PAPERS AND ORIGINALS

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Iron-binding Proteins in Milk and Resistance to Escherichia coli Infection in Infants

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Summary

Human milk contains large quantities of iron-binding protein, of which the greater proportion is lactoferrin, though small amounts of transferrin are also present. Three samples of human milk with unsaturated iron-binding capacities of between 56 and 89% had a powerful bacteriostatic effect on *Escherichia coli* O111/B4. The bacteriostatic properties of milk were abolished if the iron-binding proteins were saturated with iron. Purified human lactoferrin, in combination with specific *E. coli* antibody, strongly inhibited the growth of *E. coli*, and this effect was also abolished by saturating the lactoferrin with iron.

Guinea-pig milk also contains lactoferrin and transferrin. Newly born guinea-pigs fed on an artificial diet and dosed with *E. coli* O111 had higher counts of *E. coli* O111 in the intestine than suckled animals. The apparent supressive effect of guinea-pig milk on *E. coli* in the intestine could be reversed by feeding the iron compound haematin. It seems that iron-binding proteins in milk may play an important part in resistance to infantile enteritis caused by *E. coli*.

Introduction

It has long been held that breast-fed babies are more resistant to infectious gastroenteritis caused by *Escherichia coli* than those receiving artificial food (Levi, 1941; Alexander, 1948; Ross and Dawes, 1954; Smith, 1955). It is also well known that this disease is associated with specific serotypes of *E. coli* (Taylor, 1970). Nevertheless, it seems unlikely that the disease is due simply to infection with special types of *E. coli*, since the same serotypes can be isolated from healthy infants (Thomson, Watkins, and Gray, 1956).

If breast-feeding confers resistance to *E. coli* it is obviously important to know what factors are involved. Ross and Dawes (1954) and C. L. Bullen and Willis (1971) suggested that the predominance of *Lactobacillus bifidus*, which is associated with a low pH in the faeces of breast-fed infants, may be related to the suppression of *E. coli* in the intestine. It seems highly probable that this system makes an important contribution to resistance, especially in the large intestine. However, our experiments show that human milk can have a specific inhibitory effect on *E. coli*. Human milk contains large quantities of iron-binding protein, of which the greater proportion is lactoferrin, though small amounts of transferrin are also present (Masson and Heremans, 1971). We have evidence to suggest that lactoferrin, in combination with specific antibody to *E. coli*, is responsible for the bacteriostatic effect. These results are supported by experiments in guinea-pigs, which produce a milk with fairly high concentrations of lactoferrin, but rather more transferrin than human milk. These studies strongly suggest that the iron-binding proteins of milk may play an important part in resistance to *E. coli* infection, and this particularly applies to conditions in the small intestine.

Materials and Methods

HUMAN MILK

Samples of milk from mothers in different stages of lactation were pooled and frozen at −20°C. One sample (No. 1), from a single individual, was obtained during the first week of lactation. The milk was centrifuged at 56,000 *g* for 45 minutes and the fluid separated from the fat. For experiments with *E. coli* the samples were sterilized by filtration through a 0·45-μ Millipore filter and stored at −70°C.

MEASUREMENT OF pH

The pH of the milk filtrate was measured with a Vibret pH meter (Electronic Industries Ltd) using a dual electrode (SCDM 33C) in a vessel maintained at 37°C. The vessel was sealed with a rubber bung and the filtrate rapidly stirred

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with a magnetic follower to allow equilibration of the filtrate with 5% CO_2 in the gas phase.

MEASUREMENT OF IRON-BINDING CAPACITY OF MILK

Saturated Iron-binding Capacity.—Two 1-ml samples of milk filtrate were placed in separate tubes and 1 ml of 10 M Analar urea containing 1% Analar ascorbic acid was added to each sample. Then 0·2 ml of 1mM 2,2′-dipyridyl in Analar glacial acetic acid was added to one sample and 0·2 ml of glacial acetic acid to the other. The second sample served as a blank. After standing for 18 hours in the dark at room temperature the optical density of the sample was measured at 520 nm in a Pye Unicam SP500 spectrophotometer. The saturated iron-binding capacity was calculated by using an extinction coefficient of $8{\cdot}8\times10^3$. Reagent blanks gave readings of 0·005 or less and were ignored.

Unsaturated Iron-binding Capacity.—The optical density at 470 nm was recorded after the addition of 0·005-ml or 0·01-ml aliquots of 1·0 mM ferric nitrilotriacetate (pH 7·40) to 2 ml of the filtrate in a 1-cm glass cell (Bates, Billups, and Saltman, 1967). The unsaturated iron-binding capacity was calculated from a plot of the optical density against the volume of ferric nitrilo-triacetate added.

PREPARATION OF HUMAN LACTOFERRIN

The casein was removed from 200 ml of human milk by centrifugation (Blanc and Isliker, 1961) and the supernatant concentrated to 70 ml by pressure filtration (Sartorius high-pressure filter No. 16208 fitted with membrane No. 1233). The concentrate was dialysed against 0·005 M phosphate buffer pH 7·6 and then applied to a 2×50 cm column of DEAE Sephadex A50 equilibrated with 0·005 M phosphate buffer pH 7·6. After washing with 60-ml volumes of 0·005 M and 0·0075 M phosphate pH 7·6, the lactoferrin was eluted by means of a linear gradient from 0·01 M phosphate to 0·01 M phosphate plus 0·1 M sodium chloride pH 7·6 (400 ml each) in place of the stepwise system used by Masson and Heremans (1966). The lactoferrin (see Table) was detected by its reaction with ferric nitrilotriacetate and its relative electrophoretic mobility compared with transferrin. The observed mobility was the same as that described by Masson, Heremans, and Schonne (1969).

Iron-binding Capacities of Three Samples of Human Milk and One Sample of Purified Human Lactoferrin

Sample	Iron-binding Capacity (10^{-5}M Fe)			Saturation with Fe %
	Saturated	Unsaturated	Total	
Human milk 1	0·88	7·00	7·88	11·2
„ „ 2	1·40	3·10	4·50	31·1
„ „ 3	1·35	1·76	3·11	43·4
Human lactoferrin (71 mg/ml)	29·5	51·8	81·3	36·2

BOVINE COLOSTRUM

A sample of bovine colostrum was obtained from a normal cow within 24 hours of parturition. A sterile filtrate was prepared in the same way as that for human milk.

E. COLI

A culture of *E. coli* O111/B4/H2 was obtained from Dr. J. Taylor, Central Public Health Laboratories, Colindale. During the past few years the culture was repeatedly passaged through guinea-pigs (Bullen, Leigh, and Rogers, 1968). Between passages the organism was stored in frozen guinea-pig spleen at −20°C. Passage maintained but did not enhance the virulence of this strain for guinea-pigs (Bullen, Leigh, and Rogers, 1968).

E. COLI ANTISERUM

Antiserum against *E. coli* O111/B4/H2 was prepared in a horse. This had an O agglutinating titre of 1/20,000.

CLOSTRIDIUM WELCHII ANTISERUM

Antiserum against *Cl. welchii* type A was prepared in a horse. Antitoxins per ml: 770α 140θ 1100κ.

L. BIFIDUS

A culture of *L. bifidus* recently isolated from the faeces of a normal suckling infant was obtained from Mrs. C. L. Bullen, Public Health Laboratory, Luton.

IRON COMPOUNDS

Ferric ammonium citrate contained 20% w/w iron estimated colorimetrically by the method of Collins, Diehl, and Smith (1959).

HAEMATIN HYDROCHLORIDE

Haematin hydrochloride (British Drug Houses Ltd) (2 g) was ground in a ball mill in water (25 ml) for three hours. After centrifugation the water was removed and the haematin placed in ethyl alcohol for 30 minutes. The mixture was again centrifuged, the alcohol removed, and the haematin dried in a desiccator over $CaCl_2$.

MEDIUM 199

Sterile concentrated medium 199, without phenol red, antibiotics, or sodium bicarbonate, was obtained from the Wellcome Research Laboratories.

EXPERIMENTS IN VITRO

Experiments on the bacteriostatic properties of filtered human milk were done under controlled conditions of oxygen tension and pH. The pH was controlled by the presence of 5% CO_2 in the gases (air and N_2) passing over the surface of the milk. The oxygen tension was controlled by the technique described previously (Bullen, Rogers, and Lewin, 1971). In each experiment 8 ml of filtered milk was inoculated with a known number of bacteria. In the case of *E. coli* the inoculum was prepared from a one-hour-old, actively growing culture in papain digest broth. With *L. bifidus* the inoculum was prepared from a 48-hour culture already growing in human milk. Viable counts of *E. coli* were made on blood agar plates by the method of Bullen, Wilson, and Cordiner (1961) and those of *L. bifidus* on R.C.M. plates (Hirsch and Grinsted, 1954) adjusted to pH 5·0 and incubated anaerobically with 5% CO_2.

Experiments with purified lactoferrin were done in vitro with 1/4 strength tissue culture medium 199 in normal saline. Na HCO_3 was added to give a final concentration of 0·2%. With 5% CO_2 in the gas phase, the pH of the medium was 7·4 at 37°C. Gas flowing over the surface of the medium consisted of equal volumes of 5% CO_2 in air, and 5% CO_2 in N_2. This gave a Po_2 of about 80-90 mm Hg.

For experiments with small volumes of material (see Fig. 2) the lactoferrin and antisera were added to 1 ml of medium in siliconized 1-oz (28-ml) universal containers. These were connected in series to the gas mixture, which was bubbled

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through water before entering the bottles containing medium. The bottles were shaken on a horizontal rotary shaker at about 100 r.p.m. in a room at 37°C. The medium was allowed to equilibrate with the gas for one hour before inoculation with approximately 2×10^2 *E. coli*. After five and a half hours' incubation the contents of the bottles were diluted and homogenized for four minutes in an M.S.E. homogenizer (5 ml) cooled with iced water. For bacterial growth curves (see Fig. 3) 4 ml of medium was placed in a 20-ml siliconized and jacketed glass vessel mounted on a horizontal rotary shaker. The temperature of the medium was kept at 37°C by water pumped from a water-bath. The vessel was sealed with a silicone rubber bung and the gas mixture passed over the surface at 20 ml/min. Samples were removed at intervals, homogenized, and counted in the usual way.

VIABLE COUNTS OF BACTERIA IN GUINEA-PIG INTESTINE

Guinea-pigs were killed by a blow on the head. The small and large intestines were ligated at both ends and removed. Both specimens were weighed and homogenized for eight minutes in 10% broth saline in a 25-ml universal container (M.S.E.) immersed in an ice-bath. Suitable dilutions of the homogenates were made in broth saline and viable counts done in the usual way.

Counts of *E. coli* were made on MacConkey's medium. The serological identity of the organisms was checked by slide agglutination tests on 50 colonies selected at random, an *E. coli* O111/B4 agglutinating antiserum (Burroughs Wellcome and Co) being used.

For counts of lactobacilli the diluted homogenates were plated on M.R.S. medium (de Man, Rogosa, and Sharpe, 1960) and incubated anaerobically with 5% CO_2. Characteristic colonies of large Gram-positive bacteria which gave a negative catalase test were counted as lactobacilli.

GUINEA-PIGS

Pregnant guinea-pigs (Hartley strain) were housed in individual cages and fed on a pelleted diet (B.O.C.M. Coney Brand Cubes), hay, and fresh cabbage. The imminence of parturition was judged by separation of the pubic symphysis. Intramuscular injection of 0·01 ml of Pitocin usually led to delivery of the young within an hour, though occasionally a further dose of 0·01 ml was necessary. The newly born were either suckled in the normal way or separated from their dams and placed in an 18 by 12-in (46 by 30-cm) cage fitted with a thermostatically controlled 250-watt ceramic heater on the underside of the lid. This arrangement gave a temperature of 30-32°C on the floor of the cage immediately below the heater (Paterson, 1969). These animals were fed on guinea-pig milk substitute (Paterson, 1969), which was given twice daily in sterile 50-ml glass jars.

Results

BACTERIOSTATIC EFFECT OF HUMAN MILK AND BOVINE COLOSTRUM

Role of pH.—In a preliminary experiment a sample of bovine colostrum equilibrated with 5% CO_2 had a pH of about 6·8. This was inoculated with approximately 2×10^2 *E. coli* and had no bacteriostatic effect, the organisms growing with a generation time of about 20 minutes. The viable count after six hours was 3×10^7/ml. After the addition of $NaHCO_3$ to a concentration of 0·6% the pH of the colostrum under the same conditions was about 7·5 and it then had a powerful bacteriostatic effect, the viable count being $3{\cdot}6 \times 10^3$/ml after seven hours. Similar results were obtained with human milk. At a pH of 6·95 or below the milk filtrates had no effect on the growth of *E. coli*. At a pH of 7·20 to 7·33 there was a powerful inhibitory effect. The reason for the sensitivity of the bacteriostatic system to changes in pH is unknown, but it is comparable to similar effects observed with serum and *Cl. welchii* (Bullen, Dobson, and Wilson, 1964) and serum and *Pasteurella septica* (Bullen, Rogers, and Lewin, 1971).

Effect of Oxygen Tension.—The oxygen tension of the milk filtrate had no effect on its bacteriostatic power, the bacteria being inhibited equally well at a Po_2 of 80-90 mm Hg, 40-50 mm Hg, or zero.

Effect of Heating the Milk Filtrate.—Heating the milk filtrate to 56°C for 30 minutes had no effect on its bacteriostatic properties.

Effect of N-acetyl-L-tyrosine Ethyl Ester Monohydrate.—The addition of this ester at a concentration of 2·75 mg/ml just before the addition of the bacteria had no effect on the bacteriostatic properties of the milk filtrate.

Effect of Added Iron.—The iron-binding capacities and the percentage saturation of the three samples of human milk filtrate varied considerably (see Table) but all three had a powerful inhibitory effect on *E. coli* (Fig. 1), though it may be

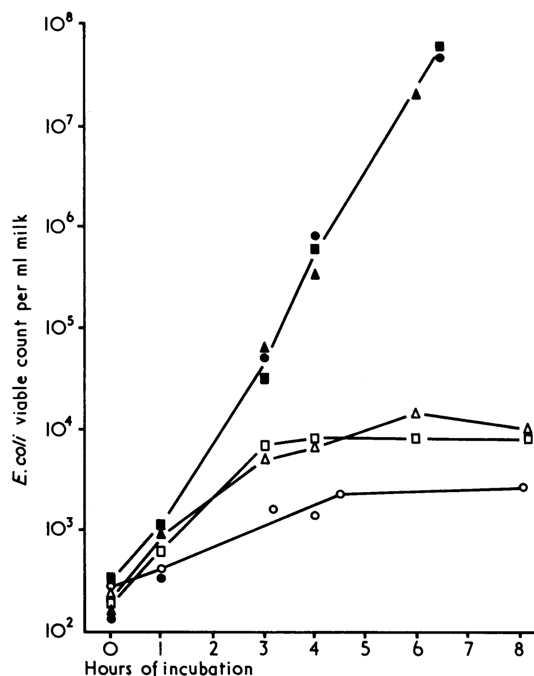

FIG. 1—Inhibition of *E. coli* O111 by human milk and the effect of adding iron. ○ △ □ viable counts in milk samples 1, 2, and 3 without added Fe. ● ▲ ■ viable counts in samples 1, 2, and 3 with 200% saturation of iron-binding protein with Fe. pH 7·20 to 7·33. Po_2 40-50 mm Hg.

significant that the most inhibitory sample (No. 1) was the least saturated with Fe. In the experiments with iron enough ferric ammonium citrate was added to doubly saturate the total iron-binding capacity of the filtrate. In every case the addition of Fe abolished the bacteriostatic properties of the filtrates (Fig. 1). The organisms grew with generation times of about 20 minutes. Thus the iron-binding capacity of the milk seemed to be essential for the inhibition of *E. coli*.

PURIFIED LACTOFERRIN AND SPECIFIC ANTIBODY

At a pH of 7·4 and a Po_2 of 80-90 mm Hg, *E. coli* O111 grew rapidly in 1/4 strength medium 199. When inoculated with approximately 2×10^2 bacteria/ml the viable count rose to $7{\cdot}6 \times 10^6$/ml in five and a half hours (Fig. 2). *Cl. welchii* antiserum (0·005 ml/ml medium) had no effect. *E. coli* O111 antiserum at the same concentration slightly reduced the viable count. Both antisera had previously been heated to 56°C for 30 minutes to inactivate complement. Lactoferrin (7·1

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mg/ml) reduced the viable count to approximately 1/50 of the control while the addition of *Cl. welchii* antiserum to the lactoferrin had little additional effect. A combination of *E. coli* antibody and lactoferrin had a pronounced effect on growth, the viable count being reduced to approximately 1/3,000 of the control (Fig. 3).

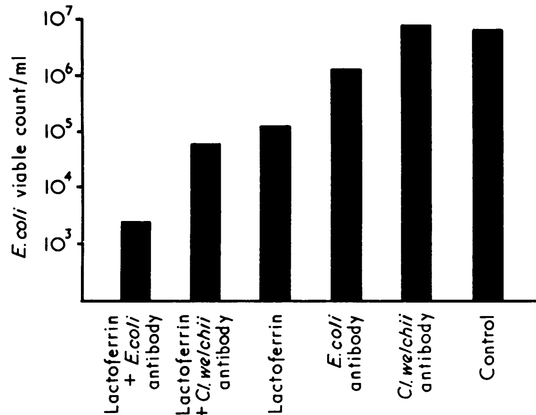

FIG. 2—Effect of purified lactoferrin and antibody on growth of *E. coli* O111 in medium 199. pH 7·4. Po_2 80-90 mm Hg. Inoculum 2×10^2 organisms. 5½ hours' incubation. (Antisera previously heated to 56°C for 30 minutes.)

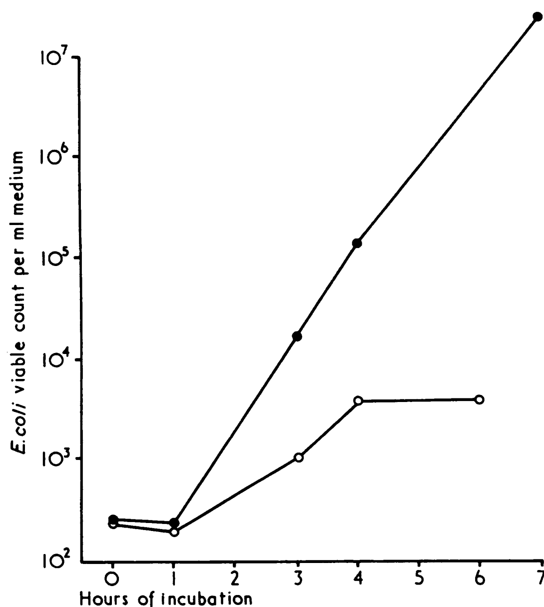

FIG. 3—Inhibition of *E. coli* O111 by mixture of purified lactoferrin and *E. coli* antibody, and the effect of adding iron. Medium 199, pH 7·4. Po_2 80-90 mm Hg. ○ viable count without added iron; ● viable count with 200% saturation of lactoferrin with Fe. (Antiserum previously heated to 56°C for 30 minutes.)

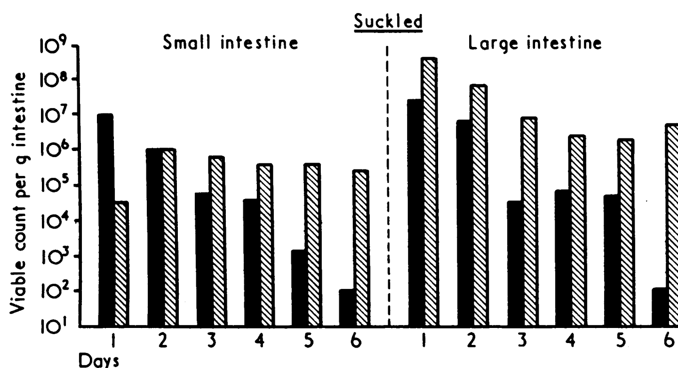

FIG. 4—*E. coli* O111 ■ and lactobacilli □ in small and large intestine of suckled guinea-pigs. Oral dose of approximately 1×10^6 *E. coli* O111 at birth.

A mixture of lactoferrin (7·1 mg/ml) and *E. coli* antibody (0·005 ml/ml) inhibited *E. coli* in a manner which closely resembled the effect of human milk (Fig. 3). When the iron-binding capacity of the lactoferrin was doubly saturated with iron the bacteriostatic effect was abolished. (Fig. 3).

EXPERIMENTS WITH L. BIFIDUS

Small inocula (about 10^2/ml) of *L. bifidus* failed to grow in human milk at pH 7·2 and a Po_2 of 40-50 mm Hg but slow growth occurred at a Po_2 of 0 mm Hg.

EXPERIMENTS WITH GUINEA-PIGS

Studies on newly born guinea-pigs were complementary to the observations made with human milk since guinea-pig milk also contains considerable quantities of lactoferrin, though the concentration of transferrin is somewhat greater than that in human milk (Masson and Heremans, 1971). With these experiments it was possible to measure the bacterial population in different parts of the alimentary tract at intervals after infection and also to carry out experiments with iron compounds.

Newly born guinea-pigs were dosed orally with approximately 1×10^6 *E. coli* in saline. Suckled animals were kept with their mothers. Artificially fed animals were separated immediately from their mothers. All the suckled guinea-pigs remained well and gained weight. Artificially fed guinea-pigs lost weight during the first 24 hours, but gained weight thereafter. Their initial weight gains were less than the suckled animals but weight gains were normal after two weeks, at which time both suckled and unsuckled animals were eating

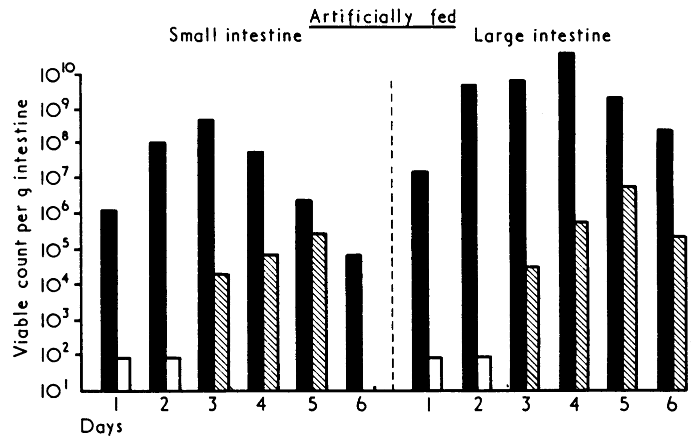

FIG. 5—*E. coli* O111 ■ and lactobacilli □ in small and large intestine of artificially-fed guinea-pigs. □ lactobacillus count $< 10^2$/g. Oral dose of approximately 1×10^6 *E. coli* O111 at birth.

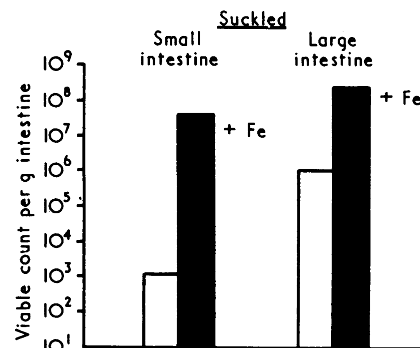

FIG. 6—Effect of feeding haematin to suckled guinea-pigs dosed with approximately 1×10^6 *E. coli* O111 at birth. □ no haematin; ■ haematin twice daily. Viable counts at three days after birth.

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considerable amounts of adult diet. A few unsuckled animals lost weight from birth onwards and died with symptoms of diarrhoea and emaciation. No counts were done on dead or dying animals, though it was of interest that many were suffering from a bacteraemia caused by *E. coli*.

The results shown in Figs. 4 and 5 were compiled from several experiments and the viable counts for each day are the means for three individuals. At each count the serological identity of 50 colonies selected at random from MacConkey plates was checked by slide agglutination tests with *E. coli* O111 antiserum. From these tests the proportion of the total *E. coli* count represented by O111 could be calculated. The counts shown in Figs. 4, 5, and 6 represent the viable counts of *E. coli* O111 only. In most cases all the colonies tested were O111. In Fig. 4 for example, all the colonies tested on days 1-5 from the small intestine were O111. On day 6 no colonies of this organism were found. In this instance the O111 count was recorded at 10^2/g since numbers below this figure could not be detected by the method used. In the large intestine (Fig. 4) all the colonies tested in 10 out of 18 counts were O111. In the remainder the proportion varied from 10 to 60%.

In the artificially fed guinea-pigs (Fig. 5) 15 out of 18 counts from the small intestine consisted entirely of O111. In the remainder 25-65% of the total count was O111. In the large intestine 14 out of 18 counts consisted entirely of O111/B4, and in the remainder 8-90%. In the experiments with haematin (Fig. 6) all the colonies tested in 22 counts from the small intestine were O111, and in the large intestine all the colonies tested in 19 counts were O111, while in three counts 16-98% were O111.

E. COLI O111 AND LACTOBACILLI IN SUCKLED GUINEA-PIGS

The viable counts of O111 were relatively high on the first day in both the small and the large intestine. Thereafter these numbers declined fairly rapidly and the organism had either disappeared or reached very low numbers by day 6 (Fig. 4). None of the animals were given lactobacilli but by the first day considerable numbers of these bacteria were present in the small intestine and very large numbers in the large intestine. By the sixth day the numbers of lactobacilli in the small intestine exceeded those of *E. coli* O111/B4 by a factor of at least 1,000-fold and in the large intestine by a factor of at least 10,000-fold.

E. COLI O111 AND LACTOBACILLI IN ARTIFICIALLY FED GUINEA-PIGS

The numbers of *E. coli* O111 in both the small and the large intestine were very much greater than those in suckled animals (Fig. 5). In addition, it was noticed that the counts rose steadily during the first three to four days whereas this did not happen in suckled animals (Fig. 4). No lactobacilli were detected in the first two days ($<10^2$/g) but these organisms appeared in considerable numbers from three days onwards (Fig. 5).

Statistical analysis showed that the numbers of *E. coli* O111 in the artificially fed guinea-pigs was significantly greater than those in suckled guinea-pigs (small intestine $0.01 < P < 0.05$, large intestine $0.01 < P < 0.05$).

EFFECT OF FEEDING HAEMATIN AND E. COLI TO SUCKLED GUINEA-PIGS

Since iron compounds can abolish the bacteriostatic effect of milk (Fig. 1) and serum (Bullen and Rogers, 1969), experiments were done to see if iron in the form of haematin could enhance the growth of *E. coli* in the intestines of suckled guinea-pigs.

Newly born guinea-pigs were dosed orally with approximately 1×10^6 *E. coli* and 50 mg of haematin. The animals were kept with their mothers, and litter mates which received *E. coli* but not haematin were kept as controls. The haematin was given twice daily for two days. All the animals were killed on the third day. The combined results of a number of experiments involving 22 animals (Fig. 6) showed that the presence of haematin in the small intestine enhanced the number of *E. coli* present by a factor of over 10,000-fold, and in the large intestine by a factor of over 100-fold. All the animals given haematin remained normal. On postmortem examination the contents of both the stomach and the caecum were dark with haematin. There was no apparent inflammation of the intestine, but it was of interest that *E. coli* O111 was often present in the spleen in large numbers, whereas this did not occur in the controls.

Statistical analysis showed that the number of *E. coli* O111 in the animals given haematin was significantly greater than that in the controls (Fig. 6) (small intestine $0.01 < P < 0.05$, large intestine $P \sim 0.02$).

Discussion

There seems to be good evidence that breast-fed infants are more resistant to enteritis caused by *E. coli* than those fed on cows' milk. In a detailed discussion on the factors involved, C. L. Bullen and Willis (1971) provided good reasons for supposing that the growth of lactobacilli in the alimentary tract of breast-fed babies and the consequent fall of pH in a relatively unbuffered medium provide conditions which are unfavourable for the growth of *E. coli*. There seems to be little doubt that this mechanism could make an important contribution to resistance, especially in the large intestine.

Nevertheless, it seems likely that other factors are also involved, especially in the small intestine. For obvious reasons it is impossible to make a quantitative bacteriological examination of different parts of the intestinal tract of healthy human infants, but analogies may be drawn from other species. Enteritis caused by *E. coli* is common among newly born calves and pigs. A characteristic feature of this infection is the rapid rise in the number of *E. coli* in the small intestine (Smith, 1962; Kohler, 1967), and there is little doubt that the highest counts of *E. coli* in the small intestine occur in colostrum-deprived animals (Smith, 1962). Sometimes, however, calves fed colostrum also suffer from severe diarrhoea, though the counts of *E. coli* in the intestine are not so high (Smith, 1962).

Ingram and Lovell (1960) and Smith (1962) make the important point that colostrum-deprived calves tend to suffer from an *E. coli* bacteraemia whereas colostrum-fed animals do not, though the latter can suffer from prolonged diarrhoea, dehydration, and collapse. Babies suffering from *E. coli* enteritis probably resemble the colostrum-fed sick calves since they can receive protective antibody via the placenta while calves can absorb antibody from colostrum. Thus artificially fed babies could receive antibodies from their mothers which would tend to prevent bacteraemia and yet suffer from serious enteritis caused by the rapid growth of *E. coli* in the small intestine.

Since there is good evidence that breast-feeding provides some protection against enteritis caused by *E. coli* the question arises whether there are any specific properties of colostrum and milk which would tend to suppress *E. coli* and thus allow sufficient time for the relatively slow-growing lactobacilli to colonize the gut.

BACTERIOSTATIC EFFECT

Human milk has a powerful bacteriostatic effect on *E. coli* (Fig. 1) provided its pH is maintained above 7·0. Human milk is unusually rich in the iron-binding protein lactoferrin, with concentrations varying from 2 to 6 mg/ml. Transferrin is also present but in smaller quantities (10-50 μg/ml) (Masson

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and Heremans, 1971). The importance of these iron-binding proteins is shown by the fact that if the iron-binding capacity of the milk is saturated with iron its bacteriostatic properties are lost (Fig. 1). Experiments with purified lactoferrin show that this protein has some inhibitory effect on *E. coli* (Fig. 2), but not to the extent of milk itself. However, a combination of purified lactoferrin and specific antibody has a very powerful effect (Figs. 2 and 3) which strongly resembles that of milk (compare Figs. 1 and 3). In addition, the bacteriostatic properties of lactoferrin and specific antibody are also abolished by saturating the lactoferrin with iron. Specific antibody by itself has only a slight effect on *E. coli* (Fig. 2) though its serological specificity seems to be important since a combination of lactoferrin and heterologous antibody (*Cl. welchii*) has an effect little greater than lactoferrin alone (Fig. 2). Complement was not involved in these results since the antisera were heated before use.

The next point is whether milk would be likely to reach the small intestine in a sufficiently undigested state to have a bacteriostatic effect. Mason (1962) pointed out that the pH of the stomach contents of breast-fed babies remains relatively high (pH 6·0-6·5) for about one hour after normal feeding. He also found that at 90 minutes after feeding no hydrolysed protein was detectable in eight specimens of gastric contents and came to the conclusion that in newborn infants the major part of the feed leaves the stomach without any digestion of protein having occurred. This conclusion was supported by the evidence of Henderson (1942), who showed by radiological methods that much of the ingested milk passed directly into the duodenum.

In the small intestine slightly alkaline conditions would be suitable for the inhibition of *E. coli* by lactoferrin and antibody, and this may be assisted by the presence of trypsin inhibitor in human milk, which would tend to delay digestion of proteins (Laskowski and Laskowski, 1951).

While the demonstration in vitro of the bacteriostatic properties of human milk was of some value the experiments in guinea-pigs provided evidence that this property of milk does operate in vivo. Guinea-pigs resemble man in that the transmission of passive immunity to the young is largely prenatal (Brambell, 1958). Guinea-pig milk contains 0·2-2 mg of lactoferrin per ml and 0·2-2 mg of transferrin per ml (Masson and Heremans, 1971). In suckled guinea-pigs dosed with *E. coli* O111 the viable counts of this organism in both the small and the large intestine declined fairly rapidly and there was a corresponding rise in the viable count of lactobacilli (Fig. 4). This situation was reversed in artificially fed animals where the *E. coli* greatly outnumber the lactobacilli, especially during the first four days of life (Fig. 5). These results support the findings of C. L. Bullen and Willis (1971) that the *E. coli* counts in the faeces of bottle-fed babies tend to be much higher than in those fed on breast milk.

Artificial feeds based on cows' milk would contain far less lactoferrin than human milk though the amount of transferrin could be greater. However, it seems quite likely that the transferrin could be partly denatured by the manufacturing process while the addition of iron to the feed would almost certainly saturate the iron-binding capacity of any protein which remained.

Evidence that guinea-pig milk actually suppresses the growth of *E. coli* is provided by the experiments done with suckled animals fed *E. coli* and haematin. In this case the presence of haematin enormously enhances the *E. coli* count and it may be significant that the greatest enhancement occurs in the small intestine, where the milk would be expected to have its greatest suppressive effect (Fig. 6). It is assumed that it is the iron in haematin that is responsible for the growth-enhancing effect (see Bullen, Leigh, and Rogers, 1968).

Much remains to be discovered about the role of iron-binding proteins in resistance to infection, but there is evidence to suggest that they can be of crucial importance. Both the bactericidal and the bacteriostatic effects of normal rabbit serum against *E. coli* can be abolished by saturating the iron-binding capacity of the serum transferrin (Bullen and Rogers, 1969). Iron compounds increase the susceptibility of guinea-pigs to *E. coli* O111 infection (Bullen, Leigh, and Rogers, 1968) and also enhance the growth of *E. coli* in the kidneys of rats and mice, with the production of kidney abscesses (Fletcher and Goldstein, 1970). Saturation of the serum transferrin with iron abolishes passive immunity to *Cl. welchii* type A in guinea-pigs (Bullen, Cushnie, and Rogers, 1967) and to *Pasteurella septica* in mice (Bullen, Wilson, Cushnie, and Rogers, 1968).

Rogers, Bullen, and Cushnie (1970) showed that *Cl. welchii* can be inhibited by mixtures of transferrin and β_2 or γ-globulin in a way that exactly resembles the effect of whole serum, while Griffiths (1971a, 1971b), working with *P. septica*, showed that specific antibody and complement seem to interfere directly with the biochemistry of the bacterial cell, which quickly leads to the inhibition of RNA accumulation and the cessation of bacterial multiplication. Interestingly enough this process can be reversed by saturating the serum transferrin with iron, which also suggests that the serum components interfere in some way with normal bacterial iron metabolism. The general hypothesis that iron-binding proteins acting in concert with specific antibody or other protein components can interfere directly with normal bacterial iron metabolism, which leads to the rapid inhibition of bacterial growth, is discussed elsewhere (Bullen, Rogers, and Lewin, 1971). Melching and Vas (1971) also reported that serum components rapidly interfere with the accumulation of RNA in *E. coli*.

So far as human milk is concerned complement does not seem to be involved, since the milk can be heated to 56°C for 30 minutes without affecting its bacteriostatic power against *E. coli* O111. In addition, bacteriostasis is not destroyed by the addition of *N*-acetyl-L-tyrosine ethyl ester, which inhibits the first component of complement (Basch, 1965). Complement is not necessary for the inhibition of *E. coli* O111 by serum (Bullen and Rogers, 1969).

In conclusion, it would seem that there is good evidence that human milk can have a powerful bacteriostatic effect against *E. coli*. It is suggested that the components involved in this reaction are the iron-binding protein lactoferrin, and possibly transferrin as well, acting in concert with specific *E. coli* antibody. It seems likely that the initial inhibition of *E. coli* by milk in the small intestine could provide ideal conditions for the eventual domination of the intestinal contents by lactobacilli. The point made by C. L. Bullen and Willis (1971) that the presence of *E. coli* could reduce the Eh in the intestine sufficiently to allow *L. bifidus* to multiply may still be valid even if comparatively small numbers of *E. coli* are present since cultures of this organism are highly reducing (Hewitt, 1950). Once established in large numbers the lactobacilli should provide yet another protective mechanism against coliform organisms.

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Precipitins to Dietary Proteins in Serum and Upper Intestinal Secretions of Coeliac Children

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Summary

We have used precipitin tests to detect antibodies to 10 dietary proteins in the serum (71 cases) and intestinal secretions (51 cases) of a group of children. Thirty-three of the patients had untreated coeliac disease. Our aims were to find out if, in coeliac patients, there was intestinal secretion of antibodies to wheat proteins only or if, as in coeliac serum, antibodies to many food proteins were present; and to confirm that secretion of antibodies to wheat or gluten was specific for coeliac disease.

Precipitins to one or more dietary antigens were detected in the intestinal secretions of 26 out of 30 coeliacs and of 11 out of 21 children who did not have coeliac disease. Most of the positive reactions were with the antigens wheat flour, gluten, oatmeal, and egg. Though precipitins to wheat flour or gluten were present in the intestinal secretions of 22 out of 30 coeliacs this was not specific for coeliac disease for these precipitins were also present in 8 out of 21 non-coeliac children.

Serum precipitins were detected in 27 out of 33 coeliacs (to the antigens wheat flour, gluten, oatmeal, rice flour, milk, bovine calf serum, sheep serum, and egg) and in 5 out of 33 non-coeliacs (mainly to milk and calf serum, but two infants aged 3 and 5 months had precipitins to several antigens).

Introduction

Though wheat gluten intolerance is a constant feature of coeliac disease there is still dispute about whether the primary defect is in the digestion and absorption of gluten or a hyper-reactivity of the lymphoid system with an abnormal immune response to gluten (Booth, 1970a; Rubin *et al.*, 1970).

The first clinical studies of antibodies to dietary proteins showed that many coeliac patients had serum antibodies to wheat or gluten extracts (Taylor *et al.*, 1961; Heiner *et al.*, 1962). This became less significant when more extensive work showed serum antibodies to other dietary antigens—for example, milk, oats, sheep, bovine, and egg proteins (Taylor *et al.*, 1961; Heiner *et al.*, 1962; Alarcon-Segovia *et al.*, 1964; Kenrick and Walker-Smith, 1970; Rossipal, 1970)—foods to which these coeliac patients were not clinically intolerant. Most authors now consider that the presence of these serum antibodies is related to increased permeability to macromolecules of the inflamed small-intestinal mucosa (Alarcon-Segovia *et al.*, 1964; Booth, 1970b; Kenrick and Walker-Smith, 1970).

Antibodies to a peptic-tryptic digest of gluten have been detected in the intestinal secretions and stools of coeliac patients, but not in control subjects (Herskovic *et al.*, 1968; Katz *et al.*, 1968). This was held to suggest that intestinal secretion of antibodies to gluten is specific for coeliac disease. Unfortunately, in these investigations of coeliac and control patients no tests were performed which would detect antibodies to dietary proteins other than wheat.

We have studied a group of children (including 33 coeliacs) further to investigate secretion of antibodies to dietary proteins. Our main purpose was to find out if, in coeliacs, there was intestinal secretion of antibodies to wheat proteins only or if, as in their serum, antibodies to many food proteins were present. We also hoped to confirm that secretion of antibodies to wheat or gluten was specific for coeliac disease.

Patients and Methods

The 71 children studied were all inpatients in the Royal Hospital for Sick Children, Glasgow; most were undergoing investigation for suspected gastrointestinal diseases; all were taking a normal gluten- and milk-containing diet. Subsequently, in 33 cases a diagnosis of coeliac disease was made on the basis of (1) upper intestinal histology of subtotal villous atrophy or severe partial villous atrophy, and (2) clinical improvement on a gluten-free diet. One of these coeliac patients also had IgA

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