Lactoferrin Binds CpG-Containing Oligonucleotides and Inhibits Their Immunostimulatory Effects on Human B Cells

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Unmethylated CpG dinucleotide motifs in bacterial DNA, as well as oligodeoxynucleotides (ODN) containing these motifs, are potent stimuli for many host immunological responses. These CpG motifs may enhance host responses to bacterial infection and are being examined as immune activators for therapeutic applications in cancer, allergy/asthma, and infectious diseases. However, little attention has been given to processes that down-modulate this response. The iron-binding protein lactoferrin is present at mucosal surfaces and at sites of infection. Since lactoferrin is known to bind DNA, we tested the hypothesis that lactoferrin will bind CpG-containing ODN and modulate their biological activity. Physiological concentrations of lactoferrin (regardless of iron content) rapidly bound CpG ODN. The related iron-binding protein transferrin lacked this capacity. ODN binding by lactoferrin did not require the presence of CpG motifs and was calcium independent. The process was inhibited by high salt, and the highly cationic N-terminal sequence of lactoferrin (lactoferricin B) was equivalent to lactoferrin in its ODN-binding ability, suggesting that ODN binding by lactoferrin occurs via charge-charge interaction. Heparin and bacterial LPS, known to bind to the lactoferricin component of lactoferrin, also inhibited ODN binding. Lactoferrin and lactoferricin B, but not transferrin, inhibited CpG ODN stimulation of CD86 expression in the human Ramos B cell line and decreased cellular uptake of ODN, a process required for CpG bioactivity. Lactoferrin binding of CpG-containing ODN may serve to modulate and terminate host response to these potent immunostimulatory molecules at mucosal surfaces and sites of bacterial infection. 


Bacterial, but not eukaryotic, DNA contains a large number of unmethylated CpG dinucleotides (1). When these unmethylated CpGs are in a particular base context (“CpG motifs”), they stimulate various innate and acquired immunological responses in murine and human systems (2). Among the effects of unmethylated CpG oligodeoxynucleotides (ODN)3 are: 1) enhancement of Ag-specific Th1 responses including enhanced macrophage and dendritic cell IL-12 production (3–7); 2) enhanced NK cell production of IFN-γ (8); 3) activation of B cell proliferation and IL-6 and Ig secretion (7); 4) stimulation of protective immunity against intracellular pathogens (9–14); 5) inhibition of allergen- and LPS-induced airway inflammation (15–17); 6) induction of direct airway inflammation (18); and 7) stimulation of immune responses to tumor Ags (19, 20). CpG-containing ODN have also been shown to be able to induce a systemic inflammatory response syndrome in animals that is analogous to that resulting from i.v. challenge with LPS (21).

These and other experimental data have suggested that immune stimulation by bacterial DNA may serve as an important signaling mechanism to activate protective immune responses to invasion by pathogenic bacteria (22). Given the potency of the response, the role of bacterial DNA and CpG ODN as potential vaccine adjuvants is being explored (3, 22). The biological effect of CpG ODN appears to involve binding and internalization of the molecule (23, 24) by a process that is mediated via a Toll-like receptor-dependent pathway (25–27) and perhaps formation of reactive oxygen species (28).

Although there has been much interest in the immunostimulatory effects of bacterial DNA, there has been relatively little attention paid to mechanisms whereby the host may limit and thereby help to regulate such responses. At many mucosal surfaces there is a normal resident microflora. Continued stimulation of the local immune system in response to the DNA of this microflora could be deleterious and it would be expected that a mechanism would have evolved to limit such events. Similarly, at a local site of bacterial infection, it would likely be beneficial to the host to have a means of terminating CpG-mediated stimulation of the immune system as the infection comes under control.

Lactoferrin is a highly cationic (isoelectric point (pl), 8.4–9.0) monomeric glycoprotein (76–80 kDa) that is found in high concentrations (1–10 mg/ml) at many mucosal surfaces and in milk (29–31). It is also a major constituent of neutrophil secondary (specific) granules (32, 33). It is secreted by local neutrophils and is present at high levels at sites of bacterial infection (34, 35). Lactoferrin contains two high-affinity ferric iron binding sites and is thought to function as host defense in part by sequestering iron from pathogenic microbes (36, 37). Lactoferrin is also thought to serve as an antioxidant since iron bound to the protein is unable to participate as a catalyst for the generation of the hydroxyl radical (38–40).

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Lactoferrin has antimicrobial activity that is independent of its iron-binding activity (41–43). Lactoferrin binds to the outer membrane of Gram-negative bacteria, leading to alterations in bacteria membrane permeability barriers, which in turn causes microbial cell injury and death (41, 44). This latter activity has been linked to the proximal N terminus of the molecule, termed lactoferricin, which contains a large number of arginine residues. These are responsible for much of the cationic nature of the protein (42, 45). The related iron-binding protein transferrin, which lacks these arginines, has a pI of 5–5.5 (46).

The cationic N-terminal component of lactoferrin has also been shown to result in the ability of the molecule to bind a variety of biologically important, negatively charged molecules, via charge-charge interactions. These include LPS (47–54), heparin, and heparan sulfates (47, 55–57) and DNA (47, 58–60). Binding of LPS to lactoferrin has been shown to modulate the bioactivity of LPS by altering how it interacts with target cells (49, 50, 52, 53). Most of the interest in the DNA-binding properties of lactoferrin has been on the interaction of the protein with eukaryotic DNA. Recently, there has been the suggestion that there may be a sequence specificity for binding of eukaryotic DNA to lactoferrin and that lactoferrin may play a role in transcriptional activation (61).

Given previous evidence that 1) lactoferrin can bind ssDNA; 2) interaction with another immunostimulatory molecule, LPS, is modified upon its binding to lactoferrin; and 3) lactoferrin is present in high concentrations at both mucosal surfaces and sites of acute bacterial infection, we hypothesized that lactoferrin will bind CpG-containing ODN and that this will lead to alterations in the biological activity of these ODN toward the immune system. Data reported herein support that hypothesis.

Materials and Methods

\(^{32}\)P-labeled ODN
The following ODN, which contained nondetectable levels of endotoxin, were provided by the Coley Pharmaceutical Group (Wellesley, MA): 2006, TCG TCG TTT TGT CGT GGT GTC GTT; 1818, GAG AAC GCT GGA CCT TCC AT; 1906, TCC ATG AGC TCT TCC ATG AGC TCC TCT CCC ATG ACG TCC TCT CTC CTC CTC; and 1958, TCC ATG CTA CGC CTA GCT CTCT CT; 1888: TCC ATG AGC TCC TCT GGT CT; 1888: TCC ATG AGC TCC TCT GGT CT. The OD of interest (200 ng) was incubated with T4 polynucleotide kinase (10 U) and \(^{32}\)P-ATP (30 μCi) in a volume of 40 μl for 30–60 min at 37°C. The solution was then centrifuged through a G-25 Sepharose column for 4 min at 2000 rpm at room temperature. The radiolabeled ODN was then harvested from the eluate, counted in a gamma counter, and then stored at −20°C until needed. For cellular uptake experiments, ODN with a 5’ fluorescein modification (Operon Technology, Alameda, CA) were used.

EMSA
Binding of ODN to lactoferrin, transferrin, and lactoferricin B was assessed by EMSA. Lactoferrin (bovine and human) as well as transferrin were purchased from Sigma (St. Louis, MO). Lactoferricin B was kindly provided by the Morinaga Milk Industry (Zama City, Japan). \(^{32}\)P-labeled ODN was incubated alone or with the protein of interest for 2–5 min in H₂O at room temperature. Samples were then subjected to native PAGE followed by overnight autoradiography (−80°C).

Ramos cell CD86 expression
The Ramos human B cell line (Burkitt lymphoma B cell line, ATCC CRL-1923) was maintained in RPMI 1640 supplemented with 1-glutamine, penicillin, streptomycin, and HEPES. Cells were harvested and incubated in RPMI 1640 containing 10% FCS at a concentration of 1–2 × 10⁶/ml. To these cell suspensions was then added ODN alone or ODN that had been preincubated with lactoferricin, lactoferricin B, or transferrin in H₂O for 1 h at 37°C. After overnight incubation in 96-well flat-bottom plates, the cells were removed and assessed for CD86 expression by surface Ag staining using FITC-conjugated Ab to CD86 (2331 (FUN-1); purchased from BD PharMingen, San Diego, CA) in conjunction with FACS analysis (62). In some experiments, polyclonal antihuman IgM (Sigma) was substituted for the ODN as an alternative stimulus for Ramos cell CD86 expression.

Cellular uptake of CpG ODN
Ramos cells were incubated at 37°C with FITC-conjugated ODN in PBS, after initial incubation of the ODN for 30 min in H₂O alone or H₂O that contained desired concentrations of lactoferrin, transferrin, or lactoferricin B. After 3 h, the cells were washed and cell-associated FITC was determined by FACS (63). To correct for cell membrane binding, parallel cell samples were also incubated with ODN on ice for 30 min to prevent uptake.

Binding of lactoferrin to Ramos cells
Ramos cells were washed twice in HBSS and suspended at a concentration of 10⁶/ml in 0.5 ml of HBSS at 4°C. To the cell suspension was added 5 μCi of \(^{125}\)I-labeled human apolactoferrin. After 30 min at 4°C, the cells were washed three times with cold HBSS and finally pelleted at 200 × g for 10 min. The tip of the centrifuge tube containing the cell pellet was cut from the tube using a clipper and placed into a tube which allowed determination of \(^{125}\)I-labeled human lactoferrin content by gamma counter. Each experiment included conditions in which \(^{125}\)I-labeled human apo-lactoferrin was added to HBSS which lacked cells and was processed in an analogous manner. This was done to control for nonspecific sticking of lactoferrin to the tubes (background). These background counts were subtracted from the cell-containing values to arrive at cell-specific lactoferrin binding.

Statistical analyses
Results obtained under different experimental conditions were compared by Student’s paired t test when independent variables were being assessed or by ANOVA when analyses of trends were being determined. For both types of analyses, results were considered to be significant at p ≤ 0.05.

Results
Lactoferrin binds CpG ODN
Lactoferrin had previously been shown to bind to both ssDNA and dsDNA (47, 58–60), we examined the ability of lactoferrin to bind synthetic ODN with or without CpG motifs. Using \(^{32}\)P-labeled ODN in conjunction with EMSA analysis, human lactoferrin was found to bind such ODN in a concentration-dependent manner following a brief (2- to 5-min) exposure (Fig. 1). The binding was saturable, as it could be inhibited by the presence of a 100-fold concentration excess of unlabeled ODN (Fig. 1A). No difference in binding to lactoferrin was distinguishable among the CPG-and non-CPG-containing ODN examined (Fig. 1B). Indicating that lactoferrin binding did not require the presence of the CpG motif. Neither was binding unique to human lactoferrin, as CpG ODN binding was also readily detectable using bovine lactoferrin (Fig. 1C).

Binding of iron to lactoferrin results in conformational changes in the molecule (64–66). However, comparison of ODN binding to apo-lactoferrin and iron-loaded forms of human lactoferrin revealed no differences in their ability to bind the CpG-containing ODN (Fig. 1D). In contrast, the related iron-binding protein transferrin was unable to bind the ODN under the same conditions (Fig. 2).

Interestingly, in studies of lactoferrin binding to ODN 1906 at relatively low ratios of lactoferrin to ODN, we observed a single band that migrated well into the gel. As the concentration of lactoferrin increased, additional slower migrating bands appeared, and with further increases a single band was eventually seen with much slower migration into the gel (Fig. 1, A–C). Interestingly, this was not seen with the other ODN examined (Fig. 1B). ODN 1906 is nearly twice as long as the other ODN studied, and we hypothesize that this banding pattern reflects the ability of the 1906...
ODN to bind several molecules of lactoferrin, resulting in the stepwise banding pattern on the gel.

**Mechanism of CpG oligonucleotide binding to lactoferrin**

The failure of the less cationic protein transferrin to bind the ODN, in conjunction with previous work by others (47, 58–60) examining the nature of lactoferrin-DNA interactions suggested that binding of CpG ODN occurred on the basis of a charge-charge interaction that likely involved the N-terminal portion of the lactoferrin molecule. To test this hypothesis, the effect of increasing salt concentration on the ability of lactoferrin to bind to CpG ODN was assessed. Consistent with a charge-charge-mediated interaction, high salt conditions (50 mM NaCl) resulted in a progressive decline in association of the ODN with lactoferrin (Fig. 3).

Previous work by others (47, 58) suggested that lactoferrin binding of eukaryotic DNA occurs at the same N-terminal portion of...
the lactoferrin molecule at which binding of other negatively charged biological molecules (e.g., LPS, heparin, and heparan sulfates) occurs (47, 48). In the present work, we found that both heparin and LPS inhibit binding of 32P-labeled ODN in a concentration-dependent manner (Figs. 4 and 5).

These data are consistent with the primary binding site for the ODN being on the N-terminal portion of lactoferrin. To confirm this, we examined the ability of purified lactoferricin B, which is comprised of a short portion of the N-terminal sequence of bovine lactoferrin (42, 67), to bind 32P-labeled ODN. As shown in Fig. 6, lactoferricin B exhibited, on a molar basis, a similar ability to bind ODN.

Lactoferricin B contains a disulfide bond (67). This bond does not appear to be necessary for the ability of lactoferricin B to bind ODN as pretreatment of the molecule with the reducing agent DTT to break those bonds had no effect on subsequent binding of 32P-labeled ODN (data not shown).

Binding to lactoferrin alters the biological activity of CpG ODN

Previous work has shown that the binding of LPS to lactoferrin alters LPS biological activity (49, 50, 52, 53). Therefore, we hypothesized that binding of CpG ODN to lactoferrin or lactoferricin B would inhibit the immunomodulatory effects of that molecule. Alternatively, it was also possible that lactoferrin would enhance the CpG activity by increasing cellular uptake of the DNA. To distinguish between these two possibilities, we examined the effect of lactoferrin/lactoferricin B binding on the ability of CpG ODN to increase CD86 expression on the human B lymphocyte-derived Ramos cell line. This parameter was chosen because our previous experience had indicated that CD86 expression in this cell line is not modulated by LPS exposure (A. M. Krieg, unpublished observation), a response which if present could potentially confound data interpretation, given the ability of lactoferrin to bind LPS as well. As shown in Fig. 7, binding of the ODN to either lactoferrin or lactoferricin B significantly decreased ODN binding at 4°C by 50 ± 28% (n = 3, p < 0.05). In contrast, transferrin (0.5 μg/ml) was without effect: MFI was 83 ± 12% of control (n = 3, p > 0.05). The same concentration of lactoferrin also decreased ODN binding at 4°C by 50 ± 28% (n = 3, p < 0.05), reflecting an effect on surface binding as well as uptake over time. Transferrin again was without effect: MFI was 107 ± 32% of control (n = 3, p > 0.05).

Lactoferrin inhibits binding and internalization of CpG ODN

The biological effect of CpG ODN on cellular systems has been shown to require their binding and internalization by a mechanism that remains ill-defined (23, 24). Accordingly, we assessed the effect of binding of CpG ODN to lactoferrin on its uptake by Ramos cells by measuring the ability of the cells to take up FITC-conjugated ODN (expressed as mean fluorescence index [MFI] by FACs). Lactoferrin (0.5 μg/ml) significantly inhibited ODN uptake over 3 h at 37°C to 35 ± 6% of control (mean MFI ± SEM, n = 3, p < 0.05). In contrast, transferrin (0.5 μg/ml) was without effect: MFI was 83 ± 12% of control (n = 3, p > 0.05). The same concentration of lactoferrin also decreased ODN binding at 4°C by 50 ± 28% (n = 3, p < 0.05), reflecting an effect on surface binding as well as uptake over time. Transferrin again was without effect: MFI was 107 ± 32% of control (n = 3, p > 0.05).

Enhancement of cell response to CpG ODN by lactoferrin

The enhancing effect of lactoferrin on cell response to CpG ODN was tested in a cell line that expresses low levels of CD86 (Ramos). The biological effect of CpG ODN on cellular systems has been shown to require their binding and internalization by a mechanism that remains ill-defined (23, 24). Accordingly, we assessed the effect of binding of CpG ODN to lactoferrin on its uptake by Ramos cells by measuring the ability of the cells to take up FITC-conjugated ODN (expressed as mean fluorescence index [MFI] by FACs). Lactoferrin (0.5 μg/ml) significantly inhibited ODN uptake over 3 h at 37°C to 35 ± 6% of control (mean MFI ± SEM, n = 3, p < 0.05). In contrast, transferrin (0.5 μg/ml) was without effect: MFI was 83 ± 12% of control (n = 3, p > 0.05). The same concentration of lactoferrin also decreased ODN binding at 4°C by 50 ± 28% (n = 3, p < 0.05), reflecting an effect on surface binding as well as uptake over time. Transferrin again was without effect: MFI was 107 ± 32% of control (n = 3, p > 0.05).
CD86 expression, anti-IgM (2 μg/ml), alone or in the presence of transferrin did not direct the ODN to a different surface receptor system. Following this incubation, the ability of the ODN to induce CD86 expression on the cell surface was assessed by FACS. Also shown in results are in which the cells were exposed to an alternative stimulus of lactoferrin (0.5 mg/ml), as a control for possible nonspecific effects of lactoferrin. Results were significantly different from ODN alone at p < 0.05. No other results were significantly different from ODN alone.

These latter data suggested that the binding of the ODN to lactoferrin did not direct the ODN to a different surface receptor system, as has been described previously with monocyes and LPS (52). ODN also altered the interaction of lactoferrin with the Ramos cells. Binding of lactoferrin to Ramos cells was decreased by 71% when lactoferrin was preincubated with the ODN, decreasing from 150 ± 18 fmol lactoferrin/10^6 cells to 43 ± 8 fmol lactoferrin/10^6 cells (mean ± SEM, n = 4) when the lactoferrin was preincubated with ODN before addition to the cells (p < 0.02). Thus, it appears that the interaction of ODN and lactoferrin decreases the ability of both agents to bind to the Ramos cell surface.

Discussion

The unmethylated CpG sequences that distinguish bacterial from eukaryotic DNA have been identified as potentially important signaling molecules whose recognition allows various aspects of the host immune system to be activated to respond to invading bacterial pathogens (1, 2). Both beneficial and deleterious consequences of such activation have been described (1, 2). Although much attention has been paid to the mechanism whereby CpG-containing ODN activate cellular immune responses in vitro and in vivo, there has been relatively little focus on the means whereby these responses are negatively modulated.

In the present work we show that lactoferrin, a protein present in high concentrations at mucosal surfaces, milk, and neutrophil-specific granules (29–34, 68), readily binds CpG-containing DNA. Although lactoferrin plays a major role as an iron-binding protein, our work and results from other investigators (47) indicate that the iron status of the protein does not influence its DNA-binding activity. The ability of high salt and other negatively charged molecules to inhibit ODN binding indicates that this most likely occurs on the basis of charge-charge interactions. The fact that lactoferrin, but not the related iron-binding protein transferrin, was able to bind the ODN suggests that the high pl of lactoferrin relative to transferrin may be an important determinant of this activity. This is further supported by that fact that a small portion of the N-terminal sequence of lactoferrin that is responsible for much of the cationic nature of the molecule (lactoferrin) (42, 67) exhibited the same ability to bind ODN as the entire lactoferrin molecule.

This observation also has important implications for the potential for this interaction to occur in vivo where lactoferrin can be cleaved through protease activity to generate lactoferrin (42, 69, 70). Thus, even at sites of high protease activity, the ability of lactoferrin to bind DNA should remain intact. Furthermore, since the oxidation state of the sulphydryl group of lactoferrin B does not appear to dramatically alter the ability of lactoferrin to bind ODN, this process should function both in highly oxidizing and reducing environments.

Previous work by others has shown that lactoferrin is capable of binding ssDNA and dsDNA (47, 58–60). Furmanski and colleague (61) indicated that there is a preferred sequence specificity for the binding of ODN by lactoferrin. These authors also reported that lactoferrin could be internalized and trafficked to cell nuclei where it could potentially serve as a regulator of gene transcription (61). Our data indicate that, at least in the case of DNA fragments that lactoferrin would initially encounter extracellularly, this process serves to inhibit rather than enhance intracellular localization.

Lactoferrin binds to cells by at least two mechanisms: 1) high capacity low-affinity binding that may occur via charge-charge interactions with surface glycolipids or other molecules such as DNA (71–76) and 2) lower capacity/higher affinity binding to one or more proteinaceous plasma membrane receptors (77–83). Lactoferrin has been reported to bind to B cells via interaction with DNA on the cell surface (74, 75) and it appears that B cell binding is mediated through the highly charged N-terminal sequence of lactoferrin (84). To our knowledge, no specific protein receptors for lactoferrin on the B cell surface have been reported. Thus, the biological response to CpG ODN bound to lactoferrin could be different in those cells possessing one or more high-affinity lactoferrin-binding mechanisms such as macrophages, hepatocytes, PHA-activated lymphocytes, and gastrointestinal epithelial cells (77–83). This requires further investigation.

Nevertheless, these data suggest that binding of bacterial DNA or DNA fragments could be a mechanism to modulate the proinflammatory effects of these compounds. Lactoferrin would seem to be ideally suited for this role as it is present at high concentrations at mucosal surfaces (1 mg/ml) and in human milk (10 mg/ml) (29–31). At these concentrations, which are far higher than those used in our experiments, it would be readily available to deal with...
bacterial DNA routinely present from these microorganisms colonizing these locations. This could serve to limit excessive immunostimulatory activity at mucosal surfaces colonized by large numbers of microbes, e.g., gut, without losing the ability to detect pathogens that have invaded into tissues past the anti-inflammatory layer of lactoferrin. Interestingly, CpG ODN are being explored as an immunoadjuvant for a variety of vaccines and as immunotherapeutic agents for cancer and allergic diseases (3, 22). The potential impact of lactoferrin in oral vaccine strategies using CpG ODN may need to be explored, particularly if they involve administration of CpG-containing vaccines to infants who are being breastfed. Likewise, the delivery of CpG ODN to other mucosal surfaces such as the nasal cavity or lungs for allergy therapy (15) may require consideration of inhibitory effects of lactoferrin to achieve optimal therapeutic effects.

In addition, as a major component of neutrophil secondary granules, lactoferrin (32, 33) would be brought to the sites of bacterial infections as part of the initial host response to that infection and would be released into the extracellular environment through neutrophil degranulation (85). Thus, as the infection was being eradicated, the ratio of bacterial DNA and LPS to lactoferrin would gradually decrease, thereby decreasing the bioactivity of both proinflammatory molecules and helping to terminate CpG-mediated inflammatory responses.

Although the interaction of lactoferrin with DNA decreases under conditions of high local salt concentrations, this would not likely be encountered under most in vivo conditions. NaCl concentrations seldom exceed 150 mM in vivo. It was not until this salt concentration was exceeded that we found evidence for a decrease in lactoferrin-ODN interactions in vitro. Interestingly, although controversial (86, 87), the salt content of airway fluid lactoferrin to bind bacterial DNA, due to a higher than fluid lactoferrin to bind bacterial DNA, due to a higher than optimal therapeutic effects.

In summary, lactoferrin is able to bind CpG-containing ODN via a mechanism that appears to involve charge-charge interactions of the N-terminal sequence of the lactoferrin molecule. The lack of specificity of the binding for CpG motifs may actually serve to enhance the effectiveness of binding of potentially immunostimulatory DNA sequences in which the CpG motif may be initially bound within a complex DNA multimer and not be immediately accessible to lactoferrin. Binding to lactoferrin results in a loss of binding and internalization of the ODN by a human B cell line and a coincident inhibition of CD86 surface expression, a marker of the N-terminal sequence of the lactoferrin molecule. The lack of specificity of the binding for CpG motifs may actually serve to enhance the effectiveness of binding of potentially immunostimulatory DNA sequences in which the CpG motif may be initially bound within a complex DNA multimer and not be immediately accessible to lactoferrin. Binding to lactoferrin results in a loss of binding and internalization of the ODN by a human B cell line and a coincident inhibition of CD86 surface expression, a marker of the proinflammatory response to CpG ODN in these cells. Our results suggest that lactoferrin normally present at mucosal surfaces or brought to sites of infection by lactoferrin-containing neutrophils could serve as a means for the host to negatively modulate the widely described proinflammatory responses to bacterial DNA and CpG ODN-containing fragments of bacterial DNA.

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