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Original article

Hemozoin triggers tumor necrosis factor alpha-mediated release of lysozyme by human adherent monocytes: new evidences on leukocyte degranulation in *P. falciparum* malaria

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Abstract

Objective: Avidly phagocytosed hemozoin (malarial pigment) alters several functions of human monocytes and stimulates generation of several cytokines. Recently, we showed that phagocytosis of hemozoin by human monocytes increases expression and activity of matrix metalloproteinase-9, a proteolytic enzyme available in specific gelatinase granules, which contain several enzymes including lysozyme. Present work investigated active lysozyme release after phagocytosis of hemozoin and its dependence on production of tumor necrosis factor alpha. **Methods**: After phagocytosis of hemozoin, hemozoin-containing trophozoites or control meals (opsonized non-parasitized red blood cells and latex particles), monocyte supernatants were monitored for 2 hours, in presence of blocking anti-human tumor necrosis factor alpha antibodies or recombinant human tumor necrosis factor alpha cytokine in selected experiments. Lysozyme release was evaluated by a specific spectrometric assay measuring lysozyme activity after coincubation of cell supernatants with suspensions of *Mycrococcus Lysodeikticus*, while levels of soluble tumor necrosis factor alpha were analyzed by specific enzyme-linked immunodsorbent assay. **Results**: Levels of lysozyme activity and soluble tumor necrosis factor alpha protein were increased in hemozoin- or trophozoites-laden monocytes supernatants. Phagocytosis per se (control meals) also increased lysozyme release, but levels were significantly lower than those obtained after phagocytosis of hemozoin or trophozoites. Interestingly, all effects on lysozyme release observed after phagocytosis were abrogated by blocking anti-human tumor necrosis factor alpha antibodies, while they were mimicked by recombinant human tumor necrosis factor alpha cytokine. **Conclusions**: Present work shows that phagocytosis of hemozoin promotes monocyte degranulation and enhances active lysozyme release. The effect requires tumor necrosis factor alpha mediation.

Keywords: Hemozoin; *Plasmodium Falciparum*; Malaria; Monocyte; Phagocytosis; Tumor necrosis factor alpha; Lysozyme; Degranulation; Gelatinase granules; Matrix metalloproteinases

INTRODUCTION

Malaria is the major human parasitic disease in the world, with a mortality of 1-3 millions people per year. Although numerous new evidences on parasite and vector biology have been recently produced, several aspects of malaria need to be more investigated, such as lethal complicances including anemia, respiratory distress and cerebral malaria. During last years, several progresses in knowledge on physiopathological mechanisms of malaria have been achieved by studying the effects of hemozoin (malarial pigment), a crystalline ferriprotoporphyrin IX polymer present in late stages of *P. falciparum*-para-

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sitized red blood cells (trophozoites), derived from incomplete digestion of host hemoglobin[1]. Hemozoin is avidly phagocytosed by mononuclear cells and alters several functions of human monocytes, including antigen presentation and oxidative burst[2,3]. Moreover, hemozoin-laden monocytes stimulate generation of several cytokines, including tumor necrosis factor alpha and interleukin-1 beta[4,5]. It has been shown previously that hemozoin-dependent enhancement of both cytokines in human monocytes induce the increase of expression and activity of matrix metalloproteinase-9, a proteolytic enzyme involved in cerebral malaria development by leading mononuclear cells extravasation and by triggering a pathological auto-alimented loop between tumor necrosis factor alpha and matrix metalloproteinase-9 themselves, after shedding soluble tumor necrosis factor alpha from its membrane-bound precursor[4]. Since hemozoin-fed monocytes produce increased amounts of peroxidation products of polyunsaturated fatty acids, as hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids[6], 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid has been proposed to be one possible mediator of matrix metalloproteinase-9 increase[5]. Besides monocytes, matrix metalloproteinase-9 is produced by other leukocytic cells, such as neutrophils; all these cells store the matrix metalloproteinase in specific gelatinase granules, which also contain several other enzymes, including lysozyme[7]. Present work investigated monocyte degranulation by analyzing active lysozyme release after phagocytosis of hemozoin or hemozoin-containing-trophozoites; addictionally, production of tumor necrosis factor was considered to verify if it could play a role in regulation of lysozyme release.

MATERIALS AND METHODS

Materials

All materials were from Sigma-Aldrich, St. Louis, MO, unless otherwise stated. Cell culture media: RPMI 1640 with or without red-phenol and macrophage-SFM media were from Invitrogen, Carlsbad, CA; Panserin 601 monocyte medium was from PAN Biotech, Aidenbach, Germany; recombinant human tumor necrosis factor alpha and blocking anti-human tumor necrosis factor alpha antibodies were from Merck, Darmstadt, Germany; enzyme-linked immunoadsorbent assay kit for tumor necrosis factor alpha assay was from Cayman, Ann Arbor, MI; anti-D IgG were from Immuno AG, Vienna, Austria; Percoll was from Pharmacia, Uppsala, Sweden; Diff-Quik parasite stain was from Baxter Dade AG, Dudingen, Switzerland; sterile plastics were from Costar, Cambridge, UK.

Cultivation of Plasmodium falciparum and isolation of trophozoite-parasitized red blood cells and hemozoin

P. falciparum parasites (Palo Alto strain, mycoplasma-free) were kept in culture as described[4]. Hemozoin and trophozoite-parasitized red blood cells (trophozoites) were isolated from cultures during the first 2 days after infection of red blood cells. After centrifugation at 5 000g on a discontinuous Percoll-mannitol density gradient, hemozoin was collected from the 0- 40% interphase and trophozoites from the 40- 80% interphase. Hemozoin was washed 5 times with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0) containing 10 mM mannitol at 4 C and once with phosphate buffered saline. Trophozoites enriched to 95-97% parasitemia were washed twice and resuspended in RPMI 1640. Before opsonization and phagocytosis experiments hemozoin and trophozoites were reincubated in RPMI 1640 for 1 h at 37°C. Nonparasitized red blood cells were treated as parasitized red blood cells.

Preparation and handling of monocytes

Human monocytes were separated by Ficoll centrifugation from freshly collected buffy coats discarded from blood donations by healthy adult donors of both sexes provided by the local blood bank (AVIS, Associazione Volontari Italiani Sangue, Torino, Italy). Separated lymphomonocytes were resuspended in RPMI 1640 medium and plated on 6-well plates. Each well received 2 mL of cell suspension containing 8×10^6 cells/mL in RPMI 1640. The plates were incubated in a humidified CO_2/air-incubator at 37°C for 60 min. Thereafter non-adherent cells were removed by 3 washes with RPMI 1640 and adherent cells (approximately 1×10^6 cells/well) reincubated at 37° C overnight in RPMI 1640. Shortly before starting phagocytosis, wells were washed with RPMI

1640 and Macrophage-SFM medium was added (2 mL/well).

Phagocytosis by adherent monocytes of opsonized trophozoites, hemozoin and nonparasitized red blood cells and latex particles

To each well of a 6-well plate with approximately 1 $\times 10^6$ adherent monocytes, 50 μL trophozoites (10% hematocrit) or hemozoin (120 nmoles hemozoin heme, an amount comparable to 50 μL trophozoites on heme content basis) or 50 μL anti-D IgG-opsonized red blood cells (10% hematocrit) or 50 μL amine-modified, red-fluorescent latex particles (2.5% solids, diameter 0.105 μm), were added. Trophozoites, hemozoin and latex particles were opsonized with fresh autologous serum, and nonparasitized red blood cells were opsonized with anti-D IgG as indicated[3]. After opsonization, all phagocytic meals were suspended in RPMI 1640. The plates were centrifuged at low speed for 5 s to start phagocytosis and incubated in a humidified CO_2/air-incubator at 37°C for 3 h. This time period maximized phagocytosis and was not sufficient to induce heme-oxygenase-mediated degradation of ingested heme[8]. As an average, each monocyte ingested approx. 8-10 trophozoites, or hemozoin equivalent to 8-10 trophozoites in terms of ingested heme, or approx. 8-10 nonparasitized opsonized RBCs, as shown previously[3]. Thereafter noningested cells, hemozoin and latex particles were removed by 4 washes with RPMI 1640. The plates were then incubated in red phenol-free RPMI 1640 or in Panserin medium in a humidified CO_2/air-incubator at 37°C for up to 2 h. In selected experiments, after the end of phagocytosis cells were incubated with recombinant human tumor necrosis factor alpha (20 ng/mL) or blocking anti-human tumor necrosis factor alpha antibodies (30 ng/mL) for 2 h.

Assay of lysozyme activity

Lysozyme released from adherent monocytes was assayed by the Sigma procedure according to the producer's specifications. Briefly, monocyte supernatants were incubated with suspensions of *Mycrococcus Lysodeikticus* in 0.4 M phosphate buffered saline, pH 6.7, at an OD_{450nm} of 1 at 37°C. The change in OD_{450nm} was measured after 30 min incubation. A standard calibration curve was generated with purified chicken egg lysozyme. One enzyme unit of enzyme activity corresponded to a decrease of 0.001 OD units each minute.

Assay of tumor necrosis factor alpha production

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37°C for 0.5, 1, 1.5 and 2 h. The level of soluble tumor necrosis factor alpha was assayed in monocyte supernatants by specific enzyme-linked immunoadsorbent assay, according the manifacturer's instructions. A standard calibration curve was generated with recombinant human tumor necrosis factor alpha.

Statistical analysis

For each set of experiments, data are showed as means SD or one representative imagine of three independent experiments. All data were analysed by Student's t test.

RESULTS

Phagocytosis of hemozoin or hemozoin-containing trophozoites enhances release of active lysozyme from human adherent monocytes

Adherent monocytes were allowed to phagocytose trophozoites, hemozoin, and nonparasitized opsonized red blood cells and latex particles (control meals) during 3 h. After termination of phagocytosis and elimination of noningested phagocytic meals by repeated washings, and further incubation during 2 h, cell supernatants were collected for following measurements. Figure 1 shows active lysozyme release from monocytes, which is indicated as enzyme activity units measured in a 2 mL-well of cell supernatants. Data are means SD of three independent experiments; all data were evaluated by Student's t test. Unstimulated cells (column 1) released basal levels of active lysozyme (less than 1 0000 activity units). Phagocytosis of control meals (opsonized red blood cells or latex particles: columns 2 and 4, respectively) induced per se higher levels of lysozyme by approximately 50% than unstimulated cells (red blood cells versus unstimulated cells: $P < 0.02$; latex versus unstimulated cells: $P < 0.002$), while

hemozoin (column 5) and hemozoin-containing trophozoites (column 3) doubled unstimulated cells levels of lysozyme (hemozoin versus unstimulated cells: $P < 0.001$; trophozoites versus unstimulated cells: $P < 0.001$). Hemozoin-dependent increases were significantly higher than those obtained with control meals (trophozoites versus red blood cells: $P < 0.01$; hemozoin versus latex: $P < 0.001$).

Phagocytosis of hemozoin or hemozoin-containing trophozoites increases time-dependently tumor necrosis factor alpha production in human adherent monocytes

After termination of phagocytosis of trophozoites, hemozoin, and control meals, and elimination of noningested phagocytic meals by repeated washings, monocytes were incubated for 0.5, 1, 1.5 and 2 h and production of tumor necrosis factor was evaluated in cell supernatants. Figure 2 shows one representative experiment of three independent with similar results. All data were evaluated by Student's t test. Production of tumor necrosis factor is expressed as pg/200 μL of cell supernatants. Unstimulated monocytes (lines with open triangle) released approximately 15 pg/200 μL of soluble tumor necrosis factor alpha at the end of the observational 2 hours-period. Hemozoin-fed and hemozoin-containing trophozoites-fed monocytes (lines with solid circle and lines with solid square, respectively) produced significantly higher levels of tumor necrosis factor alpha than those of control cells (worst p: $P < 0.05$). Production of tumor necrosis factor alpha was non linear with the time, showing a progressive acceleration within the first 2 h. Tumor necrosis factor levels produced after phagocytosis of control meals (lines with open square and lines with open circle) were not significantly different from those of unstimulated cells.

Hemozoin-triggered increase of active lysozyme release from human adherent monocytes is dependent on tumor necrosis factor alpha

After the end of a 3 hours phagocytosis of trophozoites, hemozoin, and control meals, cells were incubated with blocking anti-human tumor necrosis factor alpha antibodies (30 ng/mL) or recombinant human tumor necrosis factor alpha (20 ng/mL) for 2 h.

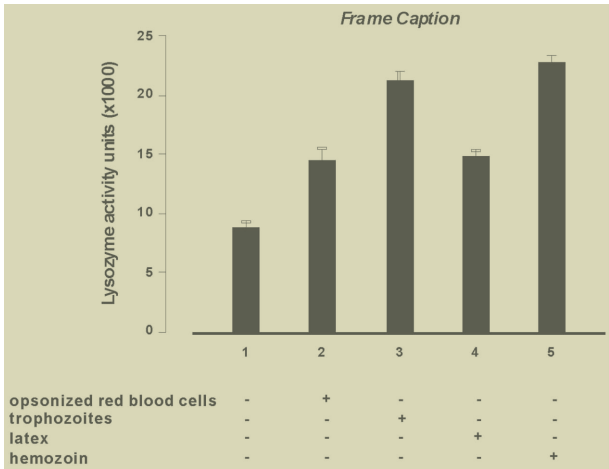

Figure 1 Hemozoin-dependent enhancement of active lysozyme release in human adherent monocytes fed with different meals.

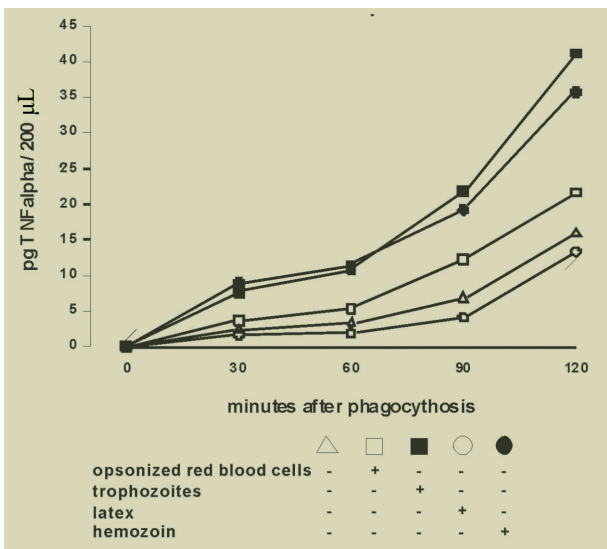

Figure 2 Time-dependent hemozoin-triggered enhancement of tumor necrosis factor alpha production in human adherent monocytes fed with different meals.

Results are resumed in Figure 3 (Panels A and B). Each panel represents means SD of three independent experiments. All data were analyzed by Student's t test. In both panels, unstimulated cells (column 1) and hemozoin-fed cells (column 2) are showed as negative and positive controls, respectively. As shown in Figure 3A, blocking anti-human tumor necrosis factor alpha antibodies did not affected control cells (column 3), while abrogated all effects induced by phagocytosis on lysozyme release (columns 4-7). Active lysozyme levels of cells treated with blocking anti-human tumor necrosis factor alpha antibodies were similar to those of unstimulated cells

without significant differences, while were significantly different from those of hemozoin-fed monocytes (worst p: $P<0.02$). Meanwhile, as shown in Figure 3B, recombinant human tumor necrosis factor alpha mimicked hemozoin effects on lysozime release. Active lysozyme levels of cells treated with recombinant human tumor necrosis factor alpha were significantly different from those of unstimulated cells (worst p: $P<0.05$) while were similar to those of hemozoin-fed monocytes without significant differences.

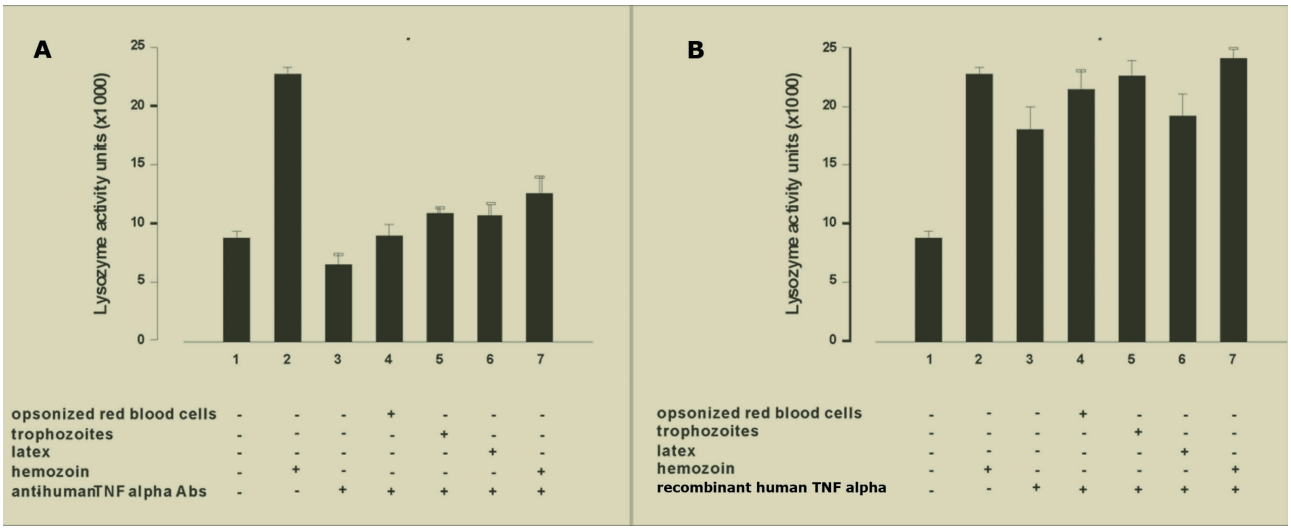

Figure 3 Role of tumor necrosis factor alpha in hemozoin-triggered higher active lysozyme release in human adherent monocytes fed with different meals.

DISCUSSION

After phagocytosis of hemozoin, the malarial pigment derived from incomplete digestion of host hemoglobin by *Plasmodium falciparum*, several monocyte abilities are impaired. For example, trophozoite/hemozoin-fed monocytes do not correctly present antigens[2] and are not able to mount oxidative burst and repeat phagocytosis[3]. Addictionally, phagocytosis of hemozoin by monocytes promotes the generation of peroxidation products of polyunsaturated fatty acids, such as hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids[6], stimulates the production of several cytokines, including tumor necrosis factor alpha and interleukin-1 beta, and enhances expression and activity of proteolytic enzymes, like matrix metalloproteinase-9[4,5]. Present study investigated hemozoin effects on monocyte degranulation, focusing on the release of active lysozyme, an important enzyme produced by several human leukocytes, including neutrophils and monocytes, and largely stored in gelatinase granules[7]. As shown in results section, phagocytosis of hemozoin or hemozoin-containing trophozoites promotes lysozyme degranulation. This finding leads to two considerations. First, as lysozyme has been indicated as a marker of monocyte activation[9] and since neutrophil activation has been previously indicated in severe malaria by observing increased lysozyme levels in plasma[10], it emerges that leukocyte activation and degranulation in malaria recovers a crucial role. Second, higher lysozyme release by hemozoin-fed monocytes must be related to matrix metalloproteinase-9 (also called gelatinase B), which is stored with lysozyme in gelatinase granules of monocytes as well as neutrophils[7]. It has been previously showed that expression and activity of matrix metalloproteinase-9 is enhanced in human monocytes after phagocytosis of malarial pigment[4,5]. Here increased lysozyme degranulation suggests that also release of matrix metalloproteinase-9 is promoted. Addictionally, higher levels of lipocalin, another marker of leukocyte activation, were found in plasma of patients with severe *falciparum* malaria[11]: interestingly, lipocalin ability to complex with gelatinases has been reported[7]. Matrix metalloproteinase-9 confers to monocytes major invasion abili-

ties, explaining the disruption of the basal lamina, the extravasation of mononuclear and red blood cells and the migration into perivascular tissues of monocytes and granulocytes observed in falciparum malaria[4]. On the other hand matrix metalloproteinase-9, whose gene is inducible by tumor necrosis factor, has been proposed to trigger an autoalimented loop between itself and the cytokine, by shedding the soluble tumor necrosis factor alpha from its membrane-bound precursor[4]. Hemozoin-dependent increase of tumor necrosis factor release is confirmed here, and previous data are extended to phagocytosis of hemozoin-containing trophozoites by human monocytes. Moreover, higher levels of soluble tumor necrosis factor seem to mediate the enhancement of monocyte degranulation, as suggested by experiments of treatment with blocking antibodies or stimulation with recombinant exogenous cytokine. Mechanisms by which hemozoin triggers enhanced monocyte production of tumor necrosis factor and following degranulation and lysozyme release are still unknown. One possible mediator could be 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid, since hemozoin-fed monocytes produce increased amounts of peroxidation products of polyunsaturated fatty acids, as hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids[6], and 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid has recently been indicated as responsible of increased production of interleukin-1 beta[5], another cytokine induced by hemozoin such as tumor necrosis factor alpha. Certainly more investigation on hemozoin-fed monocyte biology is required, and hopefully present evidences on tumor necrosis factor-dependent enhancement of monocyte degranulation and active lysozyme release may be useful in future research approach to find new therapies or vaccines against malaria.

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