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INACTIVATION OF LYSOSOMAL FUNCTION IN NORMAL CULTURED HUMAN FIBROBLASTS BY CHLOROQUINE

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Abstract—The lysosomal function of mucopolysaccharide degradation in normal cultured, living human fibroblasts can specifically be inhibited without affecting over-all cell viability or growth. In an earlier report we have shown that an increase in medium pH will progressively inhibit this function. The present paper demonstrates that chloroquine in concentrations around 1–2 × 10^{-5} M strongly interferes with mucopolysaccharide degradation in the living cell. Electron microscopy shows that a morphological picture resembling a lysosomal storage disease develops in these cells after less than 2 days exposure to the drug. We propose that the antimalarial effect of chloroquine (and preliminary results would include quinacrine and quinine) and at least some of the major side effects of the drug are due to the same basic mechanism: an inhibition of normal lysosomal activities in the parasite as well as in the human cell.

DRUGS effective in the treatment and prophylaxis of malaria have been divided into the "specific" and "nonspecific" groups.[1] Belonging to the first category are drugs such as chloroquanide, pyrimethamine and their derivatives. These drugs have a slow schizontocidal effect, resistance develops readily, and their mode of action is the inhibition of synthesis of folinic acid from para-aminobenzoic acid (PABA).

This report is concerned with the effects of the "nonspecific" group, which includes drugs such as chloroquine, quinacrine and quinine. These drugs have a rapid schizontocidal effect, resistant strains are difficult to isolate, and their mechanism of action appears to be completely non-specific.

Homewood *et al.*[2] have recently suggested that chloroquine kills the malarial parasite by being concentrated in the digestive vacuole of the infecting organism. Such an accumulation is suggested to raise the pH in this organelle to the point where the acid hydrolases are inactivated. The digestion of host material consequently stops and the parasite starves to death. The digestive vacuole of the malarial parasite is considered the analogue of the lysosome in the mammalian cell.

We have recently reported that the lysosomal function of mucopolysaccharide degradation in normal cultured human fibroblasts is progressively inhibited as the pH of the growth medium is increased.[3] A morphological picture of a severe "lysosomal storage disease" results from the same changes.[4] This paper reports that the presence

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of small (approx. 10^{-5}M) concentrations of chloroquine inactivates this lysosomal function in much the same way as an increase in medium pH. Preliminary observations suggest that low concentrations of quinacrine or quinine have the same effect. These observations suggest that these drugs may in principle have an identical effect on human cells as on the malarial parasite, and it offers an explanation for their well known side effects.

MATERIALS AND METHODS

Cell culture. Fibroblast lines were established from skin biopsies of five normal young adults (three males, two females) and maintained as monolayers in plastic culture flasks (Falcon). Growth medium was Eagles minimal essential medium with 10% fetal calf serum and $NaHCO_3$ (1·7 g/l). Penicillin and streptomycin were added to final concentrations of 100 U and 100 μg/ml, respectively. Aureomycin were included in some experiments in final concentrations of 100 μg/ml. When bicarbonate was the only buffer system, the flasks were incubated with a gas phase of 5% CO_2 in air. When a more exact pH control was necessary, organic buffers were used as described by Eagle[5] and the cells incubated with air as the gas phase. Medium was changed daily in order to keep pH as constant as possible. Cultures were periodically screened for the presence of mycoplasma, and infected cultures discarded. pH was measured with a Corning model 12 m.

Ultrastructural studies. Cells for electron microscopy were grown on coverslips and examined before reaching confluence. They were either fixed *in situ* on the coverslips, or trypsinized and fixed as a pellet with 2% glutaraldehyde in cacodylate buffer, pH 7·4, containing 0·3 M sucrose at 4° for 60–90 min. Details have been described earlier[4].

Lysosomal studies. Normal catabolic function of the lysosomes in cultured fibroblasts was studied using degradation of mucopolysaccharide (MPS) as the parameter. This technique was developed for the study of genetic mucopolysaccharidoses,[6-8] and takes advantage of the fact that when labelled inorganic sulphate is given to mammalian cells in culture, the only macromolecules to become labelled are the mucopolysaccharides. The majority of the newly synthesized macromolecules are rapidly secreted from the cell as proteoglycans, but a minor portion is transferred to the lysosomes of the cell. MPS molecules which have been trapped in the lysosomes must be degraded to small molecules before they can leave the lysosomes.[6-8] The disappearance rate of radioactivity from cells which have been labelled with inorganic sulphate is therefore an index of this lysosomal function in the living cell. (So-called "chase" experiments.)

Technical details have been given by Neufeld *et al.* [6-8] In brief, cells are labelled with inorganic sulphur (S^{35}) in a medium where $MgCl_2$ is substituted for $MgSO_4$. Macromolecules are isolated from trypsinized cells and counted in a liquid scintillation counter. The radioactivity of macromolecules recovered in the medium is determined after dialyzing the medium against 0·1 M NH_4SO_4 for 6–8 hr and against running tap water overnight.

Chloroquine, quinacrine and quinine were purchased from Sigma Chemical Co. They were dissolved in medium immediately before use and sterilized by Millipore filtration.

RESULTS

When normal cells in medium with a pH below 7·0 are grown in the presence of labelled inorganic sulphate, they accumulated labelled MPS molecules for the initial 8–12 hr.[6] After this period, there is a steady state situation, in which the removal and synthesis of MPS are in equilibrium. Synthesis takes place in the endoplasmic reticulum and Golgi apparatus, while the removal involves secretion of macromolecules from the cells as well as degradation of molecules inside the lysosomes with release of inorganic sulphate.

Figure 1 shows the effect of chloroquine on such experiments in media with a pH below 7. At concentrations of and above 1×10^{-5} M, the accumulation of labelled MPS molecules increases markedly, and there is no evidence of a steady state situation

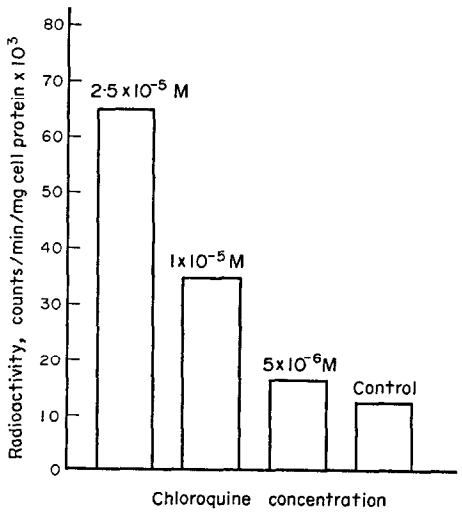

FIG. 1. The effect of chloroquine on mucopolysaccharide accumulation in normal human fibroblasts. Cells were grown in medium of pH 6·8 labelled with $H_2S^{35}O_4$ and with the chloroquin concentrations indicated. After 3 days the cells were trypsinized and intracellular radioactivity in macromolecules was determined.[6,7]

within the first 4–6 days of incubation. Chloroquine did not influence the secretion of MPS macromolecules into the medium or the amount of labelled macromolecules released from the cells by trypsin.

This picture of increased accumulation is also observed in cells carrying genetic defect in the lysosomal degradation of mucopolysaccharides.[6-8] Figure 2 shows that this similarity is found also in the "chase" experiments. Cells were labelled for 4 days and then washed and fed unlabelled medium. The disappearance rate of the intracellular label under such circumstances is a function of the lysosomal degradation of mucopolysaccharides.[6-8] It is evident that concentrations of chloroquine exceeding 1×10^{-5} M strongly interferes with this degradation.

The effects of chloroquine on the MPS degradation in cells carrying a genetic defect in mucopolysaccharide degradation, such as Hurler and Hunter cells were also studied. Studying one cell line of each of these genotypes, we found that they were not

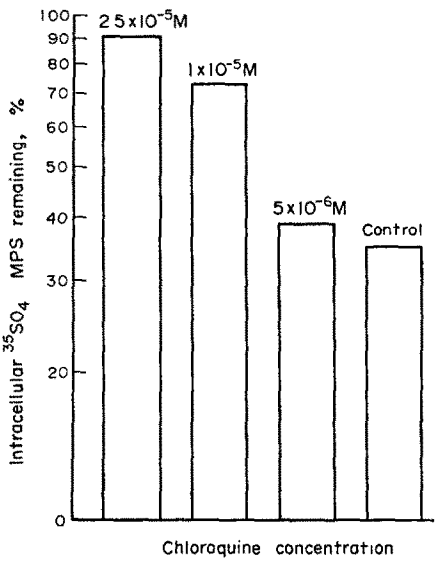

FIG. 2. The effect of chloroquine on the lysosomal degradation of mucopolysaccharides. Normal cells were labelled with S^{35} for 4 days at pH 8·0 and the disappearance of labelled macromolecules was determined after 15 hr in unlabelled medium of pH 6·8.

affected by the concentrations of chloroquine used, suggesting that chloroquine inactivates the same pathway as that affected by their genetic defect. Normal cells grown at neutral pH with 1–3 $\times$ 10^{-5} M chloroquine were thus indistinguishable from cells with genetic defects in MPS degradation. The effect of chloroquine on normal cells is therefore remarkably similar in this respect to the effect of increasing the medium pH[3].

There was no obvious toxic effect of chloroquine in concentrations up to 3 $\times$ 10^{-5} M. However, concentrations above 5 $\times$ 10^{-5} M clearly had deleterious effects on the cells.

Preliminary experiments have shown that quinacrine and quinine have essentially the same effect on the lysosomal function as chloroquine. Quinacrine inactivated the MPS degradation effectively at a concentration of 1 $\times$ 10^{-5} M while quinine required a higher concentration and had peak efficiency around 1 $\times$ 10^{-3} M.

Effect of chloroquine on the ultrastructure of cultured fibroblasts. Following 2 days exposure to 1 $\times$ 10^{-5} M chloroquine the cells contained large numbers of cytoplasmic inclusion bodies. The central or perinuclear portions of the cells appeared most affected and were almost entirely filled with abnormal structures some of which contained glycogen, mitochondria, and other cellular components (Fig. 3).

The predominant type of inclusions induced by chloroquine were round to oval in shape and bound by a single limiting membrane. The majority of these structures contained several electron dense components which usually contained a crystalline-like, tightly packed material (Fig. 4). Lipid configurations and membranous myeloid bodies were less frequently observed (Fig. 5) although these structures are characteristic of cellular chloroquine toxicity *in vivo*.[9–11]

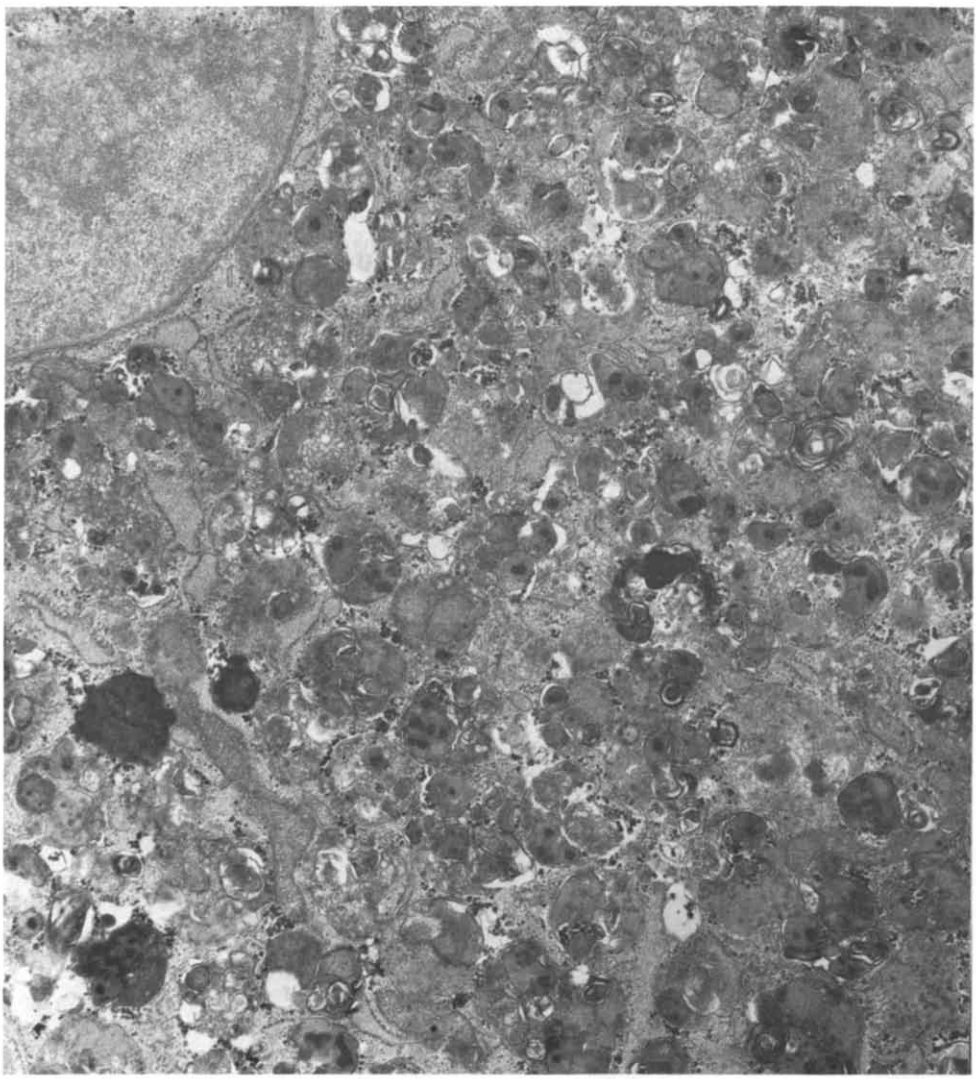

FIG. 3. Central portion of a normal human fibroblast exposed to 1×10^{-5} M chloroquine for 2 days. The nucleus is shown at the upper left, the adjacent cytoplasm is packed with inclusions. × 16,250.

Control cultures, although containing a small number of autophagic vacuoles, did not contain inclusions like those observed in the chloroquine treated cells, and appeared as previously described.[4]

Cells were examined after 7 days exposure in order to determine whether the inclusions observed at 2 days were an intermediate stage between autophagic vacuoles and myeloid bodies. If this was the case, the cells should contain predominantly myeloid figures after 7 days. Cells exposed to chloroquine for 7 days however, appeared essentially the same as at 2 days and the number of myeloid bodies was still relatively small.

These cells were structurally similar in appearance to fibroblasts maintained at a higher than normal pH[4], although the non-myeloid type of inclusion body induced by chloroquine exhibited a more complex and variable sub-structure.

DISCUSSION

This report demonstrates that chloroquine and probably also quinacrine and quinine inactivates at least one lysosomal function in cultured normal human fibroblasts, namely that of mucopolysaccharide degradation. Whether the lysosomal degradation of macromolecules other than mucopolysaccharides are likewise inhibited by chloroquine has not been demonstrated. Electron microscopy show that a picture resembling a lysosomal storage disease develops when chloroquine is present in the growth medium of the cells. The effects of low concentrations of chloroquine are thus very similar to the effects observed when the pH of the growth medium of the cells is raised above the physiological range.

Homewood *et al.*[2] have postulated that chloroquine kills the malarial parasite because it is selectively concentrated in the digestive vacuoles of the parasite[12] and lowers the proton concentrations to the point where the acid hydrolases are inactivated. Increase in medium pH had a similar effect on the parasite as addition of chloroquine. Their observations on the malarial parasite are similar to our observations in the living human cell[3] and indicate that chloroquine may have the same effect on these two cell types. Whether this pH and chloroquine sensitivity is mediated through changes in membranes or actually reflects changes in proton concentrations inside the lysosomes cannot be decided by the present observations. It is well known, however, that chloroquine does have a significant influence on membrane stability.[13–15]

The administration of chloroquine to experimental animals greatly increases the number of autophagic vacuoles in liver cells.[11] Early autophagic changes can also be detected in isolated macrophages.[16] Stimulation of autophagy by chloroquine has been offered as an explanation for these observations. However, an alternative possibility is that chloroquine inhibits the normal process of autophagy so that the increased number of vacuoles observed in the cells may reflect an accumulation rather than an increased production. The effect of chloroquine would thus be comparable to the effect of genetic deficiencies of lysosomal enzymes,[17] to the administration of antibodies to lysosomal acid hydrolases,[18] or to an increase in extracellular pH[3].

The concentrations of chloroquine needed to inactivate lysosomal functions in living fibroblasts were not too different from those which may be found in plasma of patients treated with chloroquine.[19] It is tempting to speculate that the inactivation which we have observed *in vitro* also explains some of the side effects of the drug when given to man, the most important being retinopathy.[20] For some unknown reason,

chloroquine persists in the eyes for a long time following chloroquine therapy, and has been detected up to 16 years after last intake of the drug.[21] Furthermore, it has been shown in cats,[22] rats[23] and swine,[24] that chloroquine-induced retinopathy is characterized by the appearance of large membrane-bound vacuoles in the cells in the retina, in particular the ganglion cells. These vacuoles are similar to lysosomes. Of special interest is the observation that chloroquine given in large doses to swine results in a cerebral lipodystrophy very similar to the human Tay–Sachs disease,[25] which is a lysosomal storage disease caused by a lack of hexosaminidase A.

We propose, therefore, that the retinopathy observed as a side effect of prolonged chloroquine therapy is due to a chloroquine induced inactivation of lysosomal function(s) in some sensitive cells in the retina, most likely the ganglion cells. The shizontocidal effect of the drug and the major side effects could therefore be due to the same basic mechanism: an inhibition of normal lysosomal activities in the parasite and the human cell.

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