Impairment of Macrophage Functions after Ingestion of *Plasmodium falciparum*-infected Erythrocytes or Isolated Malarial Pigment

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Summary

Human monocyte-derived macrophages ingest diamide-treated red blood cells (RBC), anti-D immunoglobulin (Ig)G-opsonized RBC, or *Plasmodium falciparum* ring-stage parasitized RBC (RPRBC), degrade ingested hemoglobin rapidly, and can repeat the phagocytic cycle. Monocytes fed with trophozoite-parasitized RBC (TPRBC), which contain malarial pigment, or fed with isolated pigment are virtually unable to degrade the ingested material and to repeat the phagocytic cycle. Monocytes fed with pigment display a long-lasting oxidative burst that does not occur when they phagocytose diamide-treated RBC or RPRBC. The phorbol myristate acetate–elicited oxidative burst is irreversibly suppressed in monocytes fed with TPRBC or pigment, but not in monocytes fed with diamide-treated or IgG-opsonized RBC. This pattern of inhibition of phagocytosis and oxidative burst suggests that malarial pigment is responsible for the toxic effects. Pigment iron released in the monocyte phagolysosome may be the responsible element. 3% of total pigment iron is labile and easily detached under conditions simulating the internal environment of the phagolysosome, i.e., pH 5.5 and 10 μM H_2O_2. Iron liberated from pigment could account for the lipid peroxidation and increased production of malondialdehyde observed in monocytes fed with pigment or in RBC ghosts and liposomes incubated at pH 6.5 in presence of pigment and low amounts of H_2O_2. Removal of the labile iron fraction from pigment by repeated treatments with 0.1 mM H_2O_2 at pH 5.5 reduces pigment toxicity. It is suggested that iron released from ingested pigment is responsible for the intoxication of monocytes. In acute and chronic falciparum infections, circulating and tissue-resident phagocytes are seen filled with TPRBC and pigment particles over long periods of time. Moreover, human monocytes previously fed with TPRBC are unable to neutralize pathogenic bacteria, fungi, and tumor cells, and macrophage responses decline during the course of human and animal malaria. The present results may offer a mechanistic explanation for depression of cellular immunity in malaria.

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Infection of nonimmune patients with *Plasmodium falciparum* has severe consequences in 1–2% of cases (1). Acute anemia, cerebral malaria, or a combination of both are the most frequent causes of death. In certain geographic areas, acute malaria outnumbers cerebral malaria as the final determinant of lethality, which amounts to one-half to two million deaths per year in Africa alone (1). The first line of defense against the parasite is provided by phagocytic cells (2–4), which recognize parasitized RBC as nonself cells and attack them by the same response adopted against any invader: extracellular generation of aggressive oxidative compounds, and phagocytosis. Phagocytes stuffed with parasitized RBC (PRBC)[1] and nonparasitized RBC (NPRBC), and pigment-containing residual bodies, are frequently seen in peripheral blood and various organs (5, 6). Increased levels of activated phagocyte products such as reactive oxygen (7) or cytokines (8) have been determined in serum of patients with acute malaria. Phagocyte activity is maximal upon first infection and declines thereafter with repeated malarial episodes (9, 10). Basically the same type of response has also been noted in animal malarial models (11–13). In vitro, PRBC are phagocytosed by human phagocytes. Phagocytosis increases with parasite maturation

[1] *Abbreviations used in this paper:* NPRBC, nonparasitized red blood cells; PRBC, parasitized RBC; RMB, RPMI 1640 supplemented with 24 mM $NaHCO_3$; SPRBC, schizont-parasitized RBC; TPRBC, trophozoite-parasitized RBC.

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and is maximal with trophozoite- and schizont-parasitized RBC (TPRBC and SPRBC, respectively) (14, 15). In vivo, the reticuloendothelial system in humans has the potential to ingest very large numbers of damaged RBC. For example, during acute anemia elicited by fava bean ingestion in glucose-6-phosphate dehydrogenase–deficient individuals (favism), destruction of up to 40–80% of total RBC mass in a few days can be accounted for by enhanced phagocytic removal (16).

In falciparum malaria the lack of total clearance of parasitized RBC from blood and the decreasing activity of macrophages with repeated infections (8) may indicate damaged function in phagocytes. For these reasons we have engaged in the study of phagocytosis of *P. falciparum*–parasitized RBC and its consequences for the activity of monocyte-derived macrophages. We show that ingestion of RBC infected with mature parasite stages or isolated pigment, but not of RPRBC, severely and permanently damages macrophages. We also provide evidence indicating that the probable cause of pigment toxicity resides in pigment-derived iron.

Materials and Methods

Cultivation of Malarial Parasites. *P. falciparum* (FCR_3) parasites were kept in culture as described previously (17). RBC parasitized with different stages of parasite maturation were isolated from nonsynchronized cultures by the Percoll-sorbitol method (18). The RPRBC fraction had a parasitemia of 60–70% and did not contain any other stage. The TPRBC fraction contained up to 5% RPRBC and no NPRBC. Fractionated cells were washed three times (200 *g*, 5 min, 20°C) in PBS supplemented with 10 mM glucose (PBS-G, pH 7.4).

Preparation of Malarial Pigment. Pigment was isolated from TPRBC-rich cultures (19). Cells were osmotically lysed by adding ~25 vol distilled H_2O (for 5 min at 4°C). Pigment and attached membrane fragments were spun down (200 *g*, 10 min, room temperature). The pellet was washed three times (200 *g*, 10 min, room temperature) with 50 ml H_2O. Pigment from different preparations was pooled and stored in PBS (5 mg protein/ml) at −20°C to obtain uniform material. No further purification of pigment was undertaken to prevent complete removal of adhered membranes, necessary for opsonization and phagocytosis. No phagocytosis could be observed when detergent-treated pigment was used (data not shown).

For iron release studies, pigment was pretreated with a large excess of acetone (10 min, 25°C) to remove lipids and residual hemoglobin from associated membranes. Pigment was quantified by protein determination (20) and by heme assay (see below). One RBC equivalent is defined as the amount of pigment containing the same quantity of heme as one RBC (2 fmol, corresponding to 7.7 pg pigment protein).

Treatment of RBC with Diamide. Washed NPRBC suspended in PBS-G (10% hematocrit) were incubated with 200 μM diamide (azodicarboxylic acid bis[dimethylamide]; Sigma Chemical Co., St. Louis, MO) for 60 min at 37°C. Thereafter, RBC were washed three times in 10 vol PBS-G as detailed above.

Opsonization of RBC with Anti-D IgG. Immediately before the phagocytosis experiment, washed NPRBC suspended in PBS-G (20% hematocrit) were incubated with 25 μg anti-D IgG/ml packed RBC (Rhesuman Berna, Institut für Serotherapeutica und Vaccine, Bern, Switzerland) for 30 min at 37°C. Thereafter, RBC were washed three times in 10 vol PBS-G and resuspended in RPMI 1640 supplemented with 24 mM $NaHCO_3$ (RMB) (10% hematocrit) and kept at 4°C until used.

Opsonization of RBC and Pigment with Serum. To a volume of packed washed NPRBC, PRBC or diamide-treated RBC were added to an equal volume of fresh serum, and cells were incubated for 30 min at 37°C. Thereafter, cells were diluted with RMB to 10% final hematocrit. Washed sonicated pigment was suspended in PBS (5 mg protein/ml) and opsonized with 1 vol human fresh serum for 30 min at 37°C. NPRBC, diamide-treated RBC, or pigment were opsonized with fresh autologous serum. PRBC from cultures were opsonized with fresh AB^+ serum.

Preparation of Adherent Monocytes. Mononuclear cells were separated from fresh human blood collected in heparin (6 IU/ml blood) (21). Separated lympho-monocytes were washed three times in PBS and resuspended in RMB containing 10% (vol/vol) autologous serum and plated at 1–2 × 10^6 cells/well (Falcon, Becton Dickinson Italia, Milano, Italy). After 1 h incubation in a humidified incubator (5% CO_2, 95% air; 37°C), nonadherent cells were removed by three gentle washes with lukewarm RMB. Adherent monocytes were reincubated with RMB for 5–10 h until beginning the phagocytosis experiments. The same protocol was followed to prepare coverslip-adherent monocytes, except that each well contained an 8-mm-diameter round glass coverslip.

Conditioning of Suspended Monocytes. Washed lympho-monocytes (see above) were resuspended at 1–2.5 × 10^7 cells/ml in RMB containing 50 mM Hepes (pH 7.4) and kept in 50 ml plastic tubes (Falcon, Becton Dickinson Italia) at 37°C. Gas exchange with the 5% CO_2, 95% air atmosphere was allowed by perforating the lid of the tube with a sterile needle.

Phagocytosis of RBC and Pigment by Monocytes. 15 μl serum-opsonized or anti-D IgG-opsonized RBC (10% hematocrit) was added to each well containing ~50,000 monocytes, as determined by DNA microassay (21). The ratio RBC/monocyte was ~300. Comparable amounts of pigment, in terms of heme content, were used (115 μg pigment protein corresponding to ~30 nmol heme/well). After 2 h of incubation at 37°C in an 5% CO_2, 95% air atmosphere, noningested pigment or noningested/nonadherent RBC were removed by aspiration and three gentle washes with lukewarm RMB. Noningested, adherent RBC were osmotically lysed by the addition of ice-cold distilled water for 20 s, followed by an additional wash with RMB to remove free hemoglobin. This treatment did not compromise the integrity of monocytes. Thereafter, monocytes containing ingested cell material were solubilized by adding to each well 1 ml solubilizing solution (0.1 N NaOH, 0.025% [vol/vol] Triton X-100 [Bio-Rad Laboratories, Richmond, CA], 3 mM EDTA [Sigma Chemical Co., St. Louis, MO]). Plates were stored for 4 h at 4°C. Phagocytosis was quantified by measuring ingested hemoglobin or pigment-derived heme by a luminescence method. Heme-associated peroxidase activity catalyzes the production of light in presence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co.) and tert-butylhydroperoxide (Merck, Darmstadt, Germany) at alkaline pH (22). The amount of emitted light is proportional to the heme concentration. Photon counts per monocyte were transformed into a number of RBC ingested per monocyte by using a calibration curve constructed for each experiment with known amounts of cells out of the same sample utilized for the phagocytosis experiment. Parallel to each phagocytosis test, opsonized NPRBC were added to monocytes and treated in the same way. The values obtained with NPRBC were subtracted from all phagocytosis values to correct for nonspecific phagocytosis or residual binding. Each phagocytosis value represents the average of data obtained from four different wells.

Effect of Ingested Material on Repeated Phagocytic Activity. The

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ability of monocytes to maintain their phagocytic activity was checked as follows: adherent monocytes were fed with RPRBC, TPRBC, pigment, and anti-D IgG-opsonized or diamide-treated RBC for 120 min. Monocytes were then washed with RMB, adherent RBC were lysed with distilled H_2O, and the monocytes were washed again with RMB and returned to the incubator. The second challenge was performed with diamide-treated RBC 24, 48, and 72 h after completion of the first phagocytic cycle. Control monocytes that had not been previously challenged were assayed in parallel.

Measurement of Oxidative Burst. Oxidative burst in suspended or plated monocytes was quantified by measuring luminol-dependent chemiluminescence (23). Monocytes adherent to 8-mm-diameter round glass coverslips (placed in a 24-well multiwell plate) were fed with diamide-treated RBC, TPRBC, or pigment at time 0. Noningested RBC or pigment were removed before luminescence measurement, at least 1 h after starting the phagocytosis, as described earlier. At the indicated times monocytes adherent to coverslips were placed into a lumimeter cuvette (Magic Lite Analyzer; Ciba Corning Diagnostic Corp., Medfield, MA) containing Krebs-Ringer phosphate buffer, supplemented with 5 mM glucose, 1 mM Ca^{2+}, and 10 μM luminol (total volume 1 ml). After measuring the basal luminescence, oxidative burst was elicited by adding PMA (100 nM final concentration). Luminescence peaking after 2.5 min was recorded. Phagocytosis of pigment by suspended monocytes was initiated by adding pigment and centrifugation (150 *g*, 5 s, room temperature) to accelerate pigment sedimentation and contact with monocytes to improve the quantification of initial burst. The amount of pigment added was equivalent to that used with plated monocytes in terms of heme content. If partially iron-stripped pigment was employed, equivalent amounts of protein was added. After resuspension, cells were further kept as described earlier. Oxidative burst was quantified as described above pipetting 10^5 to 10^6 cells into the lumimeter cuvette.

ATP Measurement. ATP levels were estimated in washed lysed monocytes by the standard luciferin/luciferase bioluminescence assay (24).

Determination of Lipoperoxides. The formation of malondialdehyde (MDA) in freshly prepared hemoglobin-free RBC ghosts and in ghost-derived liposomes in the presence of pigment was determined by a standard procedure (25). Ghosts were prepared by lysis (5 min) of washed RBC in 30 vol ice-cold 10 mM phosphate buffer (pH 8.0), containing 1 mM PMSF (Sigma Chemical Co.). After centrifugation of the lysate (40,000 *g*, 5 min, 4°C), the resulting pellet was washed three times in the same phosphate buffer at 4°C. For liposome preparation, lipids of these ghosts were extracted with 2 vol of chloroform/methanol (1:2, vol/vol) for 30 min at 25°C. The lower chloroform phase was collected and dried under nitrogen. PBS (pH 6.5, 25°C) was added to obtain the original lipid concentration of the ghost preparation after sonication for 1 min (total time) with frequent breaks for cooling. Hemoglobin-free ghosts (250 μg protein/100 μl) or ghost-derived liposomes (100 μl) were incubated with acetone-washed pigment (570 μg protein) in 1 ml 20 mM Hepes, pH 6.5, containing 140 mM NaCl in presence of 100 μM to 1 mM H_2O_2, respectively, with or without 25 μm of the iron chelator deferoxamine (deferoxamine mesylate; Sigma Chemical Co.) for 1 h at 37°C. The reaction was stopped by adding 1 ml ice-cold 13% TCA in 1 N HCl (vol/vol). After centrifugation (20,000 *g*, 5 min, 4°C), 1 ml of supernatant was used for the determination of MDA production (25). Lipoperoxides formed in monocytes after phagocytosis of pigment were quantified by a cytochrome P-450–catalyzed, luminol-dependent luminescence production (26). Approximately 25,000 monocytes were solubilized in 0.5 ml solubilizing solution (0.1 N NaOH, 0.025% Triton X-100, 3 mM EDTA). Lipids were extracted from solubilized material with 1.0 ml chloroform/methanol (1:2; vol/vol) for 30 min at 25°C. 10 μl of the lower chloroform phase was pipetted into a lumimeter cuvette and the luminescence was recorded in a Magic Lite Analyzer (Ciba Corning, Medfield, MA) 2 s after injection of 300 μl solution 1 (0.3 μg cytochrome P-450/ml and 3 mM EDTA in 0.1 N NaOH), and 300 μl solution 2 (10 μg luminol/ml and 3 mM EDTA in 0.1 N NaOH).

Removal of Iron from Pigment. Acetone-washed pigment (500 μg protein/100 μl distilled water) with a total iron content of 109 nmol, calculated from heme content in solubilized pigment measured by the luminescence method described earlier, was added to 2.9 ml Hepes-buffered saline (NaCl 140 mM, 20 mM Hepes, pH 5.5) containing 1 mM bathophenanthroline (4,7-diphenyl-1,10-phenanthroline-disulfonic acid; Sigma Chemical Co.) supplemented with different concentrations of H_2O_2. The presence of bathophenanthroline was found necessary to react with iron upon its liberation from the pigment. This protocol yielded highly reproducible results, probably because iron binding or precipitation were inconsequential once the iron had reacted with the reagent. This treatment was continued for 2 d at 37°C with frequent medium changes and sonication of pigment for 30 s at these times. Iron was determined by measuring the iron-bathophenanthroline complex at 530 nm (27). OD values were corrected for trace amounts of liberated hemoglobin/heme, measured in parallel at the same wavelength in absence of bathophenanthroline. This correction was never >0.05% of the total heme present in the system.

Results

Heme Degradation in Phagocytosed RBC and Pigment. Serum-opsonized PRBC are avidly phagocytosed by adherent monocytes. The extent of phagocytosis depends on the maturation stage of parasites, as previously reported (28). Approximately 10 TPRBC and 4 RIRBC were phagocytosed per monocyte after 2 h (Fig. 1). Digestion of PRBC was compared to two different positive controls: diamide-treated RBC, a model of oxidatively stressed cells recognized and removed via an IgG-complement mediated mechanism (29), and anti-D IgG-opsonized RBC, a model of phagocytosis exclusively mediated by IgG (30). Heme degradation in both controls was rapid (macrophages possess the enzymatic machinery for heme degradation [31]), obeying a double-exponential decay kinetics (Table 1). The majority (>90%) of the ingested RBC heme was degraded with a $t_{1/2}$ of <0.5 h, while the rest was degraded much more slowly with a $t_{1/2}$ of >10 h. This was in contrast to the digestion of RPRBC, where only 26% of the ingested heme was rapidly degraded with a $t_{1/2}$ similar to that of control cells. This could be explained if the ring stage of the parasite contains pigment in small amounts that can not be detected morphologically. This trend was further emphasized with TPRBC: 21% of the ingested heme, possibly originating from host-cell hemoglobin, was degraded with a $t_{1/2}$ of 1 h, and the rest was eliminated extremely slowly. Ingested pigment was not digested at all during the 48 h of the assay (not shown). These alterations indicate that heme degradation in this case was considerably impaired.

Monocytes that had ingested TPRBC were viable, in as much as they displayed normal ATP levels and adhered nor-

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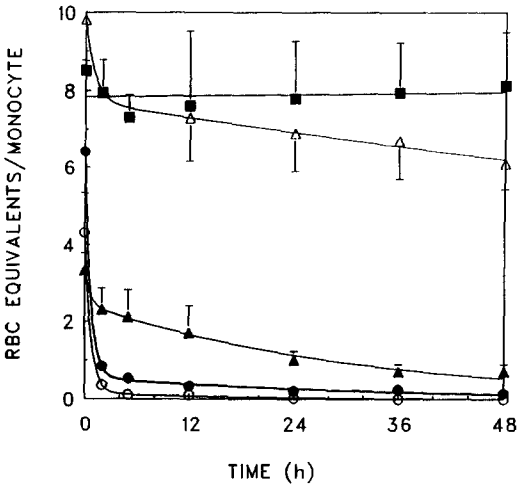

Figure 1. Extent of phagocytosis and kinetics of heme degradation in human monocytes fed with NPRBC, RPRBC, TPRBC, or malarial pigment. Anti-D IgG-opsonized RBC (●) or diamide-treated RBC (○) as nonparasitized controls were presented to the monocytes. RPRBC (▲), TPRBC (△), pigment (■), and diamide-treated RBC were opsonized with fresh serum. 5 × 10^4 adherent monocytes were challenged with 1.5 × 10^7 RBC or an equivalent amount of malarial pigment for 2 h. Thereafter, noningested material was removed by osmotic shock and washings. The experiment was started (time 0) at the end of the 2-h phagocytosis period. At different times, monocytes were lysed and phagocytosis was measured by the assay of heme-derived luminescence. Luminescence data were transformed in RBC or RBC equivalents (in case of pigment) ingested per monocyte. For details, see Materials and Methods. The curves represent the best fit to the data obtained from four independent experiments to a double-exponential decay. Means ± SD are shown.

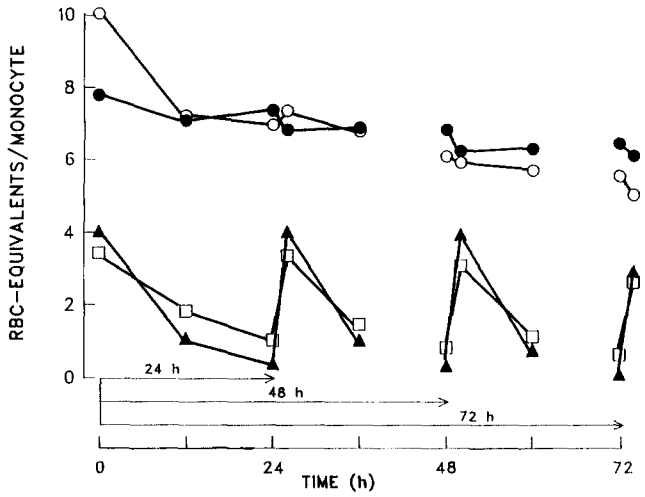

Figure 2. Phagocytic activity of human adherent monocytes after a previous challenge with parasitized or nonparasitized oxidatively damaged RBC, or pigment. 5 × 10^4 monocytes were challenged with 1.5 × 10^7 opsonized RBC (TPRBC [○[; RPRBC [□]; or diamide-treated RBC [▲]) or an equivalent amount of opsonized pigment (●). After 2 h of phagocytosis, noningested RBC or pigment were removed by washings and osmotic lysis, and the base-line heme content (in RBC equivalents) was measured (time 0). After 24, 48, and 72 h of further incubation, a second phagocytic cycle was performed with 1.5 × 10^7 opsonized, diamide-treated RBC. After 2 h, ingested RBC were quantified as described in Materials and Methods. Residual heme from the first challenge, as well as heme remaining in wells after washing and lysis of opsonized control RBC, was measured in parallel wells. Each point represents mean values of four replicas of one typical experiment.

mally for up to 72 h to the plastic dishes, as indicated by the constant DNA content in wells (not shown).

Impairment of Phagocytic Activity after Ingestion of Pigment-containing Material. Digestion of phagocytosed diamide- or anti-D IgG-treated RBC is followed by full recovery of the phagocytic ability. This was tested by performing a second phagocytic cycle 24, 48, or 72 h after the first one. Since monocytes fed with diamide- or anti-D IgG-treated RBC behave identically (not shown), only diamide-treated RBC were used as phagocytic challenge. As shown in Fig. 2, monocytes were fully responsive to a second phagocytic challenge. RPRBC were phagocytosed to the same extent as diamide-treated RBC and did not impair the ability of monocytes to perform a second cycle at 24, 48, and 72 h after the first one. By con-

Table 1. *Kinetics of Heme Degradation in Monocytes Fed with Anti-D Ig-opsonized RBC, Diamide-treated RBC, RPRBC, or TPRBC*

Cell treatment	Fraction 1	k_1	$t_{1/2}$	Fraction 2	k_2	$t_{1/2}$
Anti-D	5.85 ± 0.1 (91.4)	1.45 ± 0.14	0.48	0.55 ± 0.08 (8.6)	0.031 ± 0.008	22.35
Diamide	4.14 ± 0.05 (96.3)	1.47 ± 0.09	0.47	0.16 ± 0.04 (3.7)	0.067 ± 0.027	10.34
RPRBC	0.863 (26.1)	1.63	0.43	2.44 (73.9)	0.032	21.7
TPRBC	2.09 ± 0.14 (21.3)	0.94 ± 0.2	0.73	7.71 ± 0.1 (78.7)	0.0045 ± 0.0005	154

Data of Fig. 1 were fit to a double-exponential decay equation by nonlinear least square analysis. Values of fraction are expressed as RBC per monocyte; k values are given as hour $^{-1}$, and $t_{1/2}$ values as hours. Values in parentheses are the percentage of each fraction of total ingested RBC per monocyte.

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trast, monocytes fed with either TPRBC or pigment were totally unable to respond to a second phagocytic challenge. In all cases, the second phagocytic cycle was performed with the same number of diamide-treated RBC as in the first cycle.

Impairment of Oxidative Burst. Phagocytosis of diamide-treated RBC by adherent monocytes is associated with the generation of low amounts of luminol-elicited luminescence. When fed with pigment, suspended lymphomonocytes produce stronger and long-lasting luminescence, indicative of oxidative burst (Fig. 3). The capacity of monocytes to generate oxidative burst after PMA stimulation declined sharply after feeding with diamide-treated cells or pigment. PMA (100 nM) added to those monocytes 10 min after the beginning of the phagocytosis period triggered oxidative burst. As shown in Fig. 4, oxidative burst declined progressively until 240 min after beginning of phagocytosis irrespective of which phagocytic challenge was used. Thereafter, the ability to respond to PMA increases in monocytes challenged with diamide-treated RBC, whereas it declines further in monocytes challenged with TPRBC or with pigment, reaching very low levels after 24 h. The delayed decline in oxidant burst output observed in monocytes that had ingested TPRBC can be explained by taking into account the slow sedimentation of those low-density cells once added to the wells containing the adherent monocytes.

Lipid Peroxidation Induced by Pigment. Due to the large amount of iron in pigment (32) and its release (see below) in conjunction with oxidative burst, the peroxidation of membrane lipids has been investigated. While diamide-treated RBC caused only a temporary increase in lipid peroxides, their level increased sixfold up to 48 h in the pigment-fed monocytes (Table 2).

The peroxidizing action of pigment in model membrane systems, as measured by MDA production, was confirmed in RBC ghosts and liposomes derived thereof incubated with isolated pigment (Table 3). H_2O_2 was necessary to induce lipoperoxidation by pigment at acidic pH, indicating that iron could possibly be released from pigment. The inhibitory effect of deferioxamine on this process indicates that iron may be liberated from the pigment, although the possibility that the chelator acts as a scavenger of oxidative radicals (33, 34) can not be excluded.

Iron Release by Pigment. Experiments were performed to assess whether iron release from ingested pigment may occur in the macrophage phagolysosome. The amount and rate of iron detachment from pigment was found to be dependent

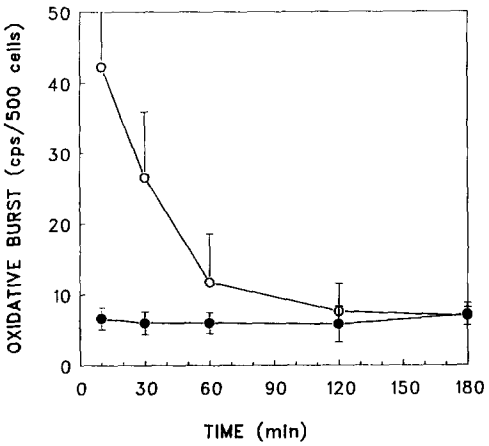

Figure 3. Initial oxidative burst induced by pigment presented to monocytes. Suspended monocytes were fed with pigment (●) at time 0, briefly spun down, resuspended, and stored at 37°C in a CO_2 incubator. About 2.5×10^5 cells were pipetted at different times in the test tube and analyzed for luminescence in presence of luminol. For details see Materials and Methods. Control monocytes without pigment (○) were treated similarly. Data shown are means ±SD of eight experiments.

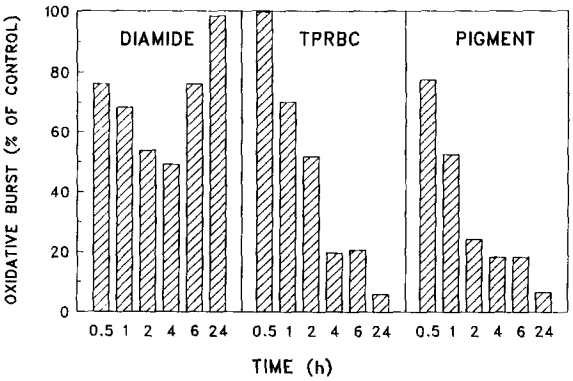

Figure 4. Irreversible inhibition of PMA-elicited oxidative burst activity in monocytes fed with malarial pigment or pigment-containing TPRBC. 2.5×10^4 adherent monocytes/coverslip were fed with 7.5×10^6 diamide-treated RBC (*left*), with 7.5×10^6 TPRBC (*middle*), or with equivalent amounts of pigment (*right*). The coverslips were placed into the luminescence cuvette at different times after the phagocytic challenge. After measuring the basal luminescence in the presence of 10 μM luminol, oxidative burst was elicited by addition 100 nM PMA. Luminescence peaking after 2.5 min was recorded and corrected for the corresponding basal luminescence. Results are expressed as percentage of PMA-elicited burst in control monocytes. Panels represent results of one typical out of four separate phagocytosis experiments.

Table 2. *Time Course of Lipoperoxide Formation in Membrane Lipids Extracted from Human Monocytes Fed with Malarial Pigment or Diamide-treated RBC*

Time after challenge	Challenge		
	None	Pigment	Diamide-treated RBC
h			
2	30,430	67,831	31,704
12	26,715	148,731	54,862
24	36,710	137,700	38,100
48	31,804	157,815	31,032

Lipoperoxides formed in macrophages after phagocytosis are given as cps/1,000 monocytes, measured by luminescence generation in monocyte lipid extracts (see Materials and Methods). Data of one typical experiment out of three with similar results are shown.

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Table 3. *MDA Formation in RBC Ghosts and Ghost-derived Liposomes Incubated with Pigment and H_2O_2, and Its Inhibition by Deferoxamine (DFX)*

Ghosts	Liposomes	Pigment	H_2O_2	DFX	OD
			mM		
+	−	+	0	−	0.004
+	−	+	0.1	−	0.023
+	−	+	0.1	+	0.014
−	+	+	0	−	0.001
−	+	+	1	−	0.058
−	+	+	1	+	0.016

After incubation of ghosts or ghost-derived liposomes originating from 100 μl packed RBC with malarial pigment, in the presence or absence of H_2O_2 or 25 μM DFX, formation of MDA was quantified by a standard method (see Materials and Methods). Data are presented as OD of a typical experiment out of three with similar results.

(at pH 5.5) on the H_2O_2 concentration, although small amounts were released also in the absence of this agent. The process of iron release is rather fast and follows a first-order kinetics (Fig. 5) with a $t_{1/2}$ decreasing from 2.6 to 1.5 h with increasing H_2O_2 concentration. The reason for this phenomenon is not well understood. The possibility that H_2O_2 is reduced by the pigment can be ruled out, because H_2O_2 was supplemented at each sampling time. Traces of iron continued to be detached at 0.1 and 1 mM H_2O_2 for as long

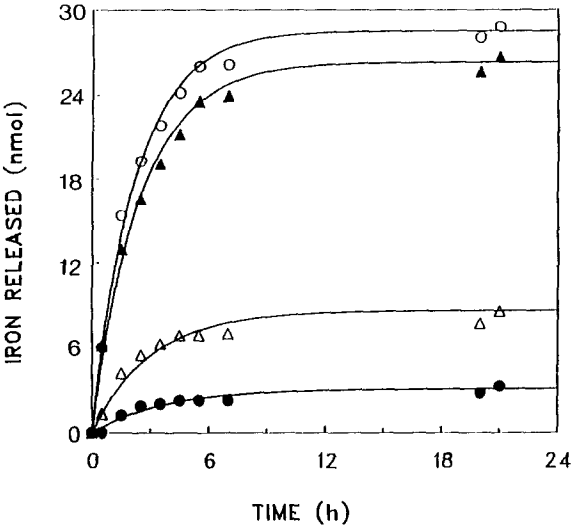

Figure 5. Kinetics of iron release from malarial pigment at different H_2O_2 concentrations. Purified pigment (109 nmol iron/500 μg protein) was incubated with 0.01 mM (△), 0.1 mM (▲), 1.0 mM. (○) H_2O_2 or without H_2O_2 (●) in 140 mM NaCl/20 mM Hepes, pH 5.5, for various periods of time at 37°C. The incubation solutions containing or not fresh H_2O_2 were changed at each sampling time. Free iron was determined by the bathophenanthroline method. The lines connecting the points for each H_2O_2 concentration were obtained by fitting the data to a first order equation by nonlinear least square analysis.

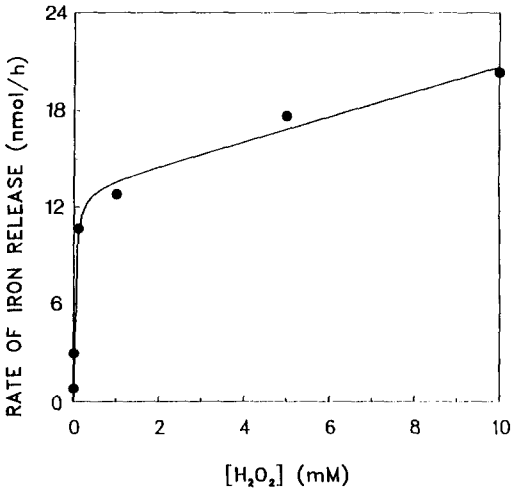

Figure 6. H_2O_2 concentration–dependent release of iron from pigment. Purified pigment (500 μg protein, 109 nmol total iron/assay) was incubated with or without different concentrations of H_2O_2 at pH 5.5, 37°C, and iron release was monitored. The initial rates of iron release (1 h) are plotted against H_2O_2 concentration and fitted to an equation that has one hyperbolic and one linear component by nonlinear least square analysis. Data obtained by this analysis are for the first component: maximal rate = 13.14 ± 0.81 nmol iron released/h and apparent Km = 0.0295 ± 0.0085 mM H_2O_2. The rate constant of the linear component is $0.75 \pm 1.2 \times 10^{-6}$/h.

as 2 d (not shown). As depicted in Fig. 6, as low as 10 μM H_2O_2 for 60 min released 3% of total pigment iron, whereas 10 mM liberated about six times as much.

Burst studies performed on monocytes that had ingested iron-stripped pigment (Fig. 7) indicate that the removal of the labile iron fraction from the pigment results in lesser inhibition of oxidative burst. This result validates the biological significance of the experiments performed in vitro. Despite the harsh iron stripping during a 2-d treatment with 1 mM H_2O_2, additional iron presumably could still be liberated in the phagolysosome as evidenced by the inhibition of PMA-elicited oxidative burst.

Discussion

The cell-mediated response in nonimmune malaria patients involves circulating and tissue-resident phagocytes (2–6). The phagocytic system has the capacity to control parasitemia or to annihilate it altogether. Phagocytes recognize and phagocytose infected RBC (15, 28); circulating and resident phagocytes in infected persons are seen filled with infected cells and pigment particles (5, 6); and the phagocytic system has the potential to dispose of up to 40–80% of the total RBC mass in few days (16). Despite these favorable conditions, frequently, malaria cannot be controlled and death occurs, the final cause being acute anemia or cerebral malaria (1, 35). The inability to control infection may be due to the incapacitation of the macrophages as evidenced in human (9, 10), rodent (11, 12), and simian (13) malarias.

In the present study we show that human monocytes fed with TPRBC (which contain large amounts of pigment) or

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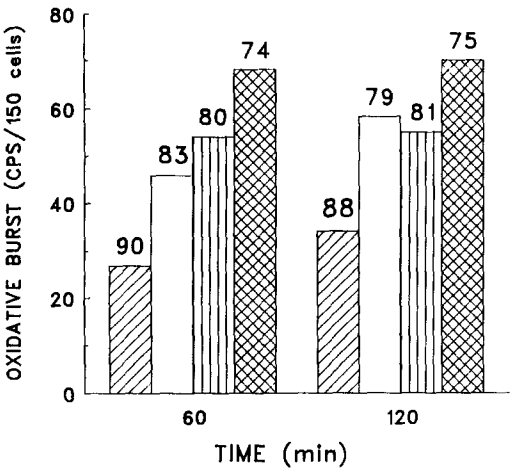

Figure 7. Effect of partial iron removal on pigment toxicity to PMA-elicited oxidative burst in monocytes. Suspended monocytes were challenged with partially iron-stripped pigment (35 μg pigment protein/1.5 $\times$ 10^5 lympho-monocytes). Iron removal was performed by treating acetone-washed pigment at pH 5.5 (▨), in the presence of 0.01 mM H_2O_2 (□), 0.1 mM H_2O_2 (▥), or 1.0 mM H_2O_2 (▩) for up to 48 h with frequent changes of H_2O_2 containing incubation solution. 1 and 2 h after challenge, 1.5 $\times$ 10^5 cells were pipetted in the luminescence cuvette, oxidative burst was elicited by 100 nM PMA, and quantified by measuring luminol-dependent luminescence. Numbers above columns indicate percent inhibition of PMA-elicited oxidative burst activity in pigment-fed cells compared with control lympho-monocytes. One typical experiment out of three with similar results is shown.

with isolated pigment are virtually unable to degrade the ingested pigment heme and to repeat the phagocytic cycle. Monocytes fed with positive controls, represented by diamide-treated, oxidatively stressed RBC, RPRBC (which contain small amounts of pigment), or anti-D IgG-opsonized RBC rapidly degrade the ingested hemoglobin-associated heme, and can repeat the phagocytic cycle. We further show that monocytes fed with TPRBC produce a long-lasting oxidative burst, which is absent when monocytes phagocytose positive controls. PMA-elicited oxidative burst, however, was irreversibly suppressed in monocytes fed with TPRBC, but was only transiently reduced when monocytes were fed with positive controls.

Membrane lipoperoxides increased up to sixfold, and for long periods of time, in monocytes fed with TPRBC, and increased only little and transiently in monocytes fed with the positive controls. Taken together, these data suggest that some essential functions of monocytes, like the ability to digest endocytosed material, to repeat the phagocytic process, and to produce PMA-elicited oxidant burst, are permanently incapacitated. Other monocyte functions apparently remained intact: monocytes fed with pigment were viable, had normal ATP levels, adhered for up to 72 h to the plastic dishes in culture, and had normal protein synthesis (E. Schwarzer, unpublished observation). The increased levels of lipid peroxides in the membranes, and the increased spontaneously generated oxidative burst, point to the presence of an oxidant species generator within the TPRBC-fed monocyte. This generator is evidently represented by ingested pigment, since all described toxic effects were identical in monocytes fed either with pigment-containing TPRBC or with isolated pigment. Malarial pigment (hemozoin) is a paracrystalline, highly compact polymeric hemoprotein, derived from host RBC hemoglobin (19, 32, 36, 37). Hemozoin heme groups are linked in high molecular weight complexes by covalent bonds established between the ferric iron of one monomer and the carboxylate side-chain of another (37) by the action of a recently discovered heme polymerase (38). We have shown here that the number of positive controls ingested during the 2-h phagocytosis time was about half that of TPRBC or isolated pigment, expressed as heme equivalents. This figure probably underestimates the total number of ingested positive controls, due to the rapidity of degradation for hemoglobin-associated heme within the monocyte. The question then arises as to why pigment should be toxic and hemoglobin-associated heme not, assuming that both are ingested in comparable amounts, and, second, why pigment should be toxic to the monocyte and not to the parasite. Macrophages are able to degrade heme of ingested oxidatively damaged RBC (31). A possible reason for the toxicity of pigment may reside in the covalent bond that crosslinks iron with carboxylate side-chains (37) and confers stability and indigestibility on the polymer in both the parasite food vacuole and the monocyte phagolysosome. A likely toxic component of pigment is iron, which can be liberated upon conditions simulating the phagolysosome or the food vacuole. While the pH of both organelles is known, the local concentrations of H_2O_2 are not (39, 40), although preliminary investigations revealed millimolar concentrations of H_2O_2 in the parasite and parasitized RBC (I.Z. Atamna and H. Ginsburg, unpublished observation). Hence, the concentrations of H_2O_2 used here were based on reasonable guesses. When pigment was incubated at pH 5.5 in the presence of increasing concentrations of H_2O_2, free iron was liberated in a biphasic dose-dependent manner, with a hyperbolic component having an apparent Michaelis-Menten constant (Km) of 30 μM and a linear component having a rate constant of 0.25 $\times$ 10^{-6}/h. However, not more than 3% of the total iron content could be released, suggesting that only the superficial pigment heme is susceptible to H_2O_2 attack. This extent of iron liberation would result in $\sim$120 μM free iron in the monocyte. Cells known to contain such concentrations of free iron were shown to be much more susceptible to peroxidative damage with H_2O_2 (41, 42). It is well established that small amounts of free iron in presence of superoxide anions or H_2O_2 can start a Haber-Weiss type of reaction, leading to the production of supra-stoichiometric amounts of hydroxyl radicals and singlet oxygen (43, 44). Such oxidative insults could surpass the antioxidant defense system of the monocyte, leading to lipid peroxidation and functional impairment.

If iron released from pigment is the causative agent of monocyte inhibition, then cells fed with pigment after the removal of labile iron should be less intoxicated. This expectation was met, in as much as pigment stripped of iron by treatment at pH 5.5 and low H_2O_2 concentration was much less inhibitory to oxidative burst production (Fig. 7). It is therefore plausible that the combined effect of free iron and H_2O_2 intoxicates monocytes.

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What then is the explanation for the selective iron toxicity against the monocyte and the relative insensitivity of the parasitized RBC? A possible explanation may reside in the different lipid composition with lower amounts of unsaturated fatty acids present in parasitized RBC. According to recent data (45), both parasite and host RBC membranes have a substantially lower saturation index, due to low arachidonic and doxohexanoic acid levels. It remains, however, to be shown that peroxidation of the monocyte membrane phospholipids can account for the defective functions.

Impairment of phagocyte activity by pigment may occur also in tissue resident macrophages, since upon liberation of the merozoites from schizonts sequestered in the deep vasculature, residual bodies containing pigment are released into the blood stream. Free floating residual bodies may reach the spleen and other organs (splenic macrophages are indeed inhibited with the progression of infection [10]). Moreover, unpublished data from our group (P.L. Fiori, P. Rappelli, S.N. Mirkarini, F. Turrini, P. Cappuccinelli, and H. Ginsburg, manuscript submitted for publication) show that human monocytes previously fed with TPRBC are unable to neutralize pathogenic bacteria, fungi, and tumor cells. Pigment production, whose main goal is to protect the parasite from heme generated during the digestion of host cell cytosol, thus provides fringe benefits to the parasite in suppressing the immune response.

Possibly, human evolution has coped with this situation. Mutations that confer resistance against falciparum malaria are all characterized by increased susceptibility to oxidants (46, 47). Since the parasite itself produces an oxidative stress on its host red cell (48), it is likely that mutant cells harboring parasites will be phagocytosed already at the early developmental stage of the parasite, i.e., when they do not contain enough pigment and are unable to incapacitate the macrophage. Preliminary results obtained in our laboratory show that RPRBC of thalassemic and glucose-6-phosphate dehydrogenase–deficient (Mediterranean variant) individuals are much more avidly phagocytosed than their normal counterparts.

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