Glutathione peroxidase (*EC* 1.11.1.9) and superoxide dismutase (*EC* 1.15.1.1) activities in riboflavin-deficient rats infected with *Plasmodium berghei* malaria

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Riboflavin deficiency interferes with the growth and multiplication of malaria parasites as well as the host response to malaria. The objective of the present work was to determine the effects of riboflavin deficiency on erythrocyte glutathione peroxidase (*EC* 1.11.1.9; GPx) and superoxide dismutase (*EC* 1.15.1.1; SOD) in rats infected with *Plasmodium berghei* malaria. Riboflavin in its co-enzyme form, FAD, is required by glutathione reductase (*EC* 1.6.4.1) to regenerate GSH and GSH is an important cellular antioxidant both in its own right and also as a substrate for the enzyme GPx. Weanling rats were deprived of riboflavin for 8 weeks before intraperitoneal injection of 1×10^6 *P. berghei* parasites. Control animals were weight-matched to the respective riboflavin-deficient group. At 10 d post-infection, parasite counts were higher in the weight-matched control group than the riboflavin-deficient group ($P = 0{\cdot}004$). GPx activity was higher in erythrocytes of rats parasitized with *P. berghei* than comparable non-infected rats regardless of riboflavin status ($P < 0{\cdot}05$). As mature erythrocytes do not synthesize new protein, the higher GPx activities were probably due to the presence of the parasite protein. In erythrocytes from riboflavin-deficient rats, GPx activity tended to be lower than in those rats fed on diets adequate in riboflavin (weight-matched controls) whether parasitized or not, but the difference was not significant. Neither riboflavin deficiency nor malaria had any effect on erythrocyte SOD activity. It was concluded that riboflavin deficiency has no marked effect on erythrocyte GPx or SOD activity in the rat.

Riboflavin: Malaria: Glutathione peroxidase: Superoxide dismutase

The importance of reactive O intermediates in host defence against parasitic infections is well known. Activated O species are toxic to plasmodia and are important components of the host's defences against malaria (Clark & Hunt, 1983; Fairfield *et al.* 1983). Malaria parasites are known to be vulnerable to pharmacological agents which generate reactive O species such as phenylhydrazine and alloxan (Pollack *et al.* 1966), t-butyl peroxide (Clark *et al.* 1983), divicine (Clarke *et al.* 1984) and primaquine (Kelman *et al.* 1982). These agents appear to work on the principle that oxidative damage affects the parasite more than the host (Clark *et al.* 1986) and reactive O species generated as part of the cell-mediated immune response of the host are now recognized as an important component of the host-defence system against malaria (Wozencraft *et al.* 1984; Jones *et al.* 1989).

In the erythrocyte, protection against oxidative damage is provided by enzymes and antioxidants. Superoxide dismutase (*EC* 1.15.1.1; SOD) catalyses the dismutation of superoxide anions (O_2^-) to H_2O_2 and O_2 while catalase (*EC* 1.11.1.6) and glutathione peroxidase (*EC* 1.11.1.9; GPx) break down H_2O_2 to water and O_2 (Fairfield *et al.* 1988). Several studies have reported on the antioxidant-defence enzymes in plasmodium-infected and uninfected erythrocytes (Suthipark *et al.* 1982; Seth *et al.* 1985; Stocker *et al.* 1985). However, the role of riboflavin has not been studied.

Riboflavin deficiency has been reported in many tropical countries where malaria is also endemic (Bates, 1987) and the deficiency has been reported to offer protection against malaria infection by inhibiting the growth and multiplication of parasites (Thurnham *et al.* 1983; Anon., 1984;

Abbreviations: EGRAC, erythrocyte glutathione reductase activation coefficient; GPx, glutathione peroxidase; GR, glutathione reductase; RD, riboflavin-deficient; SOD, superoxide dismutase, WM, weight-matched.

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Dutta *et al.* 1985). Riboflavin in its co-enzyme form, FAD, is required by glutathione reductase (*EC* 1.6.4.1; GR) to regenerate GSH from GSSG. GSH functions both to prevent radical initiation and as a substrate for GPx and thus helps to maintain erythrocyte stability. Riboflavin deficiency has been reported to lower GSH in erythrocytes (Hassan & Thurnham, 1977). The presence of riboflavin deficiency may make erythrocytes, which are infected with plasmodia species, even more vulnerable to reactive O species generated as part of cell-mediated immune responses, since even in rats without malaria, riboflavin-deficient erythrocytes have been reported to be more fragile than cells from non-deficient rats (Hassan & Thurnham, 1977). Riboflavin deficiency would therefore provide an unfavourable environment for the invading malaria parasite. In order to complete the erythrocytic stage of the life cycle, the malaria parasite needs to grow, mature and finally divide into a number of merozoites which are released to infect new cells at schizogony. A failure to complete the life cycle due to enhanced susceptibility to reactive O species resulting in premature lysis of the erythrocyte has been suggested to be the mechanism by which many of the hereditary haemoglobinopathies e.g. glucose-6-phosphate dehydrogenase deficiency, sickle cell anaemia and thalassaemia may protect against malaria (Nagel & Roth, 1989).

The present study was designed to investigate the effect of riboflavin deficiency on the oxidant defence enzymes SOD and GPx in erythrocytes of rats infected with *Plasmodium berghei*.

Materials and methods

Animals

The twenty male DNU hooded rats used in this experiment were bred on site (MRC Dunn Nutrition Centre, Milton Road, Cambridge, UK). Animals weighed 40–50 g (mean 47·8 (SD 5·4)g when selected for the studies.

Diet

The rats were weaned onto a riboflavin-deficient or -adequate diet. The composition of the basal (deficient) diet has been previously reported (Adelekan & Thurnham, 1986) and the adequate diet was prepared by addition of 5·0 mg riboflavin/kg diet (Medical Research Council, 1977). Rats were divided into two groups so that the mean weight of each group of rats was identical. One group containing ten rats was allowed free access to a diet deficient in riboflavin (RD) while a second group containing ten rats was fed on a diet which was adequate in riboflavin in a quantity that permitted the same rate of growth as the RD rats; this was termed the weight-matched (WM) group. Control rats were weight matched as a group to the RD rats. All rats were allowed free access to water. Rats were housed individually in wire-bottomed cages in a room maintained at a temperature of 26–28°. Rats were fed on the respective diets for a period of 8 weeks. After infection (see below), the control groups (adequate riboflavin) of rats were weight matched to the respective riboflavin-deficient groups. The protocol complied with the guidelines for the care and use of laboratory animals (National Research Council, 1985).

Parasite infection

At the end of the feeding period, five rats randomly selected from each group were infected with *Plasmodium berghei ANKA* (1×10^6 parasites/100 g body weight; Wynch's Farm, St Albans, Herts., UK) by the intraperitoneal route. The remaining five rats in each group were not infected. Malaria parasite development in erythrocytes was followed by measurement of the packed cell volume and parasite density on thin blood film 2 d after infection and thereafter at intervals up to 10 d (see Table 1). Rats were killed by cardiac puncture under light ether anaesthesia on the tenth day following infection with *P. berghei*.

Preparation of erythrocytes for enzyme assays

Blood (5 ml) was removed from the heart of each rat under light ether anaesthesia into heparinized tubes. The blood was mixed gently on a roller mixer and then centrifuged for 5 min at 1200 ***g*** to separate plasma and erythrocytes. Erythrocytes were then washed three times in cold normal saline (9 g NaCl/l).

Enzyme assays

GPx activity in erythrocyte haemolysates (one part washed erythrocytes plus four parts water) was assayed by the method of Wendel (1981) using H_2O_2 as substrate. Tubes containing blanks were included in the assay and the values subtracted accordingly in the calculation of enzyme activity. Results were expressed as IU per g haemoglobin. SOD was assayed by the method of Winterbourn *et al.* (1975). Results were expressed as IU per g haemoglobin. GR (NAD(P)H : glutathione oxido-reductase), in a 1 in 20 aqueous haemolysate, was used to measure riboflavin status by measuring the activity with and without FAD as previously described (Adelekan & Thurnham, 1986). Riboflavin status is expressed as a ratio of the FAD-stimulated : unstimulated activities which is called the erythrocyte GR activation coefficient (EGRAC). EGRAC values $\geq 1{\cdot}30$ are considered to represent biochemical riboflavin deficiency.

Statistical analyses

Erythrocyte GPx and SOD data were normally distributed, therefore parametric statistics were used. EGRAC values and parasite counts were non-normally distributed, therefore the data were log-transformed before analysis. Repeated measures and two-way ANOVA were used to analyse the data as indicated in the text (SPSS–PC, Statistical Package for Social Sciences, SPSS UK Ltd, Chertsey, Surrey, UK). Other tests used are indicated in the text.

Table 1. Parasite density* and packed cell volume (PCV) in riboflavin-adequate and riboflavin-deficient rats infected with *Plasmodium berghei* (Mean values and standard deviations for five rats per group)

	Riboflavin-deficient				Riboflavin-adequate‡			
	PCV§		Parasite density‖		PCV§		Parasite density‖	
Time post-infection (d)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	44[a]	2·4	0	–	45[a]	0·8	0	–
2	35[b]	3·1	0	–	37[b]	1·3	†	–
4	33[b]	3·3	1·9[a]	0·5	34[bc]	2·6	6·7[a]	1·8
6	33[bc]	3·1	3·2[bc]	1·3	33[bc]	3·1	7·2[a]	3·4
10	29[c]	1·3	4·2[c]	1·9	32[c]	2·5	13·0[b]	3·9

[a,b,c] Mean values within a column not sharing a common superscript letter were significantly different, $P < 0.05$ (*post-hoc* paired *t* tests).
* Percentage *P. berghei* parasites in 500 erythrocytes.
† Positive parasitaemia but less than 1 %.
‡ During the infection with *P. berghei*, the riboflavin-adequate rats were weight-matched to the corresponding riboflavin-deficient group.
§ Significant differences were observed between days ($P < 0.0001$) and treatments ($P = 0.082$) (repeated measures ANOVA).
‖ Differences between days ($P < 0.0001$) and treatments ($P = 0.004$) were analysed using log-transformed data (repeated measures ANOVA).

Results

Table 1 shows the mean percentage parasitaemia and packed cell volume values obtained from the different groups of rats measured at intervals up to 10 d after infection with *P. berghei*. Malaria parasites were present in the blood of all rats receiving the parasite inocculum from day 2 in the WM control rats and from day 4 in the RD rats. The parasitaemia increased steadily over the 10 d period and was significantly greater in the WM group where it reached a mean of 13 %, than in the RD group where the mean only rose to 4·2 % ($P = 0.004$). The infection was mild and no animal died during the study. The mean packed cell volume values of all the RD and all the WM rats at 8 weeks, i.e. before infection with malaria parasites, were not significantly different (43·9 (SD 2·42) and 45·3 (SD 0·82) respectively). Packed cell volume values in both groups infected with malaria parasites were significantly decreased by the infection ($P < 0.0001$) but there was no significant difference between the two treatments ($P = 0.082$, Table 1).

There were no significant differences in weight between the four groups of rats at the end of the study, i.e. 10 d post-infection. Mean weights for the *P. berghei*-infected rats were 76·0 (SD 1·3) and 78·0 (SD 3·52) g and for the non-infected controls 82·0 (SD 6·78) and 84·0 (SD 10·01) g for the RD and WM rats respectively.

Table 2 shows the riboflavin status of the malaria-infected and control rats. EGRAC measurements were significantly higher in both infected and WM control groups which had been restricted in their riboflavin intake ($P < 0.0001$). In addition there was a tendency for both the RD rats and those rats which received the diet containing adequate riboflavin to have higher EGRAC values when infected with malaria than the non-infected animals ($P = 0.07$); that is, malaria-infected rats tended to be more biochemically deficient in riboflavin, but this did not reach statistical significance. There was a very slight tendency for EGRAC values to be positively correlated with GPx and negatively correlated with SOD but neither effect was significant.

Table 3 shows the erythrocyte GPx activities of rats infected with *P. berghei* and of non-infected rats. Slightly lower mean GPx activity was associated with riboflavin deficiency by comparison with that in those rats which received adequate riboflavin, but the difference was not significant. However, the rats infected with *P. berghei* had significantly higher erythrocyte GPx activities than non-infected rats ($P < 0.05$). There was no interaction between the effects of malaria infection and riboflavin deficiency on GPx activity.

Table 2. Measurements of erythrocyte glutathione reductase activation coefficient (EGRAC) in control and *Plasmodium berghei*-infected rats fed on a riboflavin-deficient or a riboflavin-adequate diet†
(Mean values and standard deviations for five rats per group)

	EGRAC			
	Infected‡		Control	
Diet group	Mean	SD	Mean	SD
Riboflavin deficient	2·87***	0·65	2·61	0·28
Riboflavin adequate	1·40	0·21	1·14	0·05

Mean value was significantly different from that for riboflavin-adequate rats, ***$P < 0.0001$.
† For details of diets and procedures, see p. 306.
‡ EGRAC values were not different between infected and control rats ($P = 0.07$) and there were no interactive effects (two-way ANOVA on log-transformed data).

Table 3. Glutathione peroxidase (GPx) activity in erythrocytes of control and *Plasmodium berghei*-infected rats fed on a riboflavin-deficient or a riboflavin-adequate diet†
(Mean values and standard deviations for five rats per group)

	GPx activity (IU/g haemoglobin)			
	Infected*		Control	
Diet group	Mean	SD	Mean	SD
Riboflavin deficient	883	127	684	174
Riboflavin adequate	902	123	817	156

* Erythrocyte GPx activity was greater in infected rats ($P < 0.05$) irrespective of the riboflavin status (NS) (two-way ANOVA using arithmetic data).
† For details of diets and procedures, see p. 306.

Table 4. Erythrocyte superoxide dismutase (SOD) activity in control and *Plasmodium berghei*-infected rats fed on a riboflavin-deficient or a riboflavin-adequate diet*

(Mean values and standard deviations for five rats per group)

	SOD activity† (IU/g haemoglobin)			
	Infected		Control	
Diet group	Mean	SD	Mean	SD
Riboflavin deficient	654	100	676	206
Riboflavin adequate	734	145	698	231

* For details of diets and procedures, see p. 306.

† There was no significant effect of either *P. berghei* infection or riboflavin status on SOD activity (two-way ANOVA on either the arithmetic data or the log-transformed data).

Table 4 shows the erythrocyte SOD activities of rats with and without *P. berghei* infection. There were no significant effects of either riboflavin deficiency or malaria infection on SOD activities.

Discussion

Although studies have shown that riboflavin deficiency appears to suppress parasite growth both in experimental animals (Kaikai & Thurnham, 1983) and man (Das *et al.* 1988), the course of the disease and parasite disappearance are more prolonged, the fever is more severe and the formation of lipid peroxidation endproducts more pronounced in riboflavin-deficient than non-deficient human subjects (Das *et al.* 1988, 1990). The results of the present study, however, indicate that riboflavin deficiency does not significantly impair the activities of antioxidant defence enzymes in erythrocytes parasitized with *P. berghei*.

Mean GPx activities in both the infected and non-infected rats with riboflavin deficiency were slightly lower than in their respective riboflavin-adequate controls (Table 3). The differences, however, were not significant ($P = 0{\cdot}266$). Dutta *et al.* (1985) also reported lower erythrocyte GPx activity in their riboflavin-deficient rats by comparison with pair-fed controls and their results also did not reach significance. Pair-fed controls are different from WM controls since the latter get less food. In the rats receiving the riboflavin-adequate diet, the basic diet contained sufficient riboflavin as indicated by the riboflavin status of the WM control group (Table 2, mean EGRAC 1·14), however the mean EGRAC of the *P. berghei*-infected groups indicates that marginal riboflavin status was present in more than half the group. Three out of the five EGRAC values were greater than 1·30 whereas in the non-infected WM animals, all EGRAC values were within the normal range. There was no significant difference in GPx activities between the riboflavin-deficient and -sufficient groups regardless of the infection but the difference between mean GPx activities appeared to be greater in the rats without malaria (NS). It is, therefore, possible that the poor riboflavin status of both the *P. berghei*-infected, RD and WM-control groups, may have lowered the potential GPx activity in both groups but the number of rats used was not sufficient to confirm this.

Although statistically the effects of riboflavin deficiency on GPx activity were not significant, malaria infection was associated with higher erythrocyte GPx activities than in the non-infected controls ($P < 0{\cdot}05$, Table 3). It is not immediately clear from these experiments whether the higher GPx activity is due to an increase in the host enzyme or the plasmodium enzyme since there are independent enzyme defence systems in both the host erythrocyte and isolated *P. berghei* parasites (Fritsch *et al.* 1987). Mature human erythrocytes are not capable of synthesizing new GPx enzyme as they do not possess the necessary protein-synthetic machinery. However, there are other factors which may contribute to the higher GPx activity. The packed cell volume of the *P. berghei*-infected rats in the present study was significantly lower than that of the non-infected rats, therefore the proportion of reticulocytes in the circulation will probably be greater in those rats with infection than those with no infection. Erythrocyte enzyme activities are higher in young erythrocytes than in mature ones (Ganzoni *et al.* 1976; Spooner *et al.* 1979), which may partly explain the higher GPx activity in the *P. berghei*-infected rats. In addition, associated with malaria, there is increased lysis of both parasitized and non-parasitized erythrocytes (Phillips *et al.* 1976). The more fragile older cells will tend to be removed sooner thus increasing the proportion of younger cells with the greater GPx activity. Furthermore, riboflavin deficiency itself tends to increase erythrocyte fragility (Hassan & Thurnham, 1977) and tends to increase the rate of disappearance of the older cells. However, as riboflavin deficiency appears, if anything, to be associated with lower GPx activity (Table 3), the major factor influencing GPx activity in these experiments would appear to be parasite material as this enzyme has been reported in isolated *P. berghei* parasites (Fritsch *et al.* 1987).

With regard to erythrocyte SOD activity, there was no significant effect of either malaria infection or riboflavin deficiency. Similar findings in human subjects infected with *P. falciparum* malaria have been previously reported by Areekul & Boonme (1987). Riboflavin is not directly involved in erythrocyte SOD activity in the manner of its involvement with GPx activity in erythrocytes. Furthermore studies in rats have also shown that the inflammation induced by turpentine administration did not significantly affect SOD activities (DiSilvestro & Marten, 1990). The lack of influence of either malaria or turpentine may indicate that erythrocyte SOD activity is not easily influenced by stressor effects on the erythrocyte or that the capacity of existing SOD activity is fully capable of meeting the extra demands caused by the malaria infection or the stress induced by turpentine administration.

In conclusion, erythrocyte GPx activity of malaria-infected rats was significantly higher than that in the controls and this was probably due to the direct presence of the parasite GPx enzyme in the haemolysates. In addition there was a tendency for GPx activity to be lower in rat haemolysate from the RD animals than the non-deficient, but this did not achieve significance. Riboflavin status tended to be poorer in both RD and control malaria-infected

rats ($P = 0{\cdot}07$) but neither riboflavin status nor malaria had any effect on erythrocyte SOD activity.

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