# Effect of intracellular iron depletion by picolinic acid on expression of the lactoferrin receptor in the human colon carcinoma cell subclone HT29-18-$C_1$

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A lactoferrin receptor has been found on the brush-border membrane of intestinal epithelial cells of several species, including humans. A role for this receptor in intestinal iron absorption, which is well regulated in response to body iron stores, has been proposed. We have investigated the effect of intracellular iron depletion by picolinic acid, an iron chelator, on the cell surface binding of human lactoferrin to human enterocytes and its intracellular uptake, using HT29-18-$C_1$ cells, an enterocyte-like differentiable cell line. The confluent cells exhibited $5.8 \times 10^6$ specific binding sites per cell for diferric human $^{125}$I-labelled lactoferrin with relatively low affinity ($K_d$ $8.4 \times 10^{-7}$ M). The addition of picolinic acid to the culture medium resulted in a concentration- and time-dependent increase in lactoferrin binding that was correlated with a decrease in intracellular iron content. The maximum effect of picolinic acid on lactoferrin binding (approx. 2-fold increase), which appeared between 12 and 18 h after its addition, was obtained at a picolinic acid concentration of 2 mM. Scatchard analysis showed that the enhanced lactoferrin binding resulted from an increase in the number of lactoferrin receptors rather than an alteration in the binding affinity for lactoferrin. The time-dependent effect of picolinic acid was completely abolished in the presence of 1 $\mu$M anisomycin, a protein synthesis inhibitor, indicating that ongoing protein synthesis is involved in this effect. The enhanced lactoferrin binding induced by picolinic acid produced an increase of approx. 30% in the uptake of lactoferrin-bound $^{59}$Fe, indicating the existence of functional receptors. These results suggest that biosynthesis of lactoferrin receptors in intestinal epithelial cells can be regulated in response to the levels of intracellular chelatable iron, consistent with intestinal iron absorption dependent on body iron stores.

## INTRODUCTION

Iron is an essential element for normal human body function and its systemic homeostasis is achieved mainly by regulation of intestinal iron absorption, because of a lack of active excretion. Although it is well known that intestinal iron absorption is regulated in response to total body iron stores, i.e. iron deficiency or blood loss leads to an increase in iron absorption from the intestinal lumen, and excess iron stores results in decreased iron absorption [1], little is known about the mechanism of regulation of intestinal iron absorption. The biosynthesis of at least three proteins important in cellular iron metabolism, the transferrin receptor, ferritin and erythroid 5-aminolevulinic acid synthase, have been shown to be regulated post-transcriptionally by a common mechanism [2]. It seems likely that such iron-dependent regulation of biosynthesis mediated by the iron-responsive element binding protein also occurs in intestinal epithelial cells. In fact, the ferritin concentration in intestinal epithelial cells is low in iron deficiency and high in secondary iron overload [3]. Mucosal ferritin has long been suggested to regulate intestinal iron absorption by acting as an iron acceptor and by blocking the passage of iron through enterocytes [4]. The blocking function of mucosal ferritin in iron absorption is supported by the observation of abnormally low levels of mucosal ferritin expression in patients with genetic haemochromatosis whose intestinal iron absorption is still highly efficient in spite of high body iron stores [5]. The number of mucosal transferrin receptors also reflects body iron stores. Nevertheless, the transferrin receptor is unlikely to play a role in the transport of iron across enterocytes, since the receptor is found specifically localized on basolateral membranes of enterocytes, particularly those of crypt cells [6]. The expression of undefined inorganic iron carriers on brush-border membranes, which are thought to facilitate the uptake of iron across these membranes, is also regulated according to body iron stores [7]. However, the mechanism of regulation remains unclear.

Human lactoferrin, also called lactotransferrin [8], is an 80 kDa glycoprotein of the transferrin family that binds reversibly two ferric ions concomitantly with two carbonate ions per molecule. Human lactoferrin consists of a single polypeptide chain [9], which folds into two separate lobes [10], and two *N*-acetyl-lactosaminic-type glycans [11]. Lactoferrin is secreted from most exocrine glands [12] and is present in the secondary granules of polymorphonuclear neutrophils [13]. Several biological roles have been proposed for lactoferrin, such as a bacteriostatic effect, regulation of myelopoiesis, modulation of the immune system and promotion of cell growth [14]. A role in promoting intestinal iron absorption in humans has also long been proposed [8]. This hypothesis is supported by the higher bioavailability of iron in human milk than cows' milk-based infant formula [15] and the persistence of intact human lactoferrin in the gastrointestinal tract, owing to its high resistance against gastrointestinal digestion [16,17]. Furthermore, *in vitro* experiments

Abbreviations used: DPBS$^+$, Dulbecco's PBS containing 1 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$, pH 7.3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TBS, 150 mM NaCl and 20 mM Tris/HCl, pH 7.4.

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using human duodenal biopsies demonstrated that human lactoferrin can donate iron to intestinal mucosal cells [18]. The existence of a specific lactoferrin receptor has been demonstrated in rabbit [19], mouse [20,21], rhesus monkey [22] and human fetal [23] intestinal brush-border membranes, as well as in human phytohaemagglutinin-stimulated lymphocytes [24] and human platelets [25]. The presence of the lactoferrin receptor on intestinal brush-border membranes (unlike the transferrin receptor, which is localized on the basolateral membranes of intestinal epithelial cells [26]), is also compatible with a role for lactoferrin in intestinal iron absorption.

Despite the presence of a lactoferrin receptor on intestinal brush-border membranes, its role in intestinal iron absorption is still a subject of controversy. Some clinical studies failed to demonstrate improved iron absorption following lactoferrin supplementation of diets [27–29]. Another clinical study found an inhibitory effect of lactoferrin on iron absorption in normal subjects, whereas no such effect was found in patients with idiopathic haemochromatosis [30]. In these patients, it is unlikely that mucosal expression of the transferrin receptor and ferritin is regulated in response to body iron stores [5]. On the other hand, an *in vivo* study in rats showed that lactoferrin supplementation improved body iron stores, particularly in the anaemic rat [31].

In the present study, we investigated a possible regulation of the intestinal expression of the lactoferrin receptor in response to the levels of intracellular iron, using a cultured enterocyte-like differentiable cell line, HT29-18-$C_1$, as a model of intestinal absorptive cells. Intracellular iron depletion was achieved by the addition of picolinic acid to the culture medium. Furthermore, we also examined an alteration in the uptake of both lactoferrin and its bound iron in response to the levels of intracellular iron.

## MATERIALS AND METHODS

### Materials

Dulbecco's PBS containing 1 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$, pH 7.3 (DPBS$^+$), BSA and anisomycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Iodo-Gen was from Pierce (Rockford, IL, U.S.A.). Na$^{125}$I (100 mCi/ml) and $^{59}FeCl_3$ (1–50 mCi/mg of Fe) were from Amersham. PD-10 prepacked Sephadex G-25 M columns were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Chelex 100 was from Bio-Rad (Brussels, Belgium).

Lactoferrin was purified from human milk as described earlier [11] and was saturated with iron as described elsewhere [32] using 0.1 M sodium citrate/0.1 M sodium bicarbonate, pH 8.2 (saturating solution) containing appropriate amounts of $FeCl_3$ [33].

### Cell culture

HT29-18 is a clone isolated from HT29 cells derived from a human colon carcinoma cell line [34]. HT29-18-$C_1$, a subclone isolated from HT29-18 growing in a galactose-containing, glucose-depleted, medium according to characteristics of intestinal absorptive cells [34], was a gift from Dr. D. Louvard (Institut Pasteur, Paris, France) and was used between passages 10 and 30. This subclone undergoes absorptive enterocyte-like differentiation even in a glucose-containing medium [34,35]. HT29-18-$C_1$ cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 $\mu$g/ml gentamycin (Gibco BRL) at 37 °C in a humidified atmosphere of 90% air and 10% $CO_2$. Cells were subcultured weekly and seeded at $4 \times 10^4$ cells/cm$^2$ in 25 cm$^2$ Falcon flasks (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.), and the medium was changed daily. For experimental purposes, cells were plated at $8 \times 10^4$ cells/cm$^2$ in 6-well Falcon plates, or 12- or 24-well Linbro plates (Flow Laboratories, Puteaux, France). Cells reached confluence 6 days after plating under these conditions. For the experiments under iron-depleting conditions, cells were cultured in DMEM without added iron (Eurobio, Paris, France) supplemented with 10% heat-inactivated iron-depleted FBS and 50 $\mu$g/ml gentamycin. Iron depletion of FBS was performed as described by Alvarez-Hernandez et al. [36].

### Labelling of human lactoferrin

Diferric lactoferrin was iodinated using Iodo-Gen as described elsewhere [37]. The specific radioactivity of the diferric $^{125}$I-labelled lactoferrin ($^{125}$I-lactoferrin) was $(4–7) \times 10^7$ c.p.m./nmol. For dual labelling with $^{59}$Fe and $^{125}$I, native lactoferrin was first saturated with $^{59}$Fe and then radioiodinated as described elsewhere [37]. The $^{125}$I and $^{59}$Fe specific radioactivities of diferric $^{59}$Fe/$^{125}$I-dual-labelled lactoferrin ($^{59}$Fe/$^{125}$I-lactoferrin) were $(1–2) \times 10^7$ c.p.m./nmol and $(3–8) \times 10^5$ c.p.m./nmol respectively.

### Binding of human lactoferrin to the apical surface of HT29-18-$C_1$ cells

Confluent HT29-18-$C_1$ cells, cultured in 24-well plates for 9 days, were rinsed three times with cold DPBS$^+$ and equilibrated at 4 °C. Incubation media were prepared in DPBS$^+$ containing 0.5% BSA and the required final concentrations of diferric $^{125}$I-lactoferrin. Binding was initiated by aspirating the rinsing solution and adding 0.2 ml of the incubation medium to each well. After 90 min of incubation at 4 °C, 100 $\mu$l of the incubation medium was collected and the remainder was aspirated. The cell monolayers were rinsed with 0.4 ml of ice-cold DPBS$^+$ five times and scraped off in DPBS$^+$. Cell-associated and medium radioactivities were determined in an LKB 1282 CompuGamma $\gamma$-counter. Dissociation constants and numbers of binding sites were determined according to Scatchard [38] with the aid of Enzfitter, a computer program for non-linear regression data analysis (Elsevier-BIOSOFT, Cambridge, U.K.). Non-specific binding was estimated by incubation in the presence of a 100-fold molar excess of unlabelled diferric lactoferrin.

### Effect of picolinic acid on lactoferrin binding to HT29-18-$C_1$ cells

First, binding of lactoferrin to the cell monolayers were examined after incubation with various concentrations of picolinic acid. Cells were cultured in 24-well plates in standard DMEM. At 7 days after plating, the medium was replaced by iron-depleted DMEM containing 0.1–4 mM picolinic acid. The cell monolayers were incubated for 48 h in the presence of picolinic acid, using fresh medium after 24 h of incubation. Then the binding assay was carried out as described above using 500 nM diferric $^{125}$I-lactoferrin; non-specific binding was estimated as above.

Secondly, the alteration of binding of lactoferrin to the cell monolayers was determined as a function of time of incubation with picolinic acid. Cells were cultured in 24-well plates in standard DMEM. At 7 days after plating, the medium was replaced by iron-depleted DMEM containing 2 mM picolinic acid. At 6, 12, 18, 24, 30 or 36 h after the first replacement, the medium of other wells was replaced in the same manner. At 42 h the binding assay was carried out, using 500 nM diferric $^{125}$I-lactoferrin. To examine the effect of anisomycin, which reversibly inhibits protein biosynthesis, cell monolayers were incubated in the presence of 1 $\mu$M anisomycin (from a 1 mM stock in ethanol)

between 6 and 18 h after the replacement of standard DMEM by iron-depleted DMEM containing 2 mM picolinic acid. At 18 h, the cell monolayers were rinsed twice with iron-depleted DMEM, re-incubated in the presence of 2 mM picolinic acid, and the binding assay was performed.

Thirdly, the nature of lactoferrin binding was determined after incubation with picolinic acid. Cells were cultured in 24-well plates in standard DMEM for 8 days, and then incubated for 24 h in iron-depleted DMEM containing 2 mM picolinic acid. Then the binding assay was carried out as described above using various concentrations of diferric $^{125}$I-lactoferrin.

Lastly, the reversibility of the effect of picolinic acid on lactoferrin binding was examined. Cells treated with picolinic acid for 24 h were re-incubated in standard DMEM for 24 h. The binding assay was carried out with non-treated cells and 48-h-treated cells.

### Measurement of the intracellular iron content of HT29-18-C$_1$ cells

The intracellular iron content was measured to verify the iron-chelating activity of picolinic acid. Cells cultured in 6-well plates were treated with picolinic acid as described above. Monolayers were rinsed five times with 150 mM NaCl and 20 mM Tris/HCl, pH 7.4 (TBS), which had been passed through a Chelex 100 column to remove traces of iron, and were scraped off with a Coster cell scraper in iron-depleted TBS. The cells were concentrated by centrifugation, and resuspended twice in iron-depleted TBS. Then the cells were resuspended in 20 mM Tris/HCl, pH 7.65, which had also been passed through a Chelex 100 column, and were homogenized using an acid-washed tight-fitting Dounce homogenizer. The homogenate was centrifuged for 1 h at 4 °C and at 19000 *g* in a Sigma centrifuge. Aliquots of the supernatant were used for iron determinations. Iron was determined by graphite-furnace atomic absorption spectroscopy with a Perkin-Elmer Model 2380 spectrometer, an HGA 500 programmer and an AS-40 sampler.

### Measurement of lactoferrin uptake into HT29-18-C$_1$ cells

Cells were cultured in 12-well plates for 7 days, and were then incubated in iron-depleted DMEM in the presence or absence of 2 mM picolinic acid for 24 h. Two different experimental procedures were performed in order to study the effect of picolinic acid on the uptake of lactoferrin into the cells. In the first procedure, cells were rinsed with ice-cold DPBS$^+$ three times and equilibrated at 4 °C, followed by aspiration of the rinsing solution. To each well was added 0.4 ml of 500 nM diferric $^{125}$I-lactoferrin in DPBS$^+$ containing 0.5% BSA. After a 90 min incubation at 4 °C, 100 $\mu$l of the incubation medium was aspirated, and the cells were rinsed with 4 × 0.5 ml of ice-cold DPBS$^+$. The assay of lactoferrin uptake was initiated by adding 0.4 ml of prewarmed DPBS$^+$ containing 0.1% BSA to each well. After 1, 10, 20, 30, 45, 65 and 90 min of incubation at 37 °C, the incubation medium and a rinse of 0.5 ml of ice-cold DPBS$^+$ were collected. To remove cell-surface-bound lactoferrin, a dissociating solution comprising 0.5 ml of ice-cold 0.2 M acetic acid/0.5 M NaCl, pH 2.5, was added to each well, followed by 10 min incubation on ice. This dissociating solution and a 0.5 ml rinse of the same solution were collected, and cells were scraped off in DPBS$^+$. Medium, acid-extractable and intracellular $^{125}$I radioactivities were determined.

In the second procedure, cells were rinsed with DPBS$^+$ prewarmed at 37 °C and then incubated in 0.4 ml of the same medium containing 0.1% BSA and 400 nM diferric $^{59}$Fe/$^{125}$I-lactoferrin in DPBS$^+$. After 10, 30, 60 and 120 min of incubation at 37 °C, the medium was aspirated, and cells were rinsed with 4 × 0.5 ml of ice-cold DPBS$^+$ four times. Acid-extractable and intracellular radioactivities were determined as above.

## RESULTS

### Binding of human lactoferrin to the apical surface of HT29-18-C$_1$ cells

The parameters of lactoferrin binding to HT29-18-C$_1$ cells were determined by incubating the cell monolayers with diferric $^{125}$I-lactoferrin at 4 °C. According to the Scatchard analysis of the results shown in Figure 1, the cell monolayers exhibited $(5.8 \pm 1.3) \times 10^6$ binding sites for lactoferrin per cell of relatively low affinity [$K_d$ $(8.4 \pm 1.0) \times 10^{-7}$ M; $n = 4$].

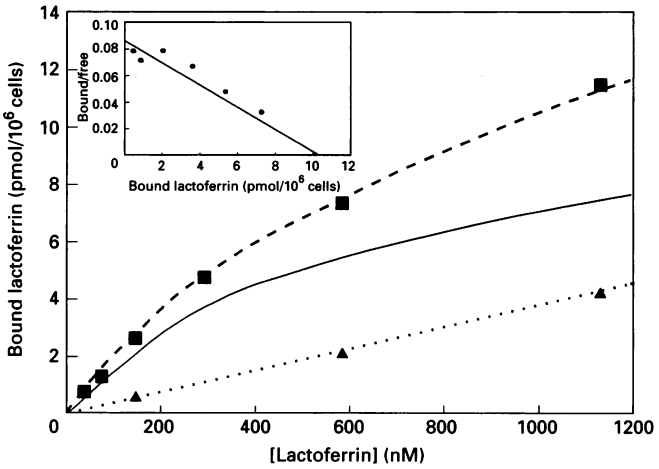


**Figure 1 Saturability of $^{125}$I-labelled diferric human lactoferrin binding to HT29-18-C$_1$ cell monolayers**

Confluent HT29-18-C$_1$ cells were incubated with the indicated concentrations of diferric $^{125}$I-lactoferrin for 90 min at 4 °C. Cell-associated and medium radioactivities were determined in the scraped cells and the collected incubation medium in a $\gamma$-radiation counter. Non-specific binding was estimated by incubation in the presence of a 100-fold molar excess of unlabelled diferric lactoferrin. ——, Specific binding; ■, total binding; ▲, non-specific binding. The inset shows the Scatchard-plot analysis of specific binding. The results are typical of four separate experiments.

**Table 1 Effect of picolinic acid concentration on $^{125}$I-lactoferrin binding to HT29-18-C$_1$ cell monolayers and on intracellular iron content**

Confluent HT29-18-C$_1$ cells were incubated at 37 °C in 10% $CO_2$ for 48 h in iron-depleted DMEM containing the indicated concentrations of picolinic acid. The cell monolayers were incubated for 90 min at 4 °C with 500 nM diferric $^{125}$I-lactoferrin. Specific binding of $^{125}$I-lactoferrin to cell monolayers was estimated as described in the legend to Figure 1. Intracellular iron content was determined by measurement in cytosolic fractions using an atomic absorption spectrometer.

| Concentration of picolinic acid (mM) | Lactoferrin binding (pmol/10$^6$ cells) | Intracellular iron contents (ng/10$^6$ cells) |
|---|---|---|
| 0 | 4.33 | 5.62 |
| 0.1 | 4.28 | 5.40 |
| 0.5 | 6.31 | 4.84 |
| 1.0 | 6.03 | 2.62 |
| 2.0 | 7.35 | 0.70 |

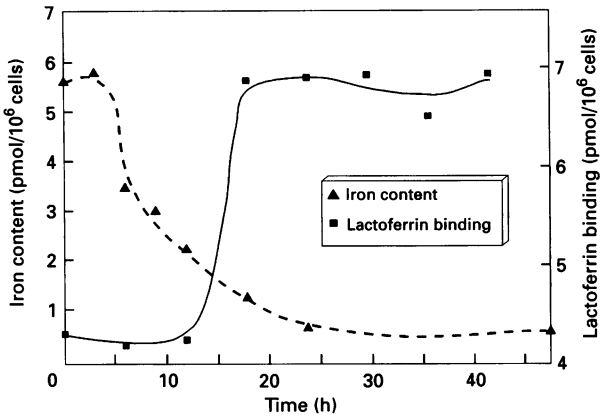


**Figure 2 Kinetics of the picolinic acid-induced alterations in $^{125}$I-lactoferrin binding to HT29-18-C$_1$ cell monolayers and in intracellular iron content**

Confluent HT29-18-C$_1$ cells were incubated at 37 °C in 10% $CO_2$ for the indicated times in iron-depleted DMEM containing 2 mM picolinic acid. The cell monolayers were incubated for 90 min at 4 °C with 500 nM diferric $^{125}$I-lactoferrin with or without a 100-fold molar excess of unlabelled diferric lactoferrin. The results are expressed as specific binding of $^{125}$I-lactoferrin to cells. Intracellular iron content was determined as described in Table 1.

## Effect of picolinic acid on intracellular iron content and binding of human lactoferrin to HT29-18-C$_1$ cells

The concentration-dependence of the effect of picolinic acid on intracellular iron content and binding of human lactoferrin to the cell monolayers was examined in the first instance. As shown in Table 1, the intracellular iron content decreased in a concentration-dependent manner after a 48 h incubation with picolinic acid. Table 1 also shows that a 48 h incubation with increasing amounts of picolinic acid resulted in enhanced lactoferrin binding to the cell surface. The maximum specific binding, which represented approx. 70% increased binding compared with control experiments, was obtained at 2 mM picolinic acid. Therefore the subsequent experiments were carried out at a picolinic acid concentration of 2 mM.

Secondly, we examined the time course of the effect of picolinic acid treatment on binding of lactoferrin to the cell monolayers in parallel with changes in intracellular iron content. As shown in Figure 2, increased lactoferrin binding of approx. 60% appeared between 12 and 18 h after addition of 2 mM picolinic acid, and an elevated level of steady-state binding was observed after 18 h. The intracellular iron content started to decrease 3 h after addition of picolinic acid and continued to decrease to approx. 13% of the control levels after 24 h.

## Basis for the increased binding of human lactoferrin to HT29-18-C$_1$ cells induced by picolinic acid treatment

The basis for the increased binding of lactoferrin induced by picolinic acid was examined first by performing the binding assay with various concentrations of lactoferrin. Table 2 shows the alteration of the parameters of lactoferrin binding to HT29-18-C$_1$ cell monolayers cultured in the presence or absence of 2 mM picolinic acid, as indicated by the Scatchard analysis. The cells treated with picolinic acid for 24 h exhibited an increase of approx. 2.1-fold in the number of lactoferrin binding sites, with an unmodified binding affinity for lactoferrin. Table 2 also shows that re-incubation of picolinic acid-treated cells with standard DMEM for 24 h decreased the expression of the lactoferrin

**Table 2 Alterations in the parameters of lactoferrin binding to HT29-18-C$_1$ cells after incubation with picolinic acid**

Diferric $^{125}$I-lactoferrin binding experiments were carried out as described in the legend to Figure 1. SM, standard DMEM; PM; iron-depleted DMEM containing 2 mM picolinic acid; *n*, number of independent experiments.

| Incubation period | *n* | $K_d$ (nM) | Binding capacity ($10^6$ sites/cell) |
|---|---|---|---|
| 24 h in SM | 4 | 840±103 | 5.76±1.29 |
| 24 h in PM | 3 | 841±209 | 12.40±3.67 |
| 24 h in PM plus 24 h in SM | 2 | 742±229 | 6.63±1.70 |

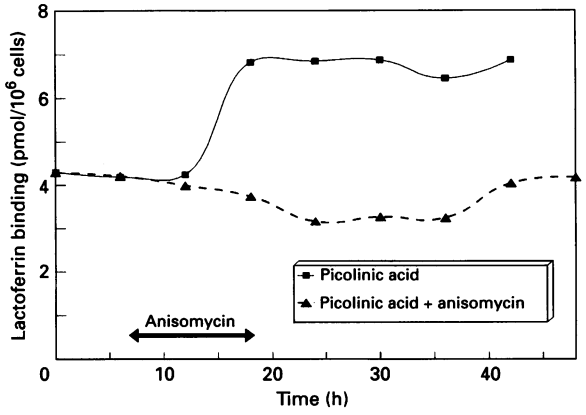


**Figure 3 Protein synthesis is required for the picolinic acid-induced alteration in $^{125}$I-lactoferrin binding to HT29-18-C$_1$ cell monolayers**

The experimental procedures was as in Figure 2, except that cells were incubated in the presence of 2 mM picolinic acid and 1 $\mu$M anisomycin for the time periods indicated. The results are expressed as specific binding of $^{125}$I-lactoferrin to cells.

receptor to the control level, indicating that the effect of picolinic acid is reversible.

Anisomycin is a reversible inhibitor of protein biosynthesis. Its effect on the time course of binding of lactoferrin after picolinic acid treatment is shown in Figure 3. The presence of anisomycin between 6 and 18 h after addition of picolinic acid totally abolished the induction of increased binding of lactoferrin, and binding of lactoferrin was decreased by approx. 20%.

## Effect of picolinic acid on the uptake of lactoferrin

Two different experimental procedures were performed to examine whether the lactoferrin receptor is functional and whether picolinic acid affects the functions of the receptor. In the first procedure, to determine the fate of cell-surface-bound lactoferrin, the cell monolayers were incubated with 500 nM diferric $^{125}$I-lactoferrin at 4 °C, rinsed with DPBS$^+$ to remove unbound labelled lactoferrin and then incubated at 37 °C. Figures 4(a) and 4(b) show the distribution of initially surface-bound lactoferrin after incubation of cells at 37 °C without and with picolinic acid treatment. In the absence of picolinic acid, cell-surface-bound lactoferrin decreased rapidly, representing 35 and 20% of total lactoferrin at 20 and 65 min respectively. Conversely, lactoferrin

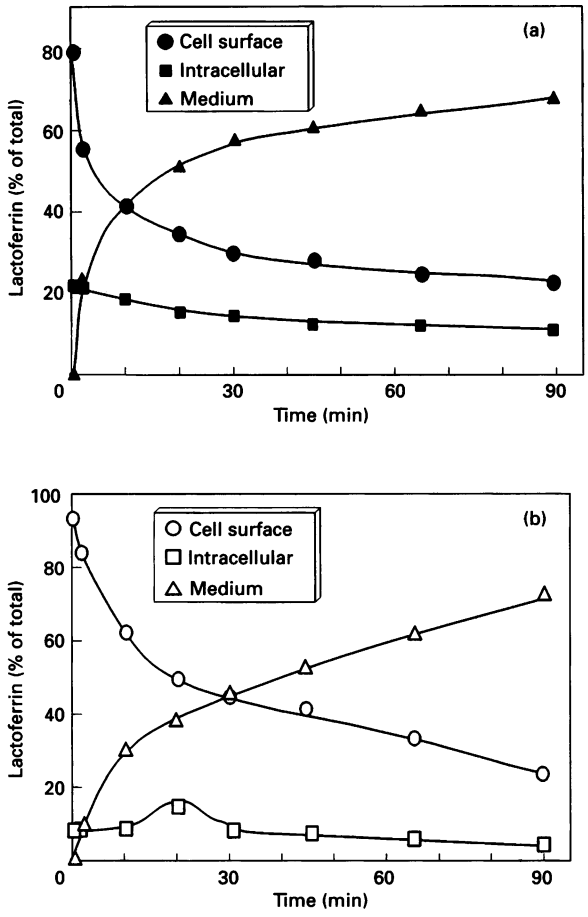


**Figure 4 Fate of $^{125}$I-lactoferrin bound to the HT29-18-C$_1$ cell surface with or without preincubation with picolinic acid**

Confluent HT29-18-C$_1$ cells were incubated for 90 min at 4 °C with 500 nM diferric $^{125}$I-lactoferrin. After removal of unbound lactoferrin, the cell monolayers were incubated at 37 °C. Released, cell-surface-bound and intracellular $^{125}$I radioactivities were determined in a $\gamma$-counter at the indicated times (**a**). The same experimental procedure was performed for the confluent HT29-18-C$_1$ cells preincubated at 37 °C in 10% $CO_2$ for 24 h in iron-depleted DMEM containing 2 mM picolinic acid (**b**). The results are expressed as a percentage of the total radioactivities.

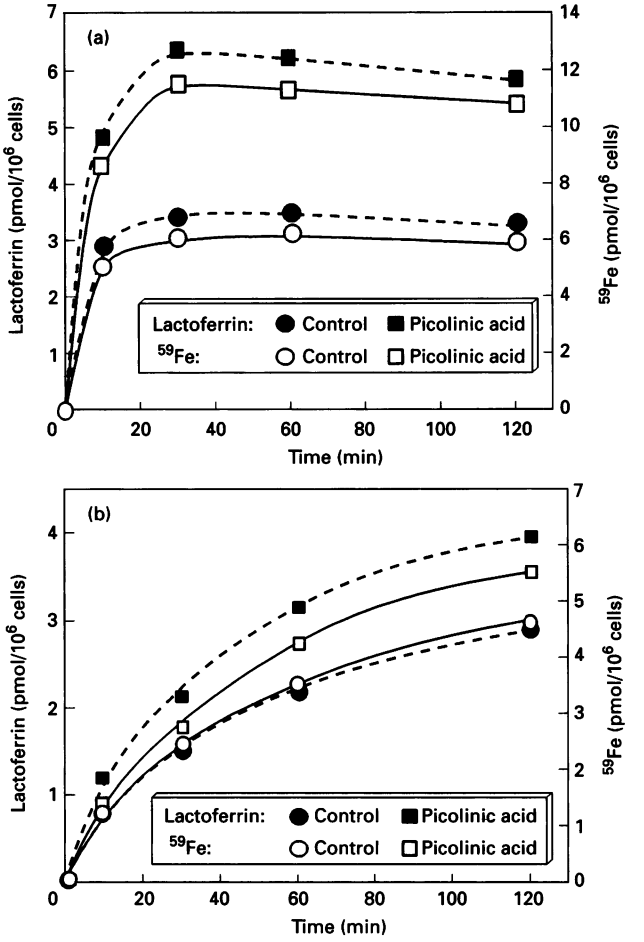


**Figure 5 Uptake of $^{59}$Fe/$^{125}$I-lactoferrin bound to the HT29-18-C$_1$ cell surface with or without preincubation with picolinic acid**

Confluent HT29-18-C$_1$ cells were preincubated at 37 °C in 10% $CO_2$ for 24 h in standard DMEM or in iron-depleted DMEM containing 2 mM picolinic acid. The cells were incubated at 37 °C with 400 nM diferric $^{59}$Fe/$^{125}$I-lactoferrin. Cell-surface-bound lactoferrin and iron originally bound to lactoferrin were determined by measuring acid-extractable $^{125}$I and $^{59}$Fe radioactivities at the indicated times (**a**). Intracellular counterparts were determined by measuring the remaining $^{125}$I and $^{59}$Fe radioactivities (**b**).

in the medium increased rapidly, representing 50 and 60% of total lactoferrin at 20 and 65 min respectively. Intracellular lactoferrin, i.e. cell-associated lactoferrin resistant to a surface acid extraction, continued to decrease progressively throughout the incubation period at 37 °C, representing 20 and 10% of total lactoferrin at 1 and 65 min respectively. These results suggest that very little internalization of surface-bound lactoferrin occurred in the cells without picolinic acid treatment. In cells treated with picolinic acid, a similar distribution of lactoferrin was observed except that a small increase peaking at 20 min was obtained for intracellular lactoferrin, i.e. 7, 15 and 7% of total lactoferrin at 1, 20 and 30 min respectively. These results suggest that a small part of the surface-bound lactoferrin was internalized in the picolinic acid-treated cells.

### Effect of picolinic acid on uptake of lactoferrin-bound iron

In the second procedure, cells were incubated at 37 °C in the presence of 400 mM diferric $^{59}$Fe/$^{125}$I-lactoferrin to examine whether the induced lactoferrin receptor functions to deliver iron into cells. Figure 5(a) shows the alteration of cell-surface-bound lactoferrin, measured by $^{125}$I-radioactivity, and of iron originally bound to lactoferrin, measured by $^{59}$Fe radioactivity, as a function of time. Figure 5(b) shows the corresponding intracellular levels. Surface-bound lactoferrin reached steady-state values within 30 min, with or without picolinic acid treatment. The levels of lactoferrin binding increased approx. 2-fold for treated cells in this experiment. The ratio of cell-surface lactoferrin to iron was unchanged throughout the incubation period at 37 °C (Figure 5a). Continuous increases in intracellular lactoferrin and iron were observed for both picolinic acid-treated and untreated cells, and treated cells internalized 20–30% more of both lactoferrin and iron than untreated cells. The ratio of intracellular lactoferrin to iron was also unchanged throughout the incubation period at 37 °C (Figure 5b).

## DISCUSSION

In the current study, we investigated the effect of picolinic acid on the following: (1) intracellular iron content, (2) lactoferrin binding to the cell surface, (3) biosynthesis of the lactoferrin

receptor, and (4) uptake of lactoferrin and lactoferrin-bound iron in HT29-18-$C_1$ cells as a model of intestinal absorptive cells. The HT29-18-$C_1$ cell monolayers exhibited $5.8 \times 10^6$ binding sites for lactoferrin per cell, with relatively low affinity ($K_d$ $8.4 \times 10^{-7}$ M). These binding parameters are similar to those found in other clones from the HT29 cell line, including HT29-D4 cells ($K_d$ $4.1 \times 10^{-7}$ M; $4.1 \times 10^6$ binding sites per cell [39]) and HT29 clone 19A ($K_d$ $8.3 \times 10^{-7}$ M; $1.5 \times 10^6$ binding sites per cell [37]). Furthermore the binding affinity is also comparable with those found in intestinal brush-border membranes of rabbit ($K_d$ $8.3 \times 10^{-7}$ M [19]), mouse ($K_d$ $2.9 \times 10^{-7}$ M [20,21]), rhesus monkey ($K_d$ $9.0 \times 10^{-6}$ M [22]) and human fetus ($K_d$ $3.3 \times 10^{-6}$ M [23]).

We observed intracellular iron depletion from HT29-18-$C_1$ cells on addition of picolinic acid in a concentration- and time-dependent manner. This iron chelator, naturally occurring as an intermediate in tryptophan metabolism, not only inhibits cellular iron uptake but also removes intracellular iron [40], and subsequently inhibits the growth of cultured normal or transformed mammalian cells by selective depletion of iron in the cells [41]. It also induces a marked increase in the number of transferrin receptors in human haematopoietic cell lines [42,43]. Furthermore, increased binding of lactoferrin to HT29-18-$C_1$ cells mirrored the decrease in intracellular iron content in a concentration- and time-dependent manner, except for a delay of approx. 10 h. These results strongly suggest that intracellular iron levels influence the binding of lactoferrin to the surface of HT29-18-$C_1$ cells. Scatchard analysis showed that the cells treated with picolinic acid exhibited a 2-fold increase in the number of lactoferrin binding sites and an unchanged binding affinity for lactoferrin, showing that increased binding of lactoferrin resulted from enhanced expression of the lactoferrin receptor on the surface of the cell, rather than an alteration of binding affinity.

The presence of anisomycin totally abolished the increased binding of lactoferrin after incubation with picolinic acid, indicating that the picolinic acid-induced increase in lactoferrin binding was not due to a shift of lactoferrin receptors from the intracellular pool to the cell surface, but a result of enhanced *de novo* synthesis of lactoferrin receptors. The decreased binding of lactoferrin by 20% during and after the addition of anisomycin was also observed in these experiments. This might now be owing to a cytotoxic effect of anisomycin but may result from an unbalanced turnover of the lactoferrin receptor, i.e. reduced receptor biosynthesis plus unchanged receptor degradation, since binding of lactoferrin returned to control levels 42 h after the addition of picolinic acid (24 h after the removal of anisomycin).

In the experiments demonstrating the fate of $^{125}$I-lactoferrin bound to the HT29-18-$C_1$ cell surface, our findings in cells without picolinic acid preincubation are consistent with results obtained with HT29-D4 cells [44]. In these cells, surface-bound lactoferrin was probably not internalized by clathrin-dependent receptor-mediated endocytotic processes, which are very efficient processes due to the concentration of ligands in clathrin-coated pits [45]. Owing to its relatively low-affinity binding, surface-bound lactoferrin might dissociate rapidly on equilibration with newly changed medium. If so, no internalization of lactoferrin would be observed, even if clathrin-independent endocytotic processes exist [46]. In picolinic acid-treated cells, a small part of the surface-bound lactoferrin was seen to be internalized. The involvement of clathrin-dependent processes in the treated cells remains to be investigated.

When cells were incubated at 37 °C with diferric $^{59}$Fe/$^{125}$I-lactoferrin, continuous increases in intracellular lactoferrin and iron were observed for both picolinic acid-treated and untreated cells. Since almost no internalization of surface-bound $^{125}$I-lactoferrin was observed in untreated cells in the previous experiments, most lactoferrin found inside such cells is likely to have been absorbed by fluid-phase endocytosis or pinocytosis. Indeed endocytosis or transcytosis of $\beta$-lactoglobulin and horseradish peroxidase by this non-specific route has been well characterized in intestinal cells [47]. The increase (20–30%) in intracellular lactoferrin and iron in treated cells as compared with untreated cells was smaller than the increase (approx. 100%) in surface-bound lactoferrin. Nevertheless, this increase may be due to the internalization of surface-bound lactoferrin, and was consistent with the previous observation that only a small part of surface-bound lactoferrin was internalized in treated cells. Therefore newly induced receptors for lactoferrin, although showing a similar binding affinity, could be another kind of molecule. Furthermore, the uptake process for lactoferrin-bound iron is likely to be different from that for transferrin-bound iron, in which receptor-bound transferrin releases its iron in early endosomes and then recycles back to the plasma membrane, while the released iron accumulates inside the cells [48]. Accumulation of both lactoferrin and iron inside cells suggested the existence of an adsorptive uptake process, as previously proposed in macrophages [49]. Our results further suggest that the lactoferrin receptor expressed after induction by picolinic acid treatment is able to mediate the cellular uptake of lactoferrin-bound iron by a mechanism different from the endocytosis of transferrin mediated by the transferrin receptor.

In summary, the present study suggests (1) that biosynthesis of lactoferrin receptors in intestinal epithelial cells may be regulated in response to intracellular iron levels, possibly in a fashion analogous to the post-transcriptional regulation of the transferrin receptor, and (2) that the lactoferrin receptor induced by intracellular iron depletion increases the uptake of lactoferrin-bound iron into enterocytes. Thus the lactoferrin receptor may play a role in iron absorption, particularly in iron deficiency.

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