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INTERACTION OF ROTAVIRUS AND ENTEROTOXIGENIC *ESCHERICHIA COLI* IN CONVENTIONALLY-REARED DAIRY CALVES

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ABSTRACT

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A study was made of the effects of rotavirus and/or enterotoxigenic *Escherichia coli* (ETEC) on dairy calves born and suckled on the farm and subsequently reared in isolation. Calves were orally inoculated at 6 days old with either rotavirus (5), ETEC (7), rotavirus and ETEC (5) or remained uninoculated controls (4), and their reactions were recorded by clinical, microbiological, and pathological observations. Rotavirus infection consistently produced diarrhoea, while ETEC inoculated alone did not colonise the intestine. In dual infections, both rotavirus and ETEC multiplied, although the severity of diarrhoea was not greater than that caused by rotavirus alone. Some ETEC-inoculated calves developed subsequent naturally-acquired rotavirus infections, but in these no ETEC multiplication occurred. The results suggest that prior or simultaneous rotavirus infection is necessary to enable ETEC colonisation of the intestine in conventional calves of this age.

INTRODUCTION

Several enteropathogens, including rotavirus and enterotoxigenic *Escherichia coli* (ETEC) have been implicated commonly in the aetiology of neonatal calf diarrhoea (Acres et al., 1975; Morin et al., 1976; Moon et al., 1978; De Leeuw et al., 1980). ETEC infections occur most commonly in calves in which diarrhoea commences by 3 days of age (Acres et al., 1975; Moon et al., 1978) and experimentally it has been possible to produce diarrhoea, with ETEC, only in calves under 48 h old (Smith and Halls, 1967). On the other hand, rotavirus infections occur mainly in calves from 4 days to several weeks of age (Acres et al., 1975; Moon et al., 1978; de Leeuw et al., 1980). However, combined natural infections with rotavirus and ETEC do occur (Acres et al., 1975; Morin et al., 1976; Moon et al., 1978) although the significance of such

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combined infections is equivocal. Although most field studies have not detected any interaction (Acres et al., 1975; Moon et al., 1978), an increased severity of decrease has been suggested by Morin et al. (1976). Experiments in gnotobiotic calves have shown both a synergistic interaction producing a fatal disease (Gouet et al., 1978), and an interaction in which rotavirus aided ETEC colonisation but with little disease enhancement (Runnels et al., 1980). However, experiments with gnotobiotic animals exaggerate the ability of microorganisms to colonise the intestine, and the interaction of two organisms can more realistically be studied against the background interaction with a normal gut flora. Preliminary experiments by Tzipori et al. (1981) showed that rotavirus and ETEC inoculated together into conventional calves produced a more severe diarrhoea than that caused by either agent alone. The present study was therefore undertaken to investigate the interaction of rotavirus and ETEC in the production of diarrhoea in conventionally-reared calves aged 6 days.

MATERIALS AND METHODS

Animals

Jersey bull calves were collected when 1 to 2 days old from four dairy farms, where they had been kept from birth with their dams, in clean paddocks away from the main herd. Calves were brought to the laboratory where they were maintained individually in fibre glass tanks in isolation blocks. The calf accommodation was cleaned and fumigated with formaldehyde between experiments. Calves were fed 2 l of ultraheat-treated milk twice a day.

Twenty-one calves remained clinically normal, and were inoculated orally at 6 days of age. Five were inoculated with rotavirus, seven with ETEC, five received rotavirus and ETEC simultaneously and there were four uninoculated control calves.

Six calves were eliminated from the experiment before inoculation due to naturally-acquired diarrhoea and rotavirus infection and a seventh was eliminated due to detection of cryptosporidia although the calf remained clinically normal.

Inoculum

(1) *Rotavirus.* A gnotobiotic calf was inoculated orally with a 0.22-μm filtrate of intestinal contents from the fourth conventional calf passage of calf rotavirus field isolate C6 (Tzipori et al., 1980). A large volume of faeces collected from this calf 3 days after inoculation was homogenized with an equal volume of phosphate-buffered saline (PBS), dispensed in 4 ml aliquots and stored at −70°C. Rotavirus particles were readily visible by electron microscopic (EM) examination of unconcentrated preparations of this material. Each calf was inoculated with one 4 ml aliquot.

(2) *E. coli.* The enterotoxigenic calf strain B44 (09:K30—K99:NM) (Smith and Halls, 1967) which produces heat-stable but not heat-labile enterotoxin, was used. The organism was grown overnight on Minca-isovitalex agar (Guinee et al., 1977) and suspended in 0.15 M NaCl. The titre was estimated by optical density readings, and subsequent titration on sheep blood agar was used to determine the viable count. The calf inoculum consisted of a 10 ml suspension with a viable count of 1.2×10^9 to 1.3×10^{10} organisms per ml.

Observations

Faecal samples were collected from calves on at least two occasions prior to inoculation, and daily thereafter for 7 days or until the calf was killed. To determine severity of diarrhoea, faecal collection harnesses were fitted on ten calves, daily faecal output was weighed and the faeces dried to constant weight for dry matter estimation. All calves were examined at least once a day, particular attention being paid to the nature of the faeces. Serum was obtained from calves prior to inoculation.

From fourteen calves under terminal anaesthesia, five segments of intestine were taken: jejunum, approximately 50 cm distal to the duodenojejunal flexure; midgut; ileum, approximately 1 metre proximal to the ileocaecal junction, where possible in an area free of Peyer's patches; caecum; and spiral colon. An opened portion of gut was fixed flat in 10% formol-saline and processed for histological examination. Mean villus height and crypt depth at each small intestinal site were determined by measuring up to ten well-orientated villi and crypts using an ocular micrometer. A 5 cm length of small intestine including contents, or contents alone in the case of large intestine, was taken for enumeration and identification of *E. coli.* A 3 cm length of gut was filled with embedding medium (Lab-Tek Products) and frozen in a dry ice-isopentane sludge for cryostat sections. Immunofluorescence was used to detect K99 or rotavirus antigen in the sections.

Neutralization Test. Serum samples were tested for the presence of neutralizing antibody to tissue culture adapted calf rotavirus (McNulty et al., 1976) on MA 104 cells grown in microtitre plates. Titres are expressed as the reciprocal of the highest dilution giving complete neutralization.

Faecal Examination. Faecal samples were examined for rotavirus by enzyme-linked-immunosorbent assay (ELISA), using a technique similar to that described by Fahey et al. (1981), except that the conjugated enzyme was horseradish peroxidase rather than alkaline phosphatase. In addition, at least one faecal sample from all scouring calves was examined by EM to detect rotavirus and other enteric viruses.

Faecal swabs were cultured on both sheep blood agar and MacConkey agar overnight. Ten colonies of *E. coli* were randomly selected from the plates and subcultured on to Minca-isovitalex agar overnight. These *E. coli* were tested

TABLE I

Occurrence of diarrhoea and infection in experimental calves

Calf Group and number		Incubation period to diarrhoea (day p.i.[a])	Preparent period to rotavirus excretion (day p.i.)	Preparent period to B44 excretion (day p.i.)	Max B44 in faeces (no. detected of 10 colonies examined)	Day of necropsy (day p.i. or age for controls)	Sites of immunofluorescence		*E. coli* Titre in ileum (log_{10} viable count/5 cm)
							Rotavirus	K99	
Control	1	N[b]	—[c]	—	0	7	—	—	3.9
	2	N	—	—	0	7	—	—	5.1
	3	N	—	—	0	6	—	—	6.2
	4	N	—	—	0	8	—	—	5.2
Rotavirus	5	4	3	—	0	. . .[d]	. . .	. . .	. . .
	6	3	4	—	0	5	1,2	—	4.3
	7	2	4	—	0	. . .	. . .	. . .	. . .
	8	2	4	—	0	. . .	. . .	. . .	. . .
	9	2	3	—	0	. . .	. . .	. . .	. . .
ETEC	10	N	—	—	0	3	—	—	3.9
	11	N	—	1	10	. . .	. . .	. . .	. . .
+naturally	12	2	4	—	0	4	1,2,3	—	5.5
acquired	13	1	1	—	0	3	1	—	3.1
rotavirus	14	4	4	—	0	6	1,2,3	—	4.9
infection	15	4	6	—	0	. . .	. . .	. . .	. . .
	16	8	6	—	0	. . .	. . .	. . .	. . .
Rotavirus	17	3	3	2	10	4	1,2,3,4	—	8.7
+ ETEC	18	2	—	2	6	2	—	—	7.1
	19	3	3	2	8	7	—	—	6.0
	20	2	—	1	10	3	1,2	2,3	9.0
	21	2	4	2	10	6	—	—	3.3

[a] p.i., post inoculation.
[b] —, not detected.
[c] N, remained normal.
[d] . . ., not examined.
Sites of immunofluorescence: 1, proximal jejunum, 2, midgut, 3, distal ileum, 4, caecum.

for the presence of K99 pilus antigen by slide agglutination with a K99 antiserum prepared in rabbits by inoculation of a K99+ mutant of a K12 strain, with subsequent absorption of the serum by the parent K12 strain (Moon et al., 1977). Each of the ten selected colonies were also subcultured onto tryptose soya agar, autoclaved for 2.5 h at 121°C, and tested for 09 antigen by a tube agglutination test using 09 antiserum prepared in rabbits (Edwards and Ewing, 1972). *E. coli* that possessed both K99 and 09 antigens were considered likely to be B44.

RESULTS

Occurrence of diarrhoea

Calves had preinoculation serum antibody titres to rotavirus of 160-1280.

The four control calves continued to pass faeces of normal colour and consistency, remained alert and had a normal appetite.

All five calves inoculated with rotavirus developed diarrhoea after 2—4 days (Table I). The mean maximum daily faecal output increased from 45 ± 8 g to 1042 ± 78 g, and dry matter decreased from 28 ± 1% to 8 ± 1% (mean ± s.e.) (Table II). Two of these calves also became dull and anorectic.

TABLE II

Maximum daily output and minimum dry matter of faeces from diarrhoeic and normal calves (Mean ± s.e.).

Group	Maximum faecal weight/24 h (g)	Minimum faecal dry matter (%)
Normal calves	45 ± 8	28 ± 1
Rotavirus	1042 ± 78	8 ± 1
Rotavirus + ETEC	1072 ± 43	9 ± 2
ETEC (+ natural rotavirus infection)	990 ± 290	8 ± 3

Only two calves inoculated with ETEC did not acquire natural rotavirus infection (Table I) and both remained normal throughout. The other five calves in this group became diarrhoeic, and were subsequently shown to be infected with rotavirus. One of them exhibited partial anorexia.

The five calves inoculated with both rotavirus and ETEC all started to scour after 2—3 days and became dull and anorectic (Table I).

The severity of diarrhoea was similar in calves infected with rotavirus, or rotavirus and ETEC, or in calves with rotavirus infections acquired naturally (Table II).

Virological Examination

All calves inoculated with rotavirus, and four of the five dually-inoculated calves, became infected with rotavirus (Table I). In spite of the isolation precautions, five of the seven ETEC-inoculated calves acquired rotavirus infections which were detected both by examination of faeces and by immunofluorescent staining of gut sections. Although all small intestinal sites sampled and caecum could be shown to be infected with rotavirus on occasion, the most consistently infected part of the gut was the anterior jejunum. Rotavirus infections were not detected in other calves. Coronaviruses or other enteric viruses were not detected by EM examination.

Bacteriological Examination

B44 were considered to have colonised the small intestine if immunofluorescence with K99 antiserum was demonstrated in gut sections, and if adherent bacteria could be observed histologically. In addition, it was considered likely that isolation of 10/10 K99+ and 09+ colonies in faeces indicated multiplication of B44 to high titre in small intestine.

None of the control calves, nor any of those inoculated with rotavirus, excreted B44. In contrast all five animals inoculated with rotavirus and ETEC became infected with B44. Of the seven calves inoculated with ETEC alone including those with naturally-acquired rotavirus infections, only one (# 20) showed evidence of infection with B44 (Table I). This animal, which was killed while K99+ bacteria were present on epithelial cells in ileum, had the highest titre of bacteria in ileum.

The titre (mean ± s.e.) $\log_{10}$ viable count/5 cm of *E. coli* in ileum of control calves was 5.1 ± 0.5 (range 3.9 to 6.2) (Table I). Titres in jejunum and midgut were also within this range. Viable bacterial counts in all three small intestinal sites of all calves inoculated separately with either rotavirus or ETEC were within this normal range. In the dually-inoculated calves killed 2 to 4 days after inoculation, ileal *E. coli* titres of 8.3 ± 0.6 were significantly greater than titres in control calves ($P < 0.01$).

Histological Examination

The small intestine of control calves had long slender villi with intact columnar epithelium. The length of villi decreased from proximal to distal end of small intestine (Table III). No significant lesions were observed in calves in this group.

There was evidence of crypt hypertrophy and a patchy villus atrophy in calf # 10, killed three days after ETEC inoculation (Table III). No other lesions were detected.

Lesions in the rotavirus-inoculated calf and in the calves with naturally-acquired rotavirus infections were similar. Villi at all small intestinal sites

TABLE III

Measurement of villus height and crypt depth in small intestine of control and infected calves (mean ± s.e.)

Group	Villus height (μm)			Crypt depth (μm)		
	Proximal jejunum	Midgut	Distal ileum	Proximal jejunum	Midgut	Distal ileum
Control (4)	972 ± 46	909 ± 41	858 ± 34	269 ± 6	235 ± 9	245 ± 7
Rotavirus (1)	497 ± 53*	543 ± 29*	389 ± 28*	501 ± 26*	554 ± 18*	447 ± 16*
Rotavirus + ETEC (5)	434 ± 14*	503 ± 26*	529 ± 38*	317 ± 9*	374 ± 14*	332 ± 8*
ETEC + naturally acquired rotavirus (3)	510 ± 32*	605 ± 30*	425 ± 16*	426 ± 13*	447 ± 8*	337 ± 13*
ETEC (1)	912 ± 75	487 ± 24*	872 ± 42	332 ± 15*	418 ± 25*	404 ± 32*

*Differ significantly ($P < 0.01$) from control measurements.

sampled were short and broad (Table III). Epithelial cells varied from columnar through low columnar to cuboidal, with most abnormal cells present near the tips of villi, particularly in midgut and ileum. Occasional fusion of villi was noted in only one animal. The lamina propria was usually infiltrated with mononuclear cells and polymorphonuclear leucocytes. Crypts were significantly lengthened (Table III) and often contained many mitotic figures.

In the five calves with rotavirus and ETEC infection, villi were blunted, shortened and broadened to an extent similar to the rotavirus-infected calves (Table III). Epithelial cells were cuboidal or even squamous near the tips of villi. Fusion of villi occurred in three calves, particularly in midgut and ileum. In calf #20, bacteria were seen adhering to epithelial cells, and tongues of epithelial cell layers protruded from the tips of many villi. The lamina propria was infiltrated with neutrophilic and eosinophilic polymorphonuclear leucocytes, and less abundantly with plasma cells, lymphocytes and macrophages. Crypts were hypertrophic (Table III) with many mitotic figures.

DISCUSSION

The major problem in using conventionally-reared calves proved, as anticipated, to be the occurrence of natural infections with enteropathogens in some calves. Experimental conditions were standardized to the greatest extent possible compatible with the inevitable variation present among conventional calves, and the microbiological status of all calves was carefully monitored. While the results must be treated with caution, they relate to the field disease in that they were obtained from conventional calves of an age in which, in our experience, diarrhoea occurs commonly.

Calves inoculated with rotavirus developed diarrhoea of moderate severity, and two of the five calves had reduced appetite. Histological lesions were similar to those described by Mebus et al. (1971). The inoculum of unfiltered

gnotobiotic calf intestinal contents containing an Australian rotavirus isolate was prepared in a similar manner to that used successfully to produce diarrhoea in calves with a rotavirus isolate from the U.K. (Snodgrass et al., 1980). The pathogenicity of these two strains in conventionally-reared calves suggest that use of a high titre inoculum may be important, and that previous failure to demonstrate substantial pathogenicity may have been due to use of lower titre filtered inoculum (Logan et al., 1979).

Of the seven calves inoculated with ETEC alone, only one (#11) showed any evidence of multiplication of the B44 strain inoculated. This is consistent with observations of others that only when given within the first 48 h of life can ETEC infection produce diarrhoea (Smith and Halls, 1967).

The five dually-inoculated calves all developed diarrhoea, all became infected with B44, and all (except #18) became infected with rotavirus. The consistent multiplication of B44 in this group was demonstrated by its excretion in faeces and by higher ileal titres. Thus simultaneous inoculation of rotavirus with ETEC enabled ETEC to multiply in all five calves while ETEC in the absence of simultaneous rotavirus inoculation colonized only one of seven calves.

This combined infection did not produce any increase in severity of disease, as measured by weight and dry matter of diarrhoeic faeces and extent of villus atrophy compared to that caused by rotavirus infection alone. However, dual infection was associated with loss of appetite in all calves, and villus fusion, which may be a feature of ETEC infection, was common (Pearson et al., 1978).

Diarrhoea occurred in five calves inoculated with ETEC, and in all these animals rotavirus infection was demonstrated. The naturally-acquired rotavirus infections were presumably responsible for the diarrhoea in these calves, as no ETEC multiplication was demonstrated. The severity of diarrhoea, the bacterial titres in the ileum and the histological findings were all similar to those of the group inoculated with rotavirus, and these calves should properly be considered with the rotavirus-inoculated group. The absence of ETEC multiplication in these calves in spite of rotavirus infection suggests that the timing of each infection may be important. In the naturally-acquired infections, rotavirus was generally detected later than in the calves inoculated with rotavirus. The establishment of ETEC infection in calves of this age may therefore require an initial or at latest a simultaneous rotavirus infection.

Other studies on combined rotavirus and ETEC infections have utilized gnotobiotic calves (Gouet et al., 1978; Runnels et al., 1980). In spite of the difference in the experimental systems, similar conclusions can be drawn. Runnels et al. (1980) demonstrated increased ileal colonisation by ETEC in the presence of rotavirus infection, and Gouet et al. (1978) showed that rotavirus infection can make a sublethal dose of ETEC become lethal. Thus in gnotobiotic calves ETEC readily colonise the intestine, and rotavirus infections enhance this colonisation. However, in conventional calves in this ex-

periment ETEC colonisation occurred in the presence of rotavirus infection while ETEC were eliminated in the absence of rotavirus infection. This supports the observations made by Tzipori et al. (1981). Thus in this study the interaction of rotavirus and ETEC in the intestine may be classed as synergistic rather than additive.

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