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Quinine Uptake by Human Polymorphonuclear Neutrophils

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The antimalarial drug quinine has been shown to impair human polymorphonuclear leukocyte (PMN) functions. To gain insight into the mechanism of this phenomenon, we investigated quinine uptake by PMN with a fluorometric assay based on the fluorescence properties of this drug. After 30 min of incubation at 37°C in the presence of 1 and 10 μg of quinine per ml, PMN-associated quinine reached 90 ± 6 and 780 ± 150 ng/2.5 × 10^6 PMN, respectively, giving a cellular-to-extracellular concentration ratio of 140 to 150. A steady state was reached within 5 min. Uptake was partially dependent on temperature, cell viability, and extracellular pH. Fractionation studies showed that 30 to 40% of the PMN-associated quinine was located in the particulate fraction. The efflux of PMN-associated quinine was rapid and complete when the incubation mixture was replaced by drug-free medium. These data suggest that several mechanisms are involved in the uptake of quinine by PMN, including a viability- and energy-independent process possibly related to reversible association of quinine to cell structures (particularly the membrane). Other mechanisms could involve trapping by protonation and/or active PMN transport systems. Thus, most of the quinine taken up by resting PMN is found in the soluble fraction of disrupted cells. This may partly explain the depressive properties of quinine.

Numerous data are available concerning the intracellular pharmacokinetic behavior of antibacterial agents (6, 7, 10, 14), whereas other anti-infective agents, in particular antimalarial agents, have been little explored in this respect, with the exception of chloroquine (22, 23). However, such studies are undoubtedly of interest for the following reasons. (i) They may provide a valuable pharmacokinetic parameter, since because of their large numbers leukocytes may represent an important drug reservoir. (ii) Since most antimalarial drugs depress leukocyte functions, knowledge of their possible uptake by phagocytes may provide insight into the mechanisms underlying such effects. (iii) Cell-penetrating antimalarial drugs may be useful tools for studying the mechanisms of phagocyte activation and function, as has been proposed for chloroquine (11, 25). We and others (3, 8, 21) have previously observed that quinine, the oldest still widely used antimalarial agent, strongly depresses polymorphonuclear neutrophil (PMN) functions in vitro at concentrations (<10 μg/ml) relevant to those therapeutically achievable in plasma (30). Quinine was one of the first drugs found to fluoresce and is widely used as a reference standard for fluorescence assays (28); this property has also been used to study the pharmacokinetic profile of quinine after extraction or high-performance liquid chromatographic separation from biologic samples (2). We used this natural property of quinine to investigate the association of quinine with PMN. Studies conducted with other fluorescing antibacterial agents, namely quinolones (19, 20), have shown that the sensitivity of this technique is similar to that of standard radioisotopic techniques or high-performance liquid chromatography (14).

MATERIALS AND METHODS

Quinine. Quinine sulfate was dissolved immediately before use in sterile distilled water (1 g/liter) and then diluted in Hanks balanced salt solution (HBSS), pH 7.4, to the desired concentration.

PMN. PMN were recovered from heparinized blood of healthy donors and purified by previously described methods (16), i.e., sedimentation on 2% dextran T-500 and centrifugation on a Ficoll-Paque density gradient (Pharmacia), followed by hypotonic lysis of residual erythrocytes. Preparations contained >97% PMN, and viability, assessed by trypan blue exclusion, was greater than 95%.

Quinine uptake by PMN. PMN (2.5×10^6) were incubated in 0.5 ml of HBSS containing concentrations of quinine therapeutically achievable in serum (0.5 to 10 μg/ml) (30). An extratherapeutic concentration of quinine (25 μg/ml) was also tested. After 30 min of incubation at 37°C, the cells were separated from the extracellular medium by velocity gradient centrifugation through a water-impermeable silicone-paraffin oil barrier (7, 10) at 12,000 × *g* for 3 min in a Beckman microcentrifuge. The cells were recovered after aspiration of the upper aqueous and oil phases. Lactate dehydrogenase (LDH) activity (1) was measured in both fractions (aspirate and cell pellet) and showed that more than 94% of the cells were recovered intact in the pellet. The fluorescence method of Udenfriend (28) was used to determine the amount of PMN-associated quinine. Briefly, the cells were suspended in 1 ml of 0.1 N sulfuric acid and sonicated (3 × 10 s; Fisher 20/200 SV TC4C). Fluorescence emission was determined in a fluorescence spectrophotometer (Aminco SPF 500); the fluorescence excitation and emission maxima of quinine in sulfuric acid at 0.1 N are 350 and 450 nm, respectively (28). The background fluorescence emitted by PMN in the absence of quinine was subtracted from the results obtained in the presence of quinine. Two standard dilution curves of quinine were made in sulfuric acid alone or in the presence of a sonicated PMN preparation. PMN extracts did not modify the fluorescence of quinine.

Measurement of cell volume. The intracellular water space was measured by using tritiated water (10 mCi/ml; Centre National de l'Energie Atomique, Saclay, France); ^{14}C-labeled polyethylene glycol was used as the extracellular

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marker (0.8 mCi/g; Dupont NEN Research Products, Boston, Mass.). PMN were incubated with these radiolabeled compounds for 5 min at 37°C before separation from the extracellular fluid by velocity gradient centrifugation, as described above, and counted in a liquid scintillation counter. The total water content of the PMN pellet was corrected by the trapped extracellular water, i.e., the polyethylene glycol space, to obtain the intracellular water space.

Characterization of quinine uptake. (i) Uptake kinetics. Uptake kinetics were studied by using incubation periods of 1 to 30 min.

(ii) Influence of temperature and metabolic inhibitors. The influence of temperature on quinine uptake was evaluated by performing incubation at 4 and 37°C. In further experiments, PMN were incubated for 10 min at 37°C with 1×10^{-3}, 2×10^{-3}, or 5×10^{-3} M sodium fluoride or potassium cyanide (Sigma Chemical Co., St. Louis, Mo.) as described by Pascual et al. (19) and Ishiguro et al. (7). Quinine was then added, and uptake was measured as described above.

(iii) Influence of extracellular pH. Quinine uptake was determined after adjusting the pH of the medium to 6, 7.4, or 9. The effect of collapsing the proton gradient was studied by using the proton ionophore carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) (Sigma) as described by Klempner and Styrt (10). Briefly, PMN were incubated at 37°C in HBSS containing 50 μM CCCP for 10 min; quinine was then added, and uptake was measured as described above. With each of these variables (temperature, KCN, NaF, pH, and CCCP), cell viability, determined by measuring LDH activity and trypan blue exclusion, was consistently greater than 92%.

(iv) Influence of cell viability. Quinine uptake by nonviable PMN was measured after exposure of the cells to 10% formaldehyde (E. Merck AG, Darmstadt, Federal Republic of Germany) for 30 min, followed by washing and suspension in fresh HBSS.

(v) Efflux and reversibility of binding of PMN-associated quinine. PMN were incubated for 30 min at 37°C with 10 μg of quinine per ml, collected by centrifugation, and immediately suspended in drug-free HBSS at 37°C. PMN-associated quinine was measured at defined intervals (5, 10, 15, 30, and 60 min). In other experiments, drug-free medium was renewed after the first 10 min of incubation and cell-associated quinine was measured after 10 and 20 min of incubation.

(vi) Localization of PMN-associated quinine. PMN were preincubated with quinine, isolated as described above, and then sonicated for 3 × 10 s. Soluble and insoluble materials were separated by ultracentrifugation at 100,000 × *g* for 30 min (Beckman TL 100). LDH and lysozyme activities were measured in each fraction (17). More than 97% of LDH activity and 80% of lysozyme activity, relative to those of control PMN lysed in 0.05% Triton X-100, were contained in the supernatant, showing the presence of most of the cytosol and granule content.

In other experiments, (i) PMN preincubated with quinine were sonicated in the presence of the drug and (ii) a freshly sonicated PMN preparation was incubated with 10 μg of quinine per ml for 30 min. The soluble and particulate fractions were separated by velocity gradient ultracentrifugation through an 85 to 15% (vol/vol) silicone-paraffin oil barrier. Quinine was then measured in both fractions as described above. Protein assays showed that all of the insoluble material was recovered through the 85 to 15% silicone-oil barrier by comparison with a sonicated PMN preparation centrifuged at 100,000 × *g* without the barrier (data not shown).

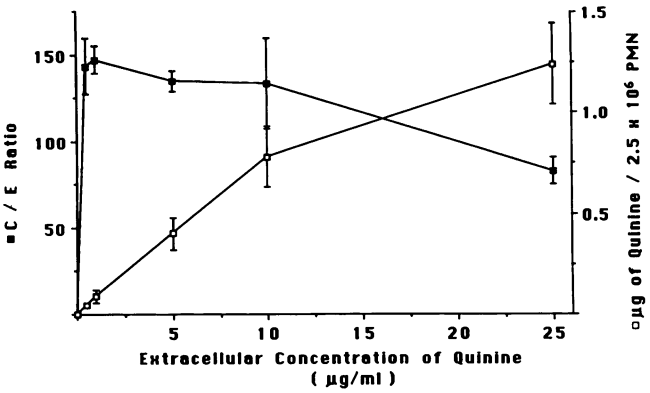

FIG. 1. Quinine uptake by PMN at different extracellular concentrations. Cells were incubated for 30 min at 37°C. Values are means ± 1 SD of four experiments. C/E, cellular/extracellular.

(vii) Control experiments. Control experiments showed that no quenching of quinine fluorescence occurred, whatever the experimental conditions.

Statistical analysis. Data were analyzed with Student's *t* test for paired data. The results are expressed as means ± standard deviations (SD) of *n* experiments.

RESULTS

Viability. PMN viability, assessed in terms of trypan blue exclusion and LDH release, was unaffected by quinine at concentrations of up to 100 μg/ml.

Determination of intracellular volume. The mean intracellular volume in eight experiments was 0.60 ± 0.08 μl/2.5 × 10^6 PMN, a value similar to those obtained by various investigators (7, 10, 14, 18, 29). This value was used to express PMN-associated quinine as the ratio of cellular-to-extracellular concentrations.

Quinine uptake by PMN. The overall amount of PMN-associated quinine is shown in Fig. 1 as a function of the extracellular concentration. After 30 min of incubation in the presence of therapeutic concentrations of the drug (1 to 10 μg/ml), the amount of PMN-associated quinine was directly proportional to the extracellular concentration. The cell/medium concentration ratios reached 143 ± 16.4 and 133 ± 25.7 with extracellular concentrations of 0.5 and 10 μg/ml, respectively. When the extracellular concentration of quinine was further increased (25 μg/ml), there was an apparent decrease in the cellular-to-extracellular concentration ratio (83 ± 7.9), which suggests a saturable phenomenon and/or complex drug entry-release mechanism with different influx-efflux velocities. All further determinations of quinine uptake were thus performed in the range of therapeutic values (1 to 10 μg/ml).

Characterization of quinine uptake. (i) Quinine uptake was rapid (Fig. 2). The maximal value was attained within the first 5 min and remained constant for at least 30 min.

(ii) Lowering the temperature from 37 to 4°C resulted in a significant decrease in maximal PMN-associated quinine (Fig. 3). Only very high concentrations (5 mM) of both metabolic inhibitors were effective in reducing quinine uptake by about 30% ($P < 0.05$; three experiments). This high concentration of inhibitors did not impair PMN viability.

(iii) Changes in the pH of the medium (pH range, 6 to 9) significantly affected quinine uptake (Fig. 4). Lowering the

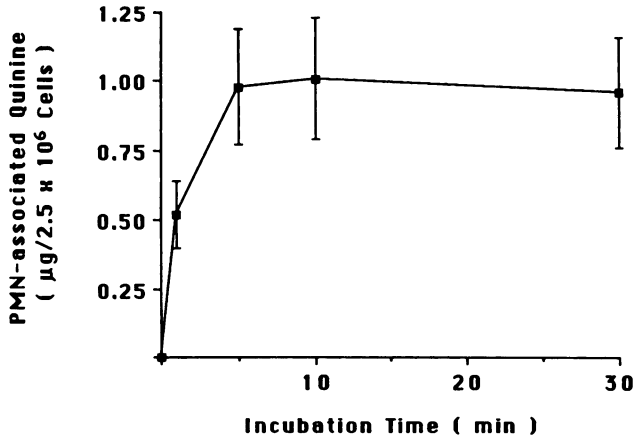

FIG. 2. Kinetics of quinine uptake by PMN. PMN were incubated with 10 µg of quinine per ml at 37°C. Results are expressed as means ± 1 SD of three experiments.

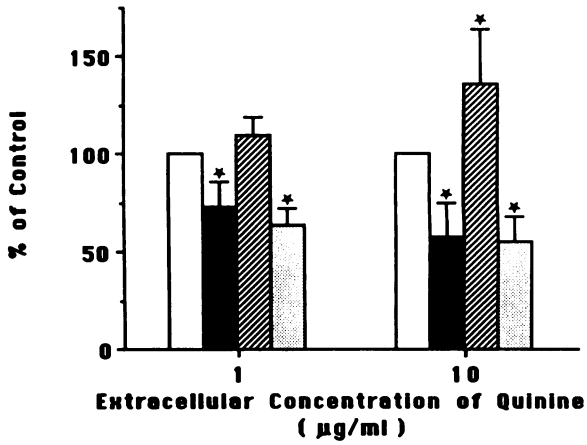

FIG. 4. Influence of pH and CCCP on quinine uptake by human PMN. PMN were incubated at 37°C with 10 or 1 µg of quinine per ml at pH 7.4 (□ [control]), 6 (■), or 9 (▨) or in the presence of CCCP (50 µM) (▩). Values are means ± 1 SD of three to five experiments. *, $P < 0.05$.

pH from 7.4 to 6 resulted in a significant decrease in uptake (about 40%); in contrast, raising the pH to 9 led to a significant increase in uptake (about 36%). Collapsing the cell proton gradient with CCCP (50 µM) also significantly reduced quinine uptake (−35 and −45% at 1 and 10 µg/ml, respectively).

(iv) The amount of PMN-associated quinine was significantly lower in Formalin-killed cells then in viable cells at both 37 and 4°C (Fig. 3).

(v) The efflux of quinine from PMN is shown in Fig. 5. When the cells were incubated in the presence of 10 µg of quinine per ml and then suspended in drug-free medium, only 31.5% of the initial cell-associated quinine remained after 10 min. This value remained relatively stable, unless fresh medium was added. When the medium was repeatedly changed (at least five times), cell-associated quinine was eliminated.

Localization of PMN-associated quinine. When PMN preincubated with quinine were first isolated through a silicone-paraffin oil barrier and then disrupted by sonication, 80.5% of PMN-associated quinine was present in the soluble material (cytosol plus intragranular content). To determine whether this value was partly due to release from insoluble material (quinine is not tightly bound to PMN), we disrupted quinine-preincubated cells in the presence of the drug. In this case, 40% of the PMN-associated quinine was found in the cell pellet (Table 1). Finally, to assess possible binding of the quinine present during cell disruption in this latter experiment, we incubated a freshly disrupted PMN preparation with quinine. Quinine binding to insoluble components of the sonicated PMN preparation was 32.5% of that associated with the intact PMN preparation. Taken together, these data suggest that quinine is partly associated with insoluble PMN components (mainly membranes) and partly concentrated in the soluble material (cytosol and/or intragranular content). The percentage of membrane-associated quinine represents at least 20% and at most 40% of the

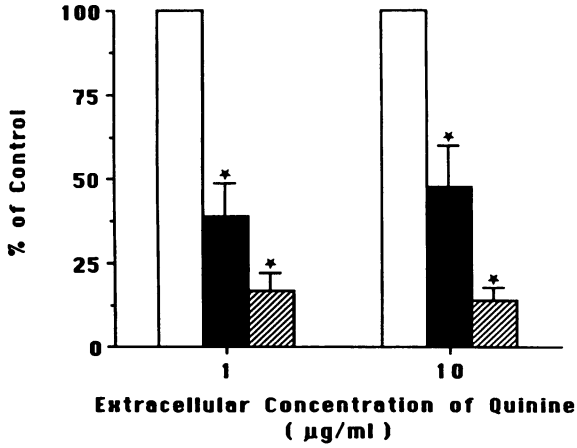

FIG. 3. Influence of temperature and cell viability on quinine uptake by PMN in the presence of 10 µg of quinine per ml. Symbols: □, viable cells incubated for 30 min at 37°C (control); ■, viable cells incubated for 30 min at 4°C; ▨, killed cells incubated for 30 min at 37°C. Values are means ± 1 SD of four experiments. *, $P < 0.01$.

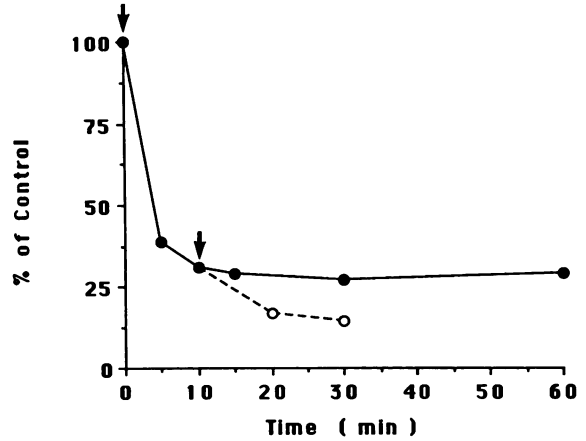

FIG. 5. Efflux of quinine from loaded PMN in drug-free medium (initial extracellular concentration of quinine, 10 µg/ml). The data are means of three experiments. Symbols: ●, single change of medium; ○, two changes. Changes of medium are indicated by arrows.

TABLE 1. Cellular localization of PMN-associated quinine

Cellular fraction[a]	Mean amt of quinine (μg) ± 1 SD[b] under condition:		
	A[c]	B[d]	C[e]
Whole PMN	0.62 ± 0.10	0.63 ± 0.17	0.71 ± 0.11
Soluble	0.51 ± 0.09 (80.5 ± 3.11)[f]		
Particulate	0.13 ± 0.03 (15.8 ± 5.44)	0.26 ± 0.09 (40.0 ± 4.07)	0.23 ± 0.04 (32.5 ± 0.80)

[a] See Materials and Methods for preparation of soluble and particulate fractions.
[b] Results are expressed as micrograms of quinine associated with 2.5×10^6 PMN (intact cells) or the corresponding soluble and particulate fractions. Means of three experiments are shown.
[c] Under condition A, PMN were preincubated with 10 μg of quinine per ml, isolated through a silicone-oil barrier, and then disrupted by sonication. Soluble and particulate fractions were separated by ultracentrifugation at 100,000 × *g*, as indicated in Materials and Methods.
[d] Under condition B, PMN preincubated with 10 μg of quinine per ml were sonicated in the presence of the drug and fractions were isolated as described in footnote *c*.
[e] Under condition C, fresh sonicated PMN samples were incubated with 10 μg of quinine per ml and fractions were isolated as described in footnote *c*. In all experiments, assays with intact PMN were performed as controls.
[f] Percentage of whole-PMN-associated quinine ([soluble- or particulate-fraction-associated quinine/whole-PMN-associated quinine] × 100).

overall cell-associated quinine. These values were similar at concentrations of 1 and 10 μg/ml.

DISCUSSION

Quinine is used widely to treat malaria. In previous studies, we and others showed that this drug impairs some PMN functions (3, 8, 21) which are thought to be important in the defense against bacterial and, possibly, parasitic infections (9, 27). To understand the mechanism of action of quinine on PMN functions, we analyzed quinine uptake by these cells. For this purpose, we developed a fluorometric assay based upon the natural fluorescence of quinine in sulfuric acid at 350 (excitation) and 450 (emission) nm (28). Similar methods have been used to measure uptake of antibiotics, including quinolones, by phagocytes (19). This technique avoids the tedious preparation required for high-performance liquid chromatography and the use of radiolabeled compounds.

Our results showed that quinine was taken up readily by resting PMN at 37°C; the cellular-to-extracellular concentration ratio of quinine was maximal within 5 min, reaching 140 to 150. Between 20 and 40% of cell-associated quinine was bound to cellular structures, mainly membranes. Our data agree with the findings of Zidovetzki et al. (31), who have shown by using nuclear magnetic resonance that the aryl methanol compounds quinine and mefloquine penetrate the phospholipid bilayer, suggesting ligand-receptor-like binding.

It is likely that several mechanisms are involved in uptake of quinine by PMN. The occurrence of an energy-independent mechanism was suggested by the following results. About 40% of control uptake was observed at 4°C, metabolic inhibitors did not impair quinine-PMN association except at very high concentrations, and the amount of quinine associated with Formalin-killed cells was approximately the same (about 30% of the quinine uptake of viable cells) as that bound by a freshly sonicated PMN preparation. However, it is possible that low temperatures and Formalin treatment not only inhibit metabolic processes but also alter the fluidity or structure of the PMN membrane (as suggested by Koga [14]). Moreover, the avid cellular concentration of quinine shown in this study also supports the notion that some type of cell membrane transport is involved.

An alternative mechanism is suggested by the fact that both acidic pH and CCCP reduced uptake. Quinine is a diprotic weak base with two pK_as (5.07 and 9.7) (24); at physiologic pH, it behaves as a monoprotic weak base, but at pH 6, it is mainly in the diprotic state. Our results suggest that quinine enters PMN better in the monoprotic state than in the diprotic state. Such a protonation trapping mechanism has been described for another antimalarial drug, chloroquine (11, 24, 25), which accumulates in lysosomes and acidic vacuoles (4, 5, 26), as well as for certain cationic antibiotics, such as clindamycin (10, 12), erythromycin (13), and roxithromycin (6). This effect of quinine ionization may also be involved in its antimalarial activity, facilitating its entry into the acidic digestive vacuole of *Plasmodium falciparum* (15).

It must be stressed that whatever the cellular localization of quinine and the mechanism of uptake, this drug is not tightly bound to PMN since in drug-free medium, quinine was quickly and completely released from the loaded cells.

Our results suggest that association of quinine with PMN impairs cell functions in two ways. (i) Binding of quinine to the membranes may alter their fluidity, and (ii) it is likely that a proportion of the cell-associated quinine induces alkalinization of cellular compartments, as is the case with other lysosomotropic weak bases (11, 12, 25).

In summary, by using a simple method we have shown that quinine accumulates strongly in PMN. The relationship between quinine uptake and inhibition of PMN functions is currently under study.

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