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INDUCTION OF SELECTIVE ACID HYDROLASE RELEASE FROM MOUSE MACROPHAGES DURING EXPOSURE TO CHLOROQUINE AND QUININE

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Abstract—Mouse peritoneal macrophages when exposed *in vitro* to doses of chloroquine and quinine of up to 300 μM, were found to release into culture media nearly 70 per cent of the total available culture activities of lysosomal acid hydrolases such as β-glucuronidase, β-galactosidase, and *N*-acetyl-β-D-glucosaminidase, by a process that did not depend on cell lysis. The kinetics of lysosomal enzyme secretion, which was maximal after 6–8 hr of incubation, were paralleled morphologically by the appearance in the perinuclear region of large autophagic vacuoles which, occasionally, were seen to link the lysosomes to the cells exterior. The possible mechanism of drug accumulation, and the apparent discrepancy between steady state therapeutic plasma levels and the doses required to induce acid hydrolase secretion, are discussed.

The 4-amino quinoline derivatives chloroquine and quinine have both been widely used in the treatment and prophylaxis of malaria [1, 2]; while chloroquine has also been used in the management of diseases of immunopathological origin such as rheumatoid arthritis [3] and systemic lupus erythematosis [4]. However, whilst their use appears to be beneficial in the treatment of some individuals, the widespread application of these drugs is somewhat limited because of their many adverse effects such as skin disorders, retinopathy, and occasionally, cardiac arrhythmia with chloroquine [1]; and headaches, myalgia and tinnitis with quinine [5].

Animal experiments have revealed further toxic effects. For instance, Gleiser *et al.* [6] have reported that the administration of large doses of chloroquine to swine results in a cerebrospinal lipodystrophy that appears very similar to human Tay–Sachs disease; Abraham *et al.* [7] have reported the finding of autophagic vacuoles in the liver cells of rats following prolonged dosing with chloroquine; and Schmalbruch [8] has observed myopathic changes in the soleus muscles of rats receiving this drug.

At the cellular level, chloroquine has been shown to trigger a plethora of diverse effects such as: an ability to inhibit the entry of the toxic lectin modeccin into cultured HeLa cells [9], and a bimodal capacity to both inhibit and potentiate the mitogenic response of human lymphocytes towards phytohaemagglutin [10]. However, perhaps the most well documented property of these drugs is their ability to become highly concentrated in the lysosomal system of a wide variety of cell types including fibroblasts [11], hepatoma cells [12], macrophages [13] and retinol ganglion cells [14]; and, in view of their basic nature, the immediate effect of this is to elevate markedly the intralysosomal pH from its basal value of around pH 4.8, to about pH 6.5 [15]. When the intralysosomal concentration of these drugs becomes sufficiently high, the lysosomes swell osmotically to form large vacuoles [11], and the consequence of the raised intralysosomal pH is to substantially inhibit the lysosomal degradation of proteins and glycosaminoglycans by the lysosomal acid hydrolases [16, 17].

In a previous communication [18] we reported that several other weak bases, such as ammonium chloride and methylamine were capable of modulating lysosomal function by triggering the selective extrusion of lysosomal acid hydrolases from mouse peritoneal macrophages into the extracellular medium. We now report that both chloroquine and quinine can also effect this response by an apparently similar process.

MATERIALS AND METHODS

Experimental animals. Adult male BALB/c strain mice weighing approximately 25 g were obtained from the Department of Immunology, Animal House, University of Birmingham.

Tissue culture materials. 30 mm diameter tissue culture grade plastic petri-dishes were obtained from Sterilin Ltd., Teddington, Middlesex; tissue culture medium 199 was obtained from Wellcome Research Laboratories, Beckenham, Kent; 1 M HEPES (*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid), streptomycin, penicillin and heat-inactivated foetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Ayrshire.

Biochemical reagents. Lactic acid, NAD^+ grade IV, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide, 4-methylumbelliferone, chloroquine phosphate and quinine hydrochloride were obtained from Sigma Chemical Co., Poole, Dorset. Triton X-100 was obtained from B.D.H., Poole, Dorset. Glutaraldehyde, sodium cacodylate, uranyl acetate, lead citrate and osmium tetroxide were obtained from EMscope, London.

Macrophage collection, purification and culture. Approximately 25 g male BALB/c mice were rapidly killed by chloroform asphyxiation. The peritoneal cells, comprising about 60 per cent macrophages, were collected by lavage of the peritoneal cavity with 3 ml portions of medium 199 supplemented with 20 mM HEPES, pH 7.2, 100 μg ml^{-1} streptomycin, 60 μg ml^{-1} penicillin and 10% v/v heat-inactivated foetal calf serum. The cell suspension was adjusted to a concentration of 2×10^6 cells ml^{-1}, and 2 ml aliquots were dispensed into 30 mm diameter plastic petri-dishes. After incubation at 37° for 2 hr, the non-adherent cells were removed, and the remaining macrophage monolayers rinsed three times with PBS, before adding fresh medium 199 supplemented with 10 per cent heat-inactivated foetal calf serum. The macrophages were cultivated overnight at 37° to give a uniform monolayer of well spread cells composed of greater than 95 per cent macrophages as judged by nuclear staining with buffered Giemsa, and by their ability to phagocytose zymosan particles.

The cells were then rinsed three times with medium 199 (without serum) before being exposed to the drugs in serum-free medium 199. At the end of the incubation period, the supernatants were removed and the cells lysed with 2 ml aliquots of 0.1% Triton X-100 in 0.9% sodium chloride. Both supernatant and cell fractions were analysed for activities of various lysosomal and cytoplasmic enzymes.

Enzyme assays. Lactate dehydrogenase was assayed colorimetrically by measuring the rate of oxidation of lactic acid to pyruvic acid in the presence of NAD^+; while β-glucuronidase, *N*-acetyl-β-D-glucosaminidase and β-galactosidase were assayed fluorimetrically using the 4-methylumbelliferyl derivatives of glucuronic acid, *N*-acetylglucosamine and galactose respectively. All enzyme assays were carried out using an automated continuous-flow method (Riches and Stanworth, in preparation), and were conducted under optimal conditions which gave a linear release of reaction product with time.

Electron microscopy. Specimens for electron microscopy were fixed for 1 hr at room temperature in 200 mM cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde, and washed overnight in 200 mM cacodylate buffer, pH 7.4. The specimens were then post-osmicated in 2% aqueous osmium tetroxide for 1 hr and dehydrated with a series of graded ethanol/distilled water solutions of 50, 70 and 90%, followed by three changes of absolute ethanol. The cells were then embedded in Spurr's low viscosity resin and finally polymerized at 60° for 12 hr.

Ultrathin sections of the embedded specimens were cut using a Reichert Ultratome OMU 2 fitted with standard glass knives, and collected on uncoated copper grids. Sections were stained with ethanolic uranyl acetate followed by lead citrate and were viewed in a Siemen's Elmiskop 102 electron microscope operated at an accelerating voltage of 80 kV.

RESULTS

Induction of selective lysosomal enzyme release from macrophages by chloroquine

The effect of chloroquine concentration on the selective discharge of lysosomal enzymes from mouse macrophages was studied by exposing macrophage cultures to doses of chloroquine ranging from 50–500 μM for 5 hr at 37°. As will be seen from Fig. 1, there was a dose-dependent redistribution of the lysosomal acid hydrolases β-glucuronidase and β-galactosidase from the cells into the culture supernatants that rose steadily to almost 80 per cent release of the total available enzymic activities in the presence of 500 μM chloroquine. The absence of significant differences between the total culture activities of β-galactosidase and β-glucuronidase in treated and untreated cultures is interpreted as indicating that the lysosomal enzymes detected in the culture

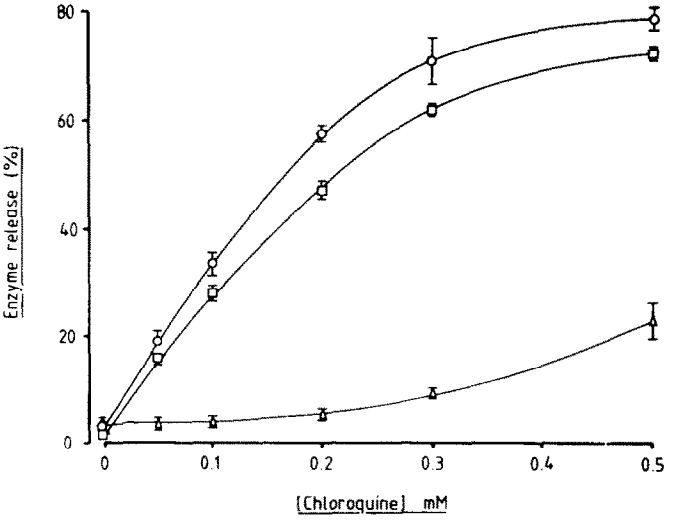

Fig. 1. Dose-dependent release of lysosomal enzymes from mouse macrophages after exposure to chloroquine for 5 hr in serum-free medium 199 at 37°. ○ = β-glucuronidase; □ = β-galactosidase; △ = lactate dehydrogenase. Each point represents the mean ± S.D. for four observations.

supernatants were derived by direct secretion from the lysosomes rather than being newly synthesized. The selectivity of the release process at lower concentrations of chloroquine (50–200 μM) is shown by the failure of cytoplasmic lactate dehydrogenase (LDH) to appear in culture supernatants. However, at higher concentrations (i.e. 500 μM) chloroquine appeared to exert a slight lytic effect on the cells leading to approximately 20 per cent loss of viability.

The time course of acid hydrolase secretion was studied by exposing macrophage cultures to a fixed dose of chloroquine of 300 μM for periods of time up to 8 hr at 37°. Figure 2 shows that small, but statistically significant ($P < 0.01$) levels of β-galactosidase and *N*-acetyl-β-D-glucosaminidase were found in the culture supernatants after only 30 min of incubation; while maximum lysosomal enzyme release, which was in the order of 50 per cent of the total available enzyme activities, occurred after approximately 6 hr of incubation. The absence of significant ($P > 0.1$) levels of LDH in the culture supernatants at any point in time confirms that the discharge of acid hydrolases occurred in the absence of cell death.

Morphologically, macrophages which had been exposed to chloroquine showed a graded, dose-related tendency to form large vacuoles in the perinuclear area. When examined under the electron microscope at a magnification of 8000 (Fig. 3) the vacuoles, which varied in diameter from 0.3–1.4 μm were found to contain cytoplasmic material and hence gave the impression of being autophagic vacuoles, in contrast to cells which had not been exposed to the drug (Figs. 3 and 4). Fusion between separate autophagic vacuoles (single arrow, Fig. 3), and between lysosomes and autophagic vacuoles (double arrow, Fig. 3) was frequently seen; but, more significantly, some cells were found to have channels connecting an autophagic vacuole to the cell's exterior (triple arrow, Fig. 3). Conceivably, these channels could be a route through which lysosomal acid hydrolases can indirectly gain access to the pericellular area.

Induction of selective lysosomal enzyme release from macrophages by quinine

Marked changes in the distribution of the activities of lysosomal acid hydrolases was also observed following exposure of mouse macrophages to the structurally related quinoline derivative, quinine (Fig. 5). After 5 hr incubation in medium containing 50 μM quinine, the levels of both β-galactosidase and β-glucuronidase were significantly greater ($P < 0.01$) than in controls that had not been exposed to the drug. This dose-dependent release of lysosomal acid hydrolases increased steadily until more than 50 per cent of the total available enzyme activities had been released following exposure to 500 μM quinine. The selectivity of the release process was analogous to that of chloroquine in that lower concentrations of quinine (50–200 μM) failed to release cytoplasmic LDH; while some loss of viability was observed at higher concentrations (500 μM).

The time course of lysosomal enzyme release was studied in macrophage cultures maintained for periods up to 8 hr in the presence of a single dose of quinine of 300 μM. As will be seen from Fig. 6, the time course of acid hydrolase secretion induced by quinine was essentially similar to that of chloroquine. Significant levels ($P < 0.01$) of β-galactosidase and *N*-acetyl-β-D-glucosaminidase were found in the culture supernatants after only 30 min incubation, while maximum enzyme release occurred after approximately 6 hr incubation.

DISCUSSION

Cells exposed to the antimalarial drugs chloroquine and quinine take up avidly these compounds, concentrating them many-fold over the surrounding

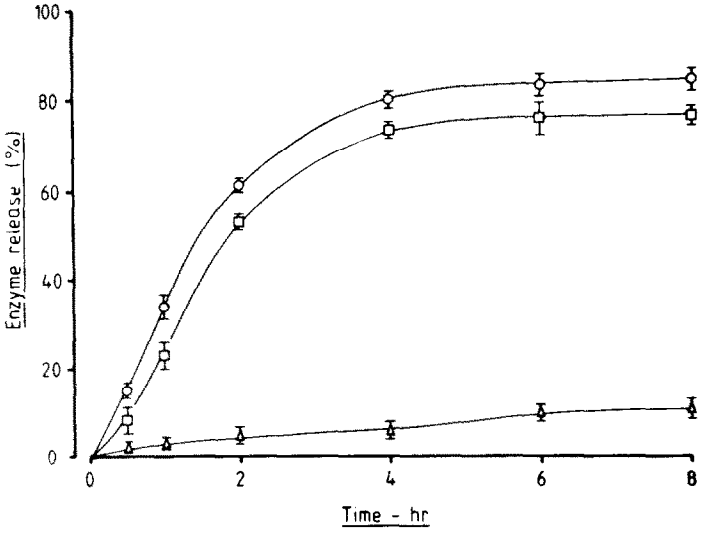

Fig. 2. Time-dependent release of lysosomal enzymes from mouse macrophages after exposure to a single dose of chloroquine (300 μM) in serum-free medium 199. ○ = *N*-acetyl-β-D-glucosaminidase; □ = β-galactosidase; △ = lactate dehydrogenase. Each point represents the mean ± S.D. for four observations.

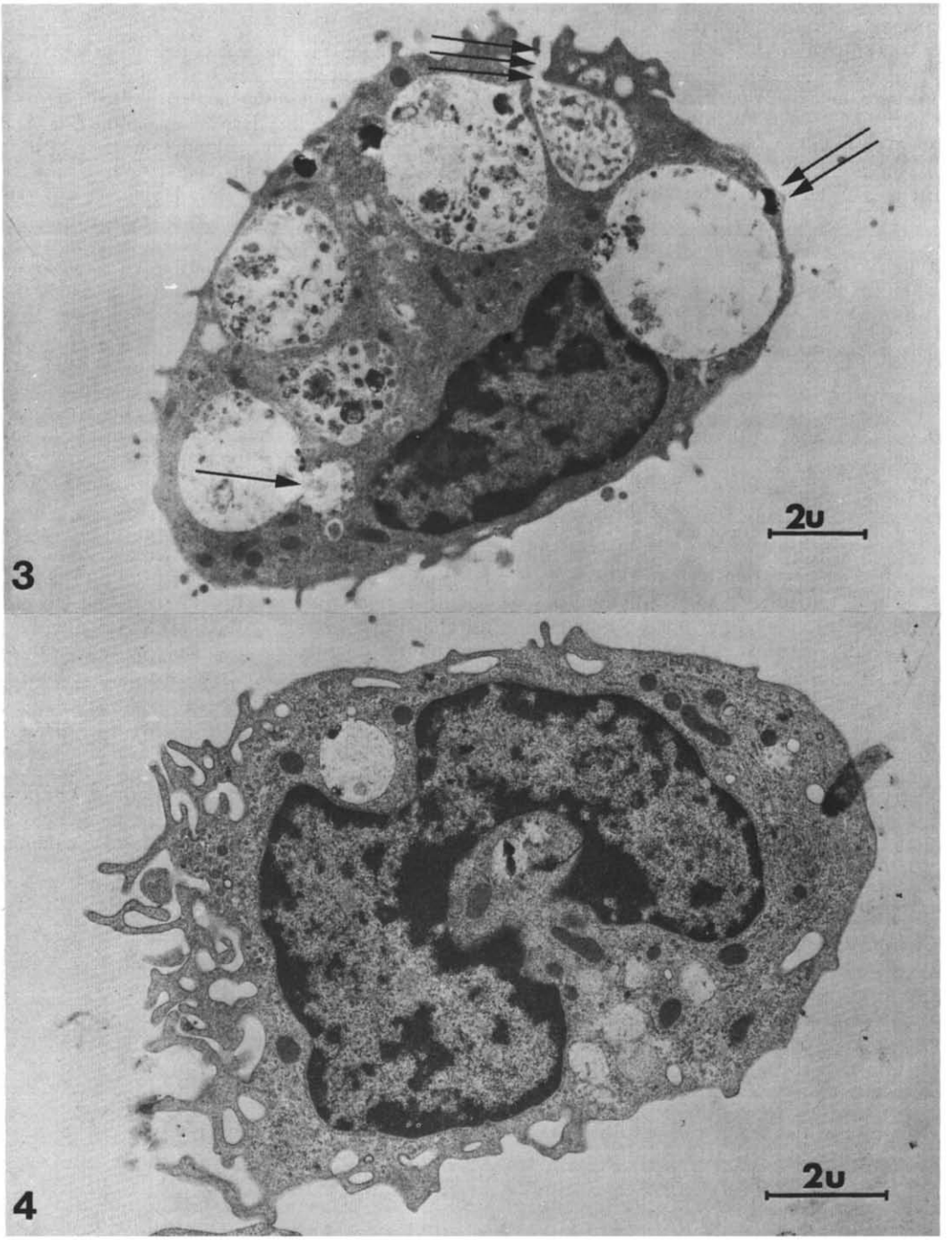

Fig. 3. Morphologic changes in mouse macrophages following exposure to chloroquine (300 μM) for 5 hr at 37° magnification ×8000.

Fig. 4. Normal mouse macrophage. Magnification ×8000.

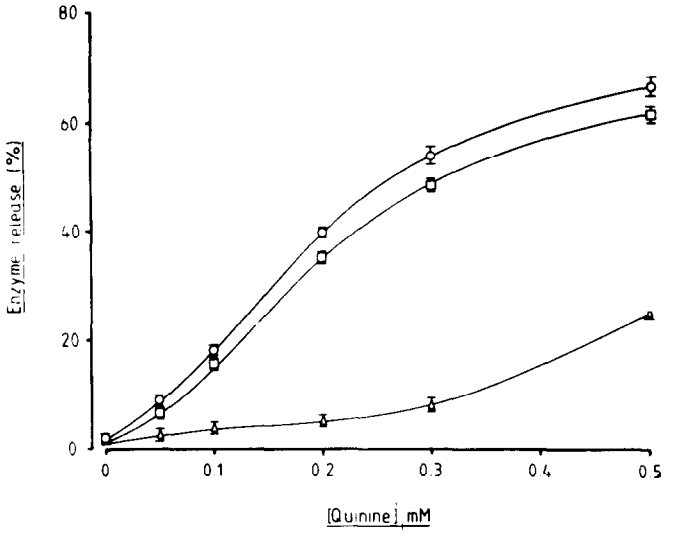

Fig. 5. Dose-dependent release of lysosomal enzymes from mouse macrophages after exposure to quinine for 5 hr in serum-free medium 199 at 37°. ○ = β-glucuronidase; □ = β-galactosidase; △ = lactate dehydrogenase. Each point represents the mean ± S.D. for four observations.

medium [19, 11]. Indeed, it has been estimated by Wibo and Poole [11] that the intralysosomal concentration of chloroquine in rat fibroblasts exposed to 100 μM of drug could be as high as 50 mM, thus representing a concentration factor of some 500-fold. Several sequelae to this phenomenon have been reported in the literature. For example, Okhuma and Poole [15] have described an increase in intralysosomal pH in mouse macrophages following brief exposure to chloroquine; while Livesey *et al.* [20] have reported that exposure of rat yolk sacs to chloroquine *in vitro*, results in a decreased rate of degradation of endogenous and pinocytically ingested exogenous proteins. As far as we are aware, our findings of the capacity of chloroquine and quinine to induce the selective extracellular release of lysosomal acid hydrolases from mouse macrophages, represents a previously undescribed property of these compounds.

The exocytotic process initiated by chloroquine and quinine showed gross similarities to one another in terms of both the dose–response and time–course characteristics with maximal selective release of lysosomal enzymes being effected by exposure of the macrophages to 300 μM drug for 6 hr. It was noted, however, that on a weight for weight basis, the amplitude of maximum release effected by 300 μM drug was higher for chloroquine than for quinine; although the reasons for this apparent difference are not at present clear.

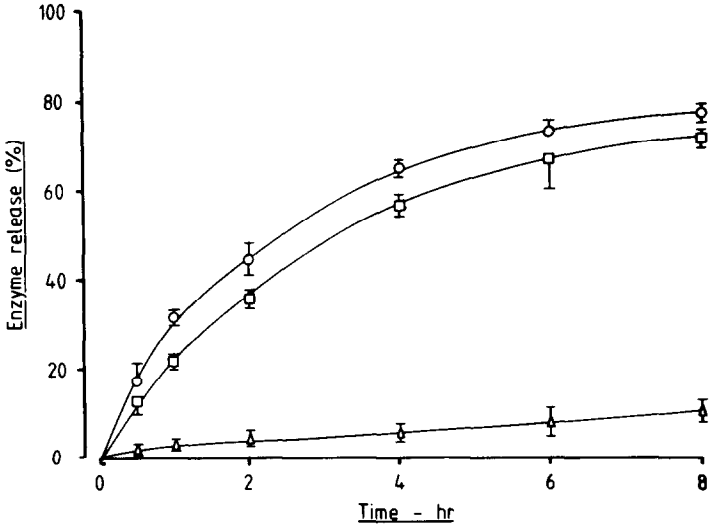

Fig. 6. Time-dependent release of lysosomal enzymes from mouse macrophages after exposure to a single dose of quinine (300 μM) in serum-free medium 199. ○ = *N*-acetyl-β-D-glucosaminidase; □ = β-galactosidase; △ = lactate dehydrogenase. Each point represents the mean ± S.D. for four observations.

One doubt that might be raised about the chemical relevance of our findings is that the doses of chloroquine and quinine required to elicit selective lysosomal enzyme release *in vitro* are considerably higher than the steady state therapeutic plasma levels for these compounds when they are employed as anti-rheumatic and anti-malarial agents; these being approximately 1.5 μM for chloroquine [21], and 17.7 μM for quinine [5]. However, it should be pointed out that in a study of the extra-vascular accumulation of several 4-aminoquinoline derivatives, Berliner *et al.* [1] reported that the levels of chloroquine in tissues such as liver and kidney exceeded the plasma level of the drug by some 600-fold. Thus, the levels of chloroquine which we have found necessary to induce hydrolase secretion from macrophages *in vitro* seem to compare favourably with those attained in the tissues of animals and individuals undergoing chloroquine chemotherapy.

Parallel studies of the morphological changes of chloroquine treated macrophages confirmed the earlier findings of Fedorko *et al.* [13, 23] of large autophagic vacuoles evenly distributed throughout the cytoplasm. However, whilst cells frequently showed evidence of fusion between separate autophagic vacuoles and between lysosomes and enclosed autophagic vacuoles, other cells were seen to have channels connecting autophagic vacuoles to the cells exterior; and, in the light of work reviewed by Goldstein [24], we believe that these channels could be a route through which acid hydrolases can indirectly gain access to the extracellular milieu.

The biochemical mechanism underlying the selective release of lysosomal enzymes from macrophages is poorly understood. Schorlemmer *et al.* [24] have drawn attention to the fact that most substances that have been found to induce selective hydrolase secretion from macrophages share in common a capacity to activate the alternative pathway of complement; and, since the macrophage is capable of secreting components of the alternative pathway [25], it could comprise a self-activating unit producing C3b which has also been found to be an effective stimulant of hydrolase secretion [26]. However, whilst we have found that several primary aliphatic monoamines are also capable of inducing selective lysosomal enzyme release [18] and interacting with components of the alternative pathway (unpublished observations), we have been unable to demonstrate that either chloroquine or quinine likewise activate the alternative complement pathway. Thus the ability of these drugs to induce acid hydrolase secretion from macrophages would appear to be due to their lysosomotropic properties rather than due to any effect on endogenous macrophage complement component C3, as seems to be the case with some other stimulants of this response such as zymosan particles.

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