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Bacteriostasis of *Escherichia coli* by milk I. Colonization of breast-fed infants by milk resistant organisms

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SUMMARY

Human milk has a bacteriostatic effect on *Escherichia coli in vitro*. The milks of 40 mothers were tested for this effect against *E. coli* isolated from their stools, from those of their own babies, and from those of babies not breast-fed. The milks had a direct bacteriostatic effect, not dependent on complement, on some but not all the strains of *E. coli*. Breast-fed babies receiving supplementary bottle feeds were colonized with milk-resistant strains, whereas bottle-fed babies and, surprisingly, babies completely breast-fed were colonized equally with milk-sensitive and milk-resistant strains, as were the mothers. These results suggest that the bacteriostatic effect of human milk, demonstrable *in vitro* does sometimes operate *in vivo*.

The antibacterial activity of human milk is not influenced by the O, H or K antigens of *E. coli* and is effective against other Gram-negative organisms, e.g. *Salmonella*, *Klebsiella*, *Proteus*.

INTRODUCTION

Breast-fed babies are more resistant to gastro-enteritis due to pathogenic *Escherichia coli* than babies receiving cow's-milk preparations. The mechanism by which human milk confers this protection is not known but the stools of breast-fed infants are more acid and contain many bifidobacteria which may inhibit the growth of *E. coli in vivo* (Bullen & Willis, 1971). In addition, fresh human milk has a direct, bacteriostatic effect, demonstrable *in vitro*, on intestinal *E. coli* (Bullen, Rogers & Leigh, 1972). Cow's milk has some bacteriostatic activity but this is lost during the commercial preparation of baby feeds.

Recent studies have shown that breast-fed babies do not necessarily have a low number of *E. coli* in their stools (Hewitt & Rigby, 1976), and this has prompted us to study the bacteriostatic effect in detail, to determine whether it could operate in the upper gut. Our preliminary observations on the in-vitro bacteriostatic effect of human milk on intestinal *E. coli* isolated from adults showed that some strains are sensitive and some are resistant. If the bacteriostatic system was effective in the baby's gut, a breast-fed infant should be colonized with milk-resistant strains whereas a bottle-fed baby receiving a milk substitute might possess either resistant or sensitive strains. In studying the characteristics of the bacteriostatic system, we have explored this hypothesis.

METHODS

Milk

Specimens were provided by about 40 nursing mothers whose babies were born 5–7 days previously. The milk was stored at −30° C until it was examined. It was then thawed, heated to 56° C. for 30 min. to reduce the bacterial content, adjusted with sodium hydroxide to approximately pH 7·5, and then stored at 4° C. This heat treatment and storage at 4° C for up to 18 months does not reduce the antibacterial activity, which must therefore be independent of complement. Where indicated milk was heated in a boiling water bath for 5 min in volumes of not more than 2 ml.

Infant feeding regimes

Babies fed wholly at the breast are referred to as breast-fed babies, those fed at the breast but receiving supplementary feeds as breast-fed bottle-supplemented, and those receiving only the commercial preparation from the bottle as bottle fed. The commercial baby milk used throughout was SMA Gold Cap S 26 (John Wyeth and Brother Ltd, Taplow, Maidenhead, Berks.). The supplementary SMA given immediately after each breast-feed or instead of the night breast-feed amounted to 50–250 ml per 24 h for 2–3 days; after this the babies were breast-fed except for one nightly feed of SMA. The groups were:

(*a*) 32 breast-fed bottle-supplemented babies,
(*b*) 11 babies wholly breast-fed,
(*c*) 21 babies bottle-fed.

Stools

Specimens were collected from the nursing mothers and their babies at about the same time as the milk specimens, from 21 bottle-fed babies aged 5–7 days and from some of their mothers. The specimens were gently homogenized at an approximate 1/10 (w/v) dilution in 10 % glycerol-broth and frozen immediately at −30° C until examination within 1 month (Crowther, 1971).

The culture of aerobic and anaerobic bacteria was as described by Hewitt & Rigby (1976).

Bacterial strains

Strains of *E. coli* and other Enterobactereaceae were isolated from plate cultures of the higher dilutions of stool specimens from the mothers and babies described above; other strains were from Saint Bartholomew's Hospital (Bettelheim *et al.* 1974*a*, *b*). Strains of pathogenic serotype were from the stools of babies ill with enteritis. One strain (no. QE 8) was isolated from the duodenal aspirate of a baby; it was not of pathogenic serotype. *Salmonella newport* was from the stools of a symptomless adult excreter.

Table 1. *Bacterial flora of the stools of babies, 5–7 days old*

Feeding of babies	No. of babies	Enterobacteriacea present in stools: *E. coli*	Enterobacteriacea present in stools: Other	Viable count (per g of stools) of lactobacilli and bifidobacteria
Breast, bottle-supplemented	11	+	−	$< 10^2$–10^{11}
	7	−	−	$< 10^2$–10^9
	4	−	+	$< 10^2$–10^{10}
Bottle	5	+	−	$< 10^2$–10^9
	4	−	−	$< 10^2$–10^9
	2	−	+	$< 10^2$–10^9
Breast	6	+	−	10^7–10^{10}
	2	−	−	10^7–10^{11}
	1	−	+	10^9

+ = present (10^7–10^{11}) per g; − = not detected ($< 10^2$ per g).

Measurement of the bacteriostatic activity of milk

The milk, treated as above, was dispensed in 0·08 ml amounts into capped, glass tubes and inoculated with 0·02 ml of a 3 h peptone–water culture of *E. coli* diluted to 10^{-3} in 0·9% NaCl. Colony counts by the method of Miles, Misra & Irwin (1938) were made on 10-fold saline dilutions of the inoculum, and on the inoculated milk after incubation for 3 h at 37° C. The results were expressed as the number of times the inoculum increased. A strain was regarded as being sensitive if it increased to less than 10 times the inoculum, moderately sensitive if the increase was 10–12 times, and resistant if greater than this, i.e. usually 50-200 times increase.

Iron was added to the system as ferric ammonium citrate. The concentration of the iron-binding protein of milk (lactoferrin) was increased by adding human serum transferrin (Sigma laboratories, Kingston-on-Thames) in 0·2 M, pH 7·8, phosphate buffer.

The iron-binding capacity of milk was measured by the method of Ramsay (1973).

RESULTS

Intestinal colonization of infants

E. coli was found in only about half of the stools whether the infants were breast-fed or bottle-fed (Table 1). Counts of lactobacilli and bifidobacteria in the breast-fed bottle-supplemented babies were in the range $< 10^2$–10^{11} per g. In the seven babies from whom bifids were absent the *E. coli* count was high in two but in the other five *E. coli* was absent. In these breast-fed babies, therefore, there was not a consistent inverse relation between the numbers of bifidobacteria and of *E. coli*. In the babies fed only at the breast there was again no inverse relation between the numbers of *E. coli* and bifidobacteria but *all* these babies had a high lactobacillus or bifid count, in contrast to those receiving any or all SMA.

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Table 2. *Bacteriostasis of* E. coli *(strain no. V12.1) by one human milk (V12, 5 days post-partum) alone and with added transferrin and ferric ammonium citrate (Fe Cit)*

Growth of *E. coli* for 3 h in:	Added transferrin (mg/ml)	Number of times inoculum increased with Added Fe Cit (μg/ml) 0	4	20	100
Human milk (56 °C)	0	5	2	70	100
	2	0	0	0	6
	8	0	0	0	0
1% peptone water	0	80	.	100	.
	8	75	.	.	.
Human milk (100 °C)	0	100	.	.	.
	8	35	.	.	.
SMA	0	140	.	.	.
	4	145	.	.	.

Table 3. *Bacteriostasis of six strains of* E. coli *by milk no. V 28 showing the variations in sensitivity to human milk*

Growth for 3 h in	Number of times inoculum increased for strain no. V12.1	VB21.2	QE8	O112 (3)	O128 K67.H12	O128 K67.H2
Milk	6	42	57	6	40	1
Milk + TF*	6	35	2	4	60	1
Milk heated 100° C for 5 min.	200	80	100	200	180	200
1% peptone water	100	150	80	250	200	330

* Transferrin 2 mg/ml

The bacteriostatic system of milk

Table 2 shows the inhibitory effect of milk *in vitro* on a milk-sensitive strain of *E. coli* isolated from the milk donor. The bacteriostatic activity was progressively decreased by the addition of iron but the iron antagonism was reversed by the addition of transferrin at 2 mg/ml, the average concentration of lactoferrin in human milk. Transferrin, even at 8 mg/ml, was completely inactive in the absence of milk, indicating that milk components other than iron-binding proteins participate in the inhibitory process. This was confirmed by measurements of the iron-binding capacity of the milks of five mothers; this varied 4-fold but all the milks were equally bacteriostatic. Heating the milk to 100° C destroyed the bacteriostatic activity, only a little of which was replaced by added transferrin; SMA was inactive and addition of transferrin did not alter this.

Milk-sensitive and milk-resistant bacteria

Table 3 shows that bacteriostatic activity of one milk varied, depending on the strain of *E. coli* against which it was tested. A mother-strain no. V12.1 and two of

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Table 4. *The milk sensitivity of serotyped, biotyped* E. coli

Donor of strain*	Serotype	Biotype	Milk sensitivity
Mother 1	O1:H−	F/S; Du, Ma, Rh, Sc	−
Baby 1 (day 4)	O1:H−	F/S; Du, Ma, Rh, Sc	+
Mother 10	O162:H10	F/S; Ma	−
Baby 10 (day 5)	O162:H10	T; Ma	−
Baby 12 (Day 6)	O162:H10	F/S; Ma	−
Baby 23 (day 5)	O162:H10	T; Ma	−
Baby 23 (day 5)	O162:H10	F/S; Ma	+

F/S = fully sensitive to nine antibiotics.
T = resistant to tetracycline.
Du, Ma, Rh, Sc = ability to ferment dulcitol, maltose, rhamnose and sucrose respectively.
* Mother 1 passed her O1 strain to her baby, and mother 10 her O162 to her baby. Babies 12 and 23 were colonized with O162 from ward sources, baby 12 the day after mother and baby 10 left, and baby 23 eight days later. Baby 10 was bottle-fed, the others were breast-fed and bottle supplemented (Bettelheim *et al.* 1974*a*,*b*).

pathogenic serotype, O112 and O128, were milk-sensitive but were resistant in the presence of iron. The baby-strain no. VB21.2, another O128 serotype, and strain no. QE8 were all resistant; no. QE8 is an example of strains in which the sensitivity can be increased by the addition of transferrin.

Milks from about 100 mothers were all active against one of two milk-sensitive indicator strains. Milks from about 50 mothers were inactive against one of two milk-resistant indicator strains. Numerous other strains had consistent sensitivities against several randomly chosen milks. Occasionally – in three out of about 50 strains investigated extensively – a strain resistant to one milk was sensitive to others or more sensitive to the same milk when the test was repeated; these seemed to be strains of variable sensitivity.

Two of the strains shown in Table 3 were of pathogenic serotype O128 and with the same K and O antigens; their sensitivities were however quite different. It therefore seems that sensitivity of a strain is independent of O and K serotype. For these two strains the H antigen was different, but (Table 4), two H10 strains had different sensitivities and so did two strains without H, so that sensitivity must also be independent of the H antigen.

Extensive testing of the antigenic and biochemical properties of strains of *E. coli* isolated from mothers and their babies was done by Bettelheim and colleagues (1974*a*, *b*). The milk-sensitivities of a few of these strains are shown in Table 4, which demonstrates not only the irrelevance of O and H antigens to milk sensitivity as described above, but also the lack of relation between milk-sensitivity and sensitivity to nine antibiotics and six fermentation reactions.

The bacteriostatic system of milk acts similarly against other Enterobactereaceae, e.g. *Proteus*, *Enterobacter*, *Klebsiella* and *Salmonella*, that is, it is reversed by iron and destroyed at 100° C. Resistant and sensitive strains of the first three of these have been found; only two strains of *Salmonella* have been tested and were sensitive. The anti-salmonella activity of milk was first demonstrated in two mothers who were found to be carriers of *Salmonella newport* just

Table 5. *The milk sensitivity of* E. coli *from mothers and 1-week-old babies; two single-colony cultures from each were tested*

Source of *E. coli*	Number of strains			
	Total	Sensitive	Moderately sensitive	Resistant
26 mothers	52	20	14	18
12 breast-fed bottle-supplemented babies	24	3	5	16
10 bottle-fed babies	20	9	3	8
7 breast-fed babies	14	8	0	6

Table 6. *The milk-sensitivity of* 5–6 *single colony cultures of* E. coli *isolated from each of three mothers and their breast-fed, bottle-supplemented babies*

Source of *E. coli*	Number of strains			
	Total	Sensitive	Moderately sensitive	Resistant
3 mothers	15	7	3	5
3 babies	18	0	5	13

before the birth of their babies. Their milk was bacteriostatic for that strain of *S. newport* but so was the milk of mothers who had not been in contact and who were not infected. The infected mothers breast-fed their babies and *S. newport* was not isolated from their babies who remained healthy.

Milk sensitivity of E. coli *from breast-fed and bottle-fed infants*

Cultures (each called a strain) were grown from two single colonies selected from the stool-culture plates inoculated with the highest dilution of the specimen giving growth of *E. coli*. In this way, two strains were collected from each of 26 mothers, from 12 colonized, breast-fed, bottle-supplemented babies, from 7 colonized wholly breast-fed babies, and from 10 colonized bottle-fed babies. Each strain was tested against the milk of the mother of the baby or, for strains from bottle fed babies, against a milk pool. Table 5 shows that from breast-fed bottle-supplemented babies we isolated more milk-resistant than milk-sensitive strains. From the other two groups of babies, however, the bottle-fed and the breast-fed, equal numbers of milk-resistant and milk-sensitive strains were isolated, as they were from the mothers. Strains of moderate sensitivity were found less often in the babies than their mothers. For these experiments only two colonies had been tested from each stool specimen; three more mothers and their breast-fed bottle-supplemented babies were more extensively tested and the preponderance of milk-resistant strains in these babies but not their mothers confirmed (Table 6). From all the strains isolated only one strain of *E. coli* was of a pathogenic serotype; it was from a healthy breast-fed infant and was milk-resistant.

DISCUSSION

Bullen *et al.* (1972) showed that suckled, baby guinea-pigs were protected from an artificial *E. coli* infection of the small intestine, and that the bacteriostatic system of human milk tested *in vitro* and the protection of a suckled guinea-pig could be reversed by iron. This is presumptive evidence that the bacteriostatic system and protection *in vivo* are the same but because the effects of suckling and iron are complex, it is not proof. Our findings of the colonization of some breast-fed babies by milk-resistant strains and the random colonization of their mothers and bottle-fed babies suggest that the bacteriostatic system sometimes operates *in vivo*.

We have shown that in a group of breast-fed babies who were receiving supplementary bottle-feeds of commercially prepared milk, there appeared to be selection in the gut, of milk-resistant strains (70–80 %) of *E. coli* suggesting that the bacteriostatic activity of breast-milk, demonstrable *in vitro* was also effective *in vivo*. There was no such selection in bottle-fed babies. From wholly breast-fed babies we expected therefore to find only resistant strains; the occurrence of equal numbers of resistant and sensitive strains was puzzling. A possible explanation is related to the bicarbonate content of the test system and is considered in our next paper (Dolby, Stephens & Honour, 1977). Differences in milk-sensitivity of *E. coli* that we have described here can be identified only in the absence of added bicarbonate; physiological amounts of bicarbonate alter the degree of bacteriostasis.

Whether milk resistance is an in-vivo adaptation of strains with otherwise similar properties, or a selection, is not known.

An investigation similar to ours, but in which sensitivity of the *E. coli* to the complement-dependent bactericidal activity of serum was determined instead of to the bacteriostatic activity of milk has been reported (Gothefors, Olling & Winberg, 1975). The *E. coli* isolated from the stools of the breast-fed babies were more sensitive to the bactericidal action of serum than those from babies not breast-fed. The bactericidal reaction depends on exposed O antigen and its antibody and has not so far been shown to have any relevance to protection *in vivo*. The lethal effect of this antibody is maximal when an optimum, small amount of O antigen is present on the bacterial cell; it may be inversely proportional to the *amount* of K antigen present (Glynn & Howard, 1970). The relation between complement-independent milk-resistance and complement-dependent serum sensitivity is not known, but from the above, milk-resistant strains that are serum-sensitive might have low K antigen. A tendency for bottle-fed babies to be colonized with K1-positive strains has been reported (Ørskov & Sørensen, 1975).

We have shown however that milk sensitivity and resistance are not correlated qualitatively with any particular O, K and H antigen or any other previously used marker properties. Milks that were bacteriostatic for *E. coli* were also active against other Enterobacteriaceae, including *Salmonella*. They tended to be inactive, however, against *Proteus*, *Klebsiella*, etc., isolated from breast-fed, bottle-supplemented babies just as *E. coli* was, because of the greater milk-resistance of these strains. Previously, milk from mothers infected with *Salmonella* during their pregnancy even though containing antibodies was said not to be inhibitory for

Salmonella (Allardyce *et al.* 1974). Our experience was that all milk was active. These earlier tests were incubated for 18 h not 3 h and a milk-resistant indicator strain may of course have been used.

Bullen & Willis (1971) suggested from their findings that the faeces of breast-fed babies had higher bifidobacteria and lower *E. coli* counts than bottle-fed babies. We and Hewitt & Rigby (1976) have been unable to confirm this inverse relation, although the completely breast-fed babies had higher bifid counts than the others. In babies receiving SMA in addition to breast-milk the inverse relation is reduced however (Dr C. L. Bullen, private communication). From this it follows that if breast-milk is protective because of its ultimate effect on the ratio of organisms in the large intestine, 'topping-up' would negate any protective effect of the milk. It was, however, in this group of breast-fed, bottle-supplemented babies that the bacteriostatic activity of milk seemed to be acting. The correct management of infant feeding obviously depends on the clarification of the parts played by these systems in protection against intestinal infection.

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