Neutralization of Heparin Activity by Neutrophil Lactoferrin
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Lactoferrin is a prominent component of neutrophil secondary granules, and its blood concentration is increased in certain inflammatory diseases. In contrast to the well-described biochemical characterization of lactoferrin as an iron-binding protein, its physiologic role in the regulation of inflammation and other host defense mechanisms is unclear. In this report, we provide evidence that lactoferrin has a potent heparin-neutralizing activity during thrombin inhibition by the serine proteinase inhibitors (serpins) antithrombin and heparin cofactor II. Activated neutrophil supernatant, which contains lactoferrin and other heparin-binding proteins, could neutralize the heparin-dependent antithrombin-thrombin inhibition reaction. The addition of lactoferrin to plasma corrected the heparin-induced prolongation of blood plasma coagulation time as measured by the activated partial thromboplastin time (aPTT). Treatment of whole blood with specific inflammatory mediators, FMLP, lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF-α) increased the concentration of both plasma lactoferrin and platelet factor 4 while inhibiting the blood anticoagulant activity of heparin as measured by the aPTT. These results suggest that the prothrombotic sequelae of some inflammatory processes may be partly due to various agonists that release neutrophil lactoferrin, which can then neutralize glycosaminoglycan-dependent serpin-thrombin inhibition reactions. © 1995 by The American Society of Hematology.

Blood Coagulation is a host defense system that maintains the integrity of the circulatory system. In this process, thrombin serves as a critical factor by activating platelets, activating factors V and VIII, and clotting fibrinogen. After its formation, thrombin is regulated by various anticoagulant pathways. One pathway is through thrombin inhibition by the plasma serine proteinase inhibitors (serpins) antithrombin and heparin cofactor II. The thrombin-inhibiting activity of both of these serpins is greatly accelerated in the presence of glycosaminoglycans such as heparin. In homeostasis, the coagulation process is under tight control by these and other mechanisms. However, certain pathologic conditions, such as septicemia, can shift this balance to the hypercoagulable state partly as a result of the failure of these natural anticoagulant mechanisms. The resulting outcome of such septicemia could be disseminated intravascular coagulation (DIC). DIC is a clinical syndrome characterized by uncontrolled thrombin generation in the systemic circulation. The clinical management of DIC has not been well described due to the complexity of its pathogenesis. Understanding this mechanism is critical for the successful treatment of DIC. The current study suggests that one factor that may contribute to the development of septic DIC is lactoferrin liberated from neutrophils in response to bacterial lipopolysaccharide (LPS) and other inflammatory stimulation.

Lactoferrin is an iron-binding protein closely related in structure to the serum iron-transporting protein, transferrin. The amino acid sequence identity between these proteins is more than 55%. Lactoferrin is a single polypeptide chain of 692 residues containing an internal repeat, with the amino-terminal half about 40% identical with the carboxy-terminal half. Each half of the lactoferrin molecule has a single iron-binding site with a dissociation constant (kd) of approximately 10^-20 mol/L. Human lactoferrin is found in milk, tears, and saliva, and it is also a prominent component of secondary granules of neutrophils. Lactoferrin in plasma is originally derived from neutrophils. A number of studies have demonstrated that in certain pathologic circumstances, such as severe infection or autoimmune disease, the concentration of plasma lactoferrin can increase twofold to threefold. In vitro experiments with isolated neutrophils have also shown that a significant amount of lactoferrin is released from neutrophils in response to LPS and tumor necrosis factor-α (TNF-α). However, the physiologic role of plasma lactoferrin during inflammatory processes remains undefined.

Lactoferrin interacts with heparin-Sepharose, and its heparin-binding properties have been partially characterized. Inspection of the primary structure of lactoferrin shows that the N-terminus has consensus sequences (BBXB or BXXBBXB, where B is a basic residue and X is any residue) found in many other glycosaminoglycan-binding proteins. The three-dimensional structure of lactoferrin shows that an α-helical region in the N-terminus forms a positively charged surface that could bind glycosaminoglycans. Recently, Wu et al. and Mann et al. have shown that the glycosaminoglycan-binding site of lactoferrin is localized to the N-terminus of the protein.

The present study examines the role of glycosaminoglycan neutralization by neutrophil-derived lactoferrin in the regulation of blood coagulation. The results suggest that neutrophil lactoferrin is an effective inhibitor of the anticoagulant activity of heparin, and it may be involved in the development of thrombotic complications associated with inflammatory diseases.

MATERIALS AND METHODS
Materials. All chemicals used were from Sigma Chemical Co (St Louis, MO) unless otherwise indicated and were of the highest purity available.

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grade purity available. Ficol-Hypaque Monopoly medium, collagen, and polyclonal antibody against human lactoferrin conjugated with alkaline phosphatase were obtained from ICN Biochemicals (Costa Mesa, CA); TNF-α, phorbol 12-myristate-13-acetate (PMA), N-tosyl-phenylalanine chloromethyl ketone (TPCK), and purified human neutrophil elastase were from CalBiochem (San Diego, CA); transforming growth factor-β (TGF-β) was obtained from R & D Systems Inc (Minneapolis, MN); platelet factor 4 (40 kDa) and a polyclonal antibody against platelet factor 4 were obtained from American Diagnostica Inc (Greenwich, CT); polyclonal antibody against human neutrophil elastase was from Biodiagnostics (Kennewick, WA); Polybrene was from Aldrich Chemical Co (Milwaukee, WI); Nε-p-tosyl-Gly-Pro-Arg-p-nitroanilide was from Boehringer-Mannheim (Indianapolis, IN); aPTT reagents were obtained from Pacific Hemostasis (Ventura, CA); and heparin, with an average molecular weight of 15 kDa, was provided from Diosynth (Oss, The Netherlands). Human α-thrombin, antithrombin, and heparin cofactor II were purified as described previously.20,32

**Purification of lactoferrin.** Human milk lactoferrin was purified from fresh mature milk as described by Blackberg and Hennell.23 For preparation of human neutrophil lactoferrin, 150 mL of blood was drawn from a healthy volunteer with EDTA as an anticoagulant. Neutrophils were isolated using the modified Ficol-Hypaque gradient as described by Kalmar et al.33 The granulocyte fraction obtained was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by immunoblot analysis.

**Preparation of human milk lactoferrin.** Human milk lactoferrin was purified from milk obtained from 10 donors. The purity of both milk and neutrophil lactoferrin was prepared from purified milk lactoferrin as described from I L of blood (10 donors). The purity and polyclonal antibody against human lactoferrin demonstrated strong immunoreactivity against human lactoferrin in both plasma and neutrophil supernatants was determined by a double-sandwich ELISA. Briefly, microplate wells were coated with 100 μL of rabbit polyclonal antibody against human purified lactoferrin (Cappel-Organon Teknika Corp, Durham, NC) at a 1:500 dilution at 4°C overnight. The plate was then washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.5 mol/L NaCl, pH 7.5 (washing buffer). After blocking with 1% BSA in PBS for 2 hours, a neutrophil supernatant dilution of 1:200 or a plasma dilution of 1:20 in blocking solution containing 0.05% Tween 20 (incubation buffer) was added to the wells in 100-μL volume. After incubation for 2 hours at room temperature, the plate was washed twice with washing buffer. Rabbit anti-human lactoferrin antibody conjugated with alkaline phosphatase at a dilution of 1:5,000 was then added for 1 hour. Finally, the plate was washed four times with washing buffer, and 100 μL of substrate containing 1 mg/mL p-nitrophenyl-phosphate in 10 mmol/L diethanolamine, 0.5 mmol/L MgCl2, pH 9.5, was added. Substrate hydrolysis was measured kinetically in the microplate reader at 405 nmol/L. Alkaline phosphatase activity in each well was determined as millioplitic units per minute. The concentration of elastase in activated neutrophil supernatants was measured by a direct ELISA. Microplate wells were coated with the various neutrophil supernatants in PBS at 4°C overnight. After treatment with the washing buffer, the wells were blocked with 2% BSA/PBS for 2 hours at 37°C and then incubated with 100 μL rabbit polyclonal antibody against human neutrophil elastase (1:500 dilution) in the incubation buffer for 3 hours at 37°C. After washing, a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:500 dilution) was added for 2 hours at 37°C. Finally, the plate was washed four times with the washing buffer, and 100 μL of substrate containing 1 mg/mL p-nitrophenyl-phosphate in 10 mmol/L diethanolamine, 0.5 mmol/L MgCl2, pH 9.5, was added. Substrate hydrolysis was measured as described above. The concentration of platelet factor 4 in the plasma after treatment of whole blood with various inflammatory mediators was determined by a competitive ELISA. Microplate wells were coated with 100 μL of purified platelet factor 4 at 0.5 μg/mL in PBS at 4°C overnight. The plate was then washed twice with PBS and blocked with 2%
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BSA for 2 hours at 37°C. After another washing, a 100-μL incubation solution containing a constant concentration of platelet factor 4 antibody (2 μg/mL) and a diluted plasma sample (from 1:10 to 1:80 dilution) was added and incubated at 37°C for 4 hours. The plate was then washed with washing buffer. A goat anti-rabbit IgG antibody conjugated with alkaline phosphatase at a dilution of 1:500 in the incubation solution was then added for 2 hours at 37°C. Alkaline phosphatase activity was measured as described above.

For each ELISA plate, a standard curve was obtained by plotting the activity against concentration of purified protein used in each assay. Each sample was analyzed in duplicate by ELISA, and the average was calculated. The mean values of three separate experiments with standard deviation were reported.

\[ \text{aPTT assays. Coagulation assays were performed in the microplate as described previously.} \]

Briefly, 40 μL plasma (anticoagulated with sodium citrate) in each well was incubated with 40 μL buffer (20 mmol/L HEPES, 150 mmol/L NaCl, and 0.1% polyethylene glycol, pH 7.4) containing the indicated reagents for 3 minutes at 25°C. Then, 40 μL aPTT reagent was added for 5 minutes at 25°C. Clotting was initiated by adding 40 μL of 25 mmol/L CaCl2. The clotting time was determined kinetically by time to maximal velocity (Vmax) at 405 nmol/L as detailed previously.

Relative clotting time (RCT) is calculated as the clotting time in the presence of 11 nmol/L heparin divided by clotting time in the absence of added heparin for each individual plasma sample. No substantial differences in the aPTT were found for any of the plasma samples measured in the absence of heparin.

The plasmas obtained after treatment of whole blood with various agonists were prepared as follows: venous blood was collected in sodium citrate, and 2 mL aliquots were mixed with various challengers at the indicated concentrations. The samples were incubated at 37°C in a tissue culture incubator with 5% CO2. At a specific time, the incubation was terminated by centrifugation at 2,000 rpm for 10 minutes at 4°C. The plasma supernatant was collected and centrifuged again at 10,000 rpm for 5 minutes to remove contaminated platelets. Plasma samples thus obtained were used for both ELISA and aPTT assays.

For all the assays used in these studies, mean values and standard deviations (SD) were determined using Cricket Graph version III on an Apple Macintosh IIci computer (Macintosh, Cupertino, CA).

RESULTS

The heparin neutralization activity of lactoferrin in the antithrombin-thrombin inhibition reaction was compared with platelet factor 4, transferrin, and protamine sulfate. Various forms of lactoferrin, including milk lactoferrin, neutrophil lactoferrin, apolactoferrin, and hololactoferrin, were also compared for this activity. Heparin neutralization activity of the protein was expressed as the percentage of inhibition of the serpin-thrombin reaction. As shown in Fig 1, lactoferrin obtained from either neutrophils or milk had heparin neutralization activity comparable with platelet factor 4, and they were both more effective than protamine sulfate. Iron-free lactoferrin had the same activity as milk lactoferrin (data not shown), while the iron-saturated form showed a small decrease in activity. Inclusion of 3 mmol/L EDTA in the reaction did not affect heparin neutralization activity of lactoferrin. Preincubation of purified lactoferrin with a lactoferrin-polyclonal antibody significantly decreased lactoferrin
activity. Interestingly, neither transferrin nor lysozyme (data not shown) had any heparin neutralization activity. Furthermore, similar results were obtained for heparin neutralization with the various forms of lactoferrin in the thrombin-heparin cofactor II inhibition reaction (Fig 2).

The ability of the products released by activated neutrophils to inhibit the heparin-catalyzed antithrombin-thrombin inhibition reaction was examined (Fig 3). The supernatants of neutrophils after challenge with fMLP, TNF-α, leukotriene B4 (LTB4), LPS, and interleukin-8 (IL-8) significantly neutralized the heparin activity in the thrombin-antithrombin reaction (Fig 3A). In contrast, treatment of neutrophils with the chemoattractant TGF-β did not have any effect compared with untreated neutrophils. Additionally, preincubation of the neutrophil supernatants with a polyclonal antibody against lactoferrin partly reduced the antiheparin activity of neutrophil supernatants examined (Fig 3A). Normal rabbit IgG, included as a control, did not alter the heparin-neutralizing activities of any of the neutrophil supernatants examined (data not shown).

The concentrations of both lactoferrin and the serine protease elastase in neutrophil supernatants were quantitated by ELISA. As shown in Fig 3B, fMLP, TNF-α, LTB4, LPS, and IL-8 mediated significant release of lactoferrin from neutrophils. The amount of lactoferrin found in these neutrophil supernatants was correlated with the specific heparin neutralization activity (Fig 3A). Lactoferrin was also released from neutrophils in a dose-dependent manner in response to those agonists (data not shown). The release of low levels of lactoferrin from unstimulated neutrophils is probably due to a trace amount of endotoxin contamination in the in vitro system. The concentration of elastase in these neutrophil supernatants was also increased significantly after the treatment with fMLP, TNF-α, LTB4, LPS, and IL-8 (Fig 3C). However, control experiments showed that none of the neutrophil supernatants altered thrombin chromogenic substrate activity with or without heparin and that thrombin inhibition by antithrombin was not altered in the presence of either aprotinin and/or TPCK in the reaction (data not shown).

The ability of lactoferrin to neutralize heparin in the complex system of blood plasma coagulation was investigated. Prolongation of the aPTT in heparinized plasma was expressed as the ratio of the aPTT in the presence of heparin to the aPTT in the absence of heparin (RCT). Addition of purified lactoferrin to plasma did not change the aPTT in the absence of heparin (data not shown). In heparinized plasma, the RCT was reduced by addition of lactoferrin in a dose-responsive manner (Fig 4).

The concentration of plasma lactoferrin was also deter-

![Fig 3](image-url)
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Fig 4. Dose-response effect of lactoferrin on the aPTT in heparinized plasma. Normal pooled plasma was used in this assay. Each reaction contained 11 nmol/L (0.166 μg/mL) heparin and the various concentrations of lactoferrin indicated. Results are the mean values (±SD) obtained from three separate experiments performed in duplicate.

Fig 5. (A) Quantitation of plasma lactoferrin obtained from whole blood after treatment with various concentrations of LPS. LPS from Salmonella minnesota was obtained from Sigma. Whole blood (2 mL) freshly obtained was incubated with LPS for the indicated time period and at the various concentrations listed. The reactions were terminated by centrifugation. The concentration of plasma lactoferrin was determined by ELISA. (B) The aPTT of heparinized plasma derived from LPS-treated blood. For each LPS-treated blood sample, RCT represents aPTT ratio of heparinized plasma to unheparinized plasma. Results are the mean values (±SD) from three separate experiments performed in duplicate.
The concentration of lactoferrin at 500 ng/mL is about 6 nmol/L. The mean value (±SD) of three separate experiments performed in duplicate is reported. Control experiments showed that all the inflammatory mediators did not alter RCT values when incubated with pooled human plasma.

We examined the release of lactoferrin from neutrophils by various inflammatory mediators. It has been reported that LPS can stimulate the release of lactoferrin from neutrophils and that this is enhanced by TNF-α, which presumably would be secreted by monocytes. Our results show that fMLP, LPS, LTB₄, IL-8, and TNF-α could liberate significant amounts of lactoferrin from neutrophils, whereas the neutrophil chemoattractant TGF-β did not have any effect. These activated neutrophil supernatants neutralized the heparin-catalyzed antithrombin-thrombin inhibition reaction, and this is partly due to the presence of lactoferrin. It is also likely that neutrophil elastase and other neutrophil cationic proteins are involved in heparin neutralization in these assays. These results suggest that lactoferrin, as a specific neutrophil-released inflammation product, can directly contribute to the regulation of blood anticoagulation.

To further examine the contribution of lactoferrin in the regulation of blood coagulation, we measured the ability of lactoferrin in a plasma-blood-based system to neutralize heparin. The heparin-induced prolongation of the aPTT was shortened by lactoferrin in a dose-dependent manner. We also examined the effect of heparin on the aPTT of plasma obtained from whole blood after treatment with LPS and compared that with the concentration of plasma lactoferrin. Pretreatment of whole blood with LPS showed time- and dose-dependent heparin-neutralizing activities in heparinized plasma when monitored by the aPTT. There was a correlation between the lactoferrin level in plasma and the reduction in the aPTT of heparinized plasma. However, the concentration of platelet factor 4 in plasma was also increased after treatment of whole blood with various inflammatory mediators. In contrast to our results with isolated neutrophils, incubation of blood with LTB₄ or IL-8 does not result in elevation of either plasma lactoferrin or platelet factor 4. It is conceivable that in this complex system either LTB₄ and IL-8 are neutralized by other proteins before their interaction with neutrophils or their activity can only be expressed in close proximity to their target cell. These results indicate that lactoferrin and other cationic proteins like platelet factor 4 can collectively neutralize the anticoagulant activity of heparin in plasma.

A pathologic role of bacterial LPS in the development of septic DIC has been demonstrated in animal models. The contribution of leukocytes in DIC pathogenesis has also been established in animal studies where DIC did not occur in animals rendered leukopenic. However, the exact mechanism by which LPS mediates this hypercoagulable response with leukocytes is not fully known. Our results provide evidence that lactoferrin released from activated neutrophils may partly contribute to the development of septic DIC by inhibiting glycosaminoglycan-dependent serpins. This hypothesis is supported by the report that a patient with a deficiency in neutrophil lactoferrin had a severe bleeding problem after surgery. Failure of adequate thrombus formation in this patient may be related to an absence of lactoferrin, which could be otherwise localized at the site of surgical inflammation.

In summary, we conceive of an in vivo scenario where, at certain inflammatory lesions, a large amount of neutrophil lactoferrin could accumulate, which impedes glycosaminoglycan-dependent serpin activity. Other cationic protein(s) released from platelets (platelet factor 4) and neutrophils could also contribute to both glycosaminoglycan neutralization and serpin inactivation (elastase). Overall, our results suggest that some of the procoagulant state induced by inflammation is due, in part, to the ability of neutrophil lactoferrin to neutralize glycosaminoglycan-catalyzed serpin-thrombin inhibition reactions.

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Neutralization of heparin activity by neutrophil lactoferrin

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