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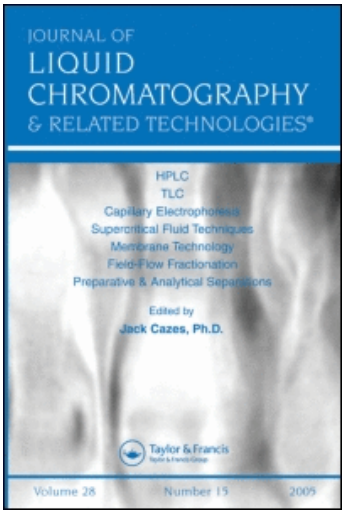

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Comparison of Methods Evaluating Lipoperoxidation in Plasma of Malaria Patients

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COMPARISON OF METHODS EVALUATING LIPOPEROXIDATION IN PLASMA OF MALARIA PATIENTS

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ABSTRACT

Lipid peroxidation is believed to be involved in malaria and plasma of malaria patients may be damaged by reactive oxygen species This study compares determinations of malondialdehyde (MDA), vitamins A and E by high performance liquid chromatography, thiobarbituric acid reactive substances (TBA-RS) by fluorometric analysis and fatty acids by gas chromatography. During malarial infection, polyunsaturated fatty acids (PUFA), vitamins A and E decreased significantly when TBA-RS increased significantly. However, there was no significant change in total or MDA bound to amino-groups. On the other hand, there was no correlation between

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TBA-RS production and PUFA decrease or between TBA-RS production and vitamin A or E consumption. These results support the concept that other compounds such as proteins and cholesterol may be involved in the formation of these TBA-RS.

INTRODUCTION

Lipid peroxidation (LPO) is believed to be intimately involved in the aetiology of a wide range of diseases including malaria (1). Highly reactive oxygen species (ROS) produced by host mononuclear phagocytes activated during the natural course of malaria can be released extracellularly and have been shown to kill intra-erythrocytic parasites (2-4) by imposing oxidant stress (5,6). It has been suggested that some aspects of severe malaria may result from exaggerated ROS effects on host tissues (3). So plasma in close contact with the host-parasite interaction might be susceptible to ROS damage and LPO.

It is widely assumed that LPO is triggered whenever conditions of increased oxidative stress and/or decreased anti-oxidant defenses occur in plasma or tissues. Therefore, the aim of the present work was to investigate techniques capable of analysing plasma LPO, including high performance liquid chromatography (HPLC) determination of malondialdehyde (MDA : LPO end product), of vitamins A and E (lipophilic antioxidants) and fluoro-

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metric determination of thiobarbituric acid reactive substances (TBA-RS : LPO breakdown products), and finally gas chromatographic (GC) determination of fatty acids (FA : LPO substrates). Results suggest that adequate assessment of LPO cannot be achieved by a single measurement of MDA or TBA-RS, and that knowledge of FA composition is required owing to the indirect and complex relationship between LPO and the TBA test.

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MATERIALS AND METHODS

Blood samples

Blood samples were obtained from 28 adult malaria patients (men and women, age range 19-59 years) infected with *Plasmodium falciparum* (23 patients), *P. vivax* (2 patients), or *P. ovale* (3 patients). Malaria was diagnosed on the basis of clinical symptoms and a parasite-positive blood determination. All patients were in an acute phase and had not received any curative treatment. Malaria patients living in Europe were chosen to avoid including individuals carrying genetically-determined abnormalities (sickle cell diseases, thalassemia, glucose-6 phosphate dehydrogenase deficiency) that increase oxidant stress on the red cell and its contents (7).

Blood was collected by veinipuncture into vacutainer tubes (Becton Dickinson, Grenoble, France) with heparin after an

overnight fast. Parasites were counted in blood and expressed as percentage of infested red blood cells (RBC).

Blood was also taken from 30 adult control subjects (men and women, age range 25-60 years). None of the controls was a heavy smoker, had a history of recent acute disease, or was taking drugs.

HPLC analyses

Free malondialdehyde of plasma was measured as previously described (8) after protein precipitation by 7 % $HClO_4$ and reaction of the clear supernatant with thiobarbituric acid (TBA) at 100°C for 30 min.

For total MDA analysis, plasma was incubated with NaOH at pH13 and 60°C for 30 min. The hydrolysed sample was then acidified to pH < 1 with $HClO_4$. After centrifugation, the clear supernatant was treated with TBA as for free MDA determination.

The difference between total MDA and free MDA determinations was considered to be the bound MDA to amino groups of proteins or nucleic acids.

Under our conditions, the mean pH value after TBA reaction was 0.75 ± 0.07 (mean ± SD). We selected a pH < 1 to minimize TBA reaction with oxidized compounds other than MDA (9,10), and because the TBA reaction with actual MDA is not pH dependent (11).

HPLC separations were done on a C18 µ Bondapak 3.9x300 mm column (Waters) with a 60/40 (v/v) mixture of 10 mmol/l phosphate

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buffer pH 5.8 and methanol as a mobile phase, with a flow rate of 1.5 ml/min. The fluorometric detector (Waters model 470) was set at excitation 515 nm and emission 553 nm with gain $\times$ 1000. 1,1,3,3 tetraethoxypropane (Aldrich-Chemie, Steinheim, Germany) was used as a standard and concentrations were calculated using a molar extinction coefficient of $1.57\ 10^5\ M^{-1}cm^{-1}$ as reported previously (8). A C18 µ Bondapak guard column was also used.

Vitamins A and E were measured as previously described (12). Briefly, proteins were precipitated with absolute ethanol containing 0.1 % ascorbic acid (wt/vol), and a mixture of retinol acetate and tocopherol acetate as an internal standard. The vitamins were subsequently extracted in hexane. This extract was concentrated and dissolved in methanol. HPLC separations were done on a C18 µ Bondapak 3.9$\times$300 mm column (Waters) with a 1.5 % deionized water/methanol mixture as a mobile phase. A spectrophotometric detector (Waters λ Max-481) was monitored at 290 nm. Vitamins were quantified using appropriate extinction coefficients with standards obtained from Sigma Chimie.

Fluorometric analysis

Thiobarbituric acid reactive substances (TBA-RS) which are a way of measuring lipid peroxidation, were estimated by the method of Yagi (13). Under these conditions, the mean pH value was 1.3 ± 0.5 (mean ± SD). Briefly, to 50 µl of plasma, 4.0 ml of 42 mmol/l

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H_2SO_4 were added followed by 0.5 ml of 35 mmol/l phosphotungstic acid (PTA). After centrifugation for 10 min at 1000 g the precipitate was resuspended in 2.0 ml of H_2SO_4 and 0.3 ml of PTA and centrifuged again. The resulting precipitate was suspended in 4.0 ml of distilled water and 1 ml of 1 % TBA/pure acetic acid (v/v) was added. The mixture was heated for 1 h at 100° C and after cooling, extracted, with 5 ml of n-butanol. Fluorescence (Jobin et Yvon JY3 spectrofluorometer) in the butanol layer was measured at 515 nm excitation and 553 nm emission (band width 10 nm). 1.1.3.3 tetraethoxypropane was used as a standard without preliminary acid hydrolysis (8,14) and plasma TBA-RS concentrations were calculated using a molar extinction coefficient of $1.5 10^5 M^{-1} cm^{-1}$ (8).

Aldehydic compounds (trans 2-hexenal, trans 2-octenal, trans 2,4-hexadienal, trans 2,4-nonadienal...) react with TBA giving yellow 450 nm-, orange 495 nm- and red 532 nm-absorbing chromophores. Moreover this TBA reaction is pH dependent. At pH <1 (HPLC method of MDA analysis), the yield of the red 532 nm-absorbing pigment produced by these aldehydes at 100°C for one hour was only 0.03 to 0.3% of that of actual MDA, while the yield was 0.4% at pH 1.3 (fluorometric method of TBA-RS analysis) and 5-10% at pH 3.5 (15).

Gas chromatography (GC) analyses

Fatty acid composition was determined from 1 ml of the lipid extract after transformation into isopropylic esters (16).

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Fatty acid esters were separated in a Carlo-Erba 6000 chromatograph equipped with a 25 m capillary column (0.32 mm internal diameter Carbowax). Column conditions were 180°C for 5 min, then rising by 7.5°C/min to 220°C for 35 min. The injector was at 60°C and the flame ionization detector at 250°C. Helium was used as a carrier gas (flow rate 2 ml/min). Peak identifications were made by comparison with reference fatty acids (Sigma Chimie) and peak areas were measured with an automatic integrator DP 700 Carlo-Erba. Quantification of each fatty acid was expressed as a percentage of the total extract.

Quantitative analyses of the two major PUFA, linolenic (omega-6 C18 : 2) and arachidonic (omega-6 C20 : 4) acids were done using the external standardization method. Peak area ratios were used for calculation following the internal standard method with pure arachidic methyl ester (Sigma Chimie) as internal standard.

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Peroxidation of fatty acid standards

For this experiment, a mixture of 2 µmol of C18 : 2 methyl ester and 0.4 µmol of C20 : 4 methyl ester was ultrasonicated in 25 ml of phosphate buffer pH 7.4 at 0°C under N_2 and for 10 min. Lipid peroxidation was induced either by 10 µM $CuSO_4$ or by 10 mM H_2O_2 and 200 µM $FeSO_4$ and was allowed to proceed for 15 h at 37°C. The amounts of MDA and TBA-RS produced were then analyzed as previously described and expressed as nmol/ml of incubation mixture.

Statistical analysis

Data are presented as means ± SD or S.E.M. (details below). For comparison between healthy controls and the malaria population, Student's t-test was chosen and $P < 0.05$ was considered significant. Linear regression analysis was assessed by Spearman rank correlation. These analyses were carried out on a computer using a statistical software package (Stat-View).

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RESULTS

All malaria patients were in an acute phase and had not received any curative treatment. They were all slightly anemic as shown by a significant ($P < 0.025$) decrease in RBC (Table 1). The amount of total plasma proteins was not significantly different in the two groups. Triglycerides were significantly ($P < 0.01$) increased when total cholesterol was significantly ($P < 0.001$) decreased in malaria patients compared to controls (Table 1).

Fatty acid composition of plasma lipids

Fatty acid (FA) composition was analysed by GC of isopropylic esters and an unsaturation index (UI) was calculated as the ratio of the sum of polyunsaturated FA percentages to the sum of saturated and monounsaturated FA percentages. Results are shown in Table 1. The unsaturation index was significantly

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TABLE 1

Biochemical data of blood in healthy subjects and malaria patients

	Erythrocytes	Plasma			
	(10^6/mm^3 of blood)	Proteins (g/l)	Triglycerides (mmol/l)	T - chol (mmol/l)	UI (mmol/l)
Healthy subjects (n=16)	5.10 ± 0.17	64.6 ± 0.1	0.96 ± 0.09	5.31 ± 0.09	0.77 ± 0.02
Malaria patients (n=28)	4.36 ± 0.14 P < 0.025	62.0 ± 0.9 NS	2.24 ± 0.32 P < 0.01	3.46 ± 0.17 P < 0.001	0.39 ± 0.04 P < 0.001

Results are expressed as means ± S.E.M.

NS : difference statistically not significant

T - chol : total cholesterol

UI : unsaturation index

decreased ($P < 0.001$) in malaria patients whatever the level of parasitaemia. This indicated without doubt an oxidation of polyunsaturated FA. Among polyunsaturated FA, only the main omega-6 ones, linoleic (C18 : 2), arachidonic (C20 : 4) and eicosatrienoic (C20 : 3) acids were significantly decreased ($P < 0.005$, $P < 0.001$ and $P < 0.01$ respectively) as shown in figure 1. Omega-3 docosahexaenoic acid (C22 : 6) was slightly decreased but not significantly so ($P > 0.1$) in spite of its high oxidation susceptibility index.

The two major PUFA in plasma, omega-6 C18 : 2 and C20 : 4 acids, were submitted to quantitative analysis in both groups. Results are shown in Table 2. During malaria ,the C18 : 2 and C20 : 4 decreases were then estimated to be 800 nmol/ml and 160 nmol/ml, respectively.

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Analytical features of LPO product determination

To measure LPO product formation, we compared two analytical methods. The LPO products were simultaneously estimated after TBA reaction by HPLC allowing separation of the MDA/TBA adduct from other TBA-reactive substances (TBA-RS), and by fluorometric measurement of whole LPO products as previously proposed by Yagi. The first method made it possible to evaluate successively free and total MDA ; the difference between total and free MDA was considered to be the bound MDA. In the second method, TBA-RS other than lipoperoxides were eliminated

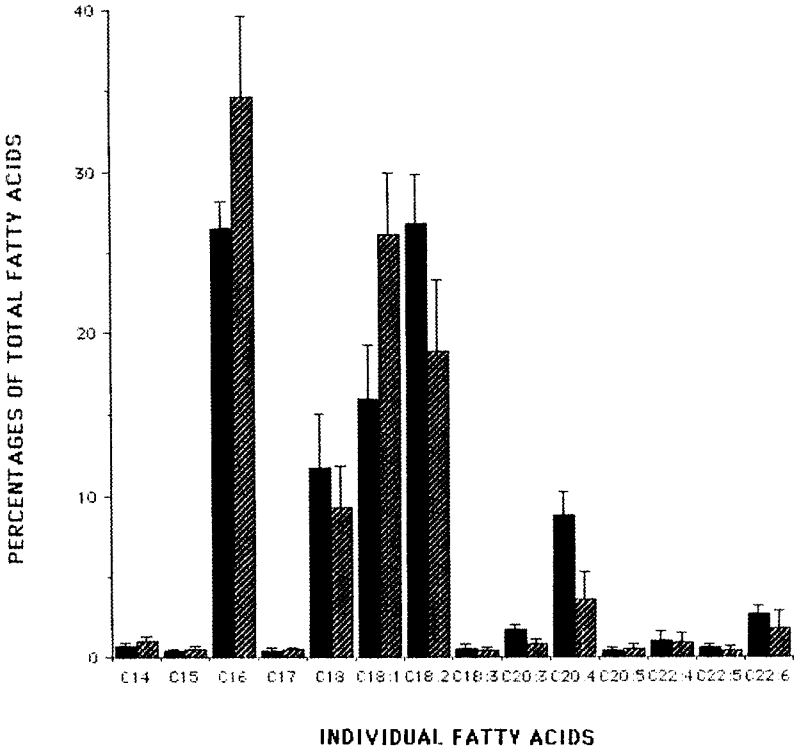

FIGURE 1 : Fatty acid profile in plasma of healthy subjects (■) and malaria patients (▨).
Loss in polyunsaturated fatty acids was statistically significant :
* $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$.

by precipitating lipids and proteins with a mixture of phosphotungstic/sulfuric acid. The TBA reaction was then performed on the pellet. The level of LPO products can be expressed in terms of MDA and this value was considered to be the plasma TBA-RS. However, under these conditions, free MDA was not taken

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TABLE 2

Decrease in plasma omega-6 C18 : 2 and omega-6 C20 : 4 acids during malarial infection

	C18 : 2 (mmol/l)	C20 : 4 (mmol/l)
Healthy subjects	8.3 ± 1.9	0.44 ± 0.06
Malaria patients	7.5 ± 1.4	0.28 ± 0.03

Results are the means ± S.E.M. of 6 independent cases examined

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into account. Therefore, we compared bound MDA measured by HPLC and TBA-RS value determined fluorometrically. The fluorescence detection used in both methods allowed more specific and sensitive detection. Results are shown in Table3. Bound MDA values were significantly lower ($P < 0.001$) than TBA-RS in both control subjects and malaria patients. Moreover, there was a significant ($P < 0.05$) positive correlation between bound MDA and TBA-RS value ($r = 0.41$; $n = 24$) in control subjects. On the other hand, the correlation was not significant ($P > 0.1$) in malaria patients ($r=0.11$; $n = 26$), as shown in figure 2.

Bound and total MDA values were not significantly different between the two groups whatever the level of parasitaemia. On the other hand, there was a significant increase ($P < 0.001$) in the TBA-RS of malaria patients compared to controls. However, there

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TABLE 3

Peroxidation product and antioxidant concentrations in plasma of healthy subjects and malaria patients

	Healthy subjects	Malaria patients		
		Total[a]	Infestation[b]	
			< 1 %	> 1 %
	(n=30)	(n=28)	(n=13)	(n=15)
TBA-RS (µmol/l)	1.89 ± 0.20	5.39 ± 0.34 P < 0.001	5.25 ± 0.41	5.53 ± 0.55
Bound MDA (µmol/l)	0.36 ± 0.006	0.28 ± 0.05 NS	0.30 ± 0.08	0.26 ± 0.07
Total MDA (µmol/l)	0.52 ± 0.05	0.39 ± 0.06 NS	0.42 ± 0.09	0.35 ± 0.08
Vitamin A (µmol/l)	1.99 ± 0.07	1.04 ± 0.16 P < 0.05	0.85 ± 0.16	1.18 ± 0.26
Vitamin E (µmol/l)	28.52 ± 1.87	16.69 ± 0.87 P < 0.05	16.37 ± 1.48	16.42 ± 1.10

Results are the means ± S.E.M.

(a) significance was calculated between healthy subjects and malaria patients

(b) difference between < 1 % - and > 1 % - infestation group means were not statistically significant

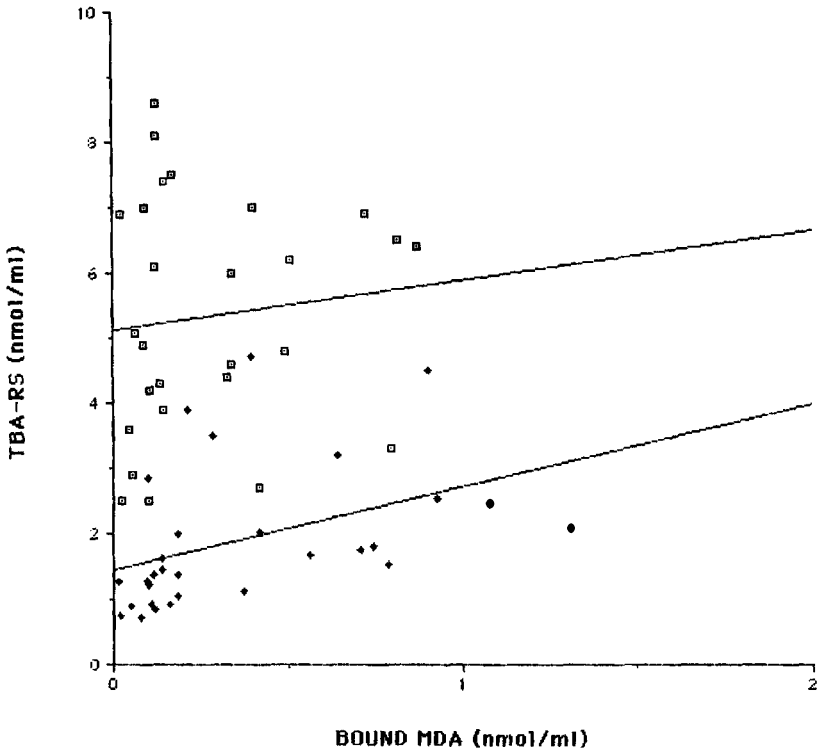

FIGURE 2 : Relationship between TBA-RS and bound MDA values in (♦) healthy subjects (y=1.44 + 1.28 x ; r=0.41 ; P < 0.05) and in (▫) malaria patients (y=5.13-0.75 x ; r=0.11 ; not significant).

was not a significant correlation between TBA-RS and the percentage (z) of erythrocytes infested by *Plasmodium,* even after Arc sin$\sqrt{z}$ conversion in order to fit a gaussian distribution.

Figure 3 shows the correlations between plasma TBA-RS values of healthy subjects or malaria patients and the fatty acid unsaturation index (FAUI). Only the former showed a statistical significance (r= -0.74 ; P < 0.01).

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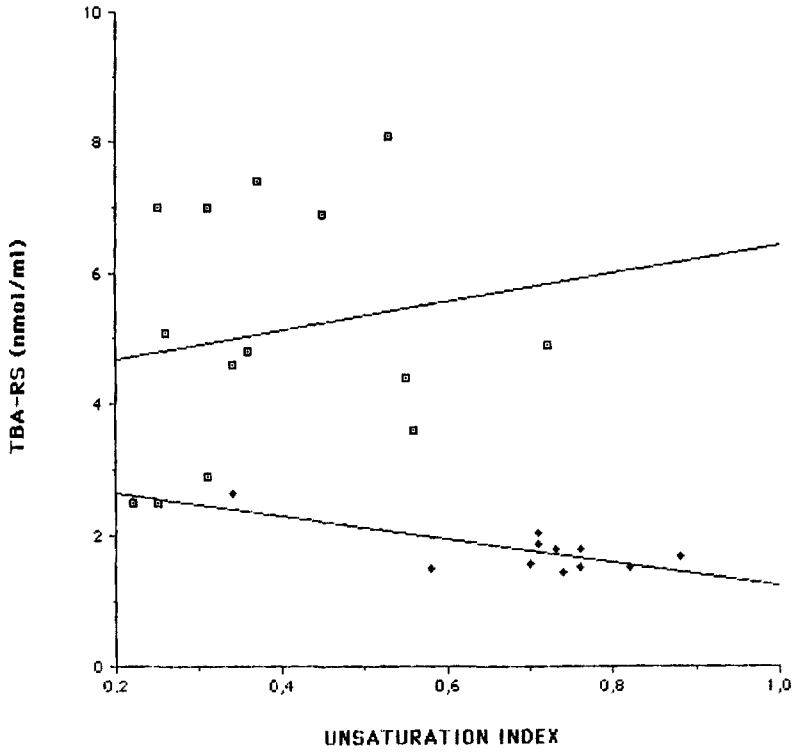

FIGURE 3 : Relationship between TBA-RS concentrations and unsaturation index
in (♦) healthy subjects (y=3.02-1.79 x ; r=-0.74 ; P < 0.01)
in (□) malaria patients (y=4.24+2.24 x ; r=0.18 ; not significant).

Comparison of MDA and TBA-RS formation from plasma and pure fatty acids submitted to *in vitro* LPO

During the 15 h oxidation of a mixture containing 80 nmol/ml C18 : 2 and 16 nmol/ml C20 : 4 acids with either 10 µM Cu^{++}or 10 mM H_2O_2 and 200 µM Fe^{++} the pure fatty acids were totally disrupted (99.9-100 %) and produced the same TBA-RS level (3.52 ± 0.1 nmol/ml and 3.99 ± 0.07 nmol/ml respectively as shown

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in Table 4. During Cu^{++} oxidation the MDA value was slightly lower ($P > 0.1$) than for TBA-RS. However, during H_2O_2/Fe^{++} oxidation, the former value was very significantly decreased ($P < 0.001$). Given the decrease in plasma PUFA during malaria infection and in *in vitro* peroxidation of the pure fatty acid reference, peroxidation products analyzed as MDA or TBA-RS could be compared. First, the decrease in PUFA during malaria infection did not increased the total MDA value (Table 3). Second, the TBA-RS production from *in vitro* LPO of the pure fatty acid preparation (Table 4) exceeded about 10-fold the increase in the TBA-RS value produced by malarial infection.

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Plasma vitamin A and E status

There was a significant ($P < 0.05$) decrease in vitamin A and E content in malaria patients compared to controls. However, this decrease was not correlated with the percentage of infested erythrocytes, as shown in Table 3. Morever, the amount of vitamin A or E was not significantly correlated with the TBA-RS value, in either group.

DISCUSSION

It has been assumed that LPO is produced by mononuclear phagocyte activation during the natural course of malaria infection

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TABLE 4

In vitro **formation of MDA and TBA-RS during 15 h incubation in the presence of 10 µM Cu^{++} or 10 mM H_2O_2 and 200 µM Fe^{++} from a mixture of pure fatty acid methyl esters.**

	Cu^{++} oxidation		H_2O_2/Fe^{++} oxidation	
	MDA	TBA-RS	MDA	TBA-RS
	(nmol/ml)		(nmol/ml)	
Incubation mixture : (80 nmol/ml C18: 2 + 16 nmol/ml C20:4)	2.81 ± 0.23 NS	3.52 ± 0.15	0.62 ± 0.04 P < 0.001	3.99 ± 0.07

Results are the means ± S.E.M. of 5 independent experiments.

Differences were statistically significant between TBA-RS and MDA values in H_2O_2/Fe^{++} oxidation.

(1-6). This LPO was clearly proved by a significant decrease in omega-6 PUFA (C18 : 2, C 20 : 3, C20 : 4) in malaria patients as compared to controls. Omega-3 C22 : 6 acid sligIty decreased but not significantly (figure 1). These results are in agreement with those of Bruna et al (17) who found that the *in vitro* oxidation rate of docosahexaenoic (C22 : 6) and eicosapentaenoic (C20 : 5) acids, two highly PUFA of the omega-3 series, were lower than the oxidation rate of linoleic (C18 : 2) and arachidonic (C20 : 4) acids, the PUFA of the omega-6 series.

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On the other hand, total cholesterol significantly decreased ($P < 0.001$) whereas triglycerides increased ($P < 0.01$), indicating that cholesterol, like PUFA, is a basic lipid structure which could be susceptible to peroxidation (18). These results have been previously shown by Stocker et al (19) in plasma of mice infected by *Plasmodium vinckei*. The plasma triglyceride increase could be the result of increased levels of triacylglyceride-rich lipoproteins which are elevated in the plasma of animals infected with various strains of *Plasmodium* (20), thereby resulting in inhibition of lipoprotein lipase which suppresses triglyceride clearance (21). Stocker et al (19) have also shown a plasma cholesterol decrease paralleling that found in red blood cells, but of a smaller magnitude.

Additional evidence for the involvement of plasma LPO in malaria patients is the significant ($P < 0.05$) decrease in plasma vitamin A and E content as compared to control values (Table3),

indicating that this protective system was overwhelmed. However, this vitamin E decrease observed in plasma could be due to its transfer to membrane RBC noted by Stocker et al. (22) in response to an oxidative stress of extra- or intracellular origin during malarial infection, or could be partly due to its consumption as plasmatic antioxidant. A similar decrease in vitamin A was also observed by Thurnham et al (23) who associated this lower vitamin A value to the oxidative conditions present in plasma of malaria patients.

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This study provides evidence for the occurence of LPO. However, total and protein-bound MDA concentrations determined by HPLC were not significantly different in the two groups. These findings suggest either that, at least, in plasma of malaria patients, LPO does not produce the end product that is MDA (24), or that MDA is rapidly oxidized and/or eliminated (25). In particular MDA is unstable in the presence of weak concentrations of hydrogen peroxide (15,26) which can be produced by the activation of macrophages by *P. falciparum* (27,28),

Our *in vitro* experiment of pure C18:2 and C20:4 acids submitted to LPO produced by H_2O_2/Fe^{++} revealed that a 10 mM concentration of H_2O_2 was sufficient to induce a significant decline ($P < 0.001$; Table 4) in the amount of MDA-TBA complexes formed as compared to the amount produced by 10 µM Cu^{++} oxidation. This decline in MDA formation may be due to its oxidation by H_2O_2. However, this oxidized MDA compound was able to

react with TBA, so the TBA-RS values were the same in both oxidation procedures.

We observed a highly significant increase ($P < 0.001$) in the amount of TBA-RS in malaria patients as compared to controls. However, the TBA-RS amount was not correlated with the percentage of infested erythrocytes, and the mean values at different parasitaemia levels were not altered significantly (Table 3). These results are in agreement with those of Mathews and Selvam (29) in *Plasmodium vivax* infected patients.

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On the other hand, PUFA are the substrate required for MDA formation, and the amount of peroxidized lipids formed may be related both to the amount of substrate and to the level of peroxidation. So in healthy subjects who have a low level of peroxidation, there is a significant correlation between TBA-RS values and the FA unsaturation index (FAUI). So the greater the unsaturation index, the lesser the decrease in PUFA, and the lesser are the TBA-RS values (figure 2). Surprisingly, in malaria patients who have a high level of peroxidation, the correlation between TBA-RS values and the FAUI was not significant (figure 3).

In this TBA test, the red pigment may not reflect MDA alone but may reflect FA oxidized components including MDA, alk-2 enals, alka-2.4 dienals and hydroperoxide functions (30) or non-lipidic compounds such as glycoproteins and other molecules containing sugar components (24). Plasma precipitation with PTA-H_2SO_4 (13) considerably increased the TBA-RS amount obtained by this method

(31) with reference values in human plasma in the range of 3-4 mmol/l (32), compared to TBA-RS amounts using untreated plasma with values in the range of 0.4 to 0.9 mmol/l with (8,33,34) or without (35-37) HPLC separation. In this respect, Warso et al. (38,39) have demonstrated that 80 % of the TBA-RS in human plasma was found not to have originated from lipid hydroperoxide conversion to MDA, and so had no relationship with PUFA peroxidation. Hydroperoxide from linoleic acid peroxidation decomposes *in vitro* into various aldehydic products including MDA, but the yield of red pigment produced during the TBA reaction was only 0.4 % (40). Morever, at pH 1.3 used in Yagi's method of TBA-RS analysis, the yield of red pigment produced by aldehydes other than MDA was only 0.4 % of that of actual MDA (see Material and Methods), and could be considered as negligible (15). In malaria patients , the TBA-RS production was significantly ($P < 0.001$) increased but was about 10-fold lower than the *in vitro* production of pure FA preparation submitted to LPO.

Vitamin E appeared to be consumed (Table3) during malaria infection, and this consumption could be explained by the chain-breaking antioxidant effect of vitamin E which inhibits the chain reaction of lipid peroxidation between PUFA attack and oxidized compounds which are able to react with TBA. Morever, Flecha et al (41) have recently suggested that radical scavenging by tocopherols could be explained by an electron transfer between the phytyl side-chain and the chromanoxyl aromatic nucleus of

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tocopherols through an electron transfer to the isoprenoid chain of carotenoids and ubiquinone. This hypothesis could explain the parallel vitamin A and E consumption observed in malaria (table 3). However, we are unable to show any significant correlation between vitamin A and E consumption and TBA-RS formation. This lack of correlation between vitamin A or E consumption and TBA-RS production may be due to protection by other antioxidants such as vitamin C (22,23) and enzymes such as glutathione peroxidase, superoxide dismutase and catalase (42).

This increased TBA-RS production which has no relationship with the loss in PUFA and the consumption in antioxidant vitamins supports the concept that other compounds such as proteins and cholesterol may contribute to the formation of these peroxidation products (43). In particular, total cholesterol was significantly lower in malaria patients. Cholesterol, like PUFA, could be susceptible to oxidation and these oxidized cholesterol compounds might act as a strong oxidant to emphasize TBA-RS formation. This hypothesis is at present under investigation in our laboratory.

Finally, the direct quantitative comparison between the loss in PUFA, the MDA amount specifically analyzed by HPLC, TBA-RS production and vitamin A and E consumption in a biological system such as human plasma takes into account the complex nature of the relationship between lipid peroxidation and the TBA test (24). As for the TBA-RS test in Yagi's method, it is better suited for the empirical indication of an oxidative injury.

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