Characterization and Biological Role of Human Lactotransferrin Complexes

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Several epidemiologic studies have indicated that breast feeding protects infants from bacterial and viral infections and from allergy (1–6). Recent prospective studies performed by Chandra (7) in an industrialized country and in a developing one confirm that in both countries, breast-fed infants have lower morbidity than those artificially fed. One of the most striking differences between breast- and bottle-fed infants concerns the fecal pH and bacterial flora. The feces of breast-fed infants contain predominantly Bifidobacterium bifidum, whereas those of bottle-fed infants are characterized by the presence of high quantities of Bacteroides, Clostridium, and Escherichia coli (8). According to Beerens et al. (9), cow’s milk as well as sheep’s milk, pig’s milk, and artificial human milks do not promote the growth of Bifidobacterium bifidum but do show activity on Bifidobacterium infantis and Bifidobacterium longum.

Human milk therefore contains two kinds of factors that contribute to the intestinal protection. The first group consists of antibacterial components such as secretory immunoglobulin A (sIgA), lactotransferrin (also called lactoferrin), lysozyme, and lactoperoxidase. The second group includes Bifidobacterium bifidum growth-promoting factors, which are essentially oligosaccharides. The na-
ture of the antibacterial and growth-promoting factors has been summarized in several review articles (10–17). Comparative study of the biochemical compositions of human and bovine milk (18,19) has shown that the essential constituents considered as protecting the infant’s intestinal tract are absent or are present in low concentration in cow’s milk.

Several procedures have been applied for isolating the oligosaccharides (20) and proteins (18,21,22) from human milk. In the course of experiments with electrophoretic separation, differences were noticed in the mobility of lactotransferrin present in milk and that of lactotransferrin extracted from this milk. The differences may be explained by the interaction of lactotransferrin with proteins and with nonprotein compounds present in the milk. Since it has not been determined whether the complexed lactotransferrin possesses biological properties similar to purified lactotransferrin, we have attempted to isolate the lactotransferrin complexes and to analyze their bacterial activities. Fractionation of milk proteins was performed by precipitation with ammonium sulfate, and the bacteriostatic activity as well as the Bifidobacterium bifidum growth-promoting activity of the different fractions were determined in vitro. Subsequently, the fraction possessing the highest antibacterial activity was selected for the treatment of infants with acute diarrhea.

The present report concerns the characterization of lactotransferrin-lysozyme and lactotransferrin-glycopeptide complexes in human milk and the description of the bacteriostatic activities of these complexes in vitro and in vivo.

MATERIALS AND METHODS

Isolation of the Milk Fractions

Samples of human milk were collected from a local milk bank, pooled, and frozen at –20°C until use. The thawed milk was defatted by centrifugation and then decaseinated. The whey proteins were fractionated by a combined concentration gradient of (NH₄)₂SO₄ and pH gradient as described by Montreuil et al. (23) and Montreuil
The lactotransferrin–lysozyme complex was isolated by ion-exchange chromatography on SP-Sephadex column and by gel filtration on an Ultrogel ACA-44 column.

Identification and Estimation of the Proteins

The proteins were submitted to electrophoresis on cellulose polyacetate strips using a barbital sodium buffer, pH 8.6. The lactotransferrin complexes were identified by cross immunoelectrophoresis using a monospecific antiserum to lactotransferrin according to the procedure described by Weeke (24).

The estimation of lactotransferrin was determined by a single radial immunodiffusion technique (25). Lysozyme activity was assessed from the enzymatic lysoplate assay, which quantitates the lysozyme-mediated lysis of killed Micrococcus lysodeikticus cells (26).

Bacterial Activities

Antibacterial activities were analyzed by measuring the inhibition of growth of Escherichia coli O111:B4 in a Ringer–tryptone medium (27) containing the different milk fractions. The optical density was followed using an automatic biophotometer.

The test for bifidus growth factors was performed by the method described by Neut et al. (28) using Bifidobacterium strains freshly isolated from infant feces. The antibacteriostatic activity of the milk fraction P7–8 was tested on five infants suffering from acute diarrhea. These infants were fed on a regimen containing 2 g of the P7–8 fraction over a period of 8 days. The intestinal flora of these infants was determined before and after treatment.

RESULTS

Analysis of the Milk Fractions

A total of seven whey precipitates was isolated after fractionation of human milk proteins with ammonium sulfate. The nature of
the proteins of each precipitate was identified by electrophoresis and by immunoelectrophoresis (Table 1).

**Bacterial Activities of the Milk Fractions**

The bacteriostatic activity of the different fractions as well as the capacity of these fractions to promote the growth of *Bifidobacterium bifidum*, *Bifidobacterium longum*, and *Bifidobacterium infantis* are given in Table 2. These results show that the most interesting fraction is the precipitate $P_{7-8}$, which contains *Bifidobacterium* growth-promoting factors and possesses bacteriostatic activity.

**Analysis of the Fraction $P_{7-8}$**

The estimation of different proteins by the radial immunodiffusion method has shown that fraction $P_{7-8}$ contains 70% lactotransferrin, 6% secretory component, and 7% lysozyme. In addition, this fraction contains 17% carbohydrates as shown by chemical

**TABLE 1. Composition of the precipitates obtained by fractionation of human milk whey with ammonium sulfate (29)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$(\text{NH}_4)_2\text{SO}_4$ saturation</th>
<th>pH</th>
<th>Nature of the proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_1$</td>
<td>0.33</td>
<td>7.0</td>
<td>Galactothermin; slgA; $\alpha$-lactalbumin; secretory component</td>
</tr>
<tr>
<td>$P_2$</td>
<td>0.33</td>
<td>4.6</td>
<td>$\alpha$-Lactalbumin; slgA; serum albumin; secretory component; lactotransferrin; $\alpha_1$-antitrypsin (traces)</td>
</tr>
<tr>
<td>$P_3$</td>
<td>0.33</td>
<td>3.8</td>
<td>Serum albumin; $\alpha$-lactalbumin; lactotransferrin (traces)</td>
</tr>
<tr>
<td>$P_1$</td>
<td>0.50</td>
<td>7.0</td>
<td>slgA; IgG; IgM; lactotransferrin (traces)</td>
</tr>
<tr>
<td>$P_{5-6}$</td>
<td>0.50</td>
<td>3.8</td>
<td>Serum albumin; lactotransferrin; slgA; $\alpha$-lactalbumin</td>
</tr>
<tr>
<td>$P_{7-8}$</td>
<td>0.75</td>
<td>4.6</td>
<td>Lactotransferrin; secretory component; slgA; lysozyme; serum albumin (traces)</td>
</tr>
<tr>
<td>$P_9$</td>
<td>0.75</td>
<td>3.8</td>
<td>Glycopeptides; peptides; lactotransferrin; $\alpha_1$-antitrypsin; lysozyme</td>
</tr>
<tr>
<td>$P_{10}$</td>
<td>1.0</td>
<td>3.8</td>
<td>Peptides; glycopeptides; $\alpha_1$-antitrypsin (traces)</td>
</tr>
</tbody>
</table>
TABLE 2. *Bacterial activities of the different fractions obtained from human milk whey by ammonium sulfate fractionation*

<table>
<thead>
<tr>
<th>Bacterial activity</th>
<th>Fraction</th>
<th>Dialyzable oligosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_1</td>
<td>P_2</td>
</tr>
<tr>
<td>Inhibition of the growth of <em>Escherichia coli</em> O_{11}B_{4} Bifidobacteria</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Bifidobacterium bifidum</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bifidobacterium infantis</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

analysis. Cross immunoelectrophoresis of the fraction P_{7-8} in the presence of a rabbit serum antihuman lactotransferrin shows that the profile is quite different from those obtained from a sample of purified lactotransferrin or from fractions P_{5-6} and P_9. In particular, two different types of lactotransferrin may be identified in this fraction (Fig. 1).

**Isolation and Properties of the Lactotransferrin–Lysozyme Complex**

In order to identify the lactotransferrin complexes present in fraction P_{7-8}, the latter was submitted to ion-exchange chromatography on an SP-Sephadex column. A fraction containing lactotransferrin and lysozyme was separated from the other components of P_{7-8}. This fraction was further submitted to gel filtration on an Ultrogel AcA-44 column, and free lysozyme was separated from a lactotransferrin–lysozyme complex. The complex has a molecular weight of 110,000 and results from the stoichiometric association of 2 moles of lysozyme per mole of lactotransferrin. As shown in Fig. 2, the electrophoretic mobility of the lactotransferrin–lysozyme
complex was quite different from those of pure lactotransferrin and of pure lysozyme. The difference in the electrophoretic mobilities explains the presence of two peaks in the profile of \( P_{7-8} \) characterized by cross immunoelectrophoresis.

**Characterization of Lactotransferrin–Glycopeptide Complexes**

Addition of increasing amounts of precipitate \( P_{10} \) containing essentially glycopeptides having a molecular weight of 20,000 to pure lactotransferrin leads to the formation of complexes between lactotransferrin and glycopeptides having an electrophoretic mobility more anodic than that of pure lactotransferrin (Fig. 3) (29,21).
FIG. 2. Polyacetate electrophoresis of lactotransferrin–lysozyme complex (1), purified human lactotransferrin (2), and purified human lysozyme (3). Arrows indicate the starting points.

FIG. 3. Electrophoretic mobility of pure lactotransferrin (LTF) compared to lactotransferrin complexes obtained by adding increasing amounts of the glycopeptides present in fraction $P_{10}$ to pure lactotransferrin. Such complexes probably exist in the fraction $P_{7-8}$ but have not been isolated. Indeed, a dissociation occurs during ion-exchange chromatography on SP-Sephadex as a result of the weakness of the ionic associations of the lactotransferrin–glycopeptides complex.
Biological Role of P$_{7-8}$ Fraction

*In vitro*, the fraction P$_{7-8}$ appears to be more bacteriostatic than pure lactotransferrin. Therefore, we have employed this fraction for the treatment of five babies suffering from acute diarrhea and intolerance to bovine milk proteins. The babies received 2 g of P$_{7-8}$ for a period of 8 days, and the effect of this diet was assessed by identifying the duodenal microflora. Results did not show a decrease of the number of enteropathogenic strains such as *Escherichia coli* or *Streptococcus*; however, a significant decrease was noted in the case of *Staphylococcus*, *Pseudomonas maltophilia*, and *Erwinia*. Subsequent to treatment, the tolerance of the children toward the bovine and human milk proteins was restored.

DISCUSSION

Lactotransferrin, by its iron-binding capacity, represents one of the most powerful bacteriostatic constituents of human milk. Its activity is retained in the gut as a result of its resistance to digestive proteases. In fact, we have demonstrated (30) that lactotransferrin is still present in the feces of breast-fed infants and that it has retained its ability to bind ferric ions reversibly. We have shown in this chapter that in human milk, lactotransferrin interacts with other constituents such as lysozyme and glycopeptides. We believe that the interaction of lactotransferrin with these constituents increases the biological activity of the lactotransferrin. In a recent publication, Perraudin and Prieels (31) have shown that the protoplasts produced by the action of lysozyme on *Micrococcus luteus* are agglutinated by free human and bovine lactotransferrins. Thus, lysis and agglutination appear to be increased by the presence of lactotransferrin–lysozyme complex.

It is generally agreed that the first step of infection is mediated by bacterial adherence to mucosal surfaces. This interaction is explained by a mechanism that involves recognition of sugars by specific bacterial lectins (32). In experiments carried out in collaboration with Dr. Mirelman (33,34), an inhibition of *Shigella flexneri* binding to the mucus extracted from colonic cells of guinea pigs
was observed in the presence of free fucose and of asialoglycopeptides containing fucose residues. The glycopeptides that form complexes with lactotransferrin are fucose-rich. Therefore, the lactotransferrin, which contains two glycans with fucose residues, and the complexes of lactotransferrin with fucose-rich glycopeptides may constitute powerful potential inhibitors for the adhesion of some bacteria.

These results represent strong arguments in favor of the use of therapeutic milks supplemented with lactotransferrin and with polysaccharides containing monosaccharide units such as mannose, which are able to inhibit the adhesion of bacteria, *Escherichia coli* in particular (35), to the mucosal surface.

In summary, the importance of fresh human colostrum and milk for the prevention of *E. coli* diarrhea (36,37) may result from the presence of several milk constituents, among which are sIgA, lactotransferrin, lysozyme, and glycopeptides. Human lactotransferrin possesses a bacteriostatic activity applicable by a ferriprivation mechanism. In addition, it has been shown *in vitro* that lactotransferrin and sIgA together have a powerful bacteriostatic effect against pathogenic strains of *Escherichia coli* responsible for diarrhea that is greater than their effect when used alone (38–40). An increase in the effect of lactotransferrin is also demonstrable *in vitro* by complex formation among this protein and lysozyme and sugars. However further experiments *in vivo* are necessary in order to assess the clinical significance of this observation.

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REFERENCES