Lactoferrin Binds to Cell Membrane DNA

ASSOCIATION OF SURFACE DNA WITH AN ENRICHED POPULATION OF B CELLS AND MONOCYTES

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ABSTRACT The binding of human $^{125}$I-labeled lactoferrin (LF) to a population of adherent mononuclear cells (ADMC) and nonrosetting lymphocytes (E−) was abolished by prior treatment of the cells with deoxyribonuclease (DNase), but not ribonuclease (RNase). When DNase-treated ADMC were incubated with exogenous DNA, the binding of $^{125}$I-LF was restored. Enzymatic digestion with other enzymes, trypsin, phospholipase D, and neuraminidase, did not significantly influence $^{125}$I-LF binding. Saturable binding of LF at 0°C was demonstrated for both E− and ADMC, with equilibrium dissociation constants of $0.76 \times 10^{-6}$ M and $1.8 \times 10^{-6}$ M, respectively. E− cells bound $2.5 \times 10^7$ and ADMC bound $3.3 \times 10^7$ molecules of LF at saturation. Cell membranes were isolated from ADMC, E− and E+ and reacted with $^{125}$I-labeled LF; significant binding was only seen with ADMC and E−. Prior treatment of the membranes with DNase abolished the binding. Immunofluorescence studies indicated that a population of ADMC and E−, but not E+, exhibited a peripheral staining pattern for LF. Prior treatment of ADMC and E− with DNase abolished the surface immunofluorescence. This study provides evidence that cell membrane DNA acts as a binding site for exogenous LF. This is a novel role for DNA that has not been previously reported. Furthermore, it points to a basic difference between E+ cells vs. ADMC and E− cells in respect to their possession of cell surface DNA.

INTRODUCTION

In 1976, Van Snick et al. (1) described the binding of human lactoferrin (LF) to mouse peritoneal macrophages. We have subsequently explored the binding of human LF to the peripheral blood cells of human subjects and shown an interaction with a population of adherent mononuclear cells (ADMC) and nonrosetting cells (E−) (2). Although the biological role of LF is poorly defined, it has been hypothesized that it may play a role in the feedback inhibition of granulopoiesis. LF is found in secondary granules of neutrophils (3) and Broxmeyer et al. (4) have shown that in vitro it will partially inhibit the production of a granulocyte-macrophage colony-stimulating factor (GM-CSF) in concentrations as low as $10^{-17}$ M (4). This inhibitory action was originally thought to be due to a direct effect of LF binding to monocytes (4, 5). However, thymus-derived lymphocytes (T cells) also produce GM-CSF (6) and, more recently, Bagby et al. (7) have shown that LF inhibits the production of a monocyte-derived soluble mediator that activates T cells to secrete GM-CSF. The facility of LF to inhibit production of a monokine raises the issue as to whether LF may have a nonspecific immunomodulatory function, and also focuses attention on the nature of the LF receptor. In this report, we present evidence that cell membrane DNA (cmDNA) acts as a binding site for LF on ADMC.

1 Abbreviations used in this paper: ADMC, adherent mononuclear cells; cmDNA, cell membrane DNA; cmS, cell membrane suspension; DNase, deoxyribonuclease; E−, nonrosetting lymphocytes; E+, rosetting lymphocytes; LF, lactoferrin; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; RNase, ribonuclease; RT, room temperature. Sig, surface membrane immunoglobulin.
and E− cells. A corollary to this study indicates that there is a fundamental difference between E+ cells vs. E− cells and ADMC in respect to their possession of cmDNA.

**METHODS**

**Agents used.** LF was isolated from pooled human breast milk, as previously described (10). Its purity was verified by polyacrylamide gel electrophoresis and immunoelectrophoresis using tropsin to whole human serum and human breast milk (10, 11). It was sterile on routine cultures and endotoxin-free in the Limulus amebocyte lysate assay (12). From the E465% 1%, it was 85% iron-saturated. LF was labeled with 125I by the chloramine-T method (13) to a 24 μCi/μg sp act. Pronase, trypsin, dextran sulfate, NP40, alpha naphthyl acetate, and deoxycholate were purchased from Sigma Chemical Co. (St. Louis, MO). Deoxynucleoside I (DNAse I) and ribonuclease (RNase) were purchased from Miles Biochemicals (Elkhart, IN). Tween 20 was purchased from Fisher Scientific Co. (Pittsburgh, PA). Calf thymus DNA was purchased from Worthington Biomedical Corp. (Freehold, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-LF antibodies were purchased from Atlantic Antibodies (Westbrook, ME)—the protein content was 19 mg/ml and the fluorescein/protein ratio 3.4. The T cell monoclonal antibody (T101) was purchased from Antibodies Incorporated (Davis, CA).

**Preparation of peripheral blood cell populations.** Human buffy coats (45 g) were obtained from the local Red Cross within 2 h of separation. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Paque as described (8). PBMC were layered onto 60-mm plastic petri dishes previously coated with heat-inactivated newborn calf serum (FCS, Microbiological Assoc., Walkersville, MD) and were incubated at 37°C for 1 h. Nonadherent cells were washed two times in Hanks' balanced salt solution (HBSS) and separated into E− and E+ subpopulations by rosetting two times with sheep erythrocytes. The E+ cells were liberated from the sheep erythrocytes by hypotonic lysis (9). ADMC were harvested, gently resuspended with a rubber policeman, and washed two times in HBSS.

All cell preparations were assessed for viability by trypan blue exclusion; it was >96% in each instance. Staining for alpha naphthyl acetate esterase showed that E+ cells contained <1% positive cells, E− cells contained 6% positive cells, and ADMC contained >91% positive cells. Staining for surface membrane immunoglobulin (Si) showed that E+ cells contained <4% Si+ cells and E− cells contained >94% Si+ cells. Staining with a T cell-specific monoclonal antibody (T101) indicated that >97% of E+ cells were positive, whereas <2% of ADMC and <8% E− cells were positive. Rosetting of E+ cells gave <5% nonrosetting cells, whereas rosetting E− cells gave <2% rosettes.

**Preparation of cell membranes.** Before disruption, the cell membranes were stabilized by the zinc chloride (ZnCl₂) method of Warren, Glick, and Nass (14). Briefly, the procedure was as follows. 1 ml of cells (2 × 10⁶) was added to 9 ml of 0.001 M ZnCl₂ at room temperature. After 10-min 0.5 ml of Tween 20 (1%) was slowly added with gentle stirring, followed by 0.5 ml of 0.01 M ZnCl₂ (at this stage, the cell membrane appears as a dark ring around swollen cells with phase-contrast microscopy). The cell suspension was cooled to 4°C and the cells disrupted with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), for 45 s at setting 8. The extent of disruption was monitored by phase-contrast microscopy; with the conditions used, ~70% of the cells were disrupted. 10 ml of 60% sucrose was added to the cell homogenate and this was then layered onto 30 ml of 45% sucrose solution and centrifuged at 400 g (4°C, 30 min). The supernatant was aspirated to within 3 mm of the interface and saved. The interface was aspirated and relayed over a 45% sucrose solution and centrifuged at 1,000 g (4°C, 20 min). The supernatant was collected and combined with the previous supernatant; further centrifugation at 8,000 g (4°C, 20 min) was performed to obtain a cell membrane pellet. The pellet was resuspended in 5 ml of 35% sucrose solution, layer onto a 45–60% sucrose gradient, and centrifuged at 25,000 g (4°C, 60 min). The cell membranes banded about half way down the tube at a sucrose density of 1.258. For further studies, the sucrose was removed by the addition of HBSS and centrifugation. In the studies reported here, the cell membrane protein content was 320 μg/ml of membrane suspension as determined by the Bio-rad protein assay (Bio-Rad Laboratories, Richmond, CA) (15), and the ratio of cell membrane to whole cell 5S ribonuclease (EC 3.1.3.5) activity was 17:1 using AMP as the substrate (16).

125I-LF binding to cells. The binding of 125I-LF to intact cells was performed at 0°C to reduce receptor turnover and internalization. Binding assays were done in 400-μl microfuge tubes (Beckman Instruments, Inc., Irvine, CA). Each tube contained 150 μl of dibutyl phthalate dinonyl phthalate (2:1 ratio) and the cell suspension was layered over this in a volume of 200 μl containing 4 × 10⁶ cells. Various concentrations of 125I-LF were added to each tube and mixed with the cell suspension. After incubation at 0°C for 1 h (prior experiments indicated binding equilibrium at ≥45 min) the tubes were centrifuged (microfuge B, Beckman Instruments, Inc.) at 10,000 g for 5 min. The tips of the tubes, containing the cells sedimented through the dibutyl phthalate, were cut off and counted. To assess non-specific binding an identical procedure was followed with the exception that a 20-fold excess of unlabelled LF was added at each concentration of 125I-LF. Nonspecific counts were subtracted from the original counts to obtain an estimate of specific LF cell binding for Scatchard plot analysis of binding affinity and number of molecules bound (18). In a comparison assay cells were incubated in the presence of DNAse (50 μg/ml) both before (15 min at room temperature [RT]) and during incubation (0°C) with LF at each concentration. Prior experiments have established that DNase does not interact with LF to inhibit specific receptor binding.

**Immunofluorescence studies.** Purified populations of E+, E−, and ADMC (2 × 10⁶ cells/ml) were suspended in HBSS containing 0.1% sodium azide. A aliquot of cells were subjected to digestion with DNase I (250 μg/ml, 1 h) and RNase (0.1% wt/vol, 25°C, 1 h). All cell suspensions were incubated with LF (10 μg/ml) in an ice bath for 30 min. After washing three times with ice-cold HBSS containing 0.1% sodium azide, the cells were incubated with FITC-conjugated anti-LF (100 μl of a 1:10 dilution/2 × 10⁶ cells) for 30 min, while remaining in an ice bath. After washing three times, the cells were resuspended in 60% glycerol in HBSS before mounting. The slides were examined with a binocular microscope using epifluorescence from a mercury vapor and a BG12 excitor filter and a 490-nm barrier filter. Photographs were taken in Ektachrome (ASA 400) film using an Orthomat automatic light monitor (Leitz Inc., Rockleigh, NJ). Control experiments were performed by omitting the incubation step with LF, by pretreatment of the cells with unfluoresceinated anti-LF and by absorption of the fluoresceinated antiserum with excess LF.
**RESULTS**

**LF binding to intact cells.** The binding of ^125^I-LF to E^- and ADMC is seen to be a saturable phenomenon (Figs. 1 and 2). Prior enzymatic treatment of the cells with DNase reduces the binding at all concentrations of LF to a level comparable to the nonspecific binding curve. The binding of LF to E^- and ADMC was not significantly influenced by prior treatment of the cells with RNase or other enzymes which might be expected to influence ligand-cell membrane binding (Table I). When DNase-treated cells were incubated with exogenous DNA (calf thymus) the binding capacity for LF was restored (Table I). E+ cells exhibited very little binding of ^125^I-LF and this appears to be of a nonspecific nature (Fig. 3). Scatchard plot analysis (18) of the data from E^- and ADMC indicate a high-binding affinity for both cell types consistent with a ligand-receptor association (Figs. 4 and 5).

**Immunofluorescence studies.** When cells incubated with LF (10 μg/ml) under conditions that should minimize capping, were studied with FITC-conjugated anti-LF, ~90% of E^- and ADMC cells but <3% E+ cells, exhibited a peripheral staining pattern (Fig. 6). Without prior incubation with LF, the occasional cell (<1 in 200) exhibited a peripheral staining pattern. At higher magnifications, the staining is seen to have a characteristic globular appearance. Prior treatment of the cells with DNase, but not RNase, completely abolished the surface staining. In control experiments in which the cells were first incubated with unfluoresceinated anti-LF or the FITC-conjugated anti-LF was absorbed with excess LF, specific surface staining was not seen.

**LF binding to isolated cell membranes.** In keeping with the observations on whole cells, ^125^I-LF bound to isolated cell membranes, and this was largely abolished by prior digestion of the membranes with DNase (Table II). It is interesting to note some binding to membranes isolated from E+ cells. We have not been able to show consistent binding to whole E+ cells (2), al-

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**Figure 1**  Dose-response curve ^125^I-LF binding (10–600 μg) to ADMC at 0°C (●). A 20-fold excess of unlabeled LF at each concentration of ^125^I-LF was added to assay nonspecific binding (▲). Enzymatic treatment of the cells with DNase virtually eliminated ^125^I-LF binding at all concentrations (■). Values represent the mean of three experiments with a range for each concentration of 8.1%. The specific activity of ^125^I-LF was 24 μCi/μg.

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though an apparent artifactual binding has been noted due to the excretion of DNA by E+ cells; the excreted DNA adheres to the sides of the plastic tubes that bind 125I-LF (unpublished observations). For this reason, it is essential to transfer the cells to a fresh tube for counting or centrifuging them through oil. Boldt et al. (19) have shown that E+ cells preferentially excrete DNA.

When isolated ADMC membranes exposed to 125I-LF were centrifuged for 1 h in a sucrose gradient, the radioactivity associated with the intact membranes was recovered from near the bottom of the gradient, whereas the radioactivity from membranes digested with DNase or subjected to deoxycholate was released to band in a position consistent with free 125I-LF (Fig. 7).

Theoretically, the deoxycholate-treated membranes should release the receptor (putatively cmDNA)-125I-LF complexes. Some evidence for this was found by an 18-h centrifugation of the deoxycholate-treated supernatants in sucrose gradients made up in either water or 1 M NaCl. We have previously noted that a 1 M NaCl solution will disrupt LF-DNA complexes (17). It is seen that in the high molarity gradient, most of the radioactivity sediments in a position consistent with 125I-LF alone, whereas in the absence of 1 M NaCl, the radioactivity sediments in the lower third of the gradient (Fig. 8). From the known susceptibility of LF-DNA complexes to changes in molarity, these results provide additional support for DNA being the cell membrane binding site for LF.

DISCUSSION

This study provides evidence that cmDNA acts as a binding site for LF; a novel role for DNA that has not been previously reported. Furthermore, it would appear that ADMC and E− cells definitely possess cmDNA, and E+ cells possess none or very little.

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**TABLE 1**

Effect of Enzymatic Digestion upon LF Cell Binding

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LF Bound/×10^6 cells</th>
<th>ADMC</th>
<th>E− Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.09</td>
<td>1.51</td>
<td></td>
</tr>
<tr>
<td>Trypsin, 250 μg/ml</td>
<td>0.69</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase, 10 μg/ml</td>
<td>0.88</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Phospholipase D, 200 μg/ml</td>
<td>0.81</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>DNase 1, 50 μg/ml</td>
<td>0.03</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>RNase, 50 μg/ml</td>
<td>0.91</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>DNase-treated cells + DNA, 100 μg/ml</td>
<td>1.98</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Enzymatic treatment of cells (2 × 10^6) was performed at 37°C for 1 h. The cells were then washed twice and incubated with 125I-LF (50 μg/ml) in a total volume of 1 ml. After a further two times, the cells were transferred to another tube for counting. In one instance, exogenous DNA (calf thymus) was added to ADMC (15 min, RT) previously treated with DNase before washing two times and incubation with 125I-LF.

* Enzymatic digestion at pH of 5.6.
Whether the very small amount of DNA apparently associated with E+ cells is membrane bound or represents excreted DNA was not determined in this study. These findings would explain the unusually high number of calculated binding sites for LF noted in a previous report (2). As each DNA molecule can bind

**FIGURE 3** Dose-response curve of $^{125}$I-LF binding (10–600 μg) to E+ cells at 0°C (●). Prior enzymatic treatment of the cells with DNase indicates that most of the apparent binding is nonspecific (●). For the sake of clarity the plot for 20-fold excess of unlabeled LF is not shown, it lies between the two plots shown here. Values represent the mean of three experiments with a range for each concentration of 6.6%. The specific activity of $^{125}$I-LF was 24 μCi/μg.

**FIGURE 4** A Scatchard plot of the specific binding of $^{125}$I-LF to ADMC using the data shown in Fig. 1. The dissociation constant ($K_d$), derived from the concentration of free ligand when half the receptors are occupied, is $1.8 \times 10^{-6}$ M. Linear regression analysis indicates that $3.3 \times 10^7$ LF molecules are bound to each cell at saturation point.

**FIGURE 5** A Scatchard plot of the specific binding of $^{125}$I-LF to E− cells using the data shown in Fig. 3. The dissociation constant ($K_d$), derived from the molar concentration of free ligand when half the receptors are occupied, is $0.76 \times 10^{-6}$ M. Linear regression analysis indicates that $2.5 \times 10^7$ LF molecules are bound to each cell at saturation point.
many LF molecules (17), the true concentration of binding sites cannot be accurately determined from our present study. Furthermore our recent observation of a calcium-dependent polymerization of LF (11) was not taken into account in our calculations: whether the tetrameric form of LF is the predominant binding species to cell cmDNA has not yet been ascertained.

Although DNA appears to be the preferential binding site on cell membranes for LF, we have shown, in competitive inhibition experiment, that RNA and dextran sulfate are both potent inhibitors (unpublished observations). This effect is due to the interaction of LF with these molecules in the extracellular medium. Whether the interaction of LF with DNA is a "non-specific" electrostatic phenomenon, as suggested by such competitive inhibition experiments, or whether unique binding regions may be necessary for the mediation of a discrete cellular response to LF is an important issue requiring further study.

The immunofluorescence studies showing a characteristic globular pattern of surface staining, rather than the punctate pattern found with most ligand receptor interactions, may be a reflection of LF reacting with multiple binding sites on a single DNA molecule. Conversely, it may reflect a partial capping phenomenon, although our experimental conditions should have minimized capping. Another explanation is that the globular fluorescence represents the aggregation of LF molecules at coated pits (receptosomes) before internalization. Further work is required to elucidate the nature of this unexpected immunofluorescence pattern.

Conventional wisdom compartmentalizes DNA within the nuclear envelope with some supercoiled DNA molecules being found in the cytoplasm in association with mitochondria. Although many observations attesting to the presence of DNA on the surface of some eukaryotic cells have been made over the past decade, its function, if any, has remained an enigma; the topic of cmDNA has been the subject of two recent review articles (20, 21). Parallel to the observations of cmDNA have been several reports concerning the excretion of DNA by mononuclear cells (22-24).

Cuatrecasas and Hallenberg (25) have suggested certain criteria that could be used to support the existence of a specific cell receptor. We have previously shown (2) that the interaction of LF with ADMC and E- cells fulfills three of these criteria: the binding is calcium-dependent, the binding is of high affinity at low molar concentrations and lastly, other surface-reactive molecules do not influence LF binding. A fourth criterion, namely, inhibition of binding by enzymatic digestion of the cell surface, has now been shown by this study.

Although the interaction of LF and monocytes has been shown to exert a biological response at very low concentrations (4, 7, 26), it has yet to be determined whether cmDNA is the receptor molecule for the inhibitory effect of LF on the production of GM-CSF. The finding of such a relationship would provide un-
equivocal evidence for a biological role for cmDNA and firmly establish its status as a functionally active receptor molecule.

The concept that ADMC and E- cells from normal subjects possess cmDNA is in accord with the observations of Bankhurst and Williams (27), who demon-

![Graph showing the effects of sucrose and NaCl on the sedimentation of Lactoferrin-receptor complexes](image)
strated the binding of exogenous DNA to B lymphocytes. These authors postulated that the DNA was interacting with a subpopulation of B lymphocytes possessing cell surface IgG directed against DNA, and noted an increased number of DNA binding cells in patients with systemic lupus erythematosus; albeit B cells from normal subjects also exhibited some DNA binding. The observation made in this study, that the addition of exogenous DNA to DNase-treated ADMC increased the amount of bound LF over the control value, is in accord with the hypothesis that there may be a specific binding site for DNA other than IgG, and that this is not normally fully saturated. A corollary of this study is the intriguing concept that the possession or absence of cmDNA is a basic difference between E+ vs. E− cells and ADMC. However, it
would appear from our immunofluorescence studies that there may be a subset of E− cells and ADMC that do not possess cmDNA. Whether cmDNA-positive lymphocytes represent a distinct subpopulation from cmDNA-negative lymphocytes, as defined by currently available monoclonal markers and functional studies, remains to be investigated.

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REFERENCES

R. M. Bennett, J. Davis, S. Campbell and S. Portnoff