Biochem. J. (1988) 249, 63–68 (Printed in Great Britain)

Detection of short-chain carbonyl products of lipid peroxidation from malaria-parasite (*Plasmodium vinckei*)-infected red blood cells exposed to oxidative stress

Gary D. BUFFINTON,*‡ Nicholas H. HUNT,*§ William B. COWDEN* and Ian A. CLARK†
Departments of *Experimental Pathology, John Curtin School of Medical Research and †Zoology, The Faculties, Australian National University, Canberra, A.C.T. 2601, Australia

Reversed-phase h.p.l.c. was used to detect 2,4-dinitrophenylhydrazine-reactive carbonyl products, which excludes malonaldehyde, in malaria-parasite (*Plasmodium vinckei*)-infected murine red blood cells (RBCs). A number of alkanals, 4-hydroxyalk-2-enals and alka-2,4-dienals were tentatively identified by comparison with authentic standards. The formation of 4-hydroxynon-2-enal, deca-2,4-dienal and hexanal was greater in *P. vinckei*-infected RBCs than in their uninfected counterparts and was increased by the presence of *t*-butyl hydroperoxide. Several of these aldehydes have previously been shown to be toxic to various types of cells, including *P. falciparum*, *in vitro*. The iron chelator desferrioxamine and the free-radical scavenger butylated hydroxyanisole inhibited the formation of these aldehydes. These experiments demonstrate that products of lipid peroxidation other than malonaldehyde are formed during the exposure of malaria-infected RBCs *in vitro* to drugs that generate reactive oxygen species and have anti-parasitic activity. The formation of products of this type during the natural course of malaria infection may have implications for the mechanisms underlying intra-RBC parasite death and the tissue damage associated with the disease.

INTRODUCTION

Traditionally, immunity to malaria has been considered to be mediated by either antibody or phagocytosis, but, more recently, serious consideration has been given to the role of soluble mediators secreted by phagocytic cells during the host response to the infection (Clark *et al.*, 1981, 1986*a*, 1987). Host macrophages release a range of mediators, including interleukin-1, tumor necrosis factor (cachectin) and reactive oxygen species (ROS) (reviewed in Clark *et al.*, 1987), which are thought to contribute to tissue damage and the appearance of degenerate intra-RBC (red blood cell) parasites ('crisis forms') during the natural course of infection (Clark *et al.*, 1981; Clark & Hunt, 1983).

Parasite crisis forms can be artificially induced by injection of oxidative drugs such as alloxan and H_2O_2 (Clark & Hunt, 1983), t-butyl hydroperoxide (t-BHP, Clark *et al.*, 1983, 1984*b*) and divicine (Clark *et al.*, 1984*a*) into malaria-parasite (*Plasmodium vinckei*)-infected mice. All these agents generate ROS and their effects may mimic the lethal oxidative stress imposed on intra-RBC parasites by stimulated phagocytes (Clark & Hunt, 1983). Exposure of *P. vinckei*-infected RBCs to these ROS-generating agents *in vitro* causes the generation of 5–10 times more malonaldehyde (MA) than in similarly treated control RBCs (Clark *et al.*, 1984*a*,*b*; N. H. Hunt, unpublished work). This phenomenon was also seen *in vivo*, for either washed RBCs or whole blood from *P. vinckei*-infected mice injected with curative doses of *t*-BHP contained significant amounts of MA, whereas none was detected in similarly treated control mice (Buffinton *et al.*, 1986; N. H. Hunt & G. D. Buffinton, unpublished work). Preinjection of infected mice with the iron chelator desferrioxamine (DF) totally inhibited MA formation *in vivo* and *in vitro*. DF and the resonance-stabilized free-radical scavenger butylated hydroxyanisole (BHA) prevent oxidative parasite killing (Clark *et al.*, 1984*a*,*b*).

In addition to MA, lipid oxidation also leads to the formation of a wide range of short-chain carbonyl species, which have been detected in peroxidizing liver microsomes (microsomal fractions) (Esterbauer *et al.*, 1982), carbon tetrachloride-treated hepatocytes (Poli *et al.*, 1985) and β-thalassaemic RBCs treated with t-BHP (Ramenghi *et al.*, 1985). These have been characterized into several major classes, including alkanals, alk-2-enals, 4-hydroxyalk-2-enals, alka-2,4-dienals, alkanones and several minor species. Many of these carbonyls, in particular the 4-hydroxyalk-2-enals, are extremely reactive toward biomolecules containing exposed thiol or ϵ-amino groups (Esterbauer *et al.*, 1975, 1976; Schauenstein & Esterbauer, 1979) and can inhibit the synthesis of DNA, RNA and protein and the activities of various enzymes, and modify macromolecules (reviewed by Esterbauer, 1985).

Since lipid peroxidation is involved in oxidative killing of malarial parasites, we have investigated the formation of long-lived short-chain carbonyl species during this process.

Abbreviations used: ROS, reactive oxygen species; t-BHP, t-butyl hydroperoxide; RBC(s), red blood cell(s); MA, malonaldehyde; DF, desferrioxamine B (Desferal); BHA, butylated hydroxyanisole [2(3)-t-butyl-4-hydroxyanisole]; 2,4-DNPHz(i/o)ne, 2,4-diphenylhydraz(i/o)ne; 4-HNE, 4-hydroxynon-2-enal; 4-HOE, 4-hydroxyoct-2-enal.

‡ Present address: Department of Pathology II, University Hospital, University of Linköping, Linköping S-58185, Sweden.
§ To whom correspondence and reprint requests should be sent.

Table 1. H.p.l.c. linear gradients

Time (min)	(Change in) solvent [methanol/water (%, v/v)] ratio
0→10	3:1→4:1
10→25	4:1→1:0
25→45	1:0

METHODS

Parasite/animals

Plasmodium vinckei subspecies *vinckei* (strain V52, originally from Dr. F. E. G. Cox, King's College, London, U.K.) was maintained by intraperitoneal syringe passage in CBA/CaH mice (6–8 weeks old). The course of infection was monitored by tail-vein-blood smears stained with either Giemsa or Harleco's Diff Quik stain.

Chemicals/equipment

Standard n-alkanals, *trans*-alk-2-enals, *trans,trans*-alka-2,4-dienals and alkan-2-ones were from EGA Chemie, Steinheim, Germany. Authentic 4-hydroxy-*trans*-alk-2-enal standards (C_6–C_{11}) were generously given by Dr. H. Esterbauer, University of Graz, Graz, Austria. BHA and t-BHP (purified by fractional distillation and stored at −40 °C) were from Sigma. DF was a gift from Ciba–Geigy, Sydney, Australia. SEP-PAK silica cartridges were from Waters Associates, Milford, MA, U.S.A. T.l.c. plates (silica-gel 60 F_{254}; pre-coated; 50 mm × 100 mm; 0.25 mm thickness) were from Merk, Darmstadt, Germany. H.p.l.c.-grade methanol was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Water for h.p.l.c. was purified by a Sybron/Barnstead NANOpure system.

All h.p.l.c. components were from Waters Associates, and consisted of a Waters 840 Data and Chromatography Control Station (incorporated into a Digital Electronics Corp. Professional 350 series computer), two 510 series h.p.l.c. pumps, a WISP 710B auto sampler (200 μl sample loop), a 490 programmable multi-wavelength detector and the Systems Interlink Module. H.p.l.c. separations were performed by using a Waters NOVA-PAK C_{18} reversed-phase column (3.9 mm int. diam. × 150 mm; 4 μm particle size; end-capped; 7% carbon loading with 100000–120000 plates/metre) and a Waters precolumn C_{18} Guard-PAK module. The void volume (1.21 ml) was determined by using uracil.

All solvent programming was controlled by the Waters 840 module using two pumps. Samples were separated by using a series of linear gradients (see Table 1); the flow rate was 0.9 ml/min.

Data was collected at two to four data points/s on each of four wavelength channels (Waters 490 detector; Channel 1, 361 nm; Channel 2, 365 nm; Channel 3, 378 nm; Channel 4, 393 nm).

Preparation of 2,4-dinitrophenylhydrazone standards

Commercially available carbonyls (n-alkanals, alk-2-enals, alka-2,4-dienals and alkan-2-ones) were allowed to react with 2,4-DNPHzine as described by Vogel (1981), to form the respective 2,4-DNPHzone. 2,4-DNPHzones of authentic 4-hydroxyalk-2-enals were prepared by allowing the carbonyl to react with 1 ml of 2,4-DNPHzine reagent (equimolar ratio). The resultant hydrazone was extracted with 2 ml of chloroform, evaporated to dryness with N_2, redissolved in 250 μl of dichloromethane and applied to silica t.l.c. plates and developed with dichloromethane. The 2,4-DNPHzone band (revealed under u.v. light) was removed and extracted into chloroform. Extracts were evaporated to dryness with N_2 and dissolved in h.p.l.c.-grade methanol.

Preparation of 2,4-DNPHzones from biological samples

Biogenic aldehydes were derivatized by a modification of the method of Esterbauer *et al.* (1982). Control and *P. vinckei*-infected (~80% parasitaemia) RBCs were prepared as described previously (Clark *et al.*, 1984*a*). Suspensions (1 ml, containing 10^9 RBCs) were pre-incubated with DF (4 mM) or BHA (25 μM; dissolved in ethanol; final concn. < 1%) in phosphate-buffered saline (g/litre of water: NaCl, 8; KCl, 0.2; $Na_2HPO_4,2H_2O$, 1.15; $NaH_2PO_4,2H_2O$, 0.2; $CaCl_2$, 0.1; $MgCl_2$, 0.1), pH 7.2, containing 2 mM-NaN_3, pH 7.2, for 30 min at 37 °C. Incubation was started by addition of t-BHP (1 mM) and terminated at 15 min by addition of 1 ml of 2,4-DNPHzine reagent, vortex-mixed and incubated at 40 °C for 3 h in the dark. The concentration of t-BHP and incubation time were chosen after preliminary experiments which examined the effects of 0.1–10 mM-t-BHP over 0–120 min. Curative doses of t-BHP (Clark *et al.*, 1984*b*) achieve millimolar concentrations in blood *in vivo*. 2,4-DNPHzones were extracted into chloroform (2 ml), vortex-mixed and centrifuged (200 *g*, 10 min). The organic (lower) phase was removed to a fresh tube, dehydrated with 0.5 g of anhydrous Na_2SO_4, and transferred to an evaporation tube. The protein and aqueous phase was then twice re-extracted with chloroform (2 ml). The pooled, dehydrated (Na_2SO_4) extract was evaporated to dryness with N_2, dissolved in 400 μl of dichloromethane and applied to a pretreated wet SEP-PAK silica cartridge (pre-washed with 5 ml of methanol, followed by 5 ml of dichloromethane). Samples were eluted with 20 ml of dichloromethane. This step removed haem-containing contaminants. The eluent was evaporated to dryness with N_2 and the residue dissolved in h.p.l.c.-grade methanol to which filtered water was added to give a 4:1 (v/v) methanol/water solvent ratio (1 ml volume). A 50 μl aliquot was analysed by h.p.l.c. Biogenic 2,4-DNPHzone peaks were compared with the retention times and absorption maxima of known standard, 2,4-DNPHzones. Identified peaks were quantified by using known absorption coefficients for specific 2,4-DNPHzones (Esterbauer *et al.*, 1982).

RESULTS

The h.p.l.c. elution profiles of control and *P. vinckei*-infected RBCs were strikingly different (Fig. 1). Treatment of normal mouse RBCs with t-BHP (Fig. 1*b*) resulted in the formation of several products, notably 4-hydroxynon-2-enal (4-HNE) and hexanal. The magnitude of peaks 13 and 14 did not vary when compared with untreated controls (Fig. 1*a*). The two peaks eluted at 21.3 and 23.3 min were also observed in the blank extraction and therefore regarded as contaminants. Fig 1(*c*), the differential plot of (control + t-BHP) − (control + phosphate-buffered saline), shows there was little net increase (positive deflection) in carbonyl formation on the basis of equal cell density.

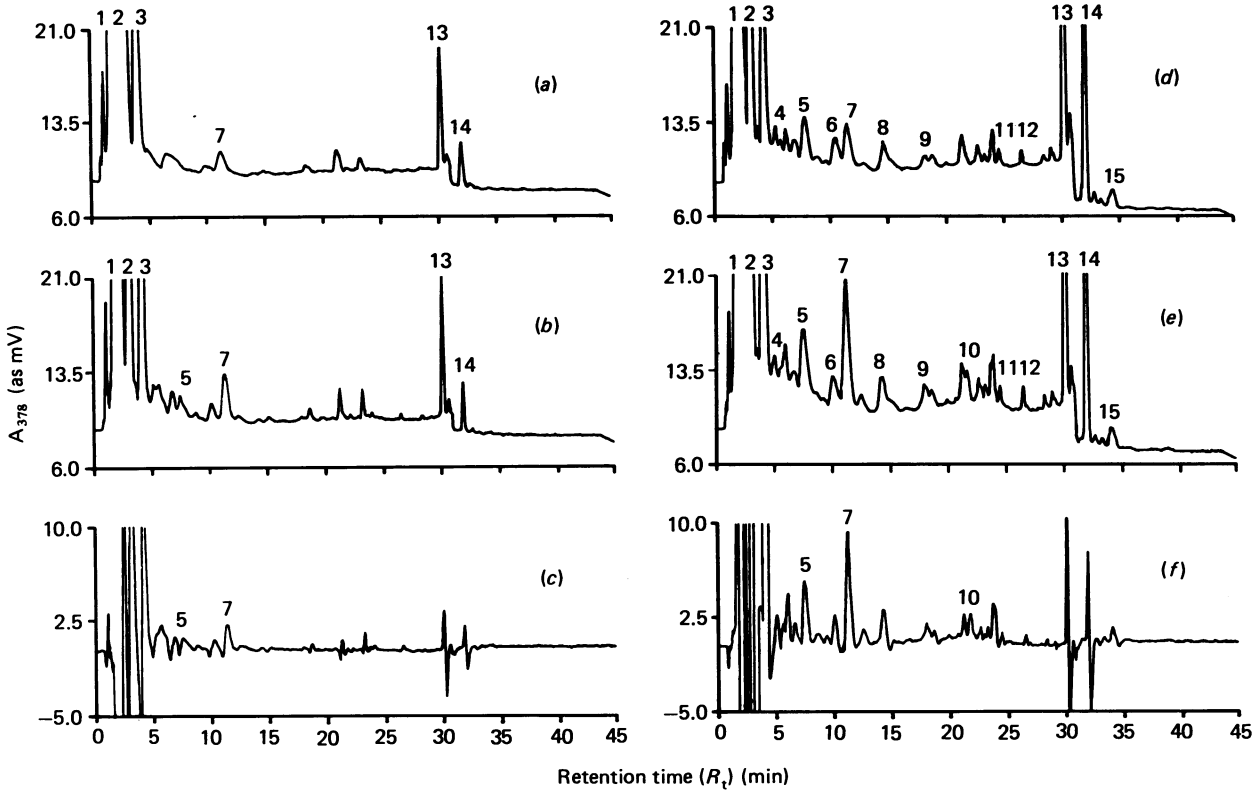

Fig. 1. H.p.l.c. chromatograms from control and *P. vinckei*-infected RBCs in the presence or absence of t-BHP

Experimental details are described in the Methods section. (*a*) Control RBCs+PBS; (*b*) control RBCs+t-BHP (1 mM); (*c*) differential plot [(*b*)–(*a*)]; (*d*) *P. vinckei*-infected RBCs+PBS; (*e*) *P. vinckei*-infected RBCs+t-BHP; (*f*) Differential plot [(*e*)–(*d*)]. Peak identification was by comparison with the retention times (R_t, in min) and absorption characteristics of known standards. Peak 1, R_t 1.89, unchanged 2,4-DNPHzine; 2, R_t 3.0, propanone; 3, R_t 4.2, propanal; 4, R_t 5.2, 4-hydroxyoctenal; 5, R_t 7.7, 4-hydroxynonenal; 6, R_t 10.5, 4-hydroxydecenal or hex-2-enal; 7, R_t 11.4, hexanal; 8, R_t 14.6, octa-2,4-dienal; 9, R_t 18.2, octanal; 10, R_t 21.7, deca-2,4-dienal; 11, R_t 24.6, undecanal; 12, R_t 26.6, dodecanal; 13, R_t 30.2, C_{16} plasmal; 14, R_t 32.1, C_{18} plasmal; 15, R_t 34.4, possibly C_{20} plasmal. Results are from a single experiment representative of several.

Without further treatment, incubation *in vitro* of RBCs from mice carrying a high intra-RBC *P. vinckei* load (Fig. 1*d*) led to the formation of many more carbonyl species than in comparatively treated control RBCs (Fig. 1*a*). The carbonyls identified included 4-hydroxyoct-2-enal (4-HOE), 4-HNE, hexanal, octanal, undecanal, dodecanal and the long-chain carbonyls, peaks 13, 14 and 15. The identity of peak 6 is unknown, the most likely candidates being 4-hydroxydec-2-enal or hex-2-enal. Peak 8 was designated as octa-2,4-dienal on the basis of its retention time and consistent absorption maxima at 393 nm, which is that of alka-2,4-dienal hydrazones.

Treatment of *P. vinckei*-infected RBCs with t-BHP (Fig. 1*e*) led to increased formation of several carbonyls and the appearance of peak 10 (deca-2,4-dienal), whereas others remained unchanged. Increases in 4-HNE and hexanal dominated the elution profile. The differential plot supports this and shows the resolution of deca-2,4-dienal (Fig. 1*f*). Formation of ozasone, described (Esterbauer *et al.*, 1982) as being eluted on the post-peak shoulder of 4-HNE, was not observed.

The identified 2,4-DNPHzones were quantified by using integrated peak area at the respective absorption maxima for each carbonyl class. t-BHP increased the levels of almost all of the carbonyl hydrazones detected (Table 1). Of particular interest were the increases in 4-HNE, hexanal and deca-2,4-dienal, which have been detected in several lipid-peroxidation systems. The inhibitory effects of the iron chelator DF and the free-radical scavenger BHA on t-BHP-initiated carbonyl formation varied, even between control and infected RBCs. Of the known lipid-peroxidation products, the formation of 4-HNE, hexanal and deca-2,4-dienal was diminished, in some cases to baseline levels, by DF or BHA or both (Table 2).

Peaks 13 and 14 are long-chain aldehydes or plasmals and acyl chain lengths of C_{16} and C_{18} respectively. From its retention time, peak 15 is estimated to be C_{20} (Fig. 1). The intensity of peak 13 decreased in both control and infected RBCs on addition of t-BHP, whereas the same treatment increased peak 14 in infected RBCs (Table 1). DF and BHA largely prevented these changes. The C_{16}/C_{18} ratio was 2.8:1 in untreated control RBCs and 7.6:1 in infected RBCs. Treatment with t-BHP decreased the ratio in infected RBCs to 2.8:1, whereas no change was observed in control RBCs. Both DF and BHA tended to prevent the decrease in C_{16}:C_{18} ratio.

The detected hydrazones are regrouped into their respective classes in Table 3. t-BHP treatment nearly doubled the formation of 2,4-DNPHzine-reactive carbonyls in both control and *P. vinckei*-infected RBCs.

Table 2. Quantification of 2,4-DNPHzine-reactive carbonyls from control and *P. vinckei*-infected RBCs: effect of t-BHP

RBCs were prepared and analysed as described in the Methods section. Concentrations of reactants were: t-BHP, 1 mM; DF, 4 mM; BHA, 25 μM. –, Not detected; 4-HDE, 4-hydroxy-2-enal; Tr, trace. Results shown are of a single experiment representative of several.

		Amount (nmol/10^9 RBCs)							
	RBCs ...	Malaria-infected				Control			
Carbonyl hydrazone derivative	Additions ...	None	t-BHP	DF + t-BHP	BHA + t-BHP	None	t-BHP	DF + t-BHP	BHA + t-BHP
Propanone		10.2	25.0	21.7	26.9	7.4	15.1	29.1	20.6
Propanal		11.2	33.8	31.6	24.0	10.0	18.7	27.3	10.9
4-HOE		1.0	1.4	0.3	1.1	—	—	1.0	0.3
4-HNE		1.3	2.3	1.3	1.1	—	0.6	0.6	0.2
4-HDE		0.4	0.5	0.4	1.1	—	—	—	0.7
Hexanal		1.0	4.0	2.5	1.4	0.2	0.9	0.6	0.4
Octa-2,4-dienal		0.5	0.6	0.6	0.7	—	Tr	Tr	Tr
Octanal		0.4	0.9	0.7	0.5	—	—	—	—
Deca-2,4-dienal		0	0.5	0.1	0.1	—	—	—	—
Undecanal		0.3	0.3	0.2	0.4		—	—	—
Dodecanal		0.2	0.3	0.2	0.2		—	—	—
Peak 13		15.9	13.7	15.2	14.4	2.5	2.1	2.2	2.0
Peak 14		2.1	4.8	3.3	4.3	0.9	0.7	0.7	0.8
Peak 15		0.5	0.8	0.1	0.3	—	—	—	—

Table 3. Distribution of the classes of carbonyl compounds from control and *P. vinckei*-infected RBCs

Total-carbonyl (columns 3 and 4) data are derived from Table 1 and expressed as nmol/10^9 RBCs. Columns 5 and 6 represent carbonyl classes expressed as percentages of the total carbonyls detected. Abbreviations: addn., addition; Tr, trace; OH-, hydroxy.

		Distribution			
		Total		Percentage	
RBCs	Carbonyl class	No addn.	t-BHP	No addn.	t-BHP
P. vinckei-infected	Alkanals	13.1	39.2	29.2	44.2
	4-OH-alk-2-enals	2.7	4.2	6.0	4.7
	Alka-2,4-dienals	0.5	1.2	1.2	1.3
	2-Alkanones	10.2	25.0	22.6	28.2
	Peaks 13, 14 and 15	18.5	19.2	41.0	21.6
	Total ...	45.0	88.8	100	100
Control	Alkanals	10.1	14.3	48.6	51.4
	4-OH-alk-2-enals	0	0.6	0	1.5
	Alka-2,4-dienals	0	Tr	0	Tr
	2-Alkanones	7.4	15.1	35.3	39.7
	Peaks 13 and 14	3.4	2.8	16.1	7.4
	Total ...	20.9	32.8	100	100

The relative amounts of each class varied considerably. Most interesting were the relatively high proportions of 4-hydroxyalk-2-enals and alka-2,4-dienals in infected RBCs, whereas in control RBCs they contributed significantly less to the detectable carbonyls.

In other experiments (Buffinton, 1986), control and *P. vinckei*-infected RBCs were exposed to H_2O_2 (1 mM) and analysed as described here. Broadly, H_2O_2 led to a smaller increase in carbonyl species than did t-BHP, and DF was more effective than BHA in preventing this carbonyl formation.

DISCUSSION

Reports of various aldehydic products from different systems indicate that 4-HNE, hexanal, deca-2,4-dienal and MA are among the most commonly detected. The changes in these products (Table 2) most closely parallel

the changes in parasite viability observed after administration of oxidative drugs (Clark & Hunt, 1983; Clark *et al.*, 1983, 1984*a*,*b*). Both DF and BHA, which block oxidative killing of *P. vinckei in vivo*, diminished the formation of several lipid-peroxidation products, including 4-HNE (Table 2). When we tested a wide range of short-chain (C_3–C_{11}) carbonyls (many of which have been detected in various lipid-peroxidation systems) against *P. falciparum in vitro*, only 4-hydroxyalk-2-enals and alka-2,4-dienals inhibited parasite growth (Clark *et al.*, 1986*b*). The mechanism of this inhibition might involve interactions with tubulin, or inhibition of DNA, RNA and protein synthesis (reviewed by Esterbauer, 1985). Intravenous injection of a mixture of aldehydes (including 4-HNE, deca-2,4-dienal and hexanal) into *P. vinckei*-infected mice led to the formation of intra-RBC parasite crisis forms (Buffinton, 1986). These crisis forms also occur when oxidative drugs are injected into *P. vinckei*-infected mice (Clark & Hunt, 1983; Clark *et al.*, 1984*a*,*b*) or when *P. falciparum* in culture is exposed to 4-hydroxyalk-2-enals and alka-2,4-dienals (Clark *et al.*, 1986*b*) and during the naturally resolving infection (Taliaferro & Taliaferro, 1944; Clark *et al.*, 1976).

Activated phagocytes secrete H_2O_2 when in intimate contact with infected erythrocytes (Ockenhouse & Shear, 1984; Ockenhouse *et al.*, 1984; Wozencraft *et al.*, 1985). This peroxide, and ROS derived from it, have the potential to initiate lipid peroxidation, thereby forming toxic aldehydes. MA from H_2O_2-stressed RBCs (Jain & Hochstein, 1980), hexanal, heptanal and deca-2,4-dienal (Beppu *et al.*, 1986) have been shown to cause cross-linking of RBC membrane proteins. Exposure of RBCs to MA leads to decreased deformability and survival *in vivo* (Jain *et al.*, 1983) and altered membrane phospholipid asymmetry (appearance of phosphotidylserine in the outer leaflet; Jain, 1984). The abnormal appearance of phosphatidylserine and increased levels of phosphatidylethanolamine in the outer-membrane leaflet occurs in non-infected and infected RBCs from *P. knowlesi*-infected monkeys (Gupta *et al.*, 1982) and in *P. falciparum*-infected human RBCs (Schwartz *et al.*, 1985*b*). *P. vinckei*-infected RBCs spontaneously generate significant levels of MDA (Clark *et al.*, 1984*a*,*b*; Stocker *et al.*, 1985), 4-HNE and hexanal (Fig. 1*d*; Table 1) *in vitro*, and these aldehydes could be involved in the aetiology of the altered phospholipid asymmetry of RBCs from malaria-infected hosts. By analogy with other reports (reviewed by Schwartz *et al.*, 1985*a*), these events would lead to an increase in the net negative surface charge, possibly contributing to disseminated intravascular coagulation (O'Leary *et al.*, 1972) and enhanced erythrophagocytosis (Greenwood *et al.*, 1978; Howard & Mitchell, 1979), both of which are well documented in malaria. Alternatively, erythrophagocytosis could result from recognition of phosphatidylserine by macrophages, as occurs in other systems (Schroit *et al.*, 1984).

Besides the direct actions of toxic aldehydes on various cell structures and intermediary-metabolic enzymes, indirect or long-range extracellular effects should also be considered. The 10-fold increase in reactivity of 4-HNE toward low-density lipoproteins compared with that of MA (Jürgens *et al.*, 1986) suggests that 4-HNE is a toxin which conceivably could be delivered by low-density lipoproteins to a wide range of cells in the infected host.

Long-chain aldehydes (plasmals) were substantially increased in infected RBC compared with controls, and there was also an elevated C_{16}:C_{18} ratio. These aldehydes originate from plasmalogens, which represent 10–30% of the total phospholipid pool and are normal constituents of cell membranes. Thus the observed changes may be related to the substantial increase in total lipids which occurs in the blood of *P. vinckei*-infected mice (Stocker *et al.*, 1987). The physiological ramifications of the increase in these long-chain aldehydes are not known.

In conclusion, biogenic cytotoxic aldehydes are formed when RBCs containing malaria parasites are exposed to oxidative stress. These compounds could damage parasites or host tissue near their site of formation and also at distant loci (reviewed in Buffinton *et al.*, 1986), contributing to the systemic tissue damage seen in malaria.

This work received support from the 'malaria' component of the United Nations Development Programme/World Bank/World Health Organisation Special Programme for Research and Training in Tropical Diseases and the Australian National Health and Medical Research Council. G.D.B. acknowledges the support of a Commonwealth Postgraduate Research Scholarshp. We also thank Professor H. Esterbauer for the gift of authentic 4-hydroxyalk-2-enal standards and for helpful advice, and Mrs. K. Rabl for expertly typing the manuscript.

REFERENCES

Beppu, M., Murakami, K. & Kikugawa, K. (1986) Chem. Pharm. Bull. **34**, 781–788

Buffinton, G. (1986) Ph.D. Thesis, Australian National University, Canberra

Buffinton, G. D., Hunt, N. H., Cowden, W. B. & Clark, I. A. (1986) in Free Radicals, Cell Damage and Disease (Rice-Evans, C., ed.), pp. 201–220, Richelieu Press, London

Clark, I. A. & Hunt, N. H. (1983) Infect. Immun. **39**, 1–6

Clark, I. A., Allison, A. C. & Cox, F. E. G. (1976) Nature (London) **259**, 309–311

Clark, I. A., Virelizier, J.-L., Carswell, E. A. & Wood, P. R. (1981) Infect. Immun. **32**, 1058–1066

Clark, I. A., Cowden, W. B. & Butcher, G. A. (1983) Lancet **i**, 234

Clark, I. A., Cowden, W. B., Hunt, N. H., Maxwell, L. E. & Mackie, E. J. (1984*a*) Br. J. Haematol. **57**, 479–487

Clark, I. A., Hunt, N. H., Cowden, W. B., Maxwell, L. E. & Mackie, E. J. (1984*b*) Clin. Exp. Immunol. **56**, 524–530

Clark, I. A., Hunt, N. H. & Cowden, W. B. (1986*a*) Adv. Parasitol. **25**, 1–44

Clark, I. A., Butcher, G. A., Buffinton, G. D., Hunt, N. H. & Cowden, W. B. (1986*b*) Biochem. Pharmacol. **36**, 543–546

Clark, I. A., Hunt, N. H. & Cowden, W. B. (1987) in Immune Responses in Parasitic Infections (Soulsby, E. J. L., ed.), vol. 4, pp. 1–34, CRC Press, Boca Raton, FL

Esterbauer, H. (1985) in Free Radicals in Liver Injury (Poli, G., Cheeseman, K. H., Dianzani, M. U. & Slater, T. F., eds), pp. 29–47, IRL Press, Oxford

Esterbauer, H., Zollner, H. & Scholz, N. (1975) Z. Naturforsch. C Biosci. **30**, 466–473

Esterbauer, H., Ertl, A. & Scholz, N. (1976) Tetrahedron **32**, 285–289

Esterbauer, H., Cheeseman, K. H., Dianzani, M. U., Poli, G. & Slater, T. F. (1982) Biochem. J. **208**, 129–140

Greenwood, B. M., Stratton, D., Williamson, W. A. & Mohammed, I. (1978) Trans. R. Soc. Trop. Med. Hyg. **72**, 378–385
Gupta, C. M., Alan, A., Mathur, P. N. & Dutta, G. P. (1982) Nature (London) **299**, 259–261
Howard, R. J. & Mitchell, G. F. (1979) Aust. J. Exp. Biol. Med. Sci. **57**, 455–457
Jain, S. K. (1984) J. Biol. Chem. **259**, 3391–3394
Jain, S. K. & Hochstein, P. (1980) Biochem. Biophys. Res. Commun. **92**, 247–254
Jain, S. K., Mohandas, N., Clark, M. J. & Shohet, S. B. (1983) Br. J. Haematol. **53**, 247–255
Jürgens, G., Lang, J. & Esterbauer, H. (1986) Biochim. Biophys. Acta **875**, 103–114
Ockenhouse, C. F. & Shear, H. L. (1984) J. Immunol. **132**, 424–431
Ockenhouse, C. F., Schulman, S. & Shear, H. L. (1984) J. Immunol. **133**, 1601–1608
O'Leary, D. S., Barr, C. F., Wellde, B. T. & Conrad, M. E. (1972) Am J. Trop. Med. Hyg. **21**, 282–287
Poli, G., Dianzani, M. U., Cheeseman, K. H., Slater, T. F., Lang, J. & Esterbauer, H. (1985) Biochem. J. **227**, 629–638
Ramenghi, V., Chiarpotto, E., Miniero, M., Piga, A., Cecchini, G., Biasi, F., Poli, G. & Esterbauer, H. (1985) IRCS Med. Sci. Libr. Compend. **13**, 273–274
Schauenstein, E. & Esterbauer, H. (1979) Ciba Found. Symp. **67**, 225–244
Schroit, A. J., Tanaka, Y., Masden, J. & Fidler, I. J. (1984) Biol. Cell. **51**, 227–238
Schwartz, R. S., Chiu, D. T.-Y. & Lubin, B. (1985*a*) Curr. Top. Hematol. **5**, 63–112
Schwartz, R. S., Olson, J. A., Raventos-Suarez, C., Yee, M., Lubin, B. & Nagel, R. L. (1985*b*) Clin. Res. **33**, 418A
Stocker, R., Hunt, N. H., Buffinton, G. D., Weidmann, M. J., Lewis-Hughes, P. & Clark, I. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 548–551
Stocker, R., Cowden, W., B., Tellam, R. L., Weidmann, M. J. & Hunt, N. H. (1987) Lipids **22**, 51–57
Taliaferro, W. H. & Taliaferro, L. G. (1944) J. Infect. Dis. **75**, 1–32
Vogel, A. (1981) Vogel's Textbook of Organic Chemistry, 4th edn., p. 1111
Wozencraft, A. O., Croft, S. L. & Sayers, G. (1985) Immunology **56**, 523–531

Received 23 February 1987/7 July 1987; accepted 3 September 1987