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Bactericidal Activity of Human Lactoferrin: Sensitivity of a Variety of Microorganisms

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Lactoferrin is an iron-binding protein that has been detected in secretions that bathe human mucosal tissues. Previous studies have shown that, when this protein is in the iron-free state, it is capable of a direct bactericidal effect on *Streptococcus mutans* and *Vibrio cholerae*. The present study demonstrates variable susceptibilities for a variety of different microorganisms. The list of susceptible organisms includes gram-positive and gram-negative microbes, rods and cocci, facultative anaerobes, and aerotolerant anaerobes. Similar morphological and physiological types are represented among the lactoferrin-resistant bacteria. *S. mutans* was more resistant to lactoferrin when grown on a sucrose-containing medium than when it was grown on brain heart infusion broth without added sucrose. When a lactoferrin-sensitive, avirulent strain of *Streptococcus pneumoniae* was passed through mice, the resultant virulent culture became lactoferrin resistant. Since organisms of the same species and even of the same strain (*S. pneumoniae*) can differ in susceptibility to lactoferrin, it appears that accessibility to the lactoferrin target site may account for differences in susceptibility. It appears that there may be a relation between virulence and resistance to lactoferrin.

Lactoferrin is an iron-binding glycoprotein synthesized by neutrophils (2, 8, 12, 17) and acinar epithelial cells (15). This protein has been detected in most of the secretions that bathe the human mucosal surfaces (14). In vitro studies have shown that lactoferrin is capable of retarding the growth of certain microorganisms (3, 18). Since this inhibition is readily reversed by the addition of iron in excess of the binding capacity of the lactoferrin, it has been suggested that lactoferrin stasis may be due to its ability to withhold iron that is essential for bacterial growth (3, 18).

In addition to this bacteriostatic effect, it has recently been shown that lactoferrin is capable of a direct bactericidal effect on *Streptococcus mutans* and *Vibrio cholerae* (1, 6; R. R. Arnold, J. Russell, W. J. Champion, M. Brewer, and J. J. Gauthier, submitted for publication). Although it is known that this action is dependent on lactoferrin being in the iron-free state (iron-saturated lactoferrin has no effect), the mechanism of this killing is unknown. Similarly, the investigations of Bullen and Wallis (5) and Gladstone and Walton (7) suggest that iron-binding proteins play an essential role in the bactericidal activity of polymorphonuclear leukocytes.

In the present study, a variety of microorganisms were examined to determine the range of susceptibility of these organisms to the bactericidal effects of lactoferrin. Data from this study extend the list of organisms that are killed by lactoferrin and suggest that resistance to the microbicidal action of lactoferrin may be associated with virulence.

MATERIALS AND METHODS

Preparation of lactoferrin. Purified lactoferrin was isolated from human colostrum by combined gel filtration and ion-exchange chromatography, as previously described (6). Purity was assessed by immunoelectrophoresis against goat anti-human colostral whey and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6). Apolactoferrin was prepared by dialysis against a saturated solution of ferrous ammonium sulfate (6). Both of these preparations were subsequently dialyzed extensively against deionized distilled water and stored at −20°C in plastic tubes.

Microorganisms. Strains of *S. mutans* representing five serotypes, AHT (*a*), BHT (*b*), NCT 10449 (*c*), NIDR 6715 (*d-g*), and LM-7 (*e*), *Streptococcus pneumoniae* ATCC 6303, *Lactobacillus casei* ATCC 4646, and *V. cholerae* 569B were from a stock collection maintained in this laboratory. *Streptococcus lactis* ATCC 11454 was provided by Donald J. LeBlanc. *Streptococcus salivarius*, *Streptococcus mitis*, and *Candida albicans* were clinical isolates from 4-day supragingival plaque samples. *Shigella sonnei*, *Salmonella newport*, *Enterobacter cloacae*, *Escherichia*

coli O126:B16, *E. coli* O11:K56:H21, non-enteropathogenic *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* were stock isolates from the clinical laboratories of Children's Hospital, Birmingham, Ala.

Growth of microorganisms. All cultures were grown to mid-exponential phase at 37°C in brain heart infusion (BHI) broth, with the exception of *V. cholerae*, which was grown in 1.5% peptone broth (pH 8.0). BHI was chosen because of the absence of sucrose, which minimized the extracellular polysaccharides of the oral streptococci. In one set of experiments, *S. mutans* 10449 was grown in BHI containing 1.0% sucrose to determine the effect of extracellular glucan on susceptibility to lactoferrin killing. Washed cells (approximately 10^6 colony-forming units [CFU] per ml) from BHI-grown and BHI-sucrose-grown cultures were incubated at 37°C in 42 μM apolactoferrin, and viable CFU were determined after 0, 30, 60, and 120 min. All cultures were washed two times and suspended in sterile saline to a concentration of approximately 10^9 CFU/ml.

Determination of the effect of lactoferrin. Samples of 100 μl of washed cells were incubated at 37°C for 60 min with 200 μl of either saline, saturated lactoferrin (83 μM), or apolactoferrin (4.2, 42, or 83 μM). Portions of these reaction mixtures were serially diluted and plated in triplicate on either blood agar or peptone agar (*V. cholerae*). Colony counts were determined after incubation at 37°C for 24 h.

Passage of *S. pneumoniae* in mice. *S. pneumoniae* was serially passed five times in 8-week-old Swiss-Webster mice. The first mouse was infected by intraperitoneal injection with 2×10^7 CFU in 0.1 ml from a culture in exponential growth. The peritoneal cavity of the near-moribund mouse was aseptically washed with 2.0 ml of sterile phosphate-buffered saline, and 0.2 ml of the peritoneal wash was injected intraperitoneally into a second mouse. This procedure was repeated through five successive passages. A 0.1-ml sample of the peritoneal wash from the final mouse was inoculated into 10 ml of BHI broth, grown to the late logarithmic phase (absorbance at 660 nm = 0.8) and then tested for susceptibility to lactoferrin as described. A control culture was successively passaged in BHI at 37°C. The fifth inoculum was grown to late exponential phase (absorbance at 660 nm = 0.8) and tested for lactoferrin susceptibility. Both the animal-passaged and BHI-passaged cells were suspended to 5×10^7 CFU/ml and treated with 42 μM apolactoferrin.

RESULTS

Lactoferrin-sensitive microorganisms. The viability of a variety of microorganisms was markedly reduced (greater than 99% reduction) by incubation of the cells with 4.2 μM apolactoferrin (Table 1). Sensitive microorganisms included several strains of oral streptococci, *S. pneumoniae*, the gram-negative organisms *V. cholerae* and *P. aeruginosa*, and the yeast *C. albicans*. The non-enteropathogenic isolate of *E. coli* required 42 μM lactoferrin to reduce the CFU by 99%. However, this strain was considerably more sensitive to the bactericidal effects of lactoferrin than were the two pathogenic strains listed in Table 2. The number of recoverable CFU was not reduced by incubation of the cells with iron-saturated lactoferrin.

Lactoferrin-insensitive microorganisms. Table 2 presents the CFU per milliliter after treatment of microorganisms that were relatively resistant to the effects of apolactoferrin

TABLE 1. *Viability of lactoferrin-sensitive microorganisms after incubation with varying concentrations of apolactoferrin, iron-saturated lactoferrin, or saline for 1 h at 37°C*

Strain	CFU/ml[a]				
	Control[b]	SAT LF[c]	Apo-LF concn[d]		
			4.2 μM	42 μM	83 μM
Streptococcus mutans AHT (*a*)	1.5×10^7	1.3×10^7	3.0×10^3	NG[e]	NG
S. mutans BHT (*b*)	3.0×10^7	3.2×10^7	1.5×10^3	NG	NG
S. mutans 10449 (*c*)	5.0×10^8	5.4×10^8	4.0×10^3	NG	NG
S. mutans 6715 (*d–g*)	7.5×10^6	8.1×10^6	3.0×10^2	NG	NG
S. mutans LM-7 (*e*)	6.0×10^8	5.8×10^8	9.0×10^4	2.0×10	NG
Streptococcus salivarius	7.0×10^7	8.1×10^7	4.0×10^4	1.2×10	NG
Streptococcus mitior	5.0×10^6	4.8×10^6	3.0×10^3	NG	NG
Streptococcus pneumoniae ATCC 6303	3.8×10^7	3.8×10^7	1.7×10^3	NG	NG
Escherichia coli (non-enteropathogen)	8.3×10^7	8.8×10^7	7.8×10^7	6.2×10	3.4×10
Vibrio cholerae 569B[f]	8.5×10^7	8.1×10^7	9.2×10^2	NG	NG
Pseudomonas aeruginosa	1.7×10^7	2.2×10^7	8.6×10^3	NG	NG
Candida albicans	7.0×10^7	7.2×10^7	3.0×10^4	NG	NG

[a] Viable counts (CFU/ml) were determined by plating serial dilutions on blood agar.
[b] Bacteria were incubated in saline.
[c] SAT LF, Iron-saturated lactoferrin (83 μM).
[d] Apo-LF, Apolactoferrin; 300-μl reaction volume.
[e] NG, No detectable growth.
[f] *V. cholerae* counts were determined on peptone agar (pH 8.0).

TABLE 2. *Viability of lactoferrin-resistant microorganisms after incubation with saline, iron-saturated lactoferrin, or apolactoferrin for 1 h at 37°C*

Strain	CFU/ml[a]		
	Control[b]	Saturated LF (83 μM)	Apo-LF (83 μM)
Streptococcus pyogenes	2.1×10^6	2.9×10^6	3.7×10^6
Streptococcus lactis 11454	8.5×10^6	1.4×10^7	1.2×10^7
Lactobacillus caseii	1.2×10^6	2.3×10^6	2.1×10^6
Staphylococcus aureus	5.0×10^7	4.8×10^7	3.6×10^7
Staphylococcus epidermidis	2.3×10^8	2.4×10^8	7.8×10^7
Escherichia coli O126:B16	5.8×10^7	9.1×10^7	6.4×10^7
Escherichia coli O111	7.6×10^7	7.9×10^7	7.5×10^7
Enterobacter cloacae	1.7×10^8	2.1×10^8	1.9×10^8
Salmonella newport	8.5×10^7	8.7×10^7	7.2×10^7
Shigella sonnei	2.1×10^8	2.7×10^8	1.9×10^8

[a] Determined on blood agar after 24 h at 37°C. LF, Lactoferrin.

[b] Bacteria were incubated in saline.

(less than a log reduction with 83 μM apolactoferrin). These included a variety of enteric bacteria, *S. pyogenes*, and two organisms capable of growth in milk, *S. lactis* and *L. casei*. The viability of the two staphylococcal cultures was partially reduced by treatment with apolactoferrin, with *Staphylococcus epidermidis* exhibiting the greater susceptibility (approximately 66% loss in recoverable CFU with 83 μM lactoferrin).

Effect of passage of *S. pneumoniae* in mice on lactoferrin sensitivity. The results obtained with *E. coli* indicate that the avirulent strain is more sensitive to lactoferrin than the two virulent strains that were tested. To determine whether this is the case for other genera, *S. pneumoniae* was serially passed in mice and tested for a change in susceptibility to lactoferrin, when compared with BHI-passed cells. There was a marked reduction in susceptibility of *S. pneumoniae* to apolactoferrin after animal passage (Fig. 1). When the animal-passed culture was tested at the same cell concentration (5×10^7 CFU/ml) and the same lactoferrin concentration (42 μM) that resulted in complete killing of the BHI-maintained bacteria, no loss in viability could be detected. However, when the cell concentration was reduced by 2 logs relative to the lactoferrin concentration, the animal-passed bacteria were detectably sensitive to lactoferrin. This increase in resistance is significant because there is greater than a 4-log reduction in viable CFU of BHI-maintained *S. pneumoniae* after 1 h of treatment with a 10-fold less concentrated lactoferrin preparation (Table 1). Resistance attained by animal passage was lost by serial passage in BHI broth.

Effect of extracellular glucan on lactoferrin sensitivity. The reduction in susceptibility of *S. pneumoniae* to apolactoferrin after animal passage suggests that the synthesis of capsule material decreases access of the cell surface to lactoferrin. To determine whether this may also be true for *S. mutans*, strain 10449 was grown on BHI containing 1.0% sucrose to determine the effect of cell surface glucans on susceptibility to lactoferrin killing (Fig. 2). The data indicate that sucrose-grown cells were markedly more resistant to lactoferrin than were BHI-grown cells. After 20 minutes, 6 logs of BHI-grown cells were killed as compared with 2 logs of sucrose-grown cells.

DISCUSSION

The present study lends support to the previous observations of a bactericidal capability

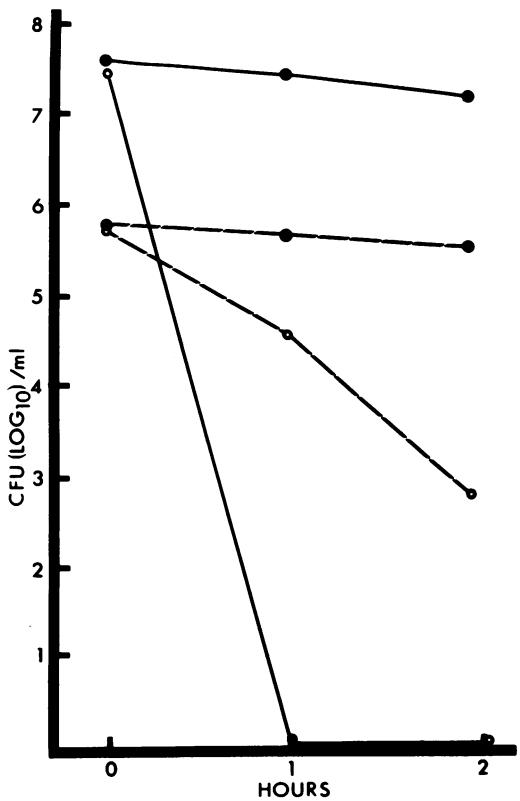

FIG. 1. *Effect of serial passage in Swiss-Webster mice on the susceptibility of S. pneumoniae to apolactoferrin. Samples (1 ml) of washed S. pneumoniae (3×10^7 CFU or 5×10^5 CFU) were treated with 42 μM apolactoferrin at 37°C. Control, BHI maintained (●——●); lactoferrin treated, BHI maintained (○——○); control, animal passed (●- - -●); lactoferrin treated, animal passed (○- - -○). When 3×10^7 CFU of animal-passed S. pneumoniae per ml was incubated with apo-lactoferrin, no loss in viability could be detected.*

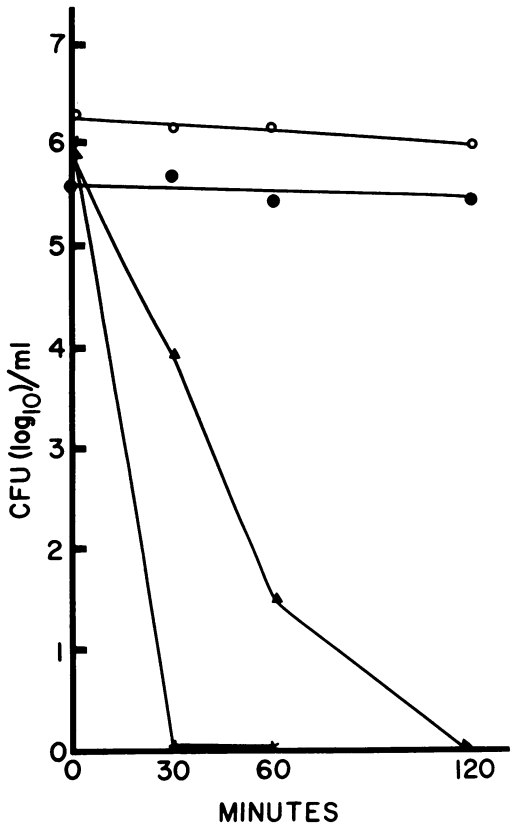

FIG. 2. *Comparison of the lactoferrin susceptibility of S. mutans grown on BHI (×) and BHI-sucrose (▲). Controls included S. mutans grown on BHI-sucrose (●) and BHI (○) and incubated with iron-saturated lactoferrin. Experimental conditions are described in the text.*

for lactoferrin and demonstrates variable susceptibilities for a range of microorganisms (1, 6; Arnold et al., submitted for publication). As in the earlier studies, saturation of lactoferrin with iron alleviates this bactericidal activity, suggesting that either the availability of the metal-binding site or the conformational state of the iron-free lactoferrin is essential for the microbiocidal effect of this protein. The differences in susceptibilities of microorganisms to killing by lactoferrin might offer a clue to the mode of lactoferrin action if the site(s) of sensitivity or mechanism(s) of resistance can be discerned.

Both lactoferrin-susceptible microorganisms (Table 1) and lactoferrin-resistant microorganisms (Table 2) include a variety of morphological and physiological types, such as gram-positive and gram-negative organisms, rods and cocci, facultative anaerobes, and aerotolerant anaerobes. The list of susceptible strains also includes a strict aerobe and a eucaryotic organism. Considering this variety of susceptible organisms, the possibility of a common target for lactoferrin is limited to the numbers of characteristics that these organisms share. It is also possible that there are different modes of action for lactoferrin on these various organisms. An interesting observation is that strains of the same species, and the same strain grown under different conditions, show differences in susceptibility to lactoferrin. This might suggest that, rather than different targets, there is a difference in the accessibility of susceptible sites.

Peptidoglycan is the only common cell wall component of all the bacteria tested. The yeast, *C. albicans*, is an exception. Although antibiotics that affect the integrity of peptidoglycan usually display a noticeable difference in their action on gram-positive versus gram-negative bacteria, this does not appear to be the case for lactoferrin. Also, interference with peptidoglycan synthesis may result in cell disruption, and this does not occur when cells are treated with lactoferrin. Although differences in peptidoglycan structure may exist between strains of the same species (10), the difference in susceptibility of different cultures of the same strain (*Streptococcus pneumoniae*) could not likely be explained by differences in peptidoglycan structure. Such differences could be attributed to varying degrees of accessibility of peptidoglycan to lactoferrin.

Although the size of the lactoferrin molecule (molecular weight 76,000) suggests that it would not penetrate the outer membrane, studies with colicins indicate that interaction with the cell membrane by proteins with a similar molecular weight (60,000 to 80,000) is a possibility (9). A number of membrane functions are essential for cell viability and are therefore potential targets for lactoferrin. These include energy metabolism, permeability, and transport mechanisms. The susceptibility of the streptococci rules out the electron transport system and oxidative phosphorylation as common targets, and the susceptibility of *Pseudomonas* rules out specific aspects of fermentative metabolism.

It has not yet been determined whether lactoferrin treatment can cause the loss of small molecules from susceptible cells. BHI broth, which was used as the growth medium for most of these cultures, contains a variety of potential carbon and energy sources. Since *P. aeruginosa* is capable of utilizing several of these different compounds, and yet is susceptible to lactoferrin, it seems unlikely that interference with the uptake of a specific source is a common mechanism of lactoferrin action. Since neither *P. aeruginosa* nor *E. coli* has specific growth factor requirements, the transport of a required organic

growth factor can be ruled out. However, interference with the uptake of inorganic nutrients such as metal ions remains a possibility.

Of the lactic acid bacteria that were tested, the *S. mutans* strains, *S. salivarius*, *S. mitis*, and *S. pneumoniae* were sensitive to apolactoferrin, whereas *S. pyogenes*, *S. lactis*, and *L. casei* were resistant. The latter two are common isolates from milk, which contains high levels of lactoferrin. It would therefore be expected that their survival in this environment would require resistance to lactoferrin.

There may be a relation between virulence and resistance to lactoferrin killing. *S. pyogenes* is considerably more resistant to lactoferrin than are the streptococci that are commonly considered to be part of the normal oral flora. The concept of resistance as a virulence factor is also supported by the variable sensitivity of the different *E. coli* strains that were tested. In addition, animal passage of *S. pneumoniae*, with consequent increase in virulence, resulted in a concomitant enhancement of resistance to lactoferrin. Also, BHI-sucrose-grown *S. mutans*, which synthesizes extracellular glucan, is more resistant to lactoferrin than a BHI-grown culture that lacks this cell surface material. In all of these examples, increased virulence may be associated with qualitative or quantitative changes in cell surface components. This may result in decreased accessibility of lactoferrin to a specific target site.

Experimental evidence suggests that resistance to the bacteriostatic effect of lactoferrin may be attributed to bacterial synthesis of iron chelators, which can compete with lactoferrin or transferrin for host iron. Previous studies have demonstrated the existence of transferrin-iron-enterochelin complexes (11). The demonstration of binding of iron-saturated lactoferrin by a resistant strain but not by sensitive strains (1) would suggest that lactoferrin is capable of forming a similar complex. The presence of iron-enterochelin external to the cell surface therefore might effectively block access of lactoferrin to surface target sites. This might explain the resistance of the enteropathogenic *E. coli* strains, since they are known to synthesize enterochelin (3, 4). However, the strain of *V. cholerae* used in this study has been shown to be capable of synthesizing enterochelin (11), yet it is very sensitive to lactoferrin. Likewise, *P. aeruginosa* has been shown to synthesize iron-chelating molecules (13). It is possible that, under the conditions employed in these studies, the test cultures were unable to overcome the bactericidal activity of lactoferrin. The inability to demonstrate surface binding of iron-saturated lactoferrin by the *V. cholerae* 569B would suggest at least a quantitative difference in its enterochelin as compared to *E. coli* O126 (1). An alternative explanation is that the bactericidal effect of lactoferrin involves a mechanism that is different from that of the bacteriostatic effect.

The demonstration of a direct bactericidal effect of lactoferrin suggests a potent role for lactoferrin in mucosal secretions and neutrophils. These studies may also offer a different interpretation of the recent data of Bullen and Wallis (5). These investigators found that iron introduced in the form of a ferritin-antiferritin complex could reverse the bactericidal activity of the neutrophils. They suggested that the iron-free environment provided by lactoferrin in the polymorph was essential for the bactericidal systems of the cell. If the cidal function reported here for purified apolactoferrin is functional in situ, then this iron reversal might be attributed to a more direct inhibition of the bactericidal mechanism.

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