Uptake of chloroquine and hydroxychloroquine by human blood leucocytes in vitro: relation to cellular concentrations during antirheumatic therapy

J K FRENCH, N P HURST, M L O'DONNELL, AND W H BETTS

From the Combined Queen Elizabeth Hospital and Royal Adelaide Hospital Rheumatology Unit, South Australia

SUMMARY The accumulation of chloroquine and hydroxychloroquine in unfractionated mononuclear cells and in purified monocytes, lymphocytes, and neutrophil polymorphonuclear leucocytes (PMN) was measured in vitro. Accumulation of both drugs in leucocytes was time and dose dependent. Cellular levels comparable to those found during antirheumatic therapy were achieved by preincubation for 60 minutes with up to 0.1 mM chloroquine or hydroxychloroquine.

Key words: monocytes, lymphocytes, polymorphonuclear leucocytes.

The 4-aminoquinolines and related antimalarial agents have a wide variety of dose related biological effects. For example, at relatively low concentrations (0.1 $\mu\text{mol/l}$) they act as schizonticidal agents by selectively binding with ferritinoporphyrin to form a toxic complex in parasitised erythrocytes.¹ Above 1 $\mu\text{mol/l}$ they affect lysosomal function in several cell types, causing accumulation of phospholipid, inhibition of lysosomal enzyme receptor recycling, and inhibition of lysosomal enzymes.² Above 0.1 mmol/l chloroquine binds and compromises the function of a wide range of cellular constituents, including proteins and DNA.¹ The cellular concentration of chloroquine and hydroxychloroquine in circulating phagocytes (0.1–0.3 mmol/l) is the highest of any cell type, except for retinal melanin containing phagocytes, and is approximately 100 times higher than therapeutic plasma levels.^{1,3}

Several studies have reported effects of chloroquine on cellular functions in vitro.^{2,4–10} In each case the extracellular concentration and period of incubation with chloroquine, rather than the intracellular level achieved, have been correlated with patient plasma rather than cellular drug levels.

To provide a guide to appropriate conditions for short term in vitro studies of functional effects of chloroquine and hydroxychloroquine on leucocytes we have measured their uptake in PMN, lympho-

cytes, monocytes, and unfractionated mononuclear cells with respect to incubation time and dose. We have correlated these data with leucocyte hydroxychloroquine levels in patients with rheumatoid arthritis taking hydroxychloroquine therapy.

Materials and methods

CHEMICALS

Hydroxychloroquine sulphate was a generous gift from Sterling-Winthrop, Rensselaer, NY. Chloroquine diphosphate was obtained from Sigma (St Louis, MO), acetonitrile (high performance liquid chromatography grade) from Waters (Milford, Mass), Percoll and Ficoll-Hypaque from Pharmacia (Uppsala, Sweden), and triton X-100 from Bio-rad (Richmond, Calif). All other chemicals were analytical grade or better.

PREPARATION OF CELL SUSPENSIONS

Venous blood was drawn from healthy human volunteers, anticoagulated with 4.5% ethylenediaminetetra-acetic acid in Dulbecco's balanced salt solution (DBSS) (pH 7.4), and mononuclear cells (20–30% monocytes, 70–80% lymphocytes) separated on a Ficoll-Hypaque gradient,¹¹ the percentage of monocytes and lymphocytes was determined by size distribution on a Coulter counter (model ZBI).¹²

Monocytes (65–80% purity) were prepared from unfractionated mononuclear cells on a Percoll gradient ($d=1.064$).¹³

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Correspondence to Dr N P Hurst, The Queen Elizabeth Hospital, Woodville, South Australia 5011.

Lymphocytes (>90% purity) were prepared from mononuclear cells by removal of adherent monocytes on plastic Petri dishes pretreated with human AB serum. After incubation at 37°C for 30 minutes non-adherent lymphocytes were gently removed with warm DBSS.

PMN were prepared by dextran sedimentation of whole blood and layering of the leucocyte rich plasma on a double Percoll gradient ($d=1.070$ and 1.092);¹⁴ PMN were obtained from the lower interface. All cell preparations were washed twice with DBSS and resuspended in Hanks's balanced salt solution (HBSS) (pH 7.4).

CHLOROQUINE AND HYDROXYCHLOROQUINE IN VITRO ACCUMULATION IN LEUCOCYTES
Mononuclear cells, monocytes, lymphocytes, or PMN (2×10^6 /ml) in 1 ml of HBSS were incubated in the presence of concentrations of chloroquine and hydroxychloroquine up to 0.1 mmol/l at 37°C. After incubation, cells were centrifuged and washed twice with HBSS. All experiments were performed in duplicate.

EXTRACTION OF CHLOROQUINE AND HYDROXYCHLOROQUINE FROM LEUCOCYTES
Water (2 ml) was added to the cell pellet followed by 250 μ l of 1 M NaOH/0.6 M borate, 150 μ l of internal standard, and 5 ml of chloroform. The internal standards for hydroxychloroquine and chloroquine were chloroquine and hydroxychloroquine respectively. The tubes were shaken for 10 minutes, centrifuged for 10 minutes at 600 g, and the chloroform layer transferred to clean glass tubes and evaporated under nitrogen at 37°C. The residue was reconstituted in 100 μ l of mobile phase 85% H_3PO_4 (0.015%)/15% CH_3CN , and 25 μ l aliquots used for high performance liquid chromatographic (HPLC) analysis.

HPLC MEASUREMENT OF CHLOROQUINE AND HYDROXYCHLOROQUINE¹⁵

HPLC was performed on a Waters system: Waters Bondapak phenyl column, length 30 cm, width 3.9 μ m, particle size 10 μ m; isocratic mobile phase, as above; flow rate 2 ml/min; UV detection at 330 nm. Standard curves were linear over the concentration range 0.001–0.1 mmol/l.

CELL VIABILITY

Cell viability was measured by trypan blue exclusion and lactic dehydrogenase (LDH) release. LDH release was measured by an established method,¹⁶ on a Technicon random access discrete analyser, RA-1000 (Technicon Instrument Corp, Tarrytown, New York). Total cellular LDH content in each

experiment was measured by incubation of cells with 0.5% triton X-100; LDH release was expressed as a percentage of total.

Results

CHLOROQUINE AND HYDROXYCHLOROQUINE IN VITRO ACCUMULATION IN LEUCOCYTES

The uptake of chloroquine and hydroxychloroquine in mononuclear cells, monocytes, lymphocytes, and PMN was measured over 60 minutes in the presence of varying concentrations of each drug (Fig. 1). Uptake was similar in lymphocytes and PMN, and was greater in purified monocytes. In mononuclear cells uptake of chloroquine and hydroxychloroquine was lower than in purified monocytes or purified lymphocytes, suggesting that cell separation procedures may have influenced the results. Uptake of chloroquine in mononuclear cells was time dependent (Fig. 2) and more rapid at 0.1 mmol/l than 0.01 mmol/l.

CELL VIABILITY

After 60 minutes' incubation with buffer alone, or 0.1 mM chloroquine or hydroxychloroquine, cell viability by trypan exclusion was >95%. There was no significant LDH release (<10%) from either

Fig. 1 Accumulation of chloroquine and hydroxychloroquine in human blood leucocytes. Uptake of chloroquine or hydroxychloroquine by monocytes (Mo), lymphocytes (Ly), PMN, and mononuclear cells (MNC) was measured after 60 minutes incubation at 37°C in the presence of chloroquine (top) or hydroxychloroquine (bottom). Each data point represents mean (SD) of separate experiments on cell samples from three separate individuals. Each experiment was performed in duplicate.

Fig. 2 Time course of accumulation of chloroquine in mononuclear cells. Mononuclear cells were incubated with (A) 0.1 mM or (B) 0.01 mM chloroquine for varying times before washing and extraction of chloroquine from cells. CQ=chloroquine.

mononuclear cells or PMN after 60 minutes' incubation in buffer, 0.1 mM chloroquine, or 0.1 mM hydroxychloroquine.

LEUCOCYTE HYDROXYCHLOROQUINE LEVELS IN RHEUMATOID ARTHRITIS

Levels of the closely related 4-aminoquinoline, hydroxychloroquine, were measured in leucocytes and plasma of five patients with rheumatoid arthritis who had been receiving 400 mg hydroxychloroquine daily for at least three months (Table 1). Cellular levels were higher in mononuclear cells than PMN. It can be seen by comparison with Fig. 1 that surprisingly high extracellular concentrations of chloroquine and hydroxychloroquine are required

Table 1 Concentrations of hydroxychloroquine in leucocytes and plasma from patients with rheumatoid arthritis

	Hydroxychloroquine concentration (mean (SD)) (n=5)
Mononuclear cells	0.6 (0.4) nmol/10 ⁶ cells
Neutrophils	0.2 (0.2) nmol/10 ⁶ cells
Plasma	1.3 (0.6) µmol/l

Patients had been treated with hydroxychloroquine 400 mg daily for at least three months.

to achieve equivalent leucocyte concentrations during short incubations in vitro.

Discussion

The purpose of these studies was to determine appropriate in vitro conditions for examining the effects of chloroquine and hydroxychloroquine on leucocyte function.

Our data show that up to 60 minutes' incubation of leucocytes with up to 100 µM chloroquine or hydroxychloroquine is necessary to generate intracellular levels similar to those in patients receiving antirheumatic therapy with hydroxychloroquine 400 mg daily. Thus during 60 minutes' incubation in vitro about 50–100 times the therapeutic plasma concentration of chloroquine is necessary to attain comparable leucocyte levels.

There have been numerous studies of the effect of hydroxychloroquine and chloroquine on stimulated leucocyte functions in vitro.^{1–10} In general three types of experimental system have been used. In the first the drug is added at the same time as the relevant stimulus; in the second the cells are preincubated with drug before addition of the stimulus and the drug remains throughout the test period; in the third the cells are preincubated with drug but then washed before stimulation to prevent further drug accumulation. Each system may give rise to problems of interpretation if no account is taken of rates of accumulation of drug within the cell. This is particularly important when functions dependent on lysosomal activities such as receptor recycling, which require time dependent accumulation of drug,^{8–10} are being studied.

For example, Salmeron and Lipsky have reported the effects of up to 25 µg/ml (50 µmol/l) chloroquine on mitogen stimulated lymphocyte transformation. Mononuclear cells were preincubated for up to 24 hours with 25 µg/ml chloroquine and then washed extensively before mitogen stimulation. Two hours' incubation had little effect, but 12 and 24 hours' preincubation caused 30% and 70% inhibition of responsiveness. Thus accumulation of drug within the cell was required for maximal effect. It is difficult to draw conclusions from these data, however, about in vivo mechanisms of action, because actual cellular concentrations of drug were unknown.

Similarly, Jones and Jayson have reported the functional and ultrastructural effects of 0.05–50 µg/ml (0.1–100 µmol/l) chloroquine on leucocytes and demonstrated time and dose related autophagosome formation and inhibition of phagocytosis. Again, although almost certainly some of the effects described are important, it is difficult to correlate

these effects with putative in vivo mechanisms in the absence of data on cellular levels of chloroquine. Similar problems arise in the interpretation of other publications.⁵⁻¹⁰

It must be emphasised that not all the effects of chloroquine and hydroxychloroquine depend on intracellular accumulation. For example, recent data from our laboratory (unpublished observations) show that inhibition of *N*-formyl-methionyl-leucyl-phenylalanine induced PMN superoxide production by these drugs is not time dependent and suggest an immediate and direct effect on the plasma membrane; this contrasts with data showing that inhibition of zymosan induced superoxide production by monocytes is time dependent.¹⁷

In conclusion, our data provide a guide to rates of accumulation of chloroquine and hydroxychloroquine in leucocytes in vitro and show that relatively high extracellular concentrations are required to achieve therapeutic leucocyte levels during short incubations. We suggest that studies which examine the effect of these drugs on leucocyte function should also measure leucocyte accumulation to ensure that relevant 'therapeutic' concentrations are being achieved. The HPLC method we have used is straightforward and suitable for study of 'batches' of cell extracts. In the absence of availability of appropriate HPLC equipment, radiolabelled chloroquine is now commercially available and will provide a sensitive and rapid alternative method of estimating cellular accumulation.

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