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HYPOTHESIS

ANTIMALARIAL ACTIVITY OF THE ETHANOL/ ALCOHOL OXIDASE SYSTEM *IN VITRO*

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Among other macrophage secretory products, H_2O_2 plays an important role in the host's defense against malaria (Wozencraft *et al.*, *Infect. Immun.*, **43**, 664, (1984)). In our *in vitro* studies on the human malaria parasite *Plasmodium falciparum*, hydrogen peroxide was produced by the alcohol oxidase-catalyzed reaction ethanol + O_2 → acetaldehyde + H_2O_2 (EC 1.1.3.13). At concentrations of 8.7 mM (= 0.5‰) ethanol and 0.1 U alcohol oxidase per ml culture, more than 95% of the parasites were irreversibly damaged. Acetaldehyde was found to be parasiticidal *per se* – probably by releasing immature forms of *P. falciparum* from erythrocytes – but CH_3CHO concentrations as high as 90 mM were required for complete elimination of the parasites. Ethanol (< 20 mM) or alcohol oxidase alone had no significant effect on parasite viability.

As discussed, the ethanol/alcohol oxidase system might be of interest as a potential chemotherapeutic principle, especially since metabolism and pharmacology of the substrates and products are well understood.

KEY WORDS: Ethanol, acetaldehyde, alcohol oxidase, antimalarial action, oxidative stress.

ABBREVIATION: ROS, reactive oxygen species

INTRODUCTION

Clinical observations and experimental evidence suggest that oxidative stress represented by hydrogen peroxide and other reactive oxygen species (ROS) plays a dominant role in the host's defense against parasites[1-6] or tumor cells.[7] H_2O_2 has been estimated to reach levels as high as 10 mM in the contact zone between a macrophage and the cell under attack.[7] The effects of macrophages and ROS on *Plasmodium falciparum*, the causative agent of malaria tropica, are well documented. Parasitized red blood cells are killed by 100 μM hydrogen peroxide, whereas non-parasitized erythrocytes are less susceptible to oxidative stress and do tolerate this H_2O_2 concentration.[4] The natural defense mechanism can be imitated by systems such as xanthine/xanthine oxidase or glucose/glucose oxidase[5] which produce reactive oxygen species according to the equation: substrate + O_2 $\xrightarrow{\text{substrate oxidase}}$ oxidized substrate + ROS. These systems are parasiticidal *in vitro*[5] but give rise to serious side effects such as urate production or hypoglycemia *in vivo*.

In this report, an analogous antimalarial system with probably less dangerous side effects is introduced. It consists of ethanol and yeast alcohol oxidase, a flavoenzyme

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catalyzing the reaction alcohol + O_2 → aldehyde + H_2O_2 (EC 1.1.3.13). This enzyme is specific for short chain linear aliphatic alcohols such as methanol (K_m = 0.7 mM), ethanol (K_m = 9 mM) and allyl alcohol.[8,9]

MATERIALS AND METHODS

Reagents and Medium

All reagents (from Merck, FRG, and Serva, FRG) were of the highest purity available. RPMI-1640 medium was obtained from Biochrom, West Berlin. Yeast alcohol oxidase (564 U/ml, suspended in 0.02% aqueous sodium azide and stored at −20°C) was provided by Phillips Chemical Company, Oklahoma, USA, and assayed according to the manufacturer's instructions.[9]

Parasite culture

Plasmodium falciparum (FCB strain) was cultured[10,11] using a 5% suspension of ARh$^+$ erythrocytes in RPMI-1640 medium supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, 50 μg/ml hypoxanthine, 100 μg/ml gentamycin and 10% compatible human serum. The pH of the culture medium was 7.4. The cultures (initial parasitemia 1%) were kept at 37°C in an incubator chamber containing a gas mixture of 75% N_2, 6% CO_2 and 19% O_2.

Cell culture experiments

The tests were performed in 24-well plastic plates (from Greiner, FRG) in culture volumes of 500 μl. Sorbitol-synchronized *P. falciparum*[12] was mainly at the late trophozoite stage of development (see ref. 5) when the cultures were exposed to various concentrations and combinations of alcohol oxidase and ethanol for 24 and/or 48 hours. Before use the compounds had been diluted with culture medium and sterilized by filtration. The culture medium was changed 24 hr after the beginning of each test. Giemsa-stained smears of cells were prepared after 24 hr and 48 hr and screened microscopically for morphological alterations. Parasitemia was determined as the percentage of intact parasites per 2000 erythrocytes. Controls, i.e. culture series where alcohol oxidase-catalyzed H_2O_2 production was not expected, contained the following additions:

i) alcohol oxidase: 0.1 U/ml, 1.0 U/ml or 10 U/ml;
ii) ethanol: 1.7 mM (0.01%), 8.7 mM (0.05%) or 17.4 mM (0.1%);
iii) acetaldehyde: 8.9 mM (0.05%), 17.9 mM (0.1%) or 90 mM (0.5%);
iv) no additions

All experiments were reproduced twice.

Other procedures

Hemolysis was quantitated according to refs[3] and [13]. For roughly estimating ethanol consumption by alcohol oxidase in the cultures, the integrated form of the Michaelis-Menten equation[14] was used; it was taken into account that 1 U alcohol oxidase

(defined at 25°C) corresponds to the consumption of approximately 2 μmol EtOH/min at 37°C.[9]

RESULTS AND DISCUSSION

Effects of the ethanol/alcohol oxidase system on the growth of P. faciparum in vitro

P. falciparum was found to be highly susceptible to the products of the ethanol/alcohol oxidase system. The viability of the parasites decreased in a dose-dependent manner both with ethanol and alcohol oxidase (Figures 1 and 2).

When the cultures were incubated with 0.1 U alcohol oxidase per ml and 1.7 mM ethanol, the parasitemia determined after 24 hr was found to be 50% in comparison

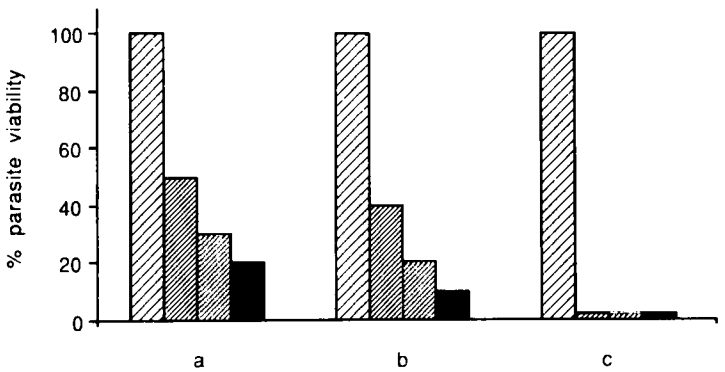

FIGURE 1 Effect of the ethanol/alcohol oxidase system on *P. falciparum* at an enzyme concentration of 0.1 U/ml. In each panel, the columns from left to right represent the effects of 0, 1.7, 8.7 and 17.4 mM EtOH (initial concentrations). (a) parasite viability after 24 hr exposure to ethanol/alcohol oxidase; (b) 24 hr exposure followed by 24 hr incubation in culture medium without additions; (c) 48 hr exposure with change of the ethanol/alcohol oxidase-containing medium at 24 hr. Viability = 100% × parasitemia of the test culture/parasitemia of the control culture.

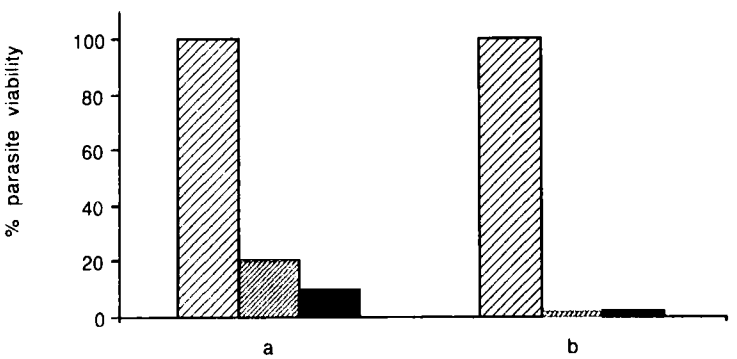

FIGURE 2 Viability of *P. falciparum* after exposure to ethanol/alcohol oxidase at an enzyme concentration of 1 U/ml. As in Figure 1, the columns from left to right represent the effects of 0, 8.7 and 17.4 mM ethanol. (a) parasite viability after 24 hr exposure to ethanol/alcohol oxidase; (b) 24 hr exposure followed by 24 hr incubation in medium without additions.

with control cultures. The corresponding values at 8.7 mM and 17.4 mM ethanol were 30% and 20%, respectively (Figure 1a). Subsequent incubation for 24 hr without ethanol and alcohol oxidase appeared to result in a further decrease of parasite viability (Figure 1b). This observation suggests that some of the parasites considered alive after 24 hr were irreversibly damaged. When exposing the parasites for a whole erythrocytic life cycle (48 hr) to ethanol and alcohol oxidase their growth was inhibited by more than 95% at all tested ethanol concentrations (Figure 1c).

In cultures containing 1 U alcohol oxidase per ml, these effects were more pronounced (Figure 2). In addition, at 17.4 mM ethanol slight hemolysis was observed after 48 hr. Exposure to 10 U alcohol oxidase per ml and 8.7 or 17.4 mM ethanol led to lysis of more than one third of the erythrocytes; no viable parasites were observed after 24 hr.

Control tests with ethanol or alcohol oxidase

In the absence of the enzyme, none of the tested ethanol concentrations (1.7 mM, 8.7 mM, 17.4 mM) had a significant effect on the growth of *P. falciparum* or on the integrity of erythrocytes. Exposure of the cultures to 0.1 or 1.0 U/ml alcohol oxidase without substrate was also found to have no obvious influence on the viability of the parasites. Hemolysis was not observed either.

It should be noted that the substrates and products of alcohol oxidase are volatile. For this reason the cultures should be kept as separate as possible. Incubation with alcohol oxidase next to an ethanol-containing well, for instance, would lead to inhibition of parasite growth since ethanol can reach the enzyme-containing culture aliquot via the vapor phase.

Effects of the reaction product acetaldehyde

Acetaldehyde was found to have an inhibitory effect *per se*. This was not surprising because a number of other aldehydes have been reported to be toxic for malaria parasites at concentrations below 0.1 mM,[15] (see also ref. 16). As shown in Figure 3, a 24 hr incubation of the parasites with 17.9 mM (0.1%) acetaldehyde reduced their

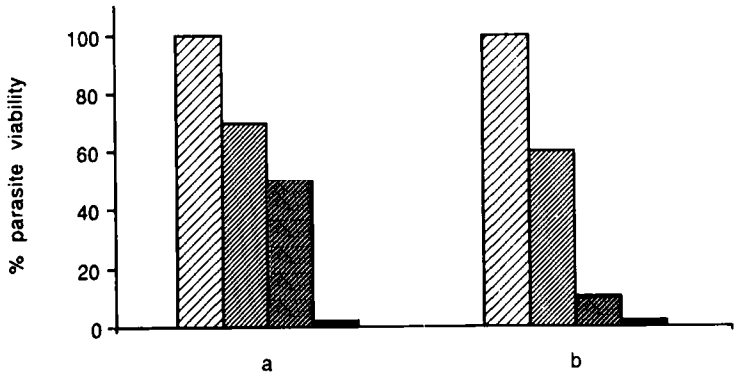

FIGURE 3 Effect of acetaldehyde on *P. falciparum in vitro*. In both panels, the columns from left to right represent the effects of 0, 8.9, 17.9 and 90 mM acetaldehyde. (a) parasite viability after 24 hr exposure to acetaldehyde; (b) 24 hr exposure followed by 24 hr incubation in medium without additions. Acetaldehyde at 90 mM led to the release of immature parasite stages from the erythrocytes.

viability by more than 50% as observed after 24 hr and by more than 90% after 48 hr. 90 mM or 180 mM acetaldehyde resulted in total inhibition of the parasites' growth after 24 hr. Predominantly at these high concentrations swelling and subsequent lysis of parasitized erythrocytes were observed, whereas non-parasitized red blood cells remained morphologically intact. As the release of immature parasites might be used in experimental malaria research the effects of acetaldehyde deserve further study.

Hydrogen peroxide

A comparison of Figure 1 and 2 shows that H_2O_2 is the more effective parasiticidal product of the alcohol oxidase-catalyzed reaction. At an ethanol concentration of 8.7 mM, an equimolar acetaldehyde concentration is the highest possible product level. 8.9 mM acetaldehyde, however, is much less effective than the combination of alcohol oxidase and 8.7 mM ethanol.

The effects of H_2O_2 on parasitized red blood cells are well documented[3-5] and were not studied systematically here. The typical H_2O_2-induced transient colour change from red to brown[5] was observed in samples with an ethanol concentration of > 8.7 mM and 1 U/ml alcohol oxidase. Under these conditions the theoretical maximal rate of H_2O_2-production is 1 μmol/ml · min.[14]

Catalase, an H_2O_2 scavenger, cannot be used for differentiating between the effects of acetaldehyde and H_2O_2 since the peroxidase activity of this enzyme (H_2O_2 + ethanol → acetaldehyde + $2H_2O$) competes with its catalatic activity.[17]

OUTLOOK

When considering the ethanol/alcohol oxidase system for the chemotherapy of malaria in animal models,[1,2,16] the conditons shown in Figure 1 (0.1 U alcohol oxidase/ml, 1.7 mM–17.4 mM ethanol, both given at 0 hr and 24 hr) could be chosen as a starting point, although the *in vivo* conditions differ from our cultures. The hematocrit, e.g., is in blood 50% instead of 5% which implies higher levels of H_2O_2-degrading enzymes; on the other hand, the O_2 supply is more constant in the bloodstream than in the culture wells. The potential problems of alcohol oxidase as an immunogen could be solved by applying the enzyme bound to a pharmaceutical carrier such as dextran[18] or polyethylene glycol.[19] These procedures would also guarantee a constant enzyme level in the blood.

If the principle of exogenous oxidative stress establishes itself in malaria chemotherapy[1-6,20] the ethanol/alcohol oxidase system might become attractive for the following reasons:

a) the required ethanol concentration is quasi-physiological (17.4 mM is the legal limit for motorcar drivers in Great Britain),

b) metabolism and pharmacology of substrates (ethanol and O_2) and products (acetaldehyde and H_2O_2) are well known,

c) serious side effects are not expected — if necessary, acetaldehyde could be detoxified enzymatically — and

d) substrate and enzyme are relatively inexpensive.[21,22]

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