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Biochemistry

Synthetic glycopeptide substrates for receptor-mediated endocytosis by macrophages

(drug targeting/carbohydrate-specific uptake/mannosyl ligands)

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ABSTRACT **Mammalian macrophages contain a transport system that binds and internalizes glycoproteins with exposed mannose residues. This system and analogous systems on other types of cells require substrates to bear multiple nonreducing terminal residues of the appropriate sugar for effective uptake. Small multivalent synthetic glycopeptides with mannose residues covalently linked through a spacer arm to the α- and ϵ-amino groups of lysine, dilysine, and trilysine are competitive inhibitors of rat alveolar macrophage uptake of the neoglycoprotein mannosyl-bovine serum albumin with inhibition constants in the μM range. Various compounds could be covalently attached to the α-carboxyl group of these glycopeptides with substantial retention of inhibitory potency. This uptake system does not recognize galactose residues, and the galactosyl analog of an inhibitory mannosylpeptide did not inhibit uptake of mannosyl-bovine serum albumin. The trimannosyldilysine ligand is not only an inhibitor but also a substrate for specific uptake by macrophages, as shown with an ^{125}I-labeled derivative. Macrophages bound 6.4×10^5 molecules per cell at 0°C with a dissociation constant of 2 μM. At 21°C the cells could internalize the labeled conjugate with an apparent Michaelis constant of 6 μM and a maximal velocity of 1.7×10^5 molecules per min per cell. The dissociation constant and Michaelis constant are similar to the inhibition constant of 9 μM determined at 21°C for inhibition by this conjugate of mannosyl-bovine serum albumin uptake. These synthetic substrates may be useful in targeting pharmacologic agents to macrophages, and analogous compounds may target such agents to other types of cell.**

Macrophages play important roles in immune responses, in chronic inflammation, and in some parasitic diseases. In each case, it may be useful to deliver pharmacologic agents selectively to macrophages, particularly subpopulations of macrophages such as those in inflamed joints or in lymph nodes. We are attempting to determine whether the carbohydrate-specific glycoprotein uptake system of macrophages (1) can be used for macrophage-specific drug targeting.

Mammalian macrophages specifically bind and internalize glycoproteins bearing exposed residues of mannose, *N*-acetylglucosamine, glucose, and L-fucose (1–3). The substrate glycoproteins are degraded within lysosomes, while the receptor component of the uptake system apparently functions repeatedly without being degraded (4). A protein that seems to be the receptor for this system has been isolated from rabbit liver (5) and serum (6) and from rat liver (7) and lymph nodes (8). The components and operation of the macrophage glycoprotein uptake system are quite similar to those of the galactose-specific mammalian hepatocyte system described by Ashwell, Morell, and coworkers (9–11). Analogous systems with other sugar specificities have also been found (12–15). The physiological role of the macrophage system is not known, but suggestions include removal from the bloodstream of lysosomal enzymes (16–19), antigen–antibody (IgM) complexes (20), and yeast (21).

In the hope that these glycoprotein uptake systems may be useful in tissue-specific delivery of pharmacologic agents, we have synthesized mannosyl-lysine conjugates that are competitive inhibitors of the macrophage mannosyl-protein uptake system. One of these glycopeptides was radiolabeled and shown to be a substrate for uptake, carrying the attached label into the cells.

METHODS

Materials. Structures of the glycopeptide ligands are shown in Fig. 1. Their syntheses will be described elsewhere (22). The iodinated conjugate ^{125}I-labeled Man_3Lys_2BH was made by reaction of $Man_3Lys_2NH_2$ with ^{125}I-labeled Bolton–Hunter reagent (ref. 23; New England Nuclear). $Man_3Lys_2NH_2$ trifluoroacetate (2 nmol), triethylamine (3 nmol), and ^{125}I-labeled Bolton–Hunter reagent (0.5 nmol) were mixed and incubated overnight at 4°C in 100 μl of *N*,*N*-dimethylformamide, followed by 1.5 hr at room temperature after addition of 10 μl of water to destroy any remaining reagent. The reaction mixture was applied to a 1.4 × 8.4 cm column of silica gel and eluted with chloroform/methanol/water/glacial acetic acid (80:20:0.5:0.5, vol/vol) until mobile radioactive material (^{125}I-labeled 4-hydroxyphenylpropionic acid) was removed. The desired product (>6×10^7 cpm/nmol) was then eluted with chloroform/methanol/water (60:40:10, vol/vol) and mixed with unlabeled Man_3Lys_2BH for individual experiments. Synthesis of $Man_3Lys_2[^3H]Raf$ followed published procedures (24), as did synthesis (25) and iodination (26) of the neoglycoprotein mannosyl-bovine serum albumin (22–27 mannose residues per molecule; hereafter referred to as mannosyl-albumin). [^{3}H]Raffinose and sodium [^{125}I]iodide were purchased from New England Nuclear.

Macrophages. Alveolar macrophages were obtained by lung washing, with conditions based on the results of Brain and Frank (27). Female Wistar rats (175–250 g, Charles River Breeding Laboratories) were anesthetized by intraperitoneal injection of 25 mg of Nembutal (Abbott). The rib cage was cut away to facilitate lung expansion, and a plastic tube was inserted into the trachea. Lungs were filled and emptied three times with 6–8 ml (varying with rat size) of warm (37°C) 0.85% sodium chloride solution from a syringe attached to the plastic tube. Upon col-

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Abbreviations: mannosyl-albumin, mannosylated bovine serum albumin; Man_3Lys_2, synthetic glycopeptide N^2-[N^2,N^6-bis[3-(α-D-mannopyranosylthio)propionyl]-L-lysyl]-N^6-[3-(α-D-mannopyranosylthio)propionyl]L-lysine. Structures and other abbreviations are in Fig. 1. Except where noted, configurations are L-lysine and D sugars.

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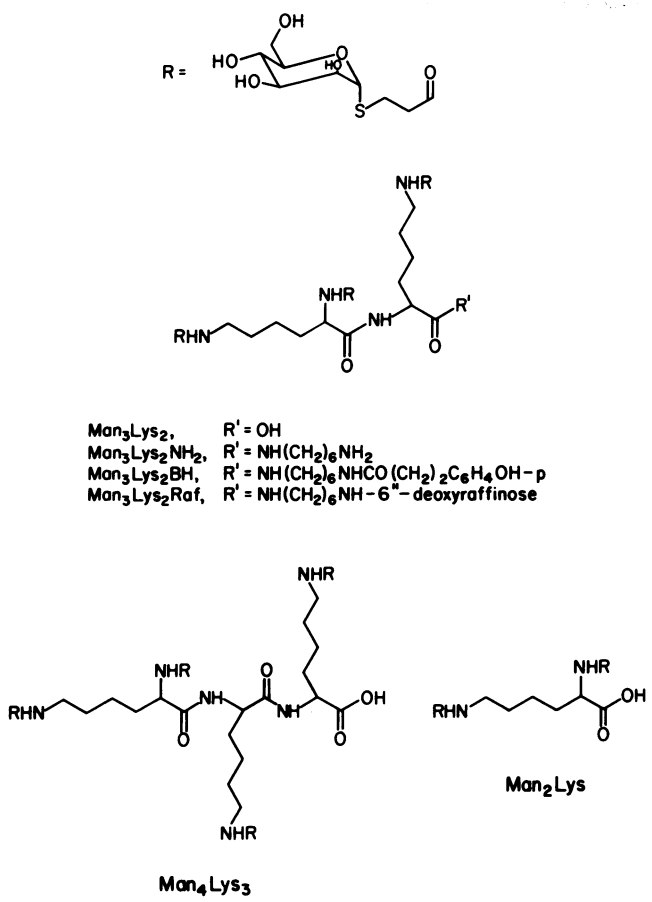

FIG. 1. Structures and abbreviations of ligands.

lection of 150 ml of rinses, cells were pelleted by centrifugation 5 min at 270 × *g* and resuspended in 10 ml of saline. Filtration of the cell suspension through gauze removed pieces of tissues before cell counting with trypan blue. The yield was 0.7–1.5 × 10^7 live cells per rat, with few or no erythrocytes unless excessive rinse volume or force had been used. The cells were centrifuged again and resuspended in buffered medium for uptake assays.

Thioglycollate-elicited mouse peritoneal macrophages were isolated and cultured as described by Bonney *et al.* (28).

Uptake Measurement. Uptake by rat alveolar macrophages was measured by the procedure of Stahl *et al.* (4) except that the medium buffers were 25 mM Hepes, 15 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (BisTris), and 10 mM [(carbamoylmethyl)imino]diacetic acid (Ada) (Sigma) and the concentration of fetal calf serum was 9%. Nonspecific uptake was defined as the cell-associated radioactivity in the presence of excess mannan (2.5 mg/ml, Sigma). Specific uptake was total minus nonspecific. Nonspecific (mannan-resistant) uptake of ^{125}I-labeled mannosyl-albumin was less than 10% of the total. Nonspecific uptake of ^{125}I-labeled Man_3Lys_2BH was higher, up to 60% of total, possibly because of the hydrophobic nature of the 4-hydroxyphenylpropionyl group. In both cases, nonspecific uptake was not saturable over the concentration range studied and nonspecifically bound label could not be released with EDTA.

For work with mouse peritoneal macrophages, the ^{3}H-labeled compounds were added directly to the normal culture medium (28) on the second day of culture. At desired times, dishes of cells were rinsed and the adherent cells were solubilized with Triton X-100 (New England Nuclear) and sodium hydroxide for scintillation counting of cell-associated radioactivity. Each 60-mm culture dish initially received 6 × 10^6 cells; because the number remaining after attachment and culture is not known, results are expressed per dish instead of per cell.

RESULTS

The synthetic glycopeptide ligands (Fig. 1) contain mannose residues linked to the α- and ε-amino groups of lysine, dilysine, and trilysine. This arrangement was chosen to expose multiple nonreducing terminal mannose residues on a small molecule. Several of these ligands were found to be competitive inhibitors of rat alveolar macrophage uptake of ^{125}I-labeled mannosyl-al-

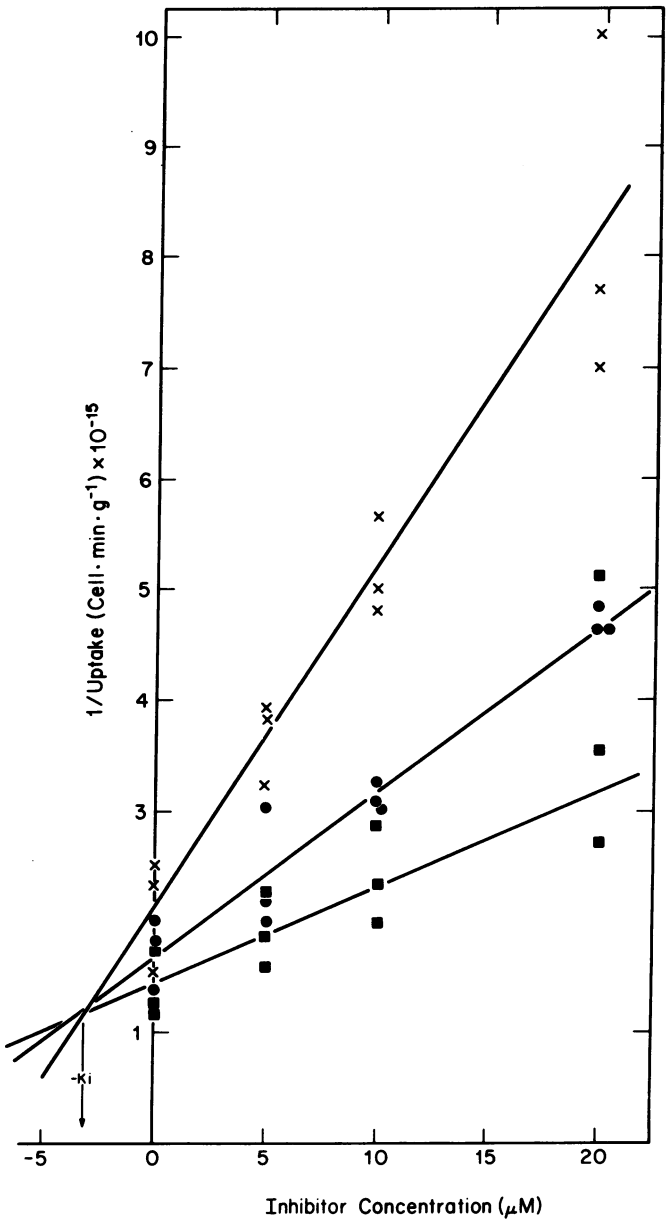

FIG. 2. Determination of K_i of Man_4Lys_3. Alveolar macrophage specific uptake of ^{125}I-labeled mannosyl-albumin was measured (10 min at 21°C) in the presence of 0–20 μM Man_4Lys_3. Concentrations of ^{125}I-labeled mannosyl-albumin were 250 (×); 500 (●); and 750 (■) ng ml^{-1}. Lines were drawn by linear regression (correlation coefficients 0.87–0.97, deleting the ■ at 20, 5). The mean abscissa value of the calculated line intersections, −3.1 μM in this experiment, was taken as $-K_i$.

bumin, as shown in Fig. 2 for Man_4Lys_3. Inhibition constants (K_i) were determined graphically as in Fig. 2. Results of several experiments are summarized in Table 1. The inhibitory potency was $Man_4Lys_3 > Man_3Lys_2 > Man_2Lys$, although the differences are not statistically significant, with all the K_i values in the μM range. The α-carboxyl group of Man_3Lys_2 could be attached to bulky groups as in Man_3Lys_2BH and Man_3Lys_2Raf with full or partial retention of inhibitory potency. Cholesterol and dexamethasone conjugates of $Man_3Lys_2NH_2$ also had K_i values of 4–6 μM (22).

A labeled ligand was prepared for binding and uptake studies by reaction of $Man_3Lys_2NH_2$ with ^{125}I-labeled Bolton–Hunter reagent. This ligand, ^{125}I-labeled Man_3Lys_2BH, binds specifically (inhibited by mannan) to the alveolar macrophages. The specific binding at 0°C is shown in Fig. 3 as a Woolf plot, which is less sensitive than a Scatchard plot to distortion by outlying points (29). There were 6.4×10^5 binding sites per cell, with an apparent dissociation constant of 2.4 μM (mean values from two experiments, one of which is shown in Fig. 3). Warming to 37°C increased the amount of ^{125}I-labeled Man_3Lys_2BH specifically associated with the cells (Table 2). Because the affinity seems not to change much with temperature, K_d at 0°C being similar to K_i and K_m (see below) at room temperature, this increase supports the hypothesis that macrophages can internalize Man_3Lys_2BH at the higher temperatures. EDTA rapidly removes glycoprotein ligands from the macrophage surface (4), and all of the ^{125}I-labeled Man_3Lys_2BH bound specifically at 0°C could be removed by EDTA (Table 2), showing its cell-surface location. Incubation at 37°C not only increased the mannan-sensitive cellular uptake of ^{125}I-labeled Man_3Lys_2BH but also permitted the cells to sequester most of it in a location, presumably internal, where it could not be released by EDTA. The specific uptake (measured as a combination of surface binding and internalization) at 21°C was saturable, with a Michaelis constant (K_m) of 6.4 μM and a maximal velocity (V_{max}) of 1.7×10^5 molecules per min per cell (mean values from two experiments, one of which is shown in Fig. 4). With the present experimental precision, the values of K_d and K_m for ^{125}I-labeled Man_3Lys_2BH are not statistically different from the K_i value of unlabeled Man_3Lys_2BH (Student's t test, $P > 0.05$).

$Man_3Lys_2NH_2$ was also labeled with [^{3}H]raffinose to give a ligand (Man_3Lys_2[^{3}H]Raf) whose label should remain in lysosomes even after enzymic digestion (24, 30). Thioglycollate-elicited mouse peritoneal macrophages in culture took up Man_3Lys_2[^{3}H]Raf much faster and to a much greater extent than they took up [^{3}H]raffinose (Fig. 5). The uptakes shown are based on initial specific activities, because metabolism of the two compounds has not yet been investigated. Mannan was not used in this long-term incubation to distinguish specific from nonspecific uptake, but preliminary short-term experiments (not shown) revealed the Man_3Lys_2[^{3}H]Raf uptake by rat lung macrophages to be largely mannan-sensitive.

Table 1. Inhibition of ^{125}I-labeled mannosyl-albumin uptake

Ligand	K_i, μM
Man_4Lys_3	2.6 ± 0.7 (3)
Man_3Lys_2	3.9 ± 1.1 (3)
Man_2Lys_1	6.2 ± 3.0 (3)
Man_3Lys_2BH	9.3 ± 4.7 (5)
Man_3Lys_2Raf	18.0 ± 2.8 (3)

Inhibition constants were determined as shown in Fig. 2. Values listed are mean ± SD (number of experiments). By Student's t test, 18 μM is significantly ($P < 0.05$) higher than the other values; the other differences are not statistically significant.

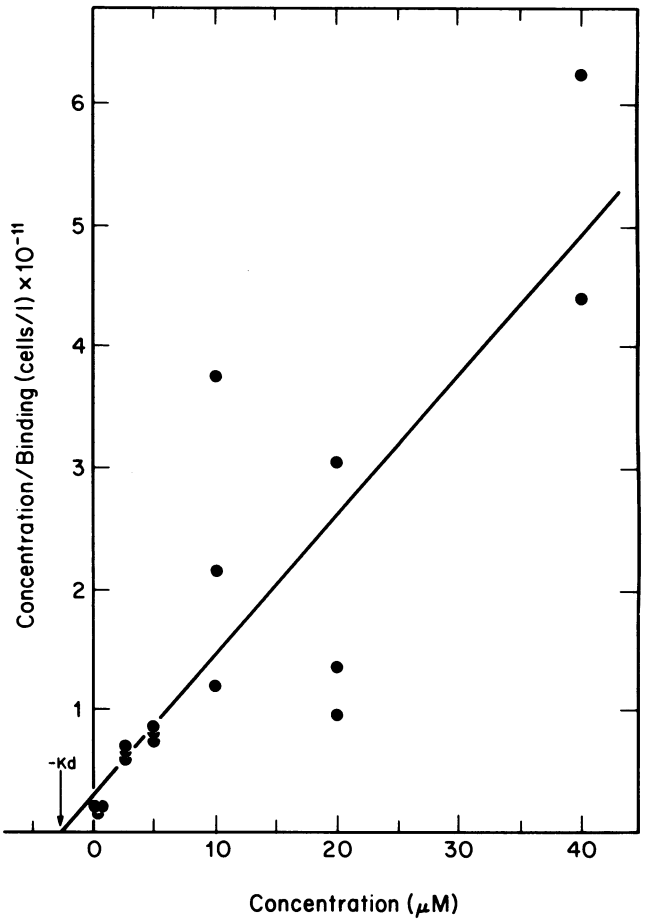

FIG. 3. Binding of ^{125}I-labeled Man_3Lys_2BH. Alveolar macrophages were incubated at 0°C with ^{125}I-labeled Man_3Lys_2BH at 8.7×10^6 cpm/ml (≤0.3 μM) and various amounts of unlabeled Man_3Lys_2BH to give the desired total concentrations, and 80 μl (1.1×10^6 cells) was centrifuged at 30 min. Shown is a Woolf plot (29) of specific binding, with the line determined by linear regression (correlation coefficient 0.86, deleting an aberrant point at 40, 12.6—not shown). This experiment gave a dissociation constant K_d [$-1 \times$ (abscissa intercept)] of 2.7 μM and a maximal binding (1/slope) of 5.2×10^5 molecules per cell. Nonspecific binding (not shown) was a constant 0.57 ± 0.03% of total radioactivity at all concentrations.

DISCUSSION

The mammalian carbohydrate-specific glycoprotein uptake systems have been studied with neoglycoproteins (generally of albumin) as well as with native and modified glycoproteins (refs. 1, 31, and 32, for example). Several workers have also attached

Table 2. Uptake of ^{125}I-labeled Man_3Lys_2BH

Postincubation addition	Specific cell-associated label, cpm	
	0°C	37°C
NaCl	3623*	6088
EDTA	−255	5751

Rat alveolar macrophages were incubated for 20 min at 0°C or 37°C in 90 μl of medium with ≈2 μM ^{125}I-labeled Man_3Lys_2BH, then chilled on ice. Fifteen microliters of 0.85% saline or 100 mM EDTA in saline was added, and 80 μl of the mixture (5.6×10^5 cells) was centrifuged through oil after 10 min. Values shown are cell pellet radioactivity minus nonspecific cpm obtained with mannan (2.5 mg/ml) present throughout the incubations. This nonspecific binding was 7994 cpm at 0°C and 10,268 cpm at 37°C.

* Approximately 2×10^5 molecules per cell.

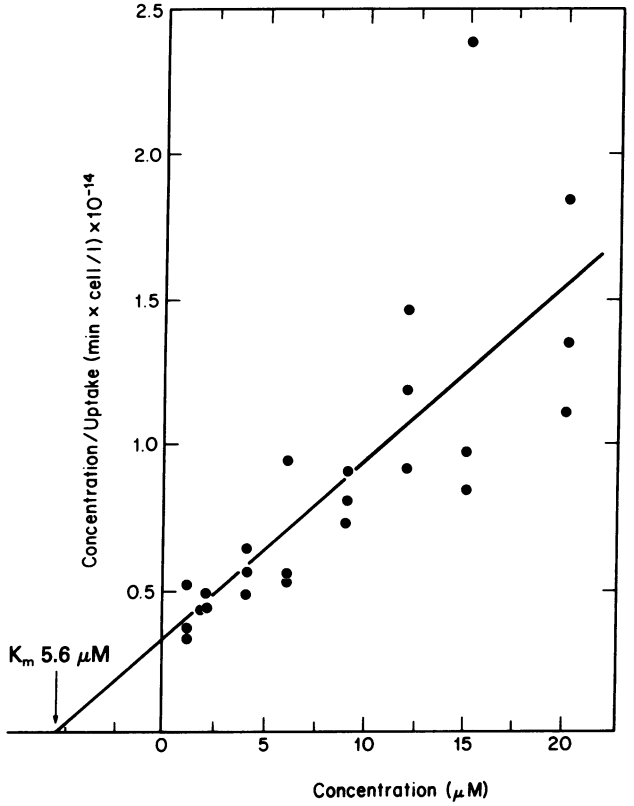

FIG. 4. Uptake of ^{125}I-labeled Man_3Lys_2BH. This experiment was similar to the binding measurement shown in Fig. 3 but was run at higher temperature to permit uptake. Cells were incubated 10 min at 21°C with ^{125}I-labeled Man_3Lys_2BH (9.42×10^4 cpm/mol at all concentrations) and 80 μl (6.9×10^5 cells) was centrifuged. The plot (correlation coefficient 0.78) indicates a K_m of 5.6 μM and a V_{max} (slope^{-1}) of 0.99×10^5 molecules per min per cell. Mannan-resistant uptake (not shown) was a constant 0.27 ± 0.01% of total radioactivity at all concentrations.

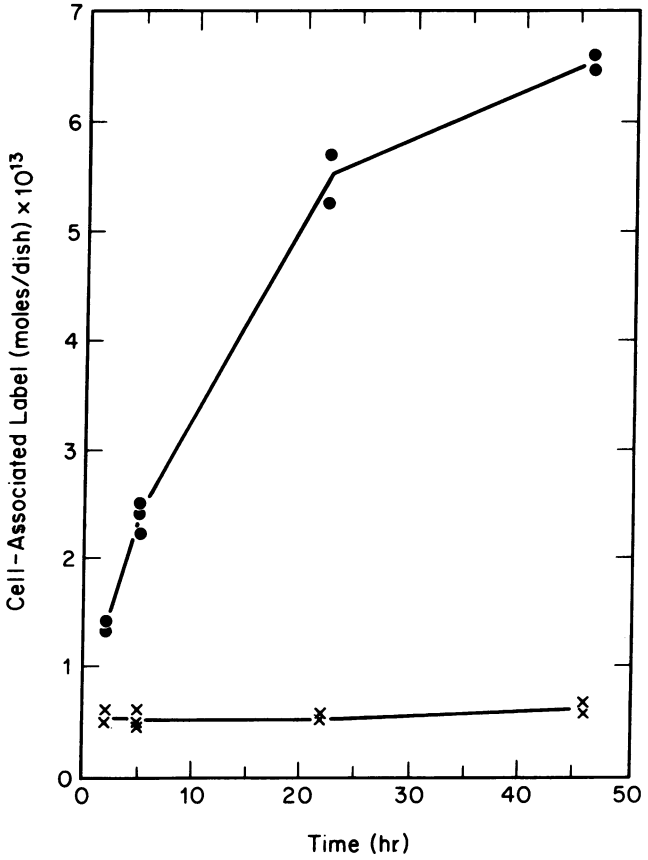

FIG. 5. Uptake of Man_3Lys_2[^{3}H]Raf. Thioglycollate-elicited mouse peritoneal macrophages were cultured in the presence of 14 nM Man_3Lys_2[^{3}H]Raf (●) or 10 nM [^{3}H]raffinose (×), and cell-associated radioactivity was measured. The calculation of mol from cpm is based on initial specific activities, assuming no metabolism.

carbohydrate residues to enzymes and low-density lipoprotein and demonstrated increased uptake by liver *in vivo* and hepatocytes *in vitro* (33–36) or by fibroblasts *in vitro* (37). We are interested in developing synthetic substrates for the carbohydrate-specific glycoprotein uptake systems that will permit the delivery of pharmacologic agents specifically to cells bearing those systems. We have chosen macrophages as the target cells for our initial work because of their involvement in inflammation, immune responses, and parasitic diseases. Earlier suggestions that multivalency is important for good substrates of the macrophage uptake system (1, 5) led us to synthesize a series of simple "branched" glycopeptides. These compounds are competitive inhibitors of ^{125}I-labeled mannosyl-albumin uptake by macrophages (Fig. 2; Table 1), with K_i values in the μM range. For comparison, the K_i of benzyl α-D-mannopyranoside is 5.7 mM (unpublished data) and the K_d of mannosyl-albumin is 10 nM (4). The macrophage uptake system does not recognize galactose (1, 5), and the galactosyl equivalent of Man_3Lys_2, Gal_3Lys_2, did not inhibit ^{125}I-labeled mannosyl-albumin uptake at 100 μM (not shown). The terminal carboxyl of Man_3Lys_2 could be derivatized with various groups with partial to complete retention of uptake affinity, as estimated by K_i, thus giving us a versatile site for attachment of pharmacologic agents to be targeted to macrophages.

Optimal performance of ligands for this targeting requires that the ligands not only bind but also are substrates of the glycoprotein uptake system, and that they can carry attached molecules into the macrophages. We have used two different radiolabeled conjugates to demonstrate that the Man_3Lys_2 ligand and the attached label are efficiently internalized. In our study ^{125}I-labeled Man_3Lys_2BH was more suitable for measurement of binding and initial rates of uptake, and Man_3Lys_2[^{3}H]Raf was better for a longer-term measurement. Stahl *et al.* (4) have demonstrated macrophage internalization of glycoproteins *via* the carbohydrate-specific uptake system by a temperature-dependent rise in cell-associated glycoprotein and its conversion from an EDTA-releasable (cell surface) to an EDTA-resistant (internal) location, followed by lysosomal degradation. Although we have not yet shown macrophage degradation of ^{125}I-labeled Man_3Lys_2BH, Table 2 and Figs. 3 and 4 show the other elements of this demonstration. The specific cell-associated amount of ^{125}I-labeled Man_3Lys_2BH was higher at 25°C and 37°C than at 0°C, and EDTA could release it from the cells only at 0°C.

At 0°C the maximal specific binding of ^{125}I-labeled Man_3Lys_2BH was 6.4×10^5 molecules per cell, with an apparent dissociation constant of 2.4 μM (Fig. 3 and *Results*). At 21°C the apparent K_m (for a combination of binding and internalization) was 6.4 μM, with a V_{max} of 1.7×10^5 molecules per min per cell (Fig. 4 and *Results*). At saturation, each cell could take up one ligand molecule per surface receptor site about every 3.8 min. Similar values of 1.7–3.3 min can be calculated from published data (4) for ^{125}I-labeled mannosyl-albumin, although fewer receptors are seen with the glycoprotein–0.5 to 1.0×10^5 per cell (ref. 4 and our unpublished data). The difference in binding may result from each glycoprotein molecule binding

simultaneously to several receptors or from receptor sites that are sterically available only to the small ligand, or both. It is possible that different types of receptors are present on the cell surface, but the linearity of Fig. 3 and the reasonably close agreement between K_i, K_d, and K_m of ^{125}I-labeled Man_3Lys_2BH suggest that the receptors are fairly homogenous in affinity for the smaller ligand. In addition, all the specific binding of ^{125}I-labeled Man_3Lys_2BH was inhibited by mannan (our operational definition of specific binding) and the binding that was not inhibited by mannan was not saturable at the concentrations used (see legends to Figs. 3 and 4).

The ligand-raffinose conjugate provided further support for internalization by macrophages. Thioglycollate-elicited mouse peritoneal macrophages in culture accumulated considerably more label from Man_3Lys_2[^{3}H]Raf than from [^{3}H]raffinose itself (Fig. 5). We do not yet have enough of this labeled material to measure initial rates and establish kinetic parameters. Uptake will be even more directly shown when we are able to demonstrate an intracellular effect or metabolism of some agent attached to the ligand Man_3Lys_2 that does not enter the macrophages well without the ligand.

Baenziger and Fiete (38) have compared the hepatocyte uptake of several galactose-terminal glycopeptides with that of glycoproteins bearing those determinants. They found similar numbers of hepatocyte surface binding sites and similar affinities for both binding and uptake of the glycopeptides and glycoproteins, despite great differences in affinity for the purified receptor. The macrophage uptake system, on the other hand, can distinguish in affinity between large and small endocytosable substrates, at least for those we have studied.

In summary, a small synthetic carbohydrate ligand is shown to be a good substrate for the macrophage mannose-specific glycoprotein uptake system. This and related ligands may be useful in the selective delivery to macrophages of antigens, adjuvants, anti-inflammatory drugs, antiparasitic compounds, and other pharmacological agents. Analogous ligands might be useful for delivery of such agents to other target cells that may contain distinctive uptake systems.

Note Added in Proof. Maynard and Baenziger (39) have demonstrated the endocytosis by rat hepatic reticuloendothelial cells of more complex glycopeptides derived from glycoproteins.

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