Coronavirus-Like Particles and *Campylobacter* in Marmosets with Diarrhea and Colitis

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Diarrhea and colitis were common clinical entities observed during a recent two-year interval in the marmoset colony located at Oak Ridge Associated Universities, Oak Ridge, Tennessee. The diarrhea occurred in sporadic episodes of several weeks duration and affected up to 5% of all animals at any one time. The incidence of the colitis is unknown, but it appeared to be widespread. Histologically, the colitis was similar to that described in cotton-top tamarins (*Saguinus oedipus oedipus*) at the New England Primate Center (1). There was infiltration of predominantly mononuclear cells into the interstitium, dilation of crypts with flattened epithelium, and accumulation of neutrophils and degenerated and necrotic epithelial cells in the lumen (2). The cause of either the diarrhea or the colitis is not known, nor is it known to what extent they are causally related.

In this report we describe studies demonstrating both coronavirus-like particles and *Campylobacter* in marmosets with diarrhea. Coronavirus-like particles were found in 24% and *Campylobacter fetus* subsp. *jejuni* in 20% of sampled animals with diarrhea. Immunoblotting studies on serum from a small number of animals having diarrhea suggested the presence in the colony of antibodies to a coronavirus antigenically related to the bovine enteric coronavirus but not the porcine transmissible gastroenteritis virus. Interestingly, all of the seven animals that died and exhibited histopathological lesions of colitis had coronavirus-like particles in their colon contents.

MATERIALS AND METHODS

Marmosets and Collection of Fecal Samples. A colony of approximately 500 marmosets is housed at the Marmoset Research Center, Oak Ridge Associated Universities, Oak Ridge, Tennessee. These include the tamarins *Saguinus oedipus oedipus*, *Saguinus fuscicollis* spp., and the common marmoset *Callithrix jacchus*. Animals are housed either singly in cages or as families in the breeding facility. Stool samples were taken from 107 animals during a two-year period over which episodes of diarrhea occurred. During one episode lasting two months, 30 samples were taken, 17 of these being collected over a three-day period. All stool samples were obtained from adult marmosets 1–15 years of age either during signs of diarrhea or within one week of recovery. Colon contents were collected at necropsy from eight marmosets that died spontaneously. The necropsy diagnosis of these animals is discussed in the Results section.

Culturing for Enteropathogenic Bacteria. Samples of feces were cultured at 37° C on MacConkeys or heptone agar, or on selenite broth followed by subcultures, for *Salmonella* and *Shigella*. Samples were also cultured at 42° C under microaerophilic

conditions on commercial *Campylobacter* enrichment medium (Campy-bap, BBL) with 10% sheep red blood cells for the isolation of *Campylobacter* sp.

Electron Microscopy. Stool samples were suspended in four volumes of distilled water, clarified at 100° g for 5 min, and 5 ml was pelleted at 15,000 g in a Beckman SW-50 rotor for 1 hr. The resulting pellet was suspended in 0.5 ml distilled water and 50 µl of this was mixed with 50 µl of 2% phosphotungstic acid (pH 6.8, adjusted to this pH by the addition of potassium hydroxide). The mixture was then sprayed from a glass nebulizer onto Formvar-coated copper grids. Electron microscopy was done on a Phillips 201 C electron microscope.

Growth and Purification of Virus, Polyacrylamide Gel Electrophoresis, and Immunoblotting. A plaque-purified stock of the bovine enteric coronavirus (BCV; Mebus strain) was grown on the human rectal tumor cell line (HRT-18) (3) using previously described methods (4). Cells were grown on Dulbecco's modified minimum essential medium (DMEM) with 10% fetal bovine serum (Sterile Systems, Inc.). Cells were infected with a multiplicity of 1, and supernatant fluids were harvested at 48–72 hr postinfection, and clarified at 10,000 g for 5 min. A plaque-purified stock of the porcine transmissible gastroenteritis virus (TGEV; Purdue strain) was grown on the swine testicle cell line (ST) (5) using previously described methods (6). Cells were grown on DMEM with 10% adult bovine serum (Sterile Systems, Inc.). Cells were infected with a multiplicity of 1, and supernatant fluids were harvested at 18–24 hr postinfection, and clarified at 10,000 g for 5 min. Each virus was separately pelleted in 38-ml tubes on a Sorval 627 rotor at 26,000 rpm, 2 hr, at 4° C through a 3-ml cushion of 20% sucrose w/w in TMEN-6 buffer [50 mM Tris acid maleate (pH 6), 0.1 M NaCl, and 1 mM EDTA], and the pellet was dissolved in sample treatment buffer [62.5 mM Tris hydrochloride (pH 6.8), 2% SDS, 5 M urea] and diluted with sample treatment buffer until the concentration of viral protein was approximately 15 µg/µl as determined by Coomassie blue staining of 1-µl aliquots on nitrocellulose (7).

Viral proteins were electrophoretically separated using the discontinuous buffer method of Laemmli (8). Approximately 1.2 mg of viral protein was spread along a continuous well of a vertical slab (0.75 × 100 × 160 mm) electrophoresis apparatus (Hoeffer Scientific Instruments) and electrophoresed through a 1-cm stacking gel of 3% polyacrylamide and an 8-cm separating gel of 9% polyacryl-

amide. Electrophoretic transfer of viral proteins from polyacrylamide gels to nitrocellulose sheets was done by the method of Burnette (7). Nitrocellulose sheets containing viral proteins were marked for alignment, cut into strips of 5 mm width, and each strip was incubated with a 1:10 dilution of serum in 15-ml, siliconized, plastic, screw-capped centrifuge tubes. The procedure of Burnette (7) was used for radiodetection of bound immunoglobulins using ^{125}I-labeled *Staphylococcus*-A protein. ^{125}I-labeled *Staphylococcus*-A protein (2–10 µCi/µg) was purchased from ICN. Strips were then exposed to Kodak X-Omat R film for autoradiography.

RESULTS

Episodes of diarrhea involved approximately 20% of all animals over a two-year period. The largest episode occurred during a two-month period and involved over 30 animals. Many animals were treated with broad-spectrum antibiotics if diarrhea was accompanied by anorexia, or if diarrhea lasted longer than three days. Because some animals were unresponsive to treatment and had no laboratory evidence of parasites or enteropathogenic bacteria, fecal samples were examined by electron microscopy for evidence of viruses.

Coronavirus-like particles were observed in feces from 24% of the 58 samples examined by electron microscopy (Table 1). The particles measured approximately 100–220 nm and had regularly spaced petal-shaped projections from the surface (Figure 1). Some also appeared to have an indentation of the surface and some were interpreted as having an empty nucleocapsid (Figure 1). In three samples, a reovirus-like particle was found, but no other virus-like agents were observed.

Fecal specimens from 65 marmosets with similar clinical signs were cultured bacteriologically. They were consistently negative for *Salmonella* and *Shigella*. *Campylobacter fetus* subsp. *jejuni* was isolated from 20% of the stool samples (Table 1). Of 16 marmosets examined for both viral and bacteriological agents, only one was found to have both coronavirus-like particles and *Campylobacter*.

The colon contents of eight animals that died were collected at necropsy and examined for the presence of coronavirus-like particles. Of these, seven had mild to moderate enterocolitis histologically (Table 2). Interestingly, all seven animals having colitis also had coronavirus-like particles in their colonic contents (Table 2).

TABLE 1. CORONAVIRUS-LIKE PARTICLES AND *Campylobacter* IN STOOL SAMPLES FROM
MARMOSETS HAVING DIARRHEA

Examination of stools by EM* only		Examination of stools by EM and bacterial culture			Examination of stools by bacterial culture only	
Number of animals	Number positive	Number of animals	Number positive corona	Number positive Campylobacter	Number of animals	Number positive
42	7	16	7	1	49	13

*EM, electronmicroscopy.

Initial attempts to grow the coronavirus-like agent in tissue culture have not been successful. The feces of five separate animals demonstrating coronavirus-like particles by electron microscopy were used as the virus source and attempts were made on cell cultures of primary marmoset kidney cells, Vero cells, the human rectal tumor cell line-18 (3), the swine testicle cell line of McClurkin and Norman (5), and human rhabdomyosarcoma cells (9).

In an initial attempt to identify the coronavirus-like agents as coronaviruses, serum from 10 marmosets having diarrhea were reacted against viral proteins of the bovine enteric coronavirus and of the porcine transmissible gastroenteritis coronavirus, representatives of the two antigenic subgroups of the mammalian coronaviruses. Coronaviral pro-

teins were electrophoretically separated, electrophoresed to nitrocellulose paper, reacted with serum and subsequently with [^{125}I]staph A protein. The results of serum from four marmosets are shown in Figure 2. Two of the five proteins of the bovine coronavirus but not of the proteins of the porcine transmissible gastroenteritis coronavirus appeared to bind antibodies in serum from six of 10 animals examined. The results suggest that a coronavirus antigenically related to the bovine coronavirus but not to the porcine transmissible gastroenteritis coronavirus exists in the marmoset colony. Whether these antibodies represent an immune response to the coronavirus-like agents observed in the feces of marmosets remains to be shown.
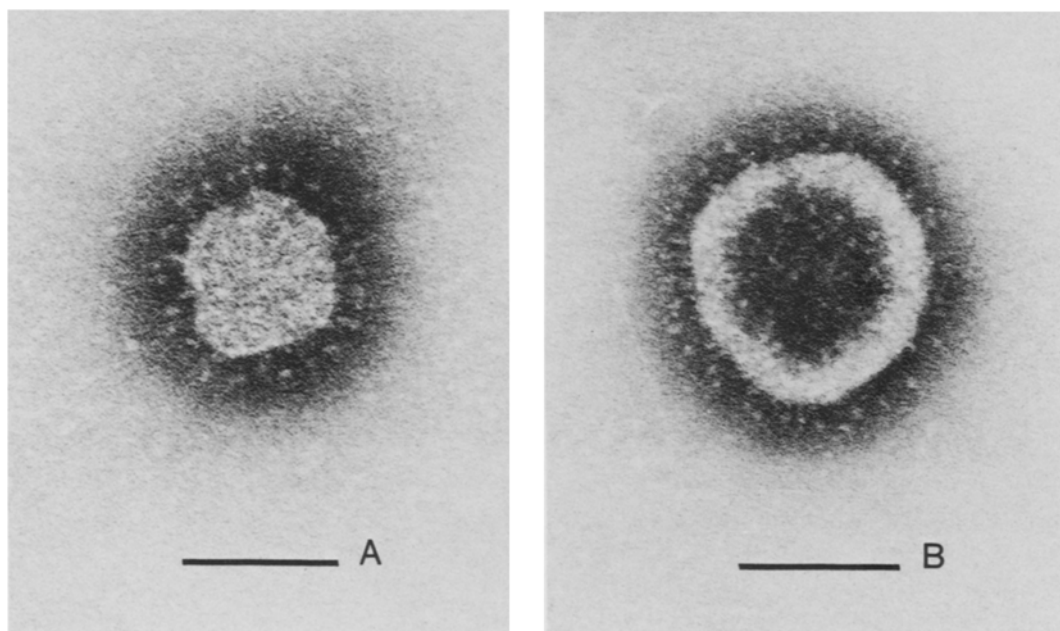

Fig 1. Negatively stained coronavirus-like particles from marmoset diarrheic feces. (A) Spherical particle showing petal-shaped projections. (B) A particle apparently devoid of a nucleocapsid. The bar represents 100 nm.

TABLE 2. SUMMARY OF CLINICAL, PATHOLOGICAL, VIRAL, AND BACTERIOLOGIC FINDINGS AMONG 8 MARMOSETS THAT DIED SPONTANEOUSLY

| Clinical Signs	Pathological Findings	Age	Species	Examination of colon contents	
				Coronavirus-like particles	Campylobacter fetus subsp. jejuni
Wasting	Emaciated, colitis	9 years	S. fusc.	+	−
Sudden onset weakness, dehydration	Emaciated, bronchopneumonia, moderate enterocolitis, mild interstitial nephritis	8 years	S. fusc.	+	−
Wasting	No significant findings	1 1/2 years	S. fusc.	−	ND
Emaciation	Renal amyloidosis, moderate colitis	11 years	S. oed.	+	ND
Emaciation	Lymphosarcoma, moderate colitis	Adult	S. oed.	+	ND
Wasting, emaciation	Colon adenocarcinoma	8 years	S. oed.	+	ND
Wasting, diarrhea	Emaciated, moderate enterocolitis, intussusception	8 years	C. jacc.	+	ND
Acute diarrhea	Acute enterocolitis	9 months	C. jacc.	+	+
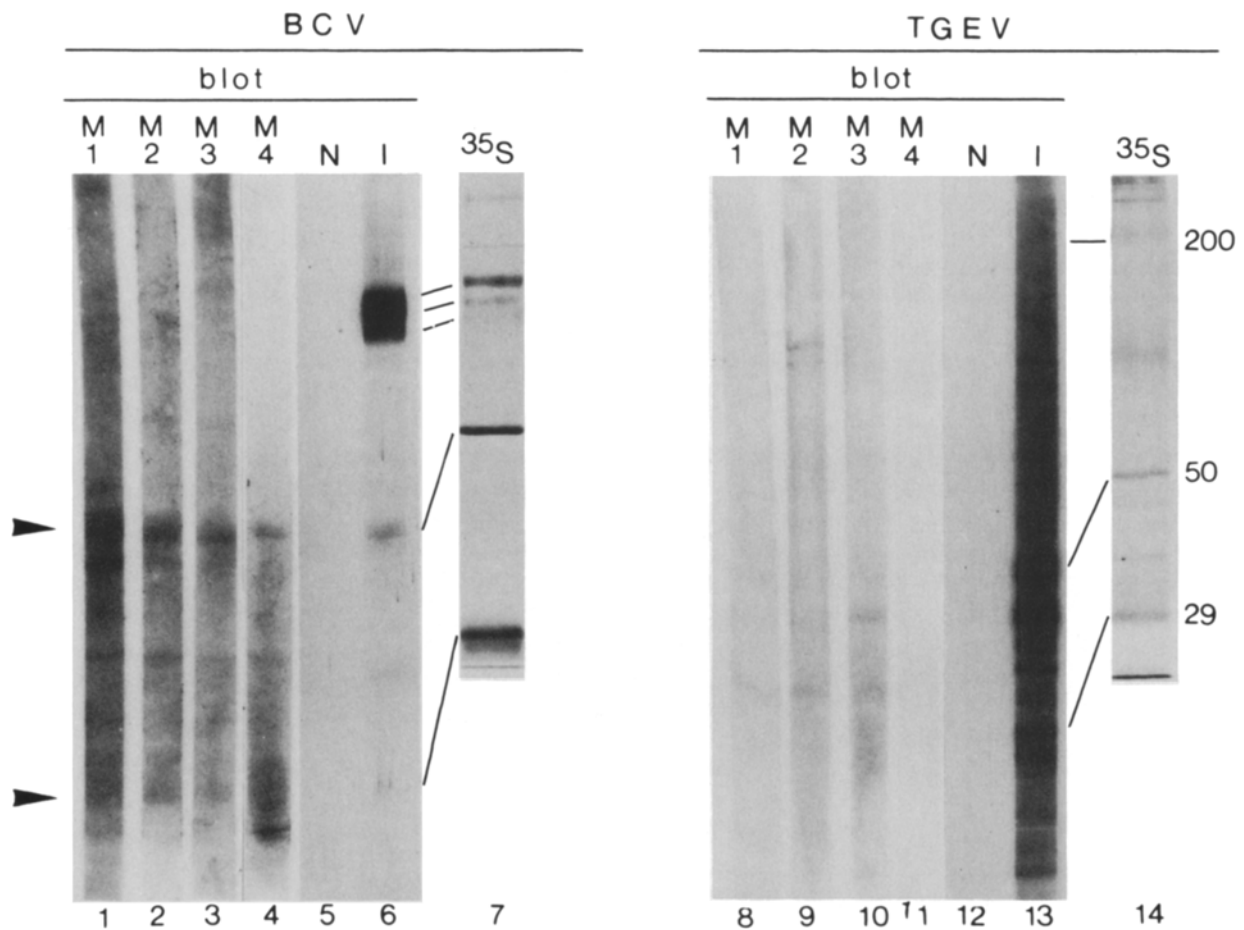
Fig 2. Immunoblots of marmoset serums against the proteins of the bovine enteric coronavirus (BCV) and the porcine transmissible gastroenteritis virus (TGEV). In lanes 1–4 and 8–11 serums from four separate marmosets who had diarrhea were used. Coronavirus-like particles were found in the feces of animals number 3 and 4. In lane 5 serum from the preimmunized rabbit and in lane 6 serum from the rabbit immunized with purified BCV were used. Lane 7 (inset) is an electropherogram of ^{35}S-labeled, purified BCV. In lane 12 serum from nonimmune newborn piglet and in lane 13 from a gnotobiotic pig hyperimmunized with TGEV were used. Lane 14 (inset) is an electropherogram of ^{35}S-labeled, purified TGEV.

DISCUSSION

We describe coronavirus-like particles and *Campylobacter* in the feces of marmosets suffering diarrhea and colitis. To date no firm association can be drawn between either agent and the diarrhea or either agent and the colitis, but our results do allow us to design approaches to investigate possible causal relationships.

The virus-like particles observed in fecal and colonic specimens were interpreted as probable coronavirus because of the ultrastructural morphology. These particles must be differentiated from fringed particles that are of nonviral origin (10). To confirm the existence of coronaviruses, two approaches should be taken: (1) The coronavirus should be grown in tissue culture and further characterized at the molecular level. Initial attempts have not yet been successful on primary marmoset cells and on cell lines of other animal species. Inasmuch as replication of coronaviruses is enhanced on transformed cells (11), transformed marmoset cells should be tried. (2) Serum from a variety of colony animals should be used to attempt precipitation of viral particles by immune electron microscopy (10). Evidence of immune clumping by antibody would support the notion that the observed coronavirus-like particles are infectious agents. Further precipitation with known antiserum to other coronaviruses should be attempted for classification of these agents.

Initial attempts have been made to identify the presence of coronavirus antibodies in the marmoset colony. Whereas no attempt has yet been made to react marmoset serum with the coronavirus-like particles observed in marmoset feces, marmoset serum was used to identify serological reactivity to the bovine enteric coronavirus and to the porcine tranmissible gastroenteritis coronavirus, each a representative of the two antigenic subfamilies of mammalian coronaviruses (12). Reactivity in six marmosets was observed for the internal 50-kd phosphoprotein and the 26-kd glycoprotein of the bovine coronavirus but not to any identifiable protein of the porcine gastroenteritis coronavirus. Since the 50-kd and 26-kd proteins are largely internal virion proteins (4), they probably represent evolutionarily conserved proteins within this antigenic subgroup of coronaviruses.

Further investigation is necessary to establish the role of the coronavirus-like particles in both the diarrhea and the colitis observed in the marmosets.

Coronaviruses have been established as the cause of acute diarrhea in many animal species (13, 14). Their presence in the stools of humans with nonbacterial gastroenteritis suggests they may be a cause of acute diarrhea in humans too (15–19). They have been found in the stools of nonhuman primates with diarrhea, but their role in this disease was not obvious since they were also found in the stools of nondiseased animals (20). The role of coronaviruses in chronic gastrointestinal disease in animals and man has been more difficult to establish. Persistent shedding of coronaviruses from many animal species is documented, but no correlation with chronic intestinal disease has yet been made (13, 21). Persistent shedding of coronavirus-like particles was observed over an eight-month period in a human individual with chronic intestinal maladsorption and suggests a possible role by coronaviruses in this disease (22).

The role of *Campylobacter* in marmoset diarrhea likewise needs further investigation. *Campylobacter* has been described as the most common bacterial cause of acute enterocolitis in man (23, 24). Histopathological findings in the large bowel for this disease are mild to moderate infiltration of mononuclear cells, neutrophils, and eosinophils in the lamina propria; cryptitis; and crypt abscesses (25–27). Age groups affected are mainly teens and adults, although cases have been reported in infants and children. Infection is usually not severe and is generally self-limiting (23, 24). *Campylobacter* has been implicated in persistent and recurrent diarrhea of infants and adults (28, 29). *Campylobacter* also has been implicated in acute and persistent diarrhea in nonhuman primates (30–34), but its high prevalence in apparently normal animals makes it difficult to establish its role in chronic disease (34, 35). Experimental infection has not been successful in some studies (36), although oral infection of young nonhuman primates with a human isolate caused mild disease of short duration (35). Histopathologic findings were absent in experimentally infected *Macaca mulatta* (35). Mild nonsuppurative enteritis was reported in *Erythrocelous patus* monkeys with persistent diarrhea from which *Campylobacter* was isolated (32).

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