Diarrhea in Gnotobiotic Calves Caused by the Reovirus-Like Agent of Human Infantile Gastroenteritis[1]

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Gnotobiotic newborn calves were found to be susceptible to infection with the reovirus-like agent of human infantile gastroenteritis (HRVL). Infection was based on (i) seroresponse using immunofluorescence and (ii) fecal shedding of virus particles using electron microscopy. Virus was detected in fecal samples for at least 2 to as long as 7 days after inoculation, although peak virus concentrations were observed on days 1 to 4. Diarrheal illness was observed in seven calves on second to fourth serial passage of HRVL in calves but in none of four animals studied on first passage. Diarrhea began 15 to 30.5 h (mean = 22.3 h) post-inoculation and lasted less than 24 h; three of the seven animals that developed diarrhea were also depressed or anorectic.

The association of a reovirus-like agent with acute nonbacterial gastroenteritis of infants and young children in several countries (1, 3, 5, 6, 13) has stimulated studies of morphologically similar agents and investigations to find suitable animal models. The human infantile gastroenteritis virus has been shown to cause diarrhea in piglets and monkeys (12, 16, 19). A morphologically similar and antigenically related calf virus causes diarrhea in calves (9, 10, 17); therefore, calves were tested for susceptibility to the human reovirus-like (HRVL) agent.

MATERIALS AND METHODS

Calves. Sixteen gnotobiotic colostrum-deprived calves (Hereford-Aberdeen Angus crossbreds) were studied. Twelve were inoculated with a reovirus-like agent, and four were controls. Equipment and procedures used to obtain and maintain the calves have been described (11). Calves were fed approximately 2,700 ml of autoclaved homogenized cow milk per day. The four control gnotobiotic calves were animals in which the pathogenicity of respiratory bovine mycoplasma was being tested during the same period.

Viruses. The origin and preparation of the HRVL strains Fh and D have been described (5, 16). Two percent filtrates of calf feces positive for the HRVL agent by electron microscopy (EM) were prepared by using methods previously described (15). The origin of the related Nebraska calf diarrhea virus (NCDV) and method of preparing fecal filtrates have been described (10).

Calf inoculation. Calves 1 through 7 were inoculated intraduodenally as outlined in Table 1. Two- to 5-ml quantities of the HRVL agent in the form of human stool filtrates were diluted to 30 ml with phosphate-buffered saline solution, pH 7.2, for intraduodenal inoculation. The HRVL agent passaged in calves was administered either intraduodenally as a 2% calf fecal filtrate (50 ml) or orally as unfiltered calf diarrheal feces (10 ml). Calf 8 received 10 ml of NCDV (Cody strain) orally.

The seven calves to be inoculated by intraduodenal injection were restrained in left lateral recumbency, and the site of incision in the right flank was infiltrated with 1% lidocaine HCl. The abomasum and duodenum were located by withdrawing the omentum through the incision. The inoculum was injected into the lumen of the duodenum by using a 20-gauge needle, and the incision was closed in the normal manner. Calves were fed 500 ml of milk before inoculation and 500 to 1,000 ml immediately thereafter to increase the intestinal volume and therefore volume of diarrheal feces if illness occurred (C. A. Mebus et al., unpublished observations). Gnotobiotic conditions for calves 1, 2, and 5 were broken when the calves were removed briefly from the isolation units for the duodenal inoculation. Immediately after surgery, these calves were given an intramuscular injection of 400,000 U of procaine penicillin U and 0.5 g of dehydrosteptomycin and were returned to the isolation units. Surgery on calves 3, 4, 6, and 7 was performed in a sterile flexible isolator attached to the isolation unit, so that the gnotobiotic conditions were maintained throughout the study.

In addition, four newborn gnotobiotic calves were inoculated orally with 10 ml of calf feces containing the HRVL agent passaged three or four times in calves. Before inoculation calves were fed 200 ml of milk, and immediately after inoculation each calf

TABLE 1. *Response of newborn gnotobiotic calves to HRBL and NCDV*

Virus (strain)	Pas-sage	Calf no.	Diar-rhea	Quantity of reovirus-like particles on indicated day postchallenge[a]							Immunofluo-rescence recip-rocal titer to HRVL agent (pre-/post-inoc-ulation sera)
				1	2	3	4	5	6	7	
HRVL (Fh)	1	1	No	M[b]	M	4+	4+	3+	0	1+	<10/320
HRVL (D)	1	2	No	M	3+	4+	>4+	0	0	0	<10/640
		3	No	M	>4+	3+	2+	0	0	0	<10/640
		4	No	M	1+	>4+	4+	2+	2+	0	<10/≥1,280
	2	5 (from 2[c])	Yes	2+	3+	2+	1+	0	1+	1+	<10/320
		6 (from 4[c])	Yes	4+	2+	1+	0	0	0	0	<10/160
	3	7 (from 6[c])	Yes	3+	4+	0	0	0	0	0	<10/640
NCDV (Cody)	18	8	Yes	4+	4+	3+	3+	3+	1+	0	<10/≥640

[a] 1+ = 1 to 9; 2+ = 10 to 49; 3+ = 50 to 499, 4+ = 500 to 999; >4+ = ≥1,000 reovirus-like particles/five squares of 400-mesh EM grid.

[b] M, Meconium.

[c] Source of inoculum.

was fed an additional 500 to 1,000 ml of milk for reasons described above.

Collection of specimens. Pre-inoculation blood samples were obtained from the umbilical cord, and post-inoculation (p.i.) blood samples were collected 16 to 33 days later from the jugular vein. Fecal samples were collected on each of the 7 days after inoculation; meconium was not collected. Firm and diarrheal fecal specimens were collected directly from the calves, placed in sterile containers, and stored at −60°C. Fecal samples at the end of the collection period were cultured aerobically on blood agar plates at 37°C.

Electron microscopic examination of feces. Two percent fecal filtrates from calves inoculated with the HRVL agent were examined by immunoelectron microscopy (4). Human immune serum globulin (Gammar, Armour Pharmaceutical Co., Phoenix, Ariz.) was used at a 1:5 dilution as a source of antibody in these tests. In an attempt to quantitate the virus, reovirus-like particles seen in five squares of a 400-mesh EM grid were counted. Fecal samples from control animals were prepared for EM exami-nation by sucrose density gradient centrifugation (14); preparations were negatively stained with 1% uranyl formate. This method is also known to be effective for the detection of reovirus-like agents (10).

Indirect immunofluorescence. Pre- and post-in-oculation sera were tested by using the indirect immunofluorescence techniques previously de-scribed (18). The source of viral antigen for these tests was frozen sections of distal small intestine from a calf infected with the HRVL agent. Fluores-cein-conjugated rabbit antibovine gamma globulin (Antibodies, Inc., Davis, Calif.) was used. As a con-trol, each serum was tested on uninfected gnoto-biotic calf distal small intestine.

RESULTS

Calf 1 was inoculated intraduodenally with fecal filtrate from patient Fh, and calves 2, 3, and 4 were inoculated by this same route with fecal filtrate from patient D. None of the four animals developed diarrhea, and all were alert and hungry throughout the observation period. The HRVL agent was detected in feces of each calf by day 3 and was shed for 3 to 5 days (Table 1). The peak virion concentrations ranged from 770 to more than 1,000 particles per five squares of a 400-mesh EM grid. *Escherichia coli* were isolated from the feces of calves 1 and 2; no bacterium was isolated from the feces of calves 3 and 4.

Calf 5 was inoculated intraduodenally with a fecal filtrate containing the HRVL agent (strain D) which had had a single passage in a calf (no. 2) that did not develop illness. At 25.5 h after inoculation the animal was not hungry, and when stimulated to defecate it passed me-conium and some liquid feces. Between 27 and 30 h p.i., 600 ml of liquid feces was collected. Forty-two hours p.i. the calf consumed all the milk offered, and when stimulated to defecate, it passed a small amount of firm feces. *E. coli* was isolated from the feces. Calf 6 developed diarrhea 30.5 h after intraduodenal inoculation of a fecal filtrate containing the HRVL agent passaged in calf 4. During the following 2 h, 300 ml of liquid feces was collected. At 44 hours p.i., the feces were firm. This calf was not anorectic during the diarrhea period. *Bacillus subtilis* was isolated from the diarrheic feces. Calf 7 was inoculated intraduodenally with a fecal filtrate containing the HRVL agent which had had two passages in calves (no. 4 and 6). The animal was depressed and anorectic 15 h p.i., and diar-rhea started 21 h p.i. Between 22 and 24 h p.i., 120 ml of liquid feces was collected. At 39 h p.i., the calf was active and hungry. No bacterium was isolated from the diarrheic feces. The HRVL agent was found in diarrheal feces from calves 5, 6, and 7 on day 1 p.i. and was shed for

2 to 7 days (Table 1). Peak virion concentrations ranged from 110 to 655 particles per five squares and occurred on days 1 or 2 p.i. All seven animals developed serological evidence of infection to the HRVL agent as determined by indirect immunofluorescence.

In a later study, oral administration of the HRVL agent also resulted in the production of diarrheal illness. Two calves administered feces containing third-calf-passage HRVL agent (D strain) developed diarrhea 15 and 18 h p.i. Two additional calves inoculated orally with feces containing fourth-calf-passage HRVL agent (D strain) developed diarrhea 22 and 24 h p.i. These four animals were sacrificed at intervals after onset of diarrhea to study pathological changes in intestinal tissues. Therefore, virus shedding and serological studies were not done.

After these studies and under similar conditions, the pathogenicity of the NCDV was also demonstrated in a single calf inoculated orally with feces containing this agent. The calf was anorectic and very depressed 14 h p.i., these signs persisted for 12 h. Diarrhea developed 15 h p.i., and 360 ml of liquid feces was collected. No bacterium was isolated from the feces.

Three of the four control calves passed their meconium more slowly than did the inoculated calves. No reovirus-like agent was found in the feces of any control calf. At 3 weeks of age, sera from the three control calves tested did not have antibody (<1:20) to the HRVL agent.

DISCUSSION

Gnotobiotic newborn calves were shown to be susceptible to infection with the HRVL agent; infection without illness developed after initial administration of the HRVL agent. However, infection and illness were observed after inoculation with calf-passaged HRVL agent. Since the serologically related NCDV also produces diarrhea in calves and since the human and calf viruses are not morphologically or serologically distinguishable, it was possible that the illness after inoculation with the HRVL agent might have been due to NCDV. However, infection without illness occurred on first passage in each of four calves given the HRVL agent, whereas infection based on virus shedding and serological methods did not occur in control calves that were housed in the same facility and were not given HRVL. Recently, the ribonucleic acid segments of the HRVL agent and the NCDV derived from the stools of some of these same animals were analyzed by polyacrylamide gel electrophoresis (A. R. Kalica et al., submitted for publication). Differences in the migration of

as many as five segments were observed between the two viruses, indicating that the agents are distinct. Since the viruses studied were derived from experimentally inoculated calves included in the study reported here, this is evidence against the diarrhea in HRVL-inoculated calves being caused by NCDV or vice versa.

Absence of diarrhea on first passage of the HRVL agent in calves may have resulted from a relatively small number of intestinal epithelial cells being infected initially and/or a slower spread of the infection through the intestine because of the small inoculum size. When a larger inoculum was used, more absorptive cells may have been simultaneously infected, thus resulting in diarrhea. In addition, adaptation of the HRVL to the calf may have occurred. It was noteworthy that one calf inoculated with NCDV under similar conditions developed a more severe illness than did HRVL-inoculated animals.

Woode and co-workers in the United Kingdom were unable to infect newborn gnotobiotic calves with a United Kingdom strain of the HRVL agent (2). This may be ascribed to a difference in route of inoculation, dosage of inoculum, host range, or virus strain.

This study and others (12, 16, 19) demonstrate the ability of this group of reovirus-like agents to produce illness in heterologous species. This approach to the study of human infantile diarrhea was reported in 1943 by Light and Hodes (7). They found that a filterable agent derived from the stools of diarrheic infants produced diarrhea in calves (7, 8). Recently, calf feces lyophilized by these workers in 1943 and obtained from calves inoculated with a diarrheal stool preparation from a human infant were shown by immunoelectron microscopy to contain reovirus-like particles (R. G. Wyatt et al., unpublished observations). If indeed these reovirus-like particles are of human origin, our study may represent the long-awaited confirmation of the studies by Light and Hodes.

It is of interest in preliminary studies that this fecal preparation lyophilized in 1943 was inoculated intraduodenally in a newborn gnotobiotic colostrum-deprived calf; diarrhea did not occur, and virus was not detected in feces (C. A. Mebus et al., unpublished studies).

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