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In Vitro Growth Inhibition of Mastitis-Causing Coliform Bacteria by Bovine Apo-Lactoferrin and Reversal of Inhibition by Citrate and High Concentrations of Apo-Lactoferrin[1]

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Bovine apo-lactoferrin (apo-Lf) was added to in vitro cultures of eight strains of coliform bacteria associated with bovine mastitis. As little as 0.02 mg of apo-Lf per ml resulted in marked inhibition of growth of all coliforms. Growth inhibition was lost if saturated Lf or iron plus apo-Lf was added to the synthetic medium. The inhibition of growth increased as the concentration of apo-Lf increased from 0.02 to 0.2 mg/ml for *Klebsiella pneumoniae* (OARDC-A1), *Klebsiella* spp. (K1-21), and *Aerobacter aerogenes* (55-12222) and 2 mg/ml for *A. aerogenes* (76-2414-1), *Escherichia coli* (60-Lilly), *E. coli* (66-S16), and *Klebsiella* spp. (K6-24). As the concentration of apo-Lf was increased above 0.2 or 2 mg/ml, there was less inhibition of growth except for *E. coli* (33-C4). Apo-Lf at 20 mg/ml was bactericidal for *E. coli* (33-C4). Results are compatible with the hypothesis that coliform bacteria respond to low-iron environments by production of iron-sequestering agents that compete effectively with apo-Lf for free iron. Addition of apo-Lf plus citrate resulted in loss of growth inhibition. The molar ratio (citrate to apo-Lf) was found to be more important than the absolute concentration of either component. A ratio of 75 resulted in 50% growth inhibition, whereas ratios of 300 and greater resulted in less than 10% growth inhibition. These results suggest that the ratio of citrate to Lf would be important in evaluating Lf as a nonspecific protective factor of bovine mammary secretions.

The ability of a variety of pathogenic bacteria to sequester iron from the host environment has been suggested as an important virulence factor (3, 25, 32, 33). Free iron occurs sparingly in body fluids, and invading bacteria are forced to compete with the naturally occurring iron-binding proteins for sufficient iron for their growth. The iron-binding protein of the internal compartment (blood serum) is transferrin (Tf), whereas the major iron-binding protein of exosecretions is generally lactoferrin (Lf), although Tf is also found in varying degrees in exosecretions (15, 16, 18, 32).

Both Lf and Tf are powerful chelators of iron, the affinity constant being approximately 10^{36}, and both have been shown to inhibit growth of a wide variety of microbial species when not fully saturated with iron (3, 19, 23, 25). Although the major biological function of Tf would appear to be transport of iron to reticulocytes, the true biological function of Lf is still in question. Masson (15) and Masson et al. (19) have suggested that Lf is a nonspecific natural protective factor of exosecretions. Evidence by Bullen and co-workers (4, 5) suggests that lactoferrin can act in conjunction with specific antibody to provide a more powerful antimicrobial system than either component alone.

The major iron-binding protein of the exosecretion of the bovine mammary gland is Lf, and the concentration varies with the functional status of the gland (28). The Lf concentration in colostrum is 2 to 5 mg/ml, whereas normal milk contains 0.1 to 0.3 mg/ml. During involution of the bovine mammary gland, the concentration increases dramatically, attaining levels of 20 to 30 mg/ml. Recently, we reported (12, 13) that the concentration of Lf increases significantly during mammary infections and that the magnitude of the increase would appear to be related to the severity of the infection. Thus an increased understanding of the interaction of Lf, iron, and bacteria in mammary exosecretions may lead to greater control of mammary infections (bovine mastitis) and intestinal infections of neonates.

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We report here our work on the in vitro growth inhibitory effects of bovine Lf on coliform bacteria known to cause bovine mastitis. In addition, the interaction of Lf and citrate (present in high concentrations in bovine colostrum and milk) was investigated.

MATERIALS AND METHODS

Isolation of bovine Lf. Isolation and purification of bovine Lf was by the method of Smith et al. (29) with the following modifications. A whey preparation of the pooled secretion from several involuted mammary glands was used as the Lf source. The bulk of the immunoglobulin was precipitated by addition of ammonium sulfate to 50% saturation (312 g/liter). The Lf fraction was precipitated from the 50% supernatant by continued addition of ammonium sulfate to 65% saturation (430 g/liter). After dialysis to remove the ammonium sulfate, the Lf was purified by ion-exchange chromatography on diethylaminoethyl-Sephadex A-50 and by gel filtration through Sephadex G-200 columns as described by Smith et al. (29). The final product was judged to be pure at a concentration of 50 mg/ml by electrophoresis, immunoelectrophoresis, and immunodiffusion. Antisera used were: (i) antibovine Lf; (ii) antibovine colostral whey; (iii) antibovine serum; (iv) antibovine immunoglobulin G (IgG); (v) antibovine serum albumin; (vi) antibovine Tf; (vii) antibovine free secretory component; (viii) antibovine secretory IgA; and (ix) antibovine IgM.

Preparation of apo-Lf. Apo-lactoferrin (apo-Lf) was prepared by the method of Groves (11) with the following modifications. The solution of Lf was adjusted to a 1% solution (10 mg/ml) by addition of deionized distilled water. The pH of the solution was lowered to 2.0 by careful addition of 1 N HCl with constant stirring. The Lf solution at pH 2.0 was allowed to stir for 3 h at room temperature. The Lf solution (50 ml) was added to a 60-ml plastic syringe fitted to the top of a column (1.5 by 30 cm) of Dowex-50W ion-exchange resin (8% cross-linked, dry mesh 200 to 500, Sigma Chemical Co., St. Louis, Mo.). The protein eluted from the column was collected in an acid-cleaned beaker. The column was flushed with 25 ml of deionized distilled water in order to recover all the protein. The pH of the eluted apo-Lf solution was immediately raised to 7.0 by careful addition of 1 N NH_4OH with constant stirring. The pH 7.0 apo-Lf solution was dialyzed against several volumes of deionized distilled water and then lyophilized under vacuum and stored at −20°C. Comparison of spectrophotometric readings at 460 and 280 nm before and after iron removal (16, 17) showed the apo-Lf product to be approximately 15% saturated with iron.

Quantitation of apo-Lf. The concentration of apo-Lf in stock solutions was determined by spectrophotometric assay. An extinction coefficient (absorbancy at 280 nm per milligram per milliliter) of 1.45 as reported by Castellino et al. (10) was used. For calculation of molar quantities of Lf, a molecular weight of 77,100 was used (10).

Coliform bacteria. The coliform bacteria used in these experiments are listed in Table 1. All organisms with the exception of *Klebsiella pneumoniae*, strain (OARDC-A1), were obtained from E. J. Carroll, Department of Clinical Pathology, University of California, Davis. The organisms provided by Dr. Carroll have been used in previous studies of bovine mastitis (1, 2, 6–9, 14). *K. pneumoniae* (OARDC-A1) was isolated from an acute case of bovine mastitis that occurred in the dairy research herd of the Ohio Agricultural Research and Development Center, Wooster.

TABLE 1. *Description of coliform bacteria*

Bacteria	Strain	Source	Serum sensitivity[a]
Klebsiella pneumoniae	OARDC-A1	Bovine mastitis	
Klebsiella spp.	K1-21	Bovine mastitis	SR
Klebsiella spp.	K6-24	Bovine mouth	SS
Aerobacter aerogenes	76-2414-1	Bovine mastitis	SR
A. aerogenes	55-1222	Bovine mastitis	SS
Escherichia coli	60-Lilly		SS
E. coli	33-C4	Bovine mastitis	SR
E. coli	66-S16	Fetal pig	SS

[a] As determined by E. J. Carroll (see references 1, 2, 6–9, 14). Abbreviations: SR, serum resistant; SS, serum sensitive.

Maintenance of bacterial cultures. Lyophilized cultures as received at the laboratory were originally suspended in brain heart infusion broth and incubated at 37°C for 4 h. Cultures were then plated for isolation on Trypticase soy agar (TSA). Single colonies were removed and stabbed into TSA tubes. They were incubated for 24 h and then stored at room temperature tightly sealed with plastic tape.

For in vitro assays, new TSA slants were grown monthly from the stabbed TSA tubes and stored at 4°C.

Synthetic medium for coliforms. The synthetic medium used for all in vitro assays was that of Young et al. (34) with the following modifications. The following amino acids were added per liter: L-glutamine (1.0 g); L(+)-valine (0.1 g); L(−)-serine (0.1 g); L(−)-proline (0.1 g); L(−)-histidine (0.1 g); and L(−)-methionine (0.1 g). The glucose was autoclaved separately and then added to the sterile medium. The final pH of the medium was 6.8. An effort was made to maintain a low iron content by using thoroughly acid-cleaned glassware, double-distilled deionized water, and the purest chemicals available. The iron concentration of the medium was 0.10 to 0.15 μg/ml. Solutions of ferric ammonium citrate, ferric sulfate, sodium citrate, Lf, and apo-Lf were sterilized by using membrane filters (13-mm diameter, filter type HA, 0.45-μm pore size, Millipore Corp., Bedford, Mass.).

Iron determination. Iron concentration was determined by atomic absorption spectrophotometer analysis (model 82-270, Jarrell-Ash Co., Waltham, Mass.). Standard iron solutions were prepared with an iron reference solution in HCl base (Fisher Scientific Co., Fairlawn, N.J.).

Growth inhibition assay. All assays were per-

formed under constant conditions to minimize variation in results. For assay, stock cultures (TSA slants) were streaked for growth on TSA plates and incubated at 37°C for 24 h. A single loopful of bacteria was transferred to 10 ml of synthetic medium and incubated at 37°C for 1.0 h. One milliliter of the culture was transferred to 200 ml of synthetic medium and incubated for 2 h at 37°C in a shaking water bath. One-half milliliter of culture was transferred to culture tubes (16 by 125 mm) containing the synthetic medium. Final volume in all assay tubes was 6.5 ml. At appropriate time intervals starting at 0 h of incubation, plate counts were determined on the cultures, using the standard plate counting technique (SPC) as previously described (30).

RESULTS

Preliminary attempts to grow *K. pneumoniae* (OARDC-A1) in the synthetic medium described by Young et al. (34) did not result in sufficient growth to adequately test the effects of added apo-Lf. Growth increased by only 0.25 log units over an 8-h incubation period. Addition of the amino acids increased growth to approximately 2.5 log units over an 8-h incubation period. In general, the coliforms used in these studies grew in a logarithmic manner up to 12 h of incubation, and at or shortly after 12 h they entered the stationary growth phase. Thus, most assays were terminated after 12 h of incubation.

The synthetic medium used was not depleted of iron for the growth inhibition studies. Analysis for iron on several batches of the synthetic medium suggested that the concentration was 1.8 to 2.7 μM. Weinberg (32) reported that the minimal quantity of iron required for growth of the bacterial species used in the present experiments was 0.3 to 0.5 μM. Thus the iron concentration of the synthetic medium should not have been rate limiting for growth. This was verified by adding up to 6.0 μmol of iron per ml as ferric ammonium citrate to the synthetic medium. The addition of iron to the medium did not improve growth.

When apo-Lf was added to the synthetic medium at a final concentration of 5 mg/ml, growth of *K. pneumoniae* (OARDC-A1) was substantially reduced (Fig. 1). The addition of 5 mg of apo-Lf (15% saturated with iron) per ml would theoretically be able to bind 107 nmol of iron per ml or some 50 times the amount of iron calculated to be present per milliliter of synthetic medium. That the growth of *K. pneumoniae* (OARDC-A1) was not completely inhibited suggests that the iron bound by the Lf was not totally unavailable to the bacteria. There was no growth inhibition when iron-saturated Lf (5 mg/ml) was added to the medium. Likewise the

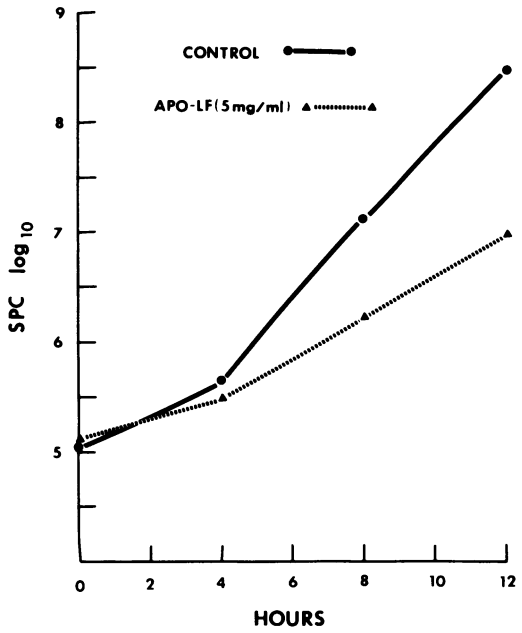

FIG. 1. *Effect of added apo-Lf (5 mg/ml) on growth of K. pneumoniae (OARDC-A1).*

simultaneous addition of apo-Lf (0.15 mg/ml) and iron (3.076 μg/ml) as ferric sulfate abolished the growth-inhibitory effects of apo-Lf (Fig. 2).

Effect of increasing apo-Lf concentration. A series of assays was conducted with *K. pneumoniae* (OARDC-A1) to determine the effect of increasing apo-Lf concentration on growth inhibition. These data were expressed as percentage of growth inhibition (%GI) calculated as follows:

$$\%GI = \left[\frac{(\Delta SPC_{0-12\ h}\ \text{control}) - (\Delta SPC_{0-12\ h}\ \text{experimental})}{\Delta SPC_{0-12\ h}\ \text{control}}\right] \times 100$$

where ($\Delta SPC_{0-12\ h}$ control) = (SPC at 12 h) − (SPC at 0 h) for the control assay and ($\Delta SPC_{0-12\ h}$ experimental) = (SPC at 12 h) − (SPC at 0 h) for the experimental assay (added apo-Lf). The arithmetic numbers and not the log values were used for these calculations.

The results (Fig. 3) showed that 0.01 mg of apo-Lf/ml did not inhibit growth, but 0.02 mg of apo-Lf/ml resulted in 93% GI. These values would suggest that the iron concentration in the medium available for bacterial growth was less than the 1.8 to 2.7 μM concentration determined to be present by atomic absorption analysis. As the concentration of apo-Lf was increased to 0.10 mg/ml, %GI increased to approximately 98%. The %GI remained rather

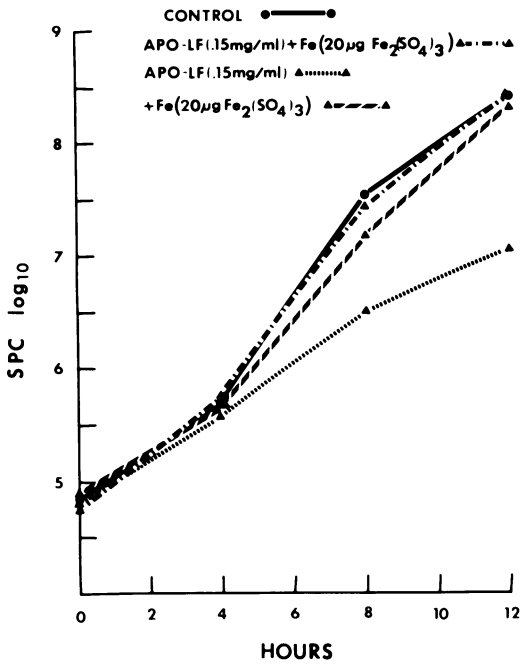

FIG. 2. *Growth of K. pneumoniae (OARDC-A1) when apo-Lf (0.15 mg/ml), ferric sulfate (final iron concentration, 3.076 μg/ml), or apo-Lf plus ferric sulfate was added to the synthetic medium.*

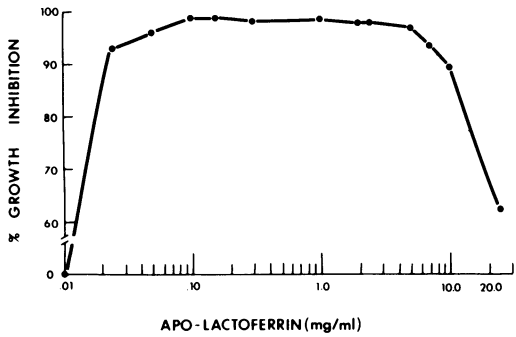

FIG. 3. *Effect of increasing apo-Lf concentration on the %GI of K. pneumoniae (OARDC-A1).*

constant at 98% up to 3 mg/ml. At concentrations of 5 mg/ml and above, the %GI declined. At 24 mg/ml apo-Lf, the %GI had decreased to approximately 60%.

The pH of cultures after 12 h of incubation was between 6.5 and 6.7, and the 12-h pH was not related to the presence or absence of apo-Lf or to apo-Lf concentration, suggesting that pH differences could not account for the decrease in %GI at high apo-Lf concentrations. After the 12-h colony count, several samples were centrifuged to remove the bacteria. The supernatant was subject to immunoelectrophoresis by using rabbit antibovine Lf. Qualitatively there was no evidence for fragmentation of the Lf or a net loss. These results suggest that the bacteria were not degrading the Lf.

A series of assays was conducted in order to determine the effect of increasing apo-Lf concentration on eight different strains of coliform bacteria (Table 2). With the exception of *E. coli* (33-C4), all the coliforms behaved in a similar manner. The inhibition of growth increased as the concentration of apo-Lf increased to 0.2 or 2.0 mg/ml. At 20 mg of apo-Lf/ml, there was less inhibition than at 0.2 or 2.0 mg/ml. The data suggest that growth of *Klebsiella* spp. (K6-24) was nearly or completely inhibited at apo-Lf concentrations of 0.2 and 2.0 mg/ml. *Klebsiella* spp. (K6-24) was the only coliform tested for which apo-Lf appeared to be truly bacteriostatic. However, apo-Lf at 20 mg/ml was markedly less inhibitory to growth.

The major deviation in these assays occurred with the *E. coli* (33-C4), with which there was increasing inhibition of growth with increasing concentration of apo-Lf. The data suggest that 20 mg of apo-Lf/ml was in fact bactericidal for *E. coli* (33-C4). The set of assays for *E. coli* (33-C4) was repeated two additional times with identical results.

Reversal of growth inhibition by citrate. The effect of citrate on growth inhibition of *K. pneumoniae* (OARDC-A1) was investigated. Results of a typical set of assays are shown in Fig. 4. In this assay, the addition of citrate (as sodium citrate) resulted in less inhibition of

TABLE 2. *Effect of increasing apo-Lf concentration on growth of coliform bacteria*

Organism	SPC[a]					
	0-h incubation[b]	12-h incubation				
		0[c]	0.02	0.2	2	20
Klebsiella pneumoniae (OARDC-A1)	4.84	6.79	6.17	5.76	6.07	6.77
Klebsiella spp. (K1-21)	4.71	8.35	8.11	6.13	6.62	6.82
Aerobacter aerogenes (76-2414-1)	4.61	8.11	6.89	6.14	6.12	7.03
A. aerogenes (55-1222)	4.81	8.43	7.79	6.95	7.62	7.97
Escherichia coli (60-Lilly)	4.99	7.47	7.06	5.69	5.64	6.59
E. coli (66-S16)	4.79	7.85	7.20	5.31	5.09	5.38
Klebsiella spp. (K6-24)	4.72	7.40	7.04	4.77	4.54	6.63
E. coli (33-C4)	4.67	8.22	8.07	5.91	5.81	1.54

[a] SPC, Standard plate count (colony-forming units per milliliter, $\log_{10}$).
[b] Mean SPC for all assays within a set.
[c] Final apo-Lf concentration in the assay.

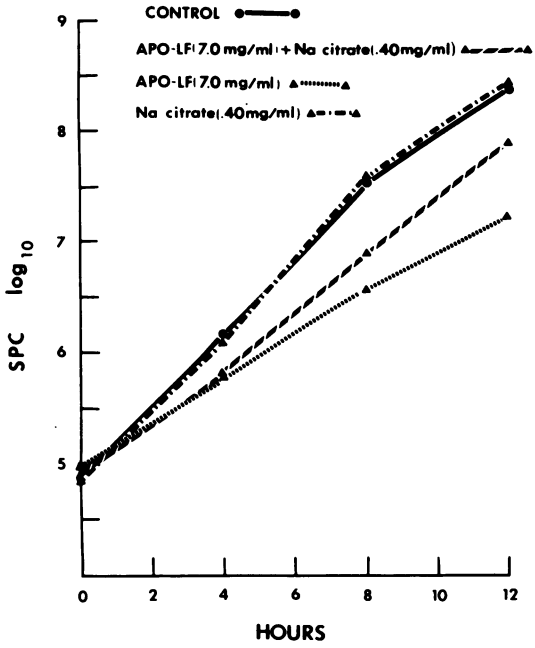

FIG. 4. *Growth of K. pneumoniae (OARDC-A1) when apo-Lf (7.0 mg/ml), sodium citrate (final citrate concentration, 0.4 mg/ml), or apo-Lf plus sodium citrate was added to the synthetic medium.*

growth than apo-Lf alone. Growth in cultures where only citrate was added did not differ from growth in control cultures.

Two types of assays were conducted. In one, the apo-Lf concentration was held constant at 0.15 mg/ml and increasing concentrations of citrate (0.002 to 0.2 mg/ml) were added. In the other, citrate was held constant at 0.25 mg/ml and increasing concentrations of apo-Lf were added (0.1 to 21 mg/ml). The results (Fig. 5) were expressed as %GI. When increasing concentrations of apo-Lf were added to a constant level of citrate, there was a biphasic increase in %GI. As apo-Lf concentration increased from 0.1 to 2.4 mg/ml, there was a rapid increase in %GI. Above 2.4 mg of apo-Lf/ml, the rate of increase was slower. Of interest was the fact that 21 mg of apo-Lf/ml resulted in 95% GI when 0.25 mg of citrate/ml was present in the assay. This was in contrast to only 60% GI for a comparable apo-Lf concentration in the absence of citrate (see Fig. 3).

When apo-Lf was held constant (0.15 mg/ml), there was a marked decline in %GI as the citrate concentration increased from 0.002 to 0.2 mg/ml. The presence of only 0.002 mg of citrate/ml reduced the %GI of 0.15 mg of apo-Lf/ml from 98 ot 89% (see Fig. 3). At 0.2 mg of citrate/ml, %GI was reduced to 4%.

The data shown in Fig. 5 suggest that the absolute concentrations of citrate or apo-Lf were not as critical to %GI as the ratio of the two components. The molar ratio of citrate to apo-Lf (micromoles of citrate per milliliter divided by micromoles of apo-Lf per milliliter) was calculated for each of the data points (Fig. 6). As the molar ratio of citrate to apo-Lf increased, the %GI decreased. Fifty percent GI was achieved at a ratio of approximately 75, whereas at ratios greater than 300, less than 10% GI occurred.

DISCUSSION

The results presented establish that bovine Lf, when not fully saturated with iron, can inhibit the growth of coliform bacteria known to cause bovine mastitis. Likewise, the results are consistent with published data suggesting that Lf exerts its growth-inhibitory effects on bacteria by depriving bacteria of the iron re-

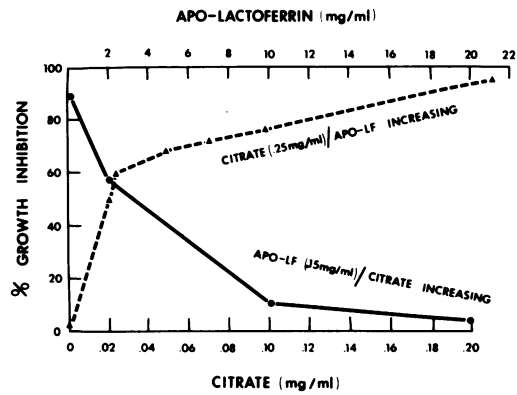

FIG. 5. *Effect of apo-Lf plus citrate on %GI of K. pneumoniae (OARDC-A1). In one set of assays, apo-Lf was constant (0.15 mg/ml) and citrate increasing. In the other, citrate was constant (0.25 mg/ml) and apo-Lf increasing.*

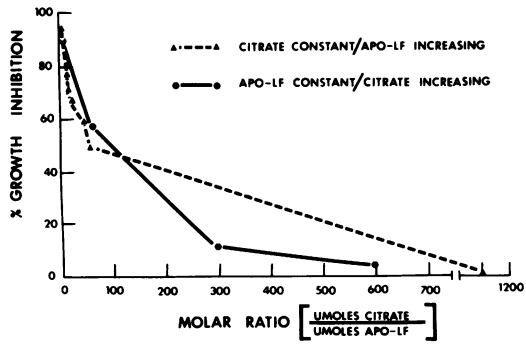

FIG. 6. *Effect of the citrate to apo-Lf molar ratio on %GI of K. pneumoniae (OARDC-A1).*

quired for normal growth (4, 15, 19, 32). The addition of apo-Lf to the culture medium at levels greater than 0.02 mg/ml inhibited growth of all eight coliform strains tested. That the growth-inhibitory effects observed were in fact due to the iron-binding function of Lf was supported by demonstrating that growth was not inhibited when exogenous iron plus apo-Lf or iron-saturated Lf was added to the culture medium.

The synthetic medium used was not iron limiting for growth and, with the exception of *Klebsiella* spp. (K6-24) and *E. coli* (33-C4), some growth occurred during the 12-h incubation period at all levels of apo-Lf tested. In addition, the results show that at apo-Lf concentrations at or above those required for maximum inhibition, further increases in concentration of apo-Lf did not increase or sustain inhibition of bacterial growth. These findings may in part be explained by the reported data pertaining to the iron-sequestering mechanisms of coliform bacteria (24, 26, 35).

Rosenberg and Young (26) have reported that enteric bacteria have at least two systems for sequestering iron. One system involves the synthesis and excretion into the medium of enterochelin, a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine. The enterochelin system is repressed by iron and expressed when iron is limiting in the growth medium. That enterochelin could compete effectively with Lf for available iron is suggested by reports that they have similar affinity constants for iron binding (3, 25). Thus the decreased growth inhibition at the higher concentrations of apo-Lf may relate to the ability of the coliform bacteria tested to produce enterochelin or enterochelin-like compounds in response to limited available iron in the medium. Additional experiments are required to determine the ability of the eight coliform strains to produce iron-sequestering compounds when grown in medium containing apo-Lf.

The results with *E. coli* (33-C4) differ significantly from those with the other organisms in that increasing apo-Lf concentrations were increasingly inhibitory to growth and 20 mg of apo-Lf/ml was bactericidal. If apo-Lf exerts its growth-inhibitory effect by limiting available iron to the bacteria, then this *E. coli* must differ significantly in its ability to sequester iron from low-iron environments. On the other hand, the bactericidal action of 20 mg of apo-Lf/ml may relate to as yet undefined mechanisms. Steel (31) reported that a human enteropathogenic strain of *E. coli* could adsorb Lf, and a specific interaction of Lf with lysozyme-treated *Micrococcus lysodeikticus* cells has been reported (F. L. Schanbacher, K. L. Smith, and L. C. Ferguson, Fed Proc. 31:936, 1972). In addition, Schade (27) observed an apparent increase in lytic activity of *Staphylococcus aureus* when cultures contained Lf as compared with cultures in basal medium alone.

The enteric bacteria possess a second iron-sequestering mechanism involving citrate (26). The citrate-mediated iron transport system is induced by the presence of citrate in the growth medium. Reiter et al. (22) have suggested that the presence of citrate in bovine colostrum counteracts the growth-inhibitory effects of lactoferrin for two strains of *E. coli* associated with neonatal diarrhea. The present data support and extend these observations to include a coliform strain associated with bovine mastitis.

Results of the present study suggest that the degree of growth inhibition when both citrate and apo-Lf were present in the medium was related to the molar ratio of the two components. A molar ratio of 75 (citrate to Lf) was found to result in 50% GI of *K. pneumoniae* (OARDC-A1), and a ratio of 300 or greater resulted in less than 10% GI. These findings may relate significantly to the potential role of Lf in providing protection of the bovine mammary gland to coliform infection.

The concentrations of citrate and Lf undergo dramatic changes during involution and lactogenesis (20, 28, 29). The changes are such that the molar ratio of citrate to Lf would decrease with involution and increase with lactogenesis. Based on reported values for the concentration of both citrate and Lf in normal milk (20, 22, 28), secretion from the involuted gland (20, 28), and colostrum (20, 28), the citrate-to-Lf molar ratio would be in excess of 1,000 for normal milk and colostrum but approximately 10 in secretion from the fully involuted bovine mammary gland. Thus, on the basis of the in vitro data presented here, Lf in secretion from fully involuted glands would be expected to inhibit growth of coliforms. This, of course, would only be true if the Lf were not fully saturated with iron.

In contrast to the secretion of the involuted gland, Lf in colostrum and milk would be expected to exert little influence on coliform growth, and this is supported by the work of Reiter et al. (22). In addition, Reiter and Bramley (21) have reported data showing that the involuted gland is more resistant than the lactating gland to coliform infection.

The ability of *E. coli* to sequester iron from the host environment has been suggested by Rogers (24, 25) as a true virulence factor. Thus,

in bovine mammary secretions, coliform bacteria would be forced to compete with Lf for iron, and as such Lf could be considered a natural factor of resistance to infection of the mammary gland. However, it is obvious from the data presented here that other factors in mammary secretions such as citrate, iron, and possible bicarbonate (17, 22) as well as the iron-sequestering mechanisms of pathogens will have to be evaluated before the true role of Lf in natural defense of the mammary gland can be fully evaluated.

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