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FACTORS THAT INFLUENCE THE UPTAKE OF β-HEXOSAMINIDASE A BY RAT PERITONEAL MACROPHAGES.

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SUMMARY: Cultured rat peritoneal macrophages contain a receptor-mediated system for the uptake of the lysosomal enzyme [^{125}I]β-hexosaminidase A (E.C.3.2.1.30). The uptake process is saturable (4.5 ng/10^6 cells) and has a Kuptake of 5.9 X 10^{-8}M. The uptake is inhibited 68% by unlabeled β-hexosaminidase A, 66% by mannan, and 24% by ahexosaminofetuin (mannose terminated). Methylamine, ammonium ion, (both 20 mM) mepacrine and chloroquine (both 0.1 mM) strongly inhibit the specific uptake of [^{125}I]-β-hexosaminidase A while cytochalasin B (5 μg/ml) and colchicine (10^{-5}M) inhibit specific uptake by 35 and 28% respectively. Mepacrine inhibits phagocytosis of latex beads 76%; methylamine chloroquine, and cytochalasin B inhibit approximately 50%; colchicine and ammonium ion have negligible effect. These results suggest that adsorptive pinocytosis and phagocytosis function independently of each other. Extrapolation of these different effects may help to elucidate the mechanism of lysosomal enzyme uptake by macrophages.

INTRODUCTION: Peritoneal macrophages are part of the mononuclear phagocyte system that are well characterized with respect to their morphology, phagocytic capacity, and receptor components. Cultured macrophages accumulate colloidal gold and poly(vinylpyrrolidone) by fluid pinocytosis (1,2), they take up glycoproteins and glycoconjugates by adsorptive pinocytosis (3), and they clear α-macroglobulin-protease complexes from the circulation by a specific receptor mediated process (4). Furthermore, rat liver sinusoidal cells, which are also part of the mononuclear phagocyte system, internalize human lysosomal enzymes by a receptor mediated process that recognizes mannose and/or N-acetylglucosamine terminated glycoproteins (5,6). We are interested in studying the mechanism of the carbohydrate mediated process in rat peritoneal macrophages for the following reasons: 1, these cells are easily prepared in large numbers and in relatively pure pop-

Abbreviations used: PBS, Dulbecco's Phosphate Buffered Saline without Ca^{++} and Mg^{++}; DMEM, Dulbecco's Minimal Essential Medium.

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ulations; 2, the macrophage system is a useful *in vitro* model for studying inherited lysosomal storage diseases (7); and 3, recent reports have shown that lysosomotropic amines interfere with the turnover of injested glycoproteins (8) and hormones (9), the clustering of α_2- macroglobulin (10) and cell spreading (11). This report describes the effects of some primary amines and other compounds on the uptake of β-hexosaminidase A by rat peritoneal macrophages.

MATERIALS AND METHODS: Methylamine and ammonium chloride were purchased from Fischer Scientific. Colchicine was from Boehringer Mannheim, GmbH. Cytochalasin B, mepacrine, and chloroquine were from Sigma Chemical Corp. and latex beads (0.81 μM) were from Difco Laboratories.

β-Hexosaminidase A was purified from human placenta by a modification of the method of Tallman *et al.* (12) employing concanavalin A-Sepharose affinity chromatography instead of the ammonium sulfate fractionation and substituting hydroxylapatite chromatography and butyl agarose hydrophobic interaction chromatography for the final DEAE-Sephadex ion exchange step. The purified enzyme was labeled by the use of Enzymobeads as described previously (13).

Macrophages were prepared 3 days after injection of 12 ml of 10% thioglycolate by peritoneal lavage with 30 ml of Hanks Balanced Salt Solution. The cells were plated at a density of $\sim 8 \times 10^5$ cells/cm^2 onto either Linbro multi-well plates (6 wells X 9.62 cm^2/well) for uptake studies or Petri dishes (19.62 cm^2/dish) for phagocytosis experiments. Nonadherent cells were aspirated after 1 hr. incubation at 37°; adherent cells were washed 3 times with warm Dulbecco's Phosphate Buffered Saline without Ca^{++} and Mg^{++} (prepared by the N.I.H. Media Unit, PBS*) and then incubated overnight (16 hrs.) at 37° in Dulbecco's Minimal Essential Medium (prepared by the N.I.H. Media Unit, DMEM*) containing 5% heat inactivated (56° 1/2 hr) fetal calf serum, 100 U/ml Penicillin, 100 μg/ml Streptomycin, and 2 mM glutamine.

Uptake studies were carried out in DMEM containing HEPES, BES, and PIPES buffers as described by Stahl (3). Cells were incubated for 3 hrs. at 37° with [^{125}I]β-hexosaminidase A (100 ng/ml, 2 X 10^5 cpm/ml) in the absence (total uptake) and presence (non-specific uptake) of an excess of unlabeled β-hexosaminidase A or mannan. The difference between total and non-specific uptake represents specific uptake. For the inhibition studies, cells were preincubated for 20 minutes with ammonium ion (20 mM), methylamine (20 mM), mepacrine (0.1 mM), chloroquine (0.1 mM), and cytochalasin B (5 μg/ml) or 2 hours with colchicine (10^{-5}M); uptake was then measured as described above. Reactions were terminated by aspirating the medium and washing the cells once with buffer and twice with PBS, both at 0°. The cells were then dissolved in 1 N NaOH and aliquots were counted in a Gamma 4000 Counting System (Beckman).

Phagocytosis was measured by the accumulation of latex beads essentially as described by Weisman and Korn (14). Latex beads were dispersed in buffer at a concentration of 1 mg/ml. After an appropriate incubation with cells, the media was aspirated. The cells were washed once with binding buffer,

twice with PBS, (both at 0°) scraped from the Petri dish and transferred to conical glass centrifuge tubes. After centrifugation at 1000 X g for 10 min. the cells were extracted with dioxane for 1 hr. at room temperature and the extract recentrifuged. An aliquot of the extract was measured spectrophotometrically at 259 nm and the concentration of latex calculated from its extinction coefficient (2.3 X 10^{-3}/μg).

RESULTS AND DISCUSSION: The uptake of β-hexosaminidase by rat peritoneal macrophages is a saturable process as shown in Figure 1, having a K_{uptake} of 5.9 X 10^{-8}M. The data in Table 1 indicate that the uptake is blocked only by glycoproteins having a mannose or N-acetylglucosamine terminated oligosacchride structure. These results indicate that the uptake process is receptor mediated and specific having characteristics similar to the uptake by rat liver sinusoidal cells (5) and rat alveolar macrophages (3).

When macrophages were preincubated for 20 minutes in the presence of various amines, at concentrations known to maximally inhibit lysosomal degradative function (8,9) or clustering of membrane receptors (11), and then assayed for their ability to take up labeled hexosaminidase, almost complete inhibition of specific uptake was noted with the exception of

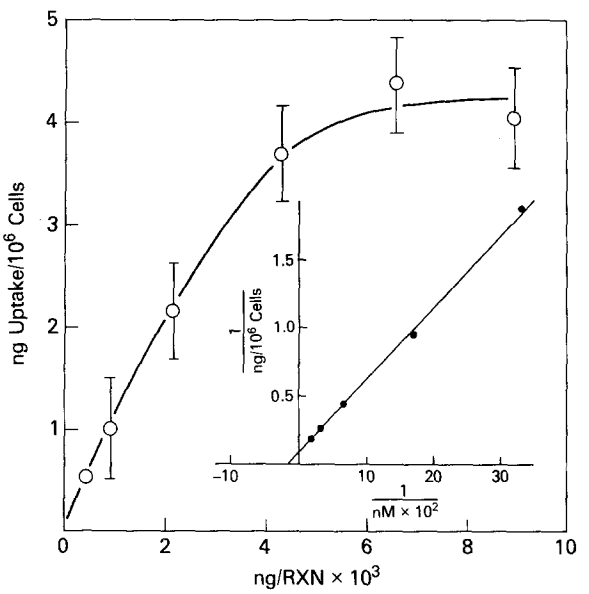

FIGURE 1

Macrophages were incubated in the presence of increasing amounts of hexosaminidase and cell associated radioactivity was determined as outlined in the METHODS. Results are the average of four experiments ± 1 S.D. Insert: Double reciprocal plot of the data. K_{uptake} = 5.9 X 10^{-8}M.

TABLE I. INHIBITION OF UPTAKE BY GLYCOPROTEINS

INHIBITOR	% INHIBITION
β-Hexosaminidase A	68 ± 12
Mannan	66 ± 12
Ahexosaminofetuin	24 ± 7.0
Agalactofetuin	8.8 ± 4.9
Asialofetuin	0.33 ± 0.58
Fetuin	0 ---

Inhibition of uptake of hexosaminidase was carried out in the presence of 100 μg of the glycoproteins. In the absence of inhibitors the uptake was 0.5 ng/10^6 cells. The results are the average of at least three experiments ± 1 S.D.

chloroquine (Table II). However under the same conditions, phagocytosis of latex beads was inhibited 76% by mepacrine, 51% by methylamine, 46% by chloroquine, and only 8.5% by ammonium ion suggesting that the two processes, adsorptive pinocytosis and phagocytosis, are independent of one another and perhaps involve different components of the cell membrane. When cells were pretreated with either cytochalasin B or colchicine, at

TABLE II. INHIBITION OF SPECIFIC UPTAKE AND PHAGOCYTOSIS

INHIBITOR	% INHIBITION	
	UPTAKE	PHAGOCYTOSIS
Methylamine HCl	100 ± 1.0	51 ± 9.0
Ammonium Chloride	89 ± 12	8.5 ± 1.0
Mepacrine	94 ± 2.2	76 ± 6.5
Chloroquine	62 ± 3.1	46 ± 3.5
Cytochalasin B	35 ± 15	54 ± 5
Colchicine	28 ± 7.0	1.0 ± 8

Macrophages were pretreated with the appropriate inhibitor at the concentrations and times indicated in METHODS. Uptake of hexosaminidase and phagocytosis of latex beads were measured in separate experiments. In the absence of inhibitors the uptake of hexosaminidase was 0.5 ng/10^6 cells and phagocytosis of latex beads was 6.5 μg/10^6 cells. The results are averages of at least three experiments ± 1 S.D.

concentrations that disrupt microfilament and microtubule assembly, approximately a 30% inhibition of hexosaminidase uptake was noted while phagocytosis was inhibited 54 and 5% by the respective agents. It is possible that these agents prevented the internalization process from distributing hexosaminidase to subcellular compartments by disrupting microfilament and microtubule assembly consequently preventing migration of endocytic vesicles.

The present results support earlier findings of two groups, Tsan and Berlin (15) using neutrophils and alveolar macrophages and the more recent work of Willinger et al. (16) using polymorphonuclear leukocytes. Both groups concluded that phagosomes may not contain a random distribution of cell surface molecules implying preferential internalization sites on the plasma membrane.

In view of some recent work dealing with the effects of amines on the uptake and binding of small peptide hormones (10,17) and toxins (18) and the results presented here, it is possible that internalization of exogenous lysosomal enzymes may occur in macrophage-like cells via clustering of plasma membrane receptors over coated pits (a process blocked by amines) and subsequent internalization via endocytic vesicles. However, there are other reports (8,19) indicating that these amines may affect processes other than those at the cell surface (i.e. secretion and/or internalization). Further work in this laboratory is aimed at resolving these possibilities.

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