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EFFECTS OF QUINOLINE-CONTAINING ANTIMALARIALS ON THE ERYTHROCYTE MEMBRANE AND THEIR SIGNIFICANCE TO DRUG ACTION ON *PLASMODIUM FALCIPARUM*

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Abstract—Quinoline-containing antimalarials are cationic amphiphiles which accumulate to high levels in lysosomes and are known to interact with membrane phospholipids. It was therefore hypothesized that they could exert their antimalarial effect by compromising the integrity of the parasite's acidic organelles. To test this hypothesis, the effects of chloroquine (CQ), quinine (Q) and mefloquine (MQ) on the osmotic stability of human red blood cells exposed to hypotonic solutions have been investigated. With CQ and Q stabilization was observed at pH 7.8 and destablization at pH 5, indicating that destabilization is caused by the protonated forms of the drugs. With MQ the pH dependence was reversed, i.e. it destabilized at pH 7.8 and stabilized at pH 5, suggesting that destabilization is caused by the unprotonated drug. MQ caused cell lysis at the tenth millimolar range by a detergent effect. The possible destabilizing effect of drugs on the membranes of *Plasmodium falciparum* acidic organelles was investigated in metabolically-labelled parasites. We expected an increase in degradation of parasite proteins if drugs did indeed cause the release of acid hydrolases from destabilized organelles to the cytoplasm. No effect of drugs on parasite protein degradation could be observed, but protein synthesis was inhibited at therapeutic drug concentrations. These results imply that quinoline-containing antimalarials do not compromise the integrity of parasite acidic organelles, and that inhibition of protein synthesis results from a limited supply of essential amino acid(s) due to the demonstrable drug-mediated suppression of parasite digestion of host cell cytosol.

One of the major reasons for malaria resurgence is the demonstrable increase of parasite resistance—to antimalarial drugs in general [1], and to the most widely used drug chloroquine (CQ), in particular. We understand neither the mode of action of the latter antimalarial nor that of other quinoline-containing compounds, such as quinine (Q) or the quinoline methanol mefloquine (MQ), and in consequence we do not understand the reason for drug resistance [2]. In a recent review on the mode of action of chloroquine, we have suggested that these drugs, which can be concentrated to high levels in the acidic compartments of malaria-infected red blood cells by virtue of their weak base and amphipatic properties, might compromise the integrity of these compartments [3]. As a result, hydrolases would be released into the parasite cytosol with consequent damage to the cellular machinery, as has been observed in liver [4] and retina cells [5] treated with CQ. The destructive effect of the drugs could result from osmotic swelling and subsequent lysis of the acidic compartments, and/or from the disruption of their membrane by a detergent-like effect. This latter presumption stems from the following observations: antimalarial drugs bind to phospholipids [6] and cell membranes [7]; Q and MQ (but not CQ) intercalate into phospholipid monolayers [8, 9]. The concentrations at which these effects are observed are compatible with the concentrations calculated to prevail in the acidic organelles of the parasite when infected red blood cells are exposed to therapeutic drug levels (taking into consideration the pH gradient and the p*K*as of the respective compounds). Once partitioned into membranes to some critical concentration, depending on the hydrophobic and ionic nature of the drug, these drugs should, like other amphiphiles, cause membrane disruption [10]. The above description suggests that membrane destabilization may be a part of the antimalarial effect of these drugs. The question then arises as to whether such a mechanism could operate *in situ*, i.e. in the presence of cellular proteins and other soluble components, compounds which are known to complex with the drugs or reduce their association with phospholipids and membranes [11].

In the present work, the effects of quinoline-containing antimalarial drugs on cell membrane stability and on parasite protein degradation have been studied. It is shown that although a considerable degree of membrane destabilization can be observed, no indication of an increased digestion of parasite proteins can be detected in the presence of therapeutic drug concentrations.

MATERIALS AND METHODS

Cells. Normal red blood cells (NRBC) were obtained from the Shaarei Zedek Hospital, Jerusa-

lem, or from healthy volunteers and kept refrigerated in ACD medium. Prior to experimentation, cells were rejuvenated by overnight incubation at 5% hematocrit at 37° in the following medium: NaCl 120 mM, KCl 10 mM, K-phosphate 10 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2 mM, glucose 10 mM and adenine 5 mM (pH 7.4).

Malaria-infected cells (IRBC) were cultivated as described before [12]. Briefly, washed NRBC were suspended at 2.5% hematocrit in culture flasks (Nunc or Sterilin) containing RPMI-1640 medium (GIBCO) supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, 10 mM glucose and 10% heat-inactivated AB^+ human plasma and inoculated with *Plasmodium falciparum* (FCR-3) infected cells. The flasks were gassed with a mixture of 90% N_2, 5% CO_2 and 5% O_2 and incubated a 37°. The medium was changed daily followed by gassing. The percentage of infected cells (parasitemia) was determined by microscopic inspection of Giemsa stained thin blood smears. Synchronization of cultures was obtained by lysing erythrocytes harbouring mature parasites with isotonic mannitol [13].

Osmotic fragility. Rejuvenated or fresh NRBC were washed once in HEPES buffered saline (HEPES 10 mM, NaCl 140 mM, pH 7.4, =HBS) and resuspended in this medium to 20% hematocrit. Twenty μl of this suspension were mixed with 1 ml of hypotonic HBS (tonicity was determined with a Wescor vapor pressure osmometer and adjusted to cause ≈50% hemolysis in absence of drugs) containing different concentrations of different drugs, as detailed in the Results section. After 30 min incubation at 37°, cells were spun down and the hemoglobin absorbance in the supernatant was determined spectrophotometrically at 540 nm. This value was compared to that obtained in the absence of drug. A ratio of test to control absorbance larger than 1 was defined as destabilization of the cell membrane and a ratio smaller than 1 was defined as stabilization [14].

Drug-induced potassium leak. Rejuvenated or fresh NRBC were suspended in isotonic HBS containing increasing concentrations of different drugs to 0.4% hematocrit. Samples were taken immediately and after 30 min incubation at 30°, centrifuged and the K^+ content of the extracellular space was determined in the supernatant after centrifugation of the cells by atomic absorption spectrometry (Varian) and related to the total K^+ content of the same number of cells.

Effect of drugs on parasite protein turnover. Infected cells at the ring stage (20% parasitemia) were treated once with isoosmotic mannitol to obtain tighter synchronization and returned to culture conditions for 24 hr in the presence of 5 μCi/ml [^{3}H]isoleucine. Cells were then washed in phosphate-buffered saline (pH 7.4, PBS) until no radioactive counts could be detected in the supernatant. The cells were resuspended in either PBS + 10 mM glucose or in growth medium containing increasing concentrations of drugs. Aliquots of 50 μl were taken in triplicate at different time intervals, mixed with 5 μl of 50% (w/v) trichloroacetic acid and centrifuged. The radioactivity in the supernatant was determined by scintillation counting.

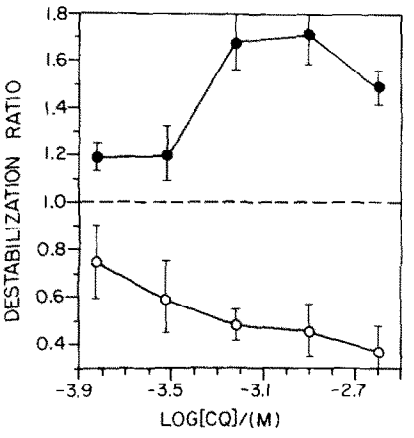

Fig. 1. Effect of CQ on the osmotic stability of human erythrocytes. Rejuvenated red blood cells were suspended at 0.4% hematocrit in hypotonic HBS which caused 50% lysis after 30 min incubation at 37°. Parallel systems containing increasing concentrations of CQ, were similarly treated and their percentage of lysis was divided by that of control to yield the destabilization ratio (see text for meaning of this term). Experiments were performed at pH 5 (closed symbols) and at pH 7.8 (open symbols).

RESULTS

Effect of antimalarial drugs on RBC membrane stability

All three drugs tested had an effect on the hypotonic lysis of human erythrocytes but this depended on the pH of the incubation medium: CQ afforded resistance to lysis at pH 7.8 but destabilized the membrane at pH 5 (Fig. 1). Q behaved similarly, but its destabilizing effect was discernible only at significantly higher concentrations (Fig. 2). This behaviour suggests that the destabilizing effect is caused by the protonated form(s) of the drugs. MQ had an opposite effect (Fig. 3): it stabilized the membrane at pH 5 and at pH 7.8 in the low concentration range but behaved as a destabilizer at higher concentrations, probably due to its lytic effect (see below).

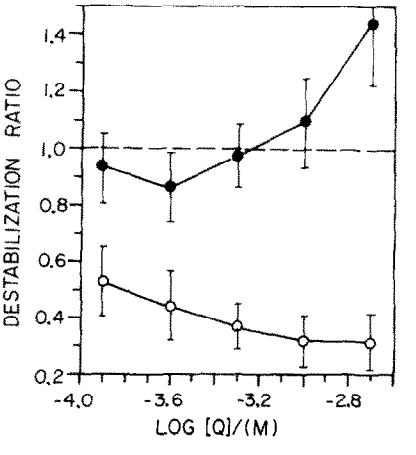

Fig. 2. Effect of Q on the osmotic stability of human erythrocytes. See legend to Fig. 1 for details.

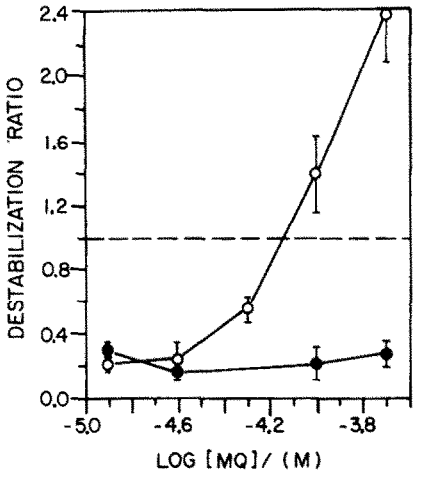

Fig. 3. Effect of MQ on the osmotic stability of human erythrocytes. See legend to Fig. 1 for details.

Effect of drugs on cell lysis and K^+-leak from NRBC

When cells were exposed to MQ at isotonic conditions and pH 7.4, total hemolysis was observed at about 0.3 mM (Fig. 4) and the addition of 50 mM sucrose did not afford any protection (data not shown), suggesting that hemolysis is not due to cation-induced leak, but rather to a detergent effect [15]. No effect of MQ could be observed at pH 5 or of CQ or Q at both pH 5 and pH 7.4.

In order to verify the nature of the drug effect on osmotic fragility, the permeability of NRBC membranes to K^+ in the presence of different drug concentrations and at different pHs was tested (Fig. 4). At pH 7.4 but not at 5, MQ induced a considerable K^+-leak at concentrations which were lower than those causing lysis. CQ had no effect whatsoever and Q induced a measurable leak above 0.25 mM at pH 5, but had no effect at pH 7.4.

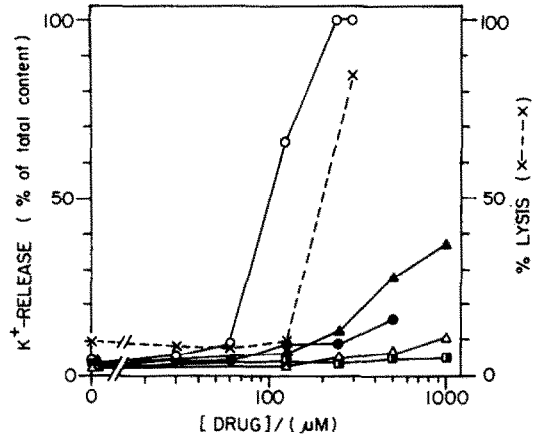

Fig. 4. Effect of drugs on cell lysis and K^+-leak from red cells. Rejuvenated cells were suspended at 0.4% hematocrit in isotonic HBS containing increasing concentrations of CQ, Q and MQ. After 30 min incubation at 30°, the K^+-content of the extracellular medium was determined by atomic absorption spectroscopy and calculated as a percentage of the total content of the suspension, ○, MQ, pH 7.4; ●, MQ, pH 5; △, Q, pH 7.4; ▲, Q, pH 5; □, CQ, pH 7.4; ■, CQ, pH 5. In the case of MQ, incubation resulted in cell lysis. This was quantified by measuring the absorbance of released hemoglobin at 540 nm and calculating the percentage of lysis with respect to the total hemoglobin content of the cell suspension – X. SD of the means in both types of experiments were smaller than 5%.

Effect of drugs on the turnover of parasite proteins

By virtue of their weak base properties, antimalarial drugs accumulate in acidic compartments of the parasite (lysosomes and food vacuole). Since the pH of the parasite food vacuole is around 4.5 to 5 [16, 17] and the extracellular pH is usually maintained at 7.4, monobasic drugs could concentrate in the acidic organelle several-hundred-fold, while the dibasic drugs could theoretically concentrate to the square of their monobasic congeners. Thus, when malaria-infected cells are exposed to therapeutic drug levels [18], the drugs could attain inside the food vacuole and the lysosomes levels that have been shown above to cause membrane destabilization.

However, the effect of the drugs on the parasite acidic organelles may be quite different from that on the erythrocyte: the effect of amphiphiles depend to a large extent on the lipid composition of the membrane [19] and the phospholipid composition of malarial parasites is distinctly different from that of their host erythrocytes [20]. It seemed therefore important to verify whether drugs could disrupt the membranes of the acidic compartments thereby releasing hydrolases into the cytosol to hydrolyse, eventually, parasite proteins. If this were to occur, one would expect to see in the cytoplasm radiolabeled free amino acids that had been previously incorporated into parasite proteins. As shown in Fig. 5, [^{3}H]isoleucine is released from metabolically

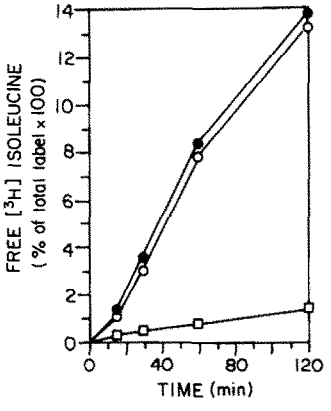

Fig. 5. Release of [^{3}H]isoleucine from malaria-infected red blood cells. Infected cells were metabolically labelled with radiolabelled isoleucine as described in Materials and Methods. After washing the cells in PBS from free label, cells were resuspended in various media at 5% hematocrit and incubated at 37°. At different time intervals aliquots were taken, deproteinized with TCA and the content of radiolabel was determined by scintillation counting. □, PBS + 10 mM glucose; ○, culture growth medium; ●, PBS + 10 mM glucose + 1 mM isoleucine. Results are depicted as label released as percentage of total label content vs. time of incubation. SD were smaller than 5% of the means.

labelled parasites incubated in PBS + glucose. This release is considerably enhanced when the cells are incubated in growth medium which contains a full complement of free amino acids or incubated in PBS + glucose containing 1 mM unlabelled isoleucine. This enhancement is undoubtedly due to competition between labelled isoleucine which is produced by protein turnover and the unlabelled isoleucine. A high rate of release was also observed when cells were incubated in PBS + glucose containing cycloheximide, an inhibitor of protein synthesis (data not shown). The presence of drugs had no effect on the rate of isoleucine release when the incubation was carried out in growth medium or in PBS + glucose and cycloheximide (not shown). Under these conditions any additional proteolytic activity, such as that caused by the release of acid hydrolases from the drug-destabilized acidic organelles, would have resulted in an increased level of free [^{3}H]isoleucine. In the PBS + glucose medium, where most of the labelled free acid produced by the natural turnover of parasite proteins is expected to be re-used in synthesis, all three drugs caused an increased release. This could have resulted from inhibition of protein synthesis.

DISCUSSION

The established effects of cationic and non-ionic amphiphiles on the osmotic behavior of erythrocytes [10], can probably explain the effects of the antimalarial drugs tested in the present work. The common feature of such compounds is that at low concentrations they protect erythrocytes against hypotonic lysis, whereas at higher concentrations they generally destabilize the cell membrane and could even induce lysis. The antihemolytic effect probably arises from their ability to intercalate into the lipid bilayer of the membrane, thereby expanding the membrane and allowing the cell to swell to a larger volume before it lyses. The hemolytic or destabilizing effect can be related to their surfactant properties which are expressed at relatively higher concentrations. At intermediate concentrations amphiphiles are known to fluidize membranes [21], and thereby could increase the ground permeability of the bilayer to small solutes. It is generally accepted that the interaction of ionic amphiphiles with membranes depends both on their hydrophobicity and their charge [22], but it is not yet established what is the relative contribution of the charged and the uncharged forms to the stabilizing, the fluidizing or the detergent effects [23].

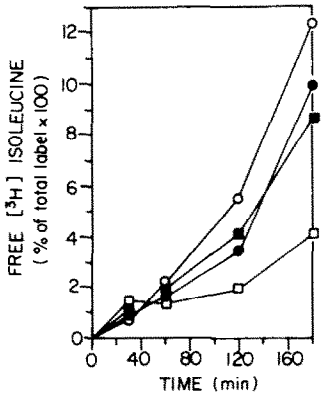

Fig. 6. Effect of drugs on the release of [^{3}H]isoleucine from malaria-infected cells. Experiments were conducted as described in the legend to Fig. 5, in (□) PBS + 10 mM glucose medium, (○) +3 μM CQ, (●) +3 μM Q, (■) +1 μM MQ. SD were smaller than 5% of the means.

The quinoline-containing antimalarial drugs are cationic amphiphiles which accumulate in acidic compartments of the parasite (lysosomes and food vacuole) by virtue of their weak base properties. Since the pH of the parasite food vacuole is ≈4.8–5 [16, 17, 24] and the extracellular pH is usually maintained at 7.4, monobasic drugs could concentrate in the acidic organelle some 250–400-fold, while the dibasic drugs could theoretically concentrate 6.3×10^4- to 1.6×10^5-fold [25]. Thus, when malaria-infected cells are exposed to therapeutic drug levels (MQ $\approx 1 \times 10^{-9}$–1×10^{-8} M; Q and CQ $\approx 1.5 \times 10^{-8}$–$1 \times 10^{-6}$ M; [18]), the drugs could attain in the lysosomes and the food vacuole levels that could cause membrane destabilization and lysis of these organelles. That this calculation holds true in malarial parasites is corroborated by the fact that when the pH of the food vacuoles is calculated according to the formalism of Schuldiner *et al.* [25] using the accumulation ratios of monobasic methylamine and the dibasic CQ, the same values are obtained [16].

From the above description it is clear that each of the drugs which were tested in this work was expected, *a priori*, to have a different potency as a membrane affector. Thus, the potency of MQ could be correlated to its demonstrable binding to phospholipid [6] and biological [7] membranes, and its intercalation into them with high affinity [8]. However, where MQ could have reached permeabilizing or lytic concentrations, the pH is acid and at this pH MQ acts as a stabilizer. Part of the stabilizing effect of MQ can be assigned to its effect on K^+-leak. In any case, however, the effects of MQ on the red blood cell membrane are observed at considerably higher concentrations than this drug could theoretically achieve in the acidic organelles of the parasite.

Q in comparison to MQ, does not appreciably accumulate in erythrocytes [26] and intercalates poorly into phospholipid monolayers [8]. Nevertheless, it induces K^+-leak without causing cell lysis, and somewhat destabilizes the membrane at pH 5. These effects are observed at the same concentrations, suggesting a common mechanism, and at drug levels which could be easily reached inside the acidic organelles. Since Q has been demonstrated to fluidize and permeabilize phospholipid membranes [21], it could have a similar effect on the erythrocyte cell membrane.

Like, Q, CQ destabilizes the membrane of red blood cells at pH 5 but not at 7.8, though this is not accompanied by an induction of a cation leak. The effect of CQ is observable in the millimolar range, a level which is lower than the theoretical maximal accumulation. Although it is obvious from the pH dependence, that CQ acts in its protonated form, its

precise mode of action is still obscure. In fact, CQ has been known for a long time to be a stabilizer of lysosomal membranes [26] and as such has been used as an anti-inflammatory drug. Conflicting data have been reported on the interaction of CQ with phospholipid membranes: binding was observed both with neutral and more so with acidic phospholipids [11, 28]; although no intercalation could be observed at low concentrations [23, 29], at higher levels the drug clearly abolished the phase transition of phosphatidylcholine [30] and this was alluded to the small amounts of free base which dissolve into the membrane [31]; fluidization could not be demonstrated with the neutral phosphatidylcholines [32], but was conspicuous with acidic phospholipids [33]. Like Ca^{2+} which binds to phospholipid interfaces (and which competes with CQ binding [9] with a 1:2 stoichiometry), CQ could also bind to two adjacent phospholipid molecules, thereby exerting its stabilizing effect. However, its higher affinity to acid phospholipids could also induce a phase separation which could induce leakage and membrane destabilization.

The difference in lipid composition of the red cell membrane vs that of the acidic parasite organelles warranted testing the effect of the antimalarial drugs on the integrity of these organelles. Lysis of their membranes should have released acidic proteases which would have degraded cytosolic proteins, but such an effect was not observed. This cannot be explained by the unfavorable neutral pH of the cytosol, since even at such pH, acidic hydrolases have a finite activity. The pH-dependence of membrane destabilization suggest that it is primarily the unprotonated forms of CQ and Q and the protonated form of MQ which exerts the effect. While this observation could explain the differential antimalarial effect of these drugs [34] and the fact that CQ-resistant parasites are cross-resistant to Q but susceptible to MQ [18], membrane destabilization is probably not an important factor in the action of these drugs.

This conclusion leaves two other alternatives for the mode of action of the quinolinic antimalarials: (a) alkalinization of the food vacuole to a pH unfavorable to the activity of acid hydrolases [35]; and (b) specific effects of the drugs on the enzymatic machinery operating in these organelles [11, 36, 37]. Eventually, the final result of drug action will be the same: inhibition of the feeding process [38, 39].

Finally, the specific antimalarial effect of the quinoline-containing drugs should be accounted for, since the same general effects could also be exerted on host cells. Although CQ demonstrably alkalinizes the lysosomes of somatic cells [40], its effect on the pH of the food vacuole of the malarial parasite is a subject of controversy: some investigators contend that all quinoline-containing antimalarials act by alkalinizing the food vacuole [24] and have demonstrated this effect in detergent-permeabilized cells. Others have clearly demonstrated that in intact infected cells, CQ does not alter the pH when cells are exposed to therapeutic drug levels [16, 17]. Wherever lies the truth, the antimalarial effects are usually observed at concentrations which do not cause alakalinization in lysosomes of somatic cells, thus explaining their specificity against the parasite. We suggest that the feeding process is so vital to the parasite, that its mere inhibition by drugs [39] could compromise parasite growth and development. Thus, if an amino acid cannot reach the parasite from without the infected cell and its supply would depend exclusively on the feeding process, the inhibition of the latter should result in the inhibition of parasite protein synthesis. This has been clearly demonstrated in this work. Inhibition of protein degradation by lysosomotropic amines, and thereby of protein synthesis due to restriction on amino acid supply, has been found in hepatocytes [41]. Most importantly, the fact that much higher CQ concentrations were needed there, and that the inhibitory effect was assigned exclusively to alkalinization, may suggest that the susceptibility of the acidic hydrolases of the parasite is substantially larger than that of the enzymes of somatic cells.

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