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Monensin inhibits recycling of macrophage mannose-glycoprotein receptors and ligand delivery to lysosomes

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1. Binding studies with cells that had been permeabilized with saponin indicate that alveolar macrophages have an intracellular pool of mannose-specific binding sites which is about 4-fold greater than the cell surface pool. 2. Monensin, a carboxylic ionophore which mediates proton movement across membranes, has no effect on binding of ligand to macrophages but blocks receptor-mediated uptake of ^{125}I-labelled β-glucuronidase. 3. Inhibition of uptake was concentration- and time-dependent. 4. Internalization of receptor-bound ligand, after warming to 37°C, was unaffected by monensin. Moreover, internalization of ligand in the presence of monensin resulted in an intracellular accumulation of receptor–ligand complexes. 5. The monensin effect was not dependent on the presence of ligand, since incubation of macrophages with monensin at 37°C without ligand resulted in a substantial decrease in cell-surface binding activity. However, total binding activity, measured in the presence of saponin, was much less affected by monensin treatment. Removal of monensin followed by a brief incubation at pH 6.0 and 37°C, restored both cell-surface binding and uptake activity. 6. Fractionation experiments indicate that ligands enter a low-density (endosomal) fraction within the first few minutes of uptake, and within 20 min transfer to the lysosomal fraction has occurred. Monensin blocks the transfer from endosomal to lysosomal fraction. 7. Lysosomal pH, as measured by the fluorescein–dextran method, was increased by monensin in the same concentration range that blocked ligand uptake. 8. The results indicate that monensin blockade of receptor-mediated endocytosis of mannose-terminated ligands by macrophages is due to entrapment of receptor–ligand complexes and probably receptors in the pre-lysosomal compartment. The inhibition is linked with an increase in the pH of acid intracellular vesicles.

Macrophages express a cell-surface receptor which recognizes and efficiently internalizes glycoprotein ligands that bear oligosaccharides terminating in mannose or *N*-acetylglucosamine (Stahl *et al.*, 1978, 1980; Stahl & Gordon, 1982). Various lysosomal glycosidases (e.g. β-glucuronidase) are rapidly taken up into macrophages via this receptor. Artificial glycoconjugates (i.e. neoglycoproteins) (Shepherd *et al.*, 1981; Hoppe & Lee, 1982) and oligosaccharides (Maynard & Baenziger, 1981) have been used to define the specificity of the macrophage receptor. These studies have disclosed that the mannose receptor has a broad specificity and is able to recognize a wide variety of glycoproteins, including glycoconjugates terminating in mannose, *N*-acetylglucosamine and L-fucose.

Abbreviations used: BSA, bovine serum albumin; Caps, cyclohexylaminopropanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid.

Previous work from our laboratory has demonstrated that ligand uptake into macrophages far exceeds the cell-surface binding capacity (Stahl *et al.*, 1980). Receptor–ligand complexes are known to be rapidly internalized at 37°C (Stahl *et al.*, 1980), and previous work has suggested that the cell-surface receptor pool is maintained by movement of unoccupied receptors from the cell interior. Experiments with trypsin, to inactivate cell-surface receptors, have suggested the presence

of an intracellular pool of receptor molecules, which may be as much as 5-fold larger than the cell-surface pool. Studies with amines have indicated that movement of the receptor–ligand complex through an acid intracellular compartment may be important in the separation of ligands from their receptors and for return of the unoccupied receptors to the cell surface (Tietze *et al.*, 1980, 1982). In the present paper we demonstrate the presence of an intracellular pool of mannose-specific binding sites in permeabilized macrophages. Moreover, we show that monensin is a potent inhibitor of receptor-mediated endocytosis, that monensin alkalinizes acid intracellular vesicles (endosomes) and reversibly decreases cell-surface binding activity while only minimally decreasing the total pool of intracellular binding sites. Fractionation studies indicate that monensin interferes with transfer of ligands from endosomes to lysosomes.

Experimental

Reagents

Monensin was purchased from Calbiochem and was dissolved in ethanol. Fluorescein–dextran (FD-40), BSA and saponin were obtained from Sigma. Foetal bovine serum was from Flow Laboratories and $Na^{125}I$ from Amersham/Searle.

Cells

Rat bone-marrow-derived macrophages were prepared by culturing rat bone marrow cells with α-minimal essential medium (Gibco manual) containing 10% (v/v) foetal bovine serum and 10% (v/v) L-cell-conditioned medium as described by Lin & Gordon (1979) and Konish *et al.* (1983). After 5 days in culture in 2 cm^2 multiwell dishes, a monolayer of well-differentiated macrophages was obtained. Rat (Stahl *et al.*, 1978) and rabbit (Shepherd *et al.*, 1981) alveolar macrophages were prepared by pulmonary lavage as described previously. Rat bone-marrow-derived macrophages were used for studies requiring attached cells; alveolar macrophages were studied in suspension, rabbit cells being used when large quantities of cells were required.

Assays

Receptor-mediated uptake by suspended cells was determined by using the oil assay described by Stahl *et al.* (1980) or the attached-cell assay described by Stahl & Gordon (1982), except that Hanks balanced salt solution buffered with 10mM-Hepes and 10mM-Tes (pH 7.4) containing 10mg of BSA/ml was used for all binding and uptake assays (Hanks/BSA medium). Protein was measured with the Miller (1959) method. Lysosomal pH measurements were performed on an Aminco SPF 500 spectrofluorimeter by a modification of the method of Ohkuma & Poole (1978). The cells were cultured on 2cm × 1cm × 0.08cm quartz rectangles; 50μl of 4mM-FD40 in Hanks balanced salt solution without bicarbonate, supplemented with 30mM-Hepes, pH 7.4, was placed directly on the quartz plate so that the cells were covered. The quartz plates were then incubated at 37°C for 30min, washed extensively with Hanks balanced salt solution + 30mM-Hepes, pH 7.4, to remove excess FD-40 and were placed in the spectrofluorimeter. The excitation spectra were scanned to measure the pH in lysosomes. During these measurements the cells were in Hanks balanced salt solution + 30mM-Hepes, pH 7.4, and the cuvette was thermostatically maintained at 37°C. Internalization and stripping methods for detection of cell-surface ligand (i.e. with EDTA/trypsin) were described by Stahl *et al.* (1980). Cell were permeabilized by suspension in Hanks balanced salt solution containing saponin (0.5%), phenylmethanesulphonyl fluoride (1mM) and leupeptin (5μg/ml). Binding studies on permeabilized cells were performed in the presence of 0.5% saponin at 4°C. After 3–4h, the cells were spun through oil in an Eppendorf Microfuge for 4min as described by Stahl *et al.* (1980).

Ligands

The neoglycoprotein mannose–BSA was prepared by the method of Lee *et al.* (1979). β-Glucuronidase was purified from rat preputial glands (Keller & Touster, 1975). Ligands were radiolabelled with carrier-free ^{125}I by the chloramine-T method, followed by dialysis or gel filtration on Sephadex G-25 (Stahl *et al.*, 1980).

Fractionation

Rabbit alveolar macrophages were suspended in in 0.25M-sucrose/3mM-imidazole, pH 7.5 (homogenization buffer), at a concentration of 1×10^7 cells/ml and homogenized by nitrogen cavitation [1.73MPa (250lb/in^2), 10min] in a cell-disruption bomb (Parr Instrument Co., Moline, IL, U.S.A.). The post-nuclear supernatant was pelleted at $2.8 \times 10^5 g$-min (17000rev./min, 10min, Beckman JA-17 rotor; $r_{av.} = 89.5$mm) and resuspended in homogenization buffer and then layered over 30ml of Percoll (1.07g/ml). Fractionation was performed by centrifugation at 17000rev./min for 60min (Beckman JA-17 rotor). Fractions (40 drops) were collected from the bottom of the resulting gradient, and the radioactivity content for each fraction was determined. The plasma-membrane marker alkaline phosphodiesterase was assayed by following the hydrolysis of dTMP *p*-nitrophenyl ester in 50mM-Caps buffered to pH 10.6 (Beaufay

et al., 1974); the lysosomal marker β-hexosaminidase was determined fluorimetrically as described by Jessup & Dean (1982).

Results

Cell-surface and total cellular binding sites for ^{125}I-labelled mannose–BSA

Previous work (Stahl *et al.*, 1980) suggested the presence of an intracellular pool of mannose receptors whose relative size could be some 5-fold larger than the cell surface pool. To allow access of ligand to intracellular binding sites, macrophages were permeabilized with 0.5% saponin. Fig. 1(*a*) shows that saponin treatment increased mannose-specific binding of ^{125}I-labelled mannose–BSA to alveolar macrophages. Scatchard plots of the binding data (Fig. 1*b*) show that permeabilized cells contain a single class of binding sites which bind approx. 4 times as much ligand (24ng/assay) as control cells (6ng/assay). Binding-site affinity, however, fell as a result of saponin treatment.

Inhibition of β-glucuronidase uptake into macrophages by monensin

Rat or rabbit alveolar macrophages were incubated at 37°C in Hanks/BSA medium containing ^{125}I-labelled β-glucuronidase and increasing concentrations of drug for 15min (Fig. 2*a*). Ligand uptake was terminated by centrifuging the cells through oil. Specific uptake of ^{125}I-labelled β-glucuronidase into macrophages was determined by incubating companion cultures with ligand plus yeast mannan (1mg/ml). Uptake in the presence of yeast mannan was considered non-specific. Monensin was found to be a potent inhibitor of uptake, with 50% inhibition at about 3 μM. Attached macrophages (bone-marrow-derived) were more sensitive to monensin, with 50% inhibition of uptake of ^{125}I-labelled β-glucuronidase at 1 μM-monensin. The ethanol carrier had no effect on uptake. To follow the time course of uptake of β-glucuronidase, alveolar macrophages were incubated with ligand for various times in standard media with or without monensin. The results in Fig. 2(*b*) show that specific uptake into cells was linear with time over the 20min uptake period. In the presence of 10 μM-monensin, uptake proceeded briskly for the first 5min, after which no further cellular accumulation of ligand occurred. The inhibition was thus found to be time-dependent.

Internalization of pre-bound ^{125}I-labelled mannose–BSA: effect of monensin

In these experiments, ^{125}I-labelled mannose–BSA was used as ligand because of its higher affinity and more predictable binding behaviour at 4°C. Monensin was found to have no effect on binding of this ligand to suspended alveolar macrophages incubated with ligand at 4°C. To investigate the effect of monensin on internalization of pre-bound ligand, cells were incubated with ^{125}I-labelled mannose–BSA at 4°C for 60–90min (Fig. 3) in the presence or absence of 10 μM-monensin. The cells were then washed free of unbound ligand and warmed to 37°C, again in the presence or absence of 10 μM-monensin. After various periods of warming, samples were cooled to 4°C to arrest further internalization and degradation. The surface-bound ligand was then determined by stripping the cells with trypsin/EDTA at 4°C. Ligand that remained cell-bound after trypsin/EDTA treatment was considered intracellular. When cells were warmed to 37°C, cell-surface ligand decreased precipitously and intracellular ligand increased correspondingly. During the warm-up period, some of the ligand, which was initially bound to the cells, slowly accumulated in the medium. This radiolabelled material released into the medium was found to be acid-soluble. Because the appearance of this component was coincidental with the loss of intracellular ligand, it was considered to be the product of lysosomal digestion. In the presence of 10 μM-monensin, the cell surface was cleared of ligand just as rapidly as that observed in control cells without monensin. However, in the presence of monensin, the intracellular component remained constant with time, and there was very little digestion.

Fractionation of rabbit alveolar macrophages: effect of monensin on transfer to lysosomes

Rabbit alveolar macrophages were allowed to take up ^{125}I-labelled β-glucuronidase for 5min in the presence or absence of monensin. Some of the cells were then washed free of ligand and incubated for another 20min at 37°C, in the presence or absence of monensin. The samples were homogenized by nitrogen cavitation and the granule fractions were layered on Percoll (1.07g/ml). The separations obtained by this method are shown in Fig. 4, where hexosaminidase was employed as a lysosomal marker and alkaline phosphodiesterase as a plasma-membrane marker. By this fractionation protocol, excellent separation of the two marker enzymes was obtained. After a 5min uptake, ^{125}I-labelled β-glucuronidase was found almost exclusively in the light membrane fraction. After a 20min chase, most of the ligand had moved into the lysosomal fraction. In the presence of monensin, transfer of ligand to the lysosomal fraction on incubation at 37°C was completely blocked.

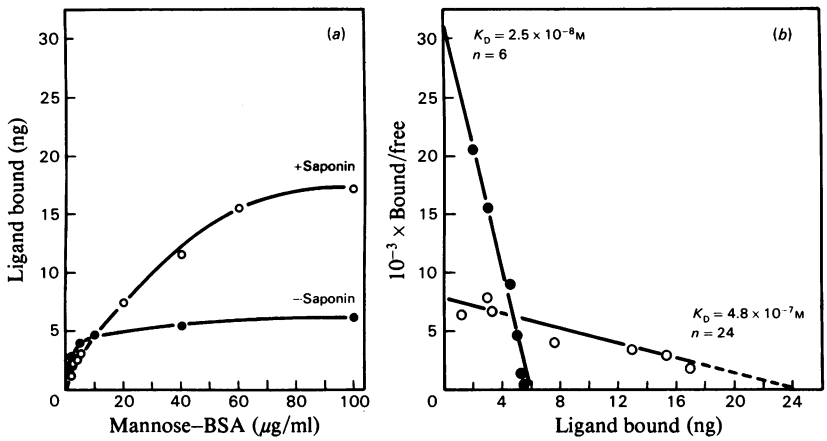

Fig. 1. *Effect of saponin on mannose–BSA binding by macrophages*

Rabbit alveolar macrophages (5×10^6/ml) were suspended in Hanks/BSA medium at 4°C. (*a*) Cell-surface binding activity (●) was determined by incubating the cells with ligand (^{125}I-labelled mannose–BSA) for 90min at 4°C as described previously (Stahl *et al.*, 1980). Total cellular binding activity (○) was determined by incubating the cells with ligand in the presence of 0.5% saponin. After 4h at 4°C, the cells were washed once in Hanks/BSA medium, resuspended to their original volume and then centrifuged through oil (Stahl *et al.*, 1980). Non-specific binding in saponin-treated cells was determined by inclusion of yeast mannan (1mg/ml) and 0.1M-α-methyl mannoside. Data points represent ng of mannose–BSA bound to 5×10^5 cells. (*b*) Scatchard plot of the binding data: n = ng of ligand bound/5×10^5 cells. ●, Control; ○, +saponin.

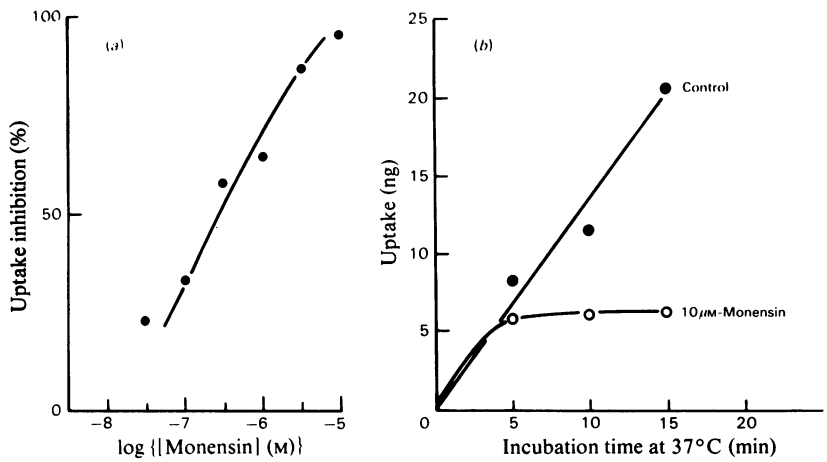

Fig. 2. (*a*) *Effect of monensin on uptake of ^{125}I-labelled β-glucuronidase by rat bone-marrow-derived macrophages, and* (*b*) *effect of monensin on time course of uptake of ^{125}I-labelled mannose–BSA*

(*a*) Rat bone-marrow-derived macrophages in 24-multiwell culture dishes were incubated in buffered Hanks/BSA medium as described in the Experimental section, along with ^{125}I-labelled β-glucuronidase (20μg/ml; 5×10^5c.p.m./μg) and increasing concentrations of the drug in a total volume of 0.4ml. Non-specific uptake of the ligand was determined by inclusion of yeast mannan (1mg/ml) in the assay. Dishes were incubated for 60min at 37°C in an air atmosphere. Uptake was terminated by removal of the ligand, followed by three sequential additions of buffered salt solution (without BSA). The cells were solubilized in 0.5% Triton X-100. Results are expresssed as percentage inhibition of uptake compared with controls. Carrier ethanol in the monensin experiment had no effect on uptake. (*b*) Rat alveolar macrophages were set up in a standard assay (5×10^5cells/0.1ml in Hanks/BSA medium) with ^{125}I-labelled mannose–BSA (5×10^5c.p.m./mg; 10μg/ml) in the presence (○) or absence (●) of 10μM-monensin. After warming to 37°C, the cells were spun through oil. The media were collected from the above oil. Then 70μl of spent medium was added to 430μl of Hanks/BSA medium, followed by 0.5ml of 20% trichloroacetic acid. The radioactivity remaining in the supernatant fraction and the pellet was then determined. The uptake curve was corrected for degradation by combining the cell-associated radioactivity with that rendered acid-soluble by incubation of cells with ligand in the absence of yeast mannan.

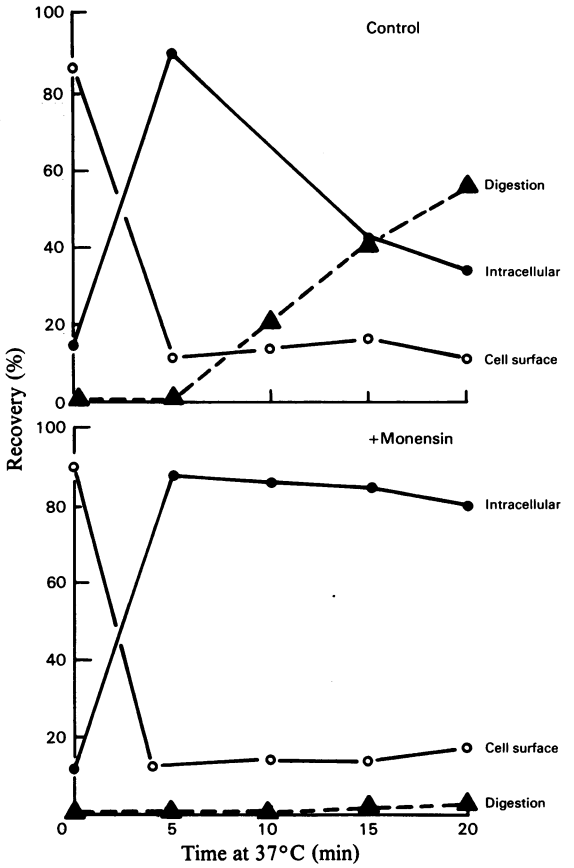

Fig. 3. *Effect of monensin on internalization of pre-bound ligand after warming to 37°C*

Rat alveolar macrophages were incubated on ice with ^{125}I-labelled mannose–BSA (7×10^6 c.p.m./μg; 4.0 μg/ml) for 90 min with or without 10 μM-monensin. Non-specific binding was determined in separate incubations by adding yeast mannan (1 mg/ml) to the cell suspension. After binding, cells were washed twice in Hanks/BSA medium and resuspended in the same medium with or without 10 μM-monensin. The cells were then warmed to 37°C for 0, 5, 10, 15 and 20 min. They were then immediately cooled and washed twice in Hanks/BSA medium. The supernatant from these two sedimentations was pooled and is referred to as 'digestion' (▲). The cells were then suspended in Ca^{2+}/Mg^{2+}-free Hanks medium without albumin, containing trypsin (1 mg/ml) and 10 mM-EGTA. After 15 min incubation at 4°C, the cells were centrifuged through oil as described by Stahl *et al.* (1980). The radioactivity found above the oil is referred to as 'cell surface' (○) and that found associated with the cells was considered 'intracellular' (●).

Does ligand internalized in the presence of monensin remain receptor-bound?

One hypothesis to explain the action of monensin on receptor-mediated endocytosis in macrophages is that neutralization of acid intracellular vesicles (possibly pre-lysosomal vesicles) prevents both the dissociation of receptor–ligand complexes and the retrieval/routing of unoccupied receptors back to the cell surface. An experiment was carried out to examine whether ligand that accumulates intracellularly in the presence of monensin remains receptor-bound. Macrophages were first loaded with ^{125}I-labelled mannose–BSA at 4°C so that only the cell-surface pool was labelled. Surface-bound ligand was then allowed to be internalized in the absence or presence of 10 μM-monensin by warming to 37°C for 10 min, similar to the protocol described in Fig. 3. Cells were then cooled to prevent further receptor and/or ligand movement [at 4°C, the dissociation of receptor–ligand complexes is very slow ($t_{\frac{1}{2}} > 3$ h)]. The cells were then permeabilized with saponin in Hanks/BSA medium containing excess unlabelled mannose–BSA. Under these conditions, free ligand would be expected to diffuse into the incubation media, whereas receptor-bound ligand should remain within the cells. Moreover, the bulk of the ligand which remains with the cells should be releasable after incubation at low pH (6.0, with EGTA at 37°C), conditions known to enhance markedly dissociation of mannose-receptor–ligand complexes. The results in Table 1 show that control and monensin-treated cells internalized approximately the same amount of ligand. However, at least twice as much ligand was released from control cells as from their monensin-treated counterparts. Most of the ligand which remained within permeabilized cells was released by the pH 6.0/EGTA treatment, indicating that it was receptor-bound.

Effect of monensin on lysosomal pH: correlation with inhibition of uptake

Earlier work (Tietze *et al.*, 1980, 1982) has suggested that neutralization of acid intracellular vesicles by weak bases prevents receptor recycling. The mechanism of inhibition by amines appears to be due to entrapment of receptors or receptor–ligand complexes within intracellular vesicles. In the present study, the pH of acid intracellular vesicles was estimated by the method of Ohkuma & Poole (1978). Cells were allowed to take up fluorescein–dextran by pinocytosis. The cells were placed in the spectrofluorimeter, and emission at 516 nm was determined at excitation wavelengths of 450 nm and 495 nm. The results in Fig. 5 show the effect of monensin pretreatment on vacuolar pH. During a preincubation of 15 min, the vacuolar pH rose as a function of monensin concentration. The inhibition of uptake of β-glucuronidase into macrophages and loss of cell-surface binding sites caused by monensin correlated with the increase in vacuolar pH. The pH response to monensin and

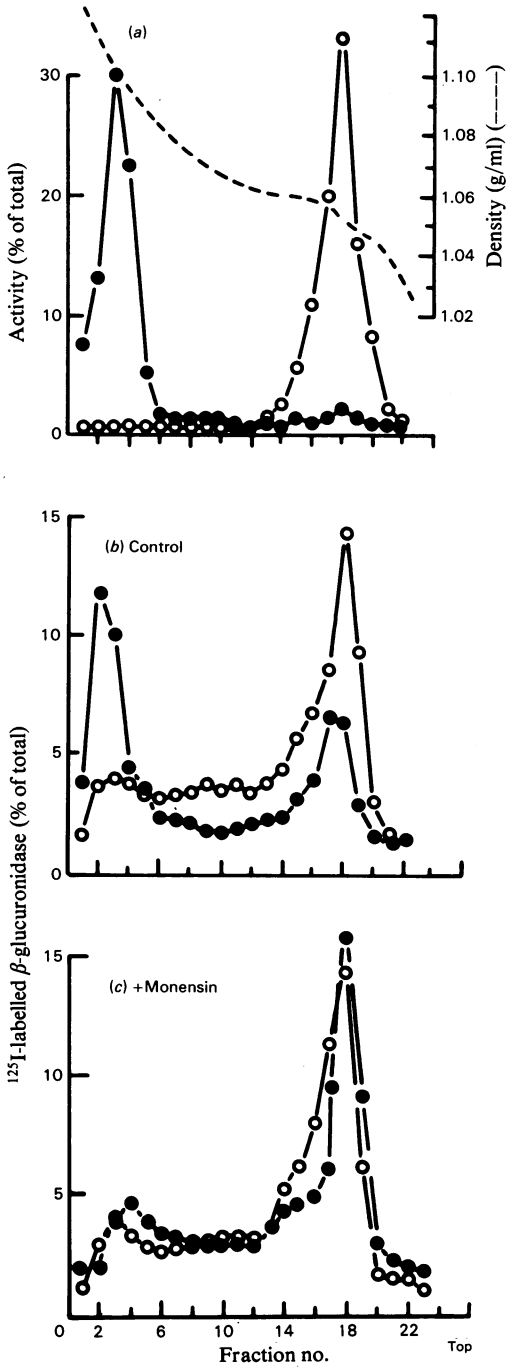

Fig. 4. *Subcellular fractionation of rabbit alveolar macrophages: effect of monensin on ligand distribution*

Macrophages were incubated with ^{125}I-labelled β-glucuronidase for 5 min at 37°C and then cooled and washed free of added ligand. Half the cells were warmed to 37°C for a further 20 min. Cells were then homogenized by nitrogen cavitation and subjected to Percoll-density-gradient centrifugation as described in the Experimental section. (*a*) Distribution of plasma-membrane marker alkaline phosphodiesterase (○) and lysosomal enzyme marker β-hexosaminidase (●). Density was determined with Pharmacia marker beads. Panels (*b*) and (*c*) compare the effects of 10 μM-monensin on ligand distribution. Monensin had no effect on marker-enzyme sedimentation. Key: ○, 5 min pulse; ●, 20 min chase.

the inhibition of uptake produced by monensin were substantially decreased in the absence of extracellular Na^+ (Table 2). When Na^+ was removed from the media and replaced with choline, the plot of vacuolar pH response to increasing concentrations of monensin was shifted to the right. Presumably this is due to the fact that monensin preferentially exchanges Na^+ for H^+ across cell membranes.

Effect of monensin on cell-surface and total binding activity after incubation at 37°C: inhibition and recovery

To determine the effect of monensin on cell-surface binding activity, rabbit alveolar macrophages were incubated with increasing concentrations of

Table 1. *Effect of monensin on dissociation of internalized mannose–BSA from receptors*

Rabbit alveolar macrophages (1×10^7 cells/ml) were incubated at 4°C for 90 min in Hanks/BSA medium containing ^{125}I-labelled mannose–BSA (40 μg/ml). The cells were washed at 4°C to remove unbound ligand and then warmed to 37°C in the presence or absence of 10 μM-monensin. Ligand internalization was arrested after 10 min by cooling the cells to 4°C. Samples were removed to estimate total cell-associated ligand and cell-surface ligand (i.e. trypsin/EGTA-releasable). The remaining cells were permeabilized with 0.5% saponin containing 100 μg of unlabelled mannose–BSA (to block unoccupied binding sites)/ml. The radioactivity released after 3 h at 4°C was used to determine the fraction of internalized ligand that had dissociated from the receptor. Receptor-bound ligand was subsequently removed by incubating the permeabilized cells at pH 6.0 in the presence of EGTA. Ligand remaining bound after pH 6/EGTA treatment was considered non-specifically bound. Data are the means of two experiments.

	Binding (ng/5×10^5 cells)	
^{125}I-labelled mannose–BSA	Control	+Monensin (10 μM)
Cell-associated ligand	5.2	4.9
Surface-bound ligand (trypsin/EGTA-releasable)	0.7	0.8
Receptor-free ligand (saponin-releasable)	2.2	1.1
Receptor-associated ligand (pH 6/EGTA-releasable)	1.7	2.3
Residual ligand	0.5	0.5

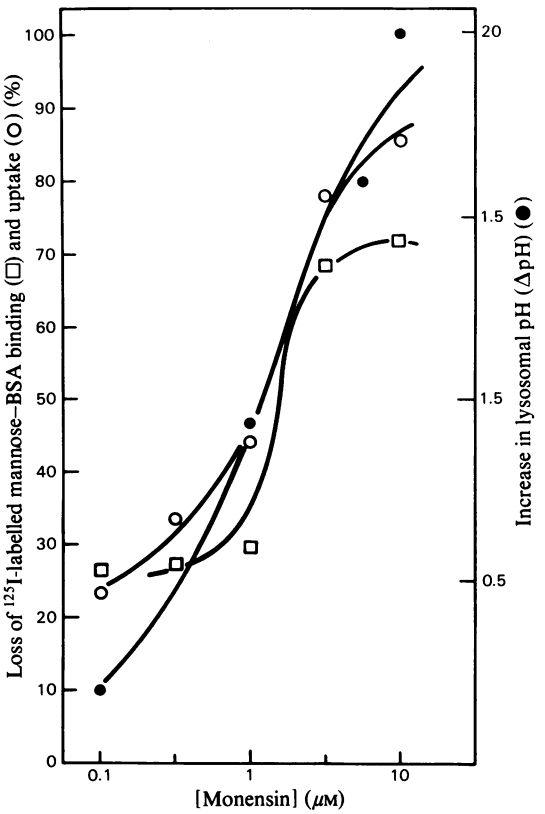

Fig. 5. *Comparison of the effects of monensin on ligand binding and uptake by macrophages and on lysosomal pH*

The effect of monensin on binding of ^{125}I-labelled mannose–BSA and uptake of ^{125}I-labelled β-glucuronidase were determined in rabbit alveolar macrophages. Lysosomal pH was measured with rat bone-marrow-derived macrophages as described in the Experimental section after uptake of fluorescein–dextran into the cells. Lysosomal pH before monensin addition was 4.96. Although addition of monensin produced a rapid change in lysosomal pH, an equilibrium period of 10–15min was allowed before the above measurements were taken.

Table 2. *Effect of extracellular Na^+ on monensin-mediated inhibition of ^{125}I-labelled β-glucuronidase uptake and rise in vacuolar pH*

Uptake studies were carried out as described in Fig. 1, but with media consisting of 2mM-$CaCl_2$, 10mM-Hepes, 10mM-Tes (pH7.4), 5mM-glucose and 10mg of BSA/ml supplemented with 0.12M-choline chloride or 0.1M-NaCl. Lysosomal pH measurements were made with *N*-methylglucamine as a substitute for Na^+.

Incubation conditions	Uptake inhibition (%)	ΔpH
$+Na^+$, 10μM-monensin	58	0.9
$-Na^+$, 10μM-monensin	28	0.4

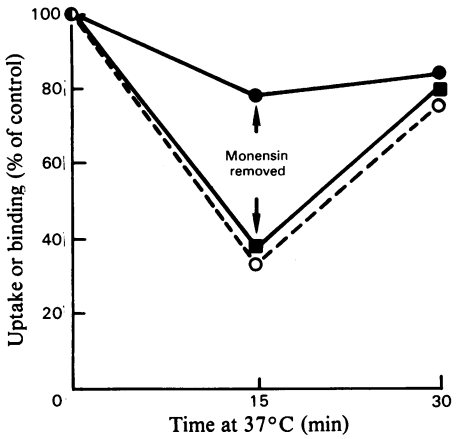

Fig. 6. *Effect of monensin on uptake and binding of mannose–BSA: inhibition and recovery*

Data are presented as the percentages of control values of surface binding, total binding and uptake before and after monensin treatment and after recovery without monensin. Rabbit alveolar macrophages (1×10^7/ml) were suspended in Hanks/BSA medium in the presence or absence of 3μM-monensin. After 15min at 37°C the cells were rapidly cooled to 4°C. Cell-surface and total cellular binding were assessed in the presence of 100μg of mannose–BSA/ml as described in the legend to Fig. 1. Cell-surface binding fell from 5.4 to 1.8ng/5×10^5 cells as a result of monensin treatment. Total binding fell only slightly, from 31.0 to 24.2ng/assay. Ligand uptake was determined in the presence or absence of 3μM-monensin by incubating cells for 15min at 37°C with 15μg of ^{125}I-labelled mannose–BSA/ml. Uptake fell from 47.2 to 18.4ng/5×10^5 cells as a result of monensin treatment. To study recovery of uptake and binding activity, cells were washed twice with ice-cold Hanks/BSA medium and incubated for 15min at 37°C in Hanks balanced salt solution, pH6.0, containing EDTA to enhance dissociation of endogenous ligand. The cells were then washed and resuspended in Hanks/BSA medium to determine mannose–BSA binding and uptake. Binding and uptake in control cells rose slightly as a consequence of washing (6.8 and 52.0ng/5×10^5 cells respectively), but total binding changed little (29.0ng/5×10^5 cells). Removal of monensin allowed a recovery of surface binding (5.2ng/5×10^5 cells) and uptake activity (41.6ng/5×10^5 cells), but only marginally affected total binding (24.5ng/5×10^5 cells). ○, Cell-surface sites; ●, total sites; ■, uptake.

monensin for 15min at 37°C in the absence of ligand. The cells were then cooled, and cell-surface binding activity was determined with ^{125}I-labelled mannose–BSA. The results indicated that monensin treatment at 37°C brought about a substantial decrease in cell-surface binding sites. (In a related study, unlabelled ligand was added to the 37°C

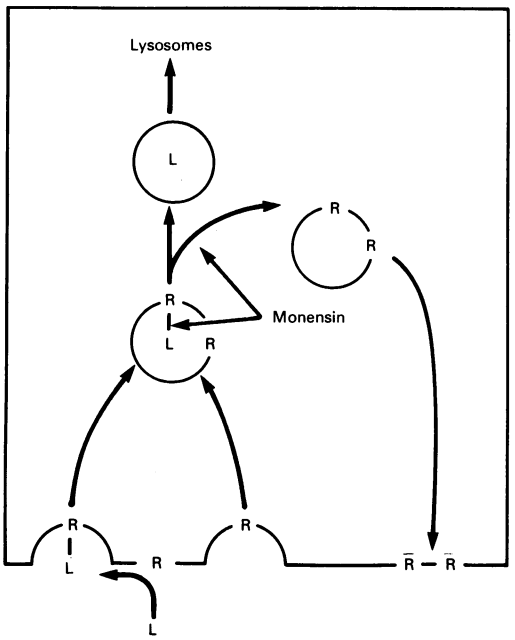

Fig. 7. *Model for receptor recycling, showing two possible sites for monensin action*

Receptor–ligand complexes and free receptor can be internalized. After dissociation of receptor–ligand complex under the influence of a low-pH environment, ligand molecules are transported to lysosomes. The rise in vacuolar pH after monensin treatment would decrease the dissociation of receptor–ligand complexes. The rise in pH may also prevent the retrieval of unoccupied receptors from this intracellular compartment and the movement of ligand to lysosomes. Key: L, ligand; R, receptor.

incubation mixture in addition to monensin to determine whether the presence of ligand would enhance the loss of cell-surface binding sites. Addition of exogenous ligand had no effect on receptor loss.) Loss of cell-surface binding activity from monensin-treated macrophages may be due to a redistribution of binding sites or to binding-site inactivation. It was therefore desirable to determine whether the loss of cell-surface binding and uptake activity after monensin treatment was reversible and whether total binding activity was affected by the drug. In this experiment, cells were incubated with or without monensin at 37°C. Some of the cells were then allowed to recover by removing the monensin, followed by incubation at 37°C. The results in Fig. 6 summarize an experiment with rabbit alveolar macrophages which shows that uptake activity and cell-surface binding activity were decreased by 61% and 68% respectively, after a brief (15 min) incubation of cells with 3 μM-monensin at 37°C. Total binding activity, on the other hand, was decreased by 22% (Fig. 6). Pretreatment with NH_4Cl resulted in a similar decrease in cell-surface binding activity. To determine whether both the loss of uptake activity and cell-surface binding activity were reversible, cells that had been treated with monensin were washed free of the drug and were then incubated at 37°C for 15 min in Hanks balanced salt solution, pH 6, containing EDTA. The latter treatment has been found to enhance cell-surface binding activity in macrophages, possibly by stripping away receptor-bound ligands. This treatment resulted in nearly complete recovery of the cell-surface binding activity and uptake activity (76% and 80% respectively) (Fig. 6). Total binding activity appeared to be unaffected by the washing procedure.

Discussion

Receptor-mediated endocytosis is now widely accepted as a highly specialized function carried out by most cell types. The process involves at least two new organelles, the coated pit (Goldstein *et al.*, 1979) and an intracellular compartment which serves to dissociate ligand from receptor. This compartment has been referred to variously as the compartment for uncoupling receptor–ligand complexes (CURL) (Geuze *et al.*, 1983), the receptosome (Willingham & Pastan, 1980) or pre-lysosomal endosomes. Other intracellular structures are undoubtedly involved in the sorting and recycling process, but they have yet to be defined morphologically. For example, structures must exist which shuttle unoccupied receptors and membrane back to the cell surface (Tietze *et al.*, 1982; Cohn & Steinman, 1982). Biochemical experiments indicate the presence of an acid intracellular compartment (Tietze *et al.*, 1980, 1982; Tycko & Maxfield, 1982), ostensibly to enhance dissociation of receptor–ligand complexes (which may be analogous to CURL) (Tietze *et al.*, 1982).

Earlier work from our laboratory has demonstrated that trypsin treatment of macrophages at 4°C resulted in a 75% loss of cell-surface binding activity (Stahl *et al.*, 1980). However, loss of 75% of cell-surface binding sites resulted in only a 10–20% loss in uptake activity measured at 37°C. This result suggested that 80% of the receptors must be inside cells, protected from the action of trypsin. The results presented in Fig. 1 indicate that total binding activity in macrophages is enhanced 4-fold by permeabilizing cells with 0.5% saponin, a technique used by Fischer *et al.* (1980) to expose intravesicular mannose 6-phosphate receptors. Saponin caused a fall in mannose-binding-site affinity, probably resulting from a detergent effect on the cell-membrane. Scatchard analysis shows a single population of binding sites in permeabilized cells, suggesting that cell-surface binding sites had been

similarly affected. These findings confirm earlier predictions (Stahl *et al.*, 1980) and suggest an intracellular/surface receptor ratio of approx. 4:1. Weigel & Oka (1983) have identified a large intracellular pool of galactose receptors in hepatocytes permeabilized with digitonin. Given that the cell-surface pool of active mannose receptors is 75000 (Stahl *et al.*, 1980), the total cellular pool must be in the neighbourhood of 375000. Further, as shown previously (Stahl *et al.*, 1980), rat alveolar macrophages can mediate the uptake of 2×10^6 molecules/h per cell. This would suggest that, on average, each receptor must complete the cycle every 11 min to carry out the above rate of uptake.

Monensin is a carboxylic ionophore (Pressman, 1976) which mediates the transfer of H^+ across cell membranes usually involving exchange with Na^+. Tartakoff & Vassalli (1978) have shown that monensin blocks transport of secretory products through the Golgi apparatus. Monensin also affects endocytosis; for example, Wilcox *et al.* (1982) describe its actions on fluid-phase pinocytosis and Basu *et al.* (1981) have shown that cell-surface low-density-lipoprotein receptor movement is impaired in monensin-treated cells. The latter group have suggested that receptors are trapped inside cells as a result of drug treatment. In the present study, we show that monensin, at low concentrations, effectively blocks receptor-mediated uptake of mannose glycoconjugates by macrophages. Monensin-dependent blockade of receptor-mediated endocytosis might occur at one of three sites: (i) ligand binding to receptor; (ii) clustering and/or internalization of receptor–ligand complexes; (iii) re-utilization of internalized receptors. Our results show that monensin had no effect on ligand binding, and when ligand was bound to the receptor, before monensin addition, no effect on internalization of the pre-bound ligand was observed when the cells were warmed to 37°C. Furthermore, when ligand was internalized in the presence of monensin, elevated amounts of receptor–ligand complexes within the cells could be demonstrated (Table 1). This indicates that the block produced by monensin is beyond the internalization step. This conclusion is also suggested by the time course of uptake of ligand at 37°C in the presence of monensin. Under standard assay conditions, uptake of ligand into cells is linear with time. In the presence of monensin, uptake proceeds linearly with time for a few minutes and then falls to a much slower rate. All the results suggest that the recycling pathway is paralysed by the action of monensin and that, in the presence of exogenous ligand, receptor–ligand complexes are trapped inside the cell. Fractionation of macrophages by using nitrogen cavitation to disrupt cells and Percoll-gradient sedimentation gives excellent separation of the endosomal and lysosomal membranes. Uptake experiments indicate that ligand first appears in the light-membrane fraction and that transfer to lysosomes requires 15–20 min. The transfer of ligand from endosomes to lysosomes is blocked by monensin.

Earlier in the Discussion section, it was suggested that receptor–ligand complexes internalized in the presence of monensin were unable to dissociate. Does this imply that ligand is required for monensin to affect the distribution and/or function of mannose receptors? Our results indicate that the presence of ligand is not required for a monensin effect. As shown in Table 1, incubation of cells with monensin in the absence of ligand produced a dramatic decrease in cell-surface binding activity. These results might be expected if receptors were continuously cycling even in the absence of exogenous ligand. Receptors might then accumulate at the site of monensin action. Saponin was used to examine more fully the effect of monensin in the absence of added ligand on total cellular binding. The results indicate that 3 μM-monensin, which caused a substantial decrease in cell-surface binding sites, only mildly decreased (22%) total cellular binding activity. We have no explanation for the loss of total binding, except that monensin may have some mild detergent effect. However, the effects of monensin were largely reversible, indicating that this dose of drug was not toxic to the cells. [Higher concentrations (>10 μM) were toxic and led to irreversible loss of binding sites.] These results suggest that cycling receptors are trapped within the cells as a result of monensin treatment. An alternative explanation is that only cell-surface sites are inactivated (Fiete *et al.*, 1983). We consider this unlikely, because during the 15 min incubation with monensin virtually all the receptors would have appeared at the cell surface, and it they were inactivated a substantial fall in total cell binding would have been observed. Our results are more in agreement with observations on low-density-lipoprotein and asialoglycoprotein receptors. Using indirect immunofluorescence with an antibody directed against the receptor, Basu *et al.* (1981) showed that monensin caused a loss of low-density-lipoprotein receptors from the cell surface and an accumulation of receptors within perinuclear vacoules. Harford *et al.* (1983) have demonstrated that monensin causes an accumulation of asialoglycoprotein-receptor–ligand complexes within the cell and inhibits delivery of the ligand to lysosomes.

Since monensin is known to mediate proton exchange, we examined the effect of monensin on the pH of intracellular vesicles. The effect of monensin on acid intracellular vacuolar pH was determined by the fluorescein–dextran method described by

Ohkuma & Poole (1978). Our results indicate that maximal pH changes were observed in the same concentration range that blocked uptake, suggesting that inhibition of receptor recycling may be a consequence of altered pH within intracellular vesicles. These findings are consistent with those of Marnell *et al.* (1982), who found that monensin blocked the transport of diphtheria toxin into cytoplasm and that the inhibitory effect of monensin was reversed by lowering extracellular pH. Since monensin mediates exchange preferentially between Na^+ and H^+, the effect of low extracellular Na^+ on the response to monensin was tested. When Na^+ was replaced with choline, total uptake was substantially decreased and the dose/response to monensin was displaced to 5-fold higher concentrations. Similarly, the lysosomal pH response was substantially decreased by monensin. Other agents that affects Na^+ transport, i.e. ouabain and amiloride, had no effect on uptake even in the absence of extracellular Na^+, nor did they influence the response to monensin. These results suggest that receptor movement is linked to the acidification of certain intracellular compartments, perhaps generated by proton pumps, and that monensin blocks receptor movement through these compartments. Tycko & Maxfield (1982) have shown that α_2-macroglobulin enters an acid environment very soon after internalization of receptor–ligand complexes has occurred, but before the ligand is transported to lysosomes. Moreover, Maxfield (1982) reported that monensin reversibly raises the pH of these pre-lysosomal acidic vesicles.

An important question raised by the present study is why incubation with monensin, in the absence of added ligand, causes receptors to be trapped within the cells. The studies with permeabilized cells indicate that, after monensin treatment, almost all the intracellular mannose-binding sites are available to ligand. Moreover, Basu *et al.* (1981) have shown that 50% of low-density-lipoprotein receptors are trapped within cells treated with monensin in the absence of low-density lipoprotein. These results appear to rule out the possibility that receptors immobilized under the influence of monensin are engaged with endogenous ligands. Rather, it suggests that acidification may be required for the collection and retrieval of unoccupied receptors in preparation for transport back to the cell surface (e.g. the clustering of unoccupied receptors and subsequent formation of a receptor-rich intracellular vesicle may be driven by a transmembrane gradient). Acidification is also required for receptor–ligand dissociation, but this event must precede receptor retrieval. The simplest rationalization for our findings (Fig. 7) is that receptor molecules recycle through an acid intracellular compartment whether or not ligand is present. When these acid intracellular compartments are neutralized (e.g. by the action of monensin), receptor retrieval from the acid compartment is blocked and receptors accumulate. Since receptor–ligand dissociation precedes receptor retrieval, cells incubated with monensin plus ligand accumulate receptor–ligand complexes. The morphological description of the acid intracellular compartment within macrophages which mediates receptor recycling remains to be elucidated.

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