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CHLOROQUINE AND AMMONIUM ION INHIBIT RECEPTOR-MEDIATED ENDOCYTOSIS OF MANNOSE-GLYCOCONJUGATES BY MACROPHAGES: APPARENT INHIBITION OF RECEPTOR RECYCLING

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SUMMARY: ^{125}I-Mannose-BSA is taken up by macrophages by receptor-mediated pinocytosis. Previous studies indicated that uptake is rapid and proceeds linearly with time in the absence of protein synthesis suggesting that receptors are conserved and recycled. Chloroquine and NH_4^+ inhibit uptake of ^{125}I-Mannose-BSA and its subsequent digestion by macrophages. Inhibition of uptake is inhibitor-concentration and time dependent. Both binding of ligand (4°) and internalization of pre-bound ligand (4°) following warm-up are unaffected by the inhibitors. However, brief incubation of cells at 37° with inhibitor in the absence of ligand results in a substantial reduction in cell surface binding sites. Our interpretation of these results is that chloroquine and NH_4^+ inhibit receptor recycling and thereby inhibit ligand uptake.

Receptor-mediated endocytosis is employed by most eucaryotic animal cells to retrieve and assimilate macromolecules from their immediate environment with high selectivity (1). This mode of macromolecular transport is exemplified by the LDL-pathway in fibroblasts (2), galactose-specific recognition in hepatocytes (3), phosphomannosyl-recognition by fibroblasts (4-5) and mannose/N-acetylglucosamine-specific recognition in macrophages (6-7). The latter two pathways retrieve lysosomal enzymes and may play a role in lysosome biogenesis. A common feature of these uptake mechanisms is the apparent ability to recycle receptors following uptake of the receptor-ligand complex (2,8-9).

Chloroquine, ammonium chloride and other amines have been utilized in the study of lysosomal function largely because of their well documented effects on lysosomal pH (10) and digestion (11) of internalized macromolecules. Previously, chloroquine and ammonium chloride have been shown to inhibit uptake of lysosomal enzymes into cultured fibroblasts (12-13). In this report, we present evidence that chloroquine and ammonium chloride inhibit uptake and degradation of mannose-terminal glycoconjugates by macrophages.

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Inhibition of uptake is time dependent and appears to be due to an inhibition of receptor-recycling.

METHODS

Materials

Yeast mannan and chloroquine were obtained from Sigma. Fetal calf serum was obtained from GIBCO. Na ^{125}I was obtained from Amersham. Rats were purchased from Harlan Industries, Cumberland, IN. Silicon oil was from Accumetric Inc., Elizabethtown, Ky. Mannose-BSA (Man-BSA) (28 moles sugar/mole BSA) was prepared (and kindly donated by Y.C. Lee) by the method of Lee et al. (14). Man-BSA was labelled with ^{125}I by the chloramine T method (15) to specific radioactivities of 5-10 μCi/μg. For uptake experiments labelled ligand was diluted with unlabelled ligand as described (6).

Standard Assay Conditions.

The binding and uptake assays are described in detail elsewhere (7). Briefly, alveolar macrophages were isolated by lung lavage (6). Viability was unaffected by chloroquine or NH_4Cl and was always greater than 90% as determined by staining with 0.1% Trypan blue. Cells, in 0.1 ml media, were layered over 0.15 ml silicon oil. For uptake, cells (5 x 10^6/ml) were incubated at 37° in MEM containing 20% fetal calf serum, 10 mM Hepes, 10 mM Pipes, 10 mM Bes, pH 7.0. The reaction was terminated by centrifugation in a Beckman microfuge for 30 sec. The microfuge tube was cut and the pellet was counted in a Packard gamma-counter. For binding, the same assay was used except cells were incubated for 90 min at 4°. In both assays, non-specific cell associated ratioactivity was obtained by incubating the cells as above in the presence of a large excess unlabelled yeast mannan (1.25 mg/ml). The non-specific radioactivity were subtracted from the total cell associated radioactivity and was always less than 20%.

RESULTS

Effect of chloroquine on binding and uptake of ^{125}I-Mannose-BSA.

The results in Fig. 1A show the effects of increasing concentrations of chloroquine on uptake of ^{125}I-Man-BSA by cells. Qualitatively similar results were obtained with NH_4Cl. Binding was essentially unaffected by the addition of chloroquine or NH_4Cl. In Fig. 1B, the inhibitory effects of chloroquine are shown to be time dependent. Uptake in the absence of inhibitor was linear over ten min. and increasing concentrations of chloroquine prevented uptake in a dose-related way. The chloroquine effect on uptake at two min was minimal at all concentrations. Therefore, a temperature and time dependent process is required for chloroquine and ammonium chloride to produce their effects.

Effect of chloroquine on degradation of ^{125}I-Man-BSA following uptake.

Cells, pre-loaded with ^{125}I-Man-BSA as described in Table 1, were suspended in media with or without chloroquine and warmed to 37° for 20 min.

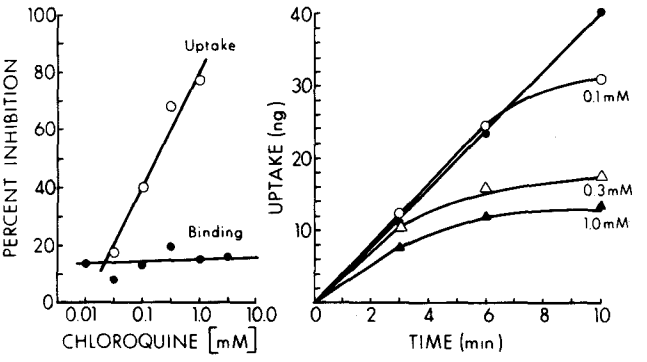

Figure 1. Effect of chloroquine on binding and uptake of ^{125}I-Mannose-BSA by alveolar macrophages. Uptake and binding (A) of ^{125}I-Man-BSA was followed as described in Methods. Chloroquine was added to cold media containing cells at least 10 min prior to warm-up. Uptake was allowed to proceed for 10 min at 37°. Binding was studied at 4° with 90 min incubations. Results are expressed as % inhibition of uptake. Time course (B) of chloroquine inhibition was determined with ^{125}I-Man-BSA (1 µg/assay) as ligand with 0.1, 0.3 and 1.0 mM chloroquine added approximately 10 min prior to warm-up.

The experiment was terminated by adding 1% sodium dodecyl sulfate to the incubation mixture. After incubation with SDS (37°, 60 min) the debris was spun away and the supernatant was chromatographed on Sephadex G-25 to determine low molecular weight degradation products. The results (Table 1) show that chloroquine sharply impaired the degradation of ^{125}I-Man-BSA.

TABLE 1

Effect of Chloroquine on Degradation of ^{125}I-Mannose-BSA by Macrophages

Treatment		Total Radioactivity		H/L
Chloroquine	Warmed to 37°	Peak I (H) Total CPM	Peak II (L) Total CPM	
-	-	94772	6359	14.9
-	+	21482	77987	0.3
+	-	90134	6207	14.5
+	+	112475	17254	6.5

Cells were pre-incubated with ^{125}I-Man-BSA (11.5 µg/ml) for 10 min at 37° after which they were rapidly cooled and washed by sedimentation. The ^{125}I-Man-BSA-loaded cells were suspended in media ± 0.5 mM chloroquine and incubated for 20 min at 37°. Degradation was terminated by the addition of an equal volume of 1% SDS in ammonium acetate, pH 7.5. After incubation for 60 min at 37° and centrifugation, the total extracted radioactivity (media + cells) was separated into high (H) and low (L) molecular weight components by Sephadex G-25 chromatography.

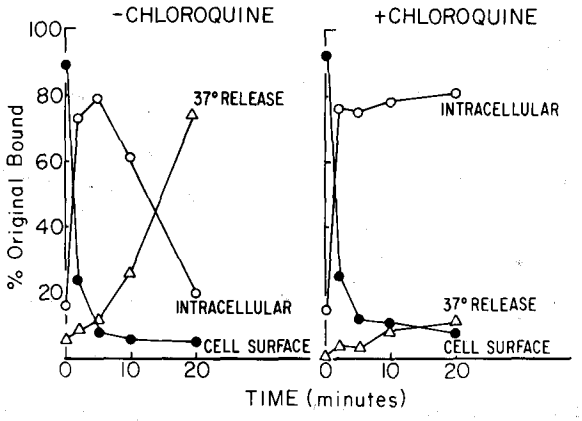

Figure 2. Effect of chloroquine on internalization of ^{125}I-Man-BSA. Cells (5 x 10^6/ml) were incubated at 4° with ^{125}I-Man-BSA (2.5 µg/ml) in MEM supplemented with 10 mg/ml BSA for 60 min with or without 1 mM chloroquine. Specific binding was determined as described in Methods. Following binding, the cells were washed by sedimentation and samples (5 x 10^5 cells) were warmed to 37° with or without chloroquine for 0, 2, 5, 10 and 20 minutes. The cells were then rapidly cooled and sedimented. The supernatant from this spin is referred to as 37° release. The cells were washed 1x, resuspended in Ca^{++}-Mg^{++}-free Hanks balanced salt solution containing 10 mg/ml BSA, pH 7.0, 0.1% trypsin and 10 mM EGTA, incubated on ice for 15 min and then sedimented through oil. Virtually all the cell surface radioactivity was released by trypsin/EGTA treatment (7). In this particular experiment, control cells bound 1.2 ng and chloroquine-treated cells, 0.8 ng of ^{125}I-Man-BSA.

Effect of chloroquine on the internalization of pre-bound ^{125}I-Man-BSA.

Cells were incubated with ^{125}I-Man-BSA at 4° where only cell surface receptors were occupied (7). Following removal of free ligand, the cell-bound radioactivity very slowly dissociates from the cells (t 1/2 > 60 min). However, cell surface radioactivity can be rapidly removed by trypsin/EGTA treatment at 4°. Cells, pre-labelled at 4°, were warmed to 37° for 0, 2, 5, 10 and 20 min after which they were rapidly cooled (Fig. 2). The addition of chloroquine (1.0 mM) to both pre-incubation and warm-up media did not alter the rate that radioactivity became resistant to trypsin/EGTA. Ammonium ion (30 mM) produced effects indistinguishable from chloroquine. The addition of CN^-/2-deoxyglucose (not shown) to the cells in another experiment impaired the internalization process. These results suggest that the mechanism of inhibition of ligand uptake by chloroquine is not an inhibition of internalization per se, but is after the internalization step.

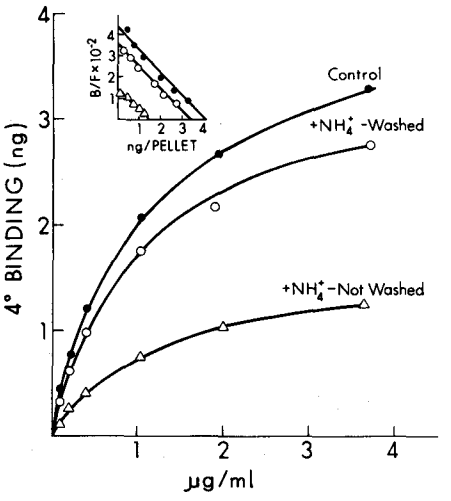

Figure 3. Effect of ammonium chloride preincubation on binding of ^{125}I-Man-BSA. Cells (5 x 10^6/ml) in standard media were preincubated for 10 min with or without NH_4^+ (30 mM) at 37°, washed by sedimentation and then incubated for an additional 10 min at 37° with or without NH_4^+. Following the second incubation, cells were cooled and tested for ^{125}I-Man-BSA binding at 4°. (a) Control cells (●) incubated without inhibitor; (b) +NH_4^+ (not washed) cells (Δ), incubated with NH_4^+ present in both incubations; (c) +NH_4^+ (washed) cells (○), incubated with NH_4^+ in the first incubation but absent from the second incubation.

Effect of pre-incubation with NH_4^+ and chloroquine on binding of ^{125}I-Man-BSA.

Considering that the inhibition of uptake is time dependent (Fig. 1B), it was reasoned that the drugs may produce their effects by reducing the availability of the receptor at the cell surface. To test this possibility, cells were incubated with NH_4^+ (Fig. 3) or chloroquine (data not shown) at 37° for ten min. Ammonium chloride was used principally in these experiments because its effects are rapidly reversed by removing the drug. Chloroquine effects, on the other hand, are only partially reversed when the drug is removed. Ohkuma and Poole (10) have shown that the effect of chloroquine on lysosomal pH is incompletely reversed by washing while the NH_4Cl effect is rapidly and completely dissipated. The effect of a brief pre-incubation of cells with NH_4^+ at 37° on binding (4°) is shown in Fig. 3. Binding was substantially reduced by the 37° preincubation with NH_4^+ and Scatchard plots (Fig. 3, inset) of the binding data show a 50-75% reduction in the number of sites with

little, if any, effect on the binding constants. The reduction in binding was rapidly reversed by washing the cells in NH_4^+-free media, followed by a brief incubation at 37° in NH_4^+-free media. Pre-incubation of the cells at 37° with chloroquine, showed a qualitatively similar reduction in the number of binding sites.

DISCUSSION

Uptake of glycoconjugates by macrophages involves binding of ligand to receptor and subsequent internalization, presumably, of the receptor-ligand complex. The mechanism of internalization is poorly understood but in several systems (e.g., LDL, EGF, α_2 macroglobulin) it is thought to take place in the coated pit region of the cell surface (2,16-17). Because uptake proceeds linearly over long periods of time, even in the presence of reduced protein synthesis (e.g., cycloheximide; 7), it has been rationalized that receptor-recycling occurs. That is, ligands are selectively transported to the lysosomes whereas receptors are spared from degradation by lysosomal enzymes and are recycled back to the cell surface. Membrane and/or receptor recycling are not new ideas and a substantial amount of experimental data, although indirect, have been generated in support of it (8,18-19).

Chloroquine and NH_4Cl have been shown to disturb the lysosomal pH gradient (10) and to inhibit lysosomal cathepsins (20). In the present study, chloroquine was shown to inhibit the degradation of ^{125}I-Man-BSA by macrophages. Moreover, Wiesmann et al. (12) and, more recently, Sando et al. (13) and Sly et al. (personal communication) have observed that chloroquine and ammonium chloride inhibit uptake of lysosomal enzymes by fibroblasts.

Inhibition of uptake of ^{125}I-Man-BSA by receptor-mediated endocytosis may be due to (i) inhibition of binding, (ii) inhibition of internalization following binding or, assuming that receptors do recycle, (iii) inhibition of receptor-return/receptor-deployment to the cell surface from an intracellular pool. Our results demonstrate that chloroquine or NH_4^+ have no effect on ligand binding or internalization of pre-bound ligand. The inhibition of

uptake is time dependent and the initial uptake of ligand is unaffected by the drugs. This result is consistent with the notion that only after the bulk of the cell surface receptors were internalized could an effect on uptake be seen. The effect of chloroquine and ammonium ion would appear to be independent of the presence of ligand since, as shown in Fig. 3, a brief incubation of cells at 37° in the presence of NH_4^+ but in the absence of added ligand, produced a substantial reduction in cell surface sites without any change in affinity. Cell surface receptors could be almost completely regenerated by washing out the drug and briefly incubating the cells at 37° in NH_4^+-free media. This result is consistent with the hypothesis that receptors are being internalized by cells whether ligand is present or not and that NH_4^+ prevents return or deployment to the cell surface. Recycling and deployment may not be mutually exclusive. Gonzalez-Noriega and Sly (personal communication) have obtained evidence that chloroquine inhibits recycling of the mannose-phosphate receptor in fibroblasts.

The mechanism by which chloroquine and NH_4^+ produce these effects remains unknown. Amines have been reported to inhibit clustering and internalization of certain ligands on the cell surface of fibroblasts (16). This explanation appears unlikely in the present case because chloroquine had no effect on internalization of pre-bound ligand. Moreover, Maxfield et al. (16) reported chloroquine as minimally effective in inhibiting clustering. A more likely possibility is that a membrane pH gradient somewhere along the endocytic pathway is required for receptor retrieval and/or dissociation of ligand from receptor. NH_4^+ or chloroquine may produce their effects on receptor recycling by dissipating such a pH gradient.

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