Identification of a human lactoferrin-binding protein in Staphylococcus aureus

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Summary. Human lactoferrin (HLf) is an iron-binding protein with antimicrobial activity that is present in high concentrations in milk and various exocrine secretions. HLF is also an acute-phase protein secreted by polymorphonuclear leucocytes, and its binding to a large number of clinical isolates of Staphylococcus aureus has been described recently from our laboratory. We have now characterised the HLF-staphylococcal interaction in S. aureus strain MAS-89. The binding of 125I-HLF to strain MAS-89 reached saturation in <90 min and was maximal between pH 4 and 9. Unlabelled HLF displaced 125I-HLF binding. Various plasma and subepithelial matrix proteins, such as IgG, fibrinogen, fibronectin, collagen and laminin, which are known to interact specifically with S. aureus, did not interfere with HLF binding. A Scatchard plot was non-linear; this implied a low affinity (1.55 x 10^7 L/mol) and a high affinity (2.70 x 10^8 L/mol) binding mechanism. We estimated that there were c. 5700 HLF binding sites/cell. The staphylococcal HLF-binding protein (HLF-BP) was partially susceptible to proteolytic enzymes or periodate treatment and was resistant to glycosidases. An active HLF-BP with an apparent M, of c. 450 Kda was isolated from strain MAS-89 cell lysate by ion-exchange chromatography on Q-sepharose. In SDS-PAGE, the reduced HLF-BP was resolved into two components of 67 and 62 Kda. The two components demonstrated a positive reaction with HLF-HRPO in a Western blot. These data establish that there is a specific receptor for HLF in S. aureus.

Introduction

Human lactoferrin (HLF) is an iron-binding protein with anti-microbial activity that is present in milk, leucocytes and various exocrine secretions.1,2 Human plasma contains c. 1 μg of HLF/ml, with a turnover rate of 5-7% per day, processed mainly by the liver.3 HLF is a glycoprotein of c. 80 Kda and is composed of a single polypeptide chain with two heterogeneous glycans attached through N-glycosidic linkages.4,5 HLF has a capacity to bind reversibly two Fe^{3+} ions with high affinity (K_a = 10^{38} L/mol), in co-operation with two HCO_3^- ions.5,7

The involvement of HLF in various physiological pathways has been suggested.8 It regulates myelo-poiesis and thereby antibody production by causing feed-back inhibition of granulocyte-monocyte colony stimulating factor.9,10 HLF also stimulates the cidal properties of phagocytes and participates in H_2O_2-dependent and -independent bacterial killing.11,12 The antimicrobial property of HLF has been attributed mainly to its iron-scavenging capacity. A bacteriostatic, a bactericidal, and an opsonic effect for HLF has been suggested.12-14 The antimicrobial activity increases when HLF adsorbs to bacterial surfaces.15 Moreover, interaction of HLF with gram-negative bacteria has been shown to cause permeability alterations in the outer membrane and release of lipopolysaccharide.16

Other studies have revealed a specific receptor-mediated interaction of HLF with Neisseria meningitidis, N. gonorrhoeae, Haemophilus influenzae, Treponema pallidum and Trichomonas vaginalis.17-21 These studies have suggested that HLF receptors may play a role in bacterial iron acquisition. Thus, the significance of HLF-bacterial interaction has been attributed to both host defence mechanisms and bacterial virulence.

Recently, we have demonstrated the binding of HLF to various clinical isolates of S. aureus.22 Furthermore, we have also reported the interaction of bovine Lf (BLf) with S. aureus and coagulase-negative staphylococci associated with bovine mastitis.23,24 We have now examined the presence of a specific HLF-binding protein (HLF-BP) in S. aureus and have characterised the HLF-staphylococcal interaction in S. aureus strain MAS-89.
Materials and methods

Chemicals

HLf (lot 63541) purified from milk was purchased from US Biochemicals Corp., Cleveland, OH, USA. BLf purified from whey was kindly provided by Dr H. Burling, Research Division, Swedish Dairies Association, Lund, Sweden. Both Lf preparations were homogeneous when tested in ion-exchange (Mono-Q column, Pharmacia AB, Uppsala, Sweden) and in molecular-sieve (TSK G4000 SW, LKBProdukter AB, Bromma, Sweden) high-performance liquid chromatography. Fibronectin was purified from human plasma by the method of Veunoto and Vaheri,25 and was provided by Dr J. Erdei. Laminin (from basement membrane of the Engelbreth-Holm-Swarm transplanted tumour) was purchased from Collaborative Research, Inc., Bedford, MA, USA. Vitrogen-100TM (containing type I 95% and type III 5% collagens) was purchased from Collagen Corporation Inc., Palo Alto, CA, USA. Human immunoglobulin-G was obtained from KabiVitrum, Stockholm, Sweden.

The following substances were obtained from Sigma: Chemicals—phenylmethylsulphonyl fluoride, sodium-p-periodate, diaminobenzidine; Proteins—fibrinogen (from human plasma), transferrin (from human serum), fettuin (from fetal calf serum), trypsin inhibitor (from soy bean) and human serum albumin; Sugars—glucose, mannose, fucose, sorbitol, ribose, siac acid type-VIII (from sheep submaxillary glands), N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine; Enzymes—trypsin (type-XIIS), pepsin (from porcine stomach mucosa), proteasine K (type-XIS, from Tritirachium album), β-galactosidase (from almonds), β-galacosidase (from Escherichia coli), α-1 fucosidase, neuraminidase, deoxyribonuclease II (from bovine spleen), ribonuclease A (from bovine pancreas), peroxidase (from horse-radish) and lysozyme. All chemicals used in the preparation of buffer solutions were of analytical grade.

Bacteria

In a previous study,22 we examined 489 S. aureus isolates (from various clinical conditions in man) and found 417 (85-3%) strains that bound 125I-HLf. From this collection, a high-HLf-binding S. aureus strain, MAS-89, was selected for characterisation in the present study. Strain MAS-89 was associated with toxic shock syndrome, and has been extensively characterised for various biochemical properties in earlier studies.26-28 For binding experiments, bacteria were grown on blood agar at 37°C for 24 h. Cells were harvested, washed and resuspended in phosphate-buffered saline (PBS), pH 7-2, to a density of 1010 bacteria/ml (standardised spectrophotometrically at 540 nm).

125I-HLf binding assay

HLf was labelled by a modified chloramine-T method with Na125I (specific activity 629 GBq/mg; DuPont Scandinavia AB, Stockholm, Sweden) by means of Iodobeads (Pierce Chemicals Co., Rockford, IL, USA). The 125I-HLf preparation was homogeneous in autoradiography after SDS-PAGE. The binding assay was performed as described previously.22-24 Briefly, 109 bacteria (in 0.1 ml of PBS) were mixed with 0.1 ml of 125I-HLf solution [c. 8 ng, corresponding to a radioactivity of (2-5-3.0) × 104 cpm] and incubated for 1 h at room temperature. After adding 2 ml of ice-cold PBS (containing Tween 20 0.1%), tubes were centrifuged at 9500 g for 15 min and the supernate was aspirated. Radio-activity bound to the bacterial pellet was measured in a γ-counter (LKB Wallac Clinigamma 1272, Turku, Finland). Background radio-activity from incubation mixtures without bacteria was 2.5%, and the non-specific 125I-HLf binding to S. aureus (in the presence of 100-fold excess of unlabelled HLf) was < 7.5%. Therefore, a binding value of < 10% was considered to be a negative result. Samples were tested in triplicate and each experiment was repeated at least twice unless otherwise stated.

Enzymatic treatment of bacteria

A suspension of S. aureus strain MAS-89 (109 bacteria/0.1 ml) harvested from blood agar was treated with proteases or glycosidases (final volume, 0.2 ml). Tryptsin (25 000 units/ml) digestion was performed in 0.15 M PBS, pH 7.4, and the reaction was blocked with soybean trypsin inhibitor. Pepsin (10 000 units/ml) treatment was performed in 100 mM sodium acetate buffer, pH 4-5, and was stopped by increasing the pH of the reaction mixture to 7-4. Proteinase K (100 units/ml) digestion was performed in 40 mM potassium phosphate buffer, pH 7-5, and the reaction was inhibited by adding 0.5 mM phenylmethylsulphonyl-fluoride. Glycosidase treatment with neuraminidase (10 units/ml), β-galactosidase (100 units/ml) of α-1 fucosidase (0.5 units/ml) was performed in 30 mM Tris-HCl buffer, pH 7-3. After enzymatic treatment for 1 h, the bacteria were thoroughly washed and resuspended in PBS. Untreated cells suspended in appropriate buffers were tested as controls.

Heat or periodate treatment of bacteria

A 1-ml volume of bacterial suspension (1010 cells/ml) was heated at 80°C or 100°C for 1 h, with gentle shaking in a water bath. For periodate treatment, 1 ml of bacterial suspension (1011 cells) was mixed with 5 mg of sodium periodate. The mixture was protected from light and kept at 4°C with gentle agitation. After incubation for 24 h, the mixture was centrifuged at 500 rpm for 5 min (sodium periodate crystals were sedimented) and the bacteria from the supernate were carefully collected. Any excess of sodium periodate was removed by dialysis with PBS at 4°C for 24 h. The
density of the sodium periodate-free cell suspension was adjusted to $10^{10}$ cells/ml.

**Isolation of staphylococcal HLF-binding protein (HLF-BP)**

*S. aureus* strain MAS-89 cell surface components were extracted by the technique of Rydén *et al.* Briefly, 20 g (wet weight) of bacteria were suspended in 200 ml of 50 mM Tris-HCl, pH 7.5 (Tris-buffer), containing 0.145 M NaCl, 2 mg of lysozyme, 2 mg of DNAase and 2 mg of RNAase. After incubation with constant shaking (200 rpm) at 37°C for 2 h, the reaction mixture was centrifuged (18 000 g for 30 min) and the supernate (now termed "lysate") was collected. The presence of functionally active HLF-BP in the lysate was detected in an $^{125}$I-HLF binding inhibition assay with strain MAS-89 whole cells. Lysate (50 ml) was applied to a column (25 x 2.5 cm) of Q-Sepharose (Pharmacia) equilibrated with Tris-buffer. After a thorough wash with Tris-buffer, the column was eluted with a 1250-ml salt gradient (0–1–0 M NaCl in Tris-buffer) at a flow rate of 48 ml/h and fractions (10 ml) were collected. All buffers and solutions used in the chromatography contained 1 mM phenylmethylsulphonyl fluoride. Duplicate samples (0.2 ml) from each fraction were incubated overnight at 4°C, in a 96-well microtitration plate (Nunc, Roskilde, Denmark). Free sites in the well were blocked with ovalbumin 2%. Horse-radish peroxidase (HLF-HRPO) was prepared according to the method of Nakamura *et al.* and used as a probe for detecting the HLF-BP activity. Enzyme-linked ligand-binding assay was performed essentially as described for a standard ELISA. $^{125}$I-O-phenylenediamine (Sigma) prepared in 34 mM citrate buffer, pH 5.4, containing H$_2$O$_2$ 0.55% was used as the enzyme substrate. Enzyme hydrolysis was terminated with 4 N sulphuric acid (25 ml) and the colour development was measured at 450 nm in a Titertek Multiscan. Fraction(s) that gave a positive reaction were tested for blocking of $^{125}$I-HLF binding to strain MAS-89 whole cells. The active fraction was further analysed for homogeneity, on a Mono-Q (Pharmacia) column by high-performance liquid chromatography with 200-μ1 samples); the equilibration buffer was 0.2 M Tris-Bis, pH 2; the elution-equilibration buffer had a gradient of 0-0.1-4.0 M NaCl.

**Gel electrophoresis**

The molecular weight determination of the isolated HLF-BP was determined in SDS-PAGE according to the method of Studies with two electrophoretic conditions. (i) The running gel was prepared with acrylamide 5%; non-reduced samples were applied to lanes; gels were developed with silver stain after electrophoresis; and cross-linked alkaline phosphatase (Sigma) was a mol.-wt standard. (ii) The running gel consisted of a linear 5–15% acrylamide gradient; samples or a mixture of reference proteins (4 μg of each protein; Pharmacia) were applied to each lane after boiling in the presence of SDS containing mercaptoethanol; after electrophoresis, proteins were transferred to Immobilon membrane (Millipore Co., Bedford, MA, USA) by electrophoretic transfer, the free hydrophobic sites on the membrane were blocked with Tween-20 2% for 30 min and probed with HLF-HRPO (diluted 1 in 200) for detecting the binding substance, in a Western blot; and mol.-wt standards in the gel were stained with Coomassie Brilliant Blue R.

**Results**

The binding of HLF to *S. aureus* strain MAS-89 was maximal between pH 4.0 and 9.0 (c. 9.8 fmol bound). Treatment with unlabelled HLF elicited a dose-dependent displacement of $^{125}$I-labelled HLF binding and required c. 0.8 μg of unlabelled ligand for 50% inhibition (fig. 1). BLF blocked HLF binding, but other iron-binding proteins (transferrin and haemin) did not interfere in binding. Proteins that bind to distinct receptors on *S. aureus* such as immunoglobulin G, fibrinogen, fibronectin, collagen and laminin did not inhibit $^{125}$I-HLF binding (table). HLF binding reached 50% and 100% saturation within 10 and 90 min respectively (fig. 2, inset). Scatchard plot analysis was non-linear and implied that there was a low ($1.55 \times 10^7$ L/mmol) and a high affinity ($2.70 \times 10^9$ L/mmol) binding mechanism. The average number of HLF-binding sites on strain MAS-89 was estimated to be 5700/cell (fig. 2).

**Nature of HLF-bacteria interaction**

The HLF binding to strain MAS-89 was abolished by heat treatment—partially (<39%) at 80°C and
Table. Inhibitory effect of various unlabelled proteins on $^{125}$I-HLf-binding to S. aureus strain MAS-89

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mean (SD) percentage inhibition (mean ± SD) at a concentration of</th>
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<tbody>
<tr>
<td></td>
<td>10 µg/ml</td>
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<tr>
<td>HLF</td>
<td>77.3 (0.7)</td>
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<tr>
<td>BLF</td>
<td>84.5 (1.1)</td>
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<tr>
<td>Transferrin (human)</td>
<td>1.8 (1.8)</td>
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<tr>
<td>Fibronectin (human)</td>
<td>-5.4 (1.6)</td>
</tr>
<tr>
<td>Fibrinogen (human)</td>
<td>-5.2 (4.1)</td>
</tr>
<tr>
<td>Immunoglobulin-G (human)</td>
<td>-1.3 (2.0)</td>
</tr>
<tr>
<td>Laminin (murine)</td>
<td>4.1 (4.0)</td>
</tr>
<tr>
<td>Collagen type-I (bovine)</td>
<td>5.0 (2.5)</td>
</tr>
<tr>
<td>Fetuin</td>
<td>3.7 (0.8)</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>4.4 (0.4)</td>
</tr>
<tr>
<td></td>
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<tr>
<td>HLF</td>
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<tr>
<td>BLF</td>
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</tr>
<tr>
<td>Fibrinogen (human)</td>
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<tr>
<td>Immunoglobulin-G (human)</td>
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<td>Laminin (murine)</td>
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<tr>
<td>Collagen type-I (bovine)</td>
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<tr>
<td>Fetuin</td>
<td>4.6 (0.5)</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>5.1 (3.6)</td>
</tr>
</tbody>
</table>

completely at 100°C after 1 h. Treatment with proteinase K or trypsin reduced HLF binding by 50% and 45%, respectively. A high concentration of pepsin reduced the binding by 25%, but at a lower concentration, the HLF binding capacity was increased, due perhaps to the exposure of cryptic binding sites. Digestion with neuraminidase, α-1 fucosidase, β-glucosidase or β-D-galactosidase did not change the HLF-binding ability. Oxidation of carbohydrate moieties on the staphylococcal cell surface by sodium periodate treatment did not change the HLF binding.

Isolation of staphylococcal HLF-BP

Strain MAS-89 cell lysate prepared by lysostaphin digestion contained c. 40 mg of protein/ml. The possible interaction of HLF (pI c. 9.0) with DNA or RNA was eliminated by hydrolysis with nuclease during extraction. A 27-1 µl volume of lysate corresponding to c. 1·1 mg of protein inhibited $^{125}$I-HLF binding to strain MAS-89 whole cells by 50%, indicating the presence of free and functional HLF-BP in the preparation (fig. 3). Lysate was fractionated by Q-Sepharose (fast flow) ion-exchange chromatography, and was eluted with a salt gradient (0–1·0 M NaCl). The presence of HLF-BP in the fractions was detected by two methods: (i) by probing with HLF-HRPO conjugate in an enzyme-linked ligand-binding assay, and (ii) by testing the blocking capacity in a $^{125}$I-HLF binding-inhibition assay. A peak eluted at 0·7 M salt concentration showed HLF-binding and HLF-binding-inhibition activity (fig. 4a). Corresponding fractions were pooled and dialysed against distilled water. The isolated HLF-BP was found to be homogeneous in high-performance liquid chromatography with a Mono-Q column (fig. 4b). HLF-BP migrated as a single band of ~450 KDa in SDS-PAGE under non-reducing conditions (fig. 5A). The 450-KDa material was recovered from the gel by electro-dialysis and the binding-blocking activity was reconfirmed. However,
after reduction with β-mercaptoethanol, the HLF-BP resolved into two components of c. 62 and c. 67 KDa that reacted with HLF-HRP-O; in the Western-blot analysis a similar pattern was obtained with the cell lysate (fig. 5B). Treatment with detergents (SDS, Triton, X-100, N-octyl-β-D-glycopyranoside, Tween-20, Noviodet, Lubrol PX—all at 0.15% or 0.25% concentration) or with 6 M urea did not dissociate the 450-KDa substance. However, treatment with Tween-80 0.5% at 50°C for 2 h demonstrated moderate dissociation.

Discussion

Most staphylococcal infections originate at the skin or mucosal surface and one of the early host responses is generally acute inflammation. Various acute-phase proteins, including HLF, are secreted in large amounts by stimulated polymorphonuclear leucocytes during such an inflammatory process. HLF with iron-binding capacity may evoke physiological hyposideraemia in the inflammatory milieu, and elicit antimicrobial effects towards the invading pathogen.

The antimicrobial action could be enhanced when HLF adsorbs to the bacterial surface. We have demonstrated recently the binding of HLF to various clinical isolates of S. aureus. Now we have examined further the nature of HLF-staphylococcal interaction and identified an HLF-BP in a previously well characterised clinical isolate of S. aureus, strain MAS-89.

The HLF binding to strain MAS-89 could be saturated and was time-dependent, indicating that there was a limited number of binding sites on the cell surface. The 125I-HLF binding could be displaced by unlabelled protein, which showed that the interaction mechanism was reversible and indicated that both labelled and native ligands were recognised by the bacteria. The Scatchard-plot was non-linear and implied the presence of a low- and high-affinity HLF-binding mechanism. The binding affinity constants (Ks values) were within the range of affinity constants estimated for other staphylococcal receptors or for most antigen-antibody systems.

Our in-vitro results indicated that the interaction of HLF with staphylococci occurs over a wide pH range...
that includes the iso-electric point of HLF. Furthermore, 2 M NaCl did not dissociate the HLF-staphylococcal interaction (data not shown). Thus, the HLF binding did not seem to be electrostatic in nature and due to the basic charge of the protein. The HLF binding capacity at acidic pH may be of pathophysiological relevance in vivo, because the inflammatory milieu is acidic and is heavily infiltrated with Lf-secreting PMNLs.42

Unlabelled BLF displaced the 125I-HLF binding more effectively than the homologous ligand. Previously we demonstrated 67- and 92-Kda BLF receptors in a S. aureus strain associated with bovine mastitis.43 Although HLF and BLF are both recognised, the interaction of BLF with staphylococci seems to be of a higher affinity. Amongst the veterinary clinical isolates of S. aureus and coagulase-negative staphylococci, the receptor density per cell was 2000–6000, with affinity constants in the range (0.96–1.1) × 106 L/mmol.23,44

Specific receptors for IgG, fibrinogen, fibronectin, collagen and laminin have been identified on S. aureus.43–47 These glycoproteins, or the iron-binding proteins transferrin and haemin, did not inhibit the binding, indicating that the HLF recognition process is distinct from previously described protein-staphylococcal interactions. The staphylococcal HLF-BP was partially destroyed by heat and of limited susceptibility to proteolytic digestion, suggesting partial involvement of a protein residue. Staphylococcal cell-surface receptors involved in binding of the plasma and subepithelial matrix components are also proteins. The N-terminal sequence and the total amino-acid composition of HLF-BP protein moiety was distinct from the previously known staphylococcal receptors (unpublished observations). Chromatographic and electrophoretic data suggested a tightly aggregated or a complexed HLF-BP, with a high M, of ~450 Kda. When reduced, HLF-BP resolved into 62- and 67-Kda components, suggesting the possibility of disulphide linkages in the structure. By analogy, the native staphylococcal fibronectin-binding receptor is a protein of ~200 Kda,48 with no disulphide bonds.49 However, the cloned receptor gene appears to code for a 100-Kda protein50 suggesting that it produces a dimer containing two copies of the translation product.50,51 Two serologically distinct fibronectin-binding mechanisms have been described recently in S. aureus52 and molecular heterogeneity has also been reported in staphylococcal fibronigin-binding protein.53

Several mammalian receptors mediate glycoprotein clearance through recognition of different terminal carbohydrate units, in particular, N-acetylglucosamine, mannose and fucose in the mononuclear phagocyte system.54 Of the two fucosyl residues in the HLF molecule, the one between the α-1–3 linkage with N-acetylglucosamine could be a potential binding determinant.4,54 In the present study, none of the glycosidases or carbohydrates (including fucose or mannose) inhibited HLF binding to S. aureus.

Identification of HLF-binding receptors on bacteria and parasites opens a new dimension in the investigation of microbial pathogenesis. Peterson and Alderete19 have described 75- and 178-Kda low-affinity HLF-binding receptors (90 000 binding sites/cell) on Trichomonas vaginalis. This interaction caused a 30-fold increase in the intracellular iron accumulation and enhanced the activity of pyruvate-ferrodoxin oxidoreductase (essential for energy metabolism) by six-fold in the trichomonads. Specific lactoferrin receptors have been demonstrated on N. gonorrhoeae (101 Kda) and N. meningitidis (105 Kda)18,20 but these pathogens do not seem to recognise BLF. Neisseriae can acquire iron from HLF55 and iron-limitation seems to enhance the HLF-binding capacity in these pathogens. Schryvers'31 has demonstrated two HLF-binding proteins of 105 and 106 Kda in H. influenzae. Specific HLF binding to T. pallidum, has also been reported.17 We have shown the binding of HLF to periodontitis-associated Prevotella intermedia, P. melaninogena and Porphyromonas gingivalis.56

In conclusion, HLF has been shown to bind to specific cell-surface receptors of S. aureus. This novel receptor-ligand interaction is distinct from the staphylococcal receptors binding to plasma or connective tissue proteins described previously.

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