Phagocytosis of malarial pigment haemozoin by human monocytes: a confocal microscopy study

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SUMMARY

Haem from host erythrocyte (RBC) haemoglobin is polymerized in the digestive organelle of *Plasmodium falciparum* to haemozoin (HZ), a crystalline, insoluble substance. Human monocytes avidly ingest HZ that persists undigested for long periods of time, and generates potent bioactive lipid peroxide derivatives. Protein kinase C, an effector of signal transduction, phagolysosome formation and acidification, is inhibited in HZ-fed monocytes. Inability to digest HZ might derive from impairment in phagolysosome formation or acidification. Time-course and extent of HZ phagocytosis and acidification of phagolysosomes were studied by quantitative confocal microscopy. From 180 min until 72 h after the start of phagocytosis approximately 75–79% of the monocytes contained massive amounts of HZ. Coincidence between red (HZ) and green (acidic organelles) fluorescent compartments was very high. Confocal images showed that at 30–60 min after the start of phagocytosis, HZ was preferentially present as separated particles, with full co-localization of red and green fluorescence. Later on HZ-laden phagolysosomes tended to fuse together. In conclusion, phagolysosome formation and acidification were normal in HZ-fed monocytes during the 72-h observation time. The presence of HZ in the phagolysosome, the site of antigen processing, may offer a physical link with immunodepression in malaria.

Key words: haemozoin, malaria, phagocytosis, confocal microscopy, monocytes.

INTRODUCTION

Human phagocytic cells avidly ingest erythrocytes (RBC) parasitized by trophozoites and schizonts of *Plasmodium falciparum* as well as isolated haemozoin (HZ) (Celada, Cruchaud & Perrin, 1983; Turrini *et al*. 1992). Ingested HZ, that persists apparently unmodified within the phagocyte for long periods of time, is responsible for a number of functional alterations of the phagocyte (reviewed by Arese & Schwarzer, 1997). The long persistence of HZ may damage lysosomes through HZ-mediated inhibition of protein kinase C (Schwarzer *et al*. 1993) possibly leading to insufficient acidification and/or defective fusion of endosomes with lysosomes (Gewert *et al*. 1995; Tapper & Sundler, 1995). Of further interest is the importance of intact lysosomes for antigen presentation, in view of the HZ-mediated alteration of antigen processing reported recently (Scorza *et al*. 1999). At present it is not known whether HZ inhibits the formation of phagolysosomes; whether the phagolysosomes are acidic and if so for how long; and finally whether phagolysosomes are disrupted with HZ escaping into the cytoplasm.

We have addressed HZ phagocytosis by human monocytes making use of quantitative confocal microscopy and fluorescent indicators of organelle acidification. Our results indicate that HZ is taken up rapidly by human monocytes and is exclusively localized in normally acidified phagolysosomes during the observation period of 72 h.

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MATERIALS AND METHODS

Culture of P. falciparum *and isolation of trophozoite-parasitized RBC and HZ*

P. falciparum (Palo Alto strain, mycoplasma-free) was kept in culture as described (Schwarzer *et al*. 1992). Trophozoite-parasitized RBC (TRBC) were isolated from non-synchronized cultures by the Percoll–mannitol method (Schwarzer *et al*. 1992). HZ was isolated from TRBC by osmotic lysis as described (Schwarzer *et al*. 1992). Briefly, TRBC-enriched cultures (> 95% TRBC) were lysed by adding 25 volumes of lysis buffer containing 10 mM phosphate and 10 mM mannitol, pH 8·0, and pelleted. The pellet was washed 3 times with cold lysis buffer, suspended in PBS (5 mg protein/ml), degassed, flushed with nitrogen and stored at −80 °C.

Preparation and handling of monocytes

Human monocytes were separated by Ficoll gradient from freshly collected buffy coats discarded from blood samples of healthy adult donors of both sexes as described (Schwarzer *et al*. 1998). Separated

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lymphomonocytes were washed 3 times with RPMI 1640 medium (Sigma, St Louis, MO), resuspended at $1{\cdot}5 \times 10^6$ cells/ml in AIM V serum-free medium (Gibco BRL, Life Technologies, Gaithersburg, MD) and plated at $1{\cdot}5 \times 10^6$ cells/cover-slip on 10-mm diameter round, delipidated glass cover-slips inserted in wells of a 24-well plate. The plates were incubated in a humidified CO_2/air-incubator at 37 °C for 60 min, when non-adherent cells were removed by 3 washes with RPMI 1640 medium at 37 °C. The cells were then reincubated for at least 1 h in AIM V medium, and washed once.

Phagocytosis of HZ, TRBC, non-parasitized RBC and latex particles by adherent monocytes

Phagocytosis was started by adding to each cover-slip/well, 10 μl of TRBC (10 % haematocrit), 20 nmol HZ-haem (corresponding to the haem amount contained in 10 μl of TRBC or RBC at 10 % haematocrit), 10 μl of anti-D IgG-opsonized RBC (10 % haematocrit), or 0·3 μl of amine-modified, red-fluorescent latex beads (2·5 % solids, diameter 0·105 μm, Sigma). TRBC, HZ and latex beads were previously opsonized with fresh autologous serum as indicated (Schwarzer *et al*. 1998) and suspended in AIM V medium. Thereafter, the plates were centrifuged at low speed for 5 sec and incubated in the humidified CO_2/air-incubator at 37 °C. Except for short-term phagocytosis (see below), non-ingested HZ, TRBC, RBC and latex particles were removed 90 min after the start of phagocytosis by 3 washings with RPMI 1640 medium followed by 1 washing with ice-cold water for 20 sec to lyse residual adherent, non-ingested TRBC and RBC, and a final washing with RPMI 1640 medium. Thereafter, the cells were further incubated in AIM V medium for the indicated time-periods in the humidified CO_2/air-incubator at 37 °C. In the case of short-term phagocytosis experiments (30- and 60-min phagocytosis time-periods), non-ingested HZ, TRBC, RBC and latex particles were removed at 30 or 60 min after the start of phagocytosis by 3 washings with RPMI 1640 medium followed by 1 washing with ice-cold water for 20 sec to lyse residual adherent, non-ingested TRBC and RBC, and a final washing with RPMI 1640 medium. Thereafter cells were treated as indicated below to assess phagocytosis and acidification by confocal microscopy.

Assessment of phagocytosis and acidification of subcellular organelles in differently fed monocytes

Phagocytosis was studied taking advantage of red fluorescence of phagocytosed material, resulting from haem-mediated red fluorescence in HZ, TRBC and non-parasitized RBC, and from red-fluorescent latex beads. To assess acidification, 30 min before the indicated time the cover-slips (prepared in triplicate for each experimental point, see below) were transferred to 1 ml of RPMI 1640 medium containing 50 μM *N*-(3-((2,4-dinitrophenyl)amino)-propyl)-*N*-(3-aminopropyl)methylamine, dihydrochloride (DAMP, Molecular Probes, Eugene, OR). DAMP is a weakly basic amine that is taken up in acidic organelles of living cells where it can be detected by green-fluorescent anti-DAMP antibodies (Molecular Probes) (Anderson *et al*. 1984). It should be noted that for the 30- and 60-min phagocytosis time-periods, DAMP was supplemented together with the phagocytic substrates. DAMP did not bind unspecifically to any of the phagocytic substrates and did not interfere with the phagocytic process (not shown). After 30 min incubation in the humidified CO_2/air-incubator at 37 °C monocytes were washed with RPMI 1640 medium containing 5 % foetal calf serum (Gibco) at 37 °C, and fixed in 3 % paraformaldehyde in PBS for 10 min at 37 °C. Each cover-slip was washed once with 2 ml of 50 mM NH_4Cl in PBS and twice with 1 ml of PBS. Adherent monocytes were permeabilized with 2 ml of ice-cold PBS containing 0·1 % Triton-X100 (Sigma) for 5 min. Immediately thereafter the cover-slips were incubated with anti-DAMP antibodies applied at a 1 in 40 dilution in PBS containing 1 % BSA (Sigma), and allowed to react for 1 h at 37 °C. Thereafter the cover-slips were washed 3 times with PBS, mounted with glycerol in PBS solution (50 % glycerol) and sealed with transparent crystal glue on glass microscope slides. Slides were kept at 4 °C in the dark until confocal microscopy examination. Positive-control monocytes were maximally acidified by phorbol 13-monoacetate stimulation (100 nM, final concentration) immediately before addition of DAMP. Anti-DAMP antibodies did not react unspecifically with naive, untreated monocytes (not shown).

Confocal microscopy analysis of differently fed monocytes

Cover-slips were examined in a Bio-Rad MRC 600 confocal microscopy system using the argon laser (Bio-Rad Microscience Division, Hercules, CA). In total 5–7 focal frames were taken along the z axis at 1-μm intervals and then merged to obtain a reconstructed image. Colour images are true colour images, with green reflecting anti-DAMP antibody fluorescence, and red representing haem-dependent fluorescence of HZ and RBC haemoglobin, in terms of DAMP/haem/latex localization and concentration, or false colour images that reflect the fluorescence intensity scales in the order: blue, lowest intensity; green; red; white, highest intensity. To assess precisely the degree of co-localization between the green fluorescence (acidic organelle) and the red fluorescence (HZ, haemoglobin haem, red-fluorescent latex), 'green' and 'red' images were

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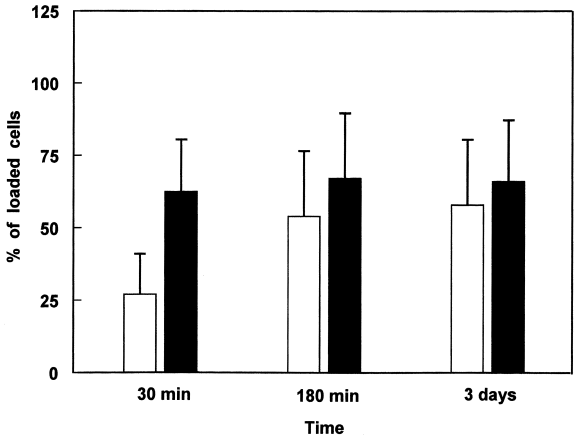

Fig. 1. Percentage of human monocytes loaded with HZ or latex beads at different time-points after the start of phagocytosis. Human monocytes adherent on glass cover-slips were allowed to phagocytose serum-opsonized HZ (□) or red-fluorescent latex beads (■). At indicated time-points after start of phagocytosis, non-ingested HZ and latex beads were removed by washing with RPMI 1640 and monocytes examined immediately (30-min time-point) or incubated further at 37 °C. Cover-slips were analysed for red fluorescence by the Bio-Rad confocal microscopy system. For each time-point, more than 400 cells from 3 distinct experiments were examined to determine ingested HZ particles or latex beads. Data are mean values ± S.D. For details, see the Materials and Methods section.

electronically fused. Assessment of percentage of monocytes with phagocytosis versus 'empty' cells (percentage of cells loaded with ingested particles/acidic organelles) was performed by image analysis of $\geqslant 400$ cells chosen at random. The intensity of background fluorescence (fluorescence intensity 1, Fi_1) and cell autofluorescence (fluorescence intensity 2, Fi_2) was quantitated and employed as a cut-off value ($Fi_1 + Fi_2$) to discriminate between negative cells and cells exhibiting patches of green or red fluorescence (identified as positive cells). A comparable approach was employed to calculate the percentage of cell volume occupied by green and red fluorescence. Single cells were selected and their volume was calculated from the number of pixels whose intensity was above the background intensity (fluorescence pixel number 1, Fn_1). Then the cut-off intensity value of cell autofluorescence (fluorescence pixel number 2, Fn_2) was subtracted and the obtained value was employed to calculate the percentage of cell volume occupied by specific fluorescence: % of volume $= (Fn_1 - Fn_2) * 100/Fn_1$.

RESULTS

Quantitative studies of phagocytosis of HZ, latex beads and anti-D IgG-opsonized RBC by human monocytes

Quantitative studies of phagocytosis took advantage of the red fluorescence associated with haem (HZ, RBC) or with red-fluorescent latex beads. Analysis of HZ-associated red fluorescence in confocal images allowed assessment of how many monocytes had phagocytosed HZ and measurement of the degree of loading at different time-points (Fig. 1). Thirty min after the start of phagocytosis 38 ± 19 % of monocytes had already ingested massive amounts of HZ. After 180 min and until 72 h after the start of phagocytosis 75 ± 31 % and 79 ± 30 %, respectively, of the examined monocytes were extensively HZ-laden. Red-fluorescent latex beads were phagocytosed very rapidly and intensely (Fig. 1). Thirty min after the start of phagocytosis, 86 ± 25 % of examined monocytes showed an intense and widespread red fluorescence. After 180 min and at 72 h after the start of phagocytosis, 92 ± 34 % and 91 ± 29 %, respectively, of the examined monocytes were laden with latex beads. Short-term analysis of RBC-haemoglobin-associated red fluorescence apparently indicated a slower kinetics and lower intensity of phagocytosis of anti-D IgG-opsonized RBC, with 16 % and 18 % RBC-laden monocytes at 30 and 60 min after the start of phagocytosis, respectively (not shown). Low levels of short-term red fluorescence may reflect a slow build-up rate of the red fluorescent haem derivative (Nagababu & Rifkin, 1998), responsible for the confocal visibility of RBC. No long-term analysis could be performed with RBC-laden monocytes, because 90 % of ingested RBC were degraded by monocytes with a t/2 of $< 0{\cdot}5$ h (Schwarzer *et al.* 1992).

Quantitative assessment of the percentage of cell volume occupied by acidic organelles (green-fluorescent compartment) and by HZ/RBC/red-fluorescent latex beads (red fluorescent compartment) was performed by computer-assisted analysis of red and green fluorescence in time-course confocal images. Fig. 2A shows that up to approximately 30 % of the cell volume was occupied by HZ or acidic organelles from 180 min to 12 h after the start of phagocytosis. A similar pattern of cellular occupancy by green and red fluorescent compartments was observed in latex bead-fed monocytes (Fig. 2B). The percentage of cell volume occupied by ingested RBC at 60 min after the start of phagocytosis was distinctly lower compared to HZ and latex, and amounted to 18 % red fluorescent and 26 % green-fluorescent compartment (not shown). At 72 h after the start of phagocytosis, cell occupancy by the green-fluorescent compartment had dropped to approximately 23 % and 20 % of the cell volume in latex-bead- and HZ-fed cells, respectively (Fig. 2A and B). These decreases were not significant. Lowering of green fluorescence by constant red fluorescence possibly indicates exhaustion of the proton-pumping machinery in phagolysosomes occupied by indigestible particles. Up to 3 h after the start of phagocytosis, the amount of cell volume occupied in any single monocyte by the red and

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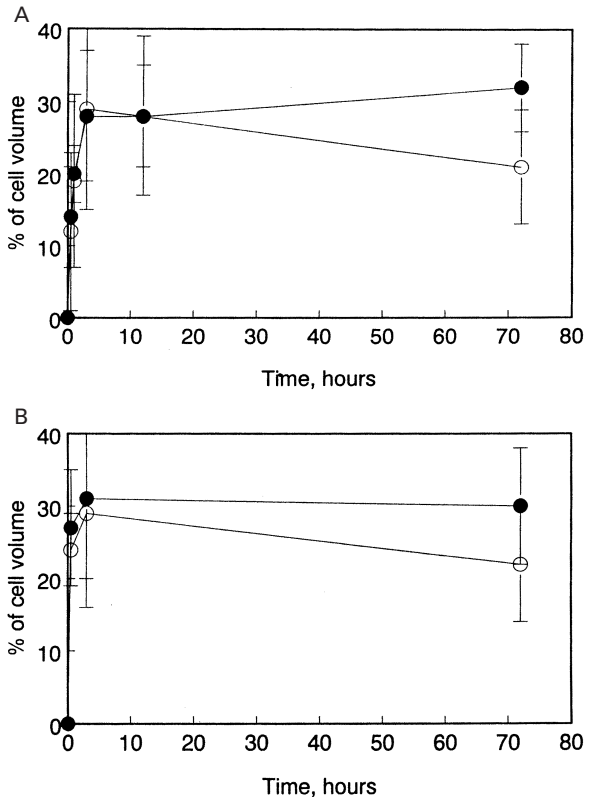

Fig. 2. Percentage of cell volume occupied by acidic organelles (○) or ingested particles (●) in human monocytes loaded with HZ (A) or red-fluorescent latex beads (B) at different time-periods after the start of phagocytosis. Human monocytes adherent on glass cover-slips were allowed to phagocytose serum-opsonized HZ or red-fluorescent latex beads. After removing non-ingested HZ and latex beads by washings with RPMI 1640, the cover-slips were transferred to RPMI 1640 medium containing 50 μM DAMP, an indicator of acidic organelles. Intracellular DAMP was allowed to react with green-fluorescent anti-DAMP antibodies. The cover-slips were examined by the Bio-Rad confocal microscopy system using the argon laser to assess green and red fluorescence. Measurement of the percentage of cell volume occupied by ingested particles or by acidic organelles was performed by image analysis of more than 400 cells chosen at random per experimental point from 3 distinct experiments. Data are mean values ± S.D. At any time-point, none of the differences was statistically significant. For details, see the Materials and Methods section.

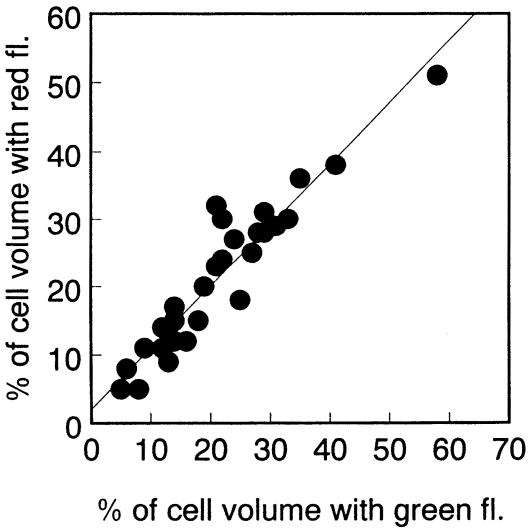

Fig. 3. Correlation between percentage of cell volume occupied by ingested particles and acidic organelles. The curve was obtained by quantifying the percentage of cell volume occupied by red-fluorescent HZ particles and green-fluorescent organelles in 28 HZ-fed monocytes chosen at random and examined 180 min after start of phagocytosis. For details, see legends of Fig. 1 and Fig. 2, and the Materials and Methods section.

green-fluorescent compartments was largely coincident, as judged by quantitative analysis of 28 HZ-fed monocytes chosen at random (Fig. 3). Visual analysis of confocal pictures constantly confirmed co-localization of green and red fluorescent compartments in HZ- and latex-bead-fed cells up to 12 h after the start of phagocytosis (for example, see Fig. 4).

High magnification confocal images of HZ-fed monocytes

Studies performed with HZ-fed monocytes observed at high magnification (Fig. 4) allowed analysis of the intensity of red and green-fluorescent compartments; the degree of co-localization of HZ and acidic organelles; form, number and volume of ingested HZ particles and acidic organelles with respect to the monocyte volume; modifications of HZ particles and acidic organelles over time, such as fusion of acidic organelles, particle loss, abrogation of acidification. HZ particles were not homogeneous in their dimensions and fluorescence output. At short time-periods after phagocytosis, HZ seemed to be preferentially present as separated particles each contained within an acidic compartment that reflected the particle's form and dimension. Without exception red- and green-fluorescent compartments were co-localized. In general, intensity of red and green fluorescence displayed parallel behaviour and larger particles were often contained within very acidic organelles of matching dimensions (Fig. 4, A1–A3). HZ-associated red fluorescence occasionally observed externally of acidic organelles occurred only in the case of non-ingested HZ particles (not shown). Frequently, ingested HZ particles were very large (Fig. 4, A1 and B1) and tended to fuse together at later time-periods (Fig. 4, B2–B4). However, at all time-points examined, the acidic content of phagolysosomes was confined within the organelles and did not leak into the cytoplasm. Remarkably, acidification of phagolysosomes persisted during long periods of time and was diminished only on the third day post-phagocytosis (Fig. 4, B4). Red and green fluorescence was co-localized also in TRBC-fed monocytes from 60 min to 12 h after the start of phagocytosis, indicating normal fusion between phagosomes and

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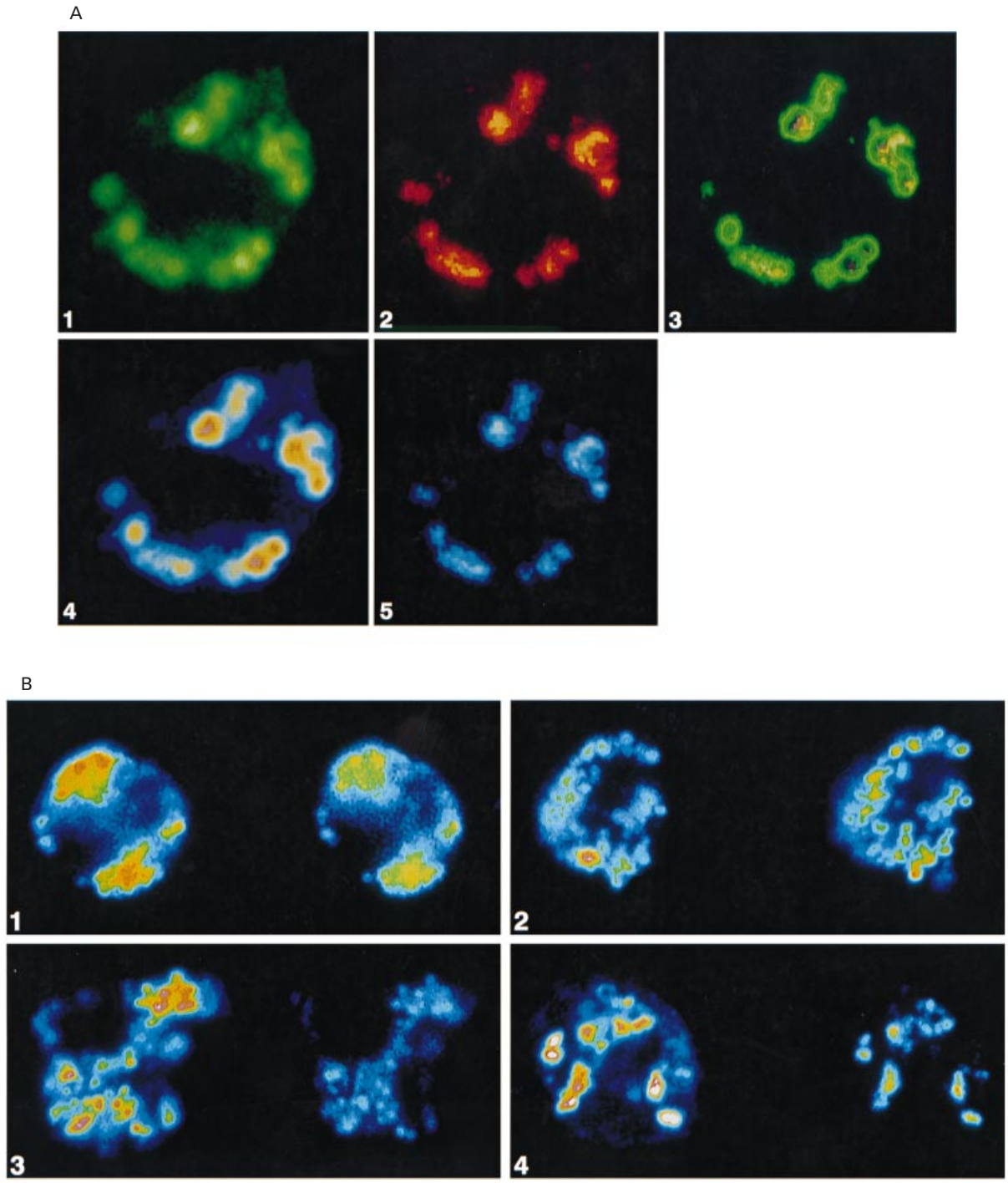

Fig. 4. (A) High-magnification confocal microscopy images of HZ-fed human monocytes. Human monocytes adherent on glass cover-slips were allowed to phagocytose serum-opsonized HZ. After 90 min, non-ingested HZ was removed by washings with RPMI 1640 and monocytes further incubated at 37 °C during 90 min. Thirty min before the end of incubation the cover-slips were transferred to RPMI 1640 medium containing 50 μM DAMP, an indicator of acidic organelles. Intracellular DAMP was then reacted with green-fluorescent anti-DAMP antibodies. Cover-slips were examined by the Bio-Rad confocal microscopy system. Five to seven focal frames were taken along the z axis at 1-μm intervals and then merged to obtain a reconstructed image. Colour images are true colour images, with green reflecting acidic organelles (A1), and red representing HZ-dependent fluorescence (A2). To assess precisely the degree of co-localization between green and red fluorescence, green and red images were electronically fused (A3). A4 and A5 correspond to A1 and A2, respectively, and represent false colour images that reflect the fluorescence intensity scales in the order: blue, lowest intensity; green; red; white, highest intensity. Magnification × 3500. For details, see the Materials and Methods section. (B) High magnification confocal microscopy images of HZ-fed human monocytes examined at 1, 2, 12 and 72 h (B1–B4) after the start of phagocytosis. Phagocytosis was stopped by washing away non-ingested HZ 60 min (B1) or 90 min (B2–B4) after the start of phagocytosis. Left and right image at each time-

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lysosomes, and normal organelle acidification (not shown).

DISCUSSION

Native HZ used in the present work was obtained by osmotic lysis of TRBC under standardized conditions. It is a crystalline insoluble mass of covalently polymerized haem (Slater, 1992; Bohle *et al*. 1997; Pagola *et al*. 2000), containing small amounts of free iron, protein and lipid remnants of parasitic and host RBC origin (Goldie *et al*. 1990; Schwarzer *et al*. 1992). Native HZ appears to be quite similar to the 'residual bodies' originated from schizont rupture, considered to be the physiological meal of phagocytes *in vivo*. Here we confirm that human monocytes avidly and rapidly ingest large quantities of native HZ, that filled up to approximately 30 % of the total cell volume and persisted unmodified throughout the observation period of 72 h.

Several intracellular micro-organisms that persist alive within the phagocytes are known to evade killing by inhibiting phagolysosome formation or blocking phagolysosomal acidification (Reiner, 1994; Sturgill-Koszycki *et al*. 1994; Bogdan & Röllinghoff, 1999). In many cases, avoidance of monocyte killing and intra-monocytic survival depend on or are accompanied by inhibition of protein kinase C (Reiner, 1994). Monocyte protein kinase C – an effector of phagolysosome formation, lysosomal acidification and lysosomal enzyme production (Gewert *et al*. 1995; Tapper & Sundler, 1995) – was inhibited after HZ phagocytosis (Schwarzer *et al*. 1993). Therefore, persistence of HZ could possibly depend on damage to phagosome–lysosome fusion, or on inability to acidify or produce proteolytic enzymes. Neither of these events was supported by present results. HZ was localized from the very beginning of the post-phagocytic phase within the same acidic organelles as opsonized RBC or latex particles. Secondly, organelles containing HZ were acidified normally and red and green fluorescence, indicators of HZ and acidified organelles, respectively, developed in parallel. Lastly, except for non-ingested particles, HZ was never localized outside the acidic compartment. Thus, the phagocytic process of HZ, opsonized latex beads or RBC seemed to follow the same route and be performed by the same mechanism.

In a recent electron-microscopy study of HZ phagocytosis (Olliaro *et al*. 2000), artificial and malarial HZ have been occasionally found free in the cytoplasm or surrounded by incomplete membranes, or perforating the plasma membrane, pointing at membrane damages similar to those elicited by ingestion of mineral asbestos fibres. This discrepancy to our data may be due to the use of deproteinized malarial HZ by Olliaro *et al*. (2000) a phagocytic meal different from the native, opsonized HZ used in the present study. We consider the latter to be very similar to the 'residual bodies', the physiological meal of phagocytes that includes the digestive vacuole with HZ inside.

Several functions appear to be modified or impaired in HZ-fed monocytes (see Arese & Schwarzer (1997) for review). Recently, the presence of HZ in monocytes has been shown to impair MHC class II expression after interferon-gamma stimulation (Schwarzer *et al*. 1998), to alter processing of protein antigens and to suppress the interleukin-2 production by the antigen-activated T-cell hybridomas (Scorza *et al*. 1999). The above HZ-related modifications may play a role in malaria-associated immunodepression. Indeed, as shown here, large amounts of HZ are localized in the very same subcellular organelles, multivesicular phagolysosomes, where loading of MHC class II molecules with antigenic peptides for immune presentation occurs (Chapman, 1998; Geuze, 1998; Dell'Angelica *et al*. 2000).

Data from our group indicate that native HZ is rich in, and generates large amounts of biologically powerful lipid derivatives such as 4-hydroxynonenal (Schwarzer *et al*. 1996), 15(S)-hydroxy-6,8,11,13-eicosatetraenoic acid (15-HETE) (Schwarzer *et al*. 1999), 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) and 9S-hydroxy-10E,12Z-octadecadienoic acid (9-HODE). Generation of the above compounds appears to occur non-enzymatically by iron-porphyrin catalysis carried out by the polymerized haem core of HZ on unsaturated fatty acids present in native HZ (H. Kühn and E. Schwarzer, unpublished results). More specifically, 4-hydroxynonenal in low micromolar concentrations was found to depress expression of MHC class II proteins in monocytes (E. Schwarzer, unpublished results). By contrast, de-lipidized HZ and artificial HZ were clearly less active when fed to monocytes (E. Schwarzer, unpublished results).

In conclusion, present studies showing persistent and massive accumulation within the phagolysosome of HZ, the generator of potent bioactive lipid derivatives, may offer a physical link between HZ and altered antigen processing, and open new avenues for the understanding of immunodepression in malaria.

point represent red and green fluorescence due to HZ and acidic organelles, respectively. Images are presented as double image of the same field with false colours that reflect the fluorescence intensity scales in the order: blue, lowest intensity; green; red; white, highest intensity. Magnification: 2400x. For details, see (A) and the Materials and Methods section.

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