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Clinical Manifestations of Diarrhea in Calves Infected with Rotavirus and Enterotoxigenic *Escherichia coli*

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The susceptibility of gnotobiotic, colostrum-deprived, or suckling calves to four bovine rotavirus isolates was found to be age dependent. Calves older than 7 days remained clinically normal, although they excreted virus in their feces and subsequently developed antibody against the virus. Enterotoxigenic *Escherichia coli*, fed to gnotobiotic, colostrum-deprived, or suckling calves ranging in age from a few hours to 26 days old, only caused diarrhea in animals younger than 24 h old. In contrast, diarrhea was consistently induced in 1- to 2-week-old calves infected with both enterotoxigenic *E. coli* and rotavirus. In general, diarrhea appeared after a rotavirus incubation period of approximately 3 days and was independent of the order in which the two microbial agents were given, the age of the calf, or the level of circulating rotavirus antibodies. The disease episode coincided with the excretion of rotavirus, rather than enterotoxigenic *E. coli*, in the feces. Infection with enterotoxigenic *E. coli* became established within 24 h of inoculation, and in older calves enterotoxigenic *E. coli* was often excreted in very small numbers and for a longer period than rotavirus.

The etiology of calf diarrhea is complex, often involving a number of infectious agents and a range of nutritional, immunological, and environmental factors. The two enteropathogens most commonly encountered in the investigation of field outbreaks of diarrhea in calves are enterotoxigenic *Escherichia coli* (ETEC) and rotavirus (3, 4). The role of rotavirus as a cause of diarrhea in calves (10-12, 24) and other mammals (9) has been established. Serological surveys have also shown that antibody against rotavirus is widespread among the cattle population (1, 14, 19, 24).

In this study we initially examined the age susceptibility of calves to diarrhea induced by several bovine rotavirus isolates and one ETEC. Secondly, we investigated the effect of combined infection with one rotavirus isolate and one ETEC on calves up to 2 weeks old. The relative importance of both enteropathogens in the manifestation of the disease was also assessed.

MATERIALS AND METHODS

Experimental animals. Sixteen gnotobiotic (GB) calves were derived by caesarean section and maintained inside polyvinyl chloride isolators throughout the experiment (T. J. Makin and S. R. Tzipori, unpublished data). Seven colostrum-deprived (CD) calves were derived by caesarean section and were maintained under strict hygienic conditions. The GB and CD calves were fed 2 liters of canned cows' milk twice daily. Sixteen suckling calves remained with their dams throughout the experiment. The cows and their calves were kept in individual open yards, and the cows were provided with water and chaffed lucerne.

Virology. Bovine rotaviruses C1, C3, and C4 were isolated from outbreaks of neonatal diarrhea in 1974, 1975, and 1978, respectively, and have been previously described (22). Bovine rotavirus C6 was isolated from a field outbreak of diarrhea which has been occurring for the last few years in a suckling beef herd of 400 in Victoria. In 1978, most of the 400 newborn calves developed diarrhea. Of the 11 fecal samples collected from scouring calves, 9 contained rotavirus, and 3 of those calves were shedding small numbers of ETEC organisms (O20:K99$^+$,ST$^+$).

Aliquots (15-ml) of fecal filtrates (20%, vol/vol) were prepared from each of the above 4 isolates. The amount of virus detected by electron microscopy was classified on an ascending scale from 1 to 4, and the preparations used contained between 3 and 4 (10^7 to 10^8 particles per ml). Except for the age susceptibility experiment of calves to rotavirus in which 4 isolates were examined, C6 was used in all the remaining experiments. The presence of virus in feces of infected calves was also determined by electron microscopy.

Bacteriology. A 4-ml portion of tryptose soya broth containing 10^6 to 10^8 serotype O20:K106:K99$^+$ organisms per ml, was used as the standard ETEC oral inoculum per calf. These organisms were found to be stable toxin and labile toxin producers as determined by the previously described mouse assay (5) and Y-1 adrenal cell culture (18), respectively. ETEC organisms in the feces were identified by their O antigen and tested for K99 by the slide agglutination test (15).

TABLE 1. *Response of GB and CD calves to inoculation with four bovine rotavirus isolates*

Status of calf	Age at inoculation (days)	Virus isolate	Clinical diarrhea[a]	Duration of diarrhea (days)	Presence (+) or absence (−) of virus in feces[b]	Antibody response	
						CF	Days after inoculation
GB	1	C3	++	3	+	32	5
CD	1	C3	++	3	+	16	12
CD	1	C4	+++	4[c]	+		
GB	4	C1	++	5	+	8	10
GB[d]	4	C6	+++	4	+	8	10
GB	7	C3	−		+	64	9
GB	9	C3	−		+	16	7
CD	9	C3	−		+	16	4
GB	9	C4	−		+	64	12
GB	10	C3	−		+	1,024	17
CD	10	C3	−		+	32	7
CD	10	C4	−		+	32	10
GB	10	C6	−		+	16	7

[a] ++, Moderate; +++, severe; −, none.
[b] As determined by electron microscopy.
[c] Calf died after 4 days of diarrhea.
[d] Calf is also included in Table 4 (calf 2).

Serology. The complement fixation (CF) test, with the SA11-cell-culture-adapted rotavirus used as antigen, was performed as described previously (21).

Clinical observations. Before and after oral inoculation, the calves were closely observed for clinical diarrhea, and fecal samples were tested daily for the presence of virus or ETEC. Serum samples were collected from the calves at the end of each experiment. Clinical diarrhea was assessed on the basis of three criteria: (i) anorexia, (ii) change of color of feces from orange to white-grey, and (iii) increased frequency of discharge and fluid content of feces.

RESULTS

Infection with a single agent. Eight GB and five CD calves between 1 and 10 days old were inoculated with one of four bovine rotavirus isolates (Table 1). Experimental inoculations with rotavirus induced diarrhea in calves less than 7 days old. Calves 7 days old or older developed subclinical infection only. Virus shedding was evident within 3 days after inoculation and persisted for up to 8 days. All surviving calves developed CF antibody against rotavirus.

TABLE 2. *The response of GB and CD calves to inoculation with ETEC (O20:K106:K99$^+$)*

Status of calf	Age at inoculation (h or days)	Clinical diarrhea (h)		Presence (+) or absence (−) of shedding of ETEC
		Incubation	Duration	
CD	2 h	12	12[a]	+
CD	6 h	15	10[a]	+
GB[b]	36 h			+
GB	12 d			+
GB	14 d			+
GB	26 d			+

[a] Calf was killed at the height of clinical diarrhea.
[b] Calf is also included in Table 4 (calf 1).

Two CD and four GB calves between 2 h and 26 days old were inoculated with ETEC. Diarrhea was evident only in calves less than 24 h of age (Table 2). Older calves showed no symptoms, although they were excreting the organism in their feces. The two CD calves were killed at the height of clinical diarrhea for pathogenesis studies; therefore the length and outcome of the infection is not recorded.

Four suckling calves between 3 and 10 days old were inoculated with ETEC, and four others between 6 and 10 days old were inoculated with rotavirus C6 (Table 3). As with the GB and CD calves, the suckling calves showed no signs of diarrhea to either of the two agents. One calf inoculated with rotavirus passed solid creamy-white feces for 1 day. The two calves that showed evidence of subclinical infections with rotavirus had lower preinoculation CF antibody (8 and <2) as compared with the remaining two (64 and 32).

Infection with rotavirus and ETEC. Two GB calves, a few days old, were inoculated with rotavirus and ETEC at long intervals (Table 4). Calf 1 developed diarrhea after inoculation with rotavirus at the age of 7 days, whereas normally it would have only developed subclinical infection (Table 1); subclinical infection with ETEC 4 days earlier must have precipitated the diarrhea (Fig. 1). Calf 2 developed diarrhea first, after inoculation with rotavirus at the age of 4

TABLE 3. *Clinical response of suckling calves inoculated orally with either ETEC or bovine rotavirus C6*

Age (days) at inoculation with:		Clinical diarrhea (days)		Presence (+) or absence (−) of the following in feces:		Antibody titer (rotavirus)	
Virus	ETEC	Incubation	Duration	Virus	ETEC	Preinoculation CF	Postinoculation CF
	3			−	+	128	64
	4			−	+	128	32
	9			−	+	64	32
	10			−	+	32	64
6				−	−	64	128
8		[a]		+	−	8	64
10				+	−	<2	16
10				−	−	32	16

[a] The calf passed creamy-white feces on the 3rd day.

TABLE 4. *Clinical response of GB calves inoculated orally with both rotavirus and ETEC given at a long interval*

Calf no.	Age (days) at inoculation with:		Clinical diarrhea (days)		Presence (+) or absence (−) of shedding of the following in feces:		Rotavirus antibody titer	
	Virus	ETEC	Incubation (days after the last inoculation)	Duration	Virus	ETEC	CF	Days after viral inoculation
1		1.5	[a]		−	+		
	7		1	7	+	+	<2	8
2	4		2	4	+	−		
		11	1	2	+	+	8	10

[a] Calf began to pass loose, orange-colored feces 24 h after inoculation; this persisted for 2 days.

days. After recovery, the calf was inoculated with ETEC at 11 days of age. It developed diarrhea again, and rotavirus shedding reoccurred in the feces along with ETEC.

Four GB calves between 8 and 13 days old were inoculated with rotavirus and ETEC at short intervals (Table 5). Three calves developed diarrhea that lasted 3 to 4 days, and they excreted both organisms in their feces. One calf failed to become infected with rotavirus even though it was inoculated twice with rotavirus C6. This calf did not develop either diarrhea or CF antibody against rotavirus, although it did become infected with ETEC.

Eight suckling calves between 5 and 15 days old were inoculated with ETEC and rotavirus either simultaneously or at short intervals (Table 6). All eight calves developed moderate to severe diarrhea. The incubation period varied from 2 to 5 days, and diarrhea lasted 5 to 6 days in calves that were not killed earlier. There was no difference in the length of the incubation period and the severity of the diarrhea between calves with high (calves 3, 6, and 9) or low (calves 4 and 10) preinoculation CF antibody titers against rotavirus. The infected calves developed moderate to severe diarrhea that included anorexia, depression, and loss of body fluid and body weight. All calves showed evidence of infection with both agents. The postinoculation antibody titers against rotavirus were difficult to assess, since no distinction could be made between maternal antibody and that resulting from infection. The relationships among the time of inoculation, the amount of ETEC and rotavirus in the feces, and the duration of diarrhea for calves 1, 6, 9, and 10 is illustrated in Fig. 1. In calves 1, 9, and 10, which were first infected with ETEC, the excretion of the organism in the feces peaked 24 h after inoculation, then dropped to a low level. The number of organisms in the feces increased in the 3 calves 24 h after viral inoculation.

Generally, in combined infections with ETEC and rotavirus, the disease appeared approximately 3 days after the rotavirus inoculations, regardless of which of the agents was given first (Tables 5 and 6). Furthermore, the disease coincided more closely with the excretion of rotavirus rather than ETEC, in the feces. Infection

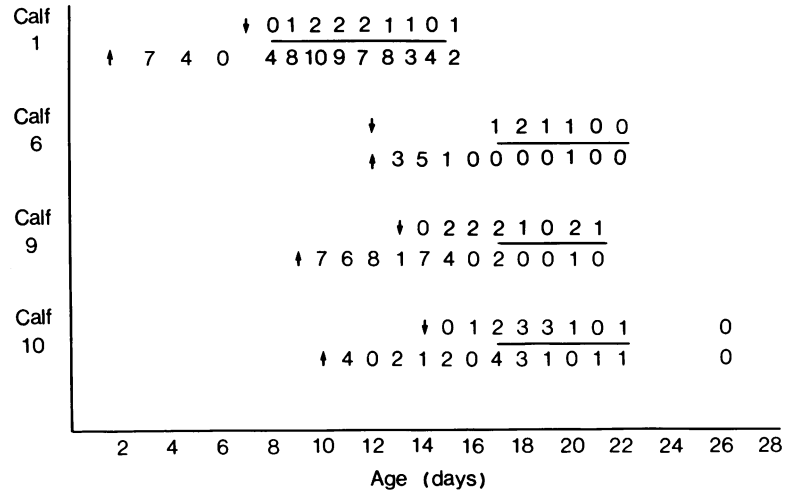

FIG. 1. *Clinical response of GB calf 1 and suckling calves 6, 9, and 10 to inoculation with bovine rotavirus C6 and ETEC (O20:K106:K99$^+$). The arrows represent the calf age at inoculation with C6 (↓) and ETEC (↑). For each calf, the level of rotavirus excretion (determined by electron microscopy and ranked on an ascending scale from 1 to 4) is represented along the top line and the level of excretion of O20 antigen (number of O20-positive E. coli colonies detected out of 10 colonies randomly selected from fecal cultures) is represented along the bottom line. —, Duration of clinical diarrhea.*

TABLE 5. *Clinical response of GB calves inoculated orally with both rotavirus C6 and ETEC*

Age (days) at inoculation with:		Clinical diarrhea (days)		Presence (+) or absence (−) of shedding of the following in feces:		Rotavirus antibody titer	
Virus	ETEC	Incubation days after the last inoculation	Duration	Virus	ETEC	CF	Days after viral inoculation
8	11	0.5	4	+	+	8	7
9	13	1	3	+	+	<2	7
9	13			−	+	<2	10
12	10	3	4	+	+	2	8

with ETEC usually became established within 24 h of inoculation, and bacterial shedding in the feces fluctuated considerably over a much longer period than did rotavirus excretion.

DISCUSSION

The results in this study demonstrate that (i) calves were susceptible to rotavirus infection only during their first week of life, and (ii) inoculation with rotavirus and ETEC induced diarrhea in calves at least 2 weeks old.

Unstressed calves 7 days or older showed evidence of subclinical infection when inoculated with rotavirus alone followed by seroconversion. Four virus isolates were used to verify whether it was a characteristic shared by more than one isolate. The four isolates were obtained from different field outbreaks of diarrhea, and, although they have not yet been shown to differ antigenically, they are genetically distinct as shown by polyacrylamide gel electrophoresis (M. Smith and S. R. Tzipori, unpublished data). These results are in disagreement with those obtained with one United Kingdom bovine rotavirus isolate (23). This isolate was shown to induce diarrhea in GB calves at least 35 days old and in nonimmune calves 8 weeks old (23, 24). On the other hand, Logan et al. (8) found little difference between the two rotaviruses when comparing the clinical reaction of CD calves to the United Kingdom isolate and a Northern Ireland field isolate (K). Both isolates induced a variable response ranging from no effect to a severe diarrhea. This discrepancy could be due either to differences in the assessment of clinical diarrhea or to the fact that individual variations

TABLE 6. *Clinical response of suckling calves inoculated orally with both bovine rotavirus and ETEC*

Calf no.	Age (days) at inoculation with:		Clinical diarrhea (days)		Presence (+) or absence (−) of the following in feces:		Rotavirus antibody titer	
	Virus	ETEC	Incubation (days after second inoculation)	Duration	Virus	ETEC	Preinoculation CF	Postinoculation CF
3	5	5	3	5	+	+	512	128
4	7	7	3	4[a]	+	+	<2	<2
5	9	9	3	1[a]	+	+	64	8
6	12	12	5	5	+	+	512	64
7	14	14	2	1[a]	+	+	64	16
8	15	15	3	6	+	+	64	16
9	13	9	4	5	+	+	256	32
10	14	10	3	5	+	+	16	8

[a] Calf was killed during the clinical illness.

between calves in response to infection with rotavirus are greater than differences in degree of virulence between viral strains. The ETEC used in these experiments was capable of inducing diarrhea only in calves less than 24 h old. Similar observations were made previously by others who used different serotypes (20). Experimental enteric colibacillosis is readily induced in calves given a large oral dose of ETEC within a few hours after birth (2, 6, 7, 16, 17); it is an unusual calf strain in that it produces labile toxin as well as stable toxin (15).

The inoculation of GB or suckling calves with both rotavirus and ETEC, simultaneously or at certain intervals, induced diarrhea which was independent of calf age. A high level of CF antibody against rotavirus appeared to be effective in preventing infection with rotavirus alone (Table 3), whereas in dual infection, it had little effect in preventing either infection or diarrhea. The relationships, however, among the level of colostral rotavirus antibodies, the quantity of ETEC present in the gut, and the challenge dose of virus is unclear. The combined action of ETEC and rotavirus has also been demonstrated in newborn GB calves (6). A more severe disease was induced when both agents were given simultaneously than when just a single agent was given. To date, the microbial agents most commonly associated with diarrhea in young calves include ETEC, rotavirus, coronavirus, and *Cryptosporidium*. We demonstrated that the concurrence of two of the above-mentioned enteropathogens can precipitate a disease in circumstances in which each one acting independently may not. It is likely that there are many more combinations which would induce a disease with a severity dependent on the nature of the organisms and on the contributing management factor involved.

The number of ETEC organisms excreted by experimentally inoculated suckling calves older than 5 days was very small and often undetected. Examination of Fig. 1 suggests that, under field conditions, diarrhea in calves 6, 9, and 10 may have been singularly attributed to rotavirus infection had we examined less than 10 colonies per sample. The role of ETEC in coinfection of older calves therefore may have been overlooked in the past.

The nature of the interaction between rotavirus and ETEC on the mucosal surface of the intestine is being investigated at present. It is probable that the physiological state of the epithelium of the small intestine is of great importance in the development of infection with ETEC. In the very young calf, it permits the adhesion of K99-possessing organisms, but does not do so in 3-day-old calves (20). Rotaviruses are known to infect mature enterocytes, causing their destruction and subsequent replacement by immature cells (14, 24). The emergence of these cells may promote the adherence of ETEC.

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