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Visualization of lactotransferrin brush-border receptors by ligand-blotting

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The uptake of iron (III) mediated by lactotransferrin to human biopsies from upper intestine has suggested the presence of specific receptors for human lactotransferrin at the brush border (Cox, T., Mazurier, J., Spik, G., Montreuil, J. and Peters, T J. (1979) Biochim. Biophys. Acta 588, 120–128). In the present data, using ^{125}I-radiolabeled transferrins, we have demonstrated that a preparation of microvillous membrane vesicles, from rabbit jejunal brush-border specifically binds human lactotransferrin. This binding is specific, saturable and calcium dependent. Scatchard plots analysis of lactotransferrin binding indicates $1.5 \cdot 10^{13}$ sites per mg of membrane proteins with an equilibrium constant of $1.2 \cdot 10^{6}$ M^{-1} Sodium dodecyl sulfate solubilization of the brush-border proteins allows the lactotransferrin receptor to retain its binding activity. Moreover, the ligand blotting of the detergent solubilized membrane proteins on nitrocellulose sheet and after incubation with ^{125}I-labeled lactotransferrin, has shown that the receptor is a protein of about 100 kDa. In the same experimental conditions, the rabbit microvillous membrane vesicles do not specifically bind rabbit serotransferrin indicating the absence of serotransferrin receptors at the brush border.

Introduction

The absorption of inorganic iron is affected by its solubility within the intestinal lumen and the binding of iron to high molecular weight-compounds has proved to be effective in preventing its precipitation The presence of serotransferrin and lactotransferrin in the duodenal fluids and at the surface of the intestinal mucosa [1,2] suggests that these two glycoproteins are involved in the mechanism of iron absorption The process of iron absorption has long been recognized as a two step mechanism, namely mucosal uptake followed by mucosal transfer [3] An important function is attributed to serotransferrin in intracellular iron transport in the mucosal cells [4–6] but the involvement of serotransferrin in the mucosal uptake has not yet been clearly established

Huebers et al [4,6] have demonstrated that iron bound serotransferrin is absorbed, in vivo, by rat intestinal mucosa while Cox et al [7] have not observed iron uptake from serotransferrin by human duodenal biopsies

The data concerning the involvement of lactotransferrin in iron intestinal uptake appear also to be conflicting The relative efficient absorption of human breast milk iron [8,9] would tend to suggest that lactotransferrin possesses an important role in the iron transport to the mucosa [10,11] This hypothesis is corroborated by the work of De Vet et al [12] and of Van Vugt et al

Abbreviations Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, phosphate-buffered saline, 0 14 NaCl/0 01 M sodium phosphate (pH 7 4), EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid

[13] who have observed that, in pathological cases, an inverse relationship exists between body stores of iron and lactotransferrin concentration of the duodenal fluid More recently, Cox et al [7] and Yoshino et al [14] have presented data suggesting that lactotransferrin has selective capacity to deliver iron to duodenal mucosa Contrarily, De Laey et al [15] have demonstrated, in vitro, that lactotransferrin inhibits the iron absorption by intestinal mucosa, and Huebers et al [16] have not observed, in vivo, an uptake of the iron bound to lactotransferrin

Nevertheless, the initial event of iron absorption in the transfer of iron from diferric transferrins to the mucosal cells, is the binding of the glycoproteins to high-affinity surface receptors Up to now, little is known about the presence or absence of receptors for transferrins on intestinal mucosa We have therefore analysed the presence of lactotransferrin and serotransferrin receptors on the brush border from rabbit upper intestine

The findings reported in this paper demonstrate the binding of human lactotransferrin to brush-border vesicles isolated from rabbit upper intestine The presence of only one protein receptor of 100 kDa as indicated by the ligand technique clearly indicates the specificity of the binding of lactotransferrin to the enterocyte

On the contrary, our data do not conclude to the presence in our homologous system (rabbit serotransferrin-rabbit intestinal membrane vesicles) of a serotransferrin brush-border receptor

Material and Methods

Material Purified iron-saturated and iron-free human lactotransferrin [17,18] and iron-free rabbit serotransferrin were prepared as described before [19] Human serotransferrin was purchased from Behring (Marburg, F R G), bovine serum albumin (transferrin-free), leucine aminopeptidase, L-leucine *p*-nitroanilide, hen ovalbumin were from Sigma Chemical Co (St Louis, U S A) Iodo-Gen and Surfasil were purchased from Pierce Chemical Co (Rotterdam, Holland), Nonidet P-40 from BDH (Pool, U K) and the nitrocellulose membranes BA85 from Schleicher and Schull (Dassel, F R G) Cellulose membrane filters (0 45 μm) were provided from Millipore (Bedford, U S A), Nunclon culture dishes from Nunc (Rodkilde, Denmark) and propylene centrifugation tubes from Beckman (Irvine, U S A) [^{125}I]Iodine (IMS 30) was obtained from Amersham International (Amersham, U K), the radioactivity was determined by gammaspectrometry using an Autologic counter (Abbot Laboratories Chicago, U S A)

Protein iodination Proteins were labeled with [^{125}I]iodine to give an average maximum of one iodine atom per protein molecule Iodo-Gen catalyzed reaction was carried out for 20 min in a 0 1 M sodium phosphate buffer (pH 7 4) at 4°C Glass vials (5 mm of diameter) were coated with 0 1 ml of a 1% solution of Iodo-Gen in chloroform Efficiency of labeling was mainly about 80% The removal of unbound [^{125}I]iodine was carried out by gel filtration on Sephadex G-25 column, equilibrated in 0 214 M NaCl or in the incubation buffer Iodinated proteins were used within 2 weeks to avoid polymerization during storage

Non-specific binding of human lactotransferrin to synthetic polymers Synthetic polymers (cellulose filters, polypropylene centrifuge tubes or nunclon culture dishes) were incubated with solutions of ^{125}I-labeled human lactotransferrin, at a concentration ranging from 1 to 100 μg per ml in phosphate-buffered saline or in 0 010 M Tris-Hepes/0 05 M NaCl/0 05 M mannitol/0 001 M glucose buffer (pH 7 3) After 20 min incubation, the synthetic polymers were washed five times with phosphate-buffered saline, cellulose filters and centrifuge tubes were then directly counted The ^{125}I-labeled human lactotransferrin bound to culture dishes was extracted overnight by 1 M NaOH

Binding of bovine serum albumin to human lactotransferrin Culture dishes (24 mm of diameter) were coated overnight with 0 5 ml solution of human lactotransferrin at a concentration of 2 mg per ml After washing five times with 1 ml of phosphate-buffered saline, the dishes were incubated with 0 3 ml of a solution of ^{125}I-labeled bovine serum albumin at a concentration ranging from 0 003 to 0 5 mg per ml After 20 min incubation, the dishes were washed five times and the complex formed by the ^{125}I-labeled bovine serum albumin bound to lactotransferrin was desorbed from the plastic support by an overnight 1 M NaOH treatment

Membrane vesicles preparation Rabbit brush-border membrane vesicles were prepared according to Kessler et al [20] after Ca^{2+} precipitation Briefly, the mucosal scrapping of the proximal one third part of the small intestine of three rabbits was suspended in a 0 002 M Tris-Hepes/ 0 050 M mannitol buffer (pH 7 3) After 4 min homogenization with a Waring blender at maximum speed, solid $CaCl_2$ was added at a final concentration of 0 010 M After 15 min standing in the cold, the suspension was spun down at $3000 \times g$ for 15 min The remaining supernatant was homogenized in a Potter-Elvehjem and was then spun down at $27000 \times g$ for 30 min The pellet was resuspended in a 0 010 M Tris-Hepes/ 0 050 M NaCl/0 05 M mannitol/0 001 M glucose buffer (pH7 3) and twice centrifuged again at $27000 \times g$ The purification was monitored by measuring the protein content, the leucine aminopeptidase and the $(Na^+ + K^+)$-ATPase activity The vesicle suspension was immediately used or frozen and stored at $-70°C$ until use

Electron microscopy Vesicle suspensions were fixed for 30 min at 4°C in 25% (v/v) glutaraldehyde in 0 1 M sodium cacodylate buffer (pH 7 4) They were then washed in cacodylate buffer and post-fixed for 2 h with osmium tetroxide in the same buffer The samples were then embedded in Epon The thin sections were viewed under a JEOL 120CX electron microscope

Binding assays Aliquots of membrane vesicles (0 040 ml) were mixed with 0 020 ml of part A (labeled protein solution) and 0 080 ml of part B in order to have after reconstitution the following composition 0 010 M Tris-Hepes/0 050 M NaCl/0 05 M mannitol/0 001 M glucose (pH 7 3) Part B of the medium mixture was used to include competing agents such as fetuin, bovine serum albumin and different transferrins The reaction mixture was made up in cellulose propionate tube centrifugation (5 × 20 mm) which have been previously coated with siliconizing agent to prevent adsorption of transferrins on the tube All incubations were performed at 37°C The reaction was terminated by adding 0 2 ml of cold saline and the tubes were immediately centrifuged for 3 min at $120000 \times g$ The resulting membrane pellets were washed three times by resuspension in 0 214 M NaCl The radioactivity of the membrane pellets and of 0 050 ml of the first supernatant was then counted The non-specific binding was determined by incubation in presence of a 100-fold molar excess of unlabeled lacto or serotransferrin

Solubilization and analysis of microvillous membranes Microvillous membrane suspensions were solubilized at 4°C for 3 h by adding a solution of SDS 5% in order to obtain a final detergent concentration of 1% and a protein concentration of 0 1% The electrophoresis was run on a gel having the following characteristics [21] length of the running gel, 10 cm, thickness, 1 mm, acrylamide concentration, 7 5%, buffer, 0 050 M Tris/0 38 M glycine/0 1% SDS Gels were calibrated with standards of known molecular weight

Electrophoretic transfer of proteins to nitrocellulose Protein transfer was performed overnight, at 4°C, from SDS slab gels to nitrocellulose sheet, according to Towbin et al [22] and Burnette [23], in a 0 02 M Tris/0 15 M glycine/20% methanol buffer at 0 3 A The lanes containing the standards were cut off and stained with Amido black and the lanes containing the membrane proteins were incubated with ^{125}I-labeled transferrins

Incubation of nitrocellulose strips with transferrins Each strip of nitrocellulose paper was soaked with 10 ml of a 0 010 M Tris-HCl/0 15 M NaCl buffer (pH 7 4) containing either 50 mg/ml of bovine serum albumin (transferrin-free) to visualize the serotransferrin receptor After a stay of 12 h at 4°C, the buffer was replaced by 10 ml of fresh buffer containing ^{125}I-labeled lacto or serotransferrin at the concentration of $12\ 10^{-9}$ M After overnight incubation at 4°C, the nitrocellulose strips were washed twice with a solution of 0 010 M Tris-HCl/0 15 M NaCl (pH 7 4), twice with the same buffer containing Nonidet P-40 at a concentration of 0 05% and once with the buffer alone

Revelation was done by autoradiography carried out by exposing Kodak X-O-mat film to the nitrocelulose for 3 to 5 days

Results and Discussion

Interaction of lactotransferrin with plastic surfaces and with bovine serum albumin

During the study of the interaction of transferrin with membrane receptors, it was found that lactotransferrin binds avidly to plastic surfaces

Further investigations revealed that this binding was not inhibited, as expected, by addition of bovine or human serum albumin, in view of these problems, the binding constant affinity of lactotransferrin to plastic surfaces and to bovine serum albumin was determined

The binding of human lactotransferrin to cellulose ester, cellulose propionate polypropylene and nunclon plastic surfaces was assessed for concentrations of protein ranging from 1 to 100 μg per ml The results obtained show that the binding is saturable and easily interpretable in terms of Scatchard plots The binding constant K_a presents value (Table I) of the same magnitude (10^6 M^{-1}) for each plastic surface and is identical to those determined for the binding of serotransferrin to polypropylene [24] However, the number of binding sites of human lactotransferrin to polypropylene was 100-fold higher than for serotransferrin [24] The binding of lactotransferrin to plastic surfaces was decreased to 5% after coating with siliconizing agent Usually, the binding of proteins to plastic surfaces is inhibited by addition of bovine serum albumin at a concentration of 1% in the incubation medium [25] However, as it can be seen from Table I, in the case of lactotransferrin, addition of bovine serum albumin produced a 2 6-fold increase of the binding parameters These results suggest the existence of interactions between lactotransferrin and bovine serum albumin

The binding of serum albumin to immobilized lactotransferrin is found to be saturable the binding affinity constant and the number of sites which have been measured according to Scatchard [26]

TABLE I

BINDING PARAMETERS OF HUMAN LACTOTRANSFERRIN TO DIFFERENT PLASTIC SURFACES

Plastic surfaces	K_a (M^{-1})	Number of sites per mm^2
Cellulose ester filter	1 00 10^5	3 82 10^{11}
Polypropylene centrifuge tubes	1 50 10^6	6 42 10^{11}
Nunclon culture dishes [a]	2 00 10^6	2 10 10^{11}
Nunclon culture dishes [b]	5 20 10^6	5 00 10^{11}

[a] The incubation was performed without bovine serum albumin

[b] The incubation was performed in presence of 1% bovine serum albumin

are, respectively, $K_a = 0\ 37\ \ 10^7\ M^{-1}$ and $n = 15\ 10^{16}\ mm^{-2}$

The findings reported herein demonstrate that lactotransferrin binds strongly to plastic surfaces and forms with bovine serum albumin a complex which leads to an increase of the background and therefore to a substantial misestimation of the binding parameters (K_a and n) of lactotransferrin to the cells [27]

Characterization of rabbit brush-border membrane vesicles

The duodenal brush border membrane vesicles preparations obtained, were essentially free from containing organelles as judged by electron microscopy (Fig 1) The vesicles were exclusively right-side-out and predominantly filled with core proteins The different preparations used in this

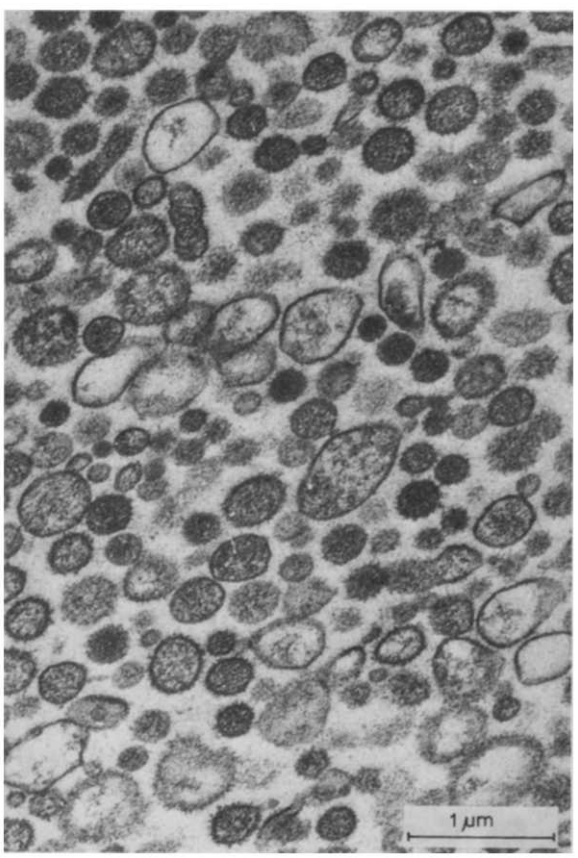

Fig 1 Electron micrograph of the isolated brush-border vesicles Magnification ×28000

study were characterized routinely by assessment of the increase of leucine aminopeptidase activity The final vesicle suspension ($n = 3$) contained 2 75 $\pm$ 5% of the homogenate proteins ($m \pm$ S E) and 40% of the leucine aminopeptidase activity corresponding to an enrichment relative to the whole homogenate of 20 The $(Na^+ + K^+)$-ATPase activity, determined as a marker of the basolateral membranes was not enriched

Transferrins binding to brush-border membrane vesicles

Serotransferrin binding Binding of ^{125}I-labeled rabbit serotransferrin to aliquots of brush-border vesicles was investigated at protein concentrations ranging from 1 to 80 μg per ml The binding, as it is shown in Fig 2A for the iron-saturated rabbit serotransferrin was not significantly different from the non-specific binding determined in presence of a 100-fold excess of unlabeled serotransferrin In the conditions used, these results demonstrate the absence of serotransferrin receptors at the surface of the rabbit brush-border vesicles

Lactotransferrin binding Binding of the ^{125}I-labeled human lactotransferrin was investigated at protein concentration ranging from 1 to 80 μg per ml An investigation time of 20 min was chosen, as a half maximal binding was observed at time less than 5 min The results shown in Fig 2B with iron-saturated human lactotransferrin, indicate that saturation of microvillous membrane lactotransferrin binding sites occurred with protein concentrations higher than 20 μg

Expression of these data in the form of Scatchard plots allowed, after subtraction of the non-specific binding as determined in presence of 100-fold excess of unlabeled lactotransferrin, the binding constant affinities and the number of receptor binding sites for human lactotransferrin to be estimated Equilibrium association constant of $K_a = 0\,56\ \ 10^6\ M^{-1}$ with $n = 1\,1\ \ 10^{13}$ binding sites per mg of membrane proteins were determined by linear estimates (correlation coefficient 0 91) The values of the binding parameters obtained for iron-free lactotransferrin were not statistically different

In order to decrease the high background and to demonstrate the specificity of the binding of lactotransferrin to vesicles, the binding was performed in presence of human serotransferrin, calf fetuin, hen ovalbumin and serum bovine albumin

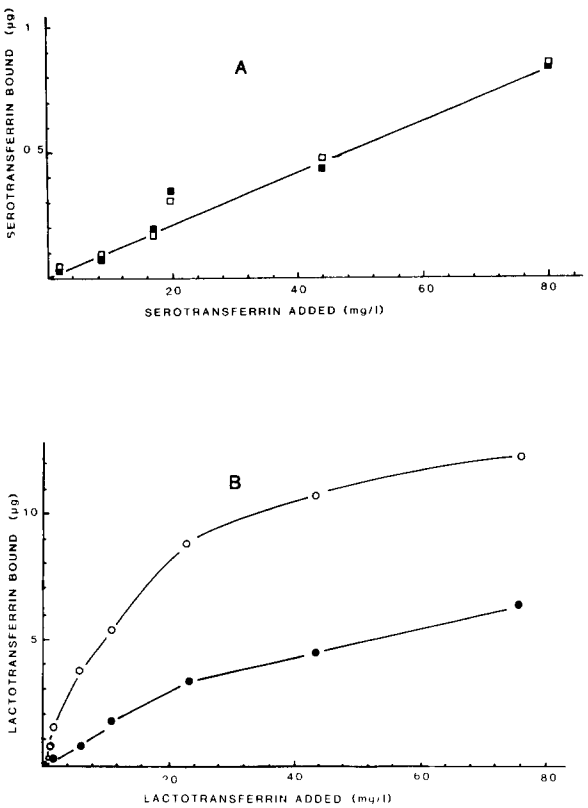

Fig 2 Binding of rabbit serotransferrin (A) and human lactotransferrin (B) to microvillous membrane vesicles The receptor-mediated binding was analyzed as a function of concentration of radiolabeled protein Reaction mixtures containing 120 μg microvillous membrane proteins and varying amount of ^{125}I-labeled transferrin (specific activities were about 30 10^3 cpm/μg protein) serotransferrin (□——□), lactotransferrin (○——○) were incubated as described in Materials and Methods Non-specific binding (■——■ ●——●) was measured by adding a 100-fold excess of unlabeled ligand to the assay mixture before addition of the membrane vesicles

Competitive binding assays were realized by incubating microvillous membrane preparations for 20 min at 37°C with increasing concentrations of ^{125}I-labeled lactotransferrin in presence of the competing proteins at the concentration of 1% In the range of lactotransferrin concentrations used (1 to 80 μg per ml), no significant inhibition was produced by serotransferrin, calf fetuin or hen ovalbumin (Table II) demonstrating the specificity

of the binding The binding affinity measured was about 1 2 10^6 M^{-1}, this value is consistent with the concentration of lactotransferrin (1 6 mg/ml) which is present in the rabbit bile [13] Contrarily, the presence of bovine serum albumin in the incubation medium, produced an increase of the apparent association constant to $K_1 = 5\,6\ 10^7$ M^{-1} and a 5-fold decrease of the number of binding sites of lactotransferrin to brush-border vesicles These modifications of the binding parameters cannot be interpreted in terms of saturation of non specific binding sites since they have not been observed in presence of serotransferrin, hen ovalbumin or calf fetuin They are more probably the reflect of the interactions between human lactotransferrin and bovine serum albumin as described above

Comparable studies of lactotransferrin binding to brush-border membranes have not been reported, but binding affinities of lactotransferrin to membrane receptors of macrophages, $K_1 = 0\,6$ 10^6 M^{-1} and $K_a = 0\,58\ 10^6$ M^{-1} [27,28] and of mononuclear cells, $K_a = 0\,7\ 10^6$ M^{-1} [29] are of the same order of magnitude The number of binding sites of human lactotransferrin to rabbit brush-border vesicles that we have to express per mg of membrane proteins $n = (1\,1–1\,5)\ 10^{13}$ per mg, cannot be compared to the values given in the literature which are expressed as number of sites per cell However, the number of lactotransferrin binding sites calculated is similar to the number of $n = 2\,5\ 10^{13}$ per mg binding sites of serotransferrin to placenta vesicles [30]

Ligand blotting of lacto- and serotransferrin receptors from proteins membranes of rabbit brush border

The rabbit brush-border vesicles were solubilized, subjected to 7 5% slab gel electrophoresis containing 0 1% SDS, and the proteins were electrophoretically transferred to nitrocellulose paper The lanes A and B of Fig 3 show an Amido black stain of the proteins in the SDS gel (Lane A) and of their transfert on nitrocellulose paper (Lane B) As it can be seen, the proteins of molecular mass lower than 100 kDa were quantitatively blotted

Conditions have been found in which lacto- and serotransferrins do not adhere non specifically to nitrocellulose paper and therefore do not cause a high background on the nitrocellulose strips More particularly, all the experiments with human lactotransferrin were performed in presence of 5% human serotransferrin one of the proteins which does not interact with human lactotransferrin (Table II)

Lane E of Fig 3 identifies the receptor-bound ^{125}I-labeled lactotransferrin as visualized by autoradiography

The ligand blotting technic has been successful since the lactotransferrin receptor retains its abil-

TABLE II

BINDING PARAMETERS OF HUMAN LACTOTRANSFERRIN TO RABBIT BRUSH-BORDER VESICLES

Competing proteins	K_a (M^{-1})	Number of sites per mg of protein
None	0 56 10^6	1 10 10^{13}
Human serotransferrin	1 20 10^6	1 30 10^{13}
Calf fetuin	1 20 10^6	1 40 10^{13}
Hen ovalbumin	1 20 10^6	1 50 10^{13}
Bovine serum albumin	5 30 10^7	2 19 10^{12}

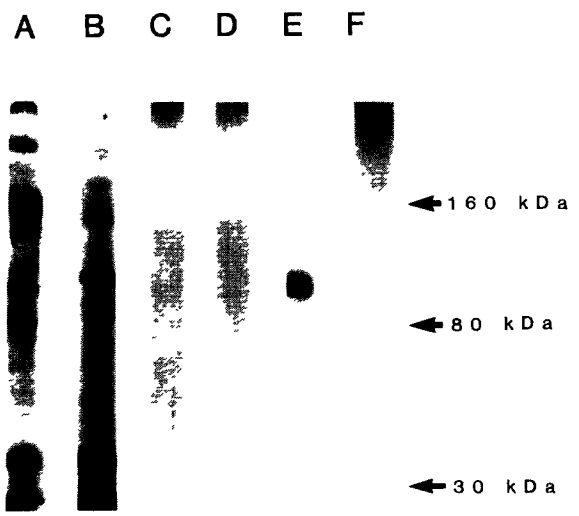

Fig 3 Ligand blotting of transferrin receptors from rabbit brush border The rabbit brush-border vesicles (30 μg) were submitted to electrophoresis in 7 5% slab gel-containing 0 1% SDS The gel strip in lane A and its electrophoretic transfer on nitrocellulose paper in lane B were stained with Amido black The nitrocellulose strips were incubated with one of the following compounds lane C, 12 10^{-9} M ^{125}I-labeled rabbit serotransferrin, lane D, 12 10^{-9} M ^{125}I-labeled human serotransferrin, lane E, 12 10^{-9} M ^{125}I-labeled human lactotransferrin, lane F, 12 10^{-9} M ^{125}I-labeled human lactotransferrin in presence of 1 10^{-3} M EGTA, as described in Materials and Methods

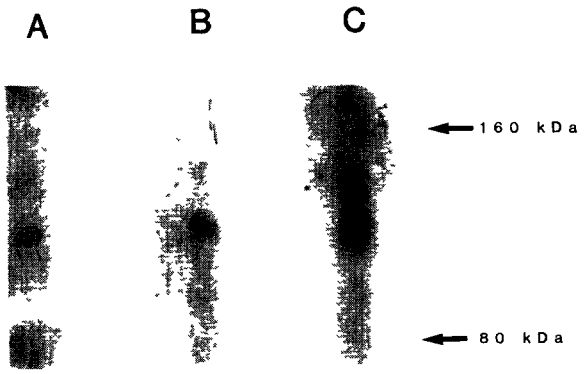

Fig 4 Ligand blotting of human lactotransferrin receptors from rabbit brush border Effect of varying concentrations of Ca^{2+} ions The experimental conditions are described in Fig 2 The nitrocellulose strips were incubated with 12 10^{-9} M ^{125}I-labeled human lactotransferrin in the absence (lane A) and in the presence of 3 mM (lane B) and 5 mM (lane C) $CaCl_2$

ity to bind lactotransferrin following SDS treatment and since the rate of dissociation of the complex permits extensive washing without removal of the ligand The ligand technic blotting can be widely used to identify the lactotransferrin receptors for which no antibody is available at the moment

Binding was markedly inhibited when EGTA was included in the incubation medium at a concentration of 1 mM (Lane F), suggesting that divalent ions are required for the binding-activity To test this hypothesis, the nitrocellulose strips were treated with ^{125}I-labeled lactotransferrin and $CaCl_2$ at concentration ranging from 0 to 5 mM By visual inspection of Fig 4, an increase of the receptor-bound lactotransferrin was apparent

The electrophoretic mobility of the lactotransferrin receptor on 0 1% SDS slab gel (Fig 3) was the same as expected for a protein of 100 kDa, in absence of reducing agent

Conclusion

The results reported in this paper indicate, in an homologous system, the absence of rabbit serotransferrin binding to brush-border vesicles isolated from rabbit upper intestine These results corroborate those reported previously by Marx et al [31] who have demonstrated the absence of binding of monoclonal antibodies (OKT_9 and B83/25) against human serotransferrin receptor at the surface of the human brush-border membranes Our results do not apparently support the interpretations of Huebers et al [6] who have observed, in rats and in vivo, an adsorption of iron bound to serotransferrin and have therefore proposed a mechanism for intestinal iron absorption by which the complex of iron and serotransferrin is taken up by the enterocytes

A species difference in the role of sero- and lactotransferrin or the fact that our study has used human lactotransferrin and rabbit membrane vesicles could explain these contradictory results An other possibility is the involvement of a non-receptor mediated uptake of serotransferrin by the enterocytes Nevertheless the involvement of the mononuclear cells in iron absorption through the macrophage-monocyte system [32,33] and the presence of receptors for both lactotransferrin and serotransferrin at the surface of macrophage and monocyte [27,29,34] highly suggest that these two iron binding glycoproteins could play a role in the iron absorption

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