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Bactericidal Activity of Human Lactoferrin: Differentiation from the Stasis of Iron Deprivation

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Previous studies have demonstrated a direct iron-irreversible inhibition of a variety of microorganisms by human apolactoferrin. The present study compared the bactericidal effects of lactoferrin on *Streptococcus mutans* with the bacteriostatic effects of iron deprivation. Growth (as determined by change in optical density) and macromolecular synthesis, as determined by incorporation of ^{14}C-labeled uracil, thymidine, and lysine, were inhibited by incubation of washed exponential-phase *S. mutans* NCTC 10449 with purified human apolactoferrin. Similarly, apolactoferrin inhibited glucose uptake and metabolism. Iron-saturated lactoferrin had no effect on bacterial growth or metabolism and was capable of serving as a source of iron in iron-depleted medium. *S. mutans* failed to grow, and there was no indication of macromolecular synthesis in iron-depleted partially defined medium; however, glucose metabolism continued, though at a reduced rate, and viability was retained for 72 h. There was no detectable metabolism of glucose by cells maintained for 18 h in iron-free medium. Metabolism was restored by transfer of iron-depleted *S. mutans* to iron-complete medium. This was in contrast to the irreversible inhibition by lactoferrin after 1 h of incubation. Inhibition could not be reversed by removal of cell surface-associated lactoferrin as detected by rhodamine isothiocyanate-labeled antilactoferrin. This inhibition of metabolism and rapid loss in viability observed with lactoferrin treatment suggest that lactoferrin has a direct bactericidal effect on *S. mutans* that cannot be attributed to simple iron deprivation.

Lactoferrin is an iron-binding glycoprotein that is synthesized by glandular epithelial cells and is present in most of the secretions that bathe human mucosal surfaces (16). Histochemical studies have demonstrated its presence in specific granules of polymorphonuclear leukocytes (15, 17). The potential antimicrobial function of lactoferrin at these sites has been suggested by the in vitro demonstration of lactoferrin-mediated inhibition of growth of a variety of microorganisms (reviewed in references 6 and 21). The inhibition observed in most of these studies was bacteriostatic in that growth could be readily restored by the addition of exogenous iron in excess of the chelating capacity of the lactoferrin. The mechanism of inhibition under these conditions has been attributed to the nutritional deprivation of essential iron (20). In addition to this static effect, several studies have suggested that the mechanisms of iron-sensitive antibacterial systems in body fluids and neutrophils may be more complex than simple nutritional deprivation (5, 7, 10, 11). These data suggest that lactoferrin and its serum counterpart, transferrin, may be, at least indirectly, essential in the bactericidal events observed with various microorganisms.

Recent investigations from this laboratory have more directly demonstrated an effect of lactoferrin on a wide range of microorganisms that is not reversed in the presence of supplemental iron (2–4, 9). In these studies, target microorganisms were incubated with iron-free (apo) lactoferrin in the absence of growth medium and subsequently inoculated into media containing concentrations of iron in excess of the binding capacity of the lactoferrin. Cell death occurs exponentially after an initial 15-min lag period, during which the bactericidal process can be stopped by dilution (4). Immunofluorescence studies indicate that apolactoferrin is capable of binding to the surface of susceptible bacteria (3). Both surface binding and inhibition due to lactoferrin are blocked in the presence of secretory immunoglobulin A with specificity for the test organism (3, 9). In addition, there is an increase in resistance to lactoferrin killing of *Streptococcus pneumoniae* that is concomitant

with an increase in capsular material (2). These data suggest that access to the bacterial cell surface is essential for the apparent microbicidal effect of lactoferrin. A possible explanation for this inhibition might be that surface-bound lactoferrin blocks sites that are essential for the transport of iron or some other essential nutrient. If so, then the inhibition should be reversed by the removal of surface-associated lactoferrin, and lactoferrin-inhibited cells should be able to continue metabolic functions that are independent of iron.

The present studies were designed to determine whether various reagents could be used to remove surface-bound lactoferrin and whether lactoferrin-mediated inhibition could be reversed by such treatment. In addition, the effects of lactoferrin on macromolecular synthesis and glucose metabolism in *Streptococcus mutans* were compared with the bacteriostatic effects of culturing this organism in an iron-depleted medium. These data suggest that lactoferrin has a direct bactericidal effect on *S. mutans* that cannot be attributed to simple iron deprivation.

MATERIALS AND METHODS

Preparation of lactoferrin. Lactoferrin was isolated and purified from pooled human colostrum by combined gel filtration and ion-exchange chromatography as previously described (9). Iron-free (apo) lactoferrin was prepared by dialysis against 0.1 M citric acid (pH 2.3), and iron-saturated lactoferrin was prepared by dialysis against a saturated solution of ferrous ammonium sulfate. Both preparations were subsequently extensively dialyzed against sterile, deionized, distilled water, and aliquots were stored at −20°C in sterile plastic tubes. Purity was confirmed by quantitative two-dimensional immunoelectrophoresis against anti-human colostrum and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Goat anti-human lactoferrin. Goat antiserum to lactoferrin was prepared by intramuscular injection of the purified protein preparation in an emulsion with Freund complete adjuvant. Specificity was confirmed by immunoelectrophoresis against human colostral whey. A single band could be detected by immunoelectrophoresis, and a single peak was apparent by quantitative two-dimensional immunoelectrophoresis. The ammonium sulfate precipitate was conjugated with rhodamine isothiocyanate (RITC) for immunofluorescence studies.

Media. Partially defined (PD)-glucose medium was prepared as previously described (9). Iron-depleted PD-glucose medium was prepared by deleting the $FeCl_3$ and removing iron contaminants by salting with $MgCO_3$ (18). Brain heart infusion agar (Difco) prepared with 1.0% sucrose was used for quantitation of colony-forming units (CFU). A characteristic morphology for *S. mutans* was apparent on this medium.

Microorganisms. *S. mutans* NCTC 10449 (Bratthall serotyoe c) was maintained in lyophile. For all analyses, cultures were grown at 37°C to early exponential phase (absorbance at 660 nm of 0.2) in PD medium containing 0.5% glucose. The cells were washed twice by centrifugation (10,000 × *g*, room temperature, 15 min) and resuspended to an appropriate concentration before experimental treatment.

Lactoferrin treatment of *S. mutans*. Unless otherwise stated, the washed bacteria were incubated with a concentration (300 μg/10^8 CFU in 1.0 ml of distilled water [~4 μM]) of apolactoferrin which resulted in greater than 99.9% killing within 1 h at 37°C. Reductions in relative apolactoferrin concentrations resulted in a decrease in rate of killing with loss in viability being detected only after longer incubation periods (4). Controls were incubated with an equivalent concentration of saturated lactoferrin or with distilled water.

Washing reagents. Lactoferrin-treated and control bacteria were washed with one of the following: 0.01% sodium lauryl sulfate, 20 mM sodium dithiothreitol, 0.1% Tween 80, 0.1 M dibasic sodium phosphate, 1.0 M sodium chloride, 0.2 M ferrous ammonium sulfate, 0.1 M citric acid, 0.01% sodium desoxycholate, or 0.21 mg of disodium ethylenediamine tetraacetate and 10 mg of trypsin per ml in 0.01 M phosphate buffer (pH 7.6). Washing was accomplished by resuspending the treated bacterial cell pellet in 1.0 ml of prewarmed (37°C) reagent and incubating for 10 min. The bacterial suspension was then washed with 50 ml of sterile distilled water on a 0.45-μm-pore size Millipore filter. The retained cells were suspended in 1.0 ml of sterile distilled water, serially diluted, and plated on brain heart infusion-sucrose agar to determine CFU. Samples were retained for immunofluorescence analysis with RITC-labeled anti-lactoferrin globulin.

The treated and washed bacteria were incubated with an optimal concentration of RITC-labeled goat anti-lactoferrin for 45 min at 37°C and washed against three changes of phosphate-buffered saline at 4°C. This antibody preparation will react with either apo- or iron-saturated lactoferrin (3). The bacterial suspensions were observed with a fluorescence microscope equipped with a vertical illuminator according to Ploem (Orthoplan; Lietz, Wetzlar, W. Germany). The light source was a mercury lamp (Osram HBO, 100 W) equipped with a filter system for narrow-band excitation.

Uptake studies. Samples of the lactoferrin-treated and washed *S. mutans* were inoculated into PD-glucose medium containing 2 μCi of either [^{14}C]thymidine, [^{14}C]uracil, or [^{14}C]lysine (New England Nuclear Corp., Boston, Mass.) in a total volume of 10 ml and incubated at 37°C. Samples were removed at selected intervals and transferred to cold 10% trichloroacetic acid containing unlabeled uracil, thymidine, or lysine and stationary-phase carrier cells. After 1 h, the suspension was filtered on 0.45-μm Millipore filters and washed three times with trichloroacetic acid. The filters were air dried, transferred to 10 ml of ACS cocktail, and counted with a Beckman LS 7000 Liquid Scintillation Counter.

Quantitation of glucose utilization and lactic acid synthesis. Apolactoferrin-treated *S. mutans* and controls were inoculated into PD medium containing 10 mg of glucose per 100 ml and were incubated at 37°C. Samples of 0.2 ml were removed at 30-min intervals for glucose and lactic acid determinations. Glucose disappearance was followed by quantitating the glucose remaining in the supernatant by using PGO enzymes (stock no. 501-6; Sigma Chemical Co., St. Louis, Mo.) and *o*-dianisidine hydrochloride (Sigma;

TABLE 1. Effect of washing apolactoferrin-treated *S. mutans* with various agents

Washing agent	CFU/ml			
	Control[a]	Reagent control[b]	Lactoferrin treated[c]	RITC anti-LF[d]
Sodium lauryl sulfate (0.01%)	7.0×10^7	6.3×10^6	NG	Neg
Ferrous ammonium sulfate (0.2 M)	1.2×10^5	2.0×10^4	NG	Neg
Citric acid (0.1 mM)	3.4×10^6	2.5×10^6	NG	Neg
EDTA-trypsin	3.7×10^6	2.8×10^6	NG	Neg
Dithiothreitol (20 mM)	4.1×10^6	1.4×10^6	NG	Pos
Tween 80 (0.1%)	4.1×10^6	1.6×10^6	NG	Pos
Dibasic phosphate (0.1 M)	1.2×10^5	1.0×10^5	NG	Pos
Sodium chloride (1.0 M)	1.2×10^5	5.0×10^4	NG	Pos
Deoxycholate (0.01%)	1.6×10^6	1.4×10^6	NG	Pos

[a] Cells were incubated with iron-saturated lactoferrin (4 μM) for 1 h at 37°C, transferred to a 0.45-μm Millipore filter, washed with 50 ml of water, serially diluted, and plated on brain heart infusion-sucrose agar.

[b] Cells that had been incubated with iron-saturated lactoferrin for 1 h at 37°C were centrifuged, suspended in 1.0 ml of prewarmed (37°C) reagent, and incubated for 10 min. These cells were then washed with water and counted as described in the text.

[c] Cells were incubated with apolactoferrin (4 μM) for 1 h at 37°C and treated for 10 min with the test reagents. The cells were washed, and the viability was determined as described in the text. NG, No growth.

[d] Apolactoferrin-treated bacteria were incubated for 30 min with RITC-labeled goat antilactoferrin, washed, and observed for fluorescence with a Leitz UV microscope equipped with epi-illumination and filters for narrow-band excitation. Negative controls were prepared in the absence of lactoferrin or by using saturated lactoferrin.

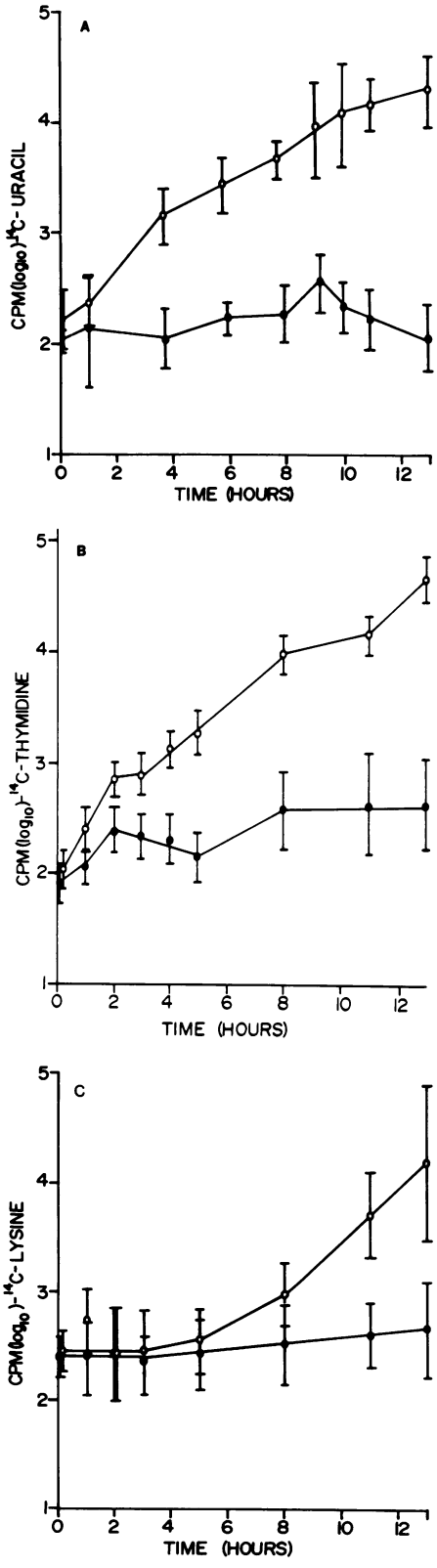
stock no. 510-50) according to Sigma Technical Bulletin no. 510. The absorbance at 450 nm was compared to that of a glucose standard curve. Lactic acid was quantitatively determined by reacting 100 μl of culture supernatant with 0.28 ml of enzyme mixture (10 μg of β-NAD^+ in 2.0 ml of glycine buffer, 4.0 ml of distilled water, and 0.1 ml of lactic dehydrogenase [Sigma]) at 37°C for 30 min. Lactic acid concentration was determined spectrophotometrically as: absorbance at 340 nm × 97.65 = mg/100 ml.

In related experiments, washed bacteria (3×10^{10} CFU) were incubated with 300 μg of apolactoferrin in 1.0 ml of saline for 1 h at 37°C. There was no detectable reduction in viable CFU at this concentration of lactoferrin. The treated bacteria were suspended in 2 ml of saline (absorbance at 660 nm = 0.3), and the pH of the stirred suspension was monitored with an Orion Ionanalyzer model 901. A 100-μl volume of glucose was added to give a final concentration in the bacterial suspension of 1%, and the change in pH was recorded on a strip chart recorder. To mimic acid production by bacteria, a precision peristaltic pump (Minipuls III; Gilson Medical Electronics, Inc., Middleton, Wis.) was calibrated gravimetrically with distilled water. A standardized solution of lactic acid (0.982 mM) was delivered at fixed rates to 2.0 ml of saline, and the pH was monitored and compared with that observed with the bacteria in glucose. The rate of pH change is logarithmically related to the rate of lactic acid synthesis and is proportional to the number of bacteria in the reaction chamber (19).

RESULTS

Removal of surface-associated lactoferrin. Previous studies have shown that apolactoferrin binds to the surface of susceptible bacteria (3). This binding could result in the inhibition of growth by blocking some essential transport site, resulting in nutritional deprivation of the organism. If this is true, then removal of the surface-bound lactoferrin should reverse this inhibition. In these experiments, a variety of washing agents were tested to determine whether the removal of bound lactoferrin from the cell surface would result in restoration of viability (Table 1). The presence of lactoferrin on the cell surface and its subsequent removal were determined by immunofluorescence. It was found that lactoferrin could no longer be detected on the bacterial surfaces after treatment with either sodium lauryl sulfate, citric acid, ferrous ammonium sulfate, or EDTA-trypsin. Similar treatment of control cell populations did not result in substantial loss of viability. Some of the other washing agents (e.g. Tween 80 and deoxycholate) could also eliminate detectable lactoferrin when used at higher concentrations than indicated in Table 1. However, such treatment caused substantial loss of viability in the control populations. Viability could not be restored even though surface-bound lactoferrin could no longer be detected.

Effect of lactoferrin on the uptake of thymidine, uracil, and lysine. To determine the effect of lactoferrin treatment on the incorporation of thymidine, uracil, and lysine, washed cells of an exponentially growing culture of *S. mutans* were incubated with a bactericidal concentration of apolactoferrin or with an equivalent concentration of iron-saturated lactoferrin for 1 h at 37°C and then transferred to PD-glucose medium containing either [^{14}C]thymidine, [^{14}C]uracil, or [^{14}C]lysine (2 μCi/10 ml of medium). At various



times, samples of these cultures were removed, and the amount of radioactivity in the trichloroacetic acid-precipitable fraction was determined. Although all three of these compounds were immediately incorporated into control cell populations that had been incubated in distilled water or in iron-saturated lactoferrin, uptake of these compounds into apolactoferrin-treated cells was markedly reduced (Fig. 1). It seems unlikely that the slight incorporation of these compounds could be accounted for by microbial growth and cell division, since there was no detectable increase either in viable plate count or in optical density at 600 nm.

Effect of iron deprivation on the uptake of thymidine, uracil, and lysine. When washed cells from an exponentially growing culture of *S. mutans* were incubated in distilled water for 1 h and then transferred to PD medium depleted of iron, the rates of uptake of [^{14}C]thymidine, [^{14}C]uracil, and [^{14}C]lysine ceased completely after 3 to 5 h, as with lactoferrin treatment.

Iron dependence of *S. mutans*. When $FeCl_3$ was omitted from PD-glucose medium, *S. mutans* showed limited growth, probably due to residual iron in the medium (8). When this residual iron was salted out of the PD medium with $MgCO_3$, no growth of *S. mutans* (inoculum, 10^8 CFU/ml) could be detected spectrophotometrically over a 72-h period at 37°C (Fig. 2). Aliquots removed at various intervals from this culture and plated indicated no loss in viability in 24 h and less than 1 log reduction in recoverable CFU after 72 h. When $FeCl_3$ was added at a concentration of 8 μM to iron-depleted medium, normal growth of *S. mutans* in iron-depleted medium was restored. Growth was also restored, though to a lesser extent, by the addition of 4 μM iron-saturated lactoferrin (Fig. 3). Apolactoferrin had no effect on growth or viability when added to the iron-depleted medium. Lactoferrin killing is not apparent at near neutral pH (4), which may account for the maintenance of viability in these experiments. Loss in bactericidal activity is possibly due to the change in conformation that occurs at neutrality.

Glucose metabolism. Pretreatment of *S. mutans* with apolactoferrin resulted in total inhibition of the bacterial uptake of glucose (Fig. 3A) as well as the production of lactic acid (Fig. 3B). Bacteria preincubated in either distilled water or

FIG. 1. Effect of 1-h preincubation of *S. mutans* 10449 (1.0×10^8 CFU/ml) with 4 μM apolactoferrin (●) and 4 μM iron-saturated lactoferrin (○) on the uptake of ^{14}C-labeled uracil (A), thymidine (B), or lysine (C) in PD-glucose medium (mean ± standard deviation). Uptake of precursors by cells preincubated in distilled water was similar to that observed with iron-saturated lactoferrin.

saturated lactoferrin were capable of immediate glucose metabolism (Fig. 3A and B). *S. mutans* was capable of glucose uptake and lactic acid synthesis in iron-free medium (Fig. 3C and D); however, the rate of glucose metabolism of the water-treated control was reduced as compared to that in the presence of iron-saturated lactoferrin.

In a parallel experiment, decrease in pH was followed as a measure of glucose metabolism (Fig. 4). Bacteria (3×10^{10} CFU/ml) were preincubated for 1 h at 37°C with either apolactoferrin (4 μM) or distilled water and suspended in saline. Glucose was added to 1% (wt/vol) once the pH of the bacterial suspension had remained stable for at least 3 min. There was an immediate drop in pH with the addition of glucose to the control bacteria. Preincubation with apolactoferrin totally inhibited glucose-induced acid production by *S. mutans* (Fig. 4). Acid synthesis was inhibited even though the concentration of apolactoferrin was not sufficient to result in a detectable loss in viability.

Effect of iron deprivation on lactate production. The following experiments were designed to determine the effects of iron depletion on the glucose metabolism of *S. mutans* 10449. Bacteria were grown to early exponential phase, washed three times in sterile distilled water, and inoculated (1.7×10^7 CFU/ml) in either iron-free or iron-complete PD medium. After incubation at 37°C for 16 h, these cultures were washed and inoculated to both iron-complete and iron-free PD-glucose media. Lactic acid levels were measured in the cell-free supernatants at hourly intervals (Fig. 5). Although there was no loss in viability in the cultures maintained in iron-free medium for 16 h, there was no evidence of lactate production when they were transferred to iron-free medium. Lactate production was delayed but evident when iron-depleted bacteria were transferred to iron-complete medium. There was a reduction in lactate production that occurred between h 4 and 5 after the transfer of bacteria grown in iron-complete medium to iron-free conditions.

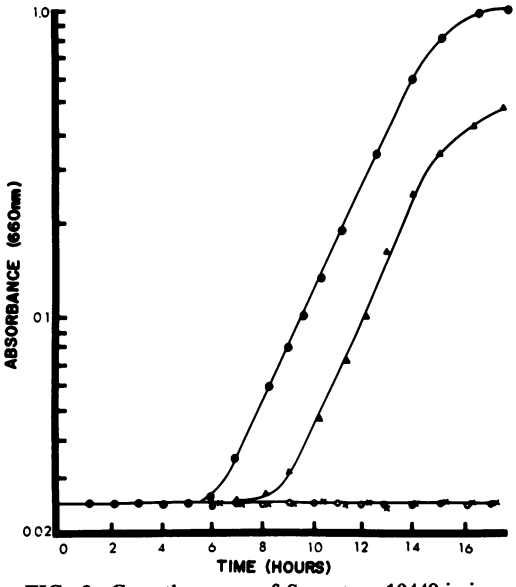


FIG. 2. Growth curves of *S. mutans* 10449 in iron-depleted (salted with $MgCO_3$) PD medium without additions (○); with $FeCl_3$ to 8.0 μM (●); with iron-saturated lactoferrin to 4.0 μM (▲); or with apolactoferrin to 4.0 μM (×). No change in absorbance was observed over a 72-h period in iron-depleted PD medium; however, viability was retained, as there was less than a log reduction in recoverable CFU as determined by plating on brain heart infusion-sucrose agar during the monitored period. The culture was inoculated to a cell concentration of 2.5×10^7 cells per ml.

DISCUSSION

Most studies of the antimicrobial action of lactoferrin have demonstrated a bacteriostatic inhibition that is readily reversed by the addition of excess iron (reviewed in references 6, 20, and 21). This indirect inhibition suggests chelation of available iron in the nutriment, rendering this essential metal inaccessible to the invading microorganisms. In contrast, when certain microorganisms are incubated directly with lactoferrin in the absence of medium, there is an iron-irreversible inhibition of subsequent growth (2, 4). Data from the present study support the interpretation that this direct antimicrobial effect of lactoferrin on *S. mutans* is a bactericidal event that is not directly related to simple iron deprivation (3, 4, 9).

The binding of lactoferrin to the bacterial cell surface might exert a bacteriostatic inhibition by blocking essential transport sites or cell wall biosynthetic events. Such inhibition might appear to be bactericidal by conventional viability plating and growth curve analyses. In the present study, however, removal of detectable cell-associated lactoferrin by techniques that retained the viability of control bacteria failed to restore viability to lactoferrin-treated *S. mutans* (Table 1). It was presumed that a loss of fluorescence in these experiments represented removal of lactoferrin from the bacterial surface. It is possible that within the limits of sensitivity of the technique undetectable lactoferrin remains attached to a few essential sites on the bacteria. A less likely possibility is that washing reagents modify the lactoferrin antigenicity, preventing detection. At least two of the reagents (ferrous ammonium sulfate and citric acid), however, when used at considerably higher concentra-

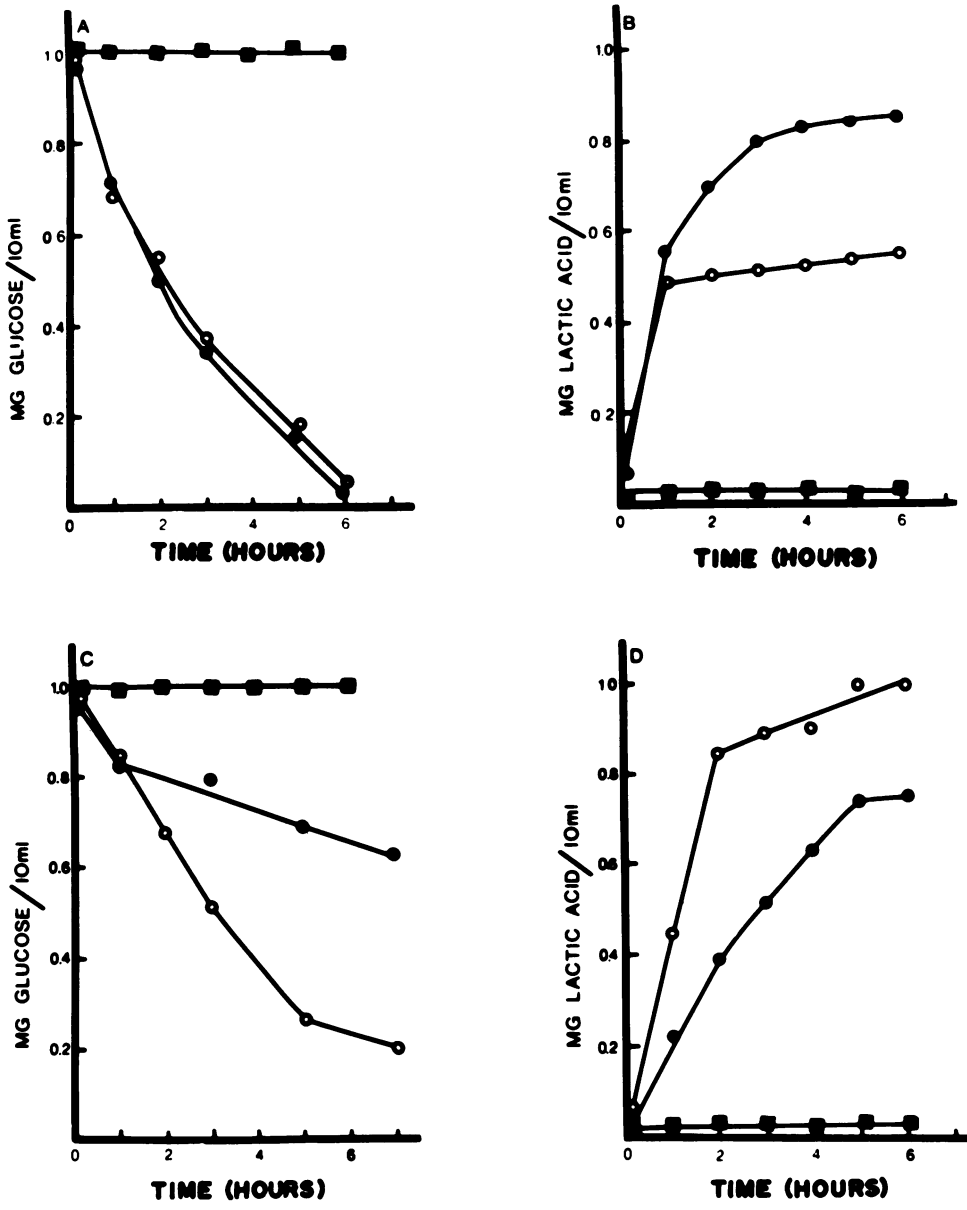


FIG. 3. Effects of lactoferrin treatment and iron deprivation on the glucose metabolism of *S. mutans*. Washed *S. mutans* 10449 (1.0×10^8 CFU/ml) were incubated for 1 h at 37°C with either 4 μM apolactoferrin (■), 4 μM iron-saturated lactoferrin (○), or distilled water (●) and inoculated to PD-glucose medium. Glucose and lactic acid levels were quantitated at hourly intervals in PD medium with iron (A and B) and in PD medium without iron (C and D).

tions have no effect on the antigenicity of free lactoferrin.

The death of the bacteria within the 1-h incubation period cannot be attributed to simple iron deprivation, since *S. mutans* maintained in an iron-depleted environment retained viability for at least 72 h (Fig. 2). This is in marked contrast to the relatively rapid loss in recoverable CFU (within 1 h) observed with lactoferrin treatment (Table 1, Fig. 2). It is interesting that *S. mutans* appears to be capable of utilizing lactoferrin-bound iron for growth and metabolism (Fig. 2 and 4). This is in contrast to the static effect reported by others. These data do not, however, preclude the possibility that lactoferrin may function by the rapid depletion of iron from a

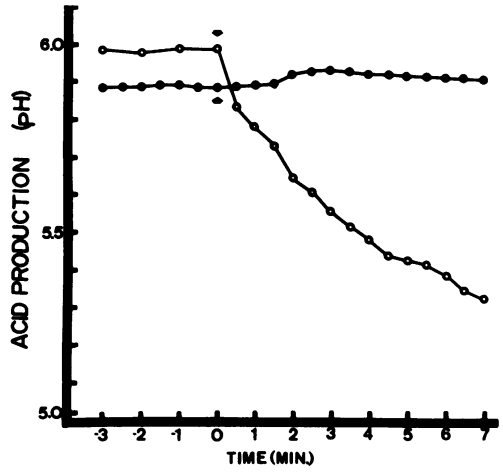


FIG. 4. Effect of preincubation of *S. mutans* (3×10^{10} CFU/ml) with 4 μM apolactoferrin (●) or 4 μM iron-saturated lactoferrin (○) on glucose metabolism. The washed bacteria were suspended in 2.0 ml of saline (5×10^8 CFU/ml), and glucose (arrows) was added to a concentration of 1% after the pH had remained stable for at least 3 min. The pH of the bacterial suspension was monitored with an Orion Ionanalyzer (model 901) and recorded on a strip chart recorder.

specific and crucial cellular role. An alternative possibility is that the binding of lactoferrin to the bacterial cell surface triggers a second process that results in the irreversible cell death. This would be similar to the mechanism postulated to interpret data obtained from studies of colicin action (14).

Although *S. mutans* viability is retained in the absence of iron, its macromolecular synthesis (DNA, RNA, and protein) and consequent growth are iron dependent. This is not surprising, as iron is known to play essential roles in microbial metabolism other than in the electron transport system, including cellular growth and division, intermediary metabolism, and various enzymes (8). The slight uptake observed in these experiments cannot be attributed to cell division but might be the result of repair mechanisms, turnover, or the completion of additional rounds of DNA replication. Lactoferrin preincubation also inhibits the uptake of uracil, thymidine, and lysine; however, interference with the energy supply would result, secondarily, in the shut down of net protein, RNA, and DNA syntheses. The microbicidal activity of lactoferrin has not been demonstrated under growth conditions, and thus this experimental design does not distinguish between the primary and secondary effects of lactoferrin on the various components of macromolecular synthesis (11, 12).

An interesting observation of these studies is the apparent requirement for iron for glucose metabolism by *S. mutans*. The rates of glucose uptake and lactic acid synthesis (Fig. 3 and 4) are reduced in iron-depleted medium as compared to those observed in media containing either free or lactoferrin-bound iron. Furthermore, lactic acid synthesis can be totally inhibited by preincubation of *S. mutans* for 16 h at 37°C under iron-depleted conditions (Fig. 5). This inhibition of glucose utilization may reflect reduced energy requirements for nongrowing cells as compared to the actively dividing cell populations in the presence of free or lactoferrin-associated iron, or it may be due to a more direct involvement of iron in glucose metabolism. Similarly, glucose uptake and lactic acid synthesis were totally inhibited by 1 h of preincubation with apolactoferrin. This inhibition of metabolism preceded a detectable loss in viability. These data suggest that an initial effect of lactoferrin treatment is the inhibition of carbohydrate metabolism or transport (active iron depletion?), and this inhibition might be essential for the initiation of killing.

Several studies have indirectly suggested that lactoferrin or transferrin may be essential for certain of the bactericidal activities of neutrophils and serum. It has been known for some time that the bactericidal activity of serum on

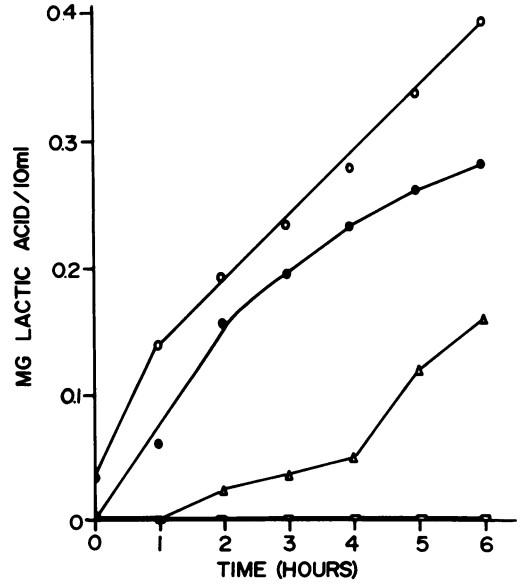


FIG. 5. Lactic acid synthesis of *S. mutans* after 16 h of maintenance under iron-free conditions and transfer to iron-free PD-glucose medium (□) or to iron-complete PD-glucose medium (△). Controls were grown for 16 h in PD-glucose medium, washed, and inoculated to iron-complete (○) and iron-free (●) PD-glucose media.

Pasteurella septica is dependent on the presence of specific antibody, complement, and transferrin (11–13). Although iron compounds interfere with the bactericidal action of antiserum on *P. septica*, an explanation based on interference with bacterial iron supply is not sufficient (12, 13). The uptake of iron compounds by polymorphonuclear leukocytes reverses their bactericidal activity against a strain of *Staphylococcus aureus* (10) and against *Pseudomonas aeruginosa* (5, 7), implicating the involvement of the iron-binding capacity in the bactericidal mechanisms of these leukocytes. Regardless of the mechanisms of action, the present studies suggest a potent bactericidal effect of purified human lactoferrin, which suggests that the iron-binding glycoproteins might make a more direct contribution to the bactericidal systems of serum, saliva, and neutrophils. Ambruso and Johnston (1) have implicated lactoferrin-associated iron with the generation of bactericidal hydroxyl radical by human neutrophils. This or a similar bactericidal product may result from apolactoferrin binding of bacterial-associated iron. This hypothesis is currently under investigation.

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