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Recombinant Human Lactoferrin and Iron Transport Across Caco-2 Monolayers: Effect of Heat Treatment on the Binding to Cells

CELIA CONESA, COLOMA POCOVÍ, MARÍA-DOLORES PÉREZ, MIGUEL CALVO, AND LOURDES SÁNCHEZ*

Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain

Recombinant human lactoferrin (rhLF) from *Aspergillus awamori* bound to Caco-2 cell membranes in a saturable manner. The dissociation constant for the apo form was (K_d) = 2.2×10^{-7} M; however, the specific binding of the iron-saturated rhLF and of lactoferrin from human milk (hLF) was too low to calculate the binding parameters. Recombinant human lactoferrin subjected to heat treatment did not lose the ability to bind to cell membranes except at high temperature and long time treatments (85 and 89 °C for 40 min) for which there was a slight decrease in the binding. No significant differences have been found in the transport of iron bound to rhLF or to hLF across Caco-2 cell monolayers. Nevertheless, the amount of iron-saturated hLF transported across Caco-2 monolayers was significantly higher than that of rhLF. For both lactoferrins, the amount of intact protein in the lower chamber was about 4.5% of the total radioactivity transported, indicating the degradation of lactoferrin in the passage across Caco-2 cells.

KEYWORDS: Recombinant human lactoferrin; heat treatment, Caco-2 cells; cell binding; iron transport.

INTRODUCTION

Lactoferrin is an iron-binding glycoprotein found in different physiological fluids of mammals such as milk, tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervicovaginal, and seminal fluid (*1*). Furthermore, lactoferrin is also present in circulating polymorphonuclear neutrophils (PMNs) (*1*). Many biological activities have been attributed to lactoferrin, including protection from pathogens, regulation of iron absorption, immune system modulation, cellular growth promoting activity (*2*) and antitumoral activity (*3*).

The presence of a high concentration of lactoferrin in human milk along the whole lactation, and the observation of a greater iron bioavailability from human than from bovine milk suggests that lactoferrin might promote iron absorption in breast-fed infants (*4*). However, whether lactoferrin increases the iron absorption in the gut is still controversial. In fact, there are studies in rats which have shown that a diet with lactoferrin as a supplement improves their iron status (*5*) while there are other works in which a clear effect of lactoferrin increasing iron absorption has not been found or even a negative effect has been observed (*6, 7*). If lactoferrin plays a role in iron absorption, a receptor would be expected to exist for this protein in the cells of the intestinal mucosa. It has been reported that lactoferrin does not interact with transferrin receptor (*8*) and some authors have characterized a receptor for lactoferrin in different types of cells such as epithelial intestinal cells (*9–11*), immune system cells, and pulmonary, hepatic, renal, and cardiac cells (*10*). However, the existence of a universal receptor is not clear due to the heterogeneity found in the characteristics of the different receptors reported. Moreover, it has been observed that the binding of lactoferrin to the cellular membranes of hepatocytes or monocytes is lower in the presence of other basic proteins (*12, 13*) which could indicate an important contribution of the highly basic charge of lactoferrin to its cell binding.

Lactoferrin levels are high in human milk throughout the whole lactation (*4*), which does not occur in bovine milk whose lactoferrin levels decrease in the first days of lactation (*14*). Hence, infant formulas made from bovine milk are practically devoid of lactoferrin. In some countries, as Japan, milk-formula manufacturers add bovine lactoferrin to infant formulas since human lactoferrin is not available for industrial scale. However, whether lactoferrin from species different than human exerts its activity in a new-born is still not well-known. Several expression systems have been used to produce recombinant human lactoferrin (rhLF) including transgenic cows, fungal cultures, and plants such as rice (*15*).

In this work, we have used rhLF from *Aspergillus awamori* kindly provided by Agennix (USA) to study some of its biological activities. We have reported in a previous work (*16*) that the behavior of this recombinant lactoferrin is similar to that of lactoferrin from human milk when subjected to calorimetry which reflects the high degree of structural similarity. However, the two lactoferrins differ in their glycosylation (*17, 18*).

* Author to whom correspondence should be addressed. Telephone: 34 976 761585. Fax: 34 976 761612. E-mail: lousanchez@unizar.es.

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In this work, we have compared the binding of human-milk and recombinant human lactoferrins to membranes of differentiated Caco-2 cells and we have also examined the effect of different heat treatments on this binding. We have also studied the passage of iron bound to these proteins and the passage of lactoferrin itself through a monolayer of differentiated Caco-2 grown in bicameral chambers.

MATERIALS AND METHODS

Cell Culture. Human colon carcinoma Caco-2 cells TC7 clone were kindly provided by the Department of Physiology from the Veterinary Faculty of the University of Zaragoza (Zaragoza, Spain). Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL Penicillin, 100 μg/mL Streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel), 25 mM glucose and 2.5% Hepes (Sigma, Poole, UK). Cells were normally grown in 25 cm^2 tissue culture flasks to confluence and maintained at 37 °C in 5% CO_2.

For the receptor binding assays, cells were seeded into plastic tissue culture dishes of 35 mm diameter at a density of 10^5 cells/cm^2 and incubated for 15 days to obtain differentiated Caco-2 cells.

For the transport studies, cells were seeded into Transwell bicameral chambers (Costar, Hygh Wycombe, UK) of 3.0 μm pore size and 6.5 mm diameter at a density of 10^5 cells/cm^2. The well contained 200 μL of medium in the upper chamber and 800 μL in the lower chamber. The polycarbonate membrane of the inserts had been previously coated with rat-tail collagen type I (Boehringer, Mannheim, Germany) as follows. A volume of 50 μL of a 2 mg/mL solution of collagen in 0.1 M acetic acid was added to each chamber and, after removing the excess, left to dry in sterile conditions before seeding the cells. Confluent cultures of differentiated cells were obtained after 21 days. Monolayer integrity was checked by measuring transepithelial electrical resistance (TEER) with an epithelial voltohmeter (World Precision Instruments, New Haven, CT) and by phenol red exclusion (*19*).

Caco-2 Cell Membranes Binding Assays. *Biotinylation of Lactoferrin.* Recombinant human lactoferrin (rhLF) was kindly provided by Agennix (Houston, TX). Human lactoferrin (hLF) was isolated from milk by Blue Cibacron chromatography (*16*) and purity checked by SDS–polyacrylamide gel electroforesis. Biotinylation of the apo- and iron-saturated forms of rhLF and hLF was performed as described by Rejman et al. (*20*). Fifty molar excess of *N*-hydroxysuccinimide-biotin (NHS-biotin; Sigma, Poole, UK) which was dissolved in dimethyl sulfoxide was added to lactoferrin at a concentration of 2 mg/mL in phosphate-buffered saline (PBS) composed by 140 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4. The reaction was allowed to proceed on ice for 2 h. Unreacted NHS-biotin was removed by dialysis against PBS for 36 h, doing three changes of buffer. To confirm that biotinylation of rhLF and hLF (B-rhLF and B-hLF, respectively) was correctly done, a transfer blotting of the proteins was performed after SDS-PAGE, followed by detection with peroxidase conjugated to streptavidin and revealed with 3-amino-9-ethilcarbazol (AEC) substrate (DakoCytomation, Carpinteria, CA).

Caco-2 Cell Membrane Preparation. Confluent monolayers of 15-day differentiated Caco-2 cells were washed, removing incubation media, using two 5 mL rinses of PBS, and the rinses were discarded. The cells were harvested into 1 mL of PBS using a rubber policeman and the plate rinsed with 5 mL of PBS. The cell suspension and wash were combined, placed on ice, pelleted at 1000*g* for 10 min at 4 °C and resuspended in 2.5 mL of cold membrane buffer composed by 25 mM Tris, 1 mM $CaCl_2$, 0.02% sodium azide (w/v), and 0.25 M sucrose, pH 7.2. The cell suspension was sonicated on ice on an output setting of 30 and a 5 min treatment/pause technique using a Branson Sonifier Model 450 (Branson Ultrasonics, Danbury, CT). The resulting membranes were pelleted at 30 000*g* for 30 min at 4 °C and washed twice in cold membrane buffer without sucrose. The final pellet was resuspended in 1 mL of cold membrane buffer without sucrose and the protein concentration determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Caco-2 Cell Membrane Binding Assays. Microtiter plates were coated with 200 ng of membrane protein per well, except two columns which were coated with standars of B-rhLF or B-hLF (1–25 ng/100 μL) and incubated overnight at 4 °C. The plates were washed three times with washing buffer consisting of 100 mM Tris, 140 mM NaCl, 0.05% Tween 20 (v/v), pH 7.4. Afterwards, plates were blocked with 200 μL of 1.0% bovine serum albumin (BSA) in blocking buffer composed by 100 mM Tris, 140 mM NaCl, pH 7.4, for 1 h at 37 °C to reduce background binding to the plastic. After four washes, 100 μL of diluted labeled or unlabeled ligands in binding buffer, 100 mM Tris, 140 mM NaCl, 0.1% BSA, 0.01% Tween 20, pH 7.4, were added to each well and incubated for 4 h at 4 °C. Unbound ligand was removed by rinsing the plates four times with washing buffer and then 100 μL of the avidin/biotin–horseradish peroxidase complex, kit ABC (Pierce, Rockford, IL) was added to each well and incubated for 1 h at room temperature. After four washes, 100 μL of tetramethylbenzidine (TMB) substrate (ZEU-Inmunotec, Zaragoza, Spain) was added to each well and incubated 20 min at room temperature in darkness. The absorbance was read at 405 nm in a Labsystems Multiskan MS microtiter photometer (Helsinki, Finland).

Binding of lactoferrin to Caco-2 cell membranes was determined by incubating increasing concentrations of B-rhLF or B-hLF (0–2.5 μM) in the absence and presence of 20-fold excess of unlabeled lactoferrin. The dissociation constant (K_d) was determined following the method of Scatchard for those assays in which the experimental points adjusted linearly. To determine lactoferrin competitive binding activity, a constant concentration of B-LF (0.4 μM) was incubated with membranes in the presence of increasing concentrations of unlabeled lactoferrin or apo-human transferrin (apo-hTF) (0–6 μM).

Competition among different proteins for the binding to Caco-2 membranes was carried out by using rhLF or hLF iron-saturated and biotinylated (B-Fe-rhLF and B-Fe-hLF, respectively) in a constant concentration (0.4 μM) and incubated with membranes in the presence of increasing concentrations (0–6 μM) of unlabeled apo-rhLF, Fe-rhLF, apo-hLF, Fe-hLF, apo-bovine lactoferrin (apo-bLF) (Fina Research, Seneffe, Belgium), apo-hTF, lysozyme, or lactoperoxidase.

The effect of heat treatment on the ability of recombinant human lactoferrin to bind to Caco-2 cell membranes was also assayed. Lactoferrin samples were treated using the capillary method. A volume of 20 μL of rhLF, at a concentration of 0.2 mg/mL in binding buffer, was introduced into glass capillary tubes (1.5–1.6 mm outer diameter, 1.1–1.2 mm inner diameter) which were sealed with a microflame and their hermeticity was checked by immersion in water. The capillaries were immersed in a temperature-controlled water bath at different temperatures and times (77, 81, 85, or 89 °C during 5, 10, 20, and 40 min). A constant concentration of biotinylated apo-recombinant human lactoferrin from *Aspergillus awamori* (B-apo-rhLF) (0.4 μM) was incubated with Caco-2 membranes coated to wells and with increasing concentrations (0–1 μM) of unlabeled heated apo-rhLF.

Iron Uptake and Transport Studies. Caco-2 monolayers grown in bicameral chambers were used for transport studies from 19 to 21 days of culture when they were differentiated and the monolayer showed integrity when tested by measuring TEER ($\approx$200 Ω cm^2) and phenol red exclusion (<1.5% per hour). After washing both upper and lower chambers with serum-free medium, solutions of recombinant human lactoferrin from *Aspergillus awamori* (rhLF) or of human lactoferrin from milk (hLF) previously radiolabeled were added to the upper chamber. For the experiments of iron transport, both proteins were previously iron saturated to 60% with [^{59}Fe] citrate (specific activity of 4.5 mCi/mg, Amersham Biosciences, Little Chalf-oril, UK) during 8 h and then unlabeled ferric nitriloacetate (FeNTA) was added to saturate completely the proteins during an overnight incubation. To eliminate free iron, the protein was applied to a desalting column, PD-10 Sephadex G-25 (Amersham Biosciences, Little Chalf-oril, UK) which was previously equilibrated with a phosphate buffer containing 1% BSA to avoid nonspecific binding of lactoferrin and washed with three column volumes.

To carry out the transport studies, the proteins were added to the upper chamber at a concentration of 50 μg/mL in serum-free medium. Transport of iron from Fe citrate was studied by adding an equivalent amount of [^{59}Fe] citrate without any protein. The lower chamber

contained culture medium with apo-human transferrin (1 mg/mL) as iron acceptor. The medium was removed from the lower chamber for analysis at 1, 5, and 24 h of incubation and replaced by fresh medium also containing apo-human transferrin. When time of incubation was over, the medium was taken from the upper chamber and the monolayer washed with Hank's balanced salt solution (Sigma, Poole, UK) mixing the washings with the previously removed upper medium. After fresh culture medium was added to the upper and lower chamber, TEER was measured in order to check the monolayer integrity. Radioactivity associated with the medium from the upper and lower chambers at the different times of incubation, and also associated with the inserts containing Caco-2 cell monolayer, was measured in a LKB Wallac 04 1282 Compugamma Universal Gammacounter (Turku, Finland). The experimental conditions used to study the transport of the proteins were the same as those used for the iron transport studies. RhLF and hLF were iodinated by the chloramine T method (*21*) and the free ^{125}I was removed by using chromatography in PD-10 Sephadex G-25 columns in the same conditions described above. Integrity of [^{125}I]-rhLF or [^{125}I]-hLF which had passed across Caco-2 monolayers was measured by precipitating 300 μL from the lower chamber medium with 10% (w/v) trichloroacetic acid, adding 25 μL of BSA at a concentration of 20 mg/mL and leaving the solution for 10 min at room temperature. After that, the solution was centrifuged at 10 000*g* for 10 min and radioactivity associated with the supernatant and the pellet determined.

RESULTS

Binding of Recombinant Human Lactoferrin and Human Milk Lactoferrin to Caco-2 Cell Membranes. The results of the binding assays to Caco-2 membranes showed specific and saturable binding of apo- and iron-saturated forms of both recombinant and human milk lactoferrin (**Figure 1**). Scatchard plot analysis was applied to data obtained for the specific binding of all the forms of lactoferrin, but we only found a good linear correlation for the apo-recombinant human lactoferrin. We obtained for this form of lactoferrin a dissociation constant of 2.2×10^{-7} M.

Competition curves for evaluation of binding of biotinylated iron-saturated recombinant human lactoferrin (B-Fe-rhLF) to Caco-2 cell membranes with increasing concentrations of different forms of lactoferrin or apo-human transferrin (apo-hTF) are shown in **Figure 2**. At a constant concentration of B-Fe-rhLF (0.4 μM), increasing concentrations of unlabeled recombinant human lactoferrin, human milk lactoferrin, and bovine milk lactoferrin, either iron-saturated or not, compete in the same manner with the iron-saturated recombinant lactoferrin for the binding to Caco-2 cell membranes. The same experiment was carried out with biotinylated apo-recombinant human lactoferrin, biotinylated iron-saturated human milk lactoferrin or biotinylated apo-human milk lactoferrin with the correspondent unlabeled protein and the results were very similar to those of **Figure 2** (data not shown). However, increasing concentrations of unlabeled apo-hTF did not decrease the binding of any of the biotinylated proteins.

Competition of lactoferrin with other cationic proteins for binding to Caco-2 membranes was also assayed. Curves of B-apo-rhLF with increasing concentrations of unlabeled lysozyme, lactoperoxidase, or apo-rhLF are shown in **Figure 3**. Lactoperoxidase markedly inhibited the binding of B-apo-rhLF and the inhibitory capacity is similar to that of unlabeled apo-rhLF, but this has not been observed with lysozyme.

The effect of heat treatment on the ability of recombinant human lactoferrin to bind to Caco-2 cell membranes was also studied and the results are shown in **Figure 4**. A constant concentration of B-apo-rhLF (0.4 μM) was incubated with the membranes and with increasing concentrations (0–1 μM) of unlabeled apo-rhLF heated at different temperatures and times

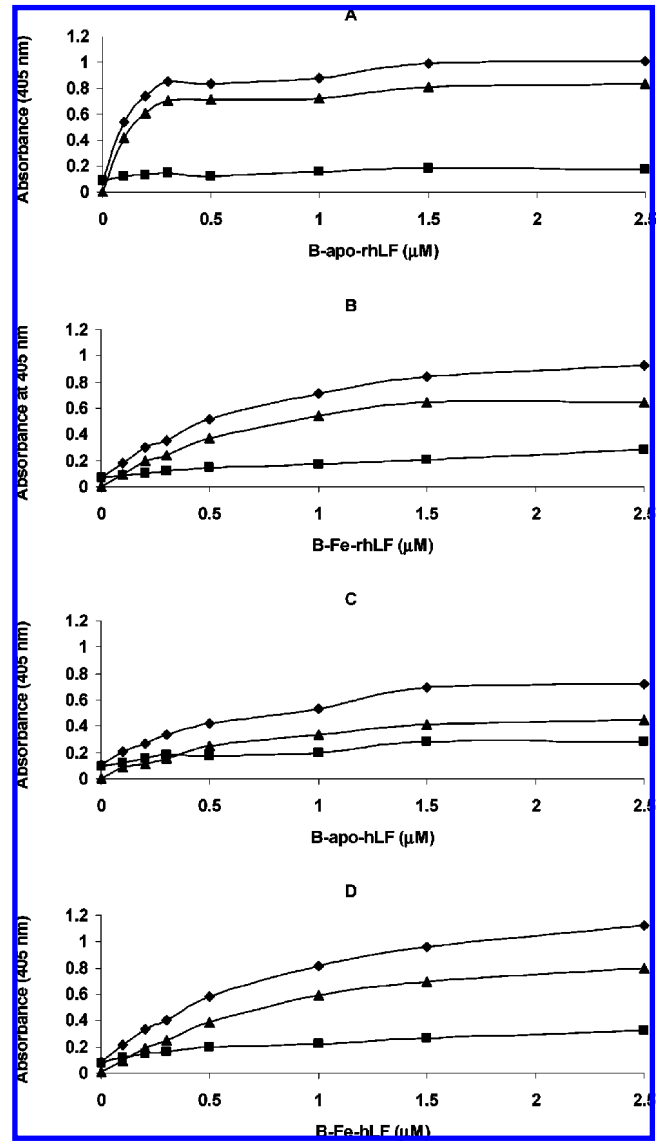

Figure 1. Specific binding (▲) of biotinylated apo-recombinant human lactoferrin (**A**), iron-saturated recombinant human lactoferrin (**B**), apo-human milk lactoferrin (**C**), and iron-saturated human milk lactoferrin (**D**) to differentiated Caco-2 cell membranes. Biotinylated lactoferrin (0–2.5 M) was incubated with Caco-2 cell membranes (200 ng of membrane protein per well) (total binding) (◆) or in the presence of 20-fold molar excess of unlabeled lactoferrin (nonspecific binding) (■). Specific binding was calculated as the difference between total binding and nonspecific binding.

(77, 81, 85, and 89 °C during 5, 10, 20, and 40 min). There was no effect of the heat treatments applied on the ability of the protein to bind to Caco-2 cell membranes, with the exception of the proteins subjected to the more severe treatments (85 and 89 °C for 40 min) as the apo-rhLF subjected to these treatments inhibited the binding of B-apo-rhLF 10–20% less than the nonheated protein

Transport Studies Across Caco-2 Monolayers. *Iron Transport Across Monolayers and Cellular Uptake.* Transfer of iron (^{59}Fe) across the monolayers was significantly greater from iron citrate than from iron-saturated rhLF or hLF ($p < 0.05$) (**Figure 5**, **Table 1**), and even for citrate the amount transported after 24 h was a small proportion, around 2%, of the total iron added. There were no significant differences in the transport of iron bound to recombinant human lactoferrin or to human milk lactoferrin. The proportion of iron associated with the cells was

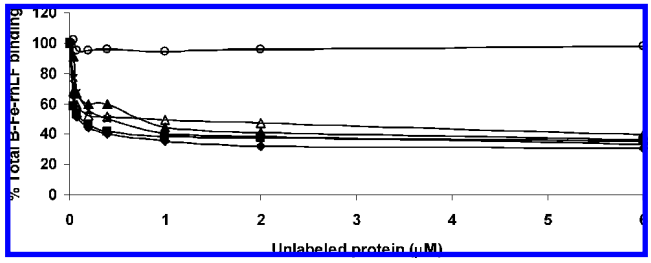

Figure 2. Competition curves on Caco-2 cell membranes. Biotinylated iron-saturated recombinant human lactoferrin (B-Fe-rhLF) (0.4 μM) was incubated with differentiated Caco-2 cell membranes (200 ng of membrane protein per well) in the presence of increasing concentrations of unlabeled iron-saturated recombinant human lactoferrin (◆) (Fe-rhLF), apo-recombinant human lactoferrin (■) (apo-rhLF), iron-saturated human lactoferrin (▲) (Fe-hLF), apo-human lactoferrin (×) (apo-hLF), apo-bovine lactoferrin (△) (apo-bLF) and apo-human transferrin (○) (apo-hTF) (0–6 μM).

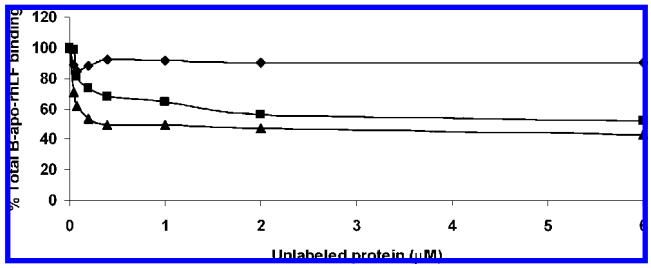

Figure 3. Competition curves on Caco-2 cell membranes. Biotinylated apo-recombinant human lactoferrin (B-apo-rhLF) (0.4 μM) was incubated with differentiated Caco-2 cell membranes (200 ng of membrane protein per well) in the presence of increasing concentrations of unlabeled lysozyme (◆), lactoperoxidase (■), or apo-recombinant human lactoferrin (▲) (apo-rhLF) (0–6 μM).

similar for the three carriers of iron. However, it has to be considered the great variability observed for the values of iron transport from citrate, compared to those of iron transport from any type of lactoferrin.

Lactoferrin Transport Across Monolayers. The passage of recombinant and human milk lactoferrin across Caco-2 monolayers was studied and it was found that the percentage of accumulated ^{125}I-activity associated with hLF in the lower chamber at 24 h was of 9.1%, significantly higher than that associated with rhLF which was of 2.1% (**Figure 6**). The amount of ^{125}I activity associated with the cells at the time of 24 h was 2.4% and 1.8% for recombinant human lactoferrin and for human milk lactoferrin, respectively. For both proteins, only about 4.5% of the radioactivity that had traversed the monolayer was TCA-precipitable which indicates that most of the protein was degraded in the passage across the cells. To check that the protein was not degraded by proteases released at the apical surface of the cells prior to transport, the integrity of lactoferrin in the upper compartment was also determined by TCA precipitation. It was confirmed that no degradation occurred over the incubation period, since all the protein in the upper compartment was intact at 1, 5, and 24 h of incubation. Furthermore, the amount of intact protein traversing the membrane was only about 0.09% and 0.34% of the total protein added to the upper chamber for human milk lactoferrin and for recombinant human lactoferrin, respectively.

DISCUSSION

Although it has been known for a long time that various types of cells can bind lactoferrin on their membranes, the nature of

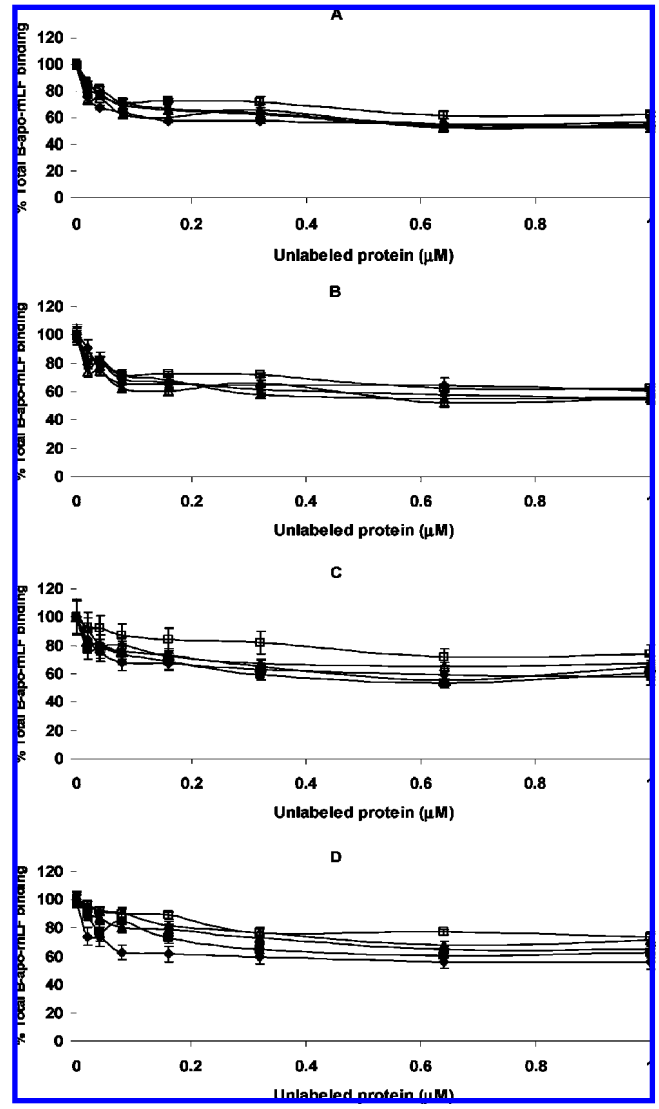

Figure 4. Competition curves on Caco-2 cell membranes. Biotinylated apo-recombinant human lactoferrin (0.4 μM) was incubated with differentiated Caco-2 cell membranes (200 ng of membrane protein) in the presence of increasing concentrations of unlabeled apo-recombinant human lactoferrin (apo-rhLF) (0–1 μM) nonheated (◆) and heated during 5 min (■), 10 min (▲), 20 min (△), and 40 min (□) at different temperatures (**A**) 77 °C, (**B**) 81 °C, (**C**) 85 °C, and (**D**) 89 °C. Each point represents the mean ± SD of four replicates from two different experiments.

the interaction and its biological consequences are still unclear (*22*). In this work, it is shown that recombinant human lactoferrin from *Aspergillus awamori* binds to Caco-2 cell membranes in a saturable manner, though the analysis by the Scatchard method of the experimental data obtained for the different forms of lactoferrin did not show the existence of a receptor with measurable affinity. Good linear correlation was only found for binding of apo-recombinant human lactoferrin to Caco-2 membranes, with a mean dissociation constant (K_d) of 2.2×10^{-7} M. This value is similar to those reported for the binding of iron-saturated recombinant human lactoferrin to the same cell line by Ward et al. (*18*) and Ashida et al. (*23*), who reported values of 1.8×10^{-7} and 1.6×10^{-7} M, respectively. In the work by Sánchez et al. (*19*), the binding of human milk lactoferrin to Caco-2 cells was also reported, though the data obtained did not support the existence of a receptor. Previous studies have shown the interaction of lactoferrin with small intestinal cells in homologous or heterologous systems in rabbit

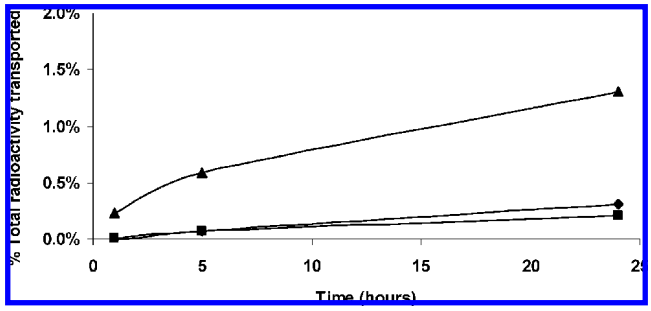

Figure 5. Iron transport across Caco-2 monolayers in bicameral chambers. Iron was added to the upper chamber as ^{59}Fe-recombinant human lactoferrin from *Aspergillus awamori* (◆), ^{59}Fe-human milk lactoferrin (■) or ^{59}Fe-citrate (▲). Values represent the percentage of total radioactivity transported to the lower chamber (mean of 18 replicates from six different experiments).

Table 1. Percentage of Total Radioactivity Associated with the Upper Chamber and with Cells and Transported to the Lower Chamber at 24 H for ^{59}Fe-Recombinant Human Lactoferrin from *Aspergillus awamori*, ^{59}Fe-Human Milk Lactoferrin, or ^{59}Fe Citrate[a]

	upper chamber	cell uptake	lower chamber
Fe-rhLF	94.89 ± 0.01	4.74 ± 0.01	0.37 ± 0.005
Fe-hLF	94.91 ± 0.01	4.80 ± 0.01	0.28 ± 0.005
Fe-citrate	91.49 ± 0.05[b]	6.37 ± 0.04	2.12 ± 0.03[b]

[a] Values are the mean ± SD of 18 replicates from six different experiments. [b] Significant differences for $p < 0.05$.

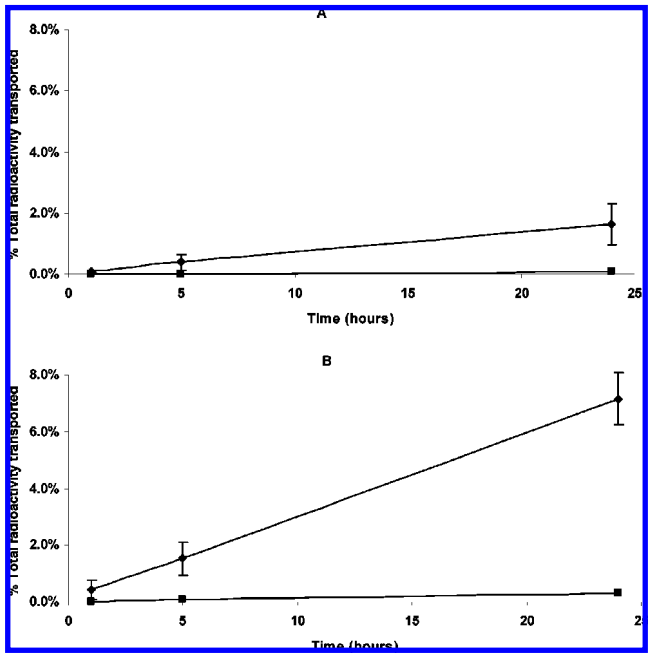

Figure 6. Passage of ^{125}I-recombinant human lactoferrin (**A**) or ^{125}I-human milk lactoferrin (**B**) across differentiated Caco-2 monolayers in bicameral chambers. Proteins (100% iron-saturated) were added at a concentration of 50 μg/mL to the upper chamber. Values represent the percentage of total radioactivity transferred to the lower chamber (◆) and the percentage corresponding to intact protein (■) (each point represents the mean ± SD of 9 replicates from three different experiments).

(*9*), mouse (*24*), monkey (*25*), human fetus (*26*), and piglet (*27*) and the values of affinity and dissociation constants were close to those obtained for the recombinant human lactoferrin (*18, 23*).

In spite of the great molecular similarities between lactoferrin and transferrin, our results show that transferrin is not able to inhibit the binding of lactoferrin to Caco-2 cell membranes. The same finding has been observed in other cell types such as mouse peritoneal cells (*28*), hepatocytes (*29*), MAC-T bovine mammary epithelial cells (*20*), or monocytes (*22*). The results of all these studies indicate clearly that lactoferrin does not bind to the well characterized transferrin receptor.

Lactoferrin from human milk, recombinant human lactoferrin from *Aspergillus awamori*, and lactoferrin from bovine milk in their apo- and iron-saturated forms had the same competition capacity for the binding to Caco-2 cell membranes. This observation indicates that the lactoferrin binding site in these human cells has no strict species specificity for the human and bovine proteins. The same fact has been observed in other cells such as those from mouse small intestinal brush border (*24*). However, although human and bovine lactoferrin bound to the human enterocyte-like cell line HT29–18-C1 following the same pattern, the mouse lactoferrin did not present the same behavior, this fact being attributed to differences in the basic residues of the N-terminal region (*30*). It has been also reported that bovine and human lactoferrin cannot compete with porcine lactoferrin in the binding to piglet small intestine cells (*27*). A similar finding has been shown for other cell lines such as the human promonocytic cell lines U937 (*31*) and THP-1 (*32*).

The existence of a putative receptor for lactoferrin in different kind of cells has been proposed, namely in intestinal epithelial cells (*9*)– (*11*), and cells from immune system, brain, lung, liver, kidney, heart, among others (*10*). However, data obtained from the characterization of the lactoferrin-binding molecules have revealed marked differences. Thus a single-chain lactoferrin-binding protein of 105 kDa has been reported in activated T cells, in platelets, and in breast carcinoma cells (*33*). On the other hand, two proteins of 35 kDa have been characterized in Jurkat human lymphoblastic T cells as the lactoferrin binding proteins (*34*), while the receptor reported in human fetal intestinal brush border membranes is composed of three subunits of 38 kDa (*26*). In human monocytes, a 43 kDa protein has been identified as the possible receptor for lactoferrin (*33*). Furthermore, some authors have characterized two classes of binding sites for lactoferrin (*20, 30, 32*) while in other cells only one type of binding site has been reported (*18, 23, 26*). This lack of homogeneity in the characteristics reported for the lactoferrin binding proteins in the membrane of different cells raises a question about the existence of a universal lactoferrin receptor.

Several reports show that the binding of lactoferrin to cells is based on electrostatic interactions, since it can be inhibited by other cationic proteins (*12, 13, 29, 31*). In the present work, we have studied the effect of some basic proteins, such as lactoperoxidase and lysozyme on the biding of lactoferrin to Caco-2 cell membranes. We have observed that lactoperoxidase markedly inhibited the binding of labeled recombinant human lactoferrin with a similar capacity as the same, unlabeled protein. However, lysozyme was not able to inhibit lactoferrin binding, in agreement with previously reported results (*29, 31*). In fact, it has been reported that only the dimerized form of lysozyme is able to inhibit the binding of lactoferrin (*12*). Since in the present work lysozyme was used in the monomer form, this may explain the lack of inhibition that produced on lactoferrin binding. Moreover, it has been reported that the removal of the basic cluster from the N-terminus of human lactoferrin causes the absence of binding of this protein to the human colon carcinoma cells HT29–18-C1, indicating the importance of electrostatic interactions (*30*). It has been also shown by El Yazidi-Belkoura et al. (*30*) that most of the human lactoferrin

binding sites on HT29–18-C1 cells contain anionic sulfate groups of glycosaminoglycans which could interact with the cationic lateral chains of lactoferrin.

The effect of heat on the structure of human lactoferrin from milk and from recombinant origin has been studied by calorimetry (*16, 35*). However, as recombinant lactoferrin has been proposed to be used as ingredient in some food products, it is necessary to evaluate the effect that heat may have on some of the properties related to its biological activities. In this work, we have studied the effect of heat treatment on the ability of apo-recombinant human lactoferrin to bind to Caco-2 cell membranes. We have observed that lactoferrin heated at different temperatures and times has not lost its ability to bind to cell membranes. However, at the higher heat treatments (85 and 89 °C for 40 min) a slight decrease, between 10 and 20%, was observed in the ability of competition respect to the nonheated lactoferrin. Therefore, from these results we can say that recombinant human lactoferrin from *Aspergillus awamori* would not lose its ability to bind to cell membranes in products subjected to heat treatments equivalent to those applied in this work. In a previous study, it was observed that bovine lactoferrin heated at 72 °C for 20 s, 85 °C for 20 min, or 137 °C for 8 s did not lose its ability to bind to the human promonocytic cell line U937 (*31*). Suzuki et al. (*36*) also found that recombinant human lactoferrin from rice heated at 62 °C for 15 min, 72 °C for 20 s, and 85 °C for 3 min did not lose its ability to bind to Caco-2 cells, although higher heat treatments such as 100 °C for 8 s did have an effect decreasing the protein binding.

We also studied the passage of iron bound to human milk lactoferrin and to recombinant human lactoferrin from *Aspergillus awamori* compared to iron citrate through a monolayer of differentiated Caco-2 cells grown in bicameral chambers. The passage of the lactoferrin molecule itself has been also studied. This system has been used as an in vitro model of the intestinal barrier to study the effect of different carrier molecules on mucosal iron transport (*19*). Differentiated Caco-2 monolayers grow polarized on the polycarbonate membranes of the upper chamber, expressing features of small intestinal cells, such as tight junctions between the cells and brush border microvilli on the apical zone as shown by transmission electron microscopy (*19*). Data obtained for iron (^{59}Fe) transport across Caco-2 cell monolayers did not show significant differences in the transport of iron bound to recombinant human lactoferrin or to human milk lactoferrin. Furthermore, the transfer of iron across the monolayer was significantly greater when bound to citrate than to the human lactoferrins. The amount of iron transported after 24 h of incubation was a small proportion even for citrate and represented around 2% of the total iron added. In the work by Sánchez et al. (*19*) it was found that the proportion of iron transported across the monolayers was lower from citrate than from human lactoferrin. These differences might be explained by the great variability that has been always found for the iron transport from citrate in all the assays done. Nevertheless, considering the iron transported in conjunction with the retained by the cells, the proportion of iron was practically the same for citrate (9%) and human milk and recombinant lactoferrins (5%) in both studies.

Suzuki et al. (*10*) found the same results that we have found in the present work, a higher iron transport from citrate than from lactoferrin. However, it has been observed that the administration of ferrous sulfate or ferrous citrate caused some injuries in the rat gastric mucosa while lactoferrin did not cause any lesion; therefore, this finding points to lactoferrin as a safer iron supplement than the iron inorganic supplements (*37*).

The role of lactoferrin in the intestinal iron absorption is still controversial and it is not clear the mechanism by which this protein may participate in that process (*37*). It has been proposed that lactoferrin is mainly endocytosed by the intestinal epithelial cells (*38*), and that the iron is released by lysosomal degradation as it happens in hepatocytes (*29*). Another possibility could be that lactoferrin would bind to a membrane protein or region, releasing the iron at the cell surface and subsequently delivering it to the cell by a nonvesicular transport pathway as it happens in other cells (*39*). The amount of intact lactoferrin transported across the monolayers for both recombinant and human milk lactoferrins was only about 4.5% of the total radioactivity transported, indicating that most of the protein was degraded in the passage across the cells, which would be consistent with an endocytosis mediated transport. Furthermore, the proportion of human milk lactoferrin transported across Caco-2 monolayers was significantly higher than that of recombinant human milk lactoferrin. It is well-known that recombinant human lactoferrin from *Aspergillus awamori* has different glycan structures from those of human milk lactoferrin (*18*). Therefore, the differences in the amount of protein transported could reflect the influence that glycosylation may have on the interaction of lactoferrin with Caco-2 cell membranes. However, if considered the iron transported across monolayers and that internalized by cells, the total amount is the same for both lactoferrins; consequently, their role in iron absorption may be comparable.

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