE 540 (MC540) is a photosensitizing dye that has been used in preclinical models and a phase I clinical trial for the purging of leukemia and lymphoma cells from autologous bone marrow grafts. The virucidal properties of MC540 have prompted investigations into its use as an agent for the inactivation of enveloped viruses in blood products.

Plasmodium falciparum is the causative agent of the most serious form of malaria and is responsible for much morbidity and mortality in the tropics. Although normally transmitted by a mosquito vector, P falciparum may also be transmitted through transfusion of infected blood from asymptomatic donors. Here we report on the ability of MC540 to preferentially photosensitize red blood cells (RBCs) parasitized by either a chloroquine-resistant or a chloroquine-sensitive clone of P falciparum.

MATERIALS AND METHODS

Parasites. Chloroquine-resistant (Dd2) and chloroquine-sensitive (HB3) clones of P falciparum were maintained in vitro using modifications of the method of Trager and Jensen. Parasitized RBCs were maintained as shallow layers in 75-cm² tissue culture flasks at 37°C in an atmosphere of 90% N₂, 5% CO₂, and 5% O₂. Parasites were passaged by daily addition of fresh human RBCs (leukocyte-free, final cell density approximately 5 x 10⁸/mL) in RPMI 1640 growth medium (GIBCO, Grand Island, NY) supplemented with 25 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 28.6 mmol/L NaHCO₃, 360 mmol/L gentamicin, and pooled human serum (360 Kmol/L). Cultures were monitored daily by microscopic examination of Giemsa-stained thin smears. Experiments were terminated when untreated control cultures reached 10% (ring stage) parasitemia.

Invasion assay. Target erythrocytes at a hematocrit of 3% were exposed to MC540 and light for 15, 30, 45, or 90 minutes, washed once, resuspended in growth medium, and cocultured at a density of 10⁸/mL with HB3-parasitized erythrocytes (10⁷/mL) that had been enriched to ≥50% parasitemia (schizont stage) using the gelatin selection technique. After 18 hours at 37°C, Giemsa-stained thin smears were prepared, and 1,000 erythrocytes were examined microscopically to determine the percentage of invaded cells.

Flow cytometry. Flow cytometry was used to quantify the concentration of parasitized (DNA-containing) erythrocytes before and after MC540-sensitized photoactivation. Erythrocytes were washed once with phosphate-buffered saline (PBS), fixed with 0.1% glutaraldehyde in PBS for 16 hours at 4°C, washed free of fixative, stained with Hoechst 33258 (10 µg/mL; Polysciences, Warrington, PA), and analyzed with a Coulter Epics V Cell Sorter (Coulter Electronics, Hialeah, FL). Normal, nonparasitized RBCs were used to determine background fluorescence. To ensure that the Hoechst stain technique for DNA was detecting parasitized cells, Hb33258-positive cells were sorted and cytocentrifuged onto microscope slides, and stained with Giemsa for microscopic analysis.

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Fluorescence microscopy. Parasitized RBCs were exposed to MC540 and light for 15 minutes, washed, and resuspended in growth medium supplemented with 10 µg/mL Hoechst 33342 (Polysciences, Warrington, PA). Wet mounts were prepared using silicon oil as the mounting medium. Cells were visualized using an Axioshot microscope (Zeiss, Thornwood, NY) equipped for both transmitted differential interference contrast (DIC) and reflected fluorescence microscopy. DIC observations were performed at 590 nm. MC540 was excited at 485 nm and its fluorescence visualized with a 515-565 nm barrier filter; Hoechst 33342-stained DNA was excited at 365 nm and visualized with a 397 nm barrier filter. Images were projected onto the faceplate of a Sony XC-77 CCD video camera (Sony Component Products Division, Cypress, CA). Video images were acquired with a DT2861 Arithmetic Frame Grabber (Data Translation, Marlboro, MA) operating in an IBM PC AT-compatible computer (ZEOS International, St Paul, MN). Image files were converted into tagged image file format for processing with Halo f/x (Media Cybernetics, Silver Spring, MD) and then converted to photographic prints with a LFR film recorder (Lasergraphics, Irvine, CA).

RESULTS

Exposure to MC540 and light inactivated the malaria parasite in a dose-dependent manner (Table 1). The chloroquine-resistant (Dd2) and the chloroquine-sensitive clones (HB3) of *P. falciparum* were equally susceptible to MC540-sensitized photoinactivation (Table 1). The rate of dye-sensitized photoinactivation of the parasites was inversely proportional to the density (hematocrit) of the cell suspension (Table 1).

Flow cytometric analyses of Hoechst 33258-stained erythrocytes confirmed that the frequency of parasitized (DNA-containing) cells was drastically reduced after exposure to MC540 and light (Table 2). When untreated, parasitized RBCs were sorted and analyzed by light microscopy, 85% (HB3) and 91% (Dd2), respectively, of all cells in the intensely staining fraction were positively identified as parasitized cells. Fluorescence microscopy also confirmed that the parasitized cells in a 7.2% parasitemic RBC suspension (Table 1).

When normal RBCs were exposed to MC540 and light for 45 minutes and then cocultured with HB3-parasitized erythrocytes, the invasion of RBCs by the parasite was close to normal (Table 3). However, when treatment with dye and light was extended to 90 minutes, invasion was inhibited by 45% (Table 3), suggesting that photodynamic damage to the cell surface of target erythrocytes interfered with parasite invasion.

DISCUSSION

In industrialized countries, concerns about the safety of the blood supply focus on the transmission of pathogenic viruses. In Third World countries where malaria and other protozoan diseases are endemic, the transmission of parasites presents an equally serious problem. We have recently shown that simultaneous exposure to MC540 and light inactivates a wide range of enveloped viruses including the human T-cell leukemia/lymphoma virus type I, the human immunodeficiency virus and cytomegalovirus, but is relatively well tolerated by RBCs, pluripotent hematopoietic stem cells, factor VIII, and von Willebrand factor. In this communication, we report that exposure to the same doses of MC540 and light also inactivates chloroquine-sensitive and chloroquine-resistant clones of *P. falciparum*.

Parasite invasion is known to induce rather drastic changes in all major components (lipids, proteins, carbohydrates) of the erythrocyte plasma membrane. These parasite-induced surface alterations were probably responsible for the increased binding of MC540 by parasitized RBCs. In our studies, MC540 fluorescence was associated with the parasite and, to a lesser extent, with the plasma membrane of the host cell. By contrast, Kara et al. had

### Table 1. MC540-Sensitized Photoinactivation of Clones HB3 and Dd2 of *P. falciparum*

<table>
<thead>
<tr>
<th>Parasitemia (%)</th>
<th>HB3</th>
<th>Dd2</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>0.2% Hct</td>
<td>3% Hct</td>
</tr>
<tr>
<td>None</td>
<td>9.8 ± 0.5</td>
<td>9.8 ± 2.1</td>
</tr>
<tr>
<td>Ethanol, light 90 min</td>
<td>10.7 ± 1.1</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td>Dye, dark</td>
<td>10.4 ± 1.6</td>
<td>9.3 ± 1.6</td>
</tr>
<tr>
<td>Dye, light 15 min</td>
<td>6.2 ± 4.5</td>
<td>6.1 ± 1.9</td>
</tr>
<tr>
<td>Dye, light 30 min</td>
<td>0.3 ± 0.4</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>Dye, light 46 min</td>
<td>0 ± 1.5</td>
<td>0.1 ± 0.1</td>
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</table>

Erythrocytes parasitized by the HB3 or the Dd2 clone of *P. falciparum* (3% to 5% parasitemia; >90% ring stage) were exposed to MC540 and light for 15, 30, 45, or 90 minutes at hematocrits (Hct) of 0.2%, 3%, or 15%, and cocultured with fresh uninfected erythrocytes. Forty-eight hours later (when control cultures had reached approximately 10% parasitemia), the degree of parasitemia was determined by examining Giemsa-stained thin smears. Data represent means ± SD of three experiments.
PHOTOINACTIVATION OF PLASMODIUM FALCIPARUM

Fig 1. Erythrocytes parasitized by P. falciparum (clone Dd2; 7.2% parasitemia). Cells were exposed to MC540 (15 μg/mL) and light (26 W/m², 15 minutes), washed, and counterstained with Hoechst 33342 (10 μg/mL). (A) Differential interference contrast. (B) MC540 fluorescence, excited at 485 nm and visualized with a 515- to 565-nm barrier filter. (C) Hoechst 33342-induced DNA fluorescence, excited at 365 nm and visualized with a 397-nm barrier filter. Bar, 10 μm.

Table 3. Invasion of MC540-Treated and Untreated Erythrocytes by HB3 Parasites

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Invasion Rate (% of total population)</th>
<th>Invasion Rate (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>30 ± 4.4</td>
<td>100</td>
</tr>
<tr>
<td>Ethanol, light 90 min</td>
<td>35 ± 0.3</td>
<td>116 ± 16.5</td>
</tr>
<tr>
<td>Dye, dark</td>
<td>29 ± 2.4</td>
<td>97 ± 6.2</td>
</tr>
<tr>
<td>Dye, light 15 min</td>
<td>31 ± 2.6</td>
<td>104 ± 6.4</td>
</tr>
<tr>
<td>Dye, light 30 min</td>
<td>27 ± 1.2</td>
<td>91 ± 17.3</td>
</tr>
<tr>
<td>Dye, light 45 min</td>
<td>25 ± 3.3</td>
<td>84 ± 1.6</td>
</tr>
<tr>
<td>Dye, light 90 min</td>
<td>16 ± 0.3</td>
<td>55 ± 6.9</td>
</tr>
</tbody>
</table>

Target erythrocytes (3% hematocrit) were exposed to MC540 and light and then cocultured with HB3-infected erythrocytes (51% parasitemia, >90% schizont stage) at a ratio of 10:1. After 18 hours, Giemsa-stained smears were prepared, and 1,000 erythrocytes were enumerated to determine the degree of parasitemia. Data represent means ± range of two experiments.

reported that MC540 preferentially binds to the parasitophorous vacuole membrane of intracellular P. falciparum while Sherman and Greenan14 found that MC540 was primarily associated with the plasma membrane of the host cell. However, the staining of cells by MC540 is known to be affected by seemingly minor differences in experimental conditions.15 Variations in type, concentration, or lot of serum or the radiation dose could easily account for reported differences in the staining of P. falciparum-infected cells. As a cell’s susceptibility to MC540-sensitized photolysis is primarily determined by the amount of dye it binds, greater dye binding was probably the major reason for the enhanced photosensitivity of parasitized RBCs.15

Erythrocytic stages of the malaria parasite are known to be vulnerable to oxidative stress,16 and the cytotoxic and antiviral activities of MC540 are at least in part mediated by singlet oxygen ($^1O_2$).17-19 It is reasonable to speculate that $^1O_2$ may also play a role in the inactivation of P. falciparum. The nearly 1,000-fold reduction in parasitized cells shown in Table 2 may underestimate the antiprotozoan activity of MC540-sensitized photolysis because samples were fixed immediately after the photolysis step whereas the MC540-mediated photolysis of cells is generally not completed until several hours post photolysis.20

MC540 has a low acute systemic toxicity, is rapidly cleared from the bloodstream, does not sensitize the skin of the recipient, and has tested negative in two standard mutagenicity tests.21,22 Because of the significant overlap between the absorption spectrum of MC540 and the absorption spectrum of hemoglobin, RBCs have to be treated either as dilute suspensions or as thin films to allow adequate light penetration. This limitation may be overcome by using one of the recently described structural analogues of MC540 that absorb at longer wavelengths and match or exceed the antiviral and cytotoxic potency of MC540 while retaining its selectivity.23

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