T he effect of artemisinin on granulocyte function assessed by flow cytometry

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The effect of dihydroartemisinin, artemisinin and artesunate (0.1, 0.5, 5 and 50 mg/L) on phagocytic function and release of reactive oxygen products by neutrophils was studied by flow cytometry. Incubation with dihydroartemisinin, artemisinin and artemether resulted in a decreased capacity to phagocytose Escherichia coli (0.1–50 mg/L: 62–40%, 66–32% and 59–47% of the control values, respectively; P < 0.001 for all). Conversely, the derivatives enhanced the intracellular generation of reactive oxygen intermediates (0.1–50 mg/L: 146–140%, 174–197% and 188–136% of the control values, respectively; P < 0.001 for all). Artemisia derivatives enhance the reactive oxygen response of neutrophils but depress their phagocytic ability at therapeutic blood levels.

Introduction

Artemisinin, a novel antimalarial drug, contains an endoperoxide (dioxygen) bridge which is thought to confer the antimalarial activity in two steps. In the first step, activation, intra-parasitic iron catalyses the cleavage of the endoperoxide bridge and the generation of free radicals. In the second step, alkylation, the artemisinin-derived free radical forms covalent bonds with parasite protein.1

Suppression of phagocyte numbers or function in malaria may result in impaired host defence.2 Immuno-modulation with antibacterial agents has shown promise as a novel strategy in the treatment of various infectious diseases.3 The current study analyses the interaction of dihydroartemisinin, artemisinin and artemether on the non-specific acute inflammatory responses of granulocytes. Flow cytometry was used to assess phagocytic function and intracellular release of reactive oxygen intermediates following phagocytosis by neutrophil granulocytes.4,5

Materials and methods

Dihydroartemisinin, artemisinin and artemether were dissolved in linolenic acid (Sigma, Munich, Germany) at a concentration of 0.1, 0.5, 5 and 50 mg/L and added to heparinized whole blood samples from eight healthy subjects aged 24–32 years. The samples were incubated for 5 min to 1 h at room temperature. No antimalarials were present in the control samples.

Flow cytometry was used to study phagocytic function and release of reactive oxygen intermediates following phagocytosis by neutrophil granulocytes (PMNL). Phagocytic capacity was assessed by measuring uptake of fluorescein isothiocyanate (FITC)-labelled Escherichia coli. Generation of reactive oxygen intermediates after phagocytosis of unlabelled bacteria was estimated by the amount of dihydrorhodamine 123 converted to rhodamine 123 intracellularly. The assays were performed as described.5 To exclude a direct effect of artemisinin (or myeloperoxidase-oxidized artemisinin) on dihydrorhodamine the fluorescence of drug-treated lymphocytes was measured under the same conditions. No fluorescence activity was seen in these experiments. Thus artemisinin alone is unlikely to convert dihydrorhodamine without PMNL.

In addition to the flow cytometry assay the oxidant production was determined by the superoxide dismutase-inhibitable cytochrome c reduction test. Briefly, isolated PMNL were purified by centrifugation of heparinized
blood from healthy donors through a layer of Ficoll-Paque (Pharmacia, Uppsala, Sweden), and dextran sedimentation of the erythrocytes followed by hypotonic lysis of residual erythrocytes. The isolation contained >98% PMNL with a cell viability of >99% as determined by Trypan Blue exclusion (10⁶). These were added to a 4 mL Falcon tube containing cytochrome c (1.2 mg/L, Sigma). Superoxide anion production was initiated by addition of N-formyl-methionyl-leucyl-phenylalanine (fMLP, 10⁻⁶ M; Sigma) and the reaction allowed to occur at 37°C for 10 min. Control tubes contained, in addition, superoxide dismutase (15 mg/L, Sigma). The reaction was stopped by placing the tubes in ice, and centrifugation at 400 g for 10 min. The absorbance at 550 nm was read (LKB U Ltr spec 4040, Wallac, Vienna, Austria). Total superoxide anion produced was calculated from a molar extinction of 21.1 mol⁻¹ cm⁻¹.

For statistical comparison between test and control samples Kruskall–Wallis and Mann–Whitney–Wilcoxon U-tests were used. Different concentrations of antimicrobials were compared with the Wilcoxon rank sum test. For correlation analysis Spearman’s test was used. All the analyses were two-sided, and differences with a P-value of <0.05 were considered statistically significant.

**Results and discussion**

Compared with results in control samples, incubation with dihydroartemisinin, artemisinin and artemether resulted in a decreased capacity to phagocytose E. coli (0.1–50 mg/L: 62–40%, 66–32% and 59–47% of the control values, respectively; P < 0.001 for all; see Figure). Conversely, in all dihydroartemisinin-, artemisinin- or artemether-treated samples the intracellular generation of reactive oxygen intermediates was significantly enhanced (0.1–50 mg/L: 146–140%, 174–197% and 188–136% of the control values, respectively; P < 0.001 for all, see Figure). These effects were independent of the incubation time (ranging from 5 to 60 min; P > 0.05 for comparisons between 5 min, 15 min, 30 min and 1 h) and occurred dose-independently. The phagocytic ability of PMNL was related to the generation of reactive oxygen intermediates in the antimalarial-free samples (r = 0.693, P < 0.001), whereas no correlation was seen in the artemisia-derivative-treated samples. The results of the superoxide dismutase-inhibitable cytochrome c reduction assays are depicted in the Table. As in the flow cytometry results, a significant increase in PMNL reactive oxygen production was seen (P < 0.01 for all comparisons).

PMNL play a role in severe malaria. The blood count of malaria-pigment-containing neutrophils significantly relates to prognosis and a modulation of their function with human granulocyte-stimulating factor has been shown to suppress parasitaemia. A rtemisinin could affect neutrophil granulocytes via iron-mediated activation of endoperoxides. This mechanism has been described in parasites and may also occur in neutrophils. The reduction in phagocytic ability could be an effect of

<table>
<thead>
<tr>
<th>Dose (mg/L)</th>
<th>Artemisinin</th>
<th>Dihydroartemisinin</th>
<th>Artemether</th>
</tr>
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<tbody>
<tr>
<td>0.1</td>
<td>53 ± 6</td>
<td>49 ± 5</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>0.5</td>
<td>59 ± 7</td>
<td>62 ± 7</td>
<td>58 ± 8</td>
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<tr>
<td>5</td>
<td>65 ± 5</td>
<td>66 ± 7</td>
<td>71 ± 7</td>
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<tr>
<td>50</td>
<td>72 ± 9</td>
<td>69 ± 12</td>
<td>74 ± 12</td>
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</tbody>
</table>
Effect of artemisinin on granulocytes

artemisinin-enhanced granulocyte auto-oxidation in vivo. A similar effect has recently been suggested to be operative in septicaemia.5

This study demonstrates that artemisinin derivatives enhance reactive radical production in neutrophils at therapeutic blood levels (peak concentrations 0.1–3 mg/L). The increase of intracellular reactive oxygen intermediate production could be advantageous in malaria and could explain the rapid parasite clearance observed in clinical studies.8

References


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