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Compstatin Cp40 blocks hematin-mediated deposition of C3b fragments on erythrocytes: Implications for treatment of malarial anemia

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ABSTRACT

During malarial anemia, 20 uninfected red blood cells (RBCs) are destroyed for every RBC infected by *Plasmodium falciparum* (Pf). Increasing evidence indicates an important role for complement in destruction of uninfected RBCs. Products of RBC lysis induced by Pf, including the digestive vacuole and hematin, activate complement and promote C3 fragment deposition on uninfected RBCs. C3-opsonized cells are then subject to extravascular destruction mediated by fixed tissue macrophages which express receptors for C3 fragments. The Compstatin family of cyclic peptides blocks complement activation at the C3 cleavage step, and is under investigation for treatment of complement-mediated diseases. We demonstrate, that under a variety of stringent conditions, second-generation Compstatin analogue Cp40 completely blocks hematin-mediated deposition of C3 fragments on naïve RBCs. Our findings indicate that prophylactic provision of Compstatin for malaria-infected individuals at increased risk for anemia may provide a safe and inexpensive treatment to prevent or substantially reduce malarial anemia.

1. Introduction

The pathogenesis of malarial anemia is associated with at least two separate mechanisms of red blood cell (RBC) destruction. RBCs infected with *Plasmodium falciparum* (Pf) are destroyed during growth and replication of the organism; the growing schizont within the RBC lyses, followed by rupture of the RBC membrane and release of the digestive vacuole (DV) as well as merozoites which invade additional RBCs and continue the pattern of RBC infection and destruction [1,2]. Secondly, severe malarial anemia occurs at relatively low parasite burdens, and thus an additional mechanism or mechanisms must operate to account for destruction of large numbers of *uninfected* RBCs [3–7].

Numerous studies have implicated complement in destruction of uninfected RBCs in malaria, but elucidation of pathways whereby complement may mediate this reaction remained elusive [2,8,9]. Now, increasing evidence indicates that in malaria, uninfected RBCs are opsonized with complement C3 fragments (C3b/iC3b/C3dg), and the opsonized RBCs can be destroyed extravascularly by fixed tissue macrophages, in spleen and liver, which express receptors specific for C3 fragments. In vitro experiments have revealed that breakdown products generated during lysis of Pf-infected RBCs, including heme/hematin and the DV, activate the alternative pathway of complement (APC) and promote C3 fragment deposition on uninfected RBCs [1,5,10–13].

These findings provide the key link by which lysis of Pf-infected RBCs leads to C3 fragment-opsonization of uninfected cells followed by their extravascular clearance and destruction. We previously reported that *anti*-C3b mAb 3E7, which blocks the APC, inhibited complement activation mediated by hematin, and prevented C3-fragment deposition on RBCs [10]. In principle mAb 3E7 could be used to treat malarial anemia, but practical/financial obstacles render this approach problematic. An alternative strategy to block the APC is centered on use of Compstatin (Cp40), a 13 amino acid cyclic peptide which binds to C3 and blocks complement activation at the C3 cleavage step in the classical and APC C3 convertases [14]. In this communication we report on a series of in vitro experiments which demonstrate that Cp40 is quite effective at inhibiting hematin-mediated C3b deposition on RBCs. Our

Abbreviations: APC, alternative pathway of complement; Cp40, second generation Compstatin analogue; DV, digestive vacuole; MESF, molecules of equivalent soluble fluorochrome; NHS, normal human serum; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell.

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findings suggest that Cp40 might find use in treatment and prevention of severe malarial anemia.

2. Materials and methods

We followed our previously reported procedures to study hematin-mediated C3b deposition on RBCs [10]. Blood was collected in EDTA, RBCs were isolated and washed, and duplicate or triplicate samples were reconstituted in polystyrene tubes (12 × 75 mm) to hematocrits of 5% or 50% in 50% autologous normal human serum (NHS), in the presence or absence of mAb 3E7 (final concentration 200 μg/ml) or Cp40 (final concentration 25 μM unless noted). Alternatively an IgG1 isotype control was used in place of mAb 3E7, or a scrambled peptide (same amino acid composition as Cp40) was used in place of Cp40. Hematin (Sigma-Aldrich, final concentration 400 μg/ml) was added and the samples were incubated for 20 min at 37 °C. The RBCs were washed once and in most experiments reconstituted under the same conditions for a second and third reaction cycle. Subsequently the cells were washed twice and probed with Al488- or Al647-labeled mAbs 7C12 or 1H8, specific for C3b/iC3b or C3b/iC3b/C3dg, respectively, and analyzed by flow cytometry. The intensities of the fluorescent signals are reported in units of molecules of equivalent soluble fluorochrome (MESF) [10], and means and SD are reported. In some experiments, blood was anticoagulated by collection in lepirudin (50 μg/ml final concentration), and either whole blood or RBCs reconstituted in 50% lepirudin plasma (±Cp40) were reacted with hematin and then processed as described above.

Unpaired single tail t tests were conducted to test the hypothesis that addition of Cp40 or mAb 3E7 would reduce C3b deposition on RBCs reacted with hematin in NHS. Significant differences between experimental samples versus controls are noted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results and discussion

3.1. Promotion and inhibition of C3b deposition mediated by hematin

Washed RBCs from donors 1 and 2 were reacted at 5% hematocrit in autologous 50% NHS with 400 μg/ml hematin, ±mAb 3E7 or varying amounts of Cp40. As previously reported, reaction with hematin for one or more treatment cycles mediates C3b deposition on RBCs, and mAb 3E7 blocks the reaction (Fig. 1A–B). There is some C3b deposition on cells reacted only in NHS, which is reduced to background in the presence of mAb 3E7. We now report that concentrations of Cp40 as low as 3 μM completely block hematin-mediated C3b deposition. Controls with an IgG1 isotype control in place of mAb 3E7, and a scrambled peptide in place of Cp40 (Fig. 1C–D, donors 3 and 2, respectively) additionally demonstrate the specific action of Cp40 and mAb 3E7, although we note that the scrambled peptide modestly reduced C3b deposition for donor 2. We previously reported that reaction of RBCs in NHS with hematin at concentrations as low as 150 μg/ml (corresponding to lysis of approximately 3% of circulating RBCs) promotes C3b deposition on the RBCs [10]. We conducted the present experiments at hematin concentrations of 400 μg/ml, thus setting a greater challenge for testing the capacity of Cp40 to block the hematin-mediated C3b deposition reaction.

Deposition of C3b on RBCs is very rapid, and if the hematin is added to the RBC-NHS mixture *first*, then the inhibitors (Cp40 or mAb 3E7) are only effective if added within the next 2 min (Fig. 1E–F, donors 1 and 2). The results illustrated in Fig. 1E–F are based on only one treatment cycle, indicating that the C3b deposition reaction is indeed quite potent.

3.2. More stringent tests for inhibition of C3b deposition

We next tested the efficacy of Cp40 in blocking hematin-mediated C3b deposition on RBCs under more stringent conditions, including

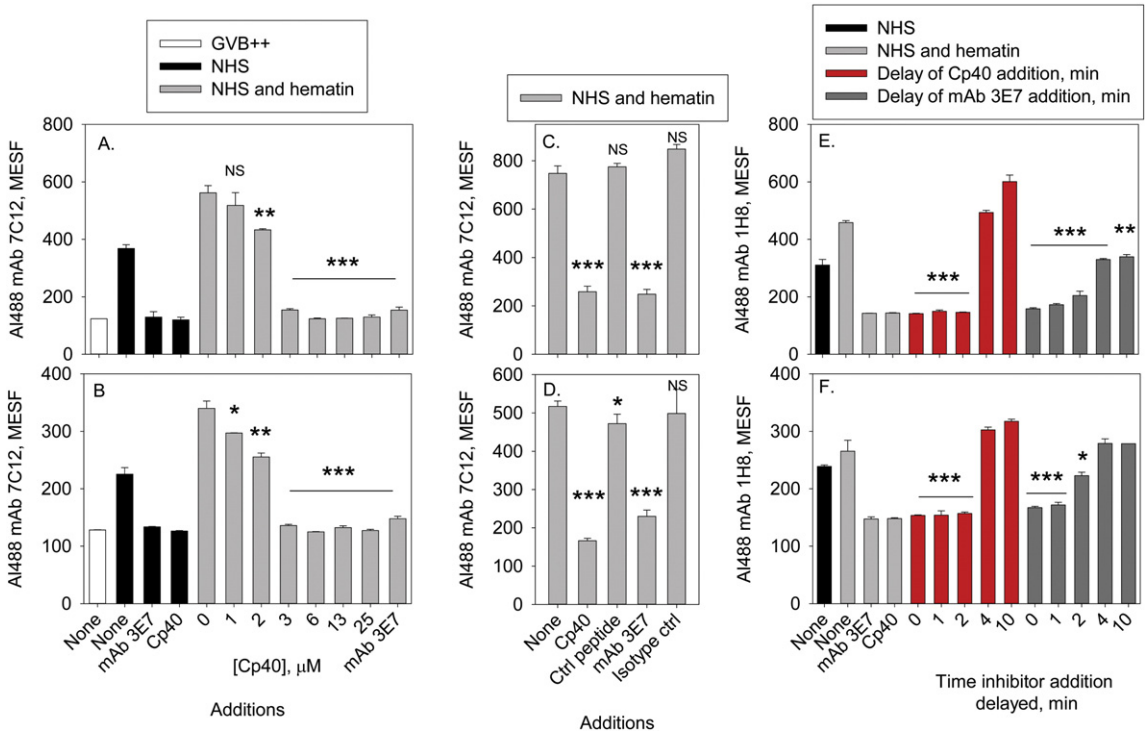

Fig. 1. In 50% NHS, hematin mediates deposition of C3b fragments on RBCs and both mAb 3E7 and Cp40 block this reaction. (A,B) Normal blood donors 1 and 2, 5% hematocrit, three cycles of reaction; cells reacted in gelatin veronal buffer (GVB++) define the background signal. Cp40 concentrations of 3 μM or more completely blocked C3b deposition. At lower concentrations of Cp40, C3b deposition is clearly evident. (C,D) For normal blood donors 3 and 2, controls demonstrate that a scrambled peptide (same amino acid composition) in place of Cp40 does not inhibit hematin-mediated C3b deposition, and similarly, an IgG1 isotype control in place of mAb 3E7 does not block C3b deposition. (E,F) For blood donors 1 and 2, a kinetic experiment indicates that treatment of samples with Cp40 or mAb 3E7 strongly inhibits C3b deposition if the reagents are added within 2 min after the hematin is added; one cycle of reaction, all at 5% hematocrit. Significant differences between treated samples compared to samples reacted with hematin alone are noted.