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L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages

(mannose-fucose lectin)

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ABSTRACT ^{125}I-Labeled L-fucose–albumin complex and rat preputial β-glucuronidase are rapidly cleared from plasma after intravenous infusion. L-Fucose–albumin retards the plasma clearance of β-glucuronidase whereas D-fucose–albumin is inactive. *In vitro*, ^{125}I-labeled L-fucose–albumin is taken up into rat or rabbit alveolar macrophages by receptor-mediated pinocytosis. Uptake (37°C) is time-dependent, is saturable with increasing ligand concentration (K_{uptake} = 4.4 × 10^{-8}M), and requires Ca^{2+}. ^{125}I-Labeled D-fucose–albumin is poorly taken up. Binding (4°C) is saturable and Ca^{2+} dependent. Binding and uptake are fully inhibited by yeast mannan. A series of neoglycoproteins, including L-fucose–albumin, were tested as inhibitors of uptake of ^{125}I-labeled β-glucuronidase into macrophages. The following order of potency was observed: L-Fuc = D-Man > GlcNAc ≈ D-Glc > D-Xyl >>> D-Gal = L-Ara = D-Fuc. L-Fucose-terminated oligosaccharides coupled to bovine serum albumin also block ^{125}I-labeled β-glucuronidase uptake into macrophages.

Recognition and plasma clearance of lysosomal glycosidases (1) and mannose/N-acetylglucosamine-terminated glycoproteins (2–5) are mediated by cell surface pinocytosis receptors associated with liver sinusoidal cells (6–11) and other cells of the mononuclear phagocyte system (12–14). *In vitro* studies with isolated macrophages have disclosed that mannose-terminated glycoproteins bind to cell surface receptors with high affinity after which they are rapidly internalized and transported to lysosomes (11–12). The liver receptor appears to be a 30,000-dalton protein that requires Ca^{2+} for binding activity (15, 16). The receptor associated with alveolar macrophages is very similar to, if not identical with, that which mediates rapid plasma clearance *in vivo* (16).

In the present study, it is shown that L-fucose-terminated glycoconjugates are recognized and internalized by macrophages via the same receptor that mediates plasma clearance and uptake of lysosomal glycosidases. We have used a family of synthetic glycoconjugates (i.e., neoglycoproteins) to investigate the specificity of lysosomal enzyme clearance *in vivo* as well as receptor-mediated pinocytosis *in vitro*. The results indicate that the macrophage receptor has broad specificity and is able to bind various glycoconjugates—most avidly those that express terminal L-fucose or D-mannose residues. Details of the binding and uptake of L-fucose–bovine serum albumin complex by macrophages are presented.

MATERIALS AND METHODS

Female Wistar rats were obtained from Harlan Industries (Cumberland, IN). White New Zealand rabbits were purchased from a local supplier. All reagents were laboratory grade from Sigma and other local vendors. Na^{125}I was obtained from Amersham or New England Nuclear. BCG was obtained from Trudeau Institute (Saranac Lake, NY).

Ligands. Neoglycoproteins were prepared by the method of Lee *et al.* (17) and Stowell and Lee (18) and contained 20–40 mol of sugar per mol of protein. Glycoconjugates of milk sugar oligosaccharides and bovine serum albumin were prepared by the method of Zopf *et al.* (19). β-Glucuronidase was purified to homogeneity from rat preputial glands by the method of Himeno *et al.* (20). The enzyme was assayed by using phenolphthalein glucuronide as described by Stahl and Touster (21). Ligands were iodinated by the chloramine-T method (22) to a specific activity of 1–10 μCi/μg (1 Ci = 3.7 × 10^{10} becquerels) as described (13).

Animal Procedures. Plasma clearance of radiolabeled neoglycoproteins and β-glucuronidase was performed in anesthetized 200-g rats as described by Stahl *et al.* (23). Alveolar macrophages were prepared from White New Zealand rabbits that had been injected intravenously with heat-killed BCG (5 mg suspended in 0.5 ml) 10 days earlier (24). The cells were washed twice by sedimentation and suspended in standard media as described (12).

Uptake and Binding. The uptake and binding of radiolabeled ligands by cells were performed exactly as described by Stahl *et al.* (13). Cells (5 × 10^5) in 0.1 ml were layered over oil (4 parts silicon oil plus 1 part mineral oil) in Microfuge (Beckman) tubes. The interaction of ligand with cells was terminated by sedimentation for 30 sec in a Beckman Microfuge.

RESULTS

Plasma Clearance of β-Glucuronidase and ^{125}I-Labeled D-Mannose–Albumin: Effect of L-Fucose–Albumin and D-Fucose-Albumin. β-Glucuronidase and ^{125}I-labeled D-mannose–albumin (^{125}I-D-Man–Alb) are rapidly cleared from plasma after intravenous infusion (6). Previous work has shown that the rate at which β-glucuronidase is cleared from plasma is sharply decreased by the simultaneous administration of mannose- or N-acetylglucosamine-terminated glycoproteins (6, 8). The results in Fig. 1 confirm the rapid clearance of the two test ligands and indicate that, when administered simultaneously with the ligand, ^{125}I-labeled L-fucose–albumin (^{125}I-L-Fuc–Alb) is effective in reducing the clearance rate of both. D-Fuc–Alb had no effect. Not shown is the observation that both L-Fuc–Alb and D-Fuc–Alb were rapidly cleared from plasma after intravenous injection.

Binding and Uptake of ^{125}I-L-Fuc–Alb by Alveolar Macrophages. Because clearance of β-glucuronidase reflects the activity of Kupffer cells (7) and other macrophages (12), it was reasonable to ask whether L-Fuc–Alb could be actively taken up

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Abbreviations: ^{125}I-D-Man–Alb, ^{125}I-labeled D-mannose–albumin; ^{125}I-L-Fuc–Alb, ^{125}I-labeled D-fucose–albumin.

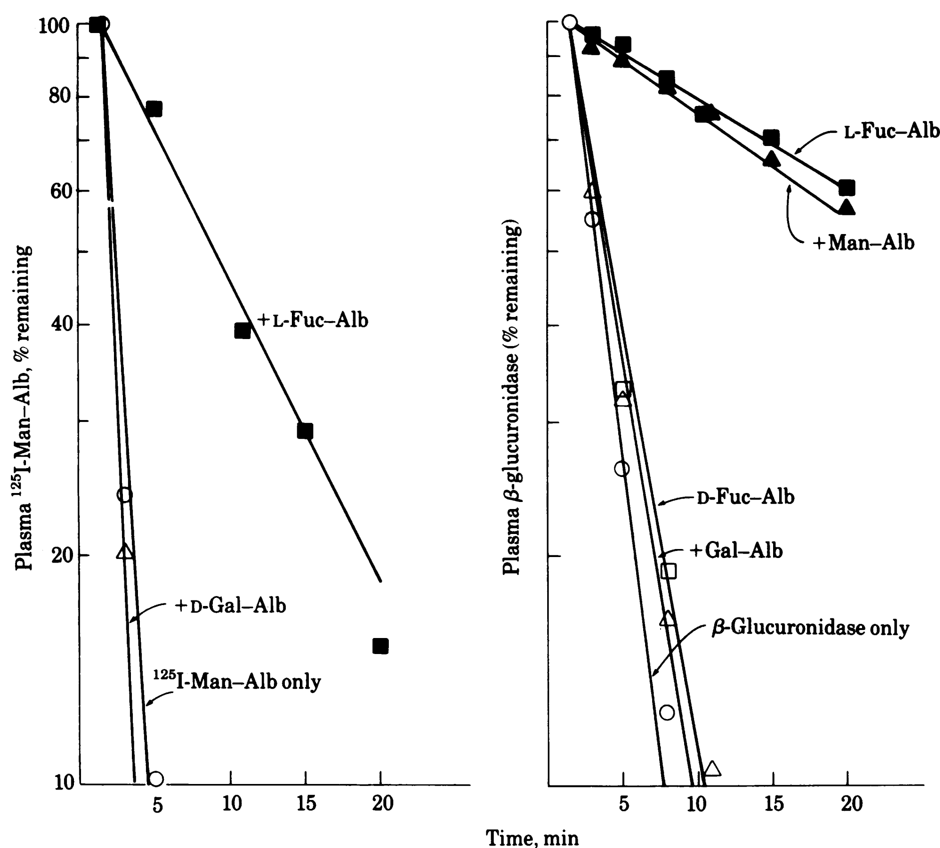

FIG. 1. Effect of neoglycoproteins on clearance of β-glucuronidase and ^{125}I-Man-Alb in the rat. Female Wistar rats were anesthetized and the femoral artery and vein were cannulated. ^{125}I-Man-Alb (*Left*) (20 μg; 10^6 cpm) or β-glucuronidase (*Right*) (75 units; 30 μg) was infused during 70 sec in a total volume of 0.5 ml. Arterial blood samples were taken commencing 20 sec after termination of the infusion. β-Glucuronidase was assayed directly in plasma as described (23). ^{125}I-Man-Alb was determined by precipitating an aliquot of plasma with 0.5% phosphotungstic acid in 2 M HCl. The precipitate was dissolved in 1 M NaOH and assayed for radioactivity. Blocking doses of L-Fuc-Alb (1 mg), D-Fuc-Alb (0.75 mg), D-Gal-Alb (2 mg), or D-Man-Alb (3 mg) were infused along with the test ligand.

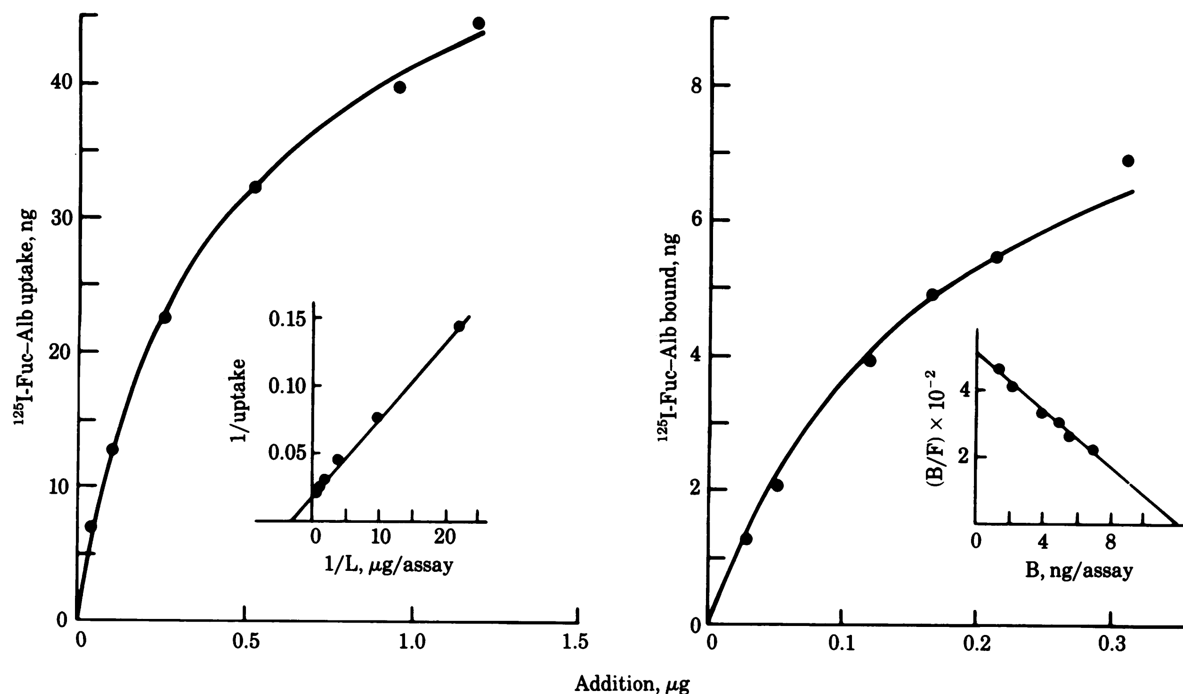

FIG. 2. Uptake (*Left*) and binding (*Right*) of ^{125}I-L-Fuc-Alb by rabbit alveolar macrophages. (*Left*) Cells (5×10^5) in 0.1 ml were incubated for 10 min at 37°C with increasing concentrations of ligand. Nonspecific uptake was determined by adding 0.125 mg of yeast mannan to companion assays. (*Right*) Binding was determined by incubating cells with ligand at 4°C. (*Inset*) Scatchard plot of the data corrected for nonspecific binding. $K_{uptake} = 4.4 \times 10^{-8}$ M.

into macrophages *in vitro* by receptor-mediated pinocytosis. Rabbit alveolar macrophages were incubated with ^{125}I-L-Fuc–Alb under standard conditions at 37°C. Binding alone was estimated after incubation at 4°C (pinocytosis blocked). Nonspecific uptake and binding were determined by running appropriate assays in the presence of an excess of yeast mannan. Yeast mannan is known to block receptor-mediated pinocytosis of mannose-glycoconjugates by macrophages (12). Uptake of L-Fuc–Alb into macrophages was linear over the 10-min assay period. The dependence of uptake at 10 min on ligand concentration is shown in Fig. 2 *Left*. The concentration of ligand required to produce half-maximal uptake (K_{uptake}) was estimated graphically as 4.4×10^{-8} M. The concentration dependence of binding of ^{125}I-L-Fuc–Alb to alveolar macrophages is shown in Fig. 2 *Right*. Binding became saturated at 60 min and required Ca^{2+} Removal of Ca^{2+} by the addition of EDTA abolished binding. A Scatchard plot of the binding data in the range tested disclosed a single class of binding sites with $K_d = 3 \times 10^{-8}$ M.

Effect of Neoglycoproteins on Macrophage Uptake of ^{125}I-Labeled β-Glucuronidase. To examine the sugar specificity of ligand binding, various neoglycoproteins were used as inhibitors and ^{125}I-labeled β-glucuronidase was used as a standard ligand. β-Glucuronidase is rapidly taken up by macrophages ($K_{uptake} = 3 \times 10^{-7}$ M) and uptake is linear with time over at least 30 min. Uptake of ^{125}I-labeled β-glucuronidase is fully inhibited by an excess of yeast mannan. The effect of increasing concentrations of neoglycoproteins on the uptake of ^{125}I-labeled β-glucuronidase is shown in Fig. 3. D-Man–Alb and L-Fuc–Alb were similar in their inhibitory potency and were clearly the best inhibitors of uptake. Less potent, but still effective, as inhibitors were D-Glc–Alb and D-GlcNAc–Alb. D-Xyl–Alb was inhibitory but only 1% as potent as D-Man–Alb or L-Fuc–Alb. L-Ara–Alb, D-Fuc–Alb, and D-Gal–Alb were inactive. The possible role of anomeric configuration was studied by testing the relative effects of two neoglycoproteins, α-L-Fuc–Alb and β-L-Fuc–Alb on uptake of ^{125}I-labeledβ-glucuronidase. The two were approximately equipotent (data not shown).

The neoglycoproteins used above are thioglycosides and may not be exactly equivalent to naturally occurring sugars in *O*-glycosidic linkage. To test the inhibitory effects of *O*-linked L-fucose terminated glycoconjugates on β-glucuronidase uptake, L-fucose and D-galactose-terminated lactose oligosaccharides coupled to albumin were studied as inhibitors. The results (Fig. 4) indicate that lacto-*N*-fucopentaose-I–Alb and lacto-*N*-fucopentaose-II–Alb were effective inhibitors of β-glucuronidase uptake whereas lacto-*N*-tetraose–Alb, a galactose-terminated glycoconjugate, was inactive.

DISCUSSION

The rapid plasma clearance of various lysosomal glycosidases (23) and perhaps other important macromolecules [e.g., IgM (25)] is mediated by a receptor that is associated with cells of the reticuloendothelial system (7–11) (reviewed in ref. 26). The uptake system has been studied in detail in alveolar macrophages (12–14). Two conclusions emerge from the present study. First, L-fucose and L-fucose-terminated oligosaccharides when coupled to bovine serum albumin are both good ligands and inhibitors (i.e., alternate ligands) for the macrophage glycoprotein pinocytosis receptor (previously referred to as the mannose receptor). Second, the receptor appears to bind glycoconjugates in following order: L-Fuc = D-Man > D-GlcNac ≅ D-Glc > D-Xyl ≫ D-Gal = L-Ara = D-Fuc.

L-Fucose is commonly found as a component of glycoproteins and glycolipids. Invariably, L-fucose is terminal and is linked

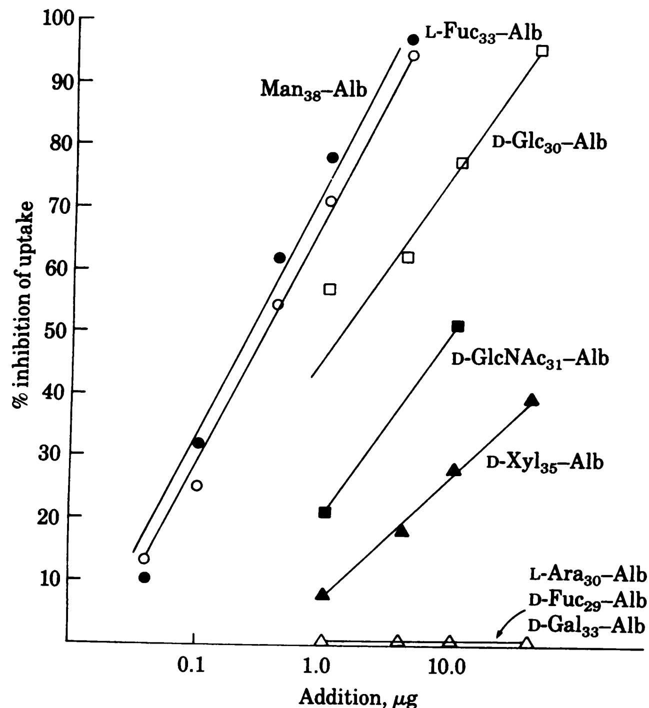

FIG. 3. Inhibition of ^{125}I-labeled β-glucuronidase uptake into macrophages by various neoglycoproteins. Cells (5×10^5/0.1 ml) in standard medium were incubated with 2.5 μg of ^{125}I-labeled β-glucuronidase and increasing concentrations of neoglycoproteins for 10 min at 37°C. The uptake of ligand was terminated by centrifugation through oil. The extent of substitution of the neoglycoprotein is indicated by the subscript. Results are the average of three to five experiments for each neoglycoprotein.

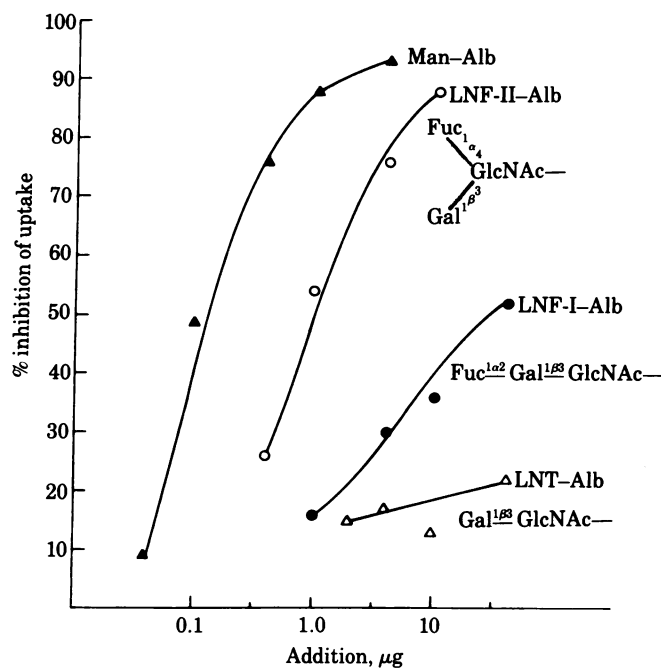

FIG. 4. Effect of lactose oligosaccharide glycoconjugates on uptake of ^{125}I-labeled β-glucuronidase by alveolar macrophages. Inhibition of uptake was exactly as described in Fig. 3 except that lactose oligosaccharide glycoconjugates were used as inhibitors. LNF-I–Alb, lacto-*N*-fucopentaose–Alb, 7 mol/mol; LNF-II–Alb, lacto-*N*-fucopentaose II–Alb, 13 mol/mol; LNT–Alb, lacto-*N*-tetraose–Alb, 20 mol/mol.

to a penultimate galactose or *N*-acetylglucosamine residue (27). Because of its terminal position, a role for L-fucose as a determinant of biologic recognition has been sought. Terminal L-fucose plays an important role in blood group specificity (28), and indirect evidence suggests that the L-fucose residues are involved in the action of macrophage inhibitory factor (29). Prieels *et al.* (30) have presented evidence for a L-fucose-specific lectin in liver hepatocytes which mediates rapid plasma clearance of α-1,3-linked L-fucose-terminal glycoproteins including lactoferrin. More recent evidence (31) with liver membranes indicates that neoglycoproteins containing fucose in the α-1,3 linkage are specifically recognized. Recently, Grant *et al.* (32) have shown that enterokinase, an L-fucose-rich glycoprotein, is rapidly cleared from plasma.

Our results indicate that L-fucose bound to protein is recognized by the same receptor on macrophages that binds D-mannose glycoconjugates. This was confirmed at three levels. (*i*) Animal experiments have indicated that L-Fuc–Alb and D-Man–Alb are potent blockers of β-glucuronidase clearance. Moreover, L-Fuc–Alb slows the clearance of D-Man–Alb. (*ii*) Experiments with alveolar macrophages indicate that the kinetics of ^{125}I-D-Man–Alb binding and uptake are similar, if not identical, to the binding and uptake of ^{125}I-L-Fuc–Alb. At 4°C L-Fuc–Alb and D-Man–Alb bind to macrophages to approximately the same extent, both require Ca^{2+} for binding, and both are inhibited by an excess of yeast mannan, a mannose-terminal glycoprotein. In short, the uptake of L-Fuc–Alb into macrophages parallels D-Man–Alb uptake in every way. (*iii*) In other studies (16) with the isolated liver mannose receptor, added L-Fuc–Alb and D-Man–Alb were very potent inhibitors of ^{125}I-labeled mannan binding. D-Fucose was inactive. In sum, L-fucose is recognized by two receptor systems *in vivo*, one associated with macrophages having broad specificity and a second having very narrow specificity and associated with hepatocytes (31).

To examine the relative inhibitory potency of a series of glycoconjugates, ^{125}I-labeled β-glucuronidase was used as the ligand. β-Glucuronidase is rapidly taken up by macrophages via the mannose pathway, and uptake is saturable and linear under the conditions of the assay. The results in Fig. 3 indicate that L-Fuc–Alb and D-Man–Alb are the most effective inhibitors of ^{125}I-labeled β-glucuronidase uptake. Sugars with the D-glucose configuration are intermediate and D-Xyl–Alb is poor. Taken together, these results suggest that the orientation of the hydroxyl about C-4 is critical to the formation of a high-affinity recognition site. C-6 appears to be less important, but still essential, because removal of C-6 (e.g., D-xylose) sharply decreased binding. Moreover, orientation of the OH group on C-2 plays some role in generating a high-affinity site because glucose and mannose, C-2 epimers, vary in inhibitory potency by an order of magnitude.

In sum, the results indicate that the receptor has a relaxed specificity and will bind a wide variety of glycoproteins but especially those with terminal mannose or fucose. This situation is similar to that found for the mammalian hepatic D-galactose-binding system (33–35). The recognition of terminal L-fucose by macrophages may play some role in transport of native glycoproteins because of its terminal position on the oligosaccharide chain.

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