Phagocytosis of Hemozoin (Native and Synthetic Malaria Pigment), and *Plasmodium falciparum* Intraerythrocyte-Stage Parasites by Human and Mouse Phagocytes

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Hemozoin, the detoxification product of hemoglobin heme, piles up as electron-dense material in the food vacuole (FV) of intraerythrocytic malaria parasites (malaria pigment). In infected individuals, pigment is internalized by both circulating and resident phagocytes, thus modulating their functions. Synthetic β-hematin, prepared in vitro from hematin (ferriprotoporphyrin IX hydroxide) in acidic condition, is spectroscopically identical to hemozoin. In this electron microscopy study, native and synthetic hemozoin also prove to be morphologically indistinguishable (large polygonal crystals with apparent transverse banding) and to undergo the same process when internalized by phagocytes (primarily a direct uptake of crystals, similar to what is described for asbestos fibers). On the contrary, whole parasites appear to follow a classical endocytic pathway. This suggests that there may be differences between the ingestion of free particles and whole parasites in terms of modulation of phagocytes' functions.

Keywords *β-hematin, hemozoin, malaria, malaria pigment, phagocytosis, red blood cells*

Hemozoin (malaria pigment) is an insoluble material resulting from the polymerization of heme (Ferri-protoporphyrin IX), the prosthetic part of hemoglobin. It resides in the food vacuole (FV) of the malaria parasite, a lysosome-like organelle. The malaria pigment contains, in addition to the hemozoin polymer, variable amounts of lipids and proteins [1–5]. Hemozoin can also be synthesized in vitro from hematin in acidic conditions; the synthetic polymer, β-hematin, retains the chemical characteristics of native hemozoin [3, 6, 7].

Hemozoin is also found in various cells of the infected host, both in the tissues where parasites sequester and in the peripheral circulation. It accumulates primarily in macrophages, monocytes, and neutrophils, as a result of the phagocytosis of either intraerythrocytic malaria parasites, "residual bodies" (the remnant of a parasite after the schizogonic cycle is completed and merozoites are released), or, possibly, free heme [8–14]. The black pigment appears to persist in the cells, possibly for their entire life-span. The amount of pigment in the mononuclear phagocyte system appears to be correlated with the duration of infection [11–13].

Hemozoin uptake occurs in vitro too. Various cell types prove able to internalize hemozoin in vitro, including monocytes [15, 16], macrophages, and endothelial cells [5, 17]; however, to our knowledge, the process has not been adequately documented microscopically thus far.

This electron microscopy study was set up to compare the morphology of natural and synthetic pigment, and to document the uptake and intracellular distribution of pigment resulting from the ingestion of pigment crystals or whole parasites.

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MATERIALS AND METHODS

Synthesis of β-hematin

Hematin (ferriprotoporphyrin IX hydroxide) (H-3505), was purchased from Sigma Milan, Italy. β-hematin (BH) was synthesized as described by Slater et al. [3]. In short, 60 μmoles of hematin were dissolved in 8 ml of 0.1 M NaOH, under N_2 then precipitated by the addition of 49 mmol of acetic acid.

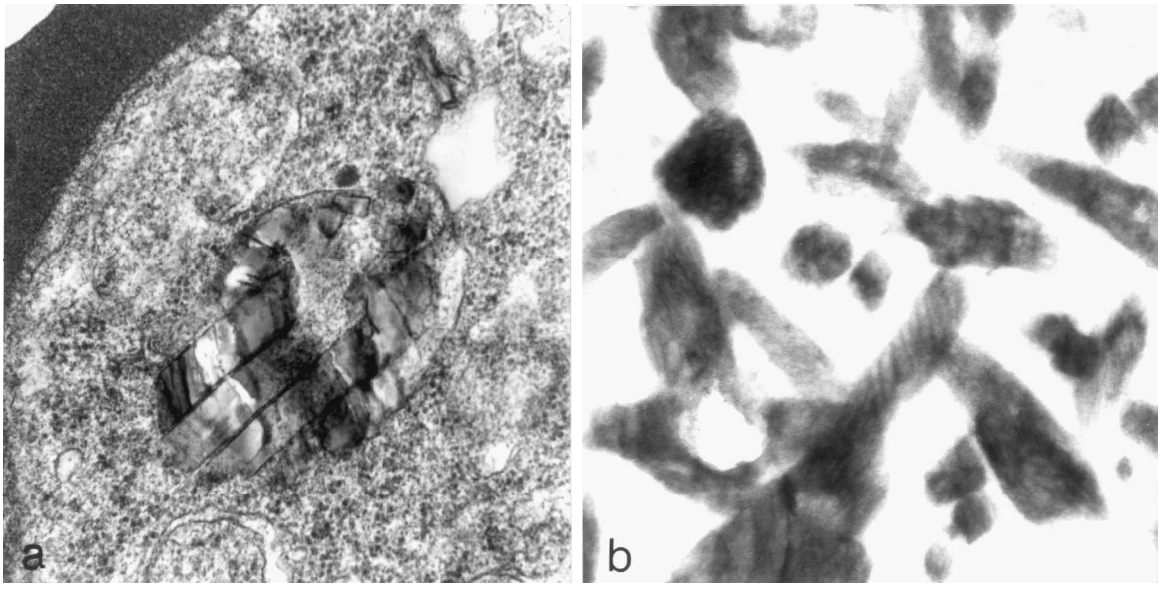

FIG. 1 *Ultrastructure of native and synthetic hemozoin. Transversal banding was observed in both native hemozoin crystals found in the parasite FV* (a) (*magnification*, ×*71.000*) *and in free particles of synthetic pigment* (β-*hematin*) (b) (*magnification*, ×*71.000*).

The suspension was heated overnight at 70°C, and the precipitate was then centrifuged and washed four times with distilled water. Unreacted hematin was removed by extracting the precipitate twice for 3 h in 0.1M sodium bicarbonate buffer at pH 9.1. The remaining insoluble material was recovered by centrifugation, washed four times in distilled water and lyophilized. The characteristics of the final product were routinely controlled by infrared spectroscopy as reported elsewhere [7, 18]. Batches of BH that did not meet standard criteria for purity were discarded. To facilitate the treatment of cell monolayers with insoluble BH, the compound was resuspended in the culture medium, mechanically microdispersed, and then sonicated.

Parasite cultures

Laboratory-adapted *P. falciparum* parasites (NF54 strain) were cultured in vitro in RPMI 1640 in human red blood cells (RBCs) according to Druilhe [19].

Purification of native hemozoin

Native hemozoin from *Plasmodium falciparum* in vitro cultures was a kind gift from Prof. S. Picot, University of Lyon, France. Parasitized red blood cells containing more than 10% schizont forms were collected and washed twice with PBS. They were lysed with 200 μl of 10% saponine in PBS for 15 min at 37°C and then washed three times in cold PBS. Crude hemozoin was then extracted by incubation for 1 h at 55°C using a lysing buffer (50 mM trisHCl, pH 8, 10 mM EDTA, 1% SDS, 10 mg/ml proteinase K). After centrifugation, the preparation was washed two times in PBS-EDTA and then exhaustively with distilled water.

Cell cultures

Monocytes

Human monocytes (MN) from healthy blood donors were separated from total blood by centrifugation on Ficoll-hypaque solution (Pharmacia, Sweden). MNs were further separated from lymphocytes by adherence to fibronectin-coated dishes (24-well culture plates) for 3 h at 37°C and then washed to remove non-adherent cells. Approximately 5×10^5 adherent cells per well were obtained with an MN:RBC ratio of 1:40.

J774 cells

A murine macrophage-like cell line (J774) [20] was maintained in MEM (Gibco-BRL-Life technologies) supplemented with 10% heat-inactivated FBS (HyClone), 1% glutammine, 1% non-essential amino acid, 2% tricine and 1% penicillin-streptomycin in 5% CO_2 at 37°C. J774 macrophages were mechanically collected with a cell lifter (Costar Italia, Milan, Italy) and transferred to a fresh medium every 3–4 days.

Electron microscopy

Specimens were fixed at 24 and 48 h of culture for MNs and at different times (25 min and 4 h) for J774 macrophages after treatment with β-hematin, native hemozoin, or carbonyl iron particles. Longer incubation times were not suitable for J774 cells due

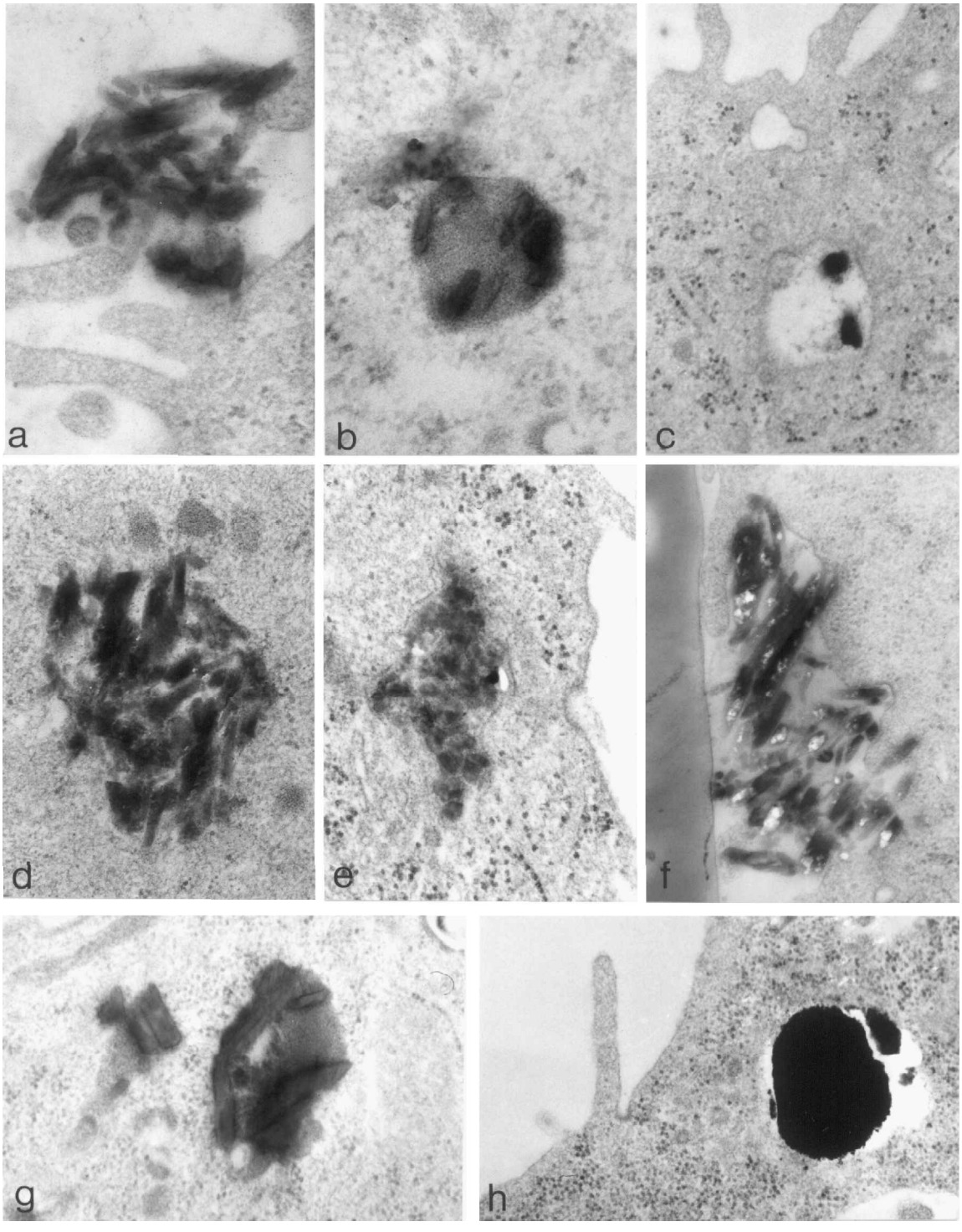

FIG. 2 *Electron micrographs of the uptake of synthetic and native hemozoin particles and carbonyl iron fillings by J774 macrophages.* (a) *An extracellular cluster of β-hematin crystals adhering to the plasma membrane and to microvilli of a macrophage. Magnification, ×40.000.* (b) *Several β-hematin particles in dense phagolysosome. Magnification, ×68.000.* (c) *Two small β-hematin particles in an early phagosome. Magnification, ×40.000.* (d) *A cluster of free, non-membrane bound intracytoplasmic β-hematin particles. Magnification, ×45.000.* (e) *A cluster of β-hematin particles surrounded by a discontinuous membrane. Magnification, ×43.000.* (f) *An extracellular cluster of β-hematin particles which appear to perforate the plasma membrane of a macrophage. Magnification, ×40.000.* (g) *Native hemozoin crystals either freely scattered in the cytoplasm or within a dense phagolysosome. Magnification, ×36.000.* (h) *Intracytoplasmic carbonyl iron particles limited by a clear halo. Magnification, ×29.000.*

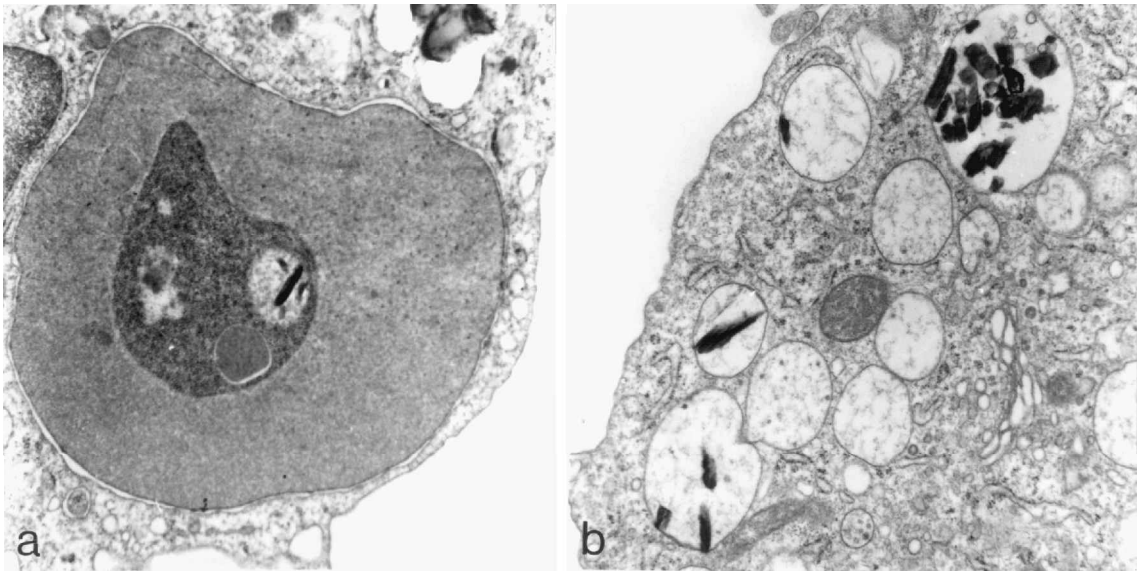

FIG. 3 *Phagocytosis of* P. falciparum*-infected red blood cells*. (a) *Human monocyte with well-developed Golgi cisterane and numerous lysosomes and an infected RBC with hemozoin crystals in the FV. Magnification*, ×*20,000*. (b) *A human monocyte containing lysosomes and phagolysosomes with remnants of RBC lysis and abundant hemozoin crystals. Magnification*, ×*30.000*.

to their fast growth rate in vitro. Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 25°C, post-fixed with osmium tetraoxide, dehydrated in ethanol and embedded in situ with Epon 812. Sections were cut perpendicularly to the substrate and stained with uranyl acetate and lead citrate.

RESULTS

In the parasite FV, hemozoin appears as polygonal crystals that, as the parasite matures, tend to stack upon each other, giving rise to larger structures with apparent transverse banding (Figure 1a). Such findings are comparable to those observed with synthetic pigment (Figure 1b).

When J774 macrophages were cultured in the presence of synthetic hemozoin, aggregates of crystal particles were seen to adhere to the cell plasma membrane (Figure 2a) and were then taken up, apparently via an endocytotic lysosomal process. Within 25 min of treatment, aggregates of small crystals were observed, mostly inside electron-dense phagolysosomes (Figure 2b), and more rarely, in early lysosomes with a clear matrix (Figure 2c).

Larger clusters of crystal particles were also observed free in the cytoplasm with no apparent delimiting membrane (Figure 2d) or surrounded by a discontinuous membrane (Figure 2e). Occasionally, synthetic hemozoin particles appeared to perforate the plasma membrane, thus penetrating into the macrophage via an endocytosis-independent pathway (Figure 2f). Similar patterns were observed with parasite-derived pigment (Figure 2g).

Macrophages fed with carbonyl iron (iron fillings) showed intracellular particles surrounded by a clear halo but no visible delimiting membrane (Figure 2h).

Monocytes co-cultured with *P. falciparum*-infected RBCs showed signs of activation, including an increased number of lysosomes, phagolysosomes, and more developed Golgi cisternae (Figures 3a and 3b) as compared to control monocytes. The various steps of the phagocytosis of infected RBCs were observed, with no apparent preference for a specific parasite stage. The only intra-monocyte structure that could be positively ascribed to the parasite after 24 h of culture was the pigment. Crystals were found inside vacuoles with a single delimiting membrane. Morphologically, crystals appeared to be identical to those found in intra-erythrocytic parasites (Figures 3a and 3b).

DISCUSSION

Synthetic β-hematin is morphologically indistinguishable from native hemozoin, whether it resides inside the parasite FV or is extracted from parasite cultures. The pictures are similar to those obtained using different materials and techniques [1, 6, 21, 22].

The uptake of free particles partially follows the classical receptor-dependent endocytotic pathways, by which substances adhere to specific plasma membrane receptors and then are internalized through pinching-off of clathrin-coated vesicles, and incorp-

orated into phagolysosomes. In our samples, obvious coated pits internalizing pigment particles are never observed. Instead, small crystal aggregates are observed in rare early phagosomes and numerous phagolysosomes. A more frequent finding is that of larger clusters of crystal particles scattered in the cytoplasm, without or with an incomplete delimiting membrane. This bears similarity to the uptake of inert material such as asbestos fibers. It has been proposed that, when ingested by lung alveolar macrophages, fibers would at first be free in the cytoplasm, and subsequently surrounded by a delimiting membrane and degraded by a phagolysosome.

The mechanism of transport of inert materials through the plasma membrane leading to the formation of clusters of free intracytoplasmic particles is still largely unknown. Our findings indicate that hemozoin could enter cells through plasma membrane ulcerations, as is the case for asbestos [23–25].

By contrast to free crystal particles, the ingestion of whole parasites results in single membrane-bound pigment. Morphologically, such structures are indistinguishable from the parasite FV.

The ingestion of hemozoin is known to affect and modulate the phagocyte response to inflammatory stimuli, although conflicting results are reported as to the direction of change with different cell types and pigment preparations [5, 15, 16, 26, 27]. Biochemical studies ascribe such variability to the cells' different membrane lipid composition and antioxidant defenses [20]. The findings of this study indicate that native and synthetic pigment are internalized by phagocytes in the same way. They also indicate that there may be differences between the ingestion of free particles and whole parasites. Studies are underway to characterize the modulation of phagocytes' functions in this latter case.

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