Neutralization of Heparin Activity by Neutrophil Lactoferrin
By Hai-feng Wu, Roger L. Lundblad, and Frank C. Church

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 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.

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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 kD) and a polyclonal antibody against platelet factor 4 were obtained from American Diagnostica Inc (Greenwich, CT); polyclonal antibody against human neutrophil elastase was from Biodesign (Kennebunk, ME); Polyclure 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 kD, was provided from Diosynth (Oss, The Netherlands). Human α-thrombin, antithrombin, and heparin cofactor II were purified as described previously.20,22

Purification of lactoferrin. Human milk lactoferrin was purified from fresh mature milk as described by Blackberg and Hernell.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 Kallmar et al.23 The granulocyte fraction obtained was composed of greater than 95% polymorphonuclear leukocytes, and it exhibited more than 99% cell viability as determined by trypan blue exclusion. After washing twice with Hanks’ Balanced Salt Solution (HBSS) and resuspending in the same solution at a density of 5 × 107 cells per milliliter and treated with 100 µg/mL PMA for 30 minutes at room temperature. After centrifugation at 2,000 rpm for 5 minutes, the supernatant was collected and stored at −20°C until assayed.

Enzyme-linked immunosorbent assay (ELISA). The concentration of 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 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 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 spectrophotometrically at 405 nm in a Vmax kinetic microplate reader. All thrombin inhibition assays were performed at least three times, and mean values of heparin neutralization and standard deviation were determined. Percentage of heparin neutralization was calculated by subtracting the molar ratio of neutralizing protein/heparin. Heparin neutralization percentage was calculated as follows: heparin neutralization % = ([B - A]/C - A) × 100%; where A is the thrombin residual activity obtained in the presence of serpin and heparin; B is the activity obtained in the presence of serpin, heparin, and lactoferrin or other indicated molecules; and C is the activity obtained in the presence of serpin only. Control experiments verified that lactoferrin did not affect (1) the activity of thrombin alone, (2) the activity of thrombin in the presence of heparin, or (3) the thrombin inhibition by the serpins in the absence of heparin.

Preparation of neutrophil-releasing products. Human neutrophils were isolated from venous blood (anticoagulated with EDTA) by centrifugation on Ficol-Hypaque gradient as described above. The isolated neutrophils were then washed twice with HBSS and resuspended in the same solution at a density of 5 × 107 cells per milliliter. A 300-µL cell suspension was incubated with various agonists at the indicated concentrations for 30 minutes at 37°C. Incubation was terminated by centrifugation at 2,000 rpm for 5 minutes. The cell-free supernatants were collected and stored at −20°C until assayed.

Thrombin inhibition assay. The assay was performed in 96-well U-bottom microplates (Becton Dickinson, Oxnard, CA) in 100 µL of 0.15 mol/L NaCl, 0.02 mol/L HEPES, 1 mg/mL polyethylene glycol (Mr = 8,000), and 1 mg/mL bovine serum albumin (BSA), pH 7.4. Inhibition reaction mixtures contained 0.1 µg/mL heparin, 50 mmol/L serpin, and various concentrations of lactoferrin or other heparin-neutralizing molecules. The reaction was initiated by adding thrombin (5 mmol/L) to the microplate well. After an incubation of 30 to 40 seconds, 50 µL of a solution containing 0.2 mmol/L tosyl-Gly-Pro-Arg-p-nitroanilide and 1.5 mg/mL Polybrene was added. Residual thrombin activity was determined by measuring the hydrolysis of the chromogenic substrate in a Molecular Devices (Menlo Park, CA) Vmax kinetic microplate reader. All thrombin inhibition assays were performed at least three times, and mean values of heparin neutralization and standard deviation were determined. Percentage of heparin neutralization was expressed by plotting extent of neutralization of anticoagulant activity against the molar ratio of neutralizing protein/heparin. Heparin neutralization percentage was calculated as follows: heparin neutralization % = ([B - A]/C - A) × 100%; where A is the thrombin residual activity obtained in the presence of serpin and heparin; B is the activity obtained in the presence of serpin, heparin, and lactoferrin or other indicated molecules; and C is the activity obtained in the presence of serpin only. Control experiments verified that lactoferrin did not affect (1) the activity of thrombin alone, (2) the activity of thrombin in the presence of heparin, or (3) the thrombin inhibition by the serpins in the absence of heparin.

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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 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.

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 challenges 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 IISi 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-polycyal 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-α 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 proteinase 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.21 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-

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**Fig 3.** (A) Neutralization of heparin by neutrophil-releasing products measured in an antithrombin-thrombin inhibition reaction. Isolated neutrophils (10⁷ cells per milliliter) were treated with 100 nmol/L fMLP, 100 U/mL TNF-α, 100 nmol/L LTB4, 1 μg/mL LPS, 500 nmol/L IL-8, or 500 nmol/L TGF-β for 30 minutes at 37°C, and the reactions were terminated by centrifugation. Each assay contained 23 ng/mL heparin (2 nmol/L), 40 nmol/L antithrombin, 20 μg/mL aprotinin, 200 μmol/L TPCK and 40 μL of each neutrophil supernatant in a total volume of 120 μL. The reaction was initiated by adding 3 nmol/L thrombin. Residual thrombin activity was measured after 90 seconds of incubation, and the percentage of heparin neutralization (B) was calculated. For the antibody blocking assay, the neutrophil supernatants were preincubated with 25 μg/mL anti-lactoferrin polyclonal antibody, 20 μg/mL aprotinin, and 200 μmol/L TPCK for 30 minutes at room temperature before addition of other reagents (B). Results are mean values (±SD) obtained from three separate experiments performed in duplicate. (B) Release of lactoferrin from isolated neutrophils after stimulation with various inflammatory mediators. The concentration of lactoferrin in these neutrophil supernatants was determined by ELISA. The results represent mean values (±SD) from three separate experiments performed in duplicate. The concentration of lactoferrin at 800 ng/mL is about 10 nmol/L. (C) Release of elastase from isolated neutrophils after stimulation with various inflammatory mediators. The concentration of elastase in these neutrophil supernatants was measured by ELISA. The results represent mean values (±SD) from three separate assays performed in duplicate.
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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.

Concentration of lactoferrin (nM)

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<th>Concentration (nM)</th>
<th>0</th>
<th>1</th>
<th>0.37</th>
<th>0.75</th>
<th>1:5</th>
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<td>1.15</td>
<td>1.05</td>
<td>1.00</td>
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mined by ELISA after incubation of whole blood with LPS. As shown in Fig 5, the plasma lactoferrin concentration increased in response to LPS treatment in a time- and dose-dependent manner. Prolongation of the aPTT by adding heparin to plasma was reduced in LPS-treated blood.

The plasma concentration of both lactoferrin and platelet factor 4 after treatment of whole blood with the various agonists was then quantitated by ELISA (Fig 6). Treatment of blood with fMLP, LPS, and TNF-α resulted in a significant increase in both lactoferrin and platelet factor 4 levels, while other neutrophil agonists, such as IL-8 and LTB4, did not increase plasma levels of either protein. We then determined the RCT of plasma obtained from these treated blood samples. A reduction of RCT was observed for blood treated with LPS, fMLP, and TNF-α (Fig 6).

DISCUSSION

It is now well established that glycosaminoglycans such as heparin and heparan sulfate catalyze certain serpin-proteinase inhibition reactions like antithrombin-thrombin, antithrombin-factor Xa, and heparin cofactor II-thrombin. These inhibition reactions are critical for the control of the hemostatic response.10,36 As there is little heparin-like activity in circulating blood in vivo, the participation of exposed glycosaminoglycan chains at the site of injured tissue is considered critical for the normal function of serpins such as antithrombin and heparin cofactor II.10,36-41 In some pathologic conditions such as infection, the thrombotic coagulopathy may occur partly as a result of the downregulation of anticoagulation. For instance, elastase released from stimulated neutrophils can contribute to the procoagulant response by directly inactivating serpins.42,43 Additionally, platelet factor 4 and other cationic proteins released from platelets and neutrophils neutralize glycosaminoglycans to attenuate glycosaminoglycan-catalyzed serpin inhibition reactions.44-47 In the present study, we demonstrated that neutrophil lactoferrin is another protein that is quite effective in neutralizing heparin-dependent serpin-thrombin inhibition reactions and in blocking the anticoagulant activity of heparin in plasma.

Neutrophil-derived lactoferrin had the same heparin-neutralizing activity in vitro as both platelet factor 4 and lactoferrin purified from milk. We also found that both apo- and holo-lactoferrin had similar heparin neutralization activities, implying that the heparin-binding domain of lactoferrin is not affected by the iron-binding status of the protein. The observed minor differences in heparin neutralization are likely due to the conformational orientation of the protein after iron binding, as demonstrated previously.29,48,49 In contrast to histidine-rich glycoprotein, which requires divalent cation binding for heparin-neutralizing activity,50,51 the addition of EDTA to lactoferrin did not affect its heparin binding. Despite the fact that lactoferrin belongs to the transferrin family of iron-binding proteins and these proteins share a sequence homology of greater than 55%, transferrin exhibited no heparin-binding properties. This unique heparin-binding function of lactoferrin suggests that, after release from neutrophils, it might play another very important role in regulating inflammation and coagulation.
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 (ase) 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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Fig 6. Relationship between plasma lactoferrin and platelet factor 4 levels with aPTT values of heparinized plasma obtained from whole blood treated with various agonists. Whole blood that was anticoagulated with sodium citrate was treated with various agonists (at the same concentrations as described in Fig 3) for 30 minutes at 37°C. The concentrations of lactoferrin (A) and platelet factor 4 (B) were measured by ELISA in the plasma obtained from each agonist-treated blood sample, and an aPTT was performed to determine RCT (C). The concentration of lactoferrin at 500 ng/mL is about 5 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 chemotactant 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...
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REFERENCES


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