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Differences in inhibition of the growth of commensal and enteropathogenic strains of *Escherichia coli* by lactotransferrin and secretory immunoglobulin A isolated from human milk

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Summary. Immunoglobulins from bovine and human colostrum and milk and lactotransferrin (LTF) from human milk were investigated for bacteriostatic activity against *Escherichia coli* growing in a tissue culture medium. When tested separately, LTF or secretory immunoglobulin A (sIgA) from pooled human milk showed only slight bacteriostatic activity against human commensal or enteropathogenic strains of *E. coli*. Together, they had a considerable bacteriostatic effect, but only against strains of enteropathogenic serotype. This activity of the sIgA from pooled human milk was consistent for all enteropathogenic serotypes tested, but sIgA isolated from individual milk samples was inactive against some serotypes, and this specificity was associated with antibody to the O antigens. The activity of the sIgA was stable to heat at 56° for 2 h but was lost progressively on heating at 65° for 10 min or longer. Bovine colostral IgG1 was without bacteriostatic effect alone. Together with LTF, it was active against a strain pathogenic to calves but not against human enteropathogenic strains. Tests on rabbit antisera raised against commensal enteropathogenic strains of *E. coli* showed that for the enteropathogens the bacteriostatic activity (in association with LTF) was high and was specific for the serotype of the eliciting strain, but bacteriostatic activity was low or absent in the antisera to commensal strains in spite of the presence of high titres of agglutinating antibodies to these strains.

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INTRODUCTION

Untreated human milk inhibits the growth of certain commensal strains of *Escherichia coli* called milk-sensitive (Dolby, Stephens & Honour, 1977). Other commensal strains are only inhibited in the presence of added bicarbonate ions (milk-resistant). After boiling, the milk is no longer inhibitory, however, the inhibitory activity against milk-sensitive commensals can be partially restored by addition of purified secretory immunoglobulin A (sIgA) and lactotransferrin (LTF) (Spik, Cheron, Montreuil & Dolby, 1978). It was not possible to demonstrate a similar inhibition of sIgA and LTF against milk-resistant commensal strains due to the interference of citrate in the boiled milk with the binding of added bicarbonate ions to the LTF (Griffiths & Humphreys, 1977). Rogers & Synge (1978) demonstrated that sIgA and LTF are involved in bacteriostasis of an enteropathogenic strain of *E. coli* using a tissue culture medium based on 199.

This sIgA/LTF activity has been studied in more detail using the medium of Rogers & Synge (1978). In this system, it has been found that the sIgA and LTF are equally inhibitory against milk-sensitive and milk resistant strains, but have a much stronger effect on strains of enteropathogenic serotype than on commensal strains.

MATERIALS AND METHODS

Strains of E. coli

A milk-sensitive commensal strain V21-1 was isolated from a mother's stools, the milk-resistant commensal strains were isolated from the stools of a healthy baby (VB71-1) and from healthy adults (JD1 and SS4). Additional strains were isolated from the stools of French 1–2 month old and English 1 week old infants. Strains of enteropathogenic serotype were as follows: 0111 K58 (B4) H2, from Dr Bullen, National Institute for Medical Research, London; 0111 (QE9) isolated at Queen Elizabeth Hospital, Hackney; 0111 K58 (B4) H-(NCTC 9703) and 0115 K? H-(NCTC 10444, a calf pathogen), both from the National Collection of Type Cultures, Colindale, London; 0128 K67 H2, 0128 K67 H12 and 0126 isolated at Northwick Park Hospital; 044 K74 H18, 055 K59 H7 and 0119 K69 H6 from Dr Rowe, Central Public Health Laboratory. Strain 0128 H2 was milk-sensitive and 0128 H12 milk-resistant.

Preparation of immunoglobulins and lactotransferrin

Samples of human milk from mothers at different stages of lactation were collected from a local milk bank and kept at $-20°$ until use. The thawed milks, delipidated and decaseinated, were fractionated combining a concentration gradient of $(NH_4)_2SO_4$ and a pH gradient as described by Montreuil, Chosson, Havez & Mullet (1960).

sIgA was isolated from fraction P_4 obtained at 50% saturation in $(NH_4)_2SO_4$ at pH 7. It was purified by gel filtration on two coupled columns of Ultrogel AcA 44 (Industrie Biologique Française) in the conditions described previously (Pierce-Cretel, Pamblanco, Strecker, Montreuil & Spik, 1980; Spik *et al.*, 1978).

LTF was isolated and purified from the precipitate P8 obtained at 75% saturation with $(NH_4)_2SO_4$ at pH 7 (Cheron, Mazurier & Fournet, 1977). The iron saturation of the purified LTF was judged by absorbance at 465 nm using $E_{465}^{1\%}$ 0·58 and the sample was found to be one-third saturated.

The bovine IgG1 was a gift from Dr A. Cheron and was prepared from colostrum as described previously (Cheron, Fournet, Spik & Montreuil, 1976).

Heat inactivation of sIgA

Purified samples of sIgA were heated as a 1% solution to 56° or 65° for 10 min to 120 min and then lyophilized.

Preparation of antisera

Four New Zealand white rabbits were injected intravenously with increasing doses of 0·2–1·0 ml of a 2×10^9 org/ml suspension of *E. coli* strain V21-1, JD1, SS4 or QE9 (0111) killed by heating to 65° for 30 min. Injections were given thrice to once weekly for 6 weeks; the rabbits were bled out from the heart 2 weeks after the last dose. Sera were inactivated at 56° for 30 min. All sera had high O agglutinating antibody titres against the homologous strain but not against the heterologous strains. A 1/50 dilution of antiserum was used in the bacteriostatic tests.

Bacteriostatic tests

A synthetic medium (Rogers & Synge, 1978) was used for testing all fractions. This consisted of half-strength 199 medium with added casein, lactose, glucose and bovine serum albumin, adjusted to pH 8·1. The tests were done in $3 \times \frac{5}{8}$ inch capped tubes in 0·2 ml volumes. To 0·08 ml medium, samples and additives to be tested (LTF, sIgA, IgG, antiserum, ferric ammonium citrate) were added in 0·02 ml volumes of stock solution. Sodium bicarbonate was added to give a final concentration of 0·005% and the volume was then made up to 0·16 ml with 0·85% saline. The inoculum of *E. coli* was added as 0·04 ml of a 10^{-3} dilution (containing about 10^4 organisms) of a 3 h peptone water culture. Tubes were incubated at 37° and sampled at 3 and 6 h. Ten-fold saline dilutions were plated on MacConkey agar for estimation of viable organisms. Tables showing results at 3 h are expressed as fold increase; less than ten-fold increase was considered as bacteriostatic. Results of 6 h tests are shown on graphs expressed as $\log_{10}$ increase.

IgA assays

A single radial immunodiffusion technique was used (Mancini, Carbonara & Heremans, 1965) with rabbit anti-human colostral IgA (Dakopatts, Denmark).

Agglutinin titres and absorptions

O-agglutinin titres of antisera were measured in two-fold dilutions in Dreyers' tubes against saline suspen-

sions of organisms at 2×10^9/ml prepared by heating in a boiling water bath for 30 min. Tests were incubated 18 h at 56°. Absorption of O-agglutinins was by similarly prepared organisms, twice, with 2×10^{11} wet deposits of cells per ml of serum (or 10 mg/ml solution sIgA) for 3 h at 37° each. K-agglutinin titres were measured similarly against saline suspensions heated 56° for 30 min, incubated for 4 h at 37° and refrigerated before reading.

RESULTS

Bacteriostasis of *E. coli* by human LTF

The effect of increasing concentrations of LTF isolated from human milk on a commensal and enteropathogenic strain of *E. coli* is shown in Table 1. There is a slight inhibition of growth against both strains but this is not substantially increased at higher concentrations.

Table 1. Bacteriostasis of *E. coli* by human lactotransferrin

Concentration in growth medium of LTF (mg/ml)	Number of times inoculum increases in 3 h for strain	
	Commensal V21-1	Enteropathogen 0111
0	47*	49
0·125	45	31
0·25	45	33
0·5	39	30
1·0	40	31
2·0	31	28
4·0	29	30

* Bacteriostatic activity is considered present if there is less than a ten-fold increase.

The activity of LTF at 2 mg/ml was examined against various strains of *E. coli* for 6 h. Under the experimental conditions described the LTF alone was not strongly bacteriostatic although there was an increase in generation time of the organisms and this was similar for all strains tested. Extension of the incubation time to 24 h showed that this slight inhibitory effect lasted only 12 h.

Table 2. Bacteriostasis of a commensal strain of *E. coli* V21-1 by LTF and sIgA

LTF mg/ml	Number of times inoculum increases in 3 h in presence of sIgA (mg/ml)		
	0	2	4
0	47*	43	44
1·0	40	45	—
2·0	30	30	24
4·0	26	23	17

* Footnote as Table 1.

Bacteriostasis of sIgA isolated from pooled human milk, alone and in association with LTF

Commensal E. coli. Table 2 shows the effect of increasing concentrations of both LTF and sIgA on the growth of strain V21-1. Secretory IgA alone is not inhibitory, but addition of sIgA to the LTF slightly enhances the inhibitory activity of the LTF. The activity of sIgA was tested at 2 mg/ml against a range of commensal strains isolated from healthy babies in Lille and Harrow. The results were all similar irrespective of the milk-sensitivity of the strains. A typical example, shown in Fig. 1a, indicates only a slight enhancement of the inhibitory effect of LTF by addition of sIgA.

Enteropathogenic E. coli *(EPEC)*. Table 3 shows the effect of increasing concentrations of LTF and

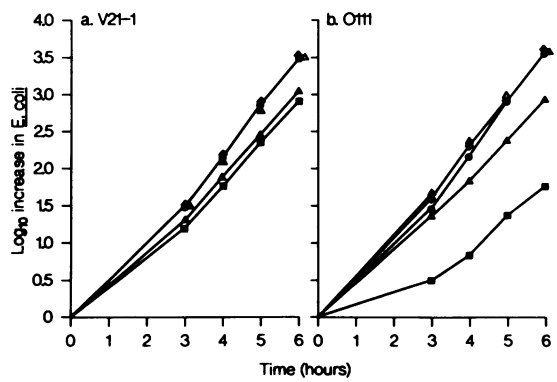

Figure 1. Comparison of bacteriostatic activity of sIgA from pooled human milk and LTF against commensal (V21-1) and enteropathogenic (0111) *E. coli*. ◆, Control; ●, sIgA (1 mg/ml); ▲, LTF (1 mg/ml); ■, LTF+sIgA; △, LTF+sIgA+Fe (200 μg/ml).

Table 3. Effect of increasing concentrations of sIgA and LTF on the growth of *E. coli* strain 0111.

LTF (mg/ml)	Number of times inoculum increases in 3 h in presence of sIgA (mg/ml)				
	0	0·25	0·5	1·0	2·0
0	47	46	35	32	17
0·125	28	33	23	11	6
0·25	30	20	15	7	4
0·5	26	24	15	9	5
1·0	27	27	14	7	5
2·0	28	25	12	8	4

Footnote as Table 1.

sIgA on the growth of strain 0111; sIgA alone shows some inhibition of growth, particularly at 2 mg/ml, but this is considerably increased in the presence of LTF. A minimum concentration of 0·25 mg/ml LTF and 1 mg/ml sIgA exerted a bacteriostatic effect (less than ten-fold increase in inoculum) and this was considerably greater than either of the activities alone. sIgA from pooled human milk was tested at 1 mg/ml alone and in the presence of LTF (1 mg/ml) against a range of EPEC O serotypes. Unlike their effect against commensal strains, these were all inhibited. An example of the results obtained is shown for an 0111 strain in Fig. 1b; maximum inhibition occurred at 3 h. The bacteriostatic activity was completely abolished by the addition of sufficient iron (ferric ammonium citrate 200 μg/ml) to saturate the LTF. Similar curves were obtained for other serotypes tested, 044, 055, 0126, both strains of 0128, and 0119.

Table 4. Comparison of bacteriostatic activity of sIgA (in association with LTF) isolated from a milk pool and from individual mothers

E. coli strain	Number of times inoculum increases in 3 h in presence of				
	Medium alone	+LTF	+LTF+IgA from		
			Pool	L3405	V202
VB 71-1	21	17	20	20	16
0 111	40	32	4	6	5
0 126	42	25	9	8	8
0 128	30	22	9	8	3
0 55	33	21	3	20	8
0 119	34	23	7	12	24

Footnote as Table 1.
LTF and IgA tested at 1 mg/ml.

Specific bacteriostatic activity of sIgA isolated from milk of individual mothers

sIgA samples V 202 and 3405 were isolated from milk collected from two individual mothers. These were tested in association with LTF against a range of enteropathogenic and commensal *E. coli* in comparison with sIgA isolated from a milk-bank pool. These results (Table 4) indicate a degree of specificity in the bacteriostatic activity of the sIgA from individual mothers. Sample 3405 was inactive against strain 055 and V202 was inactive against strain 0119. The activity against the other EPEC tested was as good as that of the sIgA from the pooled milk; none of the sIgA samples was active against the commensal strain VB71-1.

Effect of heat treatment on sIgA activity

sIgA fraction 3405 was heated to 56° or 65° for 10–120 min. It was then assayed at a concentration equivalent to 2 mg/ml of the original sample by single radial immunodiffusion, and tested for bacteriostatic activity against an EPEC strain 0111 in the presence of 1 mg/ml LTF. The results (Fig. 2) indicate that heating to 56° for 2 h did not significantly diminish the bacteriostatic activity of the sIgA although estimates by gel diffusion showed a reduction from 2 mg/ml to 1·26 mg/ml. After heating to 65° for 20 min or longer, the activity of the sIgA was not substantially greater than the activity of the LTF alone and the gel diffusion assay indicated more than 50% reduction in the sIgA.

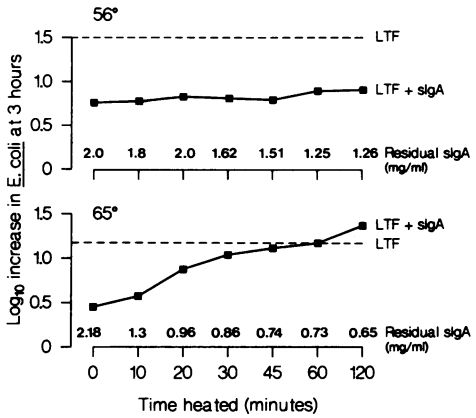

Figure 2. Effect of extended periods of heat treatment on the bacteriostatic activity of sIgA in the presence of LTF (1 mg/ml) against *E. coli* strain 0111.

Effect of IgG1 isolated from bovine colostrum

A sample of IgG1 isolated from colostrum of a single cow was tested against human commensal and enteropathogenic strains and a strain pathogenic to calves, NCTC 10444. The results shown in Fig. 3 indicate bacteriostatic activity against the calf strain, but little or no activity against the strains of human origin.

Bacteriostatic effect of specific rabbit antisera

Rabbit antisera raised against commensal strains V21-1 and JD1 and tested at a 1/50 dilution showed no bacteriostatic activity alone or in the presence of LTF against the homologous strain (Fig. 4a) or other strains of either commensal or enteropathogenic serotype. Antiserum raised against commensal strain SS4 was however inhibitory for 3 h against the homologous strain in the absence of LTF; this activity was not

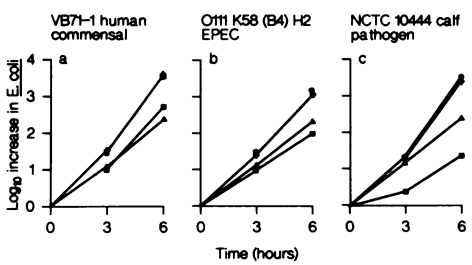

Figure 3. Bacteriostatic activity of bovine colostral IgG1 against *E. coli*. ◆, Control; ●, IgG1 (2 mg/ml); ▲, LTF (2 mg/ml); ■, LTF + IgG1.

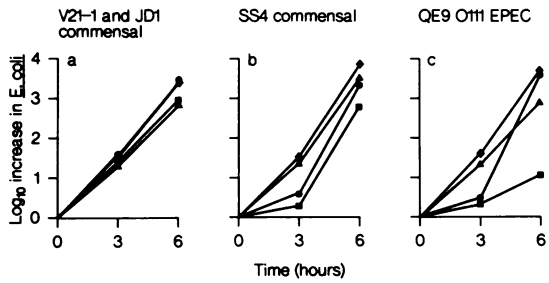

Figure 4. Bacteriostatic activity of specific antisera in association with LTF against commensal and enteropathogenic strains of *E. coli*. ◆, Control; ▲, LTF (1 mg/ml); ●, 1/50 homologous antiserum (AS) and also LTF + AS + Fe (200 μg/ml ferric ammonium citrate); ■, LTF + AS.

reversed by iron. Together with LTF this antiserum showed increased bacteriostatic activity for the homologous strain which lasted only 3 h and which was reduced by iron to the level of the activity of antiserum alone (Fig. 4b).

Antiserum raised against the pathogenic strain QE9(0111) tested alone against the homologous strain was like the SS4 antiserum—active for 3 h but not reversed by ferric ammonium citrate at 200 μg/ml (Fig. 4c). Tested in the presence of LTF however, this antiserum had a very much stronger bacteriostatic activity than the antisera raised against the commensal strains. It was active not only against the homologous strain but also against other strains of 0111 serotype with differing H but similar K or B antigens. By the addition of iron this activity was reduced to that of antiserum alone. The antiserum had no activity, alone or in the presence of LTF, either against enteropathogenic strains carrying other O serotypes (but similar H antigens) or against commensal strains.

The bacteriostatic activity of antisera against *E. coli* in the presence of LTF therefore appears to be effective only against strains of enteropathogenic serotype and could be specific for the O or K antigens.

The activity of two of the antisera alone at 3 h which was not reversed by iron, appears to be due to another antibacterial mechanism.

Effect on bacteriostasis of removing O-agglutinins

To determine whether the bacteriostatic activity of the QE9 antiserum was due to antibodies directed against the O or K antigens, absorption experiments were carried out. Absorption of O antibodies by the boiled, homologous suspension removed the bacteriostatic

Table 5. Bacteriostatic activity of rabbit antiserum for *E. coli* 0111 K58 (B4) H2 and O and K agglutinin titres

LTF with antiserum 1/50	Number of times 0111 inoculum increases in 3 h	Reciprocal of agglutinin titres to strain 0111	
		O	K
Unabsorbed	5	640	160
Absorbed 0111*	51	< 10	160
Absorbed 0128	15	320	160
Absorbed VB71-1	10	320	160

* Absorbing suspension heated at 100°.

effect but absorption with VB71-1 and 0128 did not (Table 5). The K antibody of the QE9 antiserum was not removed by this procedure which indicated that this antibody was not involved in the bacteriostatic activity. Agglutinins to the O-antigen were similarly removed from sIgA of pooled milk-bank milk and again bacteriostatic activity was lost.

DISCUSSION

Bacteriostatic activity has been attributed to lactoferrin in both human (Masson, Heremans, Prignot & Wauters, 1966) and bovine (Oram & Reiter, 1966) milk. Bullen, Rogers & Leigh (1972) found however that human lactoferrin had only a slight inhibitory effect on the growth of *E. coli* when compared with lactoferrin in the presence of specific serum antibody. This was confirmed by Rogers (1976) working with purified horse IgG and by Rogers & Synge (1978) with sIgA from human milk. The experiments reported by these workers were carried out in a CO_2/bicarbonate-buffered tissue culture medium and activity against enteropathogenic strains of *E. coli* was measured. Spik *et al.* (1978) measured the activity of purified sIgA and LTF, in boiled, inactivated milk in an attempt to mimic the *in vivo* environment more closely and activity was measured against a milk-sensitive commensal strain and not an enteropathogen. As Samson, Mirtle & McClelland (1979) have fairly pointed out, the increase in bacteriostasis achieved by Spik and colleagues by adding sIgA to LTF was very slight although consistent. Samson and colleagues showed that the addition of sIgA did not enhance the activity of human sIgA-deficient colostrum for an enteropathogenic strain of *E. coli* and have questioned the involvement of sIgA in bacteriostasis by whole milk.

The experiments described here using a synthetic growth medium have confirmed that human LTF alone has an inhibitory effect on both commensal and enteropathogenic strains of *E. coli*, but this is small in comparison with the bacteriostatic effect of whole milk. Addition of sIgA to the LTF in this medium slightly increases the bacteriostasis against commensal strains confirming the results of Spik *et al.* (1978) using a boiled milk diluent. Results with all the enteropathogenic strains tested, however, show a very strong enhancement of the LTF activity by addition of sIgA from a milk-bank pool. This bacteriostatic activity of sIgA against enteropathogenic strains of *E. coli* is stable to heat at 56° but not at 65°. The antibodies involved appear to occur widely although in individual milks, serotype specificity can be demonstrated. This specificity presumably depends on the strains of *E. coli* to which the mother's gut has been exposed, as specific sIgA in milk has been shown to be produced by lymphocytes primed in the gut, which home to the mammary gland during lactation (Goldblum, Ahlstedt, Carlsson, Hanson, Jodal, Lindin-Janson & Sohl-Akerlund, 1975).

The IgG1 fractions isolated from bovine colostrum also enhance specifically the bacteriostatic activity of LTF against the strain pathogenic to calves. No activity was found against any strain of human pathogenic serotype, presumably because the cow herd had not been exposed to them. Rabbit antisera (predominantly IgG) raised against an enteropathogenic strain also demonstrate a type-specific enhancement of the bacteriostatic activity of LTF. Absorption experiments indicate that the O and not the K antibody was involved.

The failure to demonstrate bacteriostatic activity of sIgA from pooled human milk, or of specific rabbit antisera against commensal strains of *E. coli* in the presence of LTF is interesting. In the rabbit antisera, high titres of agglutinating antibody were present although no bacteriostatic activity could be demonstrated against two strains, and only poor activity against a third. Presumably there is some important difference between pathogenic and non-pathogenic strains in their structure or function, possibly at the bacterial surface. Whole milk, on the other hand, is equally active against commensal and enteropathogenic strains (Dolby & Honour, 1979). This suggests that the *in vitro* activity of whole milk against commensals which is reversed by addition of iron may be

due to another component in addition to LTF and IgA and this possibility is now being investigated.

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REFERENCES

BULLEN J.J., ROGERS H.J. & LEIGHT L. (1972) Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br. med. J.* **8,** 69.

CHERON A., FOURNET B., SPIK G. & MONTREUIL J. (1976) Structure complète des glycopeptides isolés des immunoglobulines IgG_1 du colostrum de Vache. *Biochimie,* **58,** 927.

CHERON A., MAZURIER J. & FOURNET B. (1977) Fractionnement chromatographique et études sur la microhétérogénéité de la lactotransferrine de Vache préparée par un procéde original. *C. R. Acad. Sci. Paris,* **284,** 585.

DOLBY J.M. & HONOUR P. (1979) Bacteriostasis of *Escherichia coli* by milk. IV The bacteriostatic antibody of human milk. *J. Hyg., Camb.* **83,** 255.

DOLBY J.M., STEPHENS S. & HONOUR P. (1977) Bacteriostasis of *Escherichia coli* by milk II. Effect of bicarbonate and transferrin on the activity of milk feeds. *J., Hyg., Camb.* **78,** 235.

GOLDBLUM R.M., AHLSTEDT S., CARLSSON B., HANSON L.A., JODAL, U., LIDIN-JANSON, G. & SOHL-AKERLUND A. (1975) Antibody forming cells in human colostrum after oral immunisation. *Nature (Lond.),* **257,** 797.

GRIFFITHS E. & HUMPHREYS J. (1977) Bacteriostatic effect of human milk and bovine colostrum on *Escherichia coli*: the importance of bicarbonate. *Infect. Immun.* **15,** 396.

MANCINI G., CARBONARA A.O. & HEREMANS J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry,* **2,** 235.

MASSON P.L., HEREMANS J.F., PRIGNOT J. & WAUTERS J. (1966) Immunohistochemical localization and bacteriostatic properties of an iron-binding protein from bronchial mucus. *Thorax,* **21,** 538.

MONTREUIL J., CHOSSON A., HAVEZ R. & MULLET S. (1960) Isolement de β-2A globulines du lait de Femme. *C.R. Soc. Biol.* **154,** 732.

ORAM J.D. & REITER B. (1966) Inhibitory substances present in milk and the secretion of the dry udder. *Reports of the National Institute for Research in Dairying.* p. 93.

PIERCE-CRETEL A., PAMBLANCO M., STRECKER G., MONTREUIL J. & SPIK G. (1980) Heterogeneity of the glycans O-glycosidally linked to the hinge region of secretory immunoglobulins sIgA of human milk. *Eur. J. Biochem.* (in press)

ROGERS H.J. (1976) Ferric iron and the antibacterial effects of horse 7S antibodies to *Escherichia coli* 0111. *Immunology,* **30,** 425.

ROGERS H.J. & SYNGE C. (1978) Bacteriostatic effect of human milk on *Escherichia coli*: the role of IgA. *Immunology,* **34,** 19.

SAMSON R.R., MIRTLE C. & MCCLELLAND D.B.L. (1979) Secretory IgA does not enhance the bacteriostatic effects of iron-binding or vitamin B_{12}-binding proteins in human colostrum. *Immunology,* **38,** 367.

SPIK G., CHERON A., MONTREUIL J. & DOLBY J.M. (1978) Bacteriostasis of a milk-sensitive strain of *Escherichia coli* by immunoglobulins and iron-binding proteins in association. *Immunology,* **35,** 663.