Human lactoferrin binding in clinical isolates of Staphylococcus aureus

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Summary. Human lactoferrin (HLf) is an iron-binding protein and a host-defence component at the mucosal surface. Recently, a specific receptor for HLf has been identified on a strain of Staphylococcus aureus associated with toxic shock syndrome. We have looked for the occurrence of $^{125}$I-HLf binding among 489 strains of S. aureus isolated from various clinical sources. HLf binding was common among S. aureus strains associated with furunculosis (94.3%), toxic shock syndrome (94.3%), endocarditis (83.3%) and septicaemia (82.4%) and other (nasal, vaginal or ocular) infections (96.1%) with a mean binding (in fmol) of 29.1, 21.9, 16.9, 22.2 and 29.2 respectively; the differences between mean HLf binding values of 29.1-29.2, 21.9-22.2 and 16.9 were significant. Furunculosis-associated (low-invasive or localised) isolates were high-to-moderate binders of HLf; 50% gave positive results at a threshold of $>31$ fmol of $^{125}$I-HLf bound. In contrast, endocarditis-associated (high-invasive or systemic) isolates demonstrated low binding and did not bind $^{125}$I-HLf at the above threshold level. S. aureus recognised human or bovine Lf. However, bound $^{125}$I-HLf was more effectively inhibited in a dose-dependent manner by unlabelled bovine Lf than by homologous HLf. Binding of $^{125}$I-HLf to staphylococci was optimal with organisms grown in agar compared with those from broth cultures. The binding capacity of S. aureus was abolished when strains were grown on carbohydrate- and salt-rich agar media. HLf-binding ability of S. aureus did not correlate with fibronectin, fibrinogen, immunoglobulin G or laminin binding.

Introduction

Iron is an essential requirement for many bacteria. In mammalian tissues, this element forms complexes with various biological molecules or is bound with high affinity to transferrin in plasma and lactoferrin (Lf) in mucous secretions. For iron acquisition, certain bacteria release low-mol. wt iron chelators known collectively as siderophores. Staphylococcus aureus is associated with human illnesses ranging from localised superficial infections such as folliculitis to severe systemic diseases such as fatal endocarditis and toxic shock syndrome (TSS). This gram-positive pathogen requires approximately 1.6 μM iron for growth. Three extracellular haemolysins and a recently identified iron chelator of S. aureus may provide an iron-sufficient environment in vivo. During the early stages of pathogenesis, staphylococci confront inflammatory cell defence, mainly the polymorphonuclear leukocytes (PMNLs) at mucosal surfaces. PMNLs secrete various antibacterial compounds such as lysozyme, myeloperoxidase and lactoferrin, an iron-binding protein. Lf deprives the invading pathogen of iron and provides an important defence at the host mucosal surface. Lf concentration in plasma is elevated during inflammation, septicaemia or endotoxic shock and ultimately leads to physiological hypoferrinaemia such as in TSS. Human plasma contains approximately 1 μg of HLf per ml, with a turnover rate of 5-7 μg/day, processed mainly by the liver. This glycoprotein has a prominent role in various physiological functions. It amplifies inflammatory responses by promoting the adhesion as well as aggregation of PMNLs to the endothelial surface. It stimulates the phagocytic and cytotoxic properties of the macrophages and mediates both hydrogen-peroxide-dependent and independent bacterial killing. It has been suggested that HLf may regulate myelopoiesis by inhib-

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iting the granulocyte-monocyte colony stimulating factor. Furthermore, mouse peritoneal macrophages, human mononuclear cells, and human PMNLs demonstrate specific receptors for HLF. The molecular-interaction of acute-phase proteins secreted by inflammatory cells (LF in particular) with pathogens is still unclear.

Recently we have demonstrated a specific HLF-binding component on the TSS-associated S. aureus strain MAS-89 (Naidu et al., unpublished observation). In this study we examined a large collection of S. aureus isolates from various clinical conditions for HLF binding. We have also compared the incidence of fibronectin (Fn), fibrinogen (Fg), immunoglobulin G (IgG) and laminin (Lm) binding properties of S. aureus with HLF binding.

**Materials and methods**

**Clinical isolates**

A total of 489 S. aureus isolates from various clinical sources was examined. Strains associated with furunculosis (88) and septicemia (122, originating from complicated and uncomplicated illnesses) were isolated in Sweden and characterised in earlier studies. Strains associated with endocarditis (192) were isolated in Denmark. TSS (35, comprising menstrual and non-menstrual cases) isolates were from Europe or the USA and were characterised extensively in earlier studies. Isolates from infected nasal, vaginal or ocular sites (52, TSS toxin-positive with no manifestation of clinical TSS) from a previous study were also included. Bacterial cells were harvested, washed and resuspended in phosphate-buffered saline (PBS), pH 7.2, to a final density of c. 10^9 cells/ml. The cell density was adjusted by counting stained samples of bacterial suspensions in a Petroff-Hausser chamber.

**Media**

The effect of growth conditions on 125I-HLF binding was examined after growth of representative strains for 24 h at 37°C on Nutrient Agar, Blood Agar, Proteose Peptone Agar, Staphylococcus Medium-110, Brain-Heart Infusion Agar and Mannitol-Salt Agar and in Trypticase-Soy Broth, Brain-Heart Infusion Broth, Nutrient Broth and skimmed milk. All media were from Difco Laboratories, Detroit, MI, USA.

**Chemicals**

Human (H) LF (lot 63541) was from US Biochemicals Corp., Cleveland, OH, USA. Bovine (B) LF, purified from bovine milk whey, was kindly provided by Dr. H. Burling, Swedish Dairies Association, Malmö, Sweden. Fibronectin was purified from human plasma by the method of Veunto and Vaheri. IgG was obtained from Kabi Vitrum, Stockholm, Sweden. Laminin, purified from the basement membrane of the Engelbreth-Holm-Swarm transplantable tumour, was purchased from Collaborative Research, Inc., Bedford, MA, USA. All chemicals used for the preparation of buffer solutions were of analytical grade.

**125I-labelled protein binding assay**

HLF was labelled by a modified chloramine-T method with Na125I (specific activity 629 GBq/mg; DuPont Scandinavia AB, Stockholm, Sweden) and Iodobeads (Pierce Chemicals Co., Rockford, IL, USA). Binding assays were performed as described earlier. c. 10^9 bacterial cells (in 0.1 ml of PBS) were mixed with 0.1 ml of 125I-HLF [radioactivity adjusted to (2-2.5) x 10^6 cpm, i.e., c. 8 ng, when diluted in cold PBS] and, after incubation for 1 h at room temperature, 2 ml of ice-cold PBS (containing Tween 20·1%) was added to the tubes; the suspension was centrifuged at 4500 g for 15 min and the supernate was aspirated. Radioactivity retained in the bacterial pellet was measured in a γ-counter (LKB Wallac Clingamma 1271, Turku, Finland). Background radioactivity from incubation mixtures containing no bacteria was 2-5%, and the nonspecific 125I-HLF binding in the presence of excess unlabelled HLF was c. 7-5% of the total labelled protein added. Therefore, an 125I-HLF-binding value below 10% was considered to be a negative result. Samples were tested in triplicate and each experiment was repeated at least twice unless otherwise stated. Binding experiments with 125I-labelled Fn, Fg, IgG, and Lm were performed by the same method with a selected group of HLF-binding or non-binding strains.

**125I-HLF binding inhibition**

Increasing amounts (0-0.1 mg) of HLF and BLF diluted in PBS were mixed with 125I-HLF [c. 8 ng of protein; (2.5-3.0) x 10^4 cpm] in 0.1-ml volumes and added to c. 10^9 cells of S. aureus strain MAS-89 grown on blood agar for 24 h at 37°C. 125I-HLF binding was measured after incubation for 1 h. Logarithmic curves were plotted for each pair of LF preparations.

**Data analysis**

The HLF binding by S. aureus isolates from different clinical sources revealed normal frequency distribution. Therefore, the unpaired Student's t test was used to assess the significance of differences between the groups.

**Results**

Of the 489 human clinical isolates of S. aureus tested, 417 (85.3%) strains bound 125I-HLF (fig. 1).
Mean HLF binding values (in fmol) were 29.1, 21.9, 16.9, 22.2 and 29.2 for strains isolated from furunculosis, TSS, endocarditis, septicaemia and other (nasal, ocular or vaginal) infections respectively. The HLF binding by strains associated with septicaemia and TSS groups or with furunculosis and other infections did not differ. The differences in HLF binding between these pairs and between each pair in the endocarditis-associated strains were significant (p < 0.001).

Individual strains in each clinical group were further categorised according to the degree of HLF binding (table I). Strains associated with furunculosis demonstrated high (50.0%) or moderate (28.4%) $^{125}$I-HLF binding. Conversely, among endocarditis-associated strains, $^{125}$I-HLF binding was low in 55.2% of strains and 16.7% isolates did not bind $^{125}$I-HLF. With TSS-associated strains, binding was moderate (48.4%) or low (31.4%). Of the strains associated with septicaemia, 17% did not bind HLF; those that did bind HLF showed an even distribution among the three categories of HLF binding.

Table I. $^{125}$I-HLF binding to S. aureus isolates from different clinical sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of strains tested</th>
<th>$^{125}$I-HLF binding*, in fmol (percentage of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>Furunculosis</td>
<td>88</td>
<td>44 (50.0)</td>
</tr>
<tr>
<td>Septicaemia</td>
<td>122</td>
<td>27 (22.1)</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>192</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>TSS</td>
<td>35</td>
<td>5 (14.3)</td>
</tr>
<tr>
<td>Other diagnoses†</td>
<td>52</td>
<td>23 (44.2)</td>
</tr>
</tbody>
</table>

* Bacteria (10⁶ cells) were incubated with c. 8 ng of $^{125}$I-HLF; radio-isotope binding (fmol) was high (> 31.0), moderate (21–30), low (11–20) or negative (< 10; non-binding).
† Strains associated with nasal, vaginal or ocular infections.

Strains MAS-89 (TSS-associated, HLF-binder) and strain E-84 (endocarditis-associated, HLF-non-binder) were grown under different growth conditions and tested for $^{125}$I-HLF binding capacity (table II). Cells of strain MAS-89 expressed HLF binding capacity when grown on nutrient agar (45.3 fmol) and blood agar (37.9 fmol). However, brain-heart-infusion agar, carbohydrate-and salt-rich (mannitol-salt or staphylococcus medium-110 agar) or broth media suppressed HLF binding ability in strain MAS-89. The negative control, strain E-84, was consistently an HLF non-binder (within the limits of non-specificity) in all the growth conditions. Strain MAS-89 recognised both human and bovine Lf. However, in HLF binding-inhibition studies, bovine Lf was three times more effective than HLF. A concentration of 0.32 μg of unlabelled HLF or 0.09 μg of unlabelled BLF was required to cause 50% inhibition of $^{125}$I-HLF binding to c. 10⁶ cells of strain MAS-89 (fig. 2).

In each of the HLF high, low or non-binding categories, 30 isolates were compared for the degree...
of Fn, Fg, IgG and Lm binding (fig. 3). With HLf high binders (>40% of total ligand bound) median binding values of Fn 31.2%, Fg 35.5%, IgG 68.7% and Lm 11.3% were demonstrated. The HLf low binders (10-15.0% of total ligand bound) gave median binding values of Fn 28.8%, Fg 34.2%, IgG 66.1% and Lm 7.5%. Strains considered to be HLf non-binders (<7.5% of total ligand bound) gave median binding values of Fn 17.6%, Fg 30.0%, IgG 57.9% and Lm 6.1%. These glycoprotein interactions showed no correlation with HLf binding.

### Table II. Influence of growth media on $\text{^{125}}\text{I}-\text{HLf}$ binding to *S. aureus*

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>$\text{^{125}}\text{I}-\text{HLf}$ binding (in fmol) to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strain MAS-89</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>45.3</td>
</tr>
<tr>
<td>Blood agar</td>
<td>37.9</td>
</tr>
<tr>
<td>Proteose peptone agar</td>
<td>16.7</td>
</tr>
<tr>
<td>Staphylococcus medium-110</td>
<td>3.2</td>
</tr>
<tr>
<td>Brain heart infusion agar</td>
<td>2.3</td>
</tr>
<tr>
<td>Mannitol salt agar</td>
<td>1.9</td>
</tr>
<tr>
<td>Trypticase soy broth</td>
<td>2.8</td>
</tr>
<tr>
<td>Brain heart infusion</td>
<td>2.7</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>0.4</td>
</tr>
<tr>
<td>Skimmed milk</td>
<td>0.1</td>
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</table>

**Discussion**

Iron in mammalian systems is regulated mainly by two iron-binding proteins—transferrin in plasma and Lf at the mucosal surface. Lf is secreted by mammary, lachrymal, bronchial and salivary glands, kidney, and mucosae of the endometrium and seminal vesicles. PMNLs, the common inflammatory cell population, also secrete this acute-phase protein. Lf exerts bactericidal or bacteriostatic effects during the exponential and late stationary phases of bacterial growth respectively. The antigen-processing cells of the RES (macrophages, mononuclear cells and PMNLs) have specific surface receptors for HLf. Furthermore, HLf has a key function in various host-defence processes. Since most infections are initiated at the mucosal surface, the specific interaction of HLf with bacteria may be important. Recently, we have demonstrated a specific receptor for HLf on the TSS-associated *S. aureus* strain MAS-89 (Naidu *et al.*, unpublished observation). In this study, *S. aureus* strains isolated from various human infections were tested for this novel binding property.

Our data suggest that HLf binding is common (85% of strains gave positive results) among clinical isolates for all strains. The binding values were highest for HLf high binders (>40% of total ligand bound) and lowest for HLf non-binders (<7.5% of total ligand bound). These glycoprotein interactions showed no correlation with HLf binding.
of *S. aureus*. However, the degree of binding varied among isolates from different clinical groups. Furunculosis-associated strains demonstrated high HLf binding, in contrast to the low binding capacity of endocarditis-associated strains of *S. aureus*. Low-invasive strains of *S. aureus* cause localised pyogenic skin infections, e.g., furunculosis, whereas high invasive ability is an important requirement for staphylococci to cause deep systemic infections such as endocarditis. Whether these apparent differences in HLf binding, would influence the host defence mechanisms or the adhesive, colonising and invasive properties of staphylococci is not known. Izhar et al. have reported an inhibitory effect of HLf on bacterial adherence to epithelial cells. Our preliminary studies suggest a regulatory effect of HLf on the phenotypic expression of staphylococcal cell-surface receptors involved in fibronectin, and collagen binding (unpublished observation).

Lf from different mammalian species demonstrates chemical and antigenic heterogeneity. Human and bovine Lfs vary in Mn, gross amino-acid composition, and in glycosidyl content. Schryvers and Morris have reported that HLf receptors on Neisseria meningitidis do not recognise bovine Lf. However, *S. aureus* recognised both human and bovine Lf. Unlabelled bovine Lf caused more effective inhibition of 125I-HLf binding than unlabelled HLf, in dose-dependent HLf-binding-inhibition studies. Such recognition may not be unusual, since staphylococcal infections are common in animals.

Growth conditions influence plasma or connective tissue protein binding and other cell-surface properties of staphylococci. Cheung and Fischetti have shown that production of high-mol. wt surface proteins of *S. aureus* is selectively expressed or enhanced in agar-grown cultures compared to broth-grown cells. We observed that HLf binding was better with agar-grown than with broth-grown *S. aureus* cells. HLf binding capacity was abolished in cells grown on carbohydrate- or salt-rich agar media that favour slime or capsule production.

Various mammalian plasma or connective tissue proteins such as Fn, Fg, IgG, vitronectin, Lm and collagen are recognised by putative cell-surface receptors of *S. aureus*. Such bacteria-host molecular interactions may have an important role in the pathogenesis of staphylococcal infections. Interactions of staphylococci with such proteins vary, depending on the origin of the strain and conditions of growth. Binding of these ligands is qualitatively different from HLf binding in *S. aureus* (Naidu et al., unpublished observation). In the present study, Fn, Fg, IgG and Lm binding was quantitatively different from HLf binding to *S. aureus* grown and tested under similar conditions, indicating that the HLf binding property is an independent characteristic.

In summary, HLf, an iron-binding, acute-phase protein secreted by inflammatory cells, binds to a majority of clinical isolates of *S. aureus*. Quantitative differences in binding were apparent among isolates from various clinical conditions. Fn, Fg, IgG and Lm binding did not correlate with HLf-binding to *S. aureus*.

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