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MEMBRANE ADSORPTION AND INTERNALIZATION OF [^{14}C]CHLOROQUINE BY CULTURED HUMAN FIBROBLASTS

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Abstract—[^{14}C]chloroquine, added in concentrations between 1 to 10 μM to the medium of normal cultured fibroblasts, is rapidly adsorbed and taken up by the cells. At pH 7.40 about 15 per cent of the total uptake can be removed by washing the cells with Hank's solution, up to 24 per cent can be detached from the surface by trypsin and about 50 per cent appears to be intracellular. At an extracellular pH of 7.4 the adsorption of chloroquine to the cell surface is the highest; it approaches saturation at concentrations in the medium exceeding to 10 μM. Both at pH 6.7 and 8.1 less chloroquine is adsorbed. On the other hand the apparent *intracellular* uptake of chloroquine rises with increasing pH. Incubation at 6° greatly reduces both surface binding and even more intracellular uptake. The data suggest adsorption and accumulation of chloroquine at the cell membrane before entering the cells.

Chloroquine, a lysosomotropic antimalaria drug is efficiently taken up by cultured Hela cells [1], rat fibroblasts [2] and human fibroblasts [3]. Human fibroblasts in culture, when exposed to chloroquine show striking changes both morphological [3] and biochemical [3, 4]. Incubation with 1 to 5 μM chloroquine at pH 7.4 for 8 hr produces granular cytoplasmic inclusions, a dose-dependent release of lysosomal enzymes into the medium and decrease of intracellular lysosomal enzyme activities [4], thus mimicking part of the genetically determined biochemical alterations observed in fibroblasts of patients with mucolipidosis II or III [5, 6]. Chloroquine also inhibits the uptake of exogenous arylsulfatase A from the culture medium into fibroblasts genetically deficient in this enzyme [4], although the endocytosis of other macromolecules like macromolecular dextran is preserved [7]. Since the drug seemed to interfere with the binding of lysosomal enzymes to the cell membrane, it was suggestive that it might bind to the cell surface. We present evidence in favor of that hypothesis. The binding is dependent on extracellular pH, drug concentration and incubation temperature.

MATERIALS AND METHODS

Cultures of normal human fibroblasts were grown in Eagle MEM supplemented with 10% fetal calf serum buffered at pH 7.4 with bicarbonate as previously described [5]. Fibroblasts were subcultured into 100-mm Falcon plastic Petri dishes and grown to confluency. Protein was measured by the method of Lowry *et al.* [8], DNA was determined by the indole method [9]. Experiments were performed by incubation of the cultures with 6.0 ml of the growth medium modified in that 20 mM HEPES HCl (Serva Chemical Comp. Heidelberg, Germany) replaced bicarbonate/CO_2 as the buffer in the presence of [^{14}C]chloroquine. Incubations were performed at pH 6.7, 7.4 and 8.1 respectively. [^{14}C]chloroquine (sp. act. 3 mCi/m-mole) was purchased from NEN Chemicals (Dreichenhain, Germany). At the end of the incubation periods the medium was collected and the cultures were washed three times for 1 min (washing 1,2,3) with 6.0 ml of Hank's solution buffered to the pH of the incubation medium with 20 mM HEPES at 37° except where stated otherwise. Cells were harvested at 10 min incubation at 37° with 6 ml of 0.25% trypsin (Bactotrypsin Difco) in Hank's solution buffered with 20 mM HEPES to the pH of the incubation medium and the washing solutions. The suspension was centrifuged for 3 min at 2000 *g* and an aliquot of the supernatant was counted by liquid scintillation spectrometry. The pelleted cells were washed by resuspending them in 6 ml Hank's solution buffered with 20 mM HEPES at the pH of the incubation medium and then centrifuged at 2000 *g* for 3 min (washing 4). The cells were finally suspended in 2 ml of distilled water, cooled in an ice bath and homogenized by sonication for 1 min with a Branson ultrasonicator (Danbury, Conn. USA). Aliquots of the sonicated cells were used for determinations of radioactivity, of protein and of DNA.

RESULTS

Distribution of [^{14}C]*chloroquine in cultures.* Table 1 illustrates the distribution of the drug in cultures exposed to 2, 6 and 10 μM [^{14}C]chloroquine at pH 7.4. Up to 50 per cent of the chloroquine disappeared from the medium during the first 2 hr incubation. We refer to this as "total uptake". Repeated washing of the cultures with Hank's solution removed between

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Table 1. Distribution of drug in cultures exposed to [^{14}C]chloroquine at pH 7.4

Dpm [^{14}C]chloroquine applied/plate	196,000	584,000	953,000
Total uptake*	97,000	280,000	407,000
Washings *in situ*			
1	7,000	31,000	40,000
2	1,000	3,000	5,000
3	0,600	1,500	2,000
Pericellular pool†	22,000	66,000	83,000
Washing 4	5,000	10,000	15,000
Cellular pool‡	50,000	144,000	226,000
Recovery§ (%)	88	88	91

Replicate confluent cultures containing approximately 1 mg of cell protein were incubated for 2 hr in HEPES-buffered medium, pH 7.4 at 37°. The cultures were labelled for 2 hr with 2, 6 and 10 μM [^{14}C]chloroquine respectively. [^{14}C]chloroquine was measured in aliquots of the original media, the media after incubation, the washings, the supernatant of the trypsin and the cell homogenate. All values represent the mean of duplicate cultures. The results are expressed as dpm/mg cell protein.

* Amount of [^{14}C]chloroquine lost in 2 hr from the incubation medium.

† [^{14}C]chloroquine recovered in the supernatant of the trypsin.

‡ [^{14}C]chloroquine in the cell homogenate.

§ Per cent recovery of the drug referred to total uptake.

8 and 15 per cent of the chloroquine. Trypsinization of the cells released up to 24 per cent in soluble form ("pericellular pool"). About 50 per cent of the radioactivity was found in the sonicated suspension of the cells ("intracellular pool"). All fractions added up to about 90 per cent of the total uptake. If the cells were exposed to chloroquine, 2 μM at pH 8.1, the total uptake was much higher than at pH 7.4. Moreover when the cells were incubated prior to trypsinization Hank's solution at a lower pH than that maintained during the exposure to the drug and the washings, additional chloroquine could be eluted, reducing both pericellular and intracellular pools (Table 2).

Kinetics of the distribution of [^{14}C]chloroquine. [^{14}C]chloroquine was rapidly taken up into the pericellular as well into the intracellular pool (Fig. 1). Half-maximal uptake was reached within 30 min, at 1 and 2 μM chloroquine. A steady state was reached in both pools after 2 hr. In the incubation medium, [^{14}C]chloroquine decreased proportionally. Medium incubated in plastic Petri dishes without cells did not lose its radioactivity, thus non-specific binding of [^{14}C]chloroquine to the plastic could be excluded. At incubation with 6 μM chloroquine, although a steady state was reached in the pericellular pool after 2 hr, the intracellular pool increased further in the 6-hr incubation period.

Effect of the pH and the temperature on the distribution of [^{14}C]chloroquine. In 10-min incubation experiments at 37° with 2 μM [^{14}C]chloroquine, the effect of pH on the rate of uptake was measured. With respect to the cellular pool the rate of uptake rose with increasing pH, while in respect to the pericellular pool it was maximal around pH 7.4 (Table 3).

Incubation of cultures at 37° for 2 hr with 2 μM [^{14}C]chloroquine at pH 6.7, 7.4 and 8.1 respectively demonstrated a striking increase in the total uptake of chloroquine with increasing pH (Fig. 2). Lowering the temperature to 6° markedly reduced the intracellular uptake at pH 6.7 and 7.4, but less at pH 8.1. The adsorption of chloroquine to the surface of the cells (pericellular pool) at 37° displayed an apparent pH optimum around pH 7.4 that was not seen when the incubations were performed at low temperature (Fig. 2). Incubations with 1–10 μM chloroquine for 2 hr (Fig. 3) at 37° resulted in a linear dose-dependent

Table 2. Effect of the pH of the washing solution on the distribution of [^{14}C]chloroquine

		Plates with additional washing		
	Control plates	Hank's pH 8.1	Hank's pH 7.4	Hank's pH 6.7
		(nmole/mg cell protein)		
Total applied [^{14}C] chloroquine/plate	12.5	12.5	12.5	12.5
Total uptake	10.2	10.1	10.6	10.6
Washings	0.5	0.4	0.4	0.4
Additional washing	—	0.1	0.6	1.3
Pericellular pool	0.8	0.9	0.6	0.5
Cellular pool	7.7	7.6	7.5	6.6

Replicate confluent cultures were incubated 2 hr at 37° in presence of medium pH 8.1 and 2 μM chloroquine, [^{14}C]chloroquine distribution was determined in control plates as described in Table 1. Test plates were incubated for 15 min *in situ*, prior to trypsinization with 20 mM HEPES buffered Hank's solutions of pH 6.7, 7.4 and 8.1 respectively (additional washing). Test plates were processed otherwise as control plates. The results were expressed as nmole [^{14}C]chloroquine per mg cell protein.

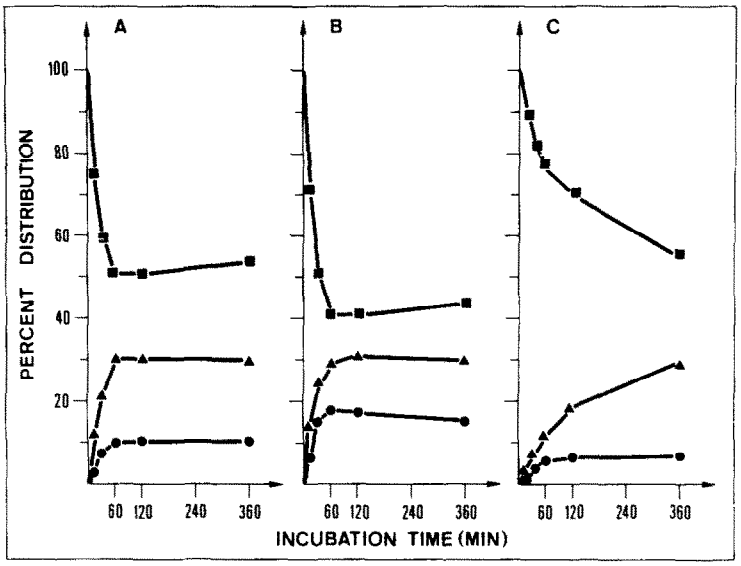

Fig. 1. Kinetics of the distribution of [^{14}C]chloroquine. Replicate confluent cultures were incubated for 2 hr at 37° in buffered medium, pH 7.4 containing 1(A), 2(B) and 6(C) μM [^{14}C]chloroquine respectively. [^{14}C]chloroquine was determined in an aliquot of the original media, the media after incubation, the supernatant of the trypsin and the cell homogenate. The results of the radioactivity were corrected for cellular proteins of the cultures and expressed as per cent of the total dose of chloroquine applied. All values are the mean of duplicate cultures. ■——■ Medium, ▲——▲ Intracellular, ●——● Pericellular.

increase of the intracellular uptake at each pH tested, being the highest at pH 8.1. The uptake into the pericellular pool at 37° was linear only at pH 6.7 and 8.1 and at 6° for all three pH. It was the highest at pH 7.4 and approached saturation above 10 μM chloroquine in the culture medium (Fig. 3).

DISCUSSION

The release of chloroquine after trypsinization of the cultures is not explained by cell damage or broken cells. Fibroblasts exposed to chloroquine for 6–8 hr and replated by trypsinization as used in our experiments were fully viable and showed no loss of protein and DNA. In addition the differences between the pericellular pool and the intracellular pool at various incubation pH speak against cell damage as a major source of the "pericellular" pool. Because of the pK_{a2} of 8.1 chloroquine exists at pH 7.4 in aqueous solutions in a mono- and in a double-protonated form and only in negligible amounts in a non-protonated form [10]. The theoretical amounts of the monoprotonated form at pH 6.7, 7.4 and 8.1 are 4, 18 and 50 per cent respectively of the total chloroquine concentration. The ratios of the intracellular uptake (Table 3) as well of the intracellular pools (from Fig. 3) at pH 6.7, 7.4 and 8.1 reflect well the theoretical values for the monoprotonated form. This suggests that chloroquine is taken up in the monoprotonated form.

Table 3. pH of incubation and rate of uptake of [^{14}C]chloroquine

pH	Cellular pool (nmole/10 min/mg cell protein)	Pericellular pool
6.7	0.5	0.3
7.4	2.0	0.8
8.1	6.5	0.7

Replicate sultures were incubated at 37° for 10 min in medium (pH 6.7, 7.4 and 8.1 respectively) containing 2 μM [^{14}C]chloroquine.

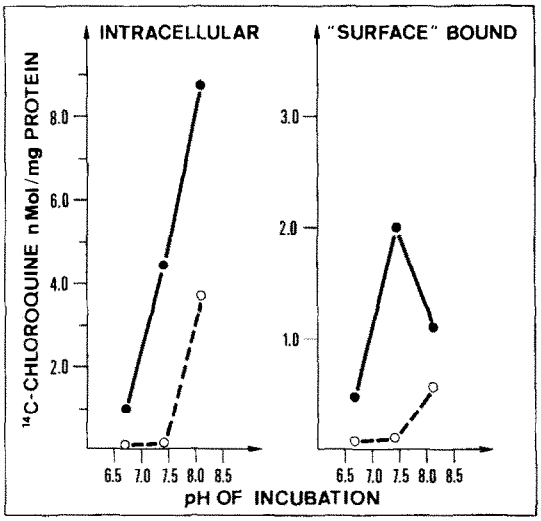

Fig. 2. Effect of the pH and the temperature on the distribution. Replicate confluent cultures containing approximately 1 mg of protein were incubated at 6° (broken line) and at 37° (solid line in buffered medium, pH 6.7, 7.4 and 8.1 respectively containing 2 μM chloroquine, 120 min. The washings were also performed at 37° and 6° respectively. At the end of each incubation time duplicate cultures were analyzed for [^{14}C]chloroquine as described in Fig. 1. The values were expressed as nmole of [^{14}C]chloroquine corrected for cellular protein.

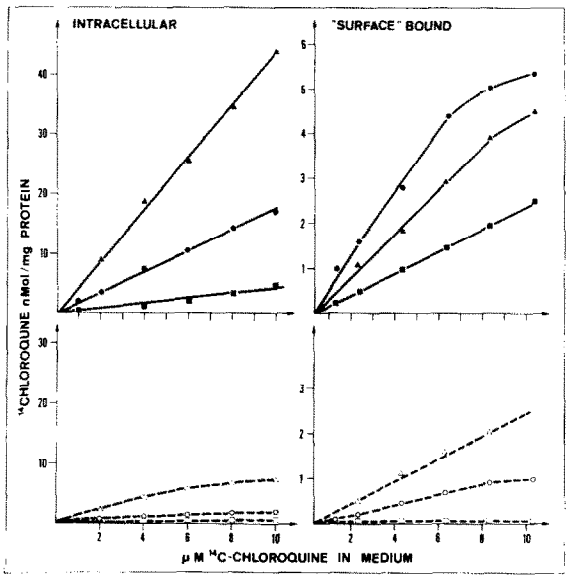

Fig. 3. Effect of pH and dose. Replicate cultures were incubated for 2 hr at pH 6.7, 7.4 and 8.1 in HEPES-buffered media containing 1 to 10 μM [^{14}C]chloroquine. After the incubation [^{14}C]chloroquine was measured in the cell homogenate and in the supernatant of the trypsin and expressed as nmole [^{14}C]chloroquine per mg cell protein. All values are mean of duplicate cultures. △--△, ▲——▲ pH 8.1; ○--○, ●——● pH 7.4; □--□, ■——■ pH 6.7: broken lines and open symbols refer to incubations performed at 6°, solid and closed symbols to incubations at 37°.

The biological effect of chloroquine to interfere with the uptake of lysosomal enzymes [4] as well as the fact that trypsin releases chloroquine from cell surfaces indicates adsorption of chloroquine to the cell membrane itself. This adsorption has an apparent pH optimum around pH 7.4 with adsorption capacity that can be partially saturated at 7 nmole/mg protein. This would account for an approximate 10^9 molecules of chloroquine adsorbed per cell.

The fact that lowering the incubation temperature to 6° reduced the adsorption could suggest an energy requiring mechanism for the binding. The nature of the binding appears to be non-covalent but rather a loose bond as evidenced by dissociation at low pH (Table 2). It could be speculated that chloroquine in the medium is adsorbed and concentrated at the cell membrane by an active process. Thus a local concentration gradient is formed and maintained between surface and inside of the cells that allows rapid permeation of chloroquine into the lysosomes of the cells as demonstrated by many investigators [11].

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