Antimicrobial mechanism of action of transferrins: Selective inhibition of H\(^+\)-ATPase

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Abstract

Two bacterial species, *Pseudomonas aeruginosa* and *Lactococcus lactis*, with different metabolic features, were used as a comparative experimental model to investigate the antimicrobial target and mechanism of transferrins. In anaerobiosis, *P. aeruginosa* cells were not susceptible to lactoferrin (hLf) or transferrin (hTf). In aerobiosis, the cells were susceptible but O$_2$-consumption was not modified, indicating that components of the electron transport chain (ETC) were not targeted. However, the respiratory chain inhibitor piericidin A significantly reduced the killing activity of both proteins. Moreover, 2,6-dichlorophenolindophenol (DCIP), a reducing agent that accepts electrons from the ETC coupled to H$^+$-extrusion, made *P. aeruginosa* susceptible to hLf and hTf in anaerobiosis. These results indicated that an active cooperation of the cell was indispensable for the antimicrobial effect. In *L. lactis* cells lacking an ETC, the absence of a detectable transmembrane electrical potential in hLf-treated cells suggested a loss of the H$^+$-ATPase activity. Furthermore, the inhibition of ATPase activity and H$^+$-translocation (inverted membrane vesicles) provided direct evidence of the ability of hLf to inhibit H$^+$-ATPase in *L. lactis*. Based on these data, we propose that hLf and hTf also inhibit the H$^+$-ATPase of respiring *P. aeruginosa* cells. Such inhibition thereby interferes with re-entry of H$^+$ from the periplasmic space to the cytoplasm, resulting in perturbation of intracellular pH and the transmembrane proton gradient. Consistent with this hypothesis, periplasmic H$^+$-accumulation was prevented by anaerobiosis or by piericidin A, or induced by DCIP in anaerobiosis. Collectively, these results indicate that transferrins target H$^+$-ATPase and interfere with H$^+$-translocation, yielding a lethal effect *in vitro*. 
Introduction

Transferrins comprise a family of proteins which include iron-binding polypeptides of diverse phylogenetic groups. Two well studied representative members of this family are transferrin and lactoferrin. These polypeptides have multiple biological functions in blood and mucosal surfaces, respectively. It is thought that both proteins contribute to defense against microbial infection in different host settings, and are considered components of innate immunity (5, 14).

The antimicrobial activity of transferrin and lactoferrin has been attributed to various causes, including nutritional deprivation of essential iron, catalytic potential in Haber-Weiss-Fenton chemistry, or outer membrane damage in Gram-negative bacteria (1, 13, 27, 33). Alternatively, we have proposed a specific interaction of human lactoferrin with a protein constituent of the microbial cytoplasmic membrane, thus explaining physiological changes associated with lactoferrin-induced cell death (24). This hypothesis derives from the fact that the antimicrobial activity of lactoferrin is tightly linked to cellular bioenergetics, and it is not due to a permeabilization of cell membranes (6, 24, 25). In addition, the antimicrobial effect may be prevented by extracellular Na$^+$ and K$^+$ (24, 25), suggesting the involvement of the homeostatic processes associated with regulation of cytoplasmic ion concentrations.

In the present study, we assessed the antimicrobial activity of lactoferrin and transferrin on *P. aeruginosa* and *Lactococcus lactis* cells to identify a putative and common target for transferrins. These species were selected in light of previously observed influences of microbial physiology on the antimicrobial activity of lactoferrin (4, 6, 24, 25), and due to their different metabolic features. *P. aeruginosa* is a Gram-negative and carbohydrate non-fermenting species that preferentially uses oxygen (aerobic) or nitrate (anaerobic) as terminal electron acceptors in respiratory metabolism (32). In the absence of electron acceptors, this opportunistic pathogen may ferment L-arginine, generating sufficient ATP for growth using substrate-level phosphorylation (11, 23). *L. lactis* is a Gram-
positive, acid-tolerant, and homo-fermentative organism that utilizes a short and oxygen-dependent respiratory chain solely when hemin is present in the growth media, thus generating a transmembrane proton gradient through aerobic electron transport (7, 8, 12).

In respiring bacterial species (i.e., *P. aeruginosa*), the electron transport chain generates a transmembrane proton gradient (ΔpH) necessary for ATP synthesis by the F$_{1}$F$_{0}$-ATPase, and transport of various solutes. However, the F$_{1}$F$_{0}$-ATPase complex is a reversible proton translocating pump that may extrude protons from the cytoplasm using energy provided by ATP hydrolysis. Such proton efflux enhances the proton gradient and assists in regulating the cytoplasmic pH (10, 18). For example, maintenance of optimal intracellular pH (pH$_{i}$) is an essential function of F$_{1}$F$_{0}$-ATPase for the survival of carbohydrate-fermenting lactic acid bacteria (i.e. *L. lactis*) (7, 10). These distinct mechanisms of ΔpH-maintenance and pH$_{i}$-regulation used by *P. aeruginosa* and *L. lactis* were finally used as comparative models to gain insights into the *in vitro* antibacterial mechanism of human lactoferrin and transferrin. Results suggest these host defense molecules selectively inhibit the H$_{i}^{+}$-ATPase complex in such bacteria. Based on this finding, we propose a model to explain the antimicrobial mechanism of action of transferrins *in vitro* under respiratory and fermentative conditions.

**Materials and methods**

**Materials.** Recombinant human apo-lactoferrin (rhLf) and human apo-transferrin (hTf) were obtained from Ventria Bioscience (Sacramento, CA) and Sigma-Aldrich Chemicals (St. Louis, MO), respectively. The peptide Lfpep was obtained from Bio-Synthesis (Lewisville, TX). The reagents 9-amino-3-chloro-7-methoxyacridine (ACMA), and DiSC$_{3}$(5) were purchased from Invitrogen-Molecular Probes (Eugene, OR). Antimycin A, Na$_{2}$ATP, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 2,6-dichlorophenolindophenol (DCIP), N,N´-dicyclohexylcarbodiimide (DCCD), 2-(N-
Bacterial strains and growth conditions. Cells were grown to mid-log phase in TSB at 37 °C (Pseudomonas aeruginosa PAO1) or in M17 broth supplemented with 0.5 % glucose (M17G) at 30 °C without shaking (Lactococcus lactis sub. lactis IL1403) as described (21). Anaerobic growth and assays under strict anaerobic conditions (80% N₂, 10% CO₂, 10% H₂) were performed in an anaerobic chamber (mod. 1024, Forma Scientific, Marietta, OH). The arginine deiminase pathway was induced under anaerobic conditions growing P. aeruginosa cells in oxygen-sensitive (OS) medium supplemented with 0.4% YE and 40 mM L-arginine (YEA medium), as described (23). When required, the denitrification system of P. aeruginosa was stimulated in 0.1x TSB containing 50 mM KNO₃ (TSBN) as previously described (16). All media and buffers used in anaerobiosis were held in the anaerobic chamber a minimum of 48 h prior to use.

Antimicrobial assays. Bacterial suspensions grown to mid-log phase in appropriate media were washed twice and resuspended in Tris buffer (10 mM Tris-HCl, pH 7.4). The cell suspensions (10⁶ cells/ml) were incubated for 90 min at 37 °C (P. aeruginosa) or 30 °C (L. lactis) with different concentrations (ranging from 0.03 to 25 µM) of rhLf or hTf, and aliquots were diluted in the same buffer and quantitatively cultured. The influence of extracellular pH on the antibacterial activity was tested using cells resuspended in 10 mM MES (pH 5.5) or 10 mM Tris-HCl (pH 7.4). Ninety and fifty percent inhibitory concentrations (IC₉₀ and IC₅₀) were defined as the protein concentrations that reduced bacterial survival by 90% and 50% of that observed in untreated controls. Microbicidal
kinetic assays were carried out incubating cells ($10^6$ cells/ml) of *P. aeruginosa* in the presence of rhLf (0.125, 0.5, 1 µM) or hTf (1, 2, 4 µM) and *L. lactis* with rhLf (0.03, 0.06, 0.125 µM) or hTf (4, 25 µM) at 37 °C. Aliquots were taken at pre-selected time intervals, serially diluted in the same buffer, and cultured quantitatively on appropriate media. When required, the assay conditions contained 5 mM KNO$_3$ or 40 mM L-arginine (*P. aeruginosa*) or 0.5% glucose (*L. lactis*), and respective quantitative culture plates contained identical concentrations with the exception of 50 mM KNO$_3$ in nitrate assay plates. The plates were incubated for 24 h at 37 °C (*P. aeruginosa*) or 30 °C (*L. lactis*) in aerobiosis or anaerobiosis, except for YEA and TSBN plates which were incubated 2 to 5 days in the anaerobic chamber. After appropriate incubation, colonies were counted to determine the percent survival of treated organisms as compared to controls.

**Permeabilization.** Cytoplasmic membrane permeabilization was investigated using the cell-impermeant fluorescent probe propidium iodide in flow cytometric analysis. Cells grown to the mid-exponential phase were washed and resuspend ($10^6$ cells/ml) in Tris buffer, and incubated with or without the above concentrations of rhLf and hTf or Lfpep (50 µM) for 90 min at 37 °C. Then, the cell suspensions were re-incubated with PI (1 µg/ml) for 5 min. Fluorescence data were acquired as monoparametric histograms using a Cytorun Absolute flow cytometer (Ortho Diagnostics Systems Inc., Raritan, NJ). Results were expressed as the percent PI-positive cells with respect to the unstained cells (control).

**Transmembrane potential.** Transmembrane electrical potential ($\Delta \psi$) was monitored using the potential-sensitive fluorescent probe DiSC$_3$(5) as described previously (20). *L. lactis* cells grown to the mid-exponential phase in M17G were washed and resuspended in 50 mM potassium phosphate (pH 5.0). Bacterial suspensions (approx. $10^7$ cells/ml) were incubated (30 min, 30 °C) with 2 µM rhLf, 20 µM hTf or 1 mM DCCD and re-incubated with 3 µM DiSC$_3$(5) to equilibrium of the...
fluorescent signal. The $\Delta \psi$ was generated upon addition of glucose (15 mM, final concentration) as source of metabolic energy. Fluorescence signals of DiSC$_3$(5) were measured ($\lambda_{ex}$= 651 nm, $\lambda_{em}$=675 nm) using a Perkin Elmer LS 50B spectrofluorometer. The K$^+$-ionophore valinomycin (2 µM) was added at the end of the reaction to elicit complete collapse of membrane potential.

**Oxygen consumption.** Dissolved oxygen in cell suspensions was measured polarographically using a Clark-type electrode (Dual digital-model 20, Rank Brothers Ltd., Cambridge, U.K.) at 25 ºC. The apparatus consisted of a twin oxygen chamber, which enabled a control experiment to be conducted concurrently. Logarithmic-phase *P. aeruginosa* cells were prepared in Tris buffer as above. The assays were performed in 1.5 ml of Tris buffer at 25 ºC. Cell suspensions (10$^7$ cells/ml) were pre-incubated for 15 min at 37 ºC with rhLf (1, 2, 4 µM) or hTf (4, 8, 12 µM). The viability of the cells was determined at 30 min by removing aliquots from the oxygen chambers and plating the subsequent dilutions on TSB agar plates.

**DCIP reducing activity.** Cultures of *P. aeruginosa* were grown in TSBN medium to mid-log phase in anaerobiosis. To maintain strict anaerobic conditions all the following steps were performed inside the anaerobic chamber. Briefly, the cells were harvested using a microcentrifuge (Spectrafuge 24D, Labnet Int., Edison, NJ), washed in Tris buffer and adjusted to ~10$^7$ cells/ml in the same buffer. Cells were pre-incubated (3 min) with or without 3 µM rhLf or 12 µM hTf before the addition of DCIP (0.2 mM, final concentration). Next, 1.2 ml aliquots were removed at pre-determined times and the cells harvested by centrifugation. The supernatant (1 ml) was retained, transferred to a sterile cuvette, and sealed in the anaerobic chamber. The absorbance ($A_{600}$) of DCIP at 25 ºC was immediately recorded by a Shimadzu UV-1700 PharmaSpec spectrophotometer. The concentration of oxidized DCIP was calculated using a molar extinction coefficient of 11.1 mM$^{-1}$ cm$^{-1}$. All colorimetric
measurements were repeated in three independent studies for each sample. The DCIP absorbance
was also measured in the presence of 3 µM rhLf or 12 µM hTf without cells.

**ATPase activity.** The influence of rhLf or hTf on ATPase activity was determined using a
colorimetric ATPase assay according to the manufacturer’s recommendations. The assays were
performed using membrane preparations (40 to 90 µg/100 µl) previously incubated with PiBind resin
to remove the free inorganic phosphate (Pi). The membrane samples were pre-incubated for 10 min
at 37 °C with rhLf and hTf (10, 20, 30 µM) or 0.5 mM DCCD to measure the effect of these
compounds on the ATPase activity. The amount of Pi-released was calculated by spectrophotometry
(A650). For all experiments, calibration was performed using a standard range of Pi concentrations,
and data were determined in a minimum of three independent assays.

**ATP-dependent proton translocation.** Inverted membrane vesicles were prepared as described
previously (7). Briefly, *L. lactis* cells were grown in M17G medium to late exponential phase,
washed, and resuspended in MMK buffer (20 mM MOPS-KOH, 10 mM MgCl₂, and 300 mM KCl,
pH 7.3), and treated with 0.1 mg/ml lysozyme for 18 h. Next, cell suspensions containing 0.2 mM
PMSF and 100 µg/ml RNAase were passed twice through a French press (15,000 p.s.i.) and
centrifuged (13,000 x g, 30 min, 4 °C) to remove cell debris. The formed vesicles were harvested by
ultracentrifugation (125,000 x g, 1 h, 4 °C) and resuspended in 10 mM Hepes-KOH buffer (pH 7.5)
supplemented with 10% glycerol, and stored at -80 °C.

Net ATP-dependent proton translocation was assessed using membrane vesicles pre-incubated
30 min on ice with rhLf and hTf (10, 20, 30 µM) or 0.5 mM DCCD (positive control), to determine
the effect of these proteins on the H⁺-translocation. ATP-dependent translocation was monitored by
fluorescence quenching of ACMA as described previously (7). Reaction mixtures contained
membrane vesicles (~10 mg/ml total protein) in MMK buffer. After addition of ACMA (0.25 µM),
the reaction was initiated by the addition of 1 mM ATP. ACMA fluorescence ($\lambda_{ex}= 410$ nm, $\lambda_{em}=490$ nm) was then recorded for 8 min in a spectrofluorometer. The proton ionophore CCCP (2 µg/ml) was added at the end of the reaction to correct for non-specific quenching.

**Other procedures.** Lactoferrin and transferrin were saturated with iron as was described by Kalmar and Arnold (17). The saturation status of lactoferrin (holo-rhLf) and transferrin (holo-hTf) was estimated by the ratio of $A_{465}/A_{280}$ (15) in 93% and 87%, respectively. Protein concentration was determined by a Bradford assay with bovine serum albumin as the standard. Nitrate utilization and arginine deaminase activity were determined with a nitrate-specific electrode (Mettler-Toledo DX262-NO3) and monitoring the production of citrulline from arginine, respectively (3, 16).

**Statistical analysis.** Data are expressed as means ± S. D., and significance was determined by using a Student’s $t$ test. A value $P < 0.05$ was considered to be significant.

**RESULTS**

**Antibacterial activity.** The concentrations of lactoferrin or transferrin that resulted in 90% ($IC_{90}$) and 50% ($IC_{50}$) reductions in cell viability were determined as summarized in Table 1. Data from killing experiments performed at pH 5.5 and pH 7.4, indicated the greatest efficacy of lactoferrin on *P. aeruginosa* and *L. lactis* at pH 5.5. A similar result was obtained with transferrin and *P. aeruginosa* cells (Table 1).

*P. aeruginosa* cells suspended in Tris buffer were susceptible to rhLf and hTf in a dose and time-dependent manner (Fig. 1). Of note, *L. lactis* cells were susceptible to lactoferrin but not to transferrin (Table 1). Such resistance to the killing activity of hTf remained unchanged even at high hTf-concentrations (25 µM) and extended (3 h) incubation time (Fig. 1D), as well as at acidic (pH
5.6) and alkaline pH (pH 7.4). The antimicrobial activity of the iron-free (apo) proteins was higher than that iron-saturated (holo) proteins (Table 1).

Impact of environmental and metabolic conditions on antimicrobial activity. Previous studies suggested an energy dependence for lactoferrin killing that might reflect a requirement for active bacterial metabolism during the cidal effect (5, 6, 25). We therefore performed bactericidal assays using *P. aeruginosa* and *L. lactis* cells under different environmental and metabolic conditions. The influence of the anaerobic respiration on the apo-rhLf and apo-hTf antimicrobial effect was determined using *P. aeruginosa* cells maintained in anaerobiosis. The antimicrobial assays were performed using cells resuspended in Tris buffer with or without 5 mM KNO$_3$ under anaerobic conditions. Cell suspensions containing KNO$_3$ were susceptible to rhLf and hTf (37% ± 9% and 52% ± 8% cell viability, respectively). Interestingly, in the absence of KNO$_3$ the number of viable cells treated with rhLf and hTf was not substantially modified versus controls (Table 2).

The efficacy of rhLf and hTf on non-respiring *P. aeruginosa* cells deriving energy (i.e. ATP) under conditions of strict anaerobiosis was also tested. Arginine-consuming *P. aeruginosa* cells were not susceptible to rhLf or hTf (Table 2). In contrast, glucose-fermenting *L. lactis* cells were susceptible to a bactericidal concentration of rhLf (0.125 µM), but not to 25 µM hTf (Table 2).

Control assays performed in parallel in 10 mM Tris-HCl (pH 7.4) further demonstrated that *P. aeruginosa* cells were able to utilize nitrate and L-arginine under these experimental conditions (data not shown), as previously described (3, 16).

Effect of transferrins on cytoplasmic membrane integrity and function. Since the antimicrobial activity on *P. aeruginosa* cells was dependent of the environmental conditions (external pH, aerobiosis vs. anaerobiosis) but independent of the cellular energetic state, we performed experiments
to determine whether membrane integrity or the function of some associated membrane elements (i.e. electron respiratory chain components) were modified by apo-transferrins.

Permeabilization. To determine whether cells exhibit elevated membrane permeability following lactoferrin or transferrin treatment, measurements for intracellular propidium iodide accumulation were undertaken in *P. aeruginosa* and *L. lactis* cells. The number of *P. aeruginosa* cells showing PI-accumulation after incubation with bactericidal concentrations of rhLf (1 µM) or hTf (4 µM) was 6% ± 5% and 8% ± 5%, respectively (Fig. 2A). *L. lactis* cells also showed a low level of PI accumulation, as observed by the increased fluorescence intensity in 9% ± 3% and 8% ± 2% of the cells following treatment with 0.125 µM rhLf and 4 µM hTf, respectively. This degree of permeabilization was not significantly different from untreated controls (Fig. 2A). However, in positive control assays, the percentage of permeabilized cells by exposure to a bactericidal concentration of the peptide Lfpep (50 µM) was high (82% ± 6%, *P. aeruginosa*; 84% ± 8%, *L. lactis*), indicating a disruption of the cytoplasmic membrane sufficient for PI permeabilization (Fig. 2A). Lfpep is an antimicrobial cationic peptide derived from lactoferrin that permeabilizes bacterial and fungal cytoplasmic membranes (24).

Transmembrane potential. Since changes in the transmembrane electrical potential (∆ψ) may reflect ion movements through cell membranes, we next studied whether the previously reported ability of lactoferrin to modify the transmembrane electrical potential (∆ψ) of *P. aeruginosa* cells and other microorganisms may also occur with *L. lactis* cells (2, 24, 25).

In *L. lactis* cells metabolizing glucose, the ∆ψ-generation depends on the H⁺-extrusion mediated by F₀F₁-ATPase (8). In our assays, the addition of glucose to *L. lactis* cells increased the transmembrane ∆ψ, resulting in the accumulation of the dye DiSC₃(5) into the cells and decreased fluorescence (Fig. 2B). As expected, the ∆ψ was negligible in cells pre-incubated with 1 mM DCCD,
a specific inhibitor of H+-ATPase used as positive control. In a similar way, cells pre-treated with a
bactericidal concentration of rhLf (2 µM) were unable to generate a detectable transmembrane
electrical potential. However, in agreement with the observed resistance of *L. lactis* to transferrin, a
\( \Delta \psi \) was detected in cells pre-incubated with 20 µM transferrin. The subsequent addition of
valinomycin (2 µM) dissipated the transmembrane \( \Delta \psi \), resulting in the release of the dye from the
cells and increased fluorescence (Fig. 2B).

**Effect of transferrins on *P. aeruginosa* respiration.** *P. aeruginosa* cells were not susceptible
to rhLf or hTf under anaerobic conditions (Fig. 3A). The effect of respiratory chain inhibitors such as
antimycin A, piericidin A and sodium azide, or the uncoupler CCCP on the bactericidal activity of
rhLf and hTf were different (Fig. 3B, 3C). The antimicrobial activity of rhLf and hTf was not
inhibited in cells previously incubated with antimycin A (10 µM) or sodium azide (1 mM) for 15 min
at 37 °C. However, *P. aeruginosa* pre-incubated (15 min, 37 °C) with piericidin A (32 µM), an
inhibitor of the bacterial type I NADH-dehydrogenase (complex I), were significantly \( P < 0.05 \) less
susceptible to the killing activity of 1 µM rhLf and 4 µM hTf (73% ± 7% and 71% ± 4% cell
survival, respectively). The pre-incubation (15 min, 37 °C) with the proton ionophore CCCP (50 µM)
also prevented the antimicrobial activity but only when the concentration of these proteins was \( \leq \text{IC}_{50} \)
(Fig. 3B, 3C).

*P. aeruginosa* cells consumed oxygen in the absence of an exogenous energy source (Fig 3D).
Oxygen utilization data obtained from cell suspensions exposed to high bactericidal concentrations of
rhLf (3 µM) or hTf (12 µM) indicated a rate of consumption of oxygen similar to the untreated cells.
Similarly, the respiration of cells energized by the addition of succinate (33 mM) was not inhibited
by these proteins (data not shown). In control assays, the pre-incubation of the cells with 32 µM
piericidin A resulted in an immediate decrease in respiration (Fig. 3D).
Importantly, cells maintained in strict anaerobiosis were susceptible to rhLf and hTf only when pre-incubated (5 min) with DCIP, an artificial electron acceptor (Fig 3E). In the presence of 0.2 mM DCIP, the cell viability of rhLf and hTf-treated cells significantly ($P < 0.05$) decreased to 27% ± 3% and 34% ± 6%, respectively. The absorbance of DCIP in *P. aeruginosa* cell suspensions treated with 3 µM rhLf or 12 µM hTf was also significantly (~70%, 90 min) decreased with respect to the control cells assayed identically in the absence of either protein (Fig. 3F). DCIP was not reduced by rhLf or hTf (Fig. 3F) and was not cytotoxic at the assayed concentrations (data not shown). These data indicated a correlation between loss in viability of rhLf-treated cells and DCIP reduction.

In conclusion, changes in the transmembrane electrical potential observed in *P. aeruginosa* (2) and *L. lactis* cells were not correlated with a permeabilization of cell membranes by transferrins, suggesting an alteration of proton homeostasis. Supporting this notion, respiratory activity of *P. aeruginosa* cells was an indispensable condition for the antimicrobial effect of transferrins. This requirement was interpreted as an event associated to changes of the pH gradient and intracellular pH due to these proteins being unable to inhibit the cellular respiration.

**Effect of transferrins on $H^+$-ATPase.** To substantiate the assertion that cytoplasmic proton homeostasis could be modified by apo- and holo-transferrins, the ATPase activity was assayed using isolated membranes. The assessment of the ATPase activity was chosen because, under our experimental conditions, the ATPase complex is the only functional and common membrane component involved in the proton flux through membranes in both studied species. This function has a critical role for the maintenance of the proton gradient and intracellular pH in many bacterial species.

**Effect of transferrins on ATPase-coupled $H^+$-transport.** Inhibition of ATP-dependent proton translocation by rhLf and hTf was determined by fluorescence quenching of ACMA using inverted
membrane vesicles from *L. lactis* cells (Table 3). The percentage of fluorescence in vesicle assays containing 20 µM apo-lactoferrin or holo-lactoferrin was almost three times higher compared to those in the absence of the protein, suggesting a decrease of the transmembrane proton gradient generated at the expense of ATP. A decrease in fluorescence quenching (~32%) was also observed in control assays using the ATPase inhibitor DCCD (0.5 mM). No significant decrease in the ACMA-quenching was detected in the presence of transferrin (Table 3).

Effect of transferrins on ATPase activity. The effect of lactoferrin or transferrin on ATP hydrolysis catalyzed by the membrane fraction of *L. lactis* cells is shown in Table 3. ATP hydrolysis decreased to approximately 24%, 27%, and 25% of the initial rate after pre-incubation with 10, 20 and 30 µM rhLf, respectively (Table 3). Similarly, the amount of hydrolyzed ATP decreased to ~35% after pre-incubation with 20 µM holo-lactoferrin. In the presence of different concentrations (10, 20, 30 µM) of hTf, ATPase activity was similar to that of the control (Table 3), and was not modified at different pH values (5.5, 6.5) or MgCl₂ concentrations (1, 2.5, 3 mM) (data not shown). In control assays, ATPase activity was significantly inhibited (~74% of inhibition) by 0.5 mM DCCD, a specific inhibitor of the bacterial F₁F₀-ATPase.

DISCUSSION

The bactericidal activity of lactoferrin, independent of iron withholding, was first reported by Arnold *et al.* (5) but the antimicrobial mechanism has not been elucidated. In this study, we assumed that in two susceptible and different bacterial species, differing in their structural and metabolic features, the possibility of a common target for transferrins was limited to the number of characteristics that these microorganisms share. Consequently, we used *P. aeruginosa* and *L. lactis* species as a comparative experimental model to identify the antimicrobial target of these innate immune proteins.
Data from distinct experiments indicated that the antimicrobial activity of lactoferrin and transferrin was independent of the energetic state of the cells (i.e. ATP synthesis). This interpretation was substantiated by the observation that *P. aeruginosa* cells obtaining energy (i.e. ATP) by anaerobic respiration (NO$_3$) were susceptible to these proteins, while cells deriving energy from substrate-level phosphorylation (L-arginine) were resistant. In addition, it seemed unlikely that interference with the energy metabolism was a common mechanism of lactoferrin action due to the fact that glucose-fermenting and resting *L. lactis* cells were both susceptible to lactoferrin. This result is in agreement with previous observations showing differences in lactoferrin susceptibility of the same bacterial strains growing under different nutritional conditions (4).

Interestingly, the bactericidal activity on *P. aeruginosa* cells was only observed in respiring cells when terminal electron acceptors (O$_2$ or NO$_3$) were available. Although these observations pointed to a blocking effect on the respiratory chain, the cellular O$_2$-consumption in the presence or absence of rhLf and hTf was similar. Moreover, *L. lactis* cells lacking a respiratory chain were also susceptible to rhLf, indicating that components of the respiratory chain were not the targets of these proteins. These findings prompted us to investigate if the bactericidal effect was associated to the proton flux mediated by respiratory chain which is involved in the generation of a transmembrane pH gradient and intracellular pH regulation. Since changes in the transmembrane electrical potential ($\Delta\psi$) may reflect modifications of pH gradient ($\Delta$pH), the previously reported ability of rhLf to modify the $\Delta\psi$ of *P. aeruginosa* cells (2) supported the above suggestion.

Despite the consumption of oxygen being similar in rhLf or hTf-treated and untreated *P. aeruginosa* cells, the functionality of all or part of the respiratory chain was essential for the antimicrobial activity. This was supported by the fact that piericidin A, a specific inhibitor of the type I NADH dehydrogenase (NADH$_d$-typeI), inhibited the bactericidal effect on respiring *P. aeruginosa* cells. The NADH$_d$ complex (complex I), couples the transfer of electrons from NADH to ubiquinone (CoQ) or menaquinone (mQ), facilitating the translocation of protons across the cytoplasmic
membrane (26). However, antimycin A or azide-treated cells were susceptible to rhLf and hTf, a finding that may be explained by the possible employment of alternative respiratory pathways (i.e. cyanide-insensitive respiratory pathway) to circumvent inhibition by such inhibitors (9, 28). Supportive evidence showing that respiratory function was indispensable for the antimicrobial activity was provided by the observation that, under anaerobic conditions, the resistance to rhLf and hTf of *P. aeruginosa* cells was reverted by an artificial electron acceptor (DCIP). This chemical agent is reduced by electrons donated from components of the respiratory chain (i.e NADH dehydrogenase and cytochromes) coupling a H⁺-translocation from the cytoplasm. Therefore, we concluded that the loss of the viability of *P. aeruginosa* cells treated with rhLf or hTf correlated with H⁺-extrusion mediated by the respiratory chain, suggesting an alteration of pH gradient and internal pH.

Since *L. lactis* species lacks a functional respiratory chain, both proton gradient and internal pH regulation depends on the H⁺-extrusion mediated by H⁺-ATPase (7, 10, 12, 19). Consequently, this complex was investigated as the possible common element in both bacterial species that might be targeted by transferrins. We assumed that the susceptibility of *L. lactis* cells to lactoferrin could be due to a lethal perturbation of the intracellular pH and proton gradient due to an inhibition of the ATPase complex (Fig. 4A). This supposition was inferred from experiments in which the Δψ-generation by rhLf-treated and untreated cells metabolizing glucose was compared. Lactoferrin-treated cells were unable to generate a membrane potential suggesting an inhibition of H⁺-ATPase. Finally, direct evidence of the inhibition of the ATPase complex by lactoferrin was provided by the inhibition of the ATPase activity and the H⁺-translocation using plasma membrane fractions and inverted membrane vesicles of *L. lactis* cells, respectively.

The identification of H⁺-ATPase as the target of lactoferrin in *L. lactis* cells suggested a similar mode of action in *P. aeruginosa* cells, and the above results obtained with this last species were interpreted as follows. The susceptibility of DCIP-treated *P. aeruginosa* cells to rhLf or hTf in the
absence of terminal electron acceptors (O$_2$ or NO$_3^-$) was compatible with DCIP reduction, performed
by components of the respiratory chain, coupled to the extrusion of protons which were unable to
return to the cytoplasm via ATPase (Fig. 4B). The reduction of DCIP was observed only in rhLf and
hTf-treated cells, further implying that this reagent was reduced by component(s) of the respiratory
chain as a response to the uncoupling effect caused by rhLf or hTf.

The suggested selective inhibition of the ATPase complex by lactoferrin and transferrin may
also explain the respective susceptibility and resistance observed in respiring and non-respiring P.
aeruginosa cells. We hypothesize that in respiring P. aeruginosa cells treated with rhLf or hTf, re-
entry of some protons previously extruded by components of the respiratory chain (i.e. NADH
dehydrogenase and cytochromes) is blocked by interactions of rhLf or hTf with the H$^+$-ATPase
complex. In this model, the subsequent loss of intracellular pH regulation and modification of the
proton gradient ultimately lead to the cell death (Fig. 4B). A similar requirement of a functional H$^+$-
ATPase to recover the protons translocated during NADH oxidation coupled with O$_2$-reduction has
recently been proposed (22).

It was of note that our findings indicate that bacterial cell death induced by transferrins is not
caused by cell damage (e.g. membrane permeabilization) but involves an active cooperation of the
cell. This observation suggest that the final antimicrobial effectiveness of transferrins depends on the
local context where these proteins are secreted. For example, in the lung infection of patientes with
cystic fibrosis the anaerobic conditions present in the bacterial biofilms could protect P.
aeruginosa of the high lactoferrin concentration accumulated in the mucosal fluid, avoiding the
erradication of this opportunistic pathogen from the airways of cystic fibrosis patients.

Since bacterial cell wall is an effective permeability barrier to large proteins, the way by which
transferrins (>78 kDa) reach a target (i.e. H$^+$-ATPase) on the cytoplasmic membrane has yet to be
elucidated. A possible explanation could be the enzymatic generation of lactoferrin-derived peptides
with antimicrobial activity. This suggestion is supported by our recent data showing that kaliocin-1, a
human lactoferrin-derived antimicrobial peptide which corresponds to the common $\gamma$-core motif found in all antimicrobial cysteine-peptides (24, 29, 30), may be obtained by enzymatic digestion from lactoferrin. Interestingly, mass spectrometry analysis has shown that this fragment maintain intact the tridimensional structure of the $\gamma$-core motif similar to that predicted in the native hLf molecule, suggesting that its potential antimicrobial activity could be also preserved (unpublished results).

It is known that apo-lactoferrin exhibit a higher bactericidal activity than that holo-lactoferrin (4, 17), also observed in our bacterial killing assays. However, measurements of the ATPase activity in vitro suggest that the mechanism of action proposed by us could be independent of the iron-saturation state of lactoferrin. Both, ATPase activity and proton translocation on membrane vesicles were inhibited less efficiently but not significantly by holo-lactoferrin. The absence of correlation between the low antimicrobial activity of holo-lactoferrin and our data from ATPase activity experiments suggest the involvement of a concomitant unknown effect associated to the iron-saturated state of lactoferrin that requires further investigation.

Beyond the current studies, work is in progress to characterize the potential molecular interactions between the transferrin proteins and the H$^+$-ATPase complex. In this respect, transferrin-resistance of L. lactis but not P. aeruginosa would be an interesting start point to determine the H$^+$-ATPase domain(s) involved in such a ligand/receptor interaction. These findings also support recent discoveries in evolutionary and phylogenetic relationships among transferrins and other endogenous host defense proteins. For example, in the transferrin protein family, we have reported the presence of evolutionarily conserved 3D-structures (i.e. $\gamma$-core motif) previously associated with the antimicrobial activity of all classes of cysteine-stabilized host defense peptides (29, 30). Given the recent discovery that $\gamma$-core motifs may mediate targeting of ion channels in microbial pathogens (31), this convergent structural motif could be involved in peptide interactions with one or more specific domains of H$^+$-ATPase and inhibition of this essential cellular component.
In summary, the body of data presented herein suggest that the *in vitro* bactericidal effect of mucosal human lactoferrin and its serum counterpart, transferrin, involves selective inhibition of the H^+-ATPase complex. As a result, H^+-ATPase-mediated flux of protons is impaired, yielding effects principally relating to deficiencies in intracellular pH homeostasis inducing cell death. To our knowledge, this is the first description of the interaction of an extracellular human protein with a bacterial H^+-ATPase. These findings suggest new opportunities to target energetic systems in bacterial pathogens as a means to discover and develop novel anti-infective agents and therapeutic strategies.

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**References**


Table 1. Determination of inhibitory concentrations of lactoferrin and transferrin

<table>
<thead>
<tr>
<th>Microorganism (pH)</th>
<th>Lactoferrin (µM)</th>
<th>Transferrin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₉₀</td>
<td>IC₅₀</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5.5)</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>(7.4)</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>(7.4)</td>
<td>2*</td>
<td>1*</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5.5)</td>
<td>0.062</td>
<td>ND</td>
</tr>
<tr>
<td>(7.4)</td>
<td>0.125</td>
<td>ND</td>
</tr>
<tr>
<td>(7.4)</td>
<td>NS*</td>
<td>NS*</td>
</tr>
</tbody>
</table>

*P. aerugiosa* or *L. lactis* cells were incubated (90 min) with different concentrations (ranging from 0.03 to 25 µM) of lactoferrin or transferrin in 10 mM MES (pH 5.5) and 10 mM Tris-HCl (pH 7.4). Cell viability was calculated with respect to the non treated cells (control) using a counting-plate method. ND, not determined; NS, no susceptible at ≥ 25 µM transferrin. (*) Values obtained with iron-saturated proteins.
Table 2. Effect of environmental conditions and substrates on the bactericidal activity of transferrins

| Incubation | Substrate (concentration) | Cell viability (% of control)
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rhLf&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;P. aeruginosa&lt;/i&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>-</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>-</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>KNO&lt;sub&gt;3&lt;/sub&gt; (5 mM)</td>
<td>37 ± 9</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>L-arginine (40 mM)</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>&lt;i&gt;L. lactis&lt;/i&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic</td>
<td>-</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>glucose (1%)</td>
<td>8 ± 4</td>
</tr>
</tbody>
</table>

<sup>a</sup> <i>P. aeruginosa</i> or <i>L. lactis</i> cells were incubated (90 min) in 10 mM Tris-HCl (pH 7.4) with or without the indicated substrates under the specified environmental conditions. <sup>b</sup> Values are means ± S. D. from duplicates of at least three independent experiments. <sup>c</sup> The IC<sub>90</sub> of lactoferrin (rhLf) and transferrin (hTf) were used. <sup>d</sup> hTf (25 µM).
Table 3. Effect of transferrins on ATP-driven proton translocation and ATPase activity of *L. lactis*.

<table>
<thead>
<tr>
<th></th>
<th>Final ACMA-fluorescence (% of initial fluorescence)</th>
<th>ATPase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32 ± 0.7</td>
<td>8.8 ± 0.6 (100)</td>
</tr>
<tr>
<td>DCCD (0.5 mM)</td>
<td>64 ± 0.5</td>
<td>2.3 ± 0.3* (26)</td>
</tr>
<tr>
<td>Lactoferrin (10 µM)</td>
<td>56 ± 0.8</td>
<td>2.1 ± 0.9* (24)</td>
</tr>
<tr>
<td>(20 µM)</td>
<td>86 ± 0.4</td>
<td>2.4 ± 0.5* (27)</td>
</tr>
<tr>
<td>(20 µM)</td>
<td>77 ± 0.7</td>
<td>3.1 ± 0.8* (35)</td>
</tr>
<tr>
<td>(30 µM)</td>
<td>ND</td>
<td>2.2 ± 1.5* (25)</td>
</tr>
<tr>
<td>Transferrin (10 µM)</td>
<td>37 ± 0.9</td>
<td>8.3 ± 0.9 (94)</td>
</tr>
<tr>
<td>(20 µM)</td>
<td>ND</td>
<td>8.2 ± 0.7 (93)</td>
</tr>
<tr>
<td>(20 µM)</td>
<td>ND</td>
<td>8.1 ± 0.2 (92)</td>
</tr>
<tr>
<td>(30 µM)</td>
<td>39 ± 0.2</td>
<td>8.7 ± 1.2 (99)</td>
</tr>
</tbody>
</table>

*Percentage of 9-amino-3-chloro-7-methoxyacridine (ACMA) fluorescence.  
*µmol Pi min⁻¹ (mg protein)⁻¹ at 30 °C.  
Iron-saturated protein. Percentage of activity with respect to the control. ND, not determined. Values are means ± S. D. of at least three experiments. *P < 0.05.
**Figure legends**

**Fig. 1. Kinetics of bactericidal activity of transferrins.** *P. aeruginosa* cells (10^6 cells/ml) were incubated (37 ºC) with 0.125 µM (○), 0.5 µM (□), 1 µM (▼) lactoferrin (A) or with 1 µM (○), 2 µM (□), 4 µM (▼) transferrin (B). *L. lactis* cells (10^6 cells/ml) were incubated (30 ºC) with 0.031 µM (○), 0.062 µM (□) or 0.125 µM (▼) lactoferrin (C), and 4 µM (○) or 25 µM (□) transferrin (D). At given timepoints, aliquots were plated and colonies were counted after 24 h. The results are the means ± S. D. from duplicates of at least three independent experiments.

**Fig. 2. Effect of transferrins on the cytoplasmic membrane.** A) *P. aeruginosa* and *L. lactis* cells incubated with or without (control) 1 µM lactoferrin (rhLf), 0.125 µM transferrin (hTf) or 50 µM Lfpep (positive control) and stained with 1 µg/ml propidium iodide (PI). B) Effect of transferrins on the Δψ of *L. lactis* cells. The addition of glucose to cell suspensions (10^7 cells/ml) resulted in the generation of a membrane potential, observed as a decrease in DiSC3(5)-fluorescence, in control assays (control). Cells were pre-incubated (30 min, 30 ºC) with 2 µM lactoferrin (rhLf), 20 µM transferrin (hTf) or 1 mM DCCD. The addition of glucose and 2 µM valinomycin (Val) are indicated (arrows). Fluorescence is expressed in arbitrary units (A. U.).

**Fig. 3. Influence of respiration on the bactericidal activity of lactoferrin.** A) Viability of *P. aeruginosa* cells (10^6 cells/ml) incubated 90 min at 37 ºC with 1 µM rhLf or 4 µM transferrin in aerobiosis and anaerobiosis. B and C) experimental conditions were the same as those described for A, except the assays were performed aerobically in the presence of the respiration inhibitors and uncouplers: 1 mM azide (■), 10 µM antimycin (■), 50 µM CCCP (■), 32 µM piericidin A (■). The activity of different concentrations of rhLf (■) or hTf (□) alone was tested. D) Consumption of oxygen in *P. aeruginosa* resting cells (○) or incubated with 3 µM rhLf (■), 12 µM hTf (△) or 32
µM piericidin A (▼), as positive control, added 15 min before the O$_2$-consumption measurement. E) Viability of *P. aeruginosa* cells treated with rhLf (■) or hTf (△) in the presence of different concentrations of the electron acceptor DCIP under anaerobic conditions. F) Concentration of oxidized DCIP in control cell suspensions (○), after addition of 3 µM rhLf (■) or 12 µM hTf (△), and in the presence of 3 µM rhLf (▼) or 12 µM hTf (◆) without cells. *, $P < 0.05$ versus untreated controls.

**Fig. 4. Hypothetical mechanism of the antimicrobial action of transferrins.** A) In *L. lactis* glucose-fermenting cells, H$^+$-pumping through the ATPase (a) is essential for generation of a proton motive force across the cytoplasmic membrane (CM) and for intracellular pH homeostasis, the latter is critical for cell survival. The blocking effect of lactoferrin (Lf) on H$^+$-ATPase (b) causes an intracellular H$^+$-accumulation, then the acidification of the bacterial cytoplasm reach levels incompatible with cell life. *L. lactis* cells lack a functional respiratory chain (NADH$_d$-typeII, mQ, and Cyt $bd$) under these experimental conditions. B) Respiration of *P. aeruginosa* coupled to ATP synthase-mediated phosphorylation (a) is uncoupled by the blocking effect of lactoferrin (Lf) or transferrin (Tf) on the ATPase complex (b). Under these experimental conditions, the H$^+$-accumulation in the periplasmic space (PS) leads to the cell death. Cellular protection was observed when protons were not pumped to PS (i.e. anaerobiosis or by inhibition of NADH$_d$-type I with piericidin A). In anaerobiosis, the presence of the electron acceptor DCIP promotes the H$^+$-pumping mediated by ETC components (e.g. NADH$_d$-type I) yielding a lethal effect in Lf or Tf-treated cells. In *P. aeruginosa*, electrons are donated from cyt $c$ to either cyt $cbb_3$I, cyt $cbb_3$II or cyt $aa_3$ (white). Cyt CIO and cyt $ba_3$ (grey) directly accept electrons from CoQ (9). Relevant steps of anaerobic respiration are indicated (green arrows).
Fig. 1
Fig. 3
Fig. 4

A. *L. lactis*

B. *P. aeruginosa*