Lactoferrin Binding by Leukemia Cell Lines

By Yasuki Yamada, Tatsuhiko Amagasaki, Donald W. Jacobsen, and Ralph Green

Monocytes and macrophages have receptors for the iron-binding protein lactoferrin. Lactoferrin acts as a potent inhibitor of granulocyte-macrophage colony stimulating factor production when it binds to these cells. Using a rosette assay and immunofluorescence, we have shown that cultured leukemia cells, including the human erythroid leukemia cell line K562, also have lactoferrin binding sites. The number of binding sites on K562 cells was estimated using soluble 56Fe-lactoferrin. Inhibition studies demonstrate that lactoferrin binding sites are distinct and unrelated to receptors for transferrin or the Fc portion of IgG, which are present on K562 cells. However, electrostatic forces may be important for lactoferrin binding, since other polycationic proteins (e.g., protamine) inhibit lactoferrin binding. Prior treatment of K562 cells with trypsin nearly abolishes lactoferrin binding. However, these cells recover their ability to bind lactoferrin when trypsin is removed. Unlike transferrin receptors, the expression of lactoferrin binding sites is not regulated by cellular iron status. Cytosine arabinoside arrests the proliferation of K562 cells and simultaneously leads to a reduction in lactoferrin surface binding, suggesting that lactoferrin binding may be dependent on cell proliferation. © 1987 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Cell preparation. K562 cells, a human erythroid leukemia cell line, were used to analyze the kinetics of lactoferrin binding. Cells were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone (Irvine Scientific, Santa Ana, CA). MOLT-4 cells and CCRF-CEM cells (human T lymphoblastic leukemia cell lines), U937 cells (human histiocytic lymphoma cell line), HL-60 cells (human acute promyelocytic leukemia cell line), CCF-Y1 cells (a human B cell line established in our laboratory from a patient with Ph1 negative juvenile chronic myelocytic leukemia), Raji cells (human Burkitt lymphoma cell line), and L1210 cells (mouse B lymphocytic leukemia cell line) were also used and were grown under similar conditions but with 10% fetal bovine serum medium. All experiments were conducted using cells at growth densities of 3 to 5 x 10⁶ cells/mL (logarithmic phase). Cells were washed once with RPMI 1640 + 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St Louis), resuspended in the same medium at a cell density of 1 x 10⁶/mL, and used for rosette formation.

Mononuclear cells were separated from heparinized normal blood by a density-gradient centrifugation technique using Ficoll-Hypaque (FH; Pharmacia, Piscataway, NJ). Cells were then passed through a nylon-wool column to enrich T lymphocytes. The resultant T lymphocytes were activated by stimulation with concanavalin A (Con A; Sigma) for 96 hours and evaluated for lactoferrin binding.

Lactoferrin and transferrin. purified human apo-lactoferrin from Colostrum (Calbiochem-Behring, La Jolla, CA) and human apo-transferrin (Sigma) were saturated with iron according to the method described by Bates and Schlabach. Iron saturation was confirmed spectrophotometrically by an increase in absorbance at 460 nm (A460/A280 = 0.045 to 0.048 for fully saturated transferrin). Monoclonal antibodies (MoAbs) against the human transferrin receptor (42/6 and B3/25) were generously supplied by Dr. Ian Trowbridge (Salk Institute, La Jolla, CA). OKT9 anti-human transferrin receptor antibody was purchased from Ortho Diagnostic Systems (Raritan, NJ).

Lactoferrin coating of bovine red blood cells and rosetting procedure. Lactoferrin was coated on bovine red blood cells (B-RBC) using CrCl₃ according to the method described originally for the protein A hemolytic plaque assay and modified subsequently by

*LaCtoFoReIN, a cationic glycoprotein that binds two atoms of ferric iron per molecule, is present in high concentration in milk (1 mg/mL). Other body secretions, and in secondary granules of myeloid cells (3 μg/10⁶ neutrophils), but is scarcely detectable in the serum (less than 1 μg/mL). It has many biochemical similarities to transferrin, which is present in plasma (2 to 4 mg/mL) and other body fluids. A major biological role for transferrin as an iron transport and delivery protein has been defined. Immature erythrocytes, activated lymphocytes, and neoplastic cells, which require iron for hemoglobin synthesis or replication, take up iron efficiently through the specific receptor for transferrin. On the other hand, the biological significance of lactoferrin remains obscure. One of its roles is in the regulation of normal myelopoiesis. Lactoferrin binding to the surface of monocytes through a putative specific receptor results in suppression of the release of granulocyte-macrophage colony stimulating factor. Another suggested role is as a bacteriostatic or bactericidal agent. Apo-lactoferrin depresses the multiplication of iron-dependent microbial strains. Little is known about the relation between lactoferrin and neoplastic cells. By applying a rosetting assay that was developed in our laboratory, we have found that neoplastic cells also express lactoferrin binding sites similar to those reported for monocytes or macrophages. We report here the nature of lactoferrin binding to cultured leukemia cells.
this laboratory for transferrin-coated B-RBC. Successful coupling of lactoferrin on B-RBC was confirmed with fluorescein isothiocyanate (FITC)-labeled antilactoferrin antibody (Cappel Worthington Biochemicals, Malvern, PA). Apo-lactoferrin, transferrin, or albumin-coated B-RBC were prepared similarly, using the same concentrations of protein. Cultured neoplastic cells or T lymphocytes (1 x 10^6/mL in 0.25 mL) were mixed with 0.1 mL of lactoferrin, transferrin, or albumin-coated B-RBC (0.5% packed cell volume) in U-bottom 12 x 75-mm tubes (Falcon #2054, Oxnard, CA). The mixtures were centrifuged at 30 x g for three minutes and then incubated at 37°C for 45 minutes (except for the time-course study). The pellets were carefully resuspended, and rosette formation was estimated visually using a hemocytometer. More than 200 cells were counted, and those cells binding more than three B-RBC on their surface were considered positive for rosette formation. All experiments were performed in triplicate.

**Immunofluorescence assay.** Lactoferrin binding was also evaluated by an immunofluorescence assay using soluble lactoferrin. After washing three times with PBS, 1 x 10^6 pelleted cells were incubated with 100 μL of lactoferrin solution (50 μg/mL in PBS) at 4°C for 60 minutes. The treated cells were washed three times with PBS, and then incubated with 50 μL of a 1/20 dilution of FITC-conjugated rabbit antimouse lactoferrin antibody (F(ab')2 fraction, Cappel Labs). After washing three times with PBS, the labeled cells were examined using a fluorescence-activated cell sorter.

**59Fe-lactoferrin binding assay.** Apo-lactoferrin was saturated with 59Fe (59FeCl2, New England Nuclear, Boston) by the method used for preparing nonradioabeled differic-lactoferrin. K562 cells (1 x 10^6) suspended in Hank’s balanced salt solution (HBSS) were incubated at 4°C for 60 minutes with various concentrations of 59Fe-lactoferrin. After incubation cells were washed three times with HBSS, and radioactivity was measured in a gamma spectrometer.

**Benzidine staining.** K562 cells were cultured in the presence of 3.6 x 10^-7 mol/L cytosine arabinoside (Sigma) to induce erythroid differentiation for assessment of differentiation-associated change in lactoferrin binding ability. Hemoglobin synthesis in these cells was estimated by benzidine staining. The proportion of cells that synthesized hemoglobin (stained blue) was assessed by light microscopy.

**Other chemicals.** Deferoxamine mesylate (Desferal, CIBA Pharmaceutical Co, Summit, NJ) or ferric ammonium citrate (Sigma) were dissolved in distilled water and added to culture media at final concentrations of 1 x 10^-7 mol/L and 10 μg/mL respectively to determine the effect of iron content of the culture solution on the expression of transferrin receptor and lactoferrin binding sites. Cells treated with 0.25% trypsin solution (Irvine Scientific) for one minute at 37°C, cells treated with 2 mg/mL of deoxyribonuclease I or ribonuclease A (DNase and RNase, Sigma) for one hour at room temperature, and cells treated with 0.1 U/mL of neuraminidase (Calbiochem-Boehringer) for 20 minutes at 37°C were used to assess the susceptibility of lactoferrin binding sites to digestion by these enzymes. Human IgG (Sigma) was incubated for 30 minutes at 60°C to prepare heat-aggregated IgG for use in experiments to study inhibition of rosette formation. Human milk lysozyme and salmon protamine (Sigma) were also used to study the specificity of lactoferrin binding.

**RESULTS**

**Time-course study of rosette formation.** Mixtures of K562 cells and lactoferrin-, apo-lactoferrin- or albumin-coated B-RBC were incubated for up to 90 minutes at 37°C. Rosette formation with lactoferrin-coated B-RBC increased rapidly and reached a plateau after 45 minutes incubation (Fig 1). Rosette formation was lower with apo-lactoferrin-coated B-RBC. Control albumin-coated B-RBCs did not form rosettes throughout the incubation time.

**Determination of lactoferrin binding sites by Scatchard analysis.** 59Fe-radiolabeled lactoferrin showed saturable binding to K562 cells (Fig 2). A Scatchard plot analysis of these data showed maximum binding of 4.9 x 10^7 molecules per cell; the dissociation constant was 7.4 x 10^-8 mol/L. A competition assay using a tenfold excess of unlabeled lactoferrin was also attempted but was unsuccessful due to the technical problem caused by cell aggregation at lactoferrin concentrations greater than 2.5 mg/mL.

**Specificity of rosette-forming assay for detection of lactoferrin binding sites.** To test the specificity of the rosette-forming assay, K562 cells were preincubated with either soluble lactoferrin, transferrin, or MoAbs against transferrin receptor for 30 minutes at room temperature before addition of lactoferrin-coated B-RBC. Rosette formation was inhibited by prior treatment with soluble lactoferrin in a dose-dependent manner (Fig 3). Soluble apo-lactoferrin also inhibited rosette formation almost to the same extent as did soluble lactoferrin (Table 1). However, neither transferrin nor three types of MoAbs against transferrin receptor inhibited rosette formation with lactoferrin-coated B-RBC (Table 1); all inhibited rosette formation with transferrin-coated B-RBC at the same concentrations.

Heat-aggregated IgG at a concentration of 64 μg/mL, which is sufficient to fully saturate all Fe receptors on K562 cells, did not inhibit rosette formation (Table 1). Since lactoferrin is a cationic protein (pI = 8.7), nonspecific electrostatic binding to the cell surface membrane was considered. To investigate this possibility, we used two kinds of cationic proteins in competition studies: human milk lysozyme and salmon protamine. Lysozyme caused no inhibition, but protamine substantially inhibited rosette formation. Furthermore, neuraminidase, an enzyme that cleaves membrane surface sialic acid resulting in decreased cell surface negative charge, also partially inhibited rosette formation.

**Trypsin sensitivity of lactoferrin binding sites.** K562 cells were examined for rosette formation with lactoferrin-coated B-RBC before and after digestion with trypsin. In three experiments, rosette formation decreased markedly...
Fig 2. Dose-response binding of ^{59}Fe-radiolabeled lactoferrin to K562 cells. The mean of three experiments is shown. A Scatchard plot analysis (inset) indicates that \( 4.9 \times 10^4 \) molecules are bound to each K562 cell at saturation. The dissociation constant \( (K_d) \) derived from the concentration of free ligand at half saturation of the binding sites is \( 7.4 \times 10^{-9} \) mol/L.

Following trypsin digestion (from 82.9% ± 0.6% to 11.8% ± 3.9%) but recovered almost to pretreatment levels following culture of the digested cells for five hours at 37°C in culture medium without trypsin (74.6% ± 2.0%). Trypsin sensitivity of lactoferrin binding by MOLT-4 cells was also shown using the immunofluorescence assay (Fig 4). Binding

Fig 3. Inhibition of rosette formation by soluble lactoferrin. K562 cells were pretreated with soluble lactoferrin before rosette formation. Each point is the mean of three assays; vertical bars indicate standard deviations.

Table 1. Specificity of Rosette-Forming Assay

<table>
<thead>
<tr>
<th>Treatment of K562 Cells*</th>
<th>Inhibition of Rosette Formation (%)</th>
<th>Mean ± SD (n = 3)</th>
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<tbody>
<tr>
<td>Lactoferrin (64 μg/mL)</td>
<td>74.0 ± 1.8</td>
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<tr>
<td>Apo-lactoferrin (64 μg/mL)</td>
<td>77.9 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Transferrin (64 μg/mL)</td>
<td>-1.9 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Apo-transferrin (64 μg/mL)</td>
<td>-3.1 ± 9.0</td>
<td></td>
</tr>
<tr>
<td>MoAbs against transferrin receptor</td>
<td>9.1 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>42/6 (1 μg/mL)</td>
<td>3.4 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>B3/25 (1 μg/mL)</td>
<td>5.4 ± 8.7</td>
<td></td>
</tr>
<tr>
<td>OKT9 (1 μg/mL)</td>
<td>1.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Heat-aggregated human IgG (64 μg/mL)</td>
<td>-3.1 ± 9.0</td>
<td></td>
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<tr>
<td>Cationic proteins</td>
<td></td>
<td></td>
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<tr>
<td>Human milk lysozyme (64 μg/mL)</td>
<td>-1.9 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Salmon protamine (64 μg/mL)</td>
<td>44.6 ± 8.9</td>
<td></td>
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<tr>
<td>Neuraminidase (0.1 U/mL)</td>
<td>31.5 ± 4.0</td>
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*K562 cells were pretreated with these agents before rosette formation for 30 minutes at room temperature except for neuraminidase treatment, which was performed at 37°C.

Fig 4. Trypsin sensitivity of lactoferrin binding sites. Labeled MOLT-4 cells (-----) were processed on a fluorescence-activated cell sorter and compared with control cells (-----) treated with FITC-conjugated antibody only. Nontreated cells (A), trypsin-digested cells (B), DNase-treated cells (C), and RNase-treated cells (D) were analyzed for lactoferrin binding.
was almost completely abrogated by trypsin digestion (Fig 4B). DNase did not remove but rather enhanced lactoferrin binding (Fig 4C). Interestingly, RNase caused slight inhibition of lactoferrin binding (Fig 4D).

**Influence of iron availability on the expression of lactoferrin binding.** K562 cells were cultured up to 96 hours in the presence of the iron chelator deferoxamine (1 × 10⁻³ mol/L) or 10 μg/mL ferric ammonium citrate. At various times lactoferrin and transferrin binding by these cells was assessed with the rosette-forming assay. Proliferation status of K562 cells was not affected by either agent at these concentrations, and the doubling times of these cells were similar to untreated control cells. Rosette formation with transferrin-coated B-RBC increased two to three times following deferoxamine treatment but decreased to less than one-half following addition of ferric ammonium citrate (Fig 5A). However, rosette formation with lactoferrin-coated B-RBC was not influenced by these agents (Fig 5B).

**Influence of cell proliferation status on expression of lactoferrin binding.** K562 cells were cultured with cytosine arabinoside (C-ara) and examined for rosette formation with lactoferrin-coated B-RBC at 24-hour intervals. The culture solution was replaced by fresh media containing C-ara every other day. Cell proliferation ceased after 24 hours in C-ara medium, but the viability remained greater than 90%. The proportion of benzidine-positive cells increased daily and reached 77.7% at 144 hours, indicating maturation to hemoglobin-producing cells. There was a corresponding inverse decline in rosette formation rate (Fig 6). This decrease in lactoferrin binding could be curtailed by removing C-ara from the culture media.

**Lactoferrin binding sites on various cells.** Expression of lactoferrin binding sites was examined on other neoplastic cell lines as well as on normal blood lymphocytes before and after activation with Con A (Table 2). The immunofluorescence assay was always more sensitive than the rosette-forming assay. Four (K562, MOLT-4, L1210, and CCF-Y1) of eight cell lines examined showed abundant lactoferrin binding sites. The rosette-forming assay failed to detect lactoferrin binding sites on activated T lymphocytes, but the immunofluorescence assay showed that approximately one half of activated T lymphocytes display lactoferrin binding.
lactoferrin. The effect was more pronounced in the presence of iron. These reports led us to study lactoferrin binding sites on neoplastic cells.

In the present studies we used a rosette-forming assay to detect lactoferrin binding. Rosette formation rate was greater with lactoferrin-coated than with apo-lactoferrin-coated B-RBC, suggesting a higher binding affinity of iron-saturated lactoferrin. Although differences in the quantity of protein coating cannot completely be excluded, it is unlikely that this is the cause of the discrepancy, since the same concentration of lactoferrin or apo-lactoferrin was used for coating. By analogy, iron-saturated transferrin has higher binding affinity to its receptor than apo-transferrin. The transferrin molecule becomes more compact and spherical after it has bound iron, and this may be advantageous for receptor binding. On the other hand, rosette formation with lactoferrin-coated B-RBC was inhibited by soluble lactoferrin irrespective of its iron content, suggesting that low affinity binding is sufficient to suppress rosette formation. However, lactoferrin-coated B-RBC rosette formation was not inhibited by transferrin, MoAbs against the transferrin receptor, or heat-aggregated human IgG. This indicates that lactoferrin occupies binding sites different from those for either the transferrin receptor or the Fc receptor for IgG, which are both present on K562 cells.

The number of lactoferrin binding sites on K562 cells was estimated by Scatchard analysis using 59Fe-lactoferrin. Binding of 59Fe-lactoferrin was saturable, and the number of binding sites per cell (4.9 × 10^5), though high, agrees with figures reported for adherent mononuclear cells (3.3 × 10^7 per cell) and nonrosetting lymphocytes (2.5 × 10^7 per cell) prepared from normal human blood. Because of problems with cell aggregation that we encountered at high lactoferrin concentrations, it was not possible to eliminate nonspecific binding, so that our figure is likely an overestimate of the number of binding sites.

Since lactoferrin is a cationic protein (pl = 8.7), we considered that binding to the negatively charged cell surface might be of a nonspecific electrostatic nature. We therefore pretreated K562 cells with two other cationic proteins to block surface-negative charges. The more cationic lysozyme (pl = 10.5 to 11.0) did not inhibit lactoferrin-B-RBC rosette formation. However, protamine caused considerable inhibition, although the inhibitory activity was less than that of soluble lactoferrin. Sialic acid, one of the major sources of surface negative charge, was removed by neuraminidase treatment of K562 cells and resulted in a decrease of rosette formation. Taken together these findings suggest that electrostatic factors are intimately involved in lactoferrin binding to cells. Similar electrostatic factors have been implicated in the binding of lactoferrin to macrophages and liver reticuloendothelial cells. Lactoferrin binds to alveolar macrophages in competition with the other cationic neutrophil granule glycoproteins, elastase and cathepsin G. Removal of sialic acid from lactoferrin increases its binding affinity to mouse peritoneal macrophages. Moreover, based on a study of lactoferrin uptake by the liver, the possible existence of common binding sites for certain cationic proteins on liver reticuloendothelial cells has been postulated.

The lactoferrin binding sites we have demonstrated on neoplastic cell lines are similar in some respects to the lactoferrin binding sites described on macrophages and liver reticuloendothelial cells. However, there are also differences, such as the sensitivity to trypsin digestion. We found marked sensitivity to trypsin digestion of lactoferrin binding by neoplastic cells. In contrast, lactoferrin binding by human peripheral blood monocytes is somewhat resistant to trypsin digestion. A further difference is the sensitivity to DNase and R.Nase. Lactoferrin binding sites on neoplastic cells were resistant to DNase but were partially sensitive to RNase, suggesting that lactoferrin binding sites may be RNase-susceptible acidic groups present on the cell surface. In contrast to neoplastic cells, lactoferrin binding sites on normal human monocytes are sensitive to DNase treatment.

Transferrin receptor expression of cultured cells is regulated by the iron concentration of the culture medium. An iron chelator, deferoxamine, increases the number of transferrin receptors, whereas excess iron decreases transferrin receptor expression. We confirmed this observation by finding a good inverse correlation between iron supply and transferrin-receptor expression using our rosette-forming assay. Lactoferrin shows close structural homology to transferrin at the iron binding site, but unlike transferrin, lactoferrin binding to cells was not influenced by cellular iron status. This finding supports the view that K562 cells cannot take up iron from lactoferrin even though cells can bind and internalize lactoferrin (unpublished observation).

Four of eight leukemic cell lines as well as activated T lymphocytes showed lactoferrin binding, but resting T lymphocytes did not. Similarly, receptor-like binding of lactoferrin to fresh leukemia cells, but not to normal lymphocytes or platelets has been reported. Furthermore, we were able to demonstrate diminished expression of lactoferrin binding by C-ara treatment of K562 cells. These results seem to indicate that there is a close relationship between lactoferrin binding and cell proliferative status. Although monocytes and activated lymphocytes also have lactoferrin binding sites, analysis in conjunction with other cell markers may prove useful for the identification of leukemic cells in blood or neoplastic cells in body fluids suspected to contain metastatic tumor.

### Table 2. Lactoferrin Binding by Neoplastic Cell Lines and Normal T Lymphocytes

<table>
<thead>
<tr>
<th>Cells</th>
<th>Rosette Formation % Mean ± SD (n = 3)</th>
<th>Immunofluorescence Assay (%)</th>
</tr>
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<tbody>
<tr>
<td>K562</td>
<td>90.0 ± 3.8</td>
<td>97.7</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>21.3 ± 5.2</td>
<td>67.3</td>
</tr>
<tr>
<td>L1210</td>
<td>28.9 ± 3.3</td>
<td>ND</td>
</tr>
<tr>
<td>CCF-Y1</td>
<td>13.0 ± 1.4</td>
<td>91.8</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>2.9 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>HL-60</td>
<td>0.7 ± 0.7</td>
<td>9.7</td>
</tr>
<tr>
<td>U937</td>
<td>0</td>
<td>14.0</td>
</tr>
<tr>
<td>Raj</td>
<td>0</td>
<td>4.9</td>
</tr>
<tr>
<td>Resting T lymphocytes</td>
<td>0</td>
<td>2.8</td>
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<tr>
<td>Mitogen-stimulated T lymphocytes</td>
<td>0</td>
<td>55.4</td>
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REFERENCES


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