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Bacteriostatic Effect of Human Milk and Bovine Colostrum on *Escherichia coli*: Importance of Bicarbonate

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At pH 7.4 and in the presence of $NaHCO_3$, human milk and bovine colostrum inhibited the growth of *Escherichia coli* O111. Adding sufficient iron to saturate the iron-binding capacity of the lactoferrin present in the milk or colostrum prevented bacteriostasis. At pH 6.8 neither milk nor colostrum inhibited *E. coli* O111. Adjusting the pH to 7.4 with $NaHCO_3$ resulted in the development of bacteriostatic activity. Adjusting the pH to 7.4 with NaOH was ineffective. Dialyzed colostrum and milk inhibited bacterial growth at pH 6.8 in the absence of added $NaHCO_3$; addition of citrate or iron abolished bacteriostasis. The chromatographic elution profile of tyrosyl-transfer ribonucleic acid (tRNA) from iron-replete *E. coli* differs significantly from that of tyrosyl-tRNA from iron-deficient organisms. Examination of the elution profile of tyrosyl-tRNA from *E. coli* O111 growing in colostrum without added $NaHCO_3$ showed that such bacteria were fully replete in iron. The nature of the elution profile of tyrosyl-tRNA also showed that iron was freely available to the bacteria when citrate was added to dialyzed colostum but not available in its absence, even at pH 6.8. Results support the idea that the bacteriostatic action of milk and colostrum, due to the combined action of antibody and lactoferrin, depends on the addition of bicarbonate to counteract the iron-mobilizing effect of the citrate normally present in these secretions.

The greater resistance of breast-fed infants to enteric infections compared to those fed artificial food is well known, and available evidence points strongly to a direct protective role for human milk in the intestinal tract (8, 17). Human milk has been shown to have a bacteriostatic effect on *Escherichia coli* O111, a strain frequently associated with diarrhea in babies, and it has been suggested that this inhibitory property of human milk may play an important part in resistance to infantile *E. coli* enteritis by suppressing bacterial growth in the small intestine (5). The bacteriostatic action of human milk (5), and also of bovine colostrum (13), on *E. coli* O111 and other serotypes has been attributed to the combined action of antibody and the iron-binding protein lactoferrin (4, 5). Human milk and bovine colostrum contain large amounts (1 to 6 mg/ml) of iron-binding protein, of which the greater proportion is lactoferrin, although small amounts of transferrin are also present (11, 13). These iron-binding proteins have an association constant for iron of about 10^{36} and are normally only partly saturated with Fe^{3+} (3, 4). Lactoferrin alone has only a slight inhibitory effect on the growth of *E. coli* O111, but, on adding specific antibody, bacteriostasis ensues. Addition of sufficient ferric iron to saturate the iron-binding capacity of the lactoferrin abolishes this inhibition (5). It has been suggested, therefore, that the bacteriostatic action of human milk and bovine colostrum on *E. Coli* O111 is due to an interference, by the antibody and lactoferrin present, with bacterial iron supply. Lactoferrin itself fails to inhibit growth because *E. coli* secrete the iron-chelating agent enterochelin, which serves to remove iron from such iron-binding proteins. Antibody is thought to block this essential process by interfering with the production of enterochelin (4).

Previous work has shown, however, that neither bovine colostrum nor human milk is bacteriostatic to *E. coli* O111 unless the pH is adjusted to 7.2 to 7.4 by addition of $NaHCO_3$ (5, 13). Bullen et al. (5) attribute the development of bacteriostasis to the change in pH. Reiter et al. (13), working with bovine colostrum, consider bicarbonate itself to be the important factor and the pH per se not to be involved. The latter workers attribute the lack of inhibition to the presence of citrate in the colostrum and suggest that citrate might compete with the lactoferrin for iron and make it available to the bacteria. They suggest that the addition of bicarbonate, which is required for the binding of

iron by transferrin and lactoferrin (3, 10), overcomes the effect of citrate. Human milk also contains citrate (12).

We have now reexamined this problem and from our results conclude that it is indeed the bicarbonate rather than the pH that is important for bacteriostasis.

MATERIALS AND METHODS

Organisms and media. *E. coli* O111 K58 H2 was originally obtained from J. Taylor, Central Public Health Laboratories, Colindale, London (5).

Organisms were stored in a 10% (wt/vol) mixture of papain digest broth in 0.15 M NaCl (10% broth-saline) at −70°C. The bacteria were grown in papain digest broth for 3 h at 37°C. Cultures were harvested by centrifugation, and the organisms were resuspended in 10% broth-saline. The total cell count per milliliter was obtained by the use of a colorimeter (E.E.L. filter no. 622) and estimating the value from a standard graph. Suitable dilutions for inoculation into milk and colostrum were made in 10% broth-saline. Viable counts were made on fresh blood agar plates. All samples from colostrum and human milk were homogenized beforehand for 1 to 2 min in an MSE homogenizer cooled in an ice bath to ensure that agglutination did not affect the measurement of the viable count.

Bovine colostrum and human milk. Colostrum was obtained from normal cows within 12 h of parturition. Whey was prepared by incubating with rennet (0.1%, wt/vol) for 40 min at 37°C and removing the clot by centrifugation at 5°C. The whey was sterilized by filtration first through a 3-μm membrane filter (Millipore Corp.) and then through a 0.45-μm membrane filter and stored at −20°C. The 3-μm filtration step greatly facilitated filtration through the 0.45-μm filter.

Samples of human milk obtained at different stages of lactation were pooled and stored at −20°C. Before use, the milk was centrifuged at 56,000 × *g* for 60 to 90 min, and the fluid was separated from the fat and sterilized by filtration first through a 3-μm membrane filter and then through a 0.45-μm membrane filter.

Unsaturated iron-binding capacities were determined according to the method of Bullen et al. (5). The optical density at 470 nm was recorded after the addition of 0.01- or 0.025-ml aliquots of ferric nitrilotriacetate (1 mM, pH 7.4) to 2 ml of colostrum or milk filtrate containing $NaHCO_3$ (0.6%, wt/vol) in a 1-cm glass cell (2). The unsaturated iron-binding capacity was calculated from a plot of the optical density against the volume of ferric nitrilotriacetate added.

Growth of bacteria under controlled conditions. Growth experiments were carried out in 10-ml amounts of bovine colostrum (whey) or human milk at 37°C in jacketed culture vessels as described previously for serum cultures (6). $NaHCO_3$ was added to give a concentration of 0.6%, wt/vol (0.071 M), and the pH was maintained at 7.4 throughout the experiment by passing a sterile gas mixture consisting of an equal volume of (95% air + 5% CO_2) and (95% N_2 + 5% CO_2) over the surface of the stirred liquid. When this gas mixture was passed over the surface of milk or colostrum not containing $NaHCO_3$, the resulting pH was 6.8.

In some experiments the pH was adjusted to and maintained at 7.4 by adding 0.1 or 1 M NaOH automatically, using an automatic titrator (Radiometer, Copenhagen). In these experiments 0.15 M NaCl was added to the milk or colostrum in place of the small volume of $NaHCO_3$ usually added, and the gas phase above the liquid surface was air.

Dialysis of bovine colostrum and human milk. Bovine colostrum and human milk contain high concentrations of citrate (12, 13). Since citrate can mediate exchange of iron between transferrin molecules (1), it was possible that direct dialysis to remove citrate might also result in the loss of iron from the iron-binding proteins present, the iron being lost as the iron-citrate complex. To prevent this, dialysis was first carried out in the presence of added $NaHCO_3$, and the $NaHCO_3$ was then removed by further dialysis.

$NaHCO_3$ was added to bovine colostrum and human milk to give a concentration of 0.6%, wt/vol (0.071 M). Each was then dialyzed first against NaCl (0.15 M), $MgCl_2$ (1 mM), $CaCl_2$ (0.3 mM), and $NaHCO_3$ (0.071 M) (two changes, 30 volumes), and then against NaCl (0.15 M), $MgCl_2$ (1 mM), and $CaCl_2$ (0.3 mM) (three changes, 40 volumes). Dialysis was carried out at 0 to 4°C. The volume of both colostrum and milk increased by 30 to 50% during dialysis. Lactose (1%, wt/vol) was added to both before use to partly replace that lost during dialysis. The pH was adjusted to 6.8 with HCl and maintained at this level during experiments by automatic addition of NaOH. In the absence of bacterial growth, no NaOH was utilized. In experiments where citrate was added back to the colostrum to give a 1 mM solution, the amount of iron added as an impurity in the reagent was extremely low, the maximum estimated level being of the order of 10^{-9} M.

Examination of tyrosyl-tRNA in *E. coli* O111 under different growth conditions. To examine the tyrosyl-transfer ribonucleic acid (tRNA) of *E. coli* O111, the bacteria were grown at 37°C in 150 ml of colostrum contained in a round-bottomed flask fitted with a condenser and gas inlet. A sterile gas mixture consisting of an equal volume of (95% air + 5% CO_2) and (95% N_2 + 5% CO_2) was passed over the surface of the stirred liquid (400 ml/min) to maintain the required pH; this was 7.4 when $NaHCO_3$ (0.071 M) was present in the colostrum and 6.8 in the absence of $NaHCO_3$. In some experiments the gas phase was air and the desired pH was maintained by automatic addition of NaOH as described before. Bacteria were added to give 10^5 to 10^6 organisms/ml at the start of the experiment.

After 5 h of growth, the bacteria were collected by centrifugation at 0 to 5°C, washed in 0.15 M NaCl, 0.01 M $NaHCO_3$, and 0.1% (wt/vol) glucose (pH 7.6), and finally resuspended in more of the same solution. tRNA was aminoacylated in vivo with [^{3}H]tyrosine (100 μCi/10 ml of incubation solution, 22 Ci/mmol; Radiochemical Centre, Amersham,

U.K.), and aminoacyl-tRNA was isolated from a mixture of labeled bacteria and "carrier cells" (unlabeled broth-grown *E. coli* O111) by procedures described previously (7). Tyrosyl-tRNA was examined by chromatography on benzoylated diethylaminoethyl-cellulose (BD-cellulose, 50 to 100 mesh; Schwartz Bioresearch). Aminoacyl-tRNA dissolved in 3 to 4 ml of 0.05 M sodium acetate, 0.01 M magnesium acetate, and 0.15 M NaCl (pH 4.5) was applied to a column of BD-cellulose (1.5 by 28 cm) preceded by a narrow band (2 to 3 ml) of the same solution. The BD-cellulose had previously been equilibrated with 0.05 M sodium acetate, 0.01 M magnesium acetate, and 1.0 M NaCl (pH 4.5). The column was then washed with 180 ml of 0.05 M sodium acetate, 0.01 M magnesium acetate, and 1.0 M NaCl (pH 4.5), and the bound aminoacyl-tRNA was eluted with a linear ethanol gradient (0 to 15%, vol/vol, in 600 ml) in the same salt solution. Column chromatography was carried out at room temperature (about 23°C), 6-ml fractions being collected at a flow rate of 47 ml/h. Radioactivity in fractions was measured as described before (7).

RESULTS

Effect of pH and bicarbonate. At pH 7.4 and in the presence of $NaHCO_3$, both human milk and bovine colostrum had a bacteriostatic effect on *E. coli* O111. Addition of sufficient iron to saturate the total iron-binding capacity of each secretion abolished its ability to induce bacteriostasis. Iron was added as ferric dicitrate (2, 18). The growth of *E. coli* O111 in human milk in the presence and absence of added iron is shown in Fig. 1. Similar results were obtained for *E. coli* O111 in bovine colostrum.

Both human milk and bovine colostrum had a pH of 6.8 under 5% CO_2 in the absence of $NaHCO_3$. Under such conditions neither secretion induced bacteriostasis. Figure 2 shows the action of bovine colostrum, under 5% CO_2, on *E. coli* O111 in the presence and absence of added $NaHCO_3$. Adding $NaHCO_3$ increased the pH of bovine colostrum and human milk, under 5% CO_2, from 6.8 to 7.4. To see whether it was the pH or bicarbonate that was responsible for inducing bacteriostasis, bovine colostrum and human milk were adjusted to and maintained at pH 7.4 by the addition of NaOH, using an automatic titrator. Neither bovine colostrum nor human milk was inhibitory to *E. coli* O111 when the pH was adjusted to 7.4 in this way.

Bacteriostatic activity of dialyzed colostrum and human milk. Human milk and bovine colostrum contain high concentrations of citrate (12, 13). To see whether removing the citrate would allow bacteriostasis to occur at pH 6.8, each was dialyzed against a salts solution as described in Materials and Methods. Dialyzed colostrum containing lactose (1%, wt/vol) was inhibitory to the growth of *E. coli* O111 in the absence of added $NaHCO_3$ and at pH 6.8 (Fig. 3). Adding citrate (final concentration, 1 mM) to the dialyzed colostrum abolished its bacteriostatic activity (Fig. 3). Addition of iron also abolished bacteriostasis. Similar results were obtained with dialyzed human milk.

Examination of tyrosyl-tRNA in *E. coli* O111 under different growth conditions. *E. coli* O111 inhibited by bovine colostrum and by human milk contains abnormal species of certain aminoacyl-tRNA's (Griffiths and Humphreys, Proc. Soc. Gen. Microbiol. III, abstr. 61, 1975; unpublished data). Tyrosyl-tRNA is one species of tRNA that appears in an abnormal form in inhibited bacteria. Addition of suffi-

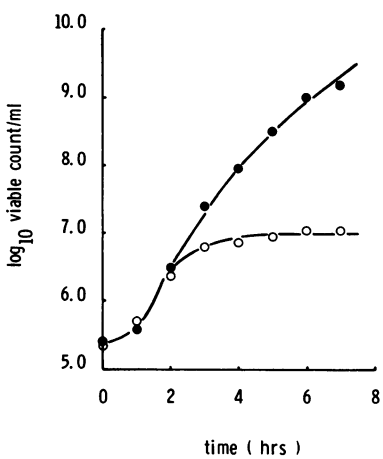

FIG. 1. *Inhibition of E. coli O111 by human milk (pH 7.4, 0.071 M $NaHCO_3$ and 5% CO_2) (○), and the effect of adding iron (●).*

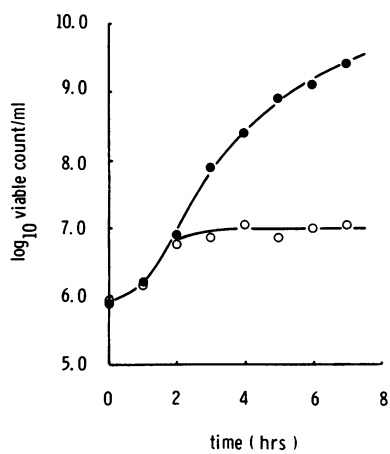

FIG. 2. *Action of bovine colostrum under 5% CO_2 on E. coli O111 with and without added $NaHCO_3$. Symbols: (●) Colostrum without $NaHCO_3$ (pH 6.8, 5% CO_2); (○) colostrum plus 0.071 M $NaHCO_3$ (pH 7.4, 5% CO_2).*

cient iron to saturate the iron-binding capacity of the milk or colostrum prevents the appearance of abnormal tRNA's and also allows bacterial growth. Figure 4 shows the elution profile of tyrosyl-tRNA from *E. coli* O111 inhibited by bovine colostrum containing $NaHCO_3$ (pH 7.4, $NaHCO_3$, 5% CO_2) and that from *E. coli* O111 growing in bovine colostrum without added $NaHCO_3$ (pH 6.8, 5% CO_2). Tyrosyl-tRNA from inhibited *E. coli* O111 showed a major peak of abnormal tRNA eluting ahead of the normal position together with a small peak of the normal type. The elution profile of tyrosyl-tRNA from *E. coli* O111 growing in colostrum at pH 6.8, however, was identical to that of the tRNA extracted from broth-grown, iron-replete *E. coli* O111. It showed only one peak, which eluted at the same position as normal tRNA. Tyrosyl-tRNA from bacteria growing in colostrum (pH 7.4, $NaHCO_3$, 5% CO_2) containing sufficient Fe^{3+} to abolish bacteriostasis also showed one peak, eluting at the same position as the tRNA from broth-grown, iron-replete *E. coli* O111 (not shown). Tyrosyl-tRNA extracted from *E. coli* O111 inhibited by dialyzed bovine colostrum at pH 6.8, in the absence of $NaHCO_3$, showed an abnormal elution profile. It was identical to that from bacteria inhibited by colostrum at pH 7.4 in the presence of $NaHCO_3$ (pH 7.4, $NaHCO_3$, 5% CO_2). The elution profile of tyrosyl-tRNA from *E. coli* O111 growing in dialyzed colostrum at pH 6.8 in the presence of added citrate (1 mM), however, showed one peak. This eluted in the same position as tyrosyl-tRNA from iron-replete *E. coli* O111.

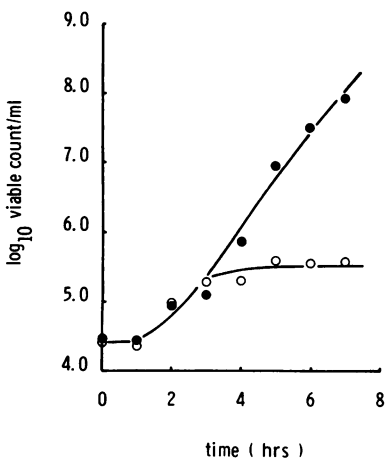

FIG. 3. *Bacteriostatic activity of dialyzed bovine colostrum in the absence of added $NaHCO_3$. Symbols: (○) E. coli O111 in dialyzed colostrum adjusted to pH 6.8; (●) E. coli O111 in dialyzed colostrum at pH 6.8 containing added citrate (1 mM).*

Tyrosyl-tRNA from *E. coli* O111 growing in undialyzed colostrum adjusted to and maintained at pH 7.4 using NaOH was mainly normal (80%), although it did contain a small amount of the abnormal species (20%).

DISCUSSION

E. coli grown in media containing less than about 10^{-7} M Fe^{3+} contain abnormal species of certain tRNA's (9, 14, 19). *E. coli* O111 inhibited by serum, bovine colostrum, and human milk also contain abnormal species of aminoacyl-tRNA's (7; Griffiths and Humphreys, Proc. Soc. Gen. Microbiol. III, abstr. 61, 1975; unpublished data). Tyrosyl-tRNA is one species of tRNA that appears in an abnormal form in *E. coli* from chemically made iron-deficient culture media and also from *E. coli* O111 inhibited by milk and colostrum. This abnormality results from a failure to thiomethylate the isopentenyl-adenosine residue adjacent to the 3′ end of the anticodon of the tRNA molecule in the absence of a plentiful supply of iron (14). Adding sufficient iron to saturate the iron-binding capacity of the milk or colostrum prevents the

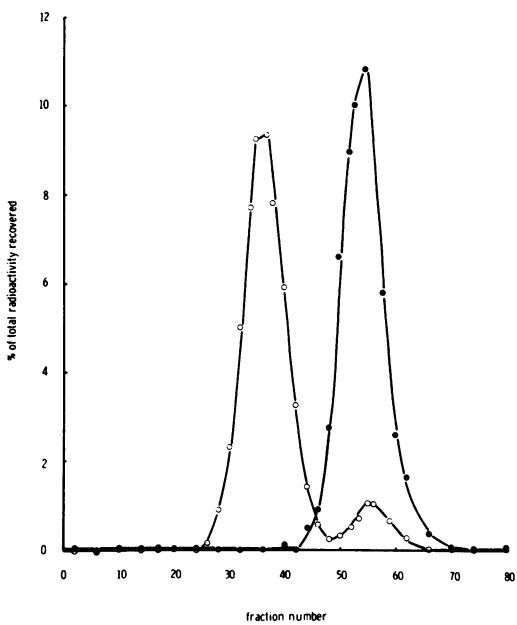

FIG. 4. *Elution profiles, on BD-cellulose, of [^{3}H]tyrosyl-tRNA extracted from : (○) E. coli O111 inhibited by bovine colostrum containing 0.071 M $NaHCO_3$ (pH 7.4, 5% CO_2) and (●) E. coli O111 growing in bovine colostrum without added $NaHCO_3$ (pH 6.8, 5% CO_2). Tyrosyl-tRNA isolated from iron-replete, broth-grown E. coli O111 eluted as a single peak in the same position as the righthand peak (not shown)*

appearance of abnormal tRNA's and also allows bacterial growth. Preliminary work suggests that although the tRNA alterations are not directly responsible for inhibiting growth, they may be necessary for inhibition to occur (Griffiths and Humphreys, Proc. Soc. Gen. Microbiol, IV, in press). The tRNA alterations themselves are, almost certainly, connected with the adaptation of *E. coli* to iron-restricted conditions. This raises the very interesting possibility that changes in tRNA's induced by iron-binding proteins in tissue fluids, resulting possibly in further phenotypic changes in the bacteria, may be of considerable importance to the pathogenicity of *E. coli* and other bacteria. This work will be discussed in detail elsewhere.

By using the fact that the chromatographic elution profile of tyrosyl-tRNA from iron-replete *E. coli* differs so much from that of tyrosyl-tRNA from iron-deficient bacteria, we have shown that *E. coli* O111 growing in colostrum at pH 6.8 without added $NaHCO_3$ are indeed iron replete. Tyrosyl-tRNA extracted from *E. coli* O111 inhibited by colostrum eluted from chromatography columns as a major abnormal peak together with a very small peak of the normal species. Tyrosyl-tRNA extracted from *E. coli* O111 growing in colostrum (pH 7.4, $NaHCO_3$, 5% CO_2) to which sufficient iron had been added to saturate the lactoferrin eluted as a single peak of the normal species. The tyrosyl-tRNA from *E. coli* O111 grown in bovine colostrum without added $NaHCO_3$ (pH 6.8, 5% CO_2) also eluted as a single peak of normal tRNA; no trace of the abnormal form was seen, thus showing that iron was freely available to the bacteria under these conditions. When iron was freely available, the bacteriostatic mechanism did not operate and the bacteria grew.

To resolve the point of whether it was pH or $NaHCO_3$ that was responsible for reducing the availability of iron and inducing bacteriostasis, colostrum and milk were adjusted to and maintained at pH 7.4 by addition of NaOH, using an automatic titrator. Neither human milk nor bovine colostrum was inhibitory to *E. coli* O111 under these conditions. By using dialyzed colostrum and milk, however, it was possible to get bacteriostasis even at pH 6.8 in the absence of added $NaHCO_3$. This inhibition was abolished by adding iron or citrate. The nature of the chromatographic elution profile of tyrosyl-tRNA clearly showed that iron was freely available to the bacteria when citrate was present in the dialyzed colostrum but not available in its absence, even at pH 6.8.

These results support the conclusions that (i) bicarbonate rather than pH is the important factor for bacteriostasis (13) and (ii) it is the presence of citrate in the milk and colostrum that allows bacterial growth and necessitates the addition of excess $NaHCO_3$. The fact that *E. coli* O111 grown in colostrum at pH 6.8 in the absence of $NaHCO_3$ is fully iron replete indicates that the citrate present makes lactoferrin-bound iron available to the bacteria. It is known that the iron-citrate complex can be utilized by *E. coli* and promotes their growth in low-iron media (15). Citrate-mediated release of protein-bound iron appears to be blocked by excess bicarbonate. Such a conclusion agrees with observations regarding the ability of increased bicarbonate levels to favor the formation of a stable Fe^{3+}-transferrin-bicarbonate ternary complex in the presence of citrate (1, 2, 16). The rapid absorption of citrate and the presence of bicarbonate in intestinal secretions would, therefore, make the local environment of the small intestine of the neonate, where it is considered that the antibacterial property of milk and colostrum operates, suitable for the inhibition of *E. coli* by the combined action of lactoferrin and antibody.

The pH itself, however, does appear to influence the efficiency of citrate-mediated release of Fe^{3+} from bovine lactoferrin in the absence of $NaHCO_3$. *E. coli* O111 growing in colostrum adjusted to pH 7.4 with NaOH were found to contain a small amount of abnormal tyrosyl-tRNA, suggesting that citrate-mediated Fe^{3+} release is slightly less efficient at pH 7.4 than at pH 6.8. Iron restriction under such conditions, however, was not sufficient to inhibit bacterial growth.

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