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The inhibition of mast cell activation by neutrophil lactoferrin: uptake by mast cells and interaction with tryptase, chymase and cathepsin G

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Abstract

Inhibitors of mast cell tryptase and chymase can be effective as mast cell stabilising compounds. Lactoferrin has been reported to inhibit tryptase activity, but its actions on other serine proteases of mast cells and its potential to alter mast cell function are not known. We have examined the ability of lactoferrin to inhibit mast cell tryptase, chymase and cathepsin G, and investigated its potential to modulate the activation of human mast cells. Enzymatically dispersed cells from human skin, lung and tonsil were challenged with anti-IgE or calcium ionophore A23187, following incubation with recombinant human lactoferrin, and histamine release determined. IgE-dependent histamine release from skin mast cells was inhibited by up to 50% following incubation with lactoferrin (50 or 500 nM). Tonsil mast cells were also stabilised by lactoferrin, but not those from lung. Calcium ionophore A23187-induced histamine release was not altered by lactoferrin. A double-labelling immunocytochemical procedure revealed the presence of lactoferrin in 4–6% of mast cells, and this proportion increased to 40% following incubation with lactoferrin. Lactoferrin did not inhibit cleavage of synthetic substrates by tryptase and chymase directly, though it was able to diminish the ability of heparin to stabilise tryptase. Cathepsin G activity was inhibited by lactoferrin. The ability of lactoferrin to inhibit IgE-dependent activation of human mast cells and modulate protease activity suggests that the release of this neutrophil product may have a role in the downregulation of allergic inflammation.

Keywords: Cathepsin G; Chymase; Mast cell; Histamine; Neutrophil; Tryptase

1. Introduction

Mast cell activation is a key event in allergic reactions and is associated with the release of a range of pharmacologically potent products, including proteases, histamine, proteoglycans, eicosanoids and various cytokines. Prominent among these are the serine proteases, tryptase, chymase and cathepsin G, which together with carboxypeptidase may be present in a quantity of up to 60 pg per mast cell [1]. These enzymes are now emerging as key mediators of inflammation and tissue remodelling and as promising targets for therapeutic intervention. There is a need for a better understanding of how their actions may be controlled at sites of inflammation.

Several peptide and protein substrates of mast cell proteases have been identified whose cleavage would be consistent with these proteases having roles in processes of neurogenic inflammation, the control of blood flow and in tissue remodelling [2]. However, of particular importance to an understanding of their function could be their ability to interact with various cell types. Human tryptase can itself provoke histamine release from mast cells, and this could represent an amplification process in allergic disease [3,4]. Moreover, tryptase can selectively induce the release of certain cytokines from epithelial and endothelial cells, stimulate the synthesis of collagen from fibroblasts and act as a potent growth factor for fibroblasts, epithelial cells and airway smooth muscle cells [5–9]. The injection of either human tryptase or chymase into animal models is associated with a rapid and prolonged increase in microvascular permeability and with the accumulation of neutrophils and eosinophils at sites of injection [3,10–12].

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Abbreviations: HBSS, Hank's balanced salt solution.

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The search for natural inhibitors of human mast cell tryptase has been unproductive. Various circulating inhibitors, including α_1-anti-trypsin, α_2-macroglobulin and C_1-esterase inhibitor, as well as several inhibitors isolated from human tissues, including bikunin, and low molecular weight elastase inhibitor are without effect on tryptase activity [2]. It has been reported that neutrophil-derived lactoferrin can inhibit tryptase activity, and this has been linked with its ability to inhibit airway responses in a naturally sensitised sheep model [13]. Lactoferrin, a 78 kDa protein that is also found in milk, tears and saliva, has anti-bacterial, anti-viral and anti-fungal activities [14–16]. It is also able to neutralise heparin and to increase natural killer cell activity [17,18]. Like its plasma homologue, transferrin, it is capable of binding iron, but this activity does not appear to be essential for its biological activity [14–18]. The potential of lactoferrin to act as an anti-allergic compound deserves investigation.

The advent of a recombinant form of human lactoferrin [19] opens the way for the anti-allergic properties of this compound to be explored. We have examined the ability of recombinant lactoferrin to inhibit tryptase activity, and have investigated its actions on other serine proteases released from human mast cells. We have found previously that inhibitors of tryptase and chymase can have potent mast cell stabilising actions [4,20], and in the present studies have investigated whether lactoferrin shares these properties. We report that lactoferrin appears to be taken up by human mast cells, and by inhibiting histamine release could represent a natural regulator of mast cell function.

2. Materials and methods

2.1. Materials

Goat anti-human IgE was purchased from Serotec. Heparin agarose was obtained from Kem-En-Tec, *p*-aminobenzamidine agarose from Affinity Chromatography Ltd and Sephacryl S-300 from Pharmacia. Human neutrophil cathepsin G and heparin (sodium salt, porcine intestinal mucosa, molecular mass in range of 13–15 kDa) were purchased from Calbiochem. Diaminobenzidine (DAB) substrate chromogen system was from DAKO. Pyroglutamyl-Pro-Arg-*p*-nitroanilide was purchased from Chromogenix, *o*-phthaldialdehyde from Fluka, foetal calf serum (FCS) from Life Technologies, and Coomassie protein assay reagent and SuperSignal chemiluminescent substrate from Pierce and Warriner. Accugel solution of acrylamide and *N*,*N*′-methylene-bis-acrylamide (19:1) was purchased from Flowgen Instruments, pre-stained molecular mass markers from Life Technologies, native molecular mass markers from Promega, and all other electrophoresis reagents from BioRad. All other chemicals were from Sigma Chemical Co or BDH. AA5 (anti-tryptase monoclonal antibody) and human skin chymase were produced as described previously [21,22]. Recombinant human lactoferrin and rabbit anti-human lactoferrin antibody were provided by Pharming BV. It was quantified by ELISA with purified human milk lactoferrin as a standard. The iron content of the preparation used in this study was 20% saturation.

2.2. Mast cell dispersion and challenge

Human tonsil, lung or skin tissues were freshly obtained at tonsillectomy, lobectomy or circumcision operations, respectively. Cells in macroscopically normal tissue were dispersed enzymatically as previously described [4]. In preparations of skin, lung or tonsil cells, mast cells represented 5.2 ± 1.0, 4.2 ± 0.9 or $0.5 \pm 0.06\%$ (mean ± SEM), respectively, of all cells present. Dispersed cells were maintained in Minimal Essential Medium (MEM) containing 10% FCS, 200 units/mL penicillin, 200 μg/mL streptomycin on a roller overnight at room temperature. After washing twice with Hank's balanced salt solution (HBSS), pH 7.4, the cells were resuspended in HBSS with 1.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (complete HBSS), and warmed at 37° for 5 min. Aliquots of 100 μL containing 4×10^3–6×10^3 mast cells were added to a 50 μL aliquot of the secretagogue or inhibitor in complete HBSS and incubated for 15 min at 37°. The reaction was terminated by the addition of 150 μL ice-cold HBSS and the tubes centrifuged immediately (500 *g*, 10 min, 4°). All experiments were performed in duplicate. For the measurement of total histamine concentration in some tubes, the suspension was boiled for 6 min. Supernatants were stored at −20° until histamine concentrations were determined.

2.3. Histamine measurement

A glass fibre-based, fluorometric assay for histamine was employed as previously described [4]. Histamine release was expressed as a percentage of total cellular histamine levels, and corrected for the spontaneous release measured in tubes in which cells had been incubated with the HBSS diluent alone under otherwise identical conditions (i.e. percentage net histamine release = [(histamine release with stimulus − spontaneous histamine release)]/total histamine content × 100. The lower limit of detection of the assay was 1 ng/mL, and inter-assay variability was less than 3%. Lactoferrin did not interfere in this assay over the range tested. Spontaneous histamine release (in the absence of added stimulus) from skin, tonsil or lung mast cells was 10.3 ± 2.4, 8.9 ± 1.1 or $6.7 \pm 1.0\%$, respectively.

In preliminary experiments, dispersed mast cell preparations from skin, tonsil or lung were incubated with a range of concentrations of anti-IgE or calcium ionophore A23187, and net histamine release calculated. Maximal histamine release without evidence of a cytotoxic effect (as investigated by preincubation with the metabolic inhibitors antimycin A and 2-deoxy-D-glucose) was observed with

1% anti-IgE or with 1 μM calcium ionophore (data not shown). These were selected as positive controls in all experiments involving mast cell challenge.

2.4. Preparation of tissues and cells for immunocytochemistry

Fresh human lung and skin tissues were obtained as described earlier and a few small pieces (approximately 10 mm × 6 mm × 3 mm) were removed from each tissue. The tissue was fixed in 85% ethanol for a week at 4° before dehydration through graded alcohols. Tissues were embedded in paraffin wax and six sections (6 μm) from each block were prepared for immunocytochemistry. To investigate whether lactoferrin can be taken up by mast cells, dispersed skin or lung cells were incubated with lactoferrin (0.05 and 0.5 μM) or with buffer alone for 60 min at 37°. Following two washes with HBSS (500 μL, 500 *g*, 6 min, 4°), the cells were resuspended in Coagulation Control Solution 1 (Sigma) before allowing to clot with thromboplastin and calcium according to the manufacturer's instructions. The cells in the clot were processed as described earlier.

2.5. Immunocytochemical localisation of lactoferrin to mast cells

A double-labelling procedure with a monoclonal antibody against human tryptase (AA5) and a polyclonal antibody against human lactoferrin was employed. Briefly, following rehydration of tissue sections, endogenous peroxidase was blocked with 0.5% H_2O_2 and 0.1% sodium azide, and non-specific protein binding was blocked with 5% BSA. Rabbit antiserum against lactoferrin and biotinylated tryptase-specific antibody AA5 was applied to the sections for 2 hr, and after washing with PBS with 0.1% Tween-20 (PBST) three times, peroxidase-labelled goat anti-rabbit IgG and streptavidin alkaline phosphatase was added for 1 hr. Cells staining for tryptase were visualised by the Fast Red TR/Naphthol AS-MX system (red colour) and lactoferrin positive cells by DAB (brown colour). Sections were counterstained with Mayer's haematoxylin for 3 min. All AA5-stained cells and double-stained cells were counted for each section.

2.6. Purification of tryptase

Tryptase was purified from human lung obtained *post mortem* by successive column chromatography with heparin agarose, benzamidine agarose and Sephacryl S-300 as described elsewhere [9]. The final product was homogeneous by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and reacted with the anti-tryptase monoclonal antibody AA5 in Western blots. Enzyme concentration was determined by titration of the active site with 4-methylumbelliferyl-*p*-guanidinobenzoate and expressed as moles of active site [23]. Comparison of these values with protein content as estimated from the absorbance at 280 nm [24] indicated the purified tryptase was more than 99% active.

2.7. Enzyme assays

During purification tryptase activity was determined by the hydrolysis of 0.9 mM *N*-benzoyl-D,L-arginine *p*-nitroanilide in 1 M glycerol, 0.1 M Tris–HCl, pH 8.0 [21], whilst for inhibition and inactivation studies, the 40-fold more sensitive substrate 0.5 mM L-pyroglutamyl-Pro-Arg-*p*-nitroanilide was used [25]. Chymase and cathepsin G were assayed with 0.5 mM *N*-succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester and 0.1 mM 5,5′-dithio-bis(2-nitrobenzoic acid) [26]. For inhibition and inactivation studies all enzymes and effectors were made up to the required dilutions in complete HBSS. Samples were taken in triplicate at $t = 0$ and after 60 min ($t = 60$) and assayed with the appropriate substrate. In the inactivation studies, BSA was included in all incubation mixtures at a final concentration of 10 μg/mL to prevent adsorptive loss of tryptase to the walls of the vessels. This concentration of BSA also approximated the residual serum content of the incubation medium of the washed cells used in the challenge studies. To accommodate the 100-fold range in tryptase activity within the sensitivity range of the assay, 150 μL of 0.5 nM tryptase samples were mixed with 50 μL of 2 mM substrate, 20 μL of 5 nM tryptase with 80 μL of 0.625 mM substrate, and 20 μL of a 10-fold dilution of 50 nM tryptase with 80 μL of 0.625 mM substrate.

2.8. Statistics

Data are shown as the mean ± SEM for the number of experiments indicated, and where appropriate the paired Student's *t*-test or regression analysis was applied, taking $P < 0.05$ as significant.

3. Results

3.1. Effect of lactoferrin on mast cell histamine release

Preincubation of dispersed skin cells with various doses of lactoferrin for 30 or 60 min prior to challenge with anti-IgE resulted in the inhibition of histamine release by up to 50% at the optimum effective dose of 50 nM (Fig. 1A). With tonsil mast cells, lactoferrin at a concentration of 500 nM inhibited IgE-dependent histamine release by approximately 30% following preincubation periods of 30 or 60 min (Fig. 1B). For both tonsil and skin cells, a shorter preincubation time with lactoferrin (10 min) was insufficient to inhibit histamine release (data not shown). In contrast, histamine release from lung mast cells was not inhibited following preincubation with lactoferrin under

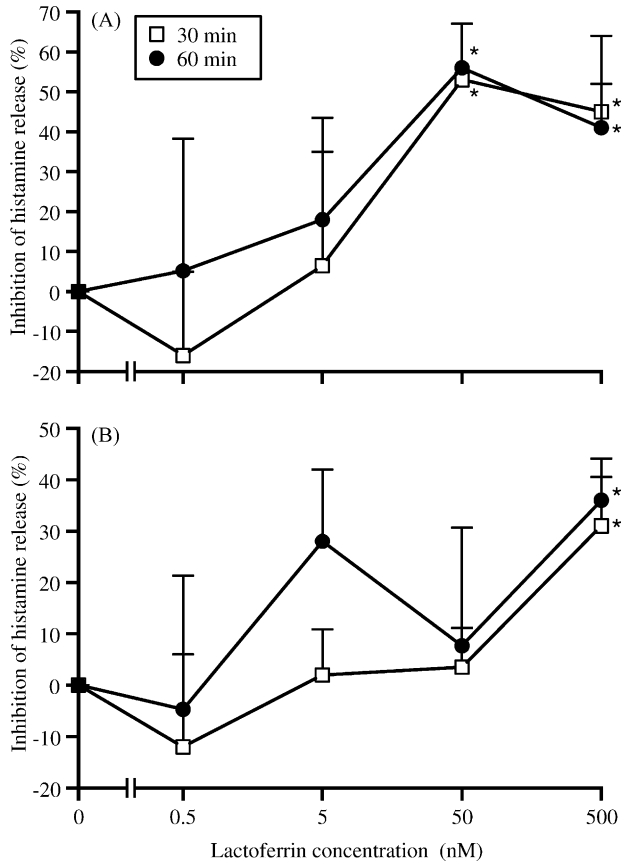

Fig. 1. Actions of lactoferrin on IgE-dependent histamine release from (A) human skin and (B) tonsil mast cells. The cells were preincubated with various concentrations of lactoferrin for 30 or 60 min before addition of stimulus. The values shown are mean ± SEM for four to six separate mast cell preparations treated in duplicate. $^{*}P < 0.05$ compared with the uninhibited controls for that time point (paired Student's *t*-test).

any of the conditions tested (data not shown). With calcium ionophore as stimulus, lactoferrin failed to alter the responsiveness of mast cells from any of the three tissues (data not shown). Lactoferrin did not itself provoke histamine release when added to cells in concentrations ranging from 0.5 to 5000 nM.

3.2. Immunocytochemical detection of lactoferrin

Lactoferrin was localised by the double-labelling immunocytochemical procedure to mast cells in human lung (Fig. 2A) and skin (Fig. 2B) tissues, as well as in preparations of enzymatically dispersed lung (Fig. 2C) or skin tissue. Both antibodies gave a granular appearance upon staining mast cells. In tissue sections, it was found that 4.0 ± 3.2% skin (mean ± SEM, N = 4) and 6.2 ± 2.5% lung mast cells (mean ± SEM, N = 6) contained immunoreactive lactoferrin. Following a 60-min incubation period with lactoferrin, the proportion of lactoferrin-containing mast cells increased by approximately 4-fold in dispersed skin preparations, and more than 7-fold in lung (Table 1).

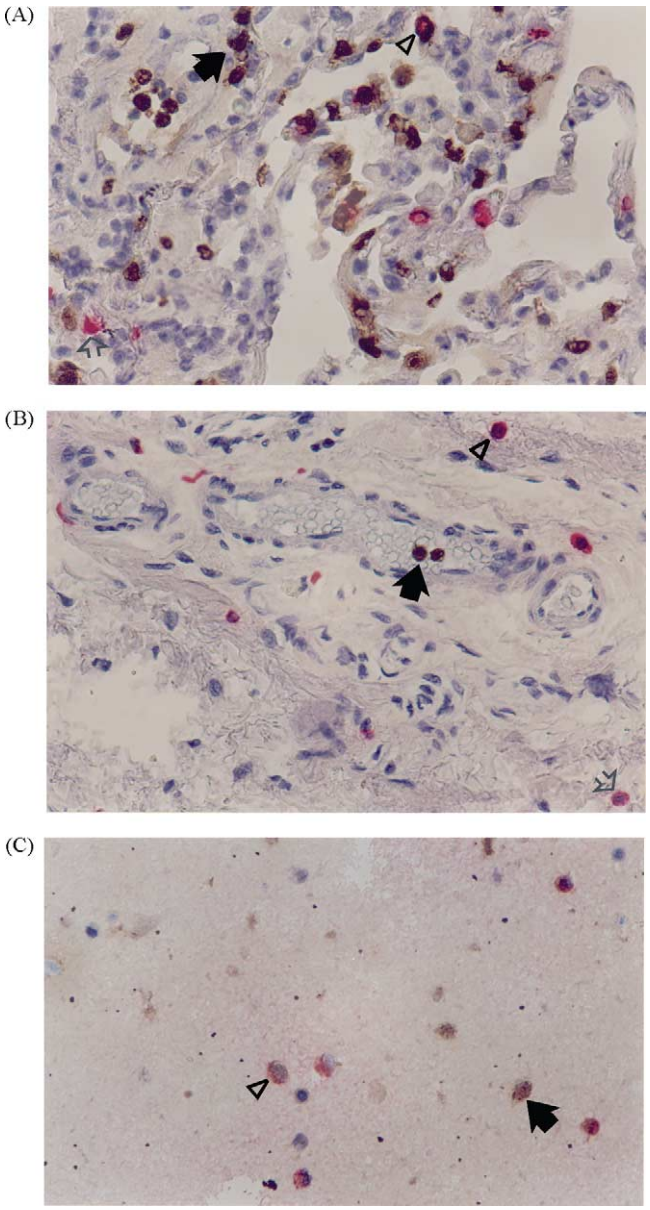

Fig. 2. Immunocytochemical double labelling of mast cells with monoclonal antibody AA5 specific for tryptase, and a polyclonal antibody specific for lactoferrin. (A) human lung tissue, (B) human skin tissue and (C) dispersed lung cells. The open and closed arrows and open arrowheads indicate tryptase-positive (red), lactoferrin-positive (brown) and double-labelled (red–brown mixture) cells, respectively.

Table 1
The percentage of mast cells staining for lactoferrin in dispersed skin or lung tissue

Treatment	Double-labelled cells (%)	
	Skin	Lung
Buffer alone	10 ± 6.2	5.5 ± 2.1
Lactoferrin		
50 nM	33 ± 11	40 ± 16
500 nM	38 ± 19	33 ± 0.7

Values shown are mean ± SEM for three or two separate experiments with skin or lung cells, respectively. Following enzymatic dispersion, cells were incubated with either lactoferrin (50 or 500 nM) or buffer alone for 60 min at 37°. All experiments were performed in duplicate.

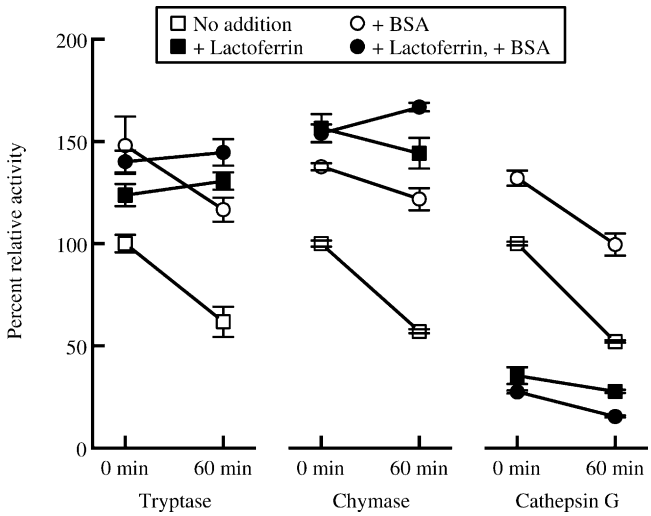

Fig. 3. Effect of lactoferrin on mast cell protease activity. Tryptase (5 nM, 170 ng/mL), chymase (4.5 mU/mL, ~5 nM, ~150 ng/mL) and cathepsin G (~18 nM, 500 ng/mL) were incubated with 5 μM (385 μg/mL) lactoferrin or 10 μg/mL BSA or both on ice. Cathepsin G was used at a higher concentration than the other two enzymes because of its relatively low activity towards the substrate. Triplicate samples were taken at 0 and 60 min and assayed with 0.5 mM pyroglutamyl-Pro-Arg-*p*-nitroanilide (tryptase) or 0.5 mM succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester (chymase and cathepsin G) in 120 mM NaCl, 50 mM Tris–HCl, pH 7.6. Readings at 410 nm were taken at 10-s intervals over 10 min and rates calculated from data within the first 7 min and a limit of change in absorbance of 0.1. Activity is expressed as percentage ± SEM of that in the absence of any additives at 0 min.

3.3. Effect of lactoferrin on mast cell proteases

Lactoferrin at the highest concentration used in the cell challenge studies (5 μM) did not inhibit mast cell tryptase or chymase under conditions where both enzymes are relatively stable in the absence of heparin (Fig. 3), but instead increased activity relative to enzyme alone. The degree of apparent stimulation was of the same magnitude as that afforded by BSA and probably represents stabilisation, possibly against adsorptive loss. In contrast, cathepsin G was significantly inhibited by lactoferrin in both the absence and presence of BSA.

3.4. Effect of lactoferrin on the stabilisation of tryptase by heparin

On the basis of dose–response curves for stabilisation of tryptase at 37° by heparin (data not shown), different concentrations of tryptase were incubated with suboptimal, adequate and excess amounts of heparin together with increasing doses of lactoferrin. At $t = 0$, lactoferrin had no effect on tryptase activity regardless of heparin concentration (results not shown). After incubation at 37° for 60 min, activity was lost almost completely in the absence of heparin, and in its presence, tryptase activity was lost in a dose-dependent manner with increasing amounts of lactoferrin. For any particular concentration of tryptase, the apparent IC_{50} of lactoferrin increased with increasing

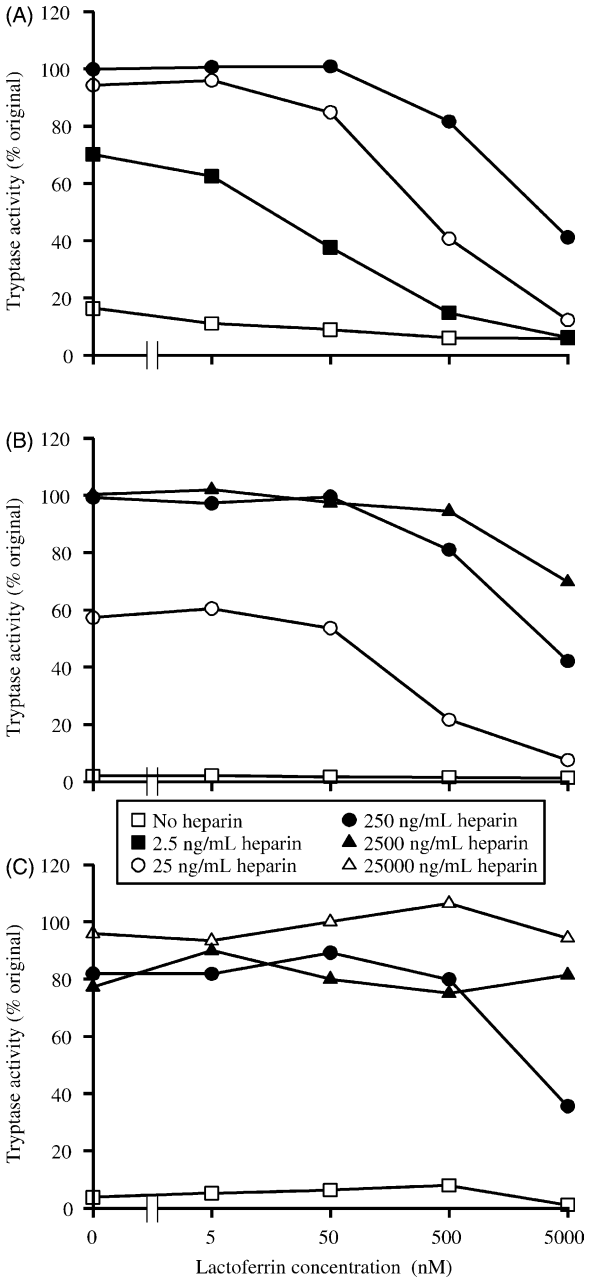

Fig. 4. Effect of lactoferrin on the stabilisation of tryptase by heparin. Lactoferrin at the indicated concentrations was incubated at 37° for 60 min with (A) 0.5 nM, (B) 5 nM or (C) 50 nM tryptase in the presence of 10 μg/mL BSA and the indicated amount of heparin (2.5–25,000 ng/mL or 0.19–1900 nM). For each tryptase concentration, a range of heparin concentrations was chosen to maintain the same molar ratios with tryptase. The SEM of triplicate assays are not shown as they are smaller than the data symbols.

concentration of heparin (Fig. 4). This inactivation was not reversed by the addition of excess heparin (100 μg/mL) prior to assay (data not shown), consistent with previous reports that tryptase undergoes an irreversible spontaneous inactivation upon dissociation from heparin at physiological ionic strength.

Table 2 shows that for the combinations of tryptase, heparin and lactoferrin for which an IC_{50} value for lactoferrin could be calculated, the values obtained at a

Table 2
Lactoferrin IC_{50} values for the destabilisation of the tryptase–heparin complex

Heparin (ng/mL) (nM)	Tryptase (nM)	Lactoferrin IC_{50} (nM)
2.5 (~0.19)	0.5	69
25 (~1.9)	0.5	342
25 (~1.9)	5	274
250 (~19)	0.5	3020
250 (~19)	5	3130
250 (~19)	50	3070

IC_{50} values were calculated by linear interpolation of the semi-logarithmic plots of Fig. 4. For suboptimal heparin concentrations, the highest tryptase activity obtained was taken as 100%. Tryptase concentrations are expressed in terms of active site.

particular concentration of heparin were nearly identical, regardless of the tryptase concentration. The IC_{50} values for lactoferrin correlated very well with heparin concentration ($r^2 = 0.999$), but their correlation with tryptase concentration ($r^2 = 0.213$) was not any greater than that between tryptase and heparin ($r^2 = 0.214$), which was set by experimental design. These results are consistent with a model whereby lactoferrin reacts with heparin, rather than directly with tryptase.

In Fig. 4, the stoichiometry of the heparin–tryptase interaction can be calculated from the tryptase activity remaining after 1-hr incubation at 37° at the suboptimal concentrations of heparin (2.5 ng/mL (~0.19 nM) heparin, 0.5 nM tryptase; 25 ng/mL (~1.9 nM) heparin, 5 nM tryptase; 250 ng/mL (~19 nM) heparin, 50 nM tryptase). If the percent tryptase activity remaining is taken to indicate the percent of total tryptase bound to heparin, then a stoichiometry of 1.9 ± 0.2 or two tryptase monomers per heparin molecule is obtained. This is equivalent to two heparin molecules required to stabilise one tryptase tetramer.

If both tryptase and lactoferrin are competing for the same ligand (heparin), then their relative affinities can be calculated. If both lactoferrin and tryptase bind to heparin in a simple, non-cooperative manner, then at the IC_{50},

$$[L_{TOT}] = \left(\frac{K_{LH}}{K_{TH}} + 1\right)([H_{TOT}] - [TH]) - [H]\left(\frac{K_{LH}}{K_{TH}} + 1\right)$$

where K_{LH} is the dissociation constant for the lactoferrin–heparin complex, K_{TH} the dissociation constant for the tryptase–heparin complex, and $[L_{TOT}]$, $[H_{TOT}]$, [H] and [TH] represent the concentrations of total lactoferrin, total heparin, free heparin and tryptase–heparin complex, respectively. A plot of $[L_{TOT}]$ vs. $([H_{TOT}] - [TH])$ should, therefore, yield a straight line with a slope of $(K_{LH}/K_{TH}) + 1$. The resultant plot (Fig. 5) indicates a reasonably good fit to this model ($r^2 = 0.855$ for all data, $r^2 = 0.996$ if the outlier is omitted). The presence of the outlier, however, does not significantly affect the slope. The calculated values of the ratio of the dissociation constants for the lactoferrin–heparin and tryptase–heparin complexes are 166 ± 34 (all data) and 161 ± 6 (outlier omitted). Thus, tryptase has approximately a 160-fold greater affinity for heparin than does lactoferrin.

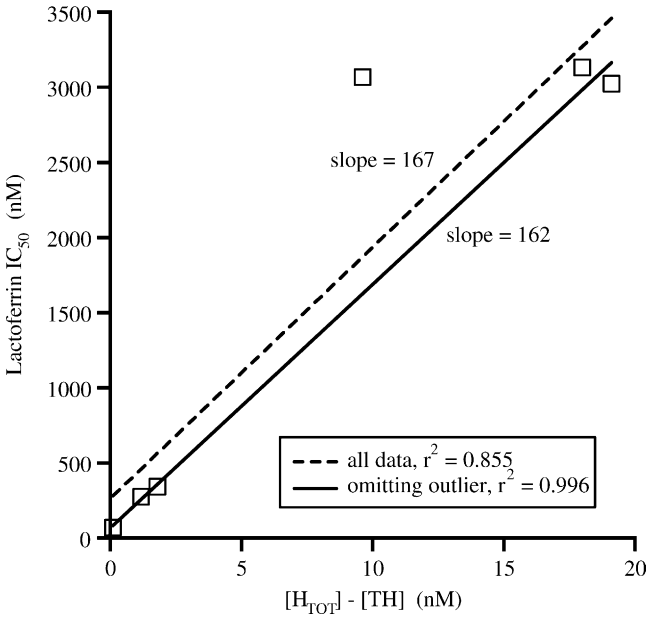

Fig. 5. Calculation of the relative binding affinities of tryptase and lactoferrin for heparin. The IC_{50} for lactoferrin (Table 2) was plotted against the total concentration of heparin minus the concentration of the tryptase–heparin complex. According to a simple, competitive, non-cooperative model of binding of lactoferrin and tryptase to heparin, this plot should be linear with a slope of $(K_{LH}/K_{TH}) + 1$.

4. Discussion

We have found that lactoferrin has the potential to counteract the actions not only of mast cell tryptase but also of cathepsin G, a product of both neutrophils and mast cells. The secretion of lactoferrin from neutrophils recruited to sites of inflammation could thus serve to limit the degree of protease-induced inflammation and tissue remodelling. The ability of lactoferrin to enter mast cells and to inhibit IgE-dependent activation of these cells could be of particular importance. Various naturally generated stimuli of mast cell activation have been identified [27,28], but this is the first report of an endogenous mast cell stabilising compound.

IgE-dependent release of histamine from skin mast cells was inhibited by approximately 50% in the presence of lactoferrin at concentrations of 50–500 nM. Lactoferrin can thus be more potent as a stabilising agent for skin mast cells than most anti-allergic drugs. Sodium cromoglycate at concentrations up to 1000 μM and ketotifen at 100 nM have failed to inhibit IgE-dependent histamine release from skin mast cells, while with salbutamol at up to 1 μM, cetirizine at up to 25 μM and terfenadine at up to 10 μM, the degree of inhibition has generally been less than 25% [29,30]. With tonsil mast cells, lactoferrin was less potent, but the potency of lactoferrin with this source

of mast cells is comparable with that of sulbutamol (some 25% inhibition with up to 1 μM), and it is more potent than ketotifen, sodium cromoglycate, terfenadine and cetirizine, which have been found to inhibit histamine release from tonsil cells by less than 15% at the concentrations employed in the present study [29]. The lack of inhibition of histamine release from lung mast cells with lactoferrin was unexpected and it would suggest heterogeneity in mast cell responsiveness to lactoferrin. In this respect there are clear parallels with previous findings with the selective inhibitor of chymase Z-Ile-Glu-Pro-Phe-CO_2Me, which can be an effective stabiliser of skin mast cells without having any apparent effect on lung mast cells [20], and also with the tryptase inhibitor APC366, which has been found to be more effective in inhibiting histamine release from skin mast cells than from tonsil mast cells [4].

The mast cell stabilising actions of lactoferrin may be related to the ability of this neutrophil product to enter mast cells. For both skin and tonsil mast cells, stabilisation was achieved when cells were preincubated with lactoferrin for 30 or 60 min, but not for 10 min. Lactoferrin was detected within a small proportion of non-stimulated mast cells, but following a 60-min incubation period, the proportion of lactoferrin-containing mast cells increased by some 4–7-fold, which strongly suggests that it is actively taken up by mast cells. The ability of mast cells to endocytose eosinophil peroxidase and major basic protein has been noted previously [31,32]. Moreover, Kido *et al*. [33] have demonstrated that soybean trypsin inhibitor and F(ab′)$_2$ fragments of a rat chymase-specific antibody may be taken up by rat peritoneal mast cells, raising the possibility that such a process could provide a means for the modulation of mast cell protease activity. How specific such a mechanism would have to be is not clear. Lactoferrin could be binding to a specific receptor or it could be binding relatively non-specifically by virtue of its strong positive charge to the negatively charged glycocalyx on the cell surface. Further work is needed to clarify the mechanism of uptake.

In view of the potent mast cell stabilising properties of inhibitors of mast cell proteases, it is tempting to speculate that the actions of lactoferrin on mast cells could involve an interaction with the proteases of this cell type. Lactoferrin has previously been described as an inhibitor of tryptase, although this designation is perhaps misleading as the proposed mechanism was displacement of tryptase from its stabilising complex with heparin followed by irreversible dissociation into inactive monomers [13]. Our present studies support this hypothesis by showing that lactoferrin does not inhibit tryptase *per se*, but could have the physiological effect of an inhibitor by competitive displacement of tryptase from its stabilising proteoglycan. However, we did not find lactoferrin to be as potent as reported by Elrod *et al*. [13]; whereas they obtained an IC_{50} of 24 nM for lactoferrin with 0.5 nM tryptase in the presence of 25 ng/mL heparin, our IC_{50} value was 14-fold greater under identical conditions. This apparent discrepancy in observed potency may be related to the use of low molecular weight heparin (5 kDa) by Elrod *et al*. [13], which has been shown to be less effective at stabilising tryptase than the high molecular weight heparin (13–15 kDa) which we employed [34]. As human mast cell heparin proteoglycan has a molecular mass of 60 kDa [35], the heparin used in the present studies is probably a better indication of the *in vivo* situation.

Hallgren *et al*. [36] did not observe inactivation of tryptase by lactoferrin at concentrations up to 11 μM. At their indicated concentrations of tryptase (8 nM active site) and heparin (625 ng/mL), one would have expected, on the basis of the results presented in Fig. 4, to have seen some, albeit modest, inactivation at the highest doses of lactoferrin. However, the incubation conditions were different. We incubated at higher temperature (37° vs. room temperature), for a longer period of time (1 hr vs. 40 min), and in the presence of 10 μg/mL BSA to prevent adsorptive loss of tryptase, a phenomenon that Hallgren *et al*. reported observing.

While not an inhibitor of tryptase, lactoferrin could nonetheless be effective in reducing tryptase activity in conditions of low heparin concentration. However, as its affinity for heparin is only 1/160 that of tryptase, then at limiting concentrations of heparin, it would need to be in 80-fold molar excess over the tryptase monomer (20-fold over the tryptase tetramer) to displace half of it from the heparin molecule. In the presence of excess heparin, even greater amounts of lactoferrin would be required. It seems unlikely that lactoferrin could accumulate within mast cells in the massive quantities necessary to alter tryptase activity in the granules. Nonetheless, lactoferrin could play an important role in controlling the activity of tryptase following its secretion from mast cells.

Elrod *et al*. [13] have examined the effect of lactoferrin on a number of proteases, including trypsin, thrombin and plasmin, and have shown that the inhibitory actions of lactoferrin are selective for tryptase. We have extended these selectivity studies to include human chymase and cathepsin G. Lactoferrin had little effect on the enzymatic activity of human chymase. In fact, it appeared to stabilise the activity by acting, in part, like BSA as a non-specific carrier for this highly charged protease. Pejler [37] has reported that lactoferrin can elicit dramatic decreases in the activity of rat chymase I-heparin complexes apparently by binding to heparin and displacing the protease. However, the effects of heparin on the enzymatic activity of human chymase are rather more modest than for the rat counterpart, inducing an apparent enhancement of 33% in activity towards the substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-NA at pH 7.5 and actually depressing activity by 40% at pH 5.5 [22]. There would seem to be little evidence to link the mast cell stabilising properties of lactoferrin with its actions on chymase. The striking decline in cathepsin G activity in the presence of lactoferrin was unexpected. Some of the inhibitors of chymotryptic enzymes found

previously to stabilise human mast cells have inhibitory actions towards cathepsin G [20], and it is thus possible that cathepsin G can participate in mast cell degranulation and that this is blocked by lactoferrin [20]. In this respect, it is of interest that cathepsin G is present in most skin mast cells, but only in a minority of mast cells in lung tissue [38].

The recruitment and activation of neutrophils is a frequent consequence of mast cell activation in allergic disease [39]. Both tryptase and chymase proved potent stimuli for the recruitment of this cell type in animal models [11,12]. Concentrations of lactoferrin as high as 20 μg/mL (260 nM) have been detected in skin blister fluid and as high as 15 μg/mL (200 nM) in nasal lavage fluid following allergen provocation [40,41]. In allergic disease mast cells are thus likely to be exposed to lactoferrin at concentrations sufficient to inhibit degranulation.

The release of lactoferrin from activated neutrophils could provide a negative feedback mechanism in allergic disease, both reducing the extent of mast cell degranulation at sites of inflammation, and counteracting the actions of mast cell proteases. Cathepsin G is also a major product of the azurophil granules of neutrophils, and the release of lactoferrin from the secondary granules could serve to regulate the actions of cathepsin G from this cellular source also. Mast cells may have a role in normal clearance of lactoferrin, though it remains to be determined. They may also become 'armed' with this neutrophil protein and secrete it subsequently. The impressive anti-allergic actions of lactoferrin in a sheep model of allergic inflammation [13], and the mast cell stabilising actions and inhibitory effects on protease activity found in the present studies must call attention to the therapeutic potential of recombinant forms of lactoferrin in allergic conditions.

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