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Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells

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Abstract

Lactoferrin is an iron-binding glycoprotein implicated in particular in the control of immune functions and cell proliferation. We have investigated its involvement, at inflammatory concentrations, in cancer progression. We report that lactoferrin has a significant effect on natural killer (NK) cell cytotoxicity against haematopoietic and breast epithelial cell lines. Lactoferrin increases cytolysis at a low concentration (10 μg/ml) while at a high concentration (100 μg/ml) it modulates cytolysis depending on the target cell phenotype. By pre-treatment of either NK cells or target cells with lactoferrin, we have demonstrated that the lactoferrin effect is due both to a modulation of NK cell cytotoxicity and the target cell sensitivity to lysis. Lactoferrin binds to 91% of the naturally heterogeneous $CD56^{dim/bright}$ NK cell population and increases the NK cell cytotoxic activity at low concentrations. High concentrations of lactoferrin seem to be toxic for the $CD56^{bright}$ NK cells and decrease NK cell cytotoxicity. Lactoferrin also exerts an effect on target cells depending on the cell phenotype. It does not modify the susceptibility to lysis of haematopoietic cells such as Jurkat and K-562 cells, but does significantly increase that of the breast and colon epithelial cells. We have also demonstrated that lactoferrin inhibits epithelial cell proliferation by blocking the cell cycle progression. © 1998 Elsevier Science B.V.

Keywords: Lactoferrin; NK cell; Cytotoxicity; Tumor cell

1. Introduction

Lactoferrin, also called lactotransferrin [1], is an iron binding glycoprotein synthesized by epithelial cells and precursors of polymorphonuclear cells. Lactoferrin is mainly found in external secretions such as breast milk and in neutrophil secondary granules [2]. More recently, some observations possibly suggest an important role for lactoferrin in the primary defense against tumorigenesis [3]. It has been shown that lactoferrin regulates the development of tumour by a direct effect on tumour cells [4]. However, the activity of lactoferrin on cell proliferation remains highly controversial. In fact, it has been reported that lactoferrin promotes the proliferation of cell lines [5] and has no effect on lymphocyte proliferation [6] or inhibits mammary cell growth [7].

Abbreviations: mAbs, monoclonal antibodies; PE, phycoerythrin; BSA, bovine serum albumin; Lf, lactoferrin; HyF-Lf, 5-({[2-(carbohydrazino)methyl]-thio}acetyl)amino-fluorescein labelled lactoferrin; NK cells, natural killer cells

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These opposite effects on tumour cells may be correlated to the phenotype and the lactoferrin receptor expression. In fact, lactoferrin binds to both high affinity specific receptors [8,9] and to low affinity binding sites such as glycosaminoglycans [10–12].

In previous works, the binding parameters of human lactoferrin to haematopoietic and epithelial cell lines of various origins were investigated. Lactoferrin binds to haematopoietic cells such as the lymphoblastic T cells Jurkat and the K-562 erythroleukemia cells. The Jurkat cell line possesses a membrane bound receptor which interacts with lactoferrin with a dissociation constant of 80 nM and 0.3×10^6 sites [13]. Garré et al. [14] found that K-562 cells exhibit 10×10^6 binding sites for lactoferrin with a high affinity (25 nM). Lactoferrin enters these cells via receptor mediated endocytosis where it is almost degraded in the lysosomal compartment [14,15]. Human lactoferrin also binds to various epithelial cells, in particular to breast non-malignant SV-40 immortalized cells (HBL-100), to breast carcinoma cells (MCF-7) [16] and to various subclones of the colon adenocarcinoma cell line HT-29 [17,18]. The dissociation constant is about 200 nM with more than 1×10^6 sites for these breast cells. HT29-18-C1 cells exhibit 5.8×10^6 binding sites with relatively low affinity (840 nM). In the colon cells, a large part of surface bound lactoferrin is not internalized under physiological conditions [17,18]. In contrast to haematopoietic cells, lactoferrin binds to these epithelial cells and remains for the most part at the cell surface [16,18,19]. Lactoferrin is not internalized in these various cells, suggesting that the intracellular effects of lactoferrin in these breast and colon epithelial cells differ from those in haematopoietic cell lines.

Recently, it was shown that the antitumoral activity of lactoferrin may be explained by the modulation of the natural killer (NK) cell and lymphokine-activated killer cell cytotoxicity [3,20]. The mechanisms by which lactoferrin modulates the cytolysis of tumour cells by NK cells is poorly understood. We have hypothesized that lactoferrin could modulate the biological responses of either NK cells or target cells by interactions with lactoferrin receptors found on these two cell types.

As the parameters for the lactoferrin binding to Jurkat cells and epithelial cell lines are different, we compared the cytolysis of these two kinds of cells by NK cells, in the presence or absence of lactoferrin. In a second step, to understand the events induced by the presence of lactoferrin, we analysed the effects of lactoferrin on cytolysis when it is incubated with NK cells or target cells, respectively. Finally, to define the mechanism of lactoferrin activity on cancerous epithelial cells, we determined its effects on proliferation and induction of cell death.

The NK cell population is naturally heterogeneous; it contains a majority of $CD56^{dim}$ cells and a minority of $CD56^{bright}$ [21]. We demonstrated that on the heterogeneous NK cell population, 91% of the cells bind lactoferrin. Low concentrations of lactoferrin lead to the activation of NK cell cytotoxicity. High concentrations of lactoferrin seem to be toxic for the $CD56^{bright}$ NK cells. Lactoferrin also acts on target cell sensitization to lysis according to the phenotype and the nature of the lactoferrin receptor. We demonstrated that lactoferrin inhibits breast and colon epithelial cell proliferation by blocking the cell cycle progression at the G1 to S transition or at the beginning of the S phase. This result points to the potential role of lactoferrin in providing protection from tumour development.

2. Materials and methods

2.1. Cells

The effector cells were prepared from the citrated blood of healthy donors by lymphoprep gradient separation [22]. Monocytic cells were extensively depleted by adherence to plastic. Preparations of $CD56^+$ NK cells (purity: $85.5 \pm 5.5\%$) were isolated by negative selection using monoclonal antibodies (mAbs) against CD3 (Immunotech, Marseille, France). The effect of lactoferrin on NK cells was measured simultaneously on this preparation and on cells that had undergone a second negative selection (purity of NK cells: $95 \pm 3.2\%$) using anti-CD20 mAbs (Immunotech) to eliminate an increase of NK cell cytotoxicity after activation of B cells by lactoferrin. Briefly, anti-CD3 or anti-CD20 mAbs were fixed on a tissue culture dish in Tris (0.05 M)–HCl buffer pH 9.5 for 4 h at 20°C. The cellular suspension was added to the fixed mAbs for 1 h at 37°C. Effector cells were finally resuspended in the RPMI medium.

Cancerous haematopoietic cells and various epithelial cells were used as target cells. Jurkat cells (lymphoblastic T-cell line) ECACC and the erythroleukemia cell line K-562 (ATCC) were grown in RPMI 1640 medium (Gibco-BRL, Eragny, France) supplemented with 2 mM L-glutamine (Eurobio, Les Ulis, France), 50 μg/ml of gentamycin (Gibco-BRL) and 10% of fetal calf serum (FCS). Human breast cancer epithelial cells MCF-7 (ATCC), and HBL-100 (SV40 immortalized human breast epithelial cell line, reportedly non-tumorigenic in nude mice), were grown in Eagle's minimal essential medium (Gibco-BRL) containing 10% FCS, 1% non-essential amino acids (Eurobio), 2 mM L-glutamine, 5 μg/ml insulin (Endopancrine, Organon, Serifontaine, France), penicillin and gentamycin at 37°C, 5% CO_2. HT29-18-C1, a subclone of the human colon adenocarcinoma cell line HT-29, was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% FCS, 2 mM L-glutamine and 50 mg/l gentamycin (Gibco-BRL) at 37°C, 10% CO_2.

2.2. Preparation of lactoferrin

Lactoferrin was prepared from a single human lactoserum by ion-exchange chromatography as previously described [23]. The degree of purity (> 99%) of human lactoferrin was checked by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and High Pressure Liquid Chromatography (HPLC). Lipopolysaccharides were removed by chromatography through a detoxi-gel column (Pierce Chemicals, Rockford, IL, USA). The effects observed with our purified lactoferrin were verified with lactoferrin purchased from Calbiochem (purity > 99%) (La Jolla, CA, USA).

Fluorescent lactoferrin (HyF-Lf) was obtained by complexing the 5-({[2-(carbohydrazino)methyl]-thio}acetyl)amino-fluorescein to lactoferrin according to the technique described by Leveugle et al. [9].

2.3. Cytotoxicity assay

Target cells were incubated with 20 μCi $^{51}CrNa_2{}^{51}CrO_4$ (ICN Biomedical, Irvine, CA, USA) at a cell density of 2×10^5 in 100 μl RPMI. The adherent epithelial cells, at a cell density of 5×10^3/well in 100 μl of medium, were labelled by addition of 1 μCi ^{51}Cr/well. Effector cells were added at effector/target ratios ranging from 5:1 to 80:1. At the end of the 12-h ^{51}Cr assay, cells were collected by centrifugation; an aliquot of supernatant was collected from each well and the ^{51}Cr released was counted using a gamma counter (1282 Compugamma, LKB Wallac, Turky, Finland). The percentage of chromium released was calculated according to the following equation: (experimental cpm − control cpm)/(maximal cpm − control cpm) × 100.

Maximum ^{51}Cr release was obtained by incubating target labelled cells (5×10^3 cells) into medium containing 1% Triton X-100. Spontaneous release (control cpm) was determined by incubation of 5×10^3 target labelled cells into medium. The ratio T_{min}/T_{max} did not exceed 3.7% for all the cytotoxic assays performed.

Global effect of lactoferrin on cytotoxicity was determined by adding lactoferrin at the beginning of the cytotoxicity assay at concentrations of 10 μg/ml or 100 μg/ml. Lactoferrin was maintained throughout the chromium release assay. The effects of lactoferrin on either NK cell activity or target cell sensitivity to lysis were also investigated. In order to determine the lactoferrin effect on NK cell cytotoxicity, cells were pre-treated with lactoferrin (concentration ranging from 0 to 100 μg/ml) for 12 h at 37°C in RPMI medium. Cells were washed just before the cytotoxicity assay to eliminate lactoferrin excess. Similar protocol was used to evaluate the effect of lactoferrin on target cell sensitivity to lysis.

2.4. Lactoferrin binding to NK cells

We first verified the lactoferrin binding to NK cells by cytofluorimetry. NK cells were washed twice with PBS and 5×10^5 cells were stained with 10 μl of mAbs against CD56 (Sigma) for 1 h at 4°C. Then, 5 μl of antibodies against mouse IgG coupled to phycoerythrin (PE), 10 μl of HyF-lactoferrin at concentrations ranging from 1 to 20 μg/ml and 50 molar excess of unlabelled lactoferrin were added for 1 h at 4°C. The non-specific control was performed by incubating the cells with Abs against mouse IgG, HyF-lactoferrin and unlabelled lactoferrin in the absence of a staining with mAbs against CD56. Stained cells were washed and analysed on a Becton-Dickin-

son FACScan flow cytofluorimeter (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

2.5. Effect of lactoferrin on target cells

Cell proliferation was evaluated by thymidine uptake. Epithelial cell lines MCF-7, HBL-100 and HT29-18-C1 were cultured in a 12 well plate at a cell density of 40,000 cells per 500 μl of medium per well. Then, cultures were incubated in the presence of lactoferrin at concentrations ranging from 0 to 100 μg/ml. After 24 h, 2 μCi of (methyl-^{3}H) thymidine (specific activity 50 Ci/mmol, ICN, Biomedical) were added to each well for 4 h at 37°C. The cells were washed with PBS, and treated with cold 5% trichloracetic acid for 45 min at 4°C. Cells were rinsed with water and solubilised with 0.3 M NaOH for 1 h at 37°C. The radioactivity of the cells was measured using a beta counter (Beckman, Fullerton, CA, USA).

The effect of lactoferrin on cell cycle progression was determined by cytofluorimetry analysis. Epithelial cell lines HBL-100, MCF-7 and HT29-18-C1 were grown to 50% confluence, and placed in a serum-free medium for 24 h for the starvation-synchronized experiments. The cells were cultured in a medium of 1% FCS in the presence of 100 μg/ml lactoferrin for 48 h. They were then trypsinized and fixed in cold 70% ethanol for 4 h. The fixed cells were washed with PBS and incubated with 5 μg of RNAse A (Sigma) per milliliter and stained with 25 μg/ml of propidium iodine for 1 h at 37°C. The stained cells were analysed on a FACscan cytofluorimeter using the cellFIT Software program.

3. Results

3.1. Effect on cytolysis of lactoferrin added simultaneously to NK cells and target cells

To test NK cell cytotoxicity in the presence of lactoferrin, lactoferrin was added to the medium and maintained throughout the chromium release assay. Divergent results were obtained depending on cellular type and lactoferrin concentration. Whatever the effector/target ratio chosen, the addition of lactoferrin (10 μg/ml) resulted in a slight but significant increase of up to 15% in the lytic ability of the NK cells with regard to the haematopoietic Jurkat and K-562 cell lines (Fig. 1A). In contrast, a high concentration of lactoferrin (100 μg/ml) decreased by about

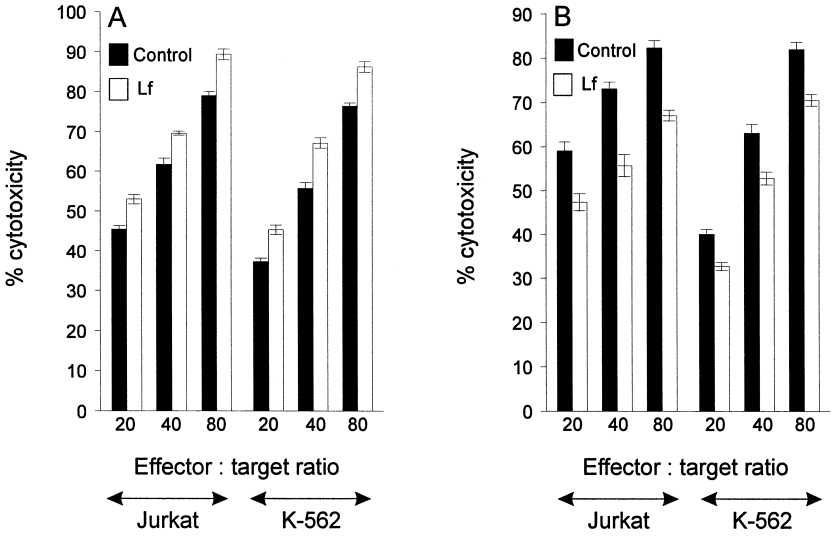

Fig. 1. Effect of lactoferrin on haematopoietic cell cytolysis by NK cells. Lactoferrin is added simultaneously to the effector and target cells at the beginning of NK cytotoxic assay. At a low concentration (10 μg/ml), lactoferrin (□) enhances spontaneous cytotoxicity on the haematopoietic cell lines (A), while at high concentration (100 μg/ml), it decreases cytolysis of the haematopoietic cells (B) as compared to the control (■). Cytolysis is measured at the three arbitrarily chosen effector/target ratios of 20, 40, and 80, respectively. Results are expressed as the mean ± SEM of three experiments using three different donors.

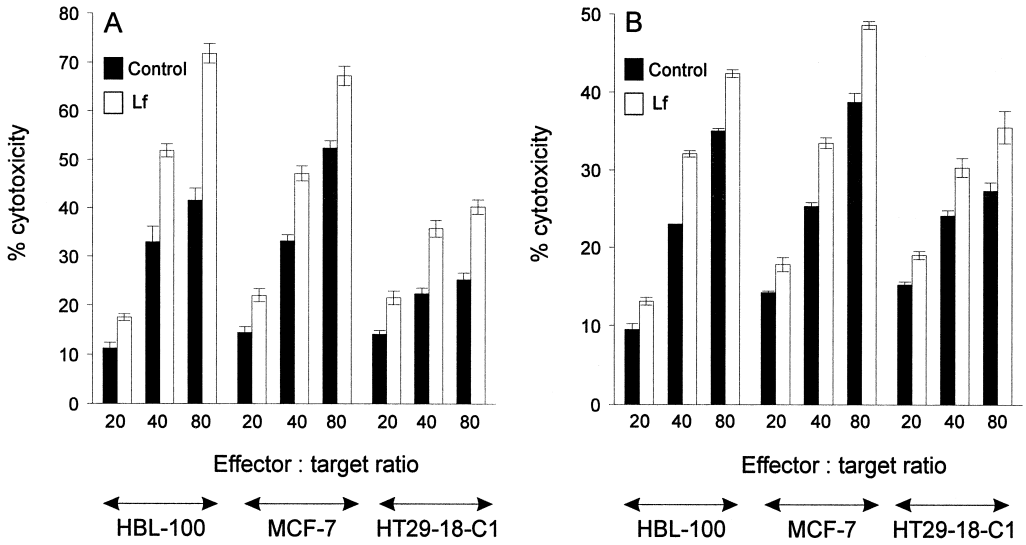

Fig. 2. Effect of lactoferrin on breast epithelial cell cytolysis by NK cells. Lactoferrin is added to the effector and the target cells at the beginning of NK cytotoxic assay. At a low concentration (10 μg/ml) (A) or high concentration (100 μg/ml) (B), lactoferrin (□) increases the cytolysis of both human breast and colon epithelial cells as compared to the control (■). Cytolysis is measured at the three arbitrarily chosen effector/target ratios, of 20, 40, and 80, respectively. The mean ± SEM of experiments with three different donors are shown.

16 to 20% the lysis of these haematopoietic cells (Fig. 1B).

Lactoferrin used at a low concentration (10 μg/ml) increased NK cell cytotoxicity against the HBL-100, MCF-7 and HT29-18-C1 epithelial cells by about 25 to 35% (Fig. 2A). A high concentration of lactoferrin (100 μg/ml) increased cytotoxicity by 40 to 55% on the MCF-7 cancer cell line and by 60 to 65% on the HBL-100 and HT29-18-C1 cell lines (Fig. 2B).

The increase of cytolysis in the presence of lactoferrin at a low concentration, whatever the target cells used in this study, may be correlated to an activation of the NK cells by lactoferrin. However, the diver-

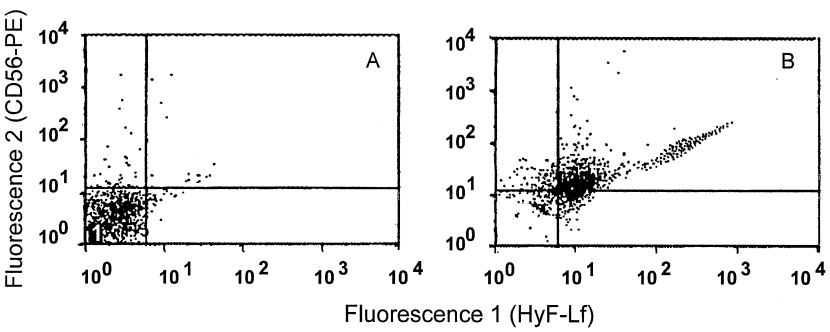

Fig. 3. FACScan detection of lactoferrin binding to NK cells. The negative control (A) is determined by incubating cells with a 100-fold molar excess of unlabelled lactoferrin and without CD56 mAbs. NK cells (CD56) are analysed for expression of lactoferrin receptor as described in Section 2 (B). Quadrants were arranged such that non-specifically lactoferrin stained cells remained in the left quadrant. Fluorescence 1 = HyF-Lf signal; Fluorescence 2 = anti-CD56 PE signal. Data are representative of separate experiments with similar results.

gent results observed on haematopoietic and epithelial cells at a high concentration of lactoferrin suggest that lactoferrin acts not only on the effector cells but also on the target cells, depending on the cell phenotype and the cell lactoferrin receptor expression. To verify this hypothesis, we pre-treated the effector cells and the target cells separately with lactoferrin.

3.2. Binding of lactoferrin to NK cells

In a first step, we checked the ability of NK cells to bind lactoferrin using a double fluorescence staining in a cytofluorimetric analysis. The staining with anti-CD56 mAbs and HyF-lactoferrin revealed that 91% of the $CD56^{+}$ cell population bound lactoferrin (Fig. 3A, B). The NK cell population was heterogeneous with a majority of $CD56^{dim}$ cells and a minority of $CD56^{bright}$ NK cells which bound more lactoferrin. The binding was reversible in the presence of unlabelled lactoferrin and was saturable at a concentration of 10 μg/ml, indicating that the binding of lactoferrin was specific with an affinity of about 10^{-8} M.

3.3. Effect of lactoferrin on NK cell cytotoxicity

To confirm the modulation of NK cell cytotoxicity in the presence of lactoferrin, the NK cells were pre-treated with lactoferrin and the cytotoxicity of NK cells with regard to the various target cells was analysed. No significant effect of lactoferrin on NK cells was observed with the haematopoietic cells which showed a high sensitivity to lysis (data not shown). In contrast, the HBL-100 and HT29-18-C1 cells showed less cytolysis than the haematopoietic cells and permitted greater observation of NK cell cytotoxicity activation by lactoferrin. Lactoferrin modulated the cytotoxic activities of NK cells as a function of its concentration. At low concentrations of lactoferrin (10–25 μg/ml), we observed a 25 to 40% increase in NK cytotoxicity. This result confirms the stimulation previously obtained in the presence of lactoferrin at 10 μg/ml added simultaneously to NK and target cells. Higher concentrations of lactoferrin (50–100 μg/ml) induced a significant decrease in NK cell activity (Fig. 4), as confirmed by the global effect of lactoferrin at 100 μg/ml on haematopoietic cell cytolysis.

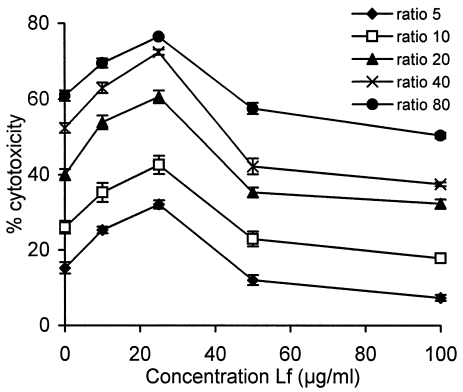

Fig. 4. Effect of lactoferrin on NK cells. ^{51}Cr release assay is performed by using HBL-100 target cells. NK cells are pre-incubated with lactoferrin (concentration ranging from 0 to 100 μg/ml) for 12 h, in RPMI medium supplemented with 10% FCS. The bars indicate the mean $\pm$ SEM of experiments performed with three donors. $p < 0.01$, Student's *t*-test.

3.4. Effect of lactoferrin on target cell sensitivity to lysis

After pre-treatment of the target cell with lactoferrin, an effect of the lactoferrin on target cell sensitivity to lysis was observed according to cell phenotype and lactoferrin concentration. Lactoferrin did not induce significant variation in the haematopoietic Jurkat (Fig. 5A) and K-562 (Fig. 5B) cell sensitivity to NK cells. In contrast, the epithelial cell lines MCF-7, HBL-100 (Fig. 5C) and HT29-18-C1 (Fig. 5D) were more sensitive to lysis in the presence of lactoferrin. The increase in sensitivity of these two types of epithelial cells to NK cell lysis was similar and dose dependent. These results were in agreement with our previous experiment in Fig. 2A, which showed that cytolysis of transformed or tumour epithelial cells was greatly increased when lactoferrin at 100 μg/ml was added to the mixture containing the effector and target cells. Furthermore, these results suggested that the effects of lactoferrin were variable depending on the cellular type.

Our results suggested that the modulation of cytolysis by lactoferrin was mainly due to a sensitization of certain target cells to NK cell lysis. In fact, lactoferrin added to the medium during the chromium release assay increased cytolysis of epithelial tumour cells at 100 μg/ml although lactoferrin slightly inhibited NK cell activity at this concentration. Thus,

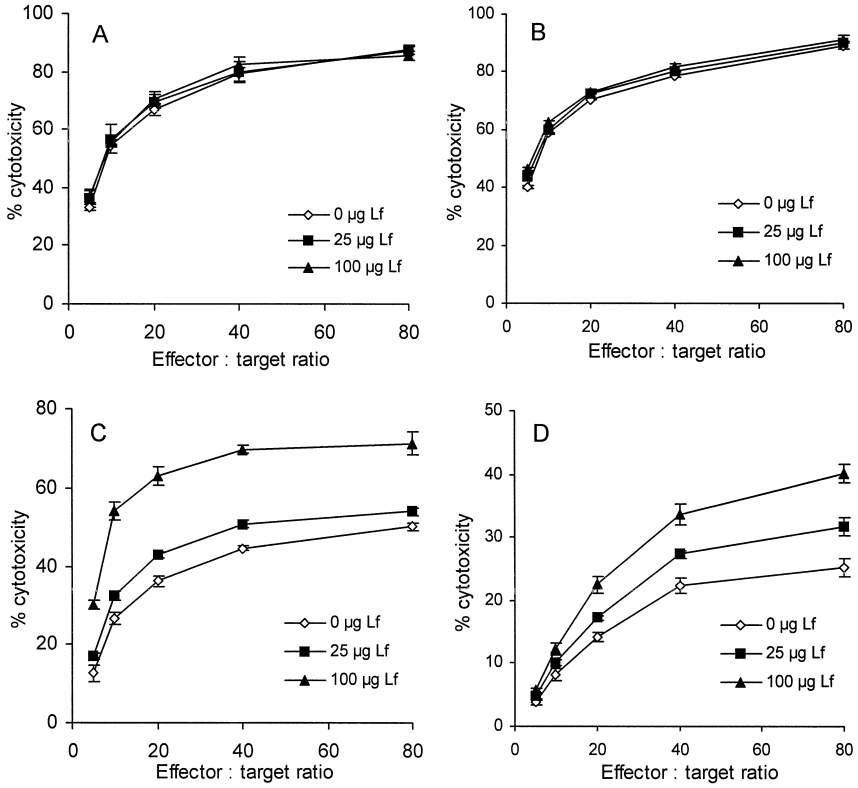

Fig. 5. Effect of lactoferrin on target cell sensitivity to lysis. Target cells are pre-incubated with lactoferrin (concentration ranging from 0 to 100 μg/ml) for 12 h in medium supplemented with 10% FCS. Target cell sensitization to lysis by lactoferrin was investigated on Jurkat (A), K-562 (B), HBL-100 (C) and HT29-18-C1 (D) cells. Error bars indicate $\pm$ SEM. $p < 0.05$, Student's t-test.

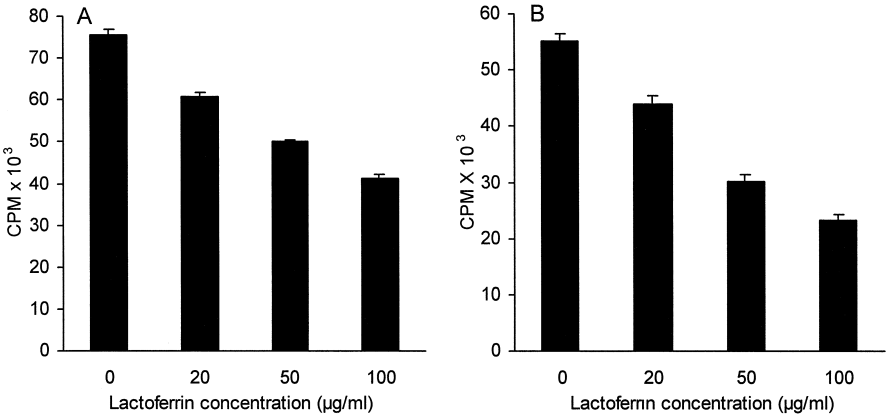

Fig. 6. Effect of lactoferrin on ^{3}H-thymidine incorporation in HBL-100 (A) and HT29-18-C1 (B) cell lines. Sub-confluent cells are cultured with lactoferrin (concentration ranging from 0 to 100 μg/ml) in 12-well plates for 24 h. After incubation with methyl ^{3}H-thymidine for 4 h, the cells are lysed and incorporated methyl ^{3}H-thymidine is counted. Each point is the mean $\pm$ SEM of three experiments.

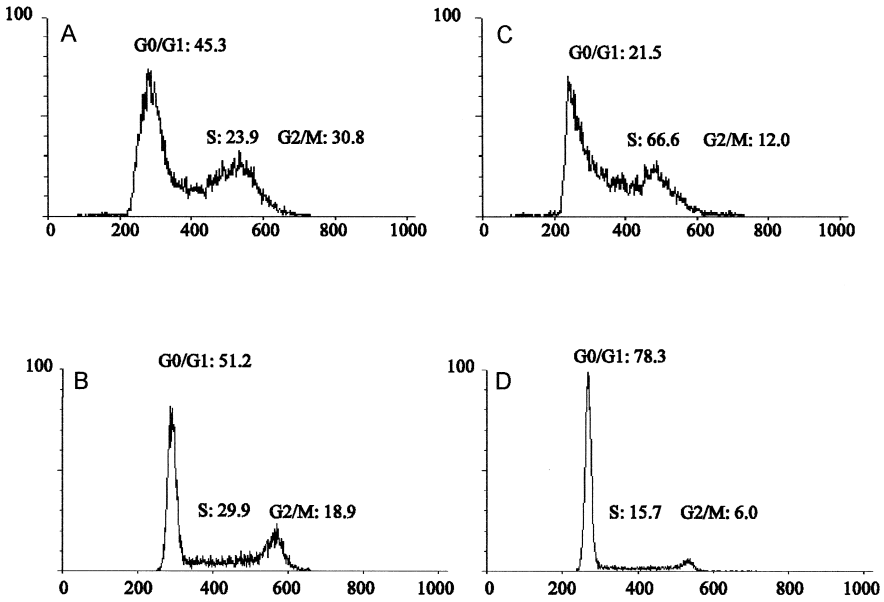

Fig. 7. FACScan analysis of untreated HBL-100 (A) and HT29-18-C1 (B) cells or lactoferrin (100 μg/ml)-treated HBL-100 (C) and HT29-18-C1 (D) cultures. Cells are treated for 48 h prior to fixation and FACS analysis, as described in Section 2. Numbers indicate the percentage of cells in the different phases of the cell cycle.

we tried to explain the mechanism of the biological activity of lactoferrin on the epithelial cells.

3.5. Effect of lactoferrin on breast epithelial cell proliferation and cell cycle

Lactoferrin (at concentrations ranging from 0 to 100 μg/ml) reduced ^{3}H-thymidine incorporation in a dose dependent manner in HBL-100, MCF-7 and HT29-18-C1 cell lines. In particular, for the HBL-100 and HT29-18-C1 cell lines, ^{3}H-thymidine incorporation was respectively decreased by about 47% (Fig. 6A) and 56% (Fig. 6B) at a lactoferrin concentration of 100 μg/ml as compared to the control (medium alone).

To understand the mechanism by which lactoferrin inhibited epithelial cell proliferation, we investigated the activity of lactoferrin on the cell cycle. Exponentially growing HBL-100 cell culture exposed to 100 μg/ml lactoferrin rapidly showed growth inhibition as indicated by the lack of cell number increase after 48 h. Trypan Blue dye exclusion did not indicate toxicity with this dose of lactoferrin ($>$ 90% viability after 48 h). FACS analysis revealed that compared to untreated cells (Fig. 7A), a 48-h treatment with lactoferrin resulted in a higher proportion of cells in S phase (Fig. 7B) consistent with a block in the S phase progression. Indeed, as described in the proliferation test, lactoferrin inhibits the DNA synthesis. The cell cycle of the MCF-7 and HT29-18-C1 cells was also modified by lactoferrin. In fact, in the absence of lactoferrin, MCF-7 and HT29-18-C1 cells proceeded to enter the G2/M phase (Fig. 7C). In contrast, lactoferrin-treated cells remained arrested in the G1 phase (Fig. 7D). These results confirm the potential antiproliferative mechanism of lactoferrin on these cells.

4. Discussion

The present findings represent the first demonstration that lactoferrin modulates cytolysis as a function of the lactoferrin concentration and the target cell phenotype, suggesting that lactoferrin acts on both NK cells and target cells.

In agreement with Nagler et al. [21], we have shown that NK cell population is heterogeneous with a majority of CD56^{dim} cells and a minority of

$CD56^{bright}$. We have demonstrated for the first time, that both the $CD56^{dim}$ and $CD56^{bright}$ sub-populations bind lactoferrin with however the $CD56^{bright}$ NK cells binding more lactoferrin than the $CD56^{dim}$ cells. The binding of lactoferrin on these cells suggests that lactoferrin may act directly and specifically on the NK cells. Indeed, we have demonstrated that pre-treatment of NK cells for 12 h with low concentrations of lactoferrin (10–25 μg/ml) enhanced NK activity. Our results differ from those of Shau et al. [20] who found an increase in cytotoxicity only when lactoferrin was added simultaneously to NK cells and tumour cells at concentrations ranging from 7.5 to 750 μg/ml. They did not observe any modification of the cytotoxic activity when the NK cells were pre-treated with lactoferrin. The differences in the results may be due to the fact that firstly, the authors did not use purified NK cells, but a total lymphocytic population which may affect the lactoferrin effect on the NK cell population, and secondly to the fact that the pre-treatment of the NK cells was carried out only for 30 min with very a high concentration of lactoferrin (500 μg/ml). This short-term treatment of the NK cells with lactoferrin did not allow the observation of the NK cell cytotoxicity modulation by lactoferrin. Indeed, in the particular case of interleukin-2, it was necessary to activate NK cells more than 3 to 6 h in order to enhance cytotoxic activity [24]. Furthermore, we have demonstrated that the increase in NK cell cytotoxicity induced by lactoferrin is only observed at low concentrations of lactoferrin; at high concentrations, lactoferrin decreased NK cell activity. The physiologic level of lactoferrin in plasma does not exceed 2 μg/ml in healthy subjects [25]. Therefore, the slight increase in lactoferrin concentration caused by degranulation of neutrophils [2] during inflammation induced by tumour development could enhance NK cell functions in vivo. In contrast, the high concentrations of lactoferrin (50–100 μg/ml) used in vitro, can exert a toxic effect on NK cells and decrease their cytotoxic activity. In fact, all NK cells have the ability to mediate spontaneous cytotoxicity. The $CD56^{bright}$ cells, which exert the highest cytotoxic activity in the total CD56 NK cell population, bind more lactoferrin than the $CD56^{dim}$ cells. These $CD56^{bright}$ NK cells are sensitive to high concentrations of lactoferrin. Thus, at a high concentration of lactoferrin, only the $CD56^{dim}$ cells maintained spontaneous cytotoxic activity, explaining the decrease in but not the disappearance of the cytotoxicity.

The antineoplastic activity of lactoferrin seems also to be due to the direct action on target cells. We have demonstrated a variability in the target cell response to pre-treatment by lactoferrin. The difference in sensitivity of the haematopoietic and the epithelial cells can be correlated to the target cell phenotype. Indeed, lactoferrin presents a variety of activities in vitro including control of proliferation and differentiation. Lactoferrin mRNA, mainly detected in normal breast epithelial tissues and in benign proliferative lesions, is down-regulated in some forms of cancer, such as human breast and colon epithelial cancers [26,27], suggesting that lactoferrin is implicated in the tumour epithelial cell progression. In fact, Hurley et al. [7] reported that lactoferrin inhibits mammary cell growth. Furthermore, this effect was also observed on the HT-29 cells. Indeed, when transferrin was replaced by lactoferrin, HT-29 cells were unable to proliferate for a long time [28]. In our work, we have confirmed these previous observations and demonstrated that lactoferrin rapidly inhibits the proliferation of the transformed and tumour epithelial cell lines by a block in the cell cycle progression at the G1 to S transition. The extensive and rapid modification of the cell cycle can explain the increase in the sensitivity to lysis of the transformed and tumour epithelial cells. In contrast, lactoferrin induces a long term differentiation of the Jurkat cell line (unpublished data). Furthermore, lactoferrin does not rapidly influence the K-562 cell proliferation, although there is a close relationship between lactoferrin binding to these cells and their proliferative status [29]. The absence of rapid cell cycle modifications in these haematopoietic cells does not modify their sensitivity to NK cell lysis.

An alternative hypothesis is that the variability in the target cell response to lactoferrin could depend on the difference in the lactoferrin remaining at the cell surface. Indeed, lactoferrin remaining at the target cell surface could increase the contact with the NK cells through its glycan moiety and lectins expressed on the NK cell surface. Such an interaction has been described for the rat c-lectin NKRP-1 and oligosaccharides of proteoglycans [30]. In the case of the Jurkat cells, lactoferrin binding to these cells occurs

mainly through a high affinity receptor which allows internalization of lactoferrin [13,15]. Therefore, the low concentration of lactoferrin at the target cell surface can not enhance contact with NK cells and does not increase the cytolysis of the Jurkat cells. In contrast, the epithelial cells showed a dose-dependent increase in their sensitivity to lysis. Lactoferrin bound at the cell surface is not internalized and remains at the cell surface [16,18,19] where it can enhance the cytolysis of these cells by NK cells.

In conclusion, the cell phenotype, the binding of lactoferrin and the internalization process can explain the variability in sensitivity of the cells studied. It appears that the binding of lactoferrin to the cells is mediated by various types of molecules which are able to induce variable effects. However, the interaction with the various lactoferrin receptors found on these cells is not yet known and remains to be determined.

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