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Clearance and binding of native and defucosylated lactoferrin

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These studies explore the role of carbohydrate recognition systems and the direct involvement of terminal α1–3-linked fucose in the clearance of lactoferrin from the murine circulation and in the specific binding of lactoferrin to receptors on murine peritoneal macrophages. As previously reported, radiolabelled lactoferrin cleared very rapidly ($t_{\frac{1}{2}} < 1$ min) after intravenous injection into mice. However, competing levels of ligands specific for the hepatic galactose receptor (asialo-orosomucoid), the hepatic fucose receptor (fucosyl-bovine serum albumin), and the mononuclear-phagocyte system pathway recognizing mannose, *N*-acetylglucosamine and fucose (mannosyl-, *N*-acetyglucosaminyl- and fucosyl-bovine serum albumin) did not block radiolabelled lactoferrin clearance *in vivo* or binding to mouse peritoneal macrophage monolayers *in vitro*. Almond emulsin α1–3-fucosidase was used to prepare defucosylated lactoferrin in which 88% of the α1–3-linked fucose was hydrolysed. No difference in clearance or receptor binding was observed between radiolabelled native and defucosylated lactoferrin. Fucoidin, a fucose-rich algal polysaccharide, completely inhibits the clearance *in vivo* and macrophage binding *in vitro* of lactoferrin. This effect, however, is probably not the result of competition for binding to the fucose receptor, since gel-filtration studies demonstrated formation of a stable complex between lactoferrin and fucoidin. The present results indicate that the lactoferrin-clearance pathway is distinct from several pathways mediating glycoprotein clearance through recognition of terminal galactose, fucose, *N*-acetylglucosamine or mannose. Furthermore, α1–3-linked fucose on lactoferrin is not essential for lactoferrin clearance *in vivo* or specific binding to macrophage receptors *in vitro*.

Lactoferrin is an iron-binding glycoprotein synthesized by neutrophils and glandular epithelial cells that is detectable in a variety of secretory fluids (Masson & Heremans, 1966; Masson *et al.*, 1969; Mason & Taylor, 1978). The native molecule is a single polypeptide chain (M_r 76500) with two asparagine-linked biantennary oligosaccharide chains per molecule, and displays considerable homology with the circulating iron-transport glycoprotein transferrin (Aisen & Leibman, 1972; Montreuil & Spik, 1975; Spik & Mazurier, 1977). Physiological activities proposed for lactoferrin include defence against mucosal infection (Arnold *et al.*, 1977; Bullen & Armstrong, 1979), and its function as a regulator of granulopoiesis (Broxmeyer *et al.*, 1978, 1980; Broxmeyer, 1979). Radiolabelled lactoferrin is rapidly removed from the circulation after intravenous injection in mice and rabbits (Prieels *et al.*, 1978; Karle *et al.*, 1979; Bennett & Kokocinski, 1979). Receptors for lactoferrin have been described on cells of the mononuclear-phagocyte system (Van Snick *et al.*, 1977; Markowetz *et al.*, 1979), and their presence has also been reported on hepatocytes (Prieels *et al.*, 1978).

Lactoferrin differs significantly from its glycoprotein homologue transferrin in possessing two fucose residues per molecule (Montreuil & Spik, 1975). The biantennary asparagine-linked oligosaccharide chains of transferrin do not contain fucose. Previous studies have demonstrated that the fucose-rich algal polysaccharide fucoidin (Percivol, 1970; Medcalf, 1978) inhibits the rapid clearance of radiolabelled lactoferrin from the circulation of mice, suggesting that terminal fucose residues mediate the clearance of lactoferrin (Prieels *et al.*, 1978). Recent

Abbreviations used: FucBSA, β-L-fucosyl$_{50}$-bovine serum albumin; GlcNAcBSA, β-*N*-acetyl-D-glucosaminyl$_{47}$-bovine serum albumin; ManBSA, β-D-mannosyl$_{50}$-bovine serum albumin; SBTI, soya-bean trypsin inhibitor; SDS, sodium dodecyl sulphate; TNBS, 2,4,6-trinitrobenzenesulphonic acid; MEM, minimal essential medium; EBSS, Earle's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

^{1}H-n.m.r. studies of lactoferrin carbohydrate structure indicate that the two fucose residues on lactoferrin are distributed between α1–3 linkage with *N*-acetylglucosamine on the terminal branches of the oligosaccharide chains, and α1–6 linkage with the innermost-core *N*-acetylglucosamine (Spik & Mazurier, 1977). Given the sterically accessible position of the α1–3-linked fucose, it would seem a potential binding determinant if lactoferrin clearance and binding were carbohydrate-mediated.

In the present study we investigated the possible role of carbohydrate-mediated clearance pathways in lactoferrin clearance and characterized the nature of fucoidin inhibition of lactoferrin clearance and receptor binding. Workers in this laboratory have recently developed an affinity-chromatographic procedure to purify the α1–3-fucosidase from almond emulsion (Imber *et al.*, 1982*b*), and we describe here its use to study directly the role of α1–3-linked fucose in lactoferrin clearance and binding.

Experimental

Materials

Bovine serum albumin, phosphorylase, enolase, SBTI, human transferrin and mannan were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-fucose, *N*-acetyl-D-glucosamine, D-mannose, D-galactose, and D-glucose from Phanstiehl Laboratories, Waukegan, IL, U.S.A.; fucoidin from Pfaltz and Bauer, Stamford, CT, U.S.A.; TNBS from Eastman Chemical Co., Rochester, NY, U.S.A.; EBSS, ten-times-concentrated, with and without Ca^{2+} and Mg^{2+}, from Gibco, Grand Island, NY, U.S.A.; and $Na^{125}I$ carrier-free in 0.1 M-NaOH, from New England Nuclear Corp., Boston, MA, U.S.A. Iron-saturated lactoferrin, purified from human milk, was a gift from Dr. Jean-Paul Prieels, Université Libre de Bruxelles, Bruxelles, Belgium. Orosomucoid was a gift of the American National Red Cross, Bethesda, MD, U.S.A. All other reagents were the highest grade commercially available. A Scientific Products AW 14120 gamma counter was used for counting radioactivity.

Methods

Macrophages. Mouse peritoneal-macrophage monolayers were generously provided by Dr. Dolph O. Adams, Department of Pathology, Duke University Medical Center, and were prepared from female inbred C57 BL/6J mice (Harlan–Sprague–Dawley, Madison, WI, U.S.A.), 8–12 weeks of age. Macrophages were obtained by intraperitoneal injection of Brewer's thioglycollate broth (Difco, Detroit, MI, U.S.A.) 3 days before peritoneal lavage. After lavage, the peritoneal-exudate cells were centrifuged at 250*g* at 4°C for 10min. The cells were resuspended in Dulbecco's MEM (Gibco) supplemented with 10% (v/v) foetal-calf serum (Sterile Systems, Logan, VT, U.S.A.). After total and differential cell counts were performed, portions of the suspension were added to 16mm-diameter-well plates (Linbro Plastics, Hamden, CT, U.S.A.). The suspensions were adjusted so that a final concentration of 2.5×10^5 adherent macrophages/cm^2 was obtained. After 2–3h the macrophage monolayers were washed three times with Hanks balanced salt solution supplemented with 5% foetal-calf serum. Serumless Neumann & Tytell (Gibco) medium (1ml/well) was then added.

Purification of almond (Amygdalus communis) emulsin fucosidase I. The almond emulsin fucosidase that specifically removes fucose in α1–3 linkage to *N*-acetylglucosamine (Ogata-Arakawa *et al.*, 1977; Yoshima *et al.*, 1979) was purified by affinity chromatography on Cibacron Blue–Sepharose as previously described (Imber *et al.*, 1982*b*). The enzyme activity was purified 1250-fold and was free of contaminating β-galactosidase and proteinase activities.

Preparation of defucosylated lactoferrin. Native lactoferrin (1.7mg, 44nmol of fucose) and purified almond emulsion fucosidase I (0.8 unit) were combined with 0.1 M-sodium acetate/0.1 M-NaCl, pH5.3 (total vol. 200μl) and incubated at 37°C for 48h. The release of L-fucose after incubation was determined on the incubation solution and a control solution of lactoferrin without added fucosidase, by the fucose dehydrogenase-linked assay of Tsay & Dawson (1977). A standard plot for this assay was determined with 5–50nmol of L-fucose.

Preparation of asialo-orosomucoid. Asialo-orosomucoid was prepared from orosomucoid as previously described (Prieels *et al.*, 1978).

Radioiodination. Native and defucosylated lactoferrin were radiolabelled by the chloramine-T procedure (Greenwood *et al.*, 1963). $Na^{125}I$ (250μCi) was added to phosphate-buffered saline (0.1 M-NaH_2PO_4/0.15 M-NaCl, pH7.4) containing 2.00μg of protein (total vol. 300μl). Iodination was initiated by adding 50μl of chloramine-T (4mg/ml) in phosphate-buffered saline. The reaction was quenched after 1min by the addition of 100μl of sodium metabisulphite (20mg/ml) in phosphate-buffered saline. The entire mixture was chromatographed on a column (0.7cm × 10cm) of Sephadex G-25 prewashed with bovine serum albumin and equilibrated in 0.15 M-NaCl. Fractions containing radiolabelled protein eluted in the void volume, were pooled, stored at 4°C and used within 2 weeks.

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed in a Bethesda Research Laboratory slab-gel apparatus with the multizonal ammediol/glycine system of Wyckoff *et al.* (1977), with a 7.5% acrylamide separating gel, and a 5% stacking gel. Protein samples were reduced

and denatured for electrophoresis by heating to 100°C for 2min in sample buffer containing 2% (w/v) SDS and 7mM-dithiothreitol. Electrophoresis was carried out at 45mA until the Bromophenol Blue tracking dye reached the bottom of the gel. Gels were fixed and stained overnight in 0.25% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (9:2:9, by vol.), and destained by diffusion in methanol/acetic acid/water (16:3:21, by vol.).

Synthesis of neoglycoproteins. Commercial bovine serum albumin was further purified by gel filtration on Sephacryl S-200 in phosphate-buffered saline, pH6.0, to remove high-molecular-weight globulin contaminants and bovine serum albumin dimer. The purified bovine serum albumin was dialysed extensively against deionized water, freeze-dried and dissolved in 0.2M-$Na_2B_4O_7$, pH8.5, at a final concentration of 10mg/ml.

The cyanomethylper-*O*-acetyl-1-thioglycopyranoside derivatives of L-fucose, D-mannose and *N*-acetyl-D-glucosamine were synthesized and crystallized by the method of Stowell & Lee (1980). Melting points for the crystalline derivatives of L-fucose (110°C), D-mannose (130°C) and *N*-acetyl-D-glucosamine (179°C) are in agreement with previously reported values (Stowell & Lee, 1980). A 1.0g sample of each cyanomethylthioglycoside was used to generate the thioimidate derivative for coupling to 100mg of purified bovine serum albumin in borate buffer as described by Stowell & Lee (1980).The modified bovine serum albumin was dialysed overnight against 0.1M-NH_4HCO_3, dialysed for 48h against frequent changes of deionized water, and then freeze-dried. More than 80% reactivity of the 59 lysine residues in bovine serum albumin was demonstrated by TNBS assays on modified and unmodified bovine serum albumin (Fields, 1972). The mean carbohydrate valency of each neoglycoprotein was L-fucosyl-bovine serum albumin, 50; D-mannosyl-bovine serum albumin, 50; and *N*-acetyl-D-glucosaminyl-bovine serum albumin, 47.

Clearance studies. Clearance studies were performed in 20-week-old CD-1 female white mice (Charles River Laboratory, Wilmington, MA, U.S.A.), as previously described (Imber & Pizzo, 1981). Samples prepared for injection included 1.5μg (approx. 20pmol) of ^{125}I-labelled native or defucosylated lactoferrin, with or without excess unlabelled competing ligand, in a total volume of 300μl of EBSS, pH7.4. After mice were first lightly anaesthetized with diethyl ether, samples were injected into the lateral tail veins with a tuberculin syringe and a 30-gauge needle. Beginning within 10s after injection, 25μl samples of blood were withdrawn at various time intervals from the retro-orbital venous plexus, using calibrated heparinized hematocrit capillary tubes. Radioactivity in drawn samples was determined by gamma counter. Radioactivity remaining in the circulation was plotted as percentage of radioactivity in the initial sample drawn.

Binding studies. Binding studies were performed at 4°C with fixed monolayers of mouse peritoneal macrophages, prepared as described above. At 1h after addition of Neumann & Tytell medium, plated macrophage monolayers were equilibrated at 4°C and then prewashed with 2 × 1.0ml of medium A (EBSS with Ca^{2+} and Mg^{2+}/25mM-Hepes/bovine serum albumin (10mg/ml), pH7.3. Portions (0.5ml) of incubation media were prepared in medium A containing the desired final concentration of radiolabelled and unlabelled ligand. Binding was initiated by aspirating the prewash medium and adding the incubation medium to the wells containing cell monolayers. Cells were gently agitated at 4°C for the desired length of time. The radioligand incubation medium was then aspirated and cells were washed with 3 × 1.0ml of medium A, followed by 3 × 1.0ml of medium B (EBSS with Ca^{2+} and Mg^{2+}/25mM-Hepes, pH7.3). Cells were dissolved overnight in 0.3ml of 0.1M-NaOH per well. Cell-bound radioactivity was determined by counting the NaOH-solubilized material in a gamma counter. Total cell protein per well was then determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Total binding was determined as radioligand bound in the absence of excess unlabelled ligand. Non-specific binding was determined in the presence of a 100-fold molar excess of unlabelled ligand. Specific binding was calculated as the difference between total and non-specific binding. Duplicate or triplicate values were determined for all experimental points. Scatchard (1949) analysis was performed as previously described (Imber *et al.*, 1982*a*) to obtain the K_d for the binding of native and defucosylated lactoferrin.

Results

Clearance studies

Fig. 1 presents the murine clearance of radiolabelled lactoferrin in the presence of a variety of carbohydrate-containing ligands. Lactoferrin clears rapidly in the absence of competing ligands, with a half-life of less than 1min (Fig. 2). Lactoferrin clearance was blocked completely, however, in the presence of a large excess of the fucose-rich algal polysaccharide fucoidin (Percivol, 1970; Medcalf, 1978) (Fig. 1). The mannose-rich yeast capsular polysaccharide mannan (Ballou & Raschke, 1974) only slightly inhibited lactoferrin clearance. Whereas the kinetics of lactoferrin clearance shows it to be a first-order process, clearance of lactoferrin in the presence of mannan was at least biphasic, sug-

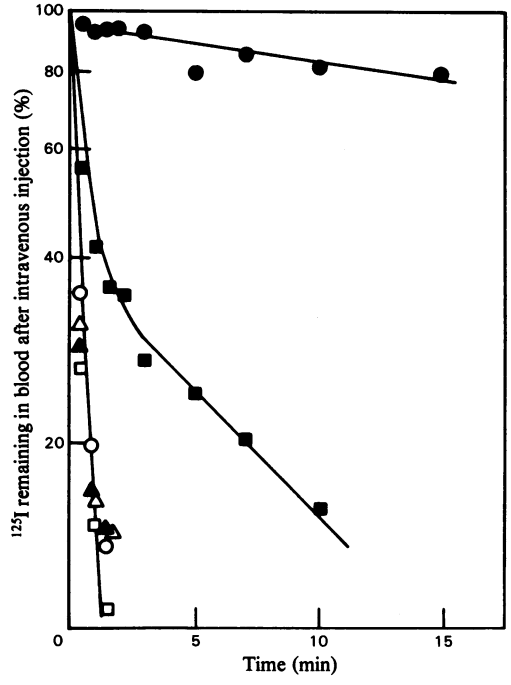

Fig. 1. *Clearance of radiolabelled lactoferrin in the presence of competing carbohydrate-containing ligands*

^{125}I-lactoferrin (20pmol; 150c.p.m./fmol) was injected in the absence or presence of a large excess of unlabelled competitor. Blood samples were taken as described in the Experimental section. Clearance studies were performed by using the following unlabelled competitors: fucoidin (5mg, ●), mannan (5mg, ■), FucBSA (45nmol, ▲), GlcNAcBSA (45nmol, ○), ManBSA (45nmol, □) and asialo-orosomucoid (55nmol, △). The clearance of radiolabelled lactoferrin in the absence of competitors was identical with that in the presence of neoglycoproteins or asialo-orosomucoid as competitors.

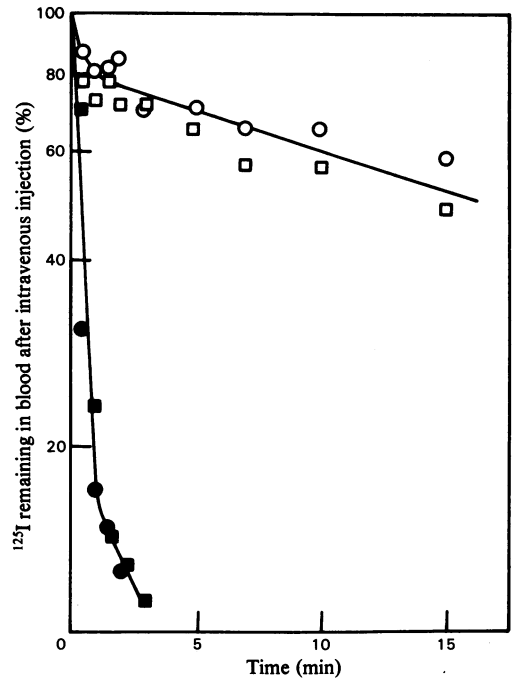

Fig. 2. *Clearance of radiolabelled native and defucosylated lactoferrin alone and in the presence of competing unlabelled native lactoferrin*

A 20pmol portion of each radiolabelled protein (150c.p.m./fmol) was injected alone or in the presence of a large excess of unlabelled native lactoferrin (approx. 65nmol). Blood samples were taken as described in the Experimental section. Clearances performed in the absence of unlabelled competing ligand: ●, ^{125}I-lactoferrin; ■, ^{125}I-defucosyl-lactoferrin. Clearances performed in the presence of unlabelled native lactoferrin: ○, ^{125}I-lactoferrin; □, ^{125}I-defucosyl-lactoferrin.

gesting heterogeneity in the ligand population or in the elimination pathway.

Transferrin, when injected at a 1000-fold molar excess to the radiolabelled lactoferrin did not alter the clearance of lactoferrin. This suggests a separate pathway for the clearance of these homologous iron-binding proteins consistent with previous observations from this laboratory (Prieels *et al.*, 1978). The β-galactosyl-terminated glycoprotein asialo-orosomucoid, a ligand specific for the hepatic galactose receptor (Pricer & Ashwell, 1971; Ashwell & Morell, 1974), did not inhibit clearance at all, nor did the synthetic neoglycoproteins FucBSA, GlcNAcBSA and ManBSA. Each of these neoglycoproteins clear by a common mononuclear-phagocyte system pathway that also recognizes mannan (Achord *et al.*, 1977; Schlesinger *et al.*, 1978; Shepherd *et al.*, 1981; Imber *et al.*, 1982*a*). FucBSA, in addition, clears by a hepatic pathway recognizing α-fucosyl linkages (Lehrman *et al.*, 1980; Furbish *et al.*, 1980; Pizzo *et al.*, 1981).

Previous studies, describing the inhibition of lactoferrin clearance by fucoidin, suggest that terminal fucose residues mediate the clearance of lactoferrin (Prieels *et al.*, 1978). The lack of clearance inhibition in the presence of FucBSA does not support this interpretation. To study directly the role of terminal fucose residues in lactoferrin clearance, a defucosylated derivative of native lactoferrin was prepared by using a highly purified preparation of the almond emulsin fucosidase I, an exoglycosidase that specifically hydrolyses fucose in α1–3 linkage (Ogata-Arakawa *et al.*, 1977; Yoshima

et al., 1979; Imber *et al.*, 1982*b*). After extensive digestion, 44% of the total fucose was released, corresponding to 88% of the terminal α1–3-linked fucose. Analysis of the defucosylated lactoferrin by SDS/polyacrylamide-gel electrophoresis demonstrated that the derivative was electrophoretically identical with the native lactoferrin molecule (Fig. 3).

Fig. 2 compares the clearance of native and defucosylated lactoferrin *in vivo*. The clearance of the radiolabelled defucosylated derivative was identical with that of native lactoferrin, and the simultaneous presence of a large excess of native unlabelled lactoferrin completely inhibited the clearance of each radioligand. These results suggest that terminal α1–3 fucose residues are not essential for the clearance of native lactoferrin.

Gel-filtration studies

Gel-filtration studies probed the possibility that direct interaction between radiolabelled lactoferrin and either polysaccharide, fucoidin or mannan, was responsible for inhibition of lactoferrin clearance demonstrated in Fig. 1. The far greater size of

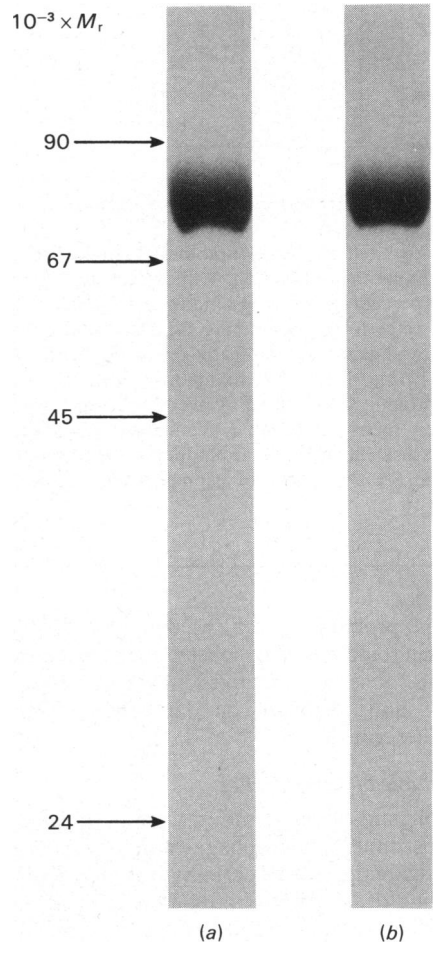

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of native and defucosylated lactoferrin*

Native lactoferrin (20μg; *a*), and defucosylated lactoferrin (also 20μg; *b*) were electrophoresed in the presence of SDS and dithiothreitol as described in the Experimental section. Protein standards used were: phosphorylase (M_r 90000), bovine serum albumin (M_r 67000), enolase (M_r 45000) and SBTI (M_r 24000).

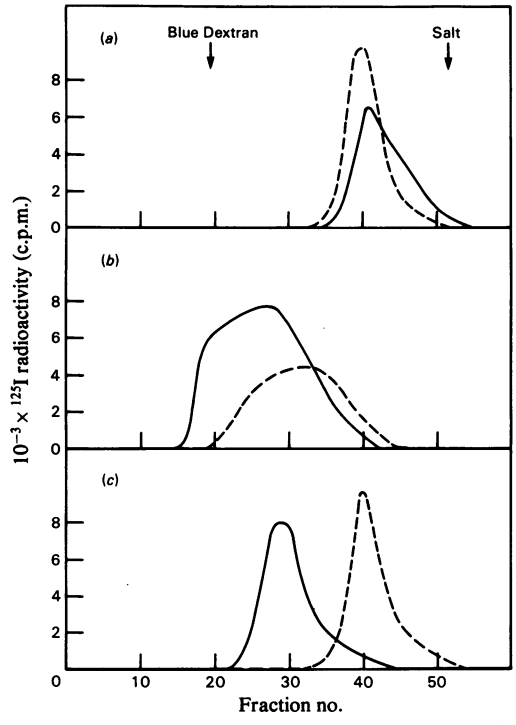

Fig. 4. *Gel filtration of radiolabelled lactoferrin alone and in the presence of fucoidin and mannan*

A 1.3nmol sample of ^{125}I-lactoferrin (77c.p.m./pmol) was prepared in total volume of 400μl of equilibration buffer alone or in the presence of a large excess of unlabelled fucoidin or mannan. Each solution was applied to a column (47cm × 1.6cm) of Sepharose 6B-CL and chromatography was performed at 20°C with a flow rate of 15ml/h per cm^2. Fractions (1.8ml) were collected and counted for radioactivity. ^{125}I-lactoferrin was eluted alone (*a*), in the presence of 10mg of fucoidin (*b*), and in the presence of 10mg of mannan (*c*). Each sample was eluted in the presence of equilibration (0.02M-NaH_2PO_4, pH7.4) buffer at two different salt concentrations: 0.15M-NaCl (——) and 0.5M-NaCl (– – – –). Column void volume was determined with Blue Dextran, and salt volume with potassium ferricyanide.

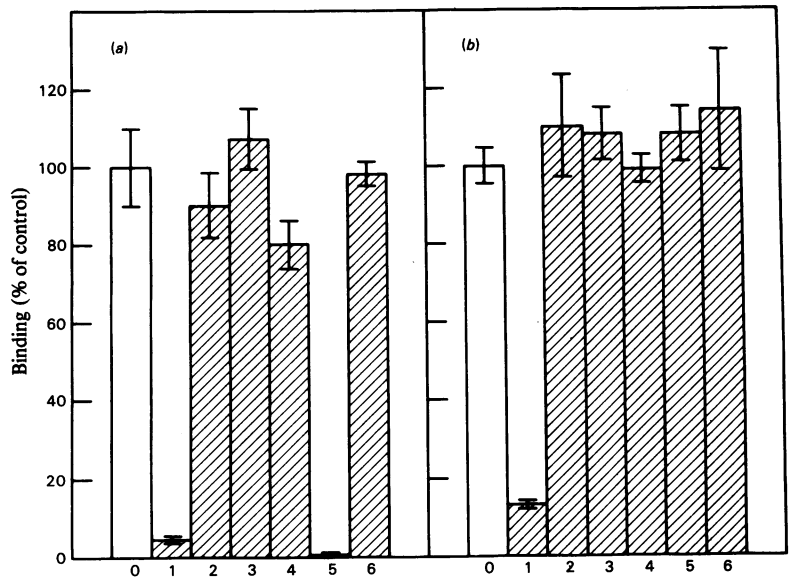

Fig. 5. *Binding of radiolabelled lactoferrin to macrophage monolayers in the presence and absence of competing carbohydrate-containing ligands and simple sugars*

^{125}I-lactoferrin (72.6 c.p.m./fmol) was incubated at a concentration of 5.0 nM at 4°C, and total binding in the absence and presence of unlabelled ligands was determined as described in the Experimental section. Values determined in the presence of competing ligands were plotted (cross-hatched bars) as a percentage of total binding determined in the absence of competing ligands (open bars). Numbers below the bars refer to the competing unlabelled ligand. (*a*) Incubations were performed for 6 h in the presence of: 0, no competing ligand; 1, lactoferrin (5.0 μM); 2, FucBSA (5.0 μM); 3, GlcNAcBSA (5.0 μM); 4, ManBSA (5.0 μM); 5, fucoidin (1.0 mg/ml); and 6, mannan (1.0 mg/ml). Total binding in the absence of unlabelled ligand was 43.9 pmol/mg of cell protein. (*b*) Incubations were performed for 2 h in the presence of: 0, no competing ligand; 1, lactoferrin (1.0 μM); 2, L-fucose (50 mM); 3, *N*-acetyl-D-glucosamine (50 mM); 4, D-mannose (50 mM); 5, D-glucose (50 mM); and 6, D-galactose (50 mM). Total binding in the absence of unlabelled ligand was 23.9 pmol/mg of cell protein. All values shown are the means of triplicate determinations. Vertical error bars represent ±1 S.D.

fucoidin and mannan would result in a decrease in the observed elution volume of radiolabelled lactoferrin if direct binding occurred.

Radiolabelled lactoferrin was eluted alone or in the presence of large excesses of fucoidin or mannan, on Sepharose 6B, with 0.15 M-NaCl, pH 7.4 (Fig. 4, ——). Elution of radiolabelled lactoferrin in the presence of a 100-fold weight excess of fucoidin or mannan resulted in a shift in the lactoferrin elution position toward the void volume of the column. Elutions were repeated in the presence of a higher salt concentration, 0.5 M-NaCl (Fig. 4, – – – –). The presence of fucoidin still resulted in a shift of lactoferrin to a higher-molecular-weight elution position. However, no shift was observed in the presence of mannan with increased ionic strength.

These results indicate that both polysaccharides directly interact with lactoferrin at physiological pH and ionic strength, probably by electrostatic forces. The disruption of mannan–lactoferrin binding by elevated salt concentration suggests that this interaction is probably much weaker than fucoidin–lactoferrin binding. This observation is consistent with the observed relative inhibitory effects of fucoidin and mannan on lactoferrin clearance already described.

Macrophage binding studies

Radioligand binding studies were performed with mouse peritoneal macrophage monolayers in order to investigate the role of carbohydrate recognition in the binding of lactoferrin to its mononuclear-phagocyte system receptor. The results of radiolabelled lactoferrin binding in the presence of a variety of carbohydrate-containing ligands are presented in Fig. 5(*a*). Non-specific binding amounted to 4.4% of total ^{125}I-lactoferrin binding, in the presence of a 1000-fold excess of unlabelled lactoferrin. Only modest variation in control lactoferrin binding was observed in the presence of the neoglycoproteins FucBSA, GlcNAcBSA and ManBSA, and the polysaccharide mannan, all of which interact at a

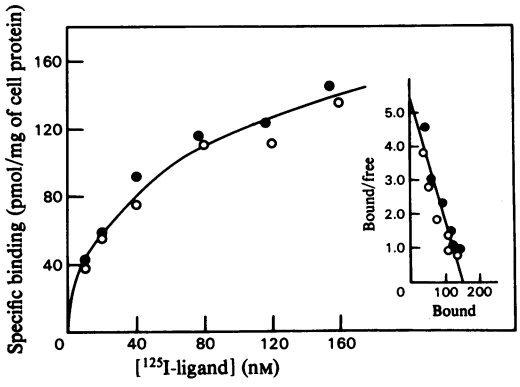

Fig. 6. *Specific binding of radiolabelled native and defucosylated lactoferrin to macrophage monolayers as a function of ligand concentration*

^{125}I-lactoferrin (137 c.p.m./fmol, O) and ^{125}I-defucosyl-lactoferrin (131 c.p.m./fmol, ●) were incubated with macrophage monolayers for 4 h at 4°C and specific binding was determined as described in the Experimental section. All values shown are the means of duplicate determinations. A Scatchard (1949) plot is shown in the insert.

common macrophage carbohydrate receptor (Achord *et al.*, 1977; Schlesinger *et al.*, 1978; Shepherd *et al.*, 1981; Imber *et al.*, 1982*a*). The polysaccharide fucoidin, however, completely blocked radiolabelled lactoferrin binding, decreasing it to 0.7% of control binding, substantially lower than the non-specific binding level of 4.4% observed in the presence of excess unlabelled lactoferrin. This observation is consistent with the gel-filtration results already described. Direct interaction of fucoidin with lactoferrin would sharply reduce the amount of free lactoferrin available to interact both specifically and non-specifically with the macrophage cell surface.

The binding of radiolabelled native and defucosylated lactoferrin were compared to determine whether α1–3 fucosyl groups are necessary for lactoferrin-receptor binding (Fig. 6). Specific and saturable binding with increasing radioligand concentration was observed for both native and defucosylated lactoferrin. There was no significant difference in the binding profiles for each radioligand, suggesting that α1–3-linked fucose is not an essential component of the receptor-binding domain on lactoferrin (Fig. 6). The K_d for native and defucosylated lactoferrin was 28 nM, in good agreement with the findings of previous studies (Imber *et al.*, 1982*a*). Further evidence for the lack of glycosyl-group involvement in lactoferrin binding is suggested by the slight variation in ^{125}I-lactoferrin binding in the presence of high concentrations (50 mM) of the simple sugars fucose, *N*-acetylglucosamine, mannose, glucose and galactose (Fig. 5*b*).

Discussion

The results of these studies suggest the need for caution in interpreting the results of radioligand clearance and receptor binding experiments. Competition studies were performed in the presence of various unlabelled ligands to investigate the uniqueness of a particular radioligand clearance or binding pathway, and/or to probe the molecular constraints that may be necessary for ligand–receptor interaction. However, the apparent inhibition of radioligand clearance or receptor binding by a second unlabelled ligand does not necessarily imply competition at a common receptor site. The possibility of direct interaction between radioligand and unlabelled ligand must first be investigated before such a conclusion is appropriate. The use of competing high-molecular-weight polysaccharides to investigate the possibility of carbohydrate-mediated glycoprotein clearance is risky, since many polysaccharides are rich in sulphate and therefore capable of electrostatic interactions with basic amino acid residues on radioligands.

In view of the fact that mannan and lactoferrin interact directly at physiological pH and ionic strength, it seems unusual that the clearance (Fig. 1) and binding (Fig. 5*a*) of radiolabelled lactoferrin should be only slightly affected by the presence of mannan. Two explanations for lack of significant mannan inhibition are possible. First, lactoferrin affinity for mannan may be far weaker than for the lactoferrin receptor. In this case, radiolabelled lactoferrin will favour the stronger receptor binding in the presence of mannan. The observation that increased ionic strength readily disrupts the interaction of lactoferrin with mannan but not with fucoidin (Fig. 4), supports this possibility. Secondly, the lactoferrin–mannan complex may still bind cell receptors for lactoferrin, whereas the binding domain of the lactoferrin–fucoidin complex may be conformationally inaccessible to the receptor site. A third explanation, that mannan-bound lactoferrin would clear or bind via the mononuclear-phagocyte-system pathway recognizing mannan, is unlikely, since the large amounts of unlabelled mannan used in these studies would adequately block clearance by this route.

From the data presented, the heterogeneity in the clearance pathway of lactoferrin in the presence of mannan (Fig. 1) most likely results from an equilibrium between lactoferrin–mannan complexes and free lactoferrin.

Since both fucoidin and mannan are negatively charged polyanions, it is likely that other similar macromolecules might also alter clearance and receptor recognition of lactoferrin, which is a very basic protein. However, this question was not addressed in the present study.

Several mammalian receptors mediate glycoprotein clearance through recognition of different terminal carbohydrate units. Competition studies with ligands specific for these pathways were performed to determine whether any of these pathways mediated the clearance of radiolabelled lactoferrin. The hepatic asialoglycoprotein receptor (Pricer & Ashwell, 1971; Ashwell & Morell, 1974) was considered a possible clearance route, since the asparagine-linked oligosaccharide chain branches on lactoferrin containing α1–3-linked fucose also contain terminal α1–4-linked galactose. The β-galactose-terminated ligand asialo-orosomucoid, which is specific for this hepatic receptor, did not inhibit lactoferrin clearance (Fig. 1). The α1–3-linked fucose on lactoferrin could potentially interact with one of two carbohydrate receptors. The possible role of a hepatic pathway that mediates the clearance of FucBSA (Lehrman *et al.*, 1980; Furbish *et al.*, 1980; Pizzo *et al.*, 1981) was ruled out by the inability of FucBSA to inhibit the clearance of native lactoferrin (Fig. 1). The possible role of a mononuclear-phagocyte-system pathway that recognizes *N*-acetylglucosamine, mannose and fucose (Achord *et al.*, 1977; Schlesinger *et al.*, 1978; Shepherd *et al.*, 1981; Imber *et al.*, 1982*a*) was ruled out by the inability of ManBSA, GlcNAcBSA, FucBSA to inhibit the clearance of radiolabelled lactoferrin (Fig. 1) or to block lactoferrin binding to macrophage monolayers (Fig. 5*a*).

The original report of a hepatic fucose receptor (Prieels *et al.*, 1978) suggested that only α1–3-linked fucose was recognized by the receptor. Subsequent studies, however, with the neoglycoprotein FucBSA, did not support the concept of such a specific recognition pathway for fucose (Lehrman *et al.*, 1980; Pizzo *et al.*, 1981). Thus the clearance and binding data for lactoferrin lacking α1–3-linked fucose and the lack of competition when excess FucBSA is injected with lactoferrin eliminates the possibility that lactoferrin clearance is mediated by either α1–3- or α1–6-linked fucose. It is suggested that clearance of lactoferrin is mediated by a unique receptor, as is also true of the homologous iron-transport protein transferrin (Hamilton *et al.*, 1979; Wada *et al.*, 1979).

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