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Effect of lactoferrin protein on red blood cells and macrophages: mechanism of parasite–host interaction

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Background: Lactoferrin is a natural multifunctional protein known to have antitumor, antimicrobial, and anti-inflammatory activity. Apart from its antimicrobial effects, lactoferrin is known to boost the immune response by enhancing antioxidants. Lactoferrin exists in various forms depending on its iron saturation. The present study was done to observe the effect of lactoferrin, isolated from bovine and buffalo colostrum, on red blood cells (RBCs) and macrophages (human monocytic cell line-derived macrophages THP1 cells).

Methods: Lactoferrin obtained from both species and in different iron saturation forms were used in the present study, and treatment of host cells were given with different forms of lactoferrin at different concentrations. These treated host cells were used for various studies, including morphometric analysis, viability by MTT assay, survivin gene expression, production of reactive oxygen species, phagocytic properties, invasion assay, and Toll-like receptor-4, Toll-like receptor-9, and MDMR1 expression, to investigate the interaction between lactoferrin and host cells and the possible mechanism of action with regard to parasitic infections.

Results: The mechanism of interaction between host cells and lactoferrin have shown various aspects of gene expression and cellular activity depending on the degree of iron saturation of lactoferrin. A significant increase (P<0.05) in production of reactive oxygen species, phagocytic activity, and Toll-like receptor expression was observed in host cells incubated with iron-saturated lactoferrin when compared with an untreated control group. However, there was no significant (P>0.05) change in percentage viability in the different groups of host cells treated, and no downregulation of survivin gene expression was found at 48 hours post-incubation. Upregulation of the Toll-like receptor and downregulation of the P-gp gene confirmed the immunomodulatory potential of lactoferrin protein.

Conclusion: The present study details the interaction between lactoferrin and parasite host cells, ie, RBCs and macrophages, using various cellular processes and expression studies. The study reveals the possible mechanism of action against various intracellular pathogens such as Toxoplasma, Plasmodium, Leishmania, Trypanosoma, and Mycobacterium. The presence of iron in lactoferrin plays an important role in enhancing the various activities taking place inside these cells. This work provides a lot of information about targeting lactoferrin against many parasitic infections which can rule out the exact pathways for inhibition of diseases caused by intracellular microbes mainly targeting RBCs and macrophages for their survival. Therefore, this initial study can serve as a baseline for further evaluation of the mechanism of action of lactoferrin against parasitic diseases, which is not fully understood to date.

Keywords: lactoferrin, phagocytosis, cytotoxicity, morphometric analysis

Introduction
Lactoferrin is a multifunctional glycoprotein belonging to the transferrin family. These proteins are capable of transferring and accepting iron ions from the surrounding...
environment depending on the iron content of the protein.\(^1\) There are three forms of lactoferrin, according to its iron saturation: apolactoferrin (iron-free), a monoferric form (containing one ferric ion), and hololactoferrin (containing two iron ions).\(^2\) Lactoferrin was first isolated by Sorensen and Sorensen from bovine milk in 1939.\(^3\) The protein is naturally present in large amounts in colostrum and milk. Lactoferrin performs many biological functions, and has antimicrobial, antitumor, antioxidant, antitumor, and immunomodulatory activity.\(^4\) The ability to keep iron bound even at low pH is important, especially at sites of infection and inflammation where, due to the metabolic activity of bacteria, the pH may fall under 4.5. In such a situation, lactoferrin also binds iron released from transferrin, which prevents its further use for bacterial proliferation.\(^5\) Lactoferrin is known to inhibit the growth of various tumor cell lines through production of oxidative stress, such as in the case of leukemia cell lines.\(^6\)

Earlier studies have shown the effect of lactoferrin on macrophages, blood cells, and tumor cell lines. This protein has been known to kill tumors when given orally to mice.\(^7\) There has been very little research on the inhibitory effect of lactoferrin protein against parasitic diseases, for which we choose the most popular host cells for parasitic infections. Red blood cells (RBCs) are host cells for Plasmodium and Babesia, and macrophages have been targeted by Leishmania, Toxoplasma, and Mycobacterium. Previous studies have shown that lactoferrin protects human RBCs from oxidative stress in its monoferric form, and macrophages show increased levels of reactive oxygen species (ROS) after treatment with bovine lactoferrin.\(^8\) These studies have yielded interesting results and created enthusiasm for further in-depth study of the various biological reactions taking place inside lactoferrin-treated cells as well as normal cells. Therefore, the present study was undertaken in an attempt to understand the mechanism of interaction between lactoferrin and host cells by studying various parameters on human RBCs and macrophages, treated as host cells. Treatment of host cells with lactoferrin and its interaction with parasitic infections has been detailed in the present paper.

Survivin is a gene that is expressed only in live tumor cells and was included in the present study through analysis of various gene expression and metabolic processes to check the survival of THP1 cells after lactoferrin treatment. Survivin expression has been studied in tumor cells treated with higher doses of lactoferrin and was found to be downregulated due to treatment of tumor.\(^9\)

The immunomodulatory and anti-inflammatory activity of lactoferrin protein has been shown to occur via signaling pathways including various Toll-like receptors (TLRs).\(^10\,11\) TLR-2, TLR-4, and TLR-9 are activated in many infections involving virus, parasites, and bacteria. Lactoferrin has been shown to reduce the activity of Epstein Bar virus\(^12\) via interaction with its coreceptor. It is also known to activate macrophages through TLR-4, involving direct and indirect signaling pathways.\(^13\) TLR signaling in macrophages that have lactoferrin on their surface can activate different TLRs against various parasitic infections, so it is necessary to study the expression of different TLRs after stimulation of the host cell with lactoferrin.

MDR1 is used to investigate the expression of P-gp, a resistance marker gene in tumor cells. As there are a lot of problems related to antibiotic drug resistance in infectious diseases, we wanted to check the expression of this gene in the presence of lactoferrin. Therefore, it is important to determine whether it is helpful in normal conditions and also if it is helpful in resistant tumor cells whether to enhance the resistance or down regulate it.

Lactoferrin protein has been isolated from five types of mammals, namely, human, cow, buffalo, goat and mare, and comparative studies have been done to differentiate between the various origins.\(^14\) The main difference lies in the structural domain, which is different from one species to another. Many studies have used the bovine and human lactoferrins, but no studies have been done using buffalo lactoferrin. This is the first study investigating the interaction between buffalo lactoferrin and host cells. How we can target this protein in parasitic diseases by studying the basic mechanism of action, and whether it differs from human lactoferrin and bovine lactoferrin has been tried to determine in the present study.

**Materials and methods**

The bovine lactoferrin used in this study was obtained from Jagat Kanwar, Deakin University, Australia, and the buffalo lactoferrin was purified from buffalo colostrum using an ion exchange chromatography method described elsewhere.\(^15\)

Three types of lactoferrin proteins of bovine and buffalo origin were prepared on the basis of their different iron saturations. Iron-saturated lactoferrin was prepared according to a method previously described by Bates et al.\(^16\) Iron estimation for all forms of lactoferrin was done using a previously described method with minor modifications.\(^17\)
Apolactoferrin was prepared using the method devised by Feng et al with minor modifications, and was found to be 8%–10% saturated with iron; the monoferic form was found to be 40%–65% saturated and the holo form was found to be 85%–95% saturated.

Host RBCs and macrophages

The RBCs were obtained from a healthy human donor (Rotary and Blood Bank Society, Sec-37C Chandigarh, India). Use of human RBCs was approved by the institutional ethics committee, Postgraduate Institute of Medical Education and Research, Chandigarh, India. The THP1 cell line was purchased from National Cell Culture Science, Pune, India. Cell line was maintained in culture medium (Roswell Park Memorial Institute [RPMI] 1640 and 10% fetal bovine serum supplemented with L-glutamine, 25 mM HEPES buffer, and penicillin-streptomycin) at 37°C with 5% CO₂ and 90% humidity. The THP1 cells were differentiated to macrophages with the assistance of phorbol myristate-13 acetate at a final concentration of 10 nM for 48 hours.

Preparation of RBCs

Fresh blood is collected from donor and collected in an anticoagulant blood collection bag. The whole blood was dispensed in aliquots into glass bottles. A small quantity of blood from each glass bottle was transferred into centrifuge tubes and spun at 1,500 rpm for 10 minutes. The plasma and buffy coat were removed aseptically. The remaining white blood cells and platelets were removed by washing with medium twice. The final blood volume was mixed with growth medium containing AlbuMAX® and stored at 4°C for further use.

Treatment of RBCs and macrophages with lactoferrin

Differentiated THP1 cells and fresh RBCs at a concentration of 1×10⁴/mL were incubated with lactoferrin in 24-well tissue culture plates and 96-well plates, respectively. Concentrations ranging from 10, 20, 40 and 50 µg/mL were used, and cells were incubated for 24 and 48 hours for initial dose standardization. Optimum concentrations were selected and RBCs and macrophages were subjected to various parameters, i.e., morphology by Giemsa staining, production of ROS, capacity of phagocytosis, and survivin gene expression by real-time polymerase chain reaction. Expression studies of TLR and drug resistance by MDR1 were studied for better understanding of the mechanism of action of lactoferrin.

Morphometric analysis by Giemsa staining

After 48 hours of incubation, the macrophages and RBCs were fixed with 100% methanol and stained with Giemsa for 30 minutes, and then observed by morphometric analysis.

Production of reactive oxygen species

The standard protocol was followed, with slight modifications. The treated macrophages and RBCs were investigated for production of ROS, detected by 2-7, dichlorofluorescein (DCFH) dye used at a final concentration of 5 µM for all experiments. The cells were incubated with the dye for 15 minutes at 37°C, washed twice to remove the extra dye, and then fixed with 4% paraformaldehyde for 1 hour at 4°C. The cells were washed twice with phosphate-buffered saline (PBS) and acquired by flow cytometry analysis using FACS Caliber. Fluorescein isothiocyanate laser at 485 nm was acquired for ROS dye. Before starting the experiment, cytometry setup tracking was run, and the voltage was set up accordingly. Macrophages were acquired in a forward-scattered light plot, fluorescence was measured in each experiment, and was calculated in all experiments.

Viability of macrophages by MTT assay

The treated macrophages were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL) at 44 hours of incubation. After 48 hours of incubation, all medium was removed from the wells and 50–100 µL of dimethyl sulfoxide was added to each well. The violet crystals were dissolved by keeping the plates at 37°C for 15–20 minutes, and reading was taken at 595 nm. Percent viability was calculated following a standard protocol described elsewhere.

Measurement of phagocytic capacity

Treated and untreated macrophages were incubated with latex beads to investigate their phagocytic properties. Phagocytosis is the process by which macrophages engulf intracellular microbes/particles. How different forms of Lactoferrin show different degree of phagocytosis has been studied in this section.

The macrophages were incubated with 1 µm fluorescent latex beads (Sigma, St Louis, MO, USA) at a concentration of 5×10⁴ beads/mL for 15 minutes at 37°C. Extra nonengulfed
beads were removed by two washes in PBS, and the cells were fixed with paraformaldehyde and observed by light microscopy. The intensity of fluorescence of the engulfed macrophages was also measured by flow cytometry to confirm the light microscopy results. The mean fluorescence intensity of the targeted macrophages was further confirmed with confocal images.

Survivin, MDR1, and TLR gene expression
The effect of lactoferrin on macrophages was investigated by studying the expression of various genes using real-time polymerase chain reaction (LC 480 Roche, Basel, Switzerland). The cells were incubated with different types of lactoferrin at optimum dose for 48 hours. After incubation, the cells were detached from the plates using Accutase enzyme solution. Total RNA was extracted using TRIzol reagent and complementary DNA was prepared (Thermo Fisher cDNA kit, Loughborough, UK). Complementary DNA was amplified for the survivin, MDR1, TLR 4, and TLR 9 genes to check the fold increase or decrease in expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene. The TLR inhibitor OxPAPC was used to inhibit TLR signaling for 24 hours. Expression studies was then conducted using the method described earlier to demonstrate the effect of lactoferrin. Primer sequences with the annealing temperatures are given in Table 1.

**Assay for intracellular invasion by Toxoplasma gondii**
After treatment with the different lactoferrins, macrophages were infected with Toxoplasma gondii tachyzoites at a ratio of 1:5 (one macrophage and 5 tachyzoites), and after 5 hours, the number of intracellular tachyzoites were used to calculate the mean number of invasive events.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivin forward</td>
<td>GCCCAGTTTCTCTGCTT</td>
<td>60°C</td>
</tr>
<tr>
<td>Survivin reverse</td>
<td>CC GACAGGATGTTTTATG</td>
<td></td>
</tr>
<tr>
<td>TLR-4 forward</td>
<td>CCAGTGGAGGATGAGCGCAAGT</td>
<td>55°C</td>
</tr>
<tr>
<td>TLR-4 reverse</td>
<td>GCCATGGCGGGTCAGACAT</td>
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</tr>
<tr>
<td>TLR-9 forward</td>
<td>GACCTCCTGTACTGCCTCCA</td>
<td>55°C</td>
</tr>
<tr>
<td>TLR-9 reverse</td>
<td>AGGCTTCTGTACACCCAGCT</td>
<td></td>
</tr>
<tr>
<td>MDR1 forward</td>
<td>GCTGG TGCTGGCTAACATT</td>
<td>55°C</td>
</tr>
<tr>
<td>MDR1 reverse</td>
<td>GCTGACAGTCAAGAAGACAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>GCCAATACGCTAGACAC</td>
<td>56°C</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>GCCCAATACGCTAACAC</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1** Primer sequences and annealing temperature for different genes

Statistical analysis
The size variation, phagocytic capacity, ROS production, and number of invasive parasites between different treatment groups were compared with the untreated group; for expression studies the difference between each group was determined by Student’s t-test and between the groups in case of MTT assay by one-way analysis of variance test. Differences between groups were accepted as being statistically significant at p<0.05, p<0.01, and p<0.005. All results are average of three experiments when performed in duplicates.

**Results**
No morphometric changes in RBCs after treatment with lactoferrin
After treatment of the human RBCs with the different types of lactoferrin, no color was observed in cultured supernatants, indicating no hemolysis of RBCs. No significant difference in the morphology of RBCs was observed on Giemsa staining at concentrations of 10–40 μg/mL on comparison with the untreated control group (Figure 1A). The results of morphological analysis by Giemsa staining were analyzed, and 40 μg/mL concentration of protein was chosen and used for further experiments, such as measurement of ROS production (Figure 1B).

Iron-saturated lactoferrin showed increased ROS production in RBCs
Lactoferrin showed different amounts of ROS production depending on its degree of iron saturation. When treated with lactoferrins at a concentration of 40 μg/mL, RBCs showed a different pattern of ROS production. The apolactoferrin form used at this concentration showed significantly decreased levels of ROS production compared with the untreated control group, whereas iron-saturated bovine lactoferrin showed a significant 2-fold increase in ROS level compared with the untreated group at 48 hours post-incubation (Figure 2).

**THP1 macrophage cells survived in a dose-/time-dependent manner**
The viability of the treated macrophages were observed using MTT viability assay. There was no significant difference observed in percent cell viability when compared with untreated macrophages at 10, 20, 40 and 50 μg/mL 48 hours posttreatment (Figure 3A). Morphological changes in the macrophages, including size and diameter, were observed.
after treatment with the lactoferrins, and no significant difference in size was found between the treated and untreated macrophages (Figure 3B and C).

Iron-saturated lactoferrin enhanced ROS production, phagocytic capacity, and expression of TLR and survivin in macrophages

When concentrations ranging from 10 to 40 μg/mL were used from all types of proteins, there was a significant increase in ROS production from a lower to a higher dose, with a 2–4-fold increase in intensity (Figure 4A and B). Iron-saturated lactoferrin protein has been shown to generate many free radical ions which can be helpful for inhibiting a variety of tumors, intracellular parasites, and microbes. After investigation of these parameters, the dose was standardized and the 20 μg/mL concentration was chosen to evaluate further parameters on the macrophages.

After treatment with the different lactoferricin at a concentration of 20 μg/mL, the phagocytic capacity of the macrophages was measured (Figure 5A). Interestingly there was a higher mean fluorescence intensity in iron-saturated lactoferrin-treated macrophages compared with
Figure 3 (A) ROS production in the differently treated groups of RBCs after 48 hours of incubation. (B) ROS production shown using bar diagrams. Each value is a replicate of three values. A significant fold increase *P<0.05 was found in the iron-treated groups when compared with the untreated group and significant P<0.05 difference was found within different groups, along with a significant *P<0.05 decrease in ROS production in the apolactoferrin-treated group compared with the untreated group.

Abbreviations: Buff, buffalo; Apo, apolactoferrin; RBCs, red blood cells; ROS, reactive oxygen species; BLF, bovine lactoferrin; BLF Apo, bovine apolactoferrin; BLF Fe, iron-saturated bovine lactoferrin; Buff, buffalo lactoferrin; Buff Apo, buffalo apolactoferrin; Buff Fe, iron-saturated buffalo lactoferrin.
No significant difference was found between the various groups.