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Metal complexes of lactoferrin and their effect on the intracellular multiplication of *Legionella pneumophila*

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Abstract

The action of bovine lactoferrin saturated with iron, zinc and manganese on the intracellular multiplication of *Legionella pneumophila* in HeLa cells has been tested. The results obtained showed that lactoferrin did not influence the invasive efficiency of *Legionella*. The intracellular multiplication of the bacterium was inhibited by apo-lactoferrin and by lactoferrin saturated with manganese and zinc, whereas lactoferrin saturated with iron enhanced the intracellular growth. Experiments in parallel were performed with iron, manganese and zinc citrate to test the effect due to the metal ions alone. Even in this condition the addition of an iron chelate enhanced the multiplication of *Legionella* while the manganese chelate produced a certain inhibition.

Introduction

Legionella species are ubiquitous bacteria, widely distributed in aquatic habitats and soil, capable of autonomous multiplication and of growing within parasitized cells (Oldham & Rodgers 1985, Fields 1993, Horwitz 1993). Similarly to other microrganisms these bacteria must obtain from their environment all the essential nutritional elements and among these iron has a particular relevance. It has been shown that in culture media the growth of *Legionella* is significantly related to the iron content of the medium, as demonstrated by varying the availability of this element or by iron sequestering by means of chelating agents (Goldoni *et al.* 1991). Significant results have been obtained by studying the multiplication of *Legionella* in the presence of iron chelating agents as desferal (Quinn & Weinberg 1988), enterochelin (Revees *et al.* 1983) and transferrins (Quinn & Weinberg 1988) in relation to their degree of iron saturation (Bortner *et al.* 1986, Byrd & Horwitz 1991b). When *Legionella pneumophila* is growing intracellularly in monocytes the iron acquisition takes place from the intermediate labile iron pool of the host cell (Byrd & Horwitz 1989, 1991a, 1991b, 1993). This pool derives its iron primarily from iron-transferrin via transferrin receptors, iron-lactoferrin via lactoferrin receptors, and the iron storage protein ferritin, which recycles iron to the pool (Shuman & Horwitz 1996). Consistent with this hypothesis, deferoxamine, an iron chelator that chelates intracellular iron from the labile iron pool, completely inhibits the intracellular multiplication of *L. pneumophila* (Green & Byrd 1997). This chelating effect of deferoxamine is completely reversed by the addition of iron-saturated transferrin but not by apotransferrin, as previously demonstrated by Byrd & Horwitz (1989).

In addition to iron, transferrin binds a number of other transition metals (Valenti *et al.* 1987) yielding complexes in which the overall physico-chemical properties of the protein are not changed, with the sole exception of a small perturbation of the metal binding-site conformation (Chasteen & Woodworth 1990, and references therein). However, the affinity of the protein for the different metals under the same conditions varies, being higher for iron ($Fe^{3+} > Cu^{2+} > Zn^{2+} > Mn^{2+}$) (Aisen 1980). In the present paper results are reported concerning the ac-

tion of apolactoferrin from bovine milk (apo-BLf) and bovine lactoferrin (BLf) saturated with iron (Fe^{3+}-BLf), manganese (Mn^{2+}-BLf) and zinc (Zn^{2+}-BLf) on intracellular multiplication of *L. pneumophila*.

Materials and methods

Bacterial strain and media

A virulent clinical isolate of *L. pneumophila* serogroup 6, strain Monza 3/1386 (Goldoni *et al.* 1995), which was subcultured only twice after isolation, was used. The strain was stored as a stock culture at −70 °C in skimmed milk, and subcultured once on buffered charcoal-yeast extract agar with α-ketoglutarate 0.1% (BCYE-α-agar) (Oxoid, Unipath Limited, Basingstoke, Hampshire, England) supplemented with Legionella growth supplement (Oxoid) before use.

A different medium was used in the experiments on the utilization of various iron sources. It consisted of yeast extract broth (Difco Laboratories, Detroit, MI, USA) 10 g l^{-1} supplemented with L-cysteine (400 mg l^{-1}) and alternatively Fe^{3+}-Lf, $FeSO_4$, $FeCl_3$. The concentration of these three compounds was calculated on the basis of the iron content of the chemically defined medium by Reeves *et al.* (1983) in order to obtain a suitable iron concentration supporting bacterial growth. A half concentration, with an iron content comparable to that present in 1 mg ml^{-1} completely saturated Fe^{3+}-BLf, was also used. In these tests the bacterial inoculum was 10^5 colony forming units (cfu) ml^{-1}. The visible growth of *Legionella* was controlled after 72 and 96 h.

Cells

HeLa S3 cells were grown at 37 °C in Eagle's minimal essential medium (MEM) containing $NaHCO_3$ 1.2 g l^{-1}, heat-inactivated foetal calf serum 10% and 2 mM glutamine. The same medium containing 2% foetal calf serum was used to maintain the cells.

Chemicals

Apo-BLf was purchased from Fluka Chemie AG, Buchs, Switzerland. Lactoferrin purity was checked by SDS-PAGE stained with silver nitrate. Fe^{3+}BLf, Mn^{2+}BLf and Zn^{2+}BLf were prepared by incubation of the apo-protein dissolved in 0.1 M sodium bicarbonate with a ten-fold excess of the citrate complex

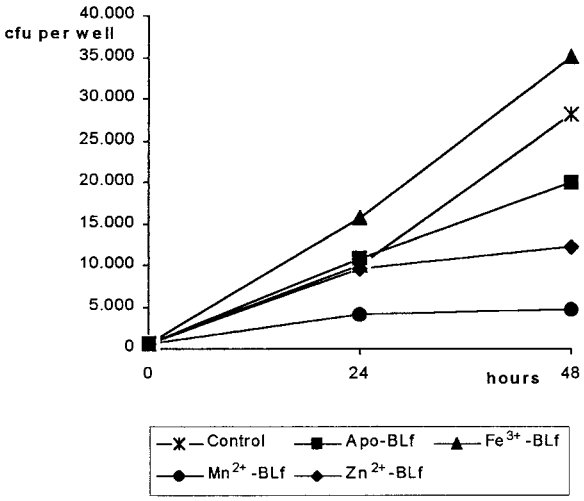

Figure 1. Effect of lactoferrin chelated with different metal ions on *Legionella pneumophila* intracellular multiplication. On the abscissa the time 0 corresponds to the first bacterial count after the removal of gentamycin (3 h after the infection).

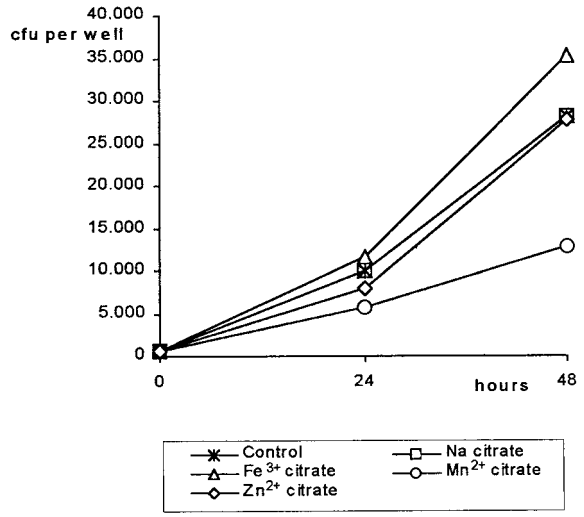

Figure 2. Effect of citrate complexed with different metal ions on *Legionella pneumophila* intracellular multiplication. On the abscissa the time 0 corresponds to the first bacterial count after the removal of gentamycin (3 h after the infection).

of the different metal ions for 12 h at room temperature, followed by extensive dialysis against 0.1 M sodium bicarbonate to remove unligated metal ions. After incubation of the protein in MEM for 1 and 2 h, in the presence or absence of cells, the metal saturation of the three BLf preparations was determined by an atomic adsorption spectrophotometer Perkin-Elmer model 360. The values obtained were 10% for apo-BLf, 95% for Fe^{3+}-BLf and 85% for both Mn^{2+}-BLf and Zn^{2+}-BLf.

To supply ferric, manganese and zinc ions to the cells at the concentration of 25 μM, corresponding to that required to saturate at 100% 1 mg ml^{-1} BLf, metal ion citrate complexes were prepared from sodium citrate and chloride or sulfate salts of the different metals.

Cell toxicity test

HeLa S3 cells (4×10^5 cells ml^{-1}) were seeded in growth medium in 96-well plates (0.1 ml per well), and allowed to adhere for 24 h at 37 °C. They were then incubated with various concentrations of the different compounds in Eagle's MEM plus 2% foetal calf serum for 2 h or for 48 h at 37 °C in a moist air atmosphere containing CO_2 5%. Monolayers treated for 2 h were incubated at 37 °C for additional 24 h in drug-free medium before being examined. Monolayers treated for 48 h were observed directly after treatment. Cells were inspected to detect changes in morphology such as swelling, granularity, rounding or floating, and their viability was determined by neutral red uptake method. In addition, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid (MTT)-based assay (Cell Proliferation Kit I) (Boehringer Mannheim, GmbH, Germany) was employed for the non radioactive quantification of cellular proliferation, viability, and cytotoxicity.

Antibacterial activity

The minimal inhibitory concentration (MIC) was determined in serial two-fold dilutions of each chemical in yeast extract broth with Legionella growth supplement (Oxoid). Dilutions were prepared from the maximal non-cytotoxic concentration. A standardised *Legionella* suspension was added to each dilution to a final concentration of approximately 10^5 cfu ml^{-1}. MIC was considered as the lowest concentration of each compound inhibiting a visible growth after incubation for 72 h at 37 °C in CO_2 2.5%. Dilutions without visible growth were plated on BCYE-α-agar to determine the minimal bactericidal concentration (MBC).

Invasion and intracellular multiplication of Legionella

A modification of the techniques described by Finlay & Falkow (1988) and Harley & Drasar (1989) was used. For the assay HeLa cells were suspended in antibiotic-free complete Eagle's MEM, plated in 24-well culture dishes (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) at 2.2×10^5 cells per well and maintained at 37 °C in a moist air atmosphere containing CO_2 2.5%. After incubation for 24 h cell monolayers were washed twice with antibiotic-free complete Eagle's MEM and 0.25 ml of a bacterial suspension in the same medium (4×10^4 cfu ml^{-1}) were added to the cells. After 1 h of centrifugation at 4000 *g* the plates were incubated for 1 h at 37 °C. The cells were then washed five times with Eagle's MEM supplemented with gentamicin (50 μg ml^{-1}) and left in the same medium for 1 h at 37 °C to kill non-internalised bacteria. Cells were then washed twice with antibiotic-free complete Eagle's MEM and disrupted by addition of sterile double distilled water (0.2 ml per well). The number of viable bacteria in the lysate was counted as described below.

Lactoferrin preparations (apo-BLf, Fe^{3+}-BLf, Mn^{2+}-BLf, Zn^{2+}-BLf) were tested at concentration of 1 mg/ml. Citrates (Na^+, Fe^{3+}, Mn^{2+}, Zn^{2+}) were used in solutions (25 μM) in which the content of the metal was identical to that present in the saturated BLf preparations. The solutions of chelating agents were added together with the inoculum in the penetration assays or after the bacterial invasion. In the invasion experiments cell monolayers were disrupted to recover intracellular bacteria three hours after the infection, immediatly after the incubation with gentamycin. In the intracellular multiplication assays the chelating agents were added three hours after the infection and maintained in contact with the infected cells for 24 h or 48 h. Appropriate controls were also included in which non-infected cells were maintained in the presence of the compounds throughout the tests. Values reported are means of triplicate experiments.

Bacterial counts

The number (cfu) of viable legionellas in the inoculum and in the cell lysates were assessed by plating the samples on BCYE-α-agar, and by counting the number of colonies developing after 72 h incubation at 37 °C in a moist air atmosphere containing CO_2 2.5%.

Fluorescent microscopy for the lactoferrin binding and internalization tests

(a) *BLf binding*
HeLa S3 cells, grown in tissue chamber slides (Lab-Tek, Miles Laboratories, Inc., Naperville, IL, USA) for 24 h at 37 °C in 5% CO_2, were treated with

1 mg ml^{-1} of differently saturated BLf for 1 h at 4 °C. After treatment, cells were washed three times in PBS at 4 °C and Lf binding to the plasma membrane was detected by indirect immunofluorescence.

Briefly, all steps were carried out at 4 °C by utilizing pre-cooled reagents. Cells were incubated with rabbit anti-Lf immunoglobulins (DAKO A/S, Denmark) for 60 min and, after washing five times with PBS, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit gammaglobulin antibodies (Sigma Chemical Co., St Louis, MO, USA). After 60 min, cells were washed again and fixed with 3.7% paraformaldehyde in PBS containing 2% sucrose for 10 min at room temperature. Cells were then washed twice in PBS and incubated for 10 min at room temperature in 0.5% Triton X-100 (Sigma) in the same buffer containing 10% sucrose. After washing five times in PBS, cells were mounted in buffered glycerol and examined with a Leitz Dialux epifluorescence microscope. Photomicrographs were made using Kodak Tri-X 400 ASA.

(b) *BLf internalization*

HeLa S3 cells, grown in tissue chamber slides for 24 h at 37 °C in 5% CO_2, were treated with 1 mg ml^{-1} of different BLf preparations for 1 h at 4 °C. After treatment, cells were washed three times with MEM and incubated at 37 °C for different lengths of time. BLf internalization was followed by indirect immunofluorescence. Prior to staining assays, the cells were washed three times with PBS, and fixed with 3.7% paraformaldehyde in the same buffer containing 2% sucrose for 10 min at room temperature. Cells were then washed twice in PBS and incubated for 10 min at room temperature in 0.5% Triton X100 (Sigma) in the same buffer containing 10% sucrose. Cells were washed again five times in PBS and then transferred to a moist chamber. For BLf detection, cells were covered with rabbit anti-human lactoferrin, cross-reacting with BLf, immunoglobulins, and incubated at 37 °C for 45 min. After incubation, cells were washed with PBS and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit gammaglobulin antibodies. After 45 min at 37 °C, cells were washed in PBS and mounted in buffered glycerol. All cells were examined with a Leitz Dialux epifluorescence microscope. Photomicrographs were made using Kodak Tri-X 400 ASA.

Table 1. Toxicity of different preparations of lactoferrin on HeLa cells and antibacterial activity on *L. pneumophila*

Drug	Maximal non-toxic concentrations on HeLa cells		MIC (48 h)	MBC (72 h)
	Time of contact			
	24 h	48 h		
Apo-BLf	8 mg ml^{-1}	1 mg ml^{-1}	>8 mg ml^{-1}	>8 mg ml^{-1}
Fe^{3+}-BLf	8 mg ml^{-1}	1 mg ml^{-1}	>8 mg ml^{-1}	>8 mg ml^{-1}
Mn^{2+}-BLf	4 mg ml^{-1}	1 mg ml^{-1}	8 mg ml^{-1}	>8 mg ml^{-1}
Zn^{2+}-BLf	4 mg ml^{-1}	1 mg ml^{-1}	8 mg ml^{-1}	8 mg ml^{-1}

Results

A first set of experiments was carried out in order to determine the maximal non-toxic concentration towards HeLa cells of bovine lactoferrin in apo- or differently saturated forms. Serial two-fold dilutions of this concentration were successively tested on the growth of *L. pneumophila* in yeast extract broth supplemented with Legionella growth supplement. The results showed that the highest concentration of BLfs which could be used in the experiments was 1 mg ml^{-1}, since this was the maximal non toxic concentration on HeLa cells (Table 1).

Another test was successively performed in order to ascertain the possible utilization of various iron sources by *Legionella* in culture medium. For this purpose *Legionella* was cultivated in yeast extract broth supplemented with L-cysteine and alternatively added with $FeSO_4$, $FeCl_3$ and Fe^{3+}-Blf at non antibacterial concentration. The results showed that growth of *Legionella* took place in the presence of $FeSO_4$ and $FeCl_3$, whereas when the iron was added as Fe^{3+}-BLf there was no bacterial multiplication.

A possible effect of the different preparations of lactoferrin on the adsorption and entry phase of *Legionella* was tested by means of penetration assays in which the number of internalized bacteria was compared with that observed in the absence of BLf. The results obtained showed that BLf did not influence the invasive efficiency of *Legionella* and no significant differences among the various preparations of Lf were observed (data not shown).

On the contrary, different results were obtained in the intracellular phase by following the multiplication of *Legionella* in the presence of apo-BLf and Lf saturated with different ions. In the experiments

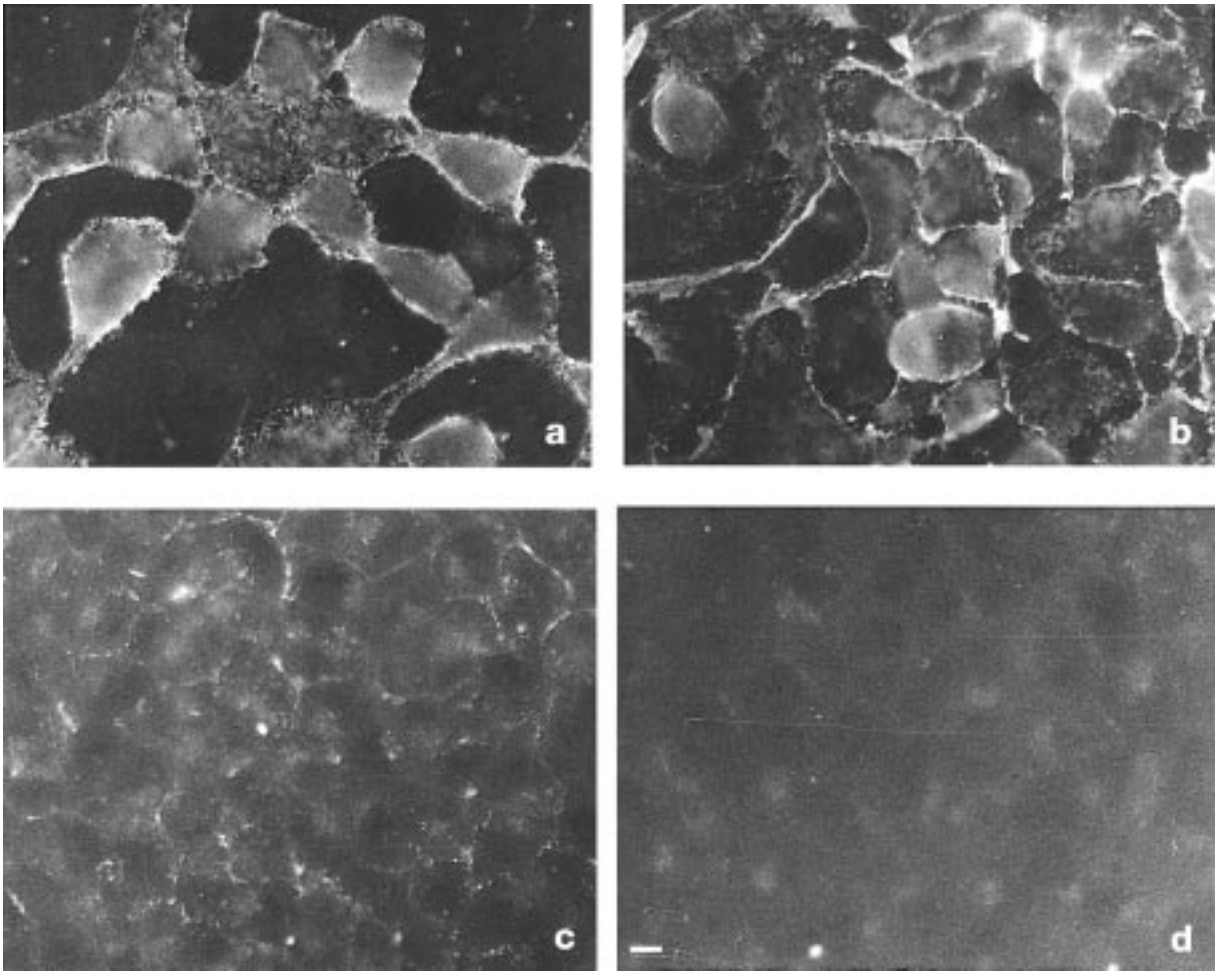

Figure 3. Bovine lactoferrin interaction with HeLa S3 cells detected by immunofluorescence. Cells, treated with Mn^{2+}-BLf for 1 h at 4 °C, were washed and incubated at 37 °C for different lengths of time. Mn^{2+}-BLf localization was examined: (a) after 1 h incubation at 4 °C (binding step); (b) 8 h after the binding step; (c) 24 h after the binding step; (d) 48 h after the binding step. Bar represents 8 μm.

prolonged for 48 h after the infection it was possible to notice that, by comparison with the controls, only in the presence of Fe^{3+}-BLf there was an increase in the multiplication of *Legionella*. The cells added with apo-BLf showed a slight inhibition of the intracellular multiplication, whereas with Zn^{2+}- and Mn^{2+}-BLf the replication of *Legionella* was inhibited with a stronger effect in the case of Mn^{2+}-BLf (Figure 1).

On the basis of these results it appeared necessary to verify whether the increase or the inhibition of bacterial multiplication exerted by Fe^{3+}-BLf or Mn^{2+}- and Zn^{2+}-BLf was due to the metal ions released into the cell's medium by the saturated Lf preparations or to the entry of Lf into the cell. For this purpose a set of experiments similar to those performed with Lf was carried out using Fe^{3+}, Mn^{2+}, Zn^{2+} citrate. Sodium citrate used as a control did not produce any effect. As observed with Fe^{3+}-BLf, the Fe^{3+} citrate increased the intracellular multiplication of *Legionella*, whereas a strong inhibition of *Legionella* replication was obtained with Mn^{2+} citrate, similarly to Mn^{2+}-BLf. Finally, and differently from Zn^{2+}-BLf, Zn^{2+} citrate did not cause any effect (Figure 2).

In order to control whether BLf added to the HeLa cells was internalized by means of a mechanism of endocytosis, experiments were performed by using an indirect immunofluorescence test. During the time of contact between BLf and cells the fluorescent antibody-conjugated lactoferrin appeared to remain outside the cells and it was not possible to demonstrate an internalization. In Figure 3 the microscopic observations obtained with Mn^{2+}-BLf are reported.

Discussion

Iron availability is a fundamental requirement for *L. pneumophila*, both *in vitro* and *in vivo*. Culture media must be supplemented with relatively large amounts of iron for optimum growth *in vitro* and the addition of chelating compounds dramatically blocks the multiplication of the microorganism. Our experiments in culture medium demonstrated that *Legionella* was unable to utilize iron when chelated to BLf. This result must obviously be put in relation to the already reported lack in *Legionella* of siderophores capable of competing with the iron chelating property of the lactoferrin molecule (Reeves *et al*. 1983).

In vivo the intracellular survival and propagation of *Legionella*, which does not produce iron-chelating siderophores, is essentially dependent on the intermediate labile iron pool of the host cell. Therefore substances that directly or indirectly deplete the intermediate iron pool inhibit *L. pneumophila* intracellular multiplication. In professional phagocytes it has been reported by Shuman & Horwitz (1996) that this effect can be produced by (a) interferon gamma, which down regulates transferrin receptor expression and intracellular ferritin concentration, shutting off the two major sources of iron to the pool.; (b) weak bases, including chloroquine and ammonium chloride, which raise the pH of endocytic vesicles and lysosomes and hence inhibit the pH dependent release of iron from iron-lactoferrin (and ferritin); (c) iron chelators, including deferoxamine and apo-lactoferrin, the latter molecule a host iron-binding protein internalized by specific receptors on the plasma membrane of mononuclear phagocytes.

Moreover, with non professional phagocytes such as HeLa cells, the inhibiting effect shown on the intracellular multiplication of *L. pneumophila* by monensin (Goldoni *et al.* 1995) and bafilomycin (Cattani *et al.* 1997) has been demonstrated in our laboratory. The former of these compounds is a carboxylic ionophore that affects receptor-mediated endocytosis (Maxfield 1982) by alkalinization of the content of lysosomes or pre-lysosomal compartments or both, with an effect which is consequent to the insertion of the ionophore into lysosomal membranes (Tartakoff 1983). The latter, i.e., bafilomycin A_1, is a potent inhibitor of the V-ATPase proton pump present in intracellular acidic compartments which pumps protons into many different acidic cell compartments and plays an important role in the acidification and protein degradation occurring in the lysosomes (Yoshimori *et al.* 1991; Furuchi *et al.* 1993; Manabe *et al.* 1993). These two compounds in fact share the capacity to raise endocytic vesicle and lysosomal pH and consequently might interfere with iron availability in the host cell.

As to the action of lactoferrin in particular, the effect of this iron-binding protein on the extracellular (Bortner *et al.* 1986, 1989) and intracellular (Byrd & Horwitz 1991b) multiplication of *L. pneumophila* has already been studied. In the former case, i.e. in culture medium, lactoferrin had a bactericidal effect and killing was dependent on the iron availability, since iron-saturated lactoferrin had no activity. In the case of intracellular growth of *Legionella* the effect of lactoferrin has been studied in human monocytes (Byrd & Horwitz 1991b). In this experimental model the situation is more complex since the growth of *Legionella* depends on the availability of iron on the intermediate labile pool iron of the host cell. Lactoferrin participates to this pool by a characteristic mechanism (Green & Byrd 1997) which takes place at the cell surface through the binding of one molecule of lactoferrin to one lactoferrin receptor. In contrast to transferrin receptors, lactoferrin receptors have similar affinity for iron-saturated and unsaturated lactoferrin. The lactoferrin-lactoferrin receptor complex is endocytized into an acidic endocytic vesicle where iron is not released in spite of the low pH. In contrast to the transferrin-transferrin receptor complex, the lactoferrin-lactoferrin receptor complex is recycled back to the cell surface (Moguilewsky *et al.* 1987). Only a proportion of lactoferrin molecules enter the lysosomial compartment where the complex with the receptor is degraded and the iron released. This iron becomes part of the intermediate labile pool and can be used for the cell metabolism or bound to the iron storage protein ferritin. Therefore the size of the intermediate pool depends also on the quantity and type of ferritin present within the cell (Green & Byrd 1997).

In experiments carried out in human monocytes (Byrd & Horwitz 1991b) it was shown that apo-lactoferrin completely inhibited *L. pneumophila* multiplication in non activated monocytes, and enhanced the capacity of IFNγ-activated monocytes to inhibit *L. pneumophila* intracellular multiplication. On the contrary, iron-saturated lactoferrin had no effect on the already rapid rate of *L. pneumophila* multiplication in non-activated monocytes. In addition, it reversed the capacity of activated monocytes to inhibit *L. pneumophila* intracellular multiplication, demonstrating that *L. pneumophila* can utilize iron from the lactoferrin-lactoferrin receptor pathway.

The results of our research carried out in non-professional phagocytes such as HeLa cells indicate that in our experimental condition the different preparations of bovine lactoferrin had no effect on the binding of *Legionella* to the cells, differently from that observed in the case of an *Escherichia coli* enteroinvasive strain (Longhi *et al.* 1993) and *Listeria monocytogenes* (Antonini *et al.* 1997). The inhibition of the invasiveness by lactoferrin in apo- and saturated forms has been ascribed to the binding of the protein to glycosaminoglycans (Wu *et al.* 1995) present on the host cell surface. The lack of invasiveness inhibition observed by us with *Legionella* suggests therefore the hypothesis that glycosaminoglycans on the HeLa cell surface should not be involved in the early phase of the interaction between *Legionella* and HeLa cells. On the other hand, the intracellular multiplication of *Legionella* was influenced by the different BLf preparations. The concentration of apo-BLf used caused only a slight reduction of the intracellular growth of *Legionella*, whereas the Fe^{3+}-BLf was capable of enhancing *Legionella* multiplication. This fact could probably be in relation to the release in the host cell of iron from bovine lactoferrin saturated form or from ferric citrate. However, the increase of iron within the cell monolayer as indicated by atomic adsorption spectrophotometry, cannot exclude a putative endocytosis of this protein, although it was not possible to demonstrate this internalization by indirect immunofluorescence assay. A more intriguing result was obtained with bovine lactoferrin preparations saturated with zinc and manganese. In both cases the saturation with a metal different from iron produced an inhibition of bacterial growth, which was higher in the case of Mn^{2+}-BLf. The comparison of these results with those of experiments in which the intracellular multiplication of *Legionella* was tested in the presence of the same metal chelated by sodium citrate showed a difference only in the case of Zn^{2+} citrate. This element which had a certain activity when bound to lactoferrin was ineffective when added as Zn^{2+} citrate. Manganese, on the other hand, was capable of inhibiting *Legionella* multiplication in both cases, either as Mn^{2+} citrate or as Mn^{2+}-BLf. These results can probably be ascribed to the intracellular release of the metals from metal complexes, or to a putative endocytosis of bovine lactoferrin. Similarly to that observed in human monocytes (Byrd & Horwitz 1991b) in which the lactoferrin-lactoferrin receptors complex releases a certain amount of iron, in our experimental model iron-saturated lactoferrin probably releases the metal which can become part of the intermediate labile iron pool of the host cell and can be used for the metabolic needs of *Legionella*. As a consequence of this the growth of *Legionella* increased as compared to the control. When, on the contrary, the element released by the saturated lactoferrin-lactoferrin receptor complex was manganese or zinc, the effect was different, and particularly in the case of manganese a certain inhibitory activity on *Legionella* was evident. This effect has been noticed both in the case of Mn^{2+} citrate and in the case of lactoferrin saturated with this element, in accordance with the higher tolerance of *L. pneumophila* towards zinc as compared to manganese (Reeves *et al.* 1981).

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References

Aisen P. 1980 The Transferrins. In Jacobs A, Worwood M, eds. *Iron in Biochemistry and Medicine*. II. London: Academic Press; 87–129.

Antonini G, Catania MR, Greco R et al. 1997 Anti-invasive activity of bovine lactoferrin against *Listeria monocytogenes*. *J Food Prot* **60**, 267–271.

Bortner CA, Miller RD, Arnold RR. 1986 Bactericidal effect of lactoferrin on *Legionella pneumophila*. *Infect Immun* **51**, 373–377.

Bortner CA, Arnold RR, Miller RD. 1989 Bactericidal effect of lactoferrin on *Legionella pneumophila*: effect of the physiological state of the organism. *Can J Microbiol* **35**, 1048–1051.

Byrd TF, Horwitz MA. 1989 Interferon gamma-activated human monocytes down-regulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J Clin Invest* **83**, 1457–1465.

Byrd TF, Horwitz MA. 1991a Chloroquine inhibits the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. A potential new mechanism for the therapeutic effect of chloroquine against intracellular pathogens. *J Clin Invest* **88**, 351–357.

Byrd TF, Horwitz MA. 1991b Lactoferrin inhibits or promotes *Legionella pneumophila* intracellular multiplication in nonactivated and interferon gamma-activated human monocytes depending upon its degree of iron saturation. Iron-lactoferrin and non-physiologic iron chelates reverse monocyte activation against *Legionella pneumophila*. *J Clin Invest* **88**, 1103–1112.

Byrd TF, Horwitz MA. 1993 Regulation of transferrin receptor expression and ferritin content in human mononuclear phagocytes: coordinate upregulation by iron-transferrin and down-regulation by interferon gamma. *J Clin Invest* **91**, 969–976.

Cattani L, Goldoni P, Castellani Pastoris M, Sinibaldi L, Orsi N. 1997 Bafilomycin A_1 and intracellular multiplication of *Legionella pneumophila*. *Antimicrob Agents Chemother* **41**, 212–214.

Chasteen ND, Woodworth RC. 1990 Transferrin and lactoferrin. In Ponka P, Shulman HM, Woodworth RC eds. *Iron Transport and Storage*. Boston: CRC Press; 67–69.

Fields BS. 1993 *Legionella* and protozoa: interaction of a pathogen and its natural host. In Barbaree JM, Breiman RF, Dufour AP eds. *Legionella Current Status and Emerging Perspectives*. Washington D.C.: American Society for Microbiology; 129–136.

Finlay BB, Falkow S. 1988 Comparison of the invasion strategies used by *Salmonella cholerae-suis*, *Shigella flexneri* and *Yersinia enterocolitica* to enter cultured animal cells: endosome acidification is not required for bacterial invasion or intracellular replication. *Biochimie* **70**, 1089–1099.

Furuchi T, Aikawa K, Arai H, Inoue K. 1993 Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, blocks lysosomal cholesterol trafficking in macrophages. *J Biol Chem* **268**, 27354–27348.

Goldoni P, Visca P, Castellani Pastoris M, Valenti P, Orsi N. 1991 Growth of *Legionella* under conditions of iron restriction. *J Med Microbiol* **34**, 113–118.

Goldoni P, Castellani Pastoris M, Cattani L, Peluso C, Sinibaldi L, Orsi N. 1995 Effect of monensin on the invasiveness and multiplication of *Legionella pneumophila*. *J Med Microbiol* **42**, 269–275.

Green GM, Byrd TF. 1997 The immune response to *Legionella* infection. In Kaufmann SHE ed. *Medical Intelligence Unit. Host response to intracellular pathogens*. Heidelberg: Springer-Verlag; 97–114.

Harley VS, Drasar BS, Forrest B, Krahn B, Tovey G. 1989 Invasion strategies and intracellular growth of bacterial pathogens. *Biochem Soc Trans* **17**, 1118 (631st Meeting, Guildford).

Horwitz MA. 1993 Toward an understanding of host and bacterial molecules mediating *Legionella pneumophila* pathogenesis. In Barbaree JM, Breiman RF, Dufour AP, eds. *Legionella Current Status and Emerging Perspectives*. Washington D.C.: American Society for Microbiology; 55–62.

Longhi C, Conte MP, Seganti L, Polidoro M, Alfsen A, Valenti P. 1993 Influence of lactoferrin on the entry process of *Escherichia coli* HB101(pRI203) in HeLa cells. *Med Microbiol Immunol* **182**, 25–35.

Manabe T, Yoshimori T, Henomatsu N, Tashiro Y. 1993 Inhibitors of vacuolar-type H^+-ATPase suppresses proliferation of cultured cells. *J Cell Physiol* **157**, 445–452.

Maxfield FR. 1982 Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J Cell Biol* **95**, 676–681.

Moguilewsky N, Massan PL, Courtoy PJ. 1987 Lactoferrin uptake and iron processing into macrophages: a study in familial hemochromatosis. *Br J Haematol* **66**, 129–136.

Oldham LJ, Rodgers FG. 1985 Adhesion, penetration and intracellular replication of *Legionella pneumophila*: an *in vitro* model of pathogenesis. *J Gen Microbiol* **131**, 697–706.

Quinn FD, Weinberg ED. 1988 Killing of *Legionella pneumophila* by human serum and iron-binding agents. *Curr Microbiol* **17**, 111–116.

Reeves MW, Pine LP, Hutner SH, George JB, Knox Harrele W. 1981 Metal requirements of *Legionella pneumophila*. *J Clin Microbiol* **13**, 688–695.

Reeves MW, Pine LP, Neilands JB, Balows A. 1983 Absence of siderophore activity in *Legionella* species grown in iron deficient media. *J Bacteriol* **154**, 324–329.

Shuman HA, Horwitz MA. 1996 *Legionella pneumophila* invasion of mononuclear phagocytes. In Virginia L Miller, ed. *Bacterial Invasiveness*. Heidelberg: Springer-Verlag; 99–112.

Tartakoff AM. 1983 Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32**, 1026–1028.

Valenti P, Visca P, Antonini G, Orsi N, Antonini E. 1987 The effect of saturation with Zn^{2+} and other metal ions on the antibacterial activity of ovotransferrin. *Med Microbiol Immunol* **176**, 123–130.

Wu HF, Monroe DM, Church FC. 1995 Characterization of the glycosaminoglycan binding region of lactoferrin. *Arch Biochem Biophys* **317**, 85–92.

Yoshimori TA, Yamamoto A, Moriyama Y, Futai M, Tashiro Y. 1991 Bafilomycin A_1, a specific inhibitor of vacuolat-type H^+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* **266**, 17707–17712.