SECRETORY IgA AGAINST ENTEROTOXINS IN BREAST-MILK

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Summary A pool of colostrum from Guatemalan mothers (Guatemalan colostrum) obtained 2–4 days post partum inhibited the induced fluid accumulation in rabbit ileal loops when incubated with Vibrio cholerae or Escherichia coli enterotoxin. There was a linear relationship between the quantity of colostrum used and the protection achieved. Pools of Guatemalan breast-milk obtained 15–30 days post partum and North American breast-milk had the same effect when tested with E. coli and V. cholerae enterotoxins, respectively. The antitoxic activity of a given pool correlated with its IgA content but not with the concentration of IgG or IgM. Guatemalan colostrum globulins were precipitated by ammonium sulphate. The globulins were filtered through a 'Biogel A5' column and fractions obtained. When tested in rabbit ileal loops the antienterotoxin activity in these fractions closely paralleled their IgA but not their detectable IgG or IgM content. We hypothesise that IgA antibody to enterotoxin, present in breast-milk of normal mothers, is probably a manifestation of natural immunity. The passive transfer of these antibodies to the infant may explain why breast-milk prevents E. coli diarrhoea in the neonate.

Introduction

INFANTILE diarrhoeas are now the leading cause of death during the first year of life in the developing world.1 Nurseries for premature infants frequently have epidemics of severe diarrhoeas caused by toxigenic Escherichia coli.2,3 Clinical observations have suggested that breast-milk could protect the neonate from infectious diseases and this has been confirmed by epidemiological studies and defined case-reports.4–6 Gindrat et al. have demonstrated that human breast-milk contains agglutinating antibodies toward the common serotypes of E. coli, but it is uncertain whether these antibodies rid the gastrointestinal tract of this organism.

The pathogenic effects of certain E. coli diarrhoeas are due to the production of an exotoxin (enterotoxin) immunologically similar to that of Vibrio cholerae.6 Since immunity to cholera is probably mediated by IgA antibodies,9 we have explored the possibility that human breast-milk contains IgA antibodies toward enterotoxins. These antibodies might inactivate E. coli enterotoxin and thereby prevent disease without eliminating the pathogenic bacteria from the host.

Materials and Methods

Breast-milk

Milk was obtained 2–4 days post partum from a group of 20 Guatemalan mothers in the vicinity of Guatemala City (Guatemalan colostrum) and 15–30 days post partum from a separate group of 12 mothers in the same locale (Guatemalan breast-milk). Both were collected by Dr Roberto Sosa, Instituto de Nutricion para centro America y Panama, Guatemala City. Milk was also obtained 15–30 days post partum from a group of 7 mothers in Cleveland, Ohio (North American breast-milk). These three pools were frozen and stored at –20°C. Pools were thawed and centrifuged for 45 min at 3000 r.p.m. to separate the fat. The defatted breast-milk was decontaminated by heating for 30 min at 56°C. Human immunoglobulins were measured by radial immunodiffusion using 'ImmunoPlates' specific for IgH, IgM, and IgA (Hyland Division, Travenol Laboratories Inc., Los Angeles).

Fractionation of Colostrum

20 ml of Guatemalan colostrum was incubated with 20 ml cold saturated ammonium sulphate at 4°C to precipitate the globulin. The precipitate was dissolved in distilled water and dialysed for 2 days against 2 litres of physiological saline. Globulin from 2.8–5 ml of pooled Guatemalan colostrum was applied to 1.5 x 9.5 cm 'Biogel A5' column (BioRad, Richmond, California), and eluted with physiological saline at a flow-rate of 12 ml/h at 4°C. The eluate was monitored at O.D.280 and the fractions assayed by radial immunodiffusion (Hyland Laboratory) for human immunoglobulins. The eluate fractions were pooled into 3–6 groups, concentrated by vacuum diafiltration against physiological saline, and the immunoglobulins again measured. The fractions were then sterilised by passage through a 0.22 µm 'Millipore' filter (Bedford, Massachusetts).

E. coli and V. cholerae Toxins

Partially purified E. coli enterotoxin, was obtained from Dr Nathaniel Pierce, Baltimore City Hospitals. The material is...
the XM-100 (Amicon Products) retentate of the culture supernatant of strain 408-3. It was prepared by Col. Joseph Metzgar at Fort Detrich, Maryland. The E.D$_{50}$ of this material in the rabbit ileal loop test$^{10,11}$ was approximately 100 µg. The stock of V. cholerae was obtained from Dr L. O. Krampitz, Department of Microbiology, Case Western Reserve University. V. cholerae cultures were grown in peptone-saline and crude toxin obtained as described by Sack et al.$^{12}$ The E.D$_{50}$ for cholera toxin was determined by the method of Kasai and Burrows.$^{10}$

**Testing for Toxin Activity**

New Zealand white male rabbits, each weighing 2–3 kg, were fasted 48 h before use. They were anaesthetised with 60 mg pentobarbitone sodium ('Diabutal') and rabbit ileal loops prepared according to Sack's modification$^{13}$ of the method of Kasai and Burrows.$^{10}$ 6 mg (3 E.D$_{50}$) of crude V. cholerae toxin (dialysed, lyophilised culture filtrate) or 300 µg (3 E.D$_{50}$) E. coli toxin was mixed with varying concentrations of breast-milk and incubated for 1 h at 37°C. Control samples of toxins were incubated with saline. 1 ml samples of the mixtures were injected into prepared rabbit ileal loops, the wound closed, and the animals killed 18 h later. At that time the loops were drained into a syringe, the volume of fluid noted, and the length of the loop measured. Toxin activity was determined by volume/length ratio.

**Results**

6 mg (3 E.D$_{50}$) of lyophilised, sterile, crude culture filtrates of V. cholerae were resuspended in various dilutions of Guatemalan colostrum, incubated, and injected into rabbit ileal loops. 0.25–1.0 ml of Guatemalan colostrum completely inhibited the cholera toxin-induced fluid accumulation (see fig. 1). Further dilutions resulted in partial inhibition. There was a linear relationship between the quantity of Guatemalan colostrum used and protection against cholera toxin. The interpolated quantity of Guatemalan colostrum, sufficient to achieve a 50% inhibition of cholera toxin (enterotoxin) activity (I$_{50}$) was 0.05 ml (fig. 1). 300 µg (3 E.D$_{50}$) of semipurified E. coli enterotoxin was also resuspended in similar dilutions of Guatemalan colostrum, incubated, and injected into intestinal loops. There was no statistically significant difference in the dilution curve obtained from that found for the biologically-equivalent quantity of cholera toxin (I$_{50}$) was 0.06 ml (table I).

When dilutions of Guatemalan breast-milk were similarly tested against 3 E.D$_{50}$ of semipurified E. coli enterotoxin, the inhibition was proportional to the quantity of breast-milk used. I$_{50}$ for E. coli enterotoxin of Guatemalan breast-milk was 0.46 ml (table I). Assays of North American breast-milk with 3 E.D$_{50}$ of cholera toxin yielded an I$_{50}$ of 0.43 ml. In two out of five experiments, 1 ml of North American breast-milk completely inhibited cholera toxin activity.

The three major classes of immunoglobulins were measured in crude colostrum and breast milks by radial immunodiffusion (table I). IgA was the predominant immunoglobulin in milk, found in highest concentration in Guatemalan colostrum and in lower concentrations in Guatemalan breast milk. The IgA content of Guatemalan breast-milk and North American breast-milk were identical. Samples of Guatemalan breast-milk and North American breast-milk had approximately the same neutralising activity when the I$_{50}$ was corrected for IgA content (table I). Concentrations of IgM and IgG in breast-milk and colostrum were variable and did not correlate with anti-enterotoxin activity.

In order to further examine the nature of the anti-toxin effect, Guatemalan colostrum was fractionated. Lactalbumin and sugars were removed by precipitation of the globulin with ammonium sulphate followed by dialysis. Then the globulins were gel-filtered through a column of biogel A5. As shown in the lower panel of fig. 2, three peaks of O.D$_{280}$ absorbing material were seen; first the fall-through peak which consisted of lipoproteins (45–65 ml), second an IgA-rich peak (65–100 ml), and finally a third peak of residual lactalbumin and lower molecular weight substances (105–140 ml). The distribution of IgA was heterogeneous (fig. 2, middle panel), possibly due to aggregation and/or presence of

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**Fig. 2.—Gel-filtration analysis of globulin from 5 ml Guatemalan colostrum upon a 1.5 x 95 biogel A5 column.**

*Volume of colostrum with an activity equivalent to 1 ml of the particular column pool.

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**TABLE I—NEUTRALISATION OF ENTEROTOXIN BY BREAST-MILK**

<table>
<thead>
<tr>
<th></th>
<th>Concentration needed to cause a 50% inhibition of E.D$_{50}$</th>
<th>IgG/ml</th>
<th>Specific activity IgA units/µg</th>
<th>E. coli toxin</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guatemalan colostrum</td>
<td>0-05 ml / 0-06 ml / 2650 µg/ml</td>
<td>750</td>
<td>3-1 units/µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guatemalan breast-milk</td>
<td>0-46 ml / N.D.</td>
<td>700</td>
<td>3-3 units/µg</td>
<td></td>
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</tbody>
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*An I$_{50}$ unit is that quantity of antibody necessary to cause a 50% decrease in the volume/length ratio induced by 3 E.D$_{50}$ of enterotoxin. It is interpolated from a dilution curve. N.D. = not determined.
TABLE II—GEL-FILTRATION ANALYSIS OF GUATEMALAN COLOSTRUM

<table>
<thead>
<tr>
<th>Column</th>
<th>Antitoxin activity of E. coli enterotoxin rabbit ileal loop assay</th>
<th>Ig content of concentrated pools (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animals in which complete inhibition was observed/animals tested</td>
<td>IgA</td>
</tr>
<tr>
<td>Physiological saline (control)</td>
<td>1.55</td>
<td>0/3</td>
</tr>
<tr>
<td>Crude colostrum</td>
<td>0.21</td>
<td>2/3</td>
</tr>
<tr>
<td>Pool A</td>
<td>0.53</td>
<td>0/3</td>
</tr>
<tr>
<td>Pool B</td>
<td>0.40</td>
<td>1/3</td>
</tr>
<tr>
<td>Pool C</td>
<td>0.39</td>
<td>2/3</td>
</tr>
<tr>
<td>Pool D</td>
<td>0.48</td>
<td>1/3</td>
</tr>
<tr>
<td>Pool E</td>
<td>1.07</td>
<td>0/3</td>
</tr>
<tr>
<td>Pool F</td>
<td>1.05</td>
<td>0/3</td>
</tr>
</tbody>
</table>

*Non-detectable by radial immunodiffusion.
IgA<100 µg/ml, IgG<20 µg/ml, and IgM<50 µg/ml.

Discussion

Paediatricians believe that breast-fed infants have fewer periods of infectious disease than those artificially fed. Epidemiological studies support this view as they show that breast-fed infants have a lower incidence of diarrheal illnesses, particularly those of bacterial origin. IgA is presumably the major effector of immunity in breast-milk. The ability of exocrine IgA to mediate resistance to viral infection by neutralisation is well established. Nonetheless, it has been difficult to definitively show that the IgA antibodies in breast-milk are capable either of directly killing bacteria, or of eliminating them from the gastrointestinal tract.

A possible insight into the interrelationships of breast-milk, IgA, and passive immunity to bacterial infections is provided by experiences in nurseries for the newborn. For the past 25–30 years, premature infants have been fed cow's milk or artificial formulas during their first few weeks of life. Life-threatening epidemics of *E. coli* induced diarrheas have periodically occurred in such nurseries. Svirsky-Gross and Tassovatz et al. stopped these epidemics by feeding premature infants with human breast-milk. They were, however, puzzled when the infants became asymptomatic long before the causative organism disappeared from their stools. Larguia et al. ameliorated *E. coli*-induced diarrheas without altering bacterial colonisation with very small quantities (5 ml/kg/day) of human colostrum. These studies suggested that the effect of breast-milk on *E. coli* diarrheas might not involve a bactericidal or bacteriostatic mechanism.

Certain strains of *E. coli* cause diarrheas by a mechanism similar to that of *V. cholerae*. They produce an enterotoxin antigenically similar to that of *V. cholerae* which induces an active outpouring of fluid into the gut. Since the most thoroughly defined biological function of IgA is its role as a neutralising antibody, we considered the possibility that IgA antibodies might stop diarrheas promptly by neutralising the enterotoxin of *E. coli*.

Incubation of human breast-milk with enterotoxin prevented accumulation of fluid in the rabbit ileum (see fig. 1). As little as 0.1 ml of colostrum had a significant inhibitory effect which explains how Larguia et al. were able to interrupt *E. coli* diarrheas with small amounts of milk. The ability of antibody to neutralise *E. coli* and *V. cholerae* enterotoxins is well established. The rabbit ileal loop model permits evaluation of in vivo protection solely on the basis of toxin neutralisation irrespective of factors conferring group-specific immunity.

IgA is probably the molecule responsible for neutralisation of these enterotoxins. The antitoxic activity of a given breast-milk preparation appeared to correlate with IgA content but not with the concentration of IgG or IgM (table I). Since the immunoglobulin content of breast-milk is known to be widely variable, this evidence is at best only suggestive. Gel filtration of breast-milk globulins revealed that antitoxin activity was closely associated with IgA (fig. 2). This analysis separated IgM from IgG and IgG cluted also contained IgA. Only minute amounts of IgG and IgM were detected in...
concentrated column eluates. The possibility that a combination of IgG and IgM antibodies could be responsible for the antitoxin effect is unlikely since these immunoglobulins were only trace contaminants of the column purified IgA.

Immunity to toxin-induced diarrheas may be either natural (occurring without any discernible antecedent infection) or acquired (occurring subsequent to a clinically and bacteriologically apparent infection). Acquired immunity to cholera is thought to be mediated by IgG and IgA antibodies present in the gut.9,20 The classical whole-cell vaccine provides transient immunity that is group-specific to the cell-wall antigens of a specific strain of *V. cholerae*.21,22 This vaccine presumably acts by inducing antibodies that bind to the surface of the vibrio and thereby prevent attachment of the bacteria to the gut mucosa.23 A second form of active immunity to cholera is mediated by anti-enterotoxin antibodies: IgA24 and IgG23 antibodies both appear to operate at the mucosal level by preventing *V. cholerae* enterotoxin from binding to the epithelial cells of the gut. Passive immunity to cholera has also been demonstrated: mice actively immunised with *V. cholerae* can passively transfer this immunity through their breast-milk.14

Acquired immunity to toxigenic *E. coli* has not yet been well documented. Active immunisation of mice with *E. coli* does not prevent bacterial colonisation or ameliorate disease.19 Although group-specific antibodies to common *E. coli* serotypic antigens have been found in human breast-milk,9 their role in immunity is uncertain.25 We have shown that antibody to enterotoxin is present in the breast-milk of normal mothers — this is probably a manifestation of what has been called "natural immunity". These antibodies are presumably produced in response to previous sporadic subclinical exposures to *E. coli* enterotoxin. The close antigenic relationship between the toxins of *E. coli* and *V. cholerae* easily explains why cholera toxin is neutralised by breast-milk from an area of North America that has not experienced a cholera epidemic in over 100 years. Although exposure to *E. coli* will not confer group specific immunity to cholera, exposure to the toxin produced by the episomally transformed *E. coli* will confer antitoxin-mediated immunity to *V. cholerae* enterotoxin.

Like most natural antibodies, anti-enterotoxin IgA is not highly active (compared to hyperimmune rabbit antiserum to enterotoxin). However, even North American breast-milk contained sufficient antibody to completely inhibit the in-vivo effect of enterotoxin. The magnitude of the effect we observed (20 · 105 units/ml in Guatemalan colostrum) is certainly sufficient to explain how Larguia et al.5 prevented diarrheae in neonates with only 5 ml/kg/day of colostrum. Kasai and Burrows19 found 100 units/ml of anti-enterotoxin activity in the serum of normal Bengalis in an area endemic for cholera. Assuming that IgG is the major serum-immunoglobulin with this antitoxin, a specific activity (7 · 7 · 105 units/mg IgG) can be calculated for this "natural" anti-enterotoxin antibody that is similar to our values (table 1). Controlled epidemiological studies must be conducted to determine whether these speculations on the nature and role of anti-enterotoxin antibodies are valid.

In conclusion, we hypothesise that inapparent exposure to toxigenic *E. coli* can induce an immune response to enterotoxin. This results in "natural" IgA antibodies in breast-milk that can inhibit *E. coli* and *V. cholerae* toxins in vivo. The passive transfer of these antibodies to the infant may explain why breast-milk prevents *E. coli* diarrheae in the neonate.

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REFERENCES