Chloroquine: its effect on leucocyte auto- and heterophagocytosis

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SUMMARY The effect of chloroquine on phagocytosis and leucocyte ultrastructure has been examined. Incubation of neutrophils in 50 $\mu\text{g/ml}$ chloroquine for 15 minutes produced a significant inhibition of latex particle uptake. After 90 minutes both 50 and 5 $\mu\text{g/ml}$ inhibited phagocytosis while 7 of 9 cases were also inhibited at 0.5 $\mu\text{g/ml}$. However, after 4 hours 50 $\mu\text{g/ml}$ chloroquine caused neutrophil granule vacuolation and a massive increase in autophagosomes in other cell types. Incubation in 5 to 0.05 $\mu\text{g/ml}$, which includes therapeutic plasma levels, had no effect on neutrophil ultrastructure but produced a dose-related increase in the number of lymphocytes containing autophagosomes, reflecting altered lysosomal function. As the antimalarial effect of chloroquine is manifested by giant autophagosome formation in *Plasmodium*, a common antirheumatic and antimalarial mechanism of action is postulated.

Antimalarials have been used to treat connective tissue diseases since 1894, when Payne¹ reported the beneficial effects of quinine in the treatment of lupus erythematosus. Various antimalarial drugs have subsequently been developed, of which those with a 4-aminoquinoline structure such as mepacrine, chloroquine, and hydroxychloroquine have been most successful in the treatment of rheumatic conditions. The efficacy of these compounds is reported to approach that of gold, penicillamine, and azothioprine.²

Although its exact mode of action is not yet understood, chloroquine is reputed to exert anti-inflammatory and immunosuppressive effects as well as the suppression of protein biosynthesis.² We have examined the in-vitro effect of chloroquine on the phagocytosis of latex particles by polymorphonuclear neutrophils, using a technique previously described.³ The bizarre alterations in cell ultrastructure following incubation in chloroquine prompted a detailed examination of the morphological changes occurring in leucocytes after exposure to varying concentrations of chloroquine, and the results have led us to propose a link between the antimalarial and antirheumatic modes of action of this drug.

Materials and methods

PHAGOCYTOSIS

Leucocytes from the heparinised blood of healthy male volunteers were isolated by dextran sedimentation at 37°C for one hour. The resulting cell-rich supernatant was washed twice in Dulbecco's modification of Eagle's medium (DMEM) at 4°C and the concentration adjusted to 10^7 cells per ml. Trypan blue exclusion indicated a viability of over 98%. Chloroquine diphosphate (Sigma) was dissolved in DMEM (1 mg/ml) and serial dilutions were made to 10^{-2} mg/ml (10^{-3} mg/ml for morphological studies). These were further diluted, and 1 ml aliquots of the cell suspension were then mixed with an equal volume of chloroquine solution, or medium only, to give final concentrations of 50, 5, and 0.5 $\mu\text{g/ml}$ chloroquine (9.7×10^{-5} M to 9.7×10^{-7} M). Incubation was carried out at 37°C in 5% CO_2 in air for 90 minutes, after which vials were transferred to a shaking water bath (160 shakes/minute) at 37°C and given 100 μl of 0.81 μm diameter latex particles (Difco) for exactly 10 minutes. Fixation, processing for electron microscopy, and analyses were carried out as previously described,³ and grids were coded so that specimens were examined blind. This experiment was performed on 9 samples of blood.

In 8 experiments incubations were carried out for 15 minutes only, followed by a 10-minute period of

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latex ingestion; cells were allowed to reach 37°C in the shaking water bath before the addition of chloroquine. Wilcoxon's matched pairs signed ranks test was used to compare counts of ingested particles in drug-treated and control populations. A phagocytic index was calculated as follows:

$$\frac{\text{Particles in 100 control cells} - \text{particles in 100 test cells}}{\text{Particles in 100 control cells}} \times 100$$

MORPHOLOGICAL STUDIES

The effect of 50 µg/ml chloroquine on the ultrastructure of normal leucocytes was examined at 1, 2, and 4 hours; cells exposed to 5, 0.5, and 0.05 µg/ml were fixed only after the full 4-hour period. Cells were isolated as above and incubated for the specified time at 37°C in 5% CO₂ in air before fixation and processing for electron microscopy. Observations were made on changes in neutrophil and lymphocyte ultrastructure with special reference to lysosomal morphology; monocytes were not analysed, as it was not always possible to distinguish between auto- and heterophagosomes owing to their tendency to ingest cell debris. In 9 experiments the number of lymphocytes containing autophagosomes per 100 cells after incubation in 5–0.05 µg/ml chloroquine was recorded on 2 separate occasions, and the totals summed to give a final result of the number present in a 200-cell sample. Wilcoxon's matched pairs signed ranks test was applied to determine whether there was any significant difference between the number of autophagosomes present in lymphocytes after incubation in the various concentrations of chloroquine.

SEPARATION OF T AND B LYMPHOCYTES

60 ml blood from a healthy male volunteer was mixed with 1.5 ml heparin, and the T and B lymphocytes were separated according to the method of Potter and Moore.⁴ The cell concentration was adjusted to 10⁷ per ml in DMEM, and 1 ml aliquots of either T or B cells were mixed with an equal volume of chloroquine solution to give a final concentration of 5 µg/ml. Medium-only controls were also prepared. Cells were incubated for 4 hours at 37°C in 5% CO₂ in air and then fixed and processed as above. Test and control grids were examined blind, the percentage of lymphocytes containing autophagosomes being recorded on 2 separate occasions and the results summed as before. This experiment was performed twice, and a chi-squared test was used to assess the presence of any difference between the 2 cell types.

ULTRAHISTOCHEMISTRY

In order to confirm the presence of lysosomes in lymphocytes cells were isolated as above and incu-

bated with either 50 or 5 µg/ml chloroquine, or medium only, for 4 hours, after which they were suspension-fixed for exactly 6 minutes in prewarmed 1.25% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4 (final concentration). After 2 washes in 0.1 M cacodylate buffer they were left overnight and then stabilised in 0.2 M acetate buffer for one hour before incubation in the medium for aryl sulphatase activity⁵ for 2 hours at 37°C. Cells were then rinsed briefly in acetate buffer before being washed in cacodylate buffer and processed as normal. Ultrathin sections were examined without further staining.

Results

PHAGOCYTOSIS

After 15 minutes' incubation in chloroquine only the highest concentration (50 µg/ml) showed a statistically significant degree of inhibition of particle uptake by neutrophils compared with controls ($p < 0.01$), with a mean phagocytic index of 30.5%. By 90 minutes, however, exposure to both 50 and 5 µg/ml resulted in a significant inhibition of phagocytosis in comparison with control preparations, with mean phagocytic indices of 39.4% and 26.5% respectively (Table 1). At 0.5 µg/ml, although 33.5% inhibition was reached in one case, 2 were slightly stimulatory with phagocytic indices of -2.8% and -0.3%, and the overall effect was not statistically significant in comparison with control cells (Table 1). In 7 of the 9 experiments the maximum degree of suppression was found at the highest concentration of chloroquine (50 µg/ml), but in 2 experiments concentrations of 5 µg/ml gave a marginally greater level of inhibition.

MORPHOLOGICAL STUDIES

After 1 hour's exposure to 50 µg/ml chloroquine, polymorphonuclear neutrophils showed slight swelling of some granules, with the formation of an electron-lucent rim or halo around the granule (Fig. 1). In some cases the normally electron-dense granule appeared to be showing signs of disruption, with a loosening of its structure to give a flocculent or fibrillar appearance; this was occasionally accompanied by a condensed core lying within the fibrillar matrix. There was more evidence of degranulation compared with control cells, and an occasional myeloid body was seen.

By 2 hours the swelling of neutrophil granules had become much more widespread, and after 4 hours' exposure to chloroquine the cells were filled with many vacuoles, their contents being in various stages of alteration (Fig. 2). Some contained electron-dense balls or flocculent material, while many granules had a loosely-woven texture similar to that found in occa-

Table 1 Particle uptake by PMNs after 15 and 90 minutes in various concentrations of chloroquine (0–50 µg/ml)

	Chloroquine concentration (µg/ml)			
	50	5	0.5	Control
No. particles in 100 cells (15 min)				
Mean ± SE (n=8)	230.2±19.6*	310.2±35.6	308.5±37.0	330.9±36.8
Mean phagocytic index	30.5	6.0	6.6	
No. particles in 100 cells (90 min)				
Mean±SE (n=9)	248.8±47.1**	302.2±56.7**	376.4±60.0	410.6±52.2
Mean phagocytic index	39.4	26.5	8.5	

* $p<0.01$, ** $p<0.005$, by Wilcoxon's matched pair signed ranks test.

Fig. 1 Neutrophil after exposure to 50 µg/ml chloroquine for 1 hour. A halo can be seen around some granules (arrows). (EM, ×10 125).

Fig. 2 A neutrophil (N) after incubation in 50 µg/ml chloroquine for 4 hours. The cell contains many large vacuoles and disintegrating granules. Within the lymphocyte (L) are 5 autophagosomes. (EM, ×6970).

sional control cells. Several of the vacuoles were devoid of contents, which may have been outside the plane of the section. In contrast the control cells showed only a very occasional incidence of degranulation after 4 hours' incubation in the medium (Fig. 3). There was minimal swelling of Golgi vacuoles in some chloroquine-treated cases, but this did not reach significant proportions. Other organelles such as nuclei and mitochondria did not appear to differ from the controls at any stage of the incubation period.

The ultrastructure of lymphocytes showed a quantitative rather than a qualitative change during the 1- to 4-hour incubation period with 50 µg/ml chloroquine, in that there was a gradual increase in the number of autophagosomes present (Fig. 2) compared with control cells. These vacuoles were filled with contents of variable electron-density, often containing one or more darkly staining inclusions. Lamellar structures with concentric membranous whorls and membranous invaginations indicative of early autophagosome formation were occasionally seen (Fig. 4). These vacuoles were present in the

Fig. 3 Control cells after 4 hours' incubation in DMEM alone. (EM, ×3100).

Fig. 4 A lymphocyte after incubation in 50 µg/ml chloroquine for 4 hours, containing large autophagosomes and a membranous invagination (arrow). (EM, $\times 10\,420$).

Fig 5 Cells after incubation in 5 µg/ml chloroquine for 4 hours. Neutrophils appear to be unaltered but autophagosomes can be seen in the lymphocytes. (EM, $\times 3500$).

control cells (see Fig. 6) but to a much lesser degree. They tended also to be smaller. Because this concentration of chloroquine appeared to be highly toxic and nonspecific in its action, no attempt was made to quantitate the number of autophagosomes found in the lymphocyte population.

After 4 hours' incubation in 5 µg/ml chloroquine there was no detectable difference in the ultrastructure of neutrophils in comparison with control cells. However, lymphocytes showed a significant increase ($p < 0.005$) in the number of cells containing autophagosomes compared with controls (Fig. 5). This feature was evident at concentrations as low as 0.05 µg/ml chloroquine and appeared to be dose-related (Fig. 6). The vacuoles were slightly smaller than those found after exposure to 50 µg/ml but contained similar features such as electron-dense inclusions, lamellar structures, and flocculent material. Instances of early stages of autophagosome formation, in which part of the cell cytoplasm was pinched off and enclosed by a membrane prior to digestion, were occasionally seen (Fig. 7). Examination of data from 9 experiments by Wilcoxon's matched pairs signed ranks test showed a very highly significant difference ($p < 0.005$) between the number of cells containing autophagosomes at each drug concentration in comparison with the controls (Fig. 6). A non-parametric analysis of variance⁶ showed there to be a significant difference ($p < 0.05$) between subjects and a very highly significant difference ($p < 0.01$) between the number of autophagosomes in lymphocytes at the various drug levels. The interaction was not significant—that is, the responses to the various chloroquine concentrations were similar for each blood sample tested.

Fig. 6 Histogram showing mean + SE of the number of autophagosomes present in a 200-cell sample of lymphocytes after exposure to 5–0.05 µg/ml chloroquine for 4 hours and controls ($n=9$). * $p < 0.005$ compared with control by Wilcoxon's matched pairs signed ranks test.

T AND B LYMPHOCYTE SEPARATION

Statistical analysis by the chi-squared test showed there to be no significant difference between the susceptibility of T and B lymphocytes to the action of

Fig. 7 Lymphocyte after incubation in 5 µg/ml chloroquine for 4 hours showing early autophagosome formation (arrow). (EM, $\times 9600$).

Fig. 8 Lymphocyte reacted for aryl sulphatase activity after exposure to 5 µg/ml chloroquine for 4 hours. Electron-dense deposits can be seen in autophagosomes (*) and granules (arrows). (EM, $\times 20\,530$).

5 µg/ml chloroquine. Both populations showed a very highly significant increase ($p < 0.001$) in the number of autophagosomes present after chloroquine treatment compared with control groups (Table 2).

ULTRAHISTOCHEMISTRY

Occasional aryl sulphatase-positive granules were found in lymphocytes from both test (50 and 5 µg/ml chloroquine) and control situations. Reaction product in the form of fine electron-dense deposits was also present in some autophagosomes, thus confirming the lysosomal nature of these inclusions (Fig. 8). Although no attempt was made to quantitate the amount of reaction product in each case, there appeared to be a decrease in enzyme activity in all cell types following incubation in chloroquine, which was more marked at the higher (50 µg/ml) concentration.

Discussion

Although the exact mode of action of chloroquine is unknown, it possesses both anti-inflammatory and

immunosuppressive effects. Its many properties have been extensively reviewed^{2,7,8} and include lysosomal stabilisation,⁹ enzyme inhibition,^{10,11} the suppression of chemotaxis¹² and particle uptake by polymorphonuclear leucocytes¹²⁻¹⁴ and inhibition of DNA, RNA, and protein biosynthesis.¹⁵ It also influences immunological reactions by suppression of in-vitro transformation of lymphocytes¹⁶ as well as reduction in cytotoxicity¹⁷ and the number of cells bearing immune associated surface markers.¹⁸

In this study we have demonstrated a significant dose-related increase in autophagosome production in lymphocytes after exposure to chloroquine, as well as an inhibition of particle uptake by polymorphonuclear leucocytes at concentrations approaching therapeutic plasma levels of the drug (1.5 µM or 0.8 µg/ml).¹⁹ After 90 minutes all the specimens showed inhibition of phagocytosis at 50 and 5 µg/ml chloroquine, and 7 out of 9 were inhibited at 0.5 µg/ml, with the 2 lower concentrations of the drug having no

Table 2 Autophagosome formation in separated T and B lymphocytes after 4 hours' incubation in 5 µg/ml chloroquine

	T cells +5 µg/ml chloroquine	T cells control	Chi- squared value	Significance (p)	B cells +5 µg/ml chloroquine	B cells control	Chi- squared value	Significance (p)
Autophagosomes per 200 lymphocytes	Expt. 1 73 Expt. 2 78	35 27	17.4 32.3	<0.001 <0.001	65 65	33 39	13.0 8.1	<0.001 <0.005

deleterious effects on neutrophil cell structure even after 4 hours. The prevention of phagocytosis may be one of the ways in which chloroquine exerts its action. This might not only have the effect of reduced release of enzymes such as collagenase²⁰ and neutral proteases capable of degrading cartilage proteoglycans,²¹ to which joint tissues would be particularly susceptible, but may also decrease the production of prostaglandins, leucotrienes, and free radicals, which occur as a direct result of phagocytosis.²² Reduced particle uptake has previously been described by other workers,¹²⁻¹⁴ but in their studies no attempt was made to evaluate the effect on the cells of the high concentrations of chloroquine required by them to inhibit phagocytosis. Our results clearly show that exposure to 50 µg/ml chloroquine for 4 hours is highly deleterious to the cell, causing swelling and vacuolation of lysosomal granules. It therefore seems unlikely that studies¹²⁻¹⁴ utilising a concentration of 10⁻³ M (516 µg/ml) chloroquine bear much resemblance to a biologically relevant situation. The importance of monitoring cells at the ultrastructural level cannot be too strongly emphasised, for moribund cells, although intact and not releasing lactate dehydrogenase, do not phagocytose, and it would be erroneous to attribute this to a specific action of the drug rather than to toxicity.

In a previous investigation³ we demonstrated a marked inhibition of phagocytosis by neutrophils after exposure to therapeutic concentrations of prednisolone which was associated with the biosynthesis of a protein or polypeptide, probably analogous to the protein isolated by Hirata *et al.*,²³ the production of which was also initiated in neutrophils by the action of glucocorticoids. This compound was found by them to inhibit phospholipase A₂ activity, an enzyme which cleaves arachidonic acid from membrane phospholipids. We suggested that phospholipase A₂ inhibition may depress phagocytosis by blocking the availability of membrane precursors necessary for the production of new plasma membrane required for the accommodation of ingested particles,²⁴ and it is interesting to note that chloroquine and its related compound mepacrine have both been shown to have a phospholipase-inhibiting action.¹¹⁻²⁵ As well as suppressing phagocytosis such an enzyme inhibition may account for the predominance of osmiophilic, membranous, electron-dense material seen in the autophagosomes of our own and other studies, which morphologically resembles undegraded phospholipid.

Autophagy is the process of sequestration of intracellular components and their subsequent degradation by lysosomes; it appears to be a normal physiological event, contributing to the turnover of cell constituents.²⁶ It can be induced by a number of

treatments, including starvation,²⁷ and a possible explanation for its occurrence in chloroquine-treated cells may be amino-acid deprivation due to the malfunction of lysosomes. Lysosomal abnormalities have previously been described in many tissues following chloroquine therapy,²⁸ and a common feature in all these cases seems to be the appearance of vacuoles containing various amounts of membranous, lamellar, or particulate matter, the accumulation of which suggests incomplete or partial digestion by lysosomal enzymes. A number of workers have demonstrated changes in lysosomal enzyme activity following chloroquine treatment, including a reduction in cathepsin B₁¹⁰ and phospholipase A₂¹¹ activity and decreased mucopolysaccharide degradation.²⁹

It has been suggested that the morphological effects of chloroquine reflect an accumulation of autophagosomes due to the inhibition of breakdown rather than to a stimulation of autophagy.²⁹ However, Read and Bay²⁸ found evidence of an increase in the synthesis of lysosomes. Our study did not conclusively support either view but would suggest the former.

The particular susceptibility of lymphocytes to autophagosome formation may account for the immunosuppressive properties of chloroquine, perhaps by stabilising lysosomes, and thus preventing the intracellular release of enzymes such as RNA-ase necessary for the derepression and subsequent transformation of lymphocytes.¹⁶⁻³⁰⁻³² An alternative mechanism to explain immunosuppression by chloroquine relates to its effects on cell surface membranes,¹⁷⁻¹⁸ within which structure it accumulates,³³ leading to a failure of cell-to-cell recognition and receptor presentation and recycling. In fibroblasts the inhibition of receptor recycling is thought to result from, at least in part, the capacity of chloroquine to raise the intralysosomal pH.³⁴⁻³⁵ A similar mechanism may account for its antimalarial activity, the increase in *Plasmodium* lysosomal pH producing enzyme inhibition and a subsequent accumulation of undigested haemoglobin in giant vacuoles²⁷ which resemble the autophagosomes found in mammalian tissues following chloroquine treatment.²⁸

In this report we have demonstrated an effect on neutrophil and lymphocyte lysosomes after only a few hours in chloroquine. How these changes affect the long-term function and metabolism of the cell in rheumatoid arthritis is difficult to assess. Most workers agree that chloroquine takes a minimum of 4 weeks before maximal benefits are achieved. This apparent anomaly may be partly explained by an effect on precursor cells as well as circulating leucocytes, as previous studies have reported the presence of abnormal granules in myelocytes.³⁶ All studies of the effects of chloroquine both in vivo and in vitro¹⁰⁻³⁷

indicate a common mode of action of the drug, leading to abnormalities of lysosomal activity which may, in rheumatoid disease, exert its effects by decreasing the amount of tissue damage caused by the action of proteases found in neutrophil granules. An inhibition of phagocytosis would also decrease enzyme release as well as cause a reduction in free radical generation and leucotriene and prostaglandin production, all of which would be beneficial in alleviating inflammation.

The hitherto undescribed increase in lymphocyte autophagosome production may also be of importance in conditions such as rheumatoid arthritis, where some populations of lymphocytes play an aggressive role, as this impedes their function and may be beneficial both by a direct effect and by allowing the restoration of normal immune responses.

It appears, therefore, that in the in-vitro situation chloroquine exerts a 2-pronged attack on leucocytes. Firstly, it has an inhibitory effect on phagocytosis, and secondly it increases autophagosome production in lymphocytes, which undoubtedly affects their function. In this respect the antirheumatic action of chloroquine parallels its anti-malarial activity. Studies are now in progress in our laboratory in which we are examining the circulating leucocytes from patients on chloroquine therapy to determine whether there is any change in the structure and frequency of lysosomes in the various cell types.

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