HOST RESPONSE TO INFECTION

Elucidation of the antistaphylococcal action of lactoferrin and lysozyme

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The cationic tear proteins lactoferrin and lysozyme exhibit co-operative antistaphylococcal properties. The purpose of this study was to determine the mechanism of action of this co-operation on *Staphylococcus epidermidis*. Following blocking of lipoteichoic acid (LTA) binding sites, the effects on binding of lactoferrin and susceptibility to lactoferrin and lysozyme were determined. The effect of lactoferrin on autolysis and LTA release was also examined. Maximal susceptibility occurred on addition of lactoferrin first followed by lysozyme. Blocking the LTA binding sites both reduced lactoferrin binding and decreased susceptibility. Autolytic activity decreased and LTA release increased in the presence of lactoferrin. These results suggest that binding of lactoferrin to LTA is important in its synergy with lysozyme and interferes with the autolysins present on the LTA. It is proposed that, on binding to the anionic LTA of *S. epidermidis*, the cationic protein lactoferrin decreases the negative charge, allowing greater accessibility of lysozyme to the underlying peptidoglycan.

Introduction

Individually, the cationic tear proteins lactoferrin and lysozyme display very different modes of antibacterial action. Lysozyme hydrolyses the β -glycosidic bonds of the repeating N-acetylmuramic acid and N-acetylglucosamine units that provide the rigidity of bacterial cell walls [1]. Most staphylococci are resistant to lysozyme [2]. A possible explanation for the lysozyme resistance of some gram-positive bacteria is the presence of anionic teichoic acids in the cell wall [3]. Lactoferrin, in its iron-limited form, displays an antimicrobial mechanism against a wide array of bacteria, including *Staphylococcus epidermidis* [4], which is unrelated to its iron-chelating ability [5]. The mode of action of lactoferrin on gram-positive bacteria is unknown. For gram-negative bacteria, lactoferrin causes the release of lipopolysaccharide (LPS) [6] leading to an increase in outer-membrane permeability [7].

In addition to their individual antibacterial mechanisms, lactoferrin and lysozyme exhibit co-operative antistaphylococcal properties for a range of *S. epidermidis* isolates which may contribute to the inability of this species to colonise contact lenses *in vivo* [8]. These

proteins also act synergically against gram-negative bacteria, possibly due to permeabilisation of the outer membrane by lactoferrin allowing entry of lysozyme to the peptidoglycan [7]. Lactoferrin also enhances the action of various antimicrobial agents against gram-negative bacteria [6, 9] and *S. epidermidis* [10].

The purpose of this study was to analyse antistaphylococcal properties displayed by lactoferrin and lysozyme.

Materials and methods

Sequential addition of lactoferrin and lysozyme

The *S. epidermidis* isolate SE5 was used in this and all subsequent assays. Bacteria and artificial tear fluid containing serum (ATFS) were prepared as described previously [8]. Bacteria (1 in 10 dilution) were added to ATFS containing lactoferrin or hololactoferrin (1.8 mg/ml) and incubated for 6 h at 37°C. Following addition of lysozyme 5.4 mg/ml, the cultures were re-incubated for a further 6 h. Similarly, ATFS containing lysozyme was incubated for 6 h, followed by addition of lactoferrin. Samples were taken at the beginning of the assay, after the addition of the second protein and at 7, 8, 10 and 12 h from commencement of the assay. Viable counts were determined as described previously [8].

Viability following pre-incubation with anti-lipoteichoic acid

Anti-lipoteichoic acid was kindly supplied by Dr D. Harty of the Sydney Dental Hospital. Prepared bacteria were incubated with anti-lipoteichoic acid (2%) raised in rabbits or rabbit serum (2%) for 1 h at 37°C and added (1 in 10 dilution) to ATFS containing lactoferrin 1.8 mg/ml. After incubation for a further 6 h, lysozyme 5.4 mg/ml was added. Viable counts were performed as above on samples taken after 6 h and 12 h and compared with those determined in ATFS initially supplemented with lactoferrin only.

Lactoferrin binding following pre-incubation with anti-lipoteichoic acid

The ELISA developed previously [11] for the detection of lactoferrin binding to *S. epidermidis* isolates was modified. Plates containing adsorbed SE5 cells were incubated with either anti-lipoteichoic acid, rabbit serum or buffer (2, 0.2 or 0.02% v/v) followed by the addition of iron-limited lactoferrin. The relative absorption of lactoferrin was determined as described previously. As controls, the binding of anti-lipoteichoic acid and serum (two-fold serial dilutions from 2.5% to 0.02% v/v) to SE5 were also assayed as above, but without the addition of lactoferrin.

Effect of lactoferrin on LTA release

Biofilm-released cells were prepared as described previously [10] and pooled. Viable counts, determined as described previously, gave *c.* 1×10^8 cfu/ml. These cells were then diluted (1 in 10) in TSB only or TSB containing lactoferrin 1 mg/ml and incubated for 4 h at 37°C. Controls containing no bacteria were treated similarly. Cells were centrifuged at 2000 *g* at 4°C for 10 min and the supernate was filter-sterilised (0.22- μ m pore size; Millipore). An ELISA was performed as above with anti-lipoteichoic acid (A) and the control rabbit serum (B) to detect the presence of lipoteichoic acid. The amount of lipoteichoic acid present was calculated by the following equation: $((A - B)/A) \times 100$. Similarly, interference was detected by the controls and subtracted from the above calculation to give the percentage release of lipoteichoic acid.

Effect of lactoferrin on autolytic activity

Supernate from the above assay was used to detect autolysin activity as described previously [12]. Briefly, ammonium sulphate was added to 75% saturation and mixed for 1 h at 4°C, then centrifuged at 12 000 *g* for 30 min at 4°C. The supernate was discarded and the pellet was resuspended in 0.1 M sodium acetate buffer (pH 6) and dialysed against four changes of this buffer. Samples (100 μ l) in duplicate were added to 96-well plates and cell walls of *Micrococcus lysodeikticus* (Sigma), diluted to a concentration of 1 mg/ml in

buffer, were added (100 μ l) to the wells. Following immediate determination of the OD₅₇₀, the plate was incubated at ambient temperature and the OD₅₇₀ was measured at 0.5-h intervals for 2.5 h.

Statistical analyses

Statistical analyses were performed by the Student's *t*-test for comparing growth in the presence of anti-lipoteichoic acid and MANOVA for all other analyses.

Results

Viability following sequential addition of lactoferrin and lysozyme

The results are shown in Fig. 1. Lactoferrin or lysozyme added after 6 h to cultures incubated in ATFS containing lysozyme or hololactoferrin, respectively, induced growth. This growth was significant ($p < 0.001$) compared with the loss in viability of the cultures initially grown in iron-limited lactoferrin with lysozyme added after 6 h.

Viability in ATFS following pre-incubation in anti-lipoteichoic acid

Growth of SE5 cells pre-treated with anti-lipoteichoic acid (0.49 net log₁₀ cfu/ml) was significantly ($p < 0.05$) greater than the minimal growth (0.16) of serum pre-treated cells (Fig. 2). Both these conditions induced greater viability of cells than the reduced net viability (−0.66) of untreated cells ($p < 0.0001$).

Lactoferrin binding following pre-incubation in anti-lipoteichoic acid

SE5 cells bound iron-limited lactoferrin with a maximum relative adsorption of 12.5 μ g/ml (Fig. 3). This bacterial strain also bound anti-lipoteichoic acid and,

Fig. 1. Growth of SE5 cells in ATFS + LZ with lactoferrin added after 6 h (■), in ATFS + holoLF with lysozyme added after 6 h (○) and ATFS + LF with lysozyme added after 6 h (◆). Results represent the mean of six experiments (bar, SD).

Fig. 2. Growth of SE5 cells, pre-treated with anti-lipoteichoic acid (■) serum (□) or with no pre-treatment (■) in ATFS + LF for 6 h. Lysozyme was added and viability monitored for a further 6 h. Results represent the mean of six experiments (bar, SD), expressed as the net growth (log₁₀ cfu/ml) following addition of lysozyme.

Fig. 3. Adsorption of lactoferrin to SE5 cells after no pre-treatment (+) and pre-treatment with anti-lipoteichoic acid (2% ■, 0.2% ●, 0.02% ◆) or rabbit serum (2% □, 0.2% ○, 0.02% ◇). Results represent the mean of four experiments expressed in relative units; SD < 42.2% of the mean.

with a reduced ($p < 0.01$) relative adsorption, rabbit serum. After pre-treatment with various dilutions of anti-lipoteichoic acid or serum, there was a significant ($p < 0.01$) reduction only in the lactoferrin-binding of cells pre-treated with anti-lipoteichoic acid 2%. No other statistically significant data were obtained. There was a general trend of increased lactoferrin binding with further dilution of anti-lipoteichoic acid, with the 0.02% concentration resulting in similar amounts of lactoferrin binding to those conditions with neither anti-lipoteichoic acid nor serum. Similarly, there was a general trend of reduced lactoferrin binding in the presence of serum 2%, but with 0.2 and 0.02% similar amounts of lactoferrin were bound as in those conditions with neither anti-lipoteichoic acid nor serum. This correlates with the results shown in Fig. 4 of anti-lipoteichoic acid and serum binding of SE5 cells in which anti-lipoteichoic acid was bound *c.* 10-fold more than serum.

Fig. 4. Adsorption of anti-lipoteichoic acid (■) and serum (○) to SE5 cells. Results represent the mean of four experiments (bar, SD) expressed in relative units.

Effect of lactoferrin on lipoteichoic acid release

Lipoteichoic acid release was detected in both TSB and TSB containing lactoferrin (Table 1). The presence of lactoferrin led to an increased release (19.82) compared with the absence of lactoferrin (7.96), although this difference was not statistically significant.

Effect of lactoferrin on autolytic activity

The autolytic activity of *S. epidermidis* was measured by incubating cell-free supernates with suspensions of *M. lysodeikticus*. As shown in Fig. 5, the autolytic

Table 1. The release of lipoteichoic acid from the surface of SE5 cells incubated in TSB or TSB containing lactoferrin (TSB + LF)

Media	Mean (SD) LTA release
TSB	7.96 (5.48)
TSB + LF	19.82 (9.54)

*Results represent the mean of four experiments expressed as the amount of lipoteichoic acid released in relative units.

Fig. 5. Autolysis of *M. lysodeikticus* by the supernate of SE5 cells grown in TSB (■) or TSB containing lactoferrin (○). Results represent the mean of four experiments (bar, SD) expressed as the percentage autolysis of *M. lysodeikticus*, as measured spectrophotometrically at 570 nm.

activity of growing *S. epidermidis* cells was reduced in the presence of lactoferrin.

Discussion

Lactoferrin and lysozyme exert co-operative antimicrobial properties *in vitro* against *S. epidermidis* which are not apparent for these proteins individually [8]. In the present study, the mechanism of action of this co-operation was investigated. As lactoferrin increases the penetration of lysozyme into gram-negative bacteria [7], the effect of sequential supplementation of ATFS with lactoferrin and lysozyme on viability was assessed. Lactoferrin added to ATFS 6 h before lysozyme resulted in a bactericidal effect. Iron-saturated lactoferrin inhibited this antimicrobial action, as did the addition of lysozyme before lactoferrin. This suggests that lactoferrin increases the penetration of lysozyme in a similar fashion to that in gram-negative bacteria.

Sensitivity of *S. aureus* to lysozyme can be induced by the removal of lipoteichoic acid and it has been suggested that teichoic acid prevents the interaction between lysozyme and its peptidoglycan substrate [13, 14]. Therefore, the present study investigated the interaction between lactoferrin and lipoteichoic acid. Occlusion of lipoteichoic acid binding sites with anti-lipoteichoic acid abolished the antimicrobial effect of lactoferrin and lysozyme, suggesting that the interaction of lactoferrin with lipoteichoic acid is important. The antibacterial synergy of lactoferrin and lysozyme against gram-negative bacteria probably occurs because of the disruption of outer-membrane permeability by lactoferrin [7]. Lactoferrin may have a similar mode of action on the lipoteichoic acid of gram-positive bacteria. Lactoferricin, a peptide generated from digestion of lactoferrin by pepsin, which probably represents the structural domain of lactoferrin responsible for its bactericidal properties [15], increases lysozyme-induced killing of *S. epidermidis* [16]. Optimal lactoferricin binding on to the cell surface of *Bacillus subtilis* occurs at 10^6 molecules/bacteria, suggesting that it binds to highly repeated surface components such as teichoic acid [16].

Direct binding of cell-surface components is integral to the antimicrobial action of lactoferricin on *B. subtilis* [15] and of lactoferrin with lysozyme synergy on gram-negative bacteria [7]. Therefore, the effect of occlusion of lipoteichoic acid binding sites by anti-lipoteichoic acid on the adsorption of lactoferrin on to the surface of SE5 cells was investigated. Lactoferrin adsorption was reduced in a dose-dependent manner by anti-lipoteichoic acid. Lactoferrin binding to the LPS of gram-negative bacteria probably occurs through the direct interaction of the cationic surface subunit of lactoferrin with the anionic core of the LPS [7] and the physical presence of lactoferrin is thought to adversely affect

normal membrane physiology, allowing greater penetration of lysozyme [7]. Other cationic proteins such as cathepsin G from neutrophils [17] and the lantibiotics Pep 5 and nisin bind to staphylococcal lipoteichoic acid as part of their antimicrobial mechanism [18].

The hydrophilic chain of *S. epidermidis* lipoteichoic acid is substituted with glucose and D-alanine [19]. Increasing the alanine ester substitution of lipoteichoic acid decreases the net negative charge and, as cationic autolysins bind to these negatively charged molecules, autolysin inhibition occurs concomitantly [20]. It is proposed that cationic autolysins *in vivo* are fixed to the negatively charged lipoteichoic acid *via* electrostatic interaction [21] similar to the electrostatic interactions which occur between cationic proteins and lipoteichoic acid [22]. In the present study, the autolytic activity of growing *S. epidermidis* cells was reduced in the presence of lactoferrin compared with that in its absence. This is contrary to previous findings that cationic polypeptides and the cationic peptide lantibiotics Pep5 and nisin displace and activate staphylococcal autolysins by association with lipoteichoic acid [18]. The reduction in autolysis induced by lactoferrin in the present study might occur by adsorption to lipoteichoic or teichoic acid sites such as the negatively charged phosphodiester groups and sterical hindrance of the release of autolysins.

Lactoferrin increased the amount of lipoteichoic acid released from the surface of SE5 cells compared with that released in media lacking lactoferrin. Leucocyte cationic proteins, heat treated and therefore acting as cationic proteins, also cause the release of lipoteichoic acid [23] and lactoferrin causes the release of LPS from the surface of gram-negative bacteria [7]. Lactoferrin-induced lipoteichoic acid release may expose more peptidoglycan residues to lysozyme, allowing lysis to occur.

In conclusion, this study has shown that lactoferrin increases the penetration of lysozyme into the cell matrix of *S. epidermidis* by binding to lipoteichoic acid. Lactoferrin also reduced autolysis and caused an increase in the release of lipoteichoic acid from the cell surface. It is proposed that lactoferrin and lysozyme exert a combined antimicrobial effect against *S. epidermidis* by the binding of lactoferrin to lipoteichoic acid, neutralising the negative charge of this structure and hence allowing greater access of lysozyme to the peptidoglycan.

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