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Original Contribution

Malarial pigment hemozoin impairs chemotactic motility and transendothelial migration of monocytes via 4-hydroxynonenal ☆

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ABSTRACT

Natural hemozoin, nHZ, is avidly phagocytosed *in vivo* and *in vitro* by human monocytes. The persistence of the undigested β-hematin core of nHZ in the phagocyte lysosome for long periods of time modifies several cellular immune functions. Here we show that nHZ phagocytosis by human primary monocytes severely impaired their chemotactic motility toward MCP-1, TNF, and FMLP, by approximately 80% each, and their diapedesis across a confluent human umbilical vein endothelial cell layer toward MCP-1 by 45 ± 5%. No inhibition was observed with latex-fed or unfed monocytes. Microscopic inspection revealed polarization defects in nHZ-fed monocytes due to irregular actin polymerization. Phagocytosed nHZ catalyzes the peroxidation of polyunsaturated fatty acids and generation of the highly reactive derivative 4-hydroxynonenal (4-HNE). Similar to nHZ phagocytosis, the exposure of monocytes to *in vivo*-compatible 4-HNE concentrations inhibited cell motility in both the presence and the absence of chemotactic stimuli, suggesting severe impairment of cytoskeleton dynamics. Consequently, 4-HNE conjugates with the cytoskeleton proteins β-actin and coronin-1A were immunochemically identified in nHZ-fed monocytes and mass spectrometrically localized in domains of protein–protein interactions involved in cytoskeleton reorganization and cell motility. The molecular and functional modifications of actin and coronin by nHZ/4-HNE may also explain impaired phagocytosis, another motility-dependent process previously described in nHZ-fed monocytes. Further studies will show whether impaired monocyte motility may contribute to the immunodepression and the frequent occurrence of secondary infections observed in malaria patients.

During its growth in the host red blood cell (RBC) the malaria parasite *Plasmodium falciparum* digests hemoglobin and accumulates natural hemozoin (nHZ) in the digestive vacuole, which is expelled as a residual body (RB) upon schizogony. nHZ consists of a biocrystallized β-hematin formed from undigested heme dimers and tightly adherent lipids and proteins from the parasite and the host [1,2]. *In vitro* and *in vivo*, nHZ-laden RB and nHZ-containing late parasite stages (trophozoites and schizonts) are avidly phagocytosed, as confirmed by high proportions of HZ-laden monocytes and granulocytes in circulation [3,4] or enriched in the microvasculature of spleen and liver [5], kidney [6], lung [7], brain [8], and possibly other organs and tissues. Several important functions are inhibited in nHZ-laden monocytes, such as oxidative burst [9], phagocytosis and extracellular killing [10], MHC class II-dependent antigen presentation [11,12], and differentiation and maturation to monocyte-derived dendritic cells (DCs) [13–15]. Cell motility in response to chemotactic stimuli and cell migration are key events for monocytes to perform phagocytosis and killing of invaders and to supply peripheral tissues with macrophages and DC precursors. Whereas the proinflammatory response to synthetic HZ (β-hematin) and nHZ is known to trigger leukocyte recruitment [16–19], the effect of nHZ phagocytosis on monocyte motility has received little attention so far.

Abbreviations: RBC, red blood cell; 4-HNE, 4-hydroxynonenal; DC, dendritic cell; FMLP, formylmethionylleucylphenylalanine; HZ, hemozoin; MCP-1, monocyte chemoattractant protein-1; M-SFM, macrophage serum-free medium; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; HUVEC, human umbilical vein endothelial cell; ECGS, endothelial cell growth factor; FACS, fluorescence-activated cell sorting; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

☆This work is dedicated to the memory of Professor Mario Umberto Dianzani, who passed away on 13 June 2014.

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This study was aimed first to elucidate the effects of nHZ on motility and transendothelial migration of monocytes and, second, to investigate the role of 4-hydroxynonenal (4-HNE), a lipoperoxidation product generated intracellularly by nHZ [20], in the motility impairment caused by phagocytosed nHZ. For these purposes, cell migration, morphology, and cytoskeleton organization in response to the chemoattractants MCP-1, formylmethionylleucylphenylalanine (FMLP), and TNF were assayed in *in vitro* cultured and nHZ-fed or 4-HNE-treated primary human monocytes, and 4-HNE modifications of cytoskeleton proteins were identified.

Constrained cell movement and defective cytoskeleton dynamics observed in nHZ-laden monocytes were caused by 4-HNE and may contribute to imperfect adaptive immunity and secondary infections frequently observed in malaria patients.

Materials and methods

Reagents

Unless otherwise stated, reagents were obtained from Sigma–Aldrich (St Louis, MO, USA).

Primary cells

All donors were healthy volunteers and gave their informed written consent in accordance with the Declaration of Helsinki and after approval from and in accordance with the local research ethics committees of the Universities of Jena (Germany) and Torino (Italy).

Monocyte preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected blood as described [9,11], washed twice, and suspended either in macrophage serum-free medium (M-SFM; Invitrogen, San Diego, CA, USA) at 3×10^7 cells/ml, for migration assays, or in RPMI 1640 medium for plating at 2×10^7 cells/well in six-well plates (Falcon, Becton–Dickinson, San Jose, CA, USA) for 4-HNE–protein conjugate identification. The plates were incubated in a humidified CO_2/air incubator at 37 °C for 30 min, nonadherent cells were removed by three washes, and M-SFM was added. To confirm data obtained from PBMCs, selected experiments were performed with immunopurified monocytes. Monocytes were enriched to $>85\%$ purity as judged by flow cytometry analysis (FACS; FACSCalibur cytofluorograph, Becton–Dickinson; of CD14 and MHC class II expression) by negative selection with Dynabeads CD2 and CD19 (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions. Monocytes were suspended at 1×10^6 cells/ml M-SFM.

Preparation, quantification, and opsonization of nHZ and latex beads

nHZ was isolated on a Percoll gradient from synchronous parasite culture supernatants after schizont rupture, quantified, and opsonized with fresh human serum as described [9,11,21,22]. Opsonized nHZ was tested negative for endotoxin [21] and DNase treatment was performed where indicated [21]. Quantification of nHZ was performed by luminescence assay [23]. For opsonization of latex beads, equal volumes of the manufacturer's latex beads suspension, phosphate-buffered saline (PBS), and fresh human serum were mixed and incubated at 37 °C for 30 min.

Phagocytosis of nHZ and latex beads

Monocytes suspended in M-SFM were fed with nHZ or DNase-treated nHZ at 25 fmol heme/cell or latex beads at 6400 beads/cell for 3 h before the start of transendothelial or two-dimensional (2D) migration assays. Alternatively, adherent monocytes were cultured at 2×10^6 cells/well in six-well plates in the presence of 25 fmol nHZ heme/cell. After 30 min the first nHZ crystals were observed inside the monocyte and phagocytosis was completed after 3 h incubation at 37 °C. At this time cells were washed with RPMI 1640 to remove noningested nHZ. At indicated time points after nHZ supplementation, cells were collected and solubilized for 2D electrophoresis and Western blotting as described below. Phagocytosis was quantified as indicated [23]. Apoptosis was tested by FACS after annexin V–FITC staining following the manufacturer's specifications (Apoptosis Detection Kit; Sigma–Aldrich).

Monocyte 2D chemotactic migration assay

Two-dimensional migration assay was performed in μ-slides from Ibidi (Martinsried, Germany). Monocytes were suspended in M-SFM and left unfed (control) or fed with latex beads (control meal), opsonized nHZ, or DNase-treated nHZ or treated with 10 μM 4-HNE (Biomol, Hamburg, Germany). After 1 h phagocytosis/treatment in suspension, 2×10^5 monocytes were seeded in the central channel of the μ-slide to adhere and complete phagocytosis during a further 2 h at 37 °C. Noningested nHZ, latex beads, and 4-HNE and nonadherent cells were then removed by three washes with RPMI 1640, and adherent cells were kept in M-SFM. Cytochalasin B was added at 25 μg/ml 30 min before the chemoattractants to block actin reorganization. The assay was started ($t=0$) by loading cell culture medium without or with MCP-1 (100 ng/ml, R&D Systems, Minneapolis, MN, USA), TNF (10 ng/ml (200 U/ml), Peprotech, Rocky Hill, NJ, USA), or FMLP (200 nM) in the lateral compartment of the μ-slide to create a chemotactic gradient. Migration of cells was assessed by microscopy at 0, 30, and 120 min. The assay allowed us to detect cell movements of ≥ 2 μm (detection limit). Motility in the absence of external chemoattractants was determined as the percentage of monocytes that (i) migrated in random directions [24] or (ii) did not migrate. In the presence of chemoattractants, motility was recorded as the percentage of monocytes that (i) migrated toward the chemotactic attractant (positive chemotaxis), (ii) migrated away from the chemotactic attractant, or (iii) did not migrate. Additionally, the motile cell subpopulations were analyzed for linear distances covered by the cells during the indicated times. Evidence from previous experiments showed that DNase treatment did not change the nHZ effect on motility, neither did the presence of lymphocytes in the assay, as inhibition of motility was reproduced with immunopurified monocytes (not shown).

Transendothelial migration assay

Migration across a human umbilical cord vein endothelial cell (HUVEC) monolayer was analyzed. Briefly, HUVECs were isolated from anonymously acquired umbilical cords within 1–24 h after donation by collagenase perfusion and were cultured in medium 199 (M199; BioWhittaker Europe, Verviers, Belgium), supplemented with 5% (w/v) human serum albumin, 15% (v/v) fetal bovine serum, 15 μg/ml human endothelial cell growth supplement (ECGS), and 7.5 U/ml heparin until reaching confluence [25]. After the first passage, the cells were seeded on transwell inserts (8.4 mm diameter, 3-μm-diameter pores) coated with 0.2% (w/v) gelatin (Greiner Bio-One, Frickenhausen, Germany) at a density of 2.5×10^5 cells/insert and grown in 24-well plates to confluence with addition of ECGS (22.5 μg/ml). Only completely confluent