Experimental Infection of Young Rabbits with a Rabbit Enteric Coronavirus

J.-P. Descôteaux and G. Lussier

ABSTRACT

The clinical signs and lesions caused by the rabbit enteric coronavirus (RECV) were studied in young rabbits orally inoculated with a suspension containing RECV particles. The inoculated animals were observed daily for evidence of diarrhea. Fecal samples and specimens from the small intestine and from the gut associated lymphoid tissue (GALT) were collected from 2 h to 29 days postinoculation (PI) and processed for immune electron microscopy (IEM) and light microscopy. Coronavirus particles were detected in the cecal contents of most inoculated animals from 6 h to 29 days PI. Lesions were first observed 6 h PI and were characterized by a loss of the brush border of mature enterocytes located at the tips of intestinal villi and by necrosis of these cells. At 48 h PI, short intestinal villi and hypertrophic crypts were noted. In the GALT, complete necrosis of the M cells as well as necrosis of the enterocytes lining the villi above the lymphoid follicules with hypertrophy of the corresponding crypts were observed in all the animals. Five inoculated rabbits had diarrhea three days PI. The presence of RECV particles in the feces of the sick animals and the microscopic lesions observed in the small intestine suggested that the virus was responsible for the clinical signs. A few inoculated rabbits remained free of diarrhea. Fecal material collected at postmortem examination contained RECV particles. The results suggest that the virus could also produce a subclinical infection.

RÉSUMÉ

Les signes cliniques et les lésions produites par le virus corona du lapin furent étudiés chez des lapereaux inoculés par voie orale. Les animaux inoculés furent observés quotidiennement. Des échantillons de matières fécales et des spécimens provenant de l'intestin grêle et des organes lymphoïdes associés furent prélevés entre deux heures et 29 jours après l'inoculation. Ce matériel fut examiné par immuno-électro-microscopie et en microscopie photonique. Des particules virales furent détectées chez presque la totalité des animaux évalués entre six heures et 29 jours après l'inoculation. Les premières lésions microscopiques furent observées 6 h après l'inoculation. Elles se caractérisaient par la perte de la bordure cilliée des entérocytes situés a l'extrémité des villosités de l'intestin grêle. Quarante-huit heures après l'inoculation, ces villosités étaient atrophiées et les cryptes correspondantes hypertrophiées. Au niveau des organes lymphoïdes associés, la nécrose des cellules M et des entérocytes recouvrant les villosités a été observée très tôt après le début de l'infection. Cinq lapins ont manifesté des signes de diarrhée trois jours après l'inoculation. La présence de particules virales dans les matières fécales de ces animaux et de lésions microscopiques caractéristiques supportent l'hypothèse d'une entérite causée par un coronavirus. Un certain nombre de lapins sont demeurés exempts de diarrhée. Toutefois, la présence de particules virales dans leurs matières fécales indique qu'ils étaient infectés.

INTRODUCTION

Infections of the gastrointestinal tract are responsible for major economic losses in rabbit production. They affect mainly young rabbits between 3 and 8 wk of age (1). Some animals die without any clinical signs while others have watery diarrhea.

A wide variety of enteric pathogens have been associated with rabbit enteritis including bacteria (2-5), parasites (1) and viruses (6-15). Among the viruses, the rabbit enteric coronavirus (RECV) was first reported in young rabbits showing signs of diarrhea in 1980, in Quebec, Canada (12). The virus has also been observed in healthy rabbits (15). Similar particles were reported in the Netherlands (13) and in the USA (14).

Coronaviruses are responsible for enteritis in various animal species (16). In order to clarify the role of coronaviruses as a cause of diarrhea in rabbits, young rabbits were inoculated with RECV particles. The results obtained are presented in this paper.

MATERIALS AND METHODS

Thirty-five New Zealand white rabbits 4-5 wk old were obtained from a commercial colony (Lapro Inc., South Stukley, Quebec) previously evaluated for the presence of RECV infection using immune electron microscopy (IEM) according to the procedure reported (15). The fecal material was diluted 1:5 in phosphate buffered saline (PBS), centrifuged at 3,000 x *g* for 30 min at 4°C and

Virology Research Center, Institut Armand-Frappier, 531, boulevard des Prairies, Laval, Quebec H7N 4Z3.

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filtered through a 0.22 μm filter to eliminate natural aggregates and bacterial contaminants. Twenty-five μL of the filtrate obtained were mixed with an equal volume of specific guinea pig antiserum diluted 1:40 and deposited on agar. A carbon formvar-coated grid was overlaid on top of the drop and when hydroextraction was almost completed, the grid was stained with 3% phosphotungstic acid (PTA) pH 6.8 and examined with a Philips EM 300 electron microscope operating at 80 kv.

The rabbits were individually housed in stainless steel cages in a room previously disinfected. No other animals were housed in the room during the study. The rabbits were kept according to the recommendations of the Canadian Council on Animal Care on the use and care of laboratory animals (17). The animals were fed regular rabbit chow (Ralston Purina Canada Inc., Montréal, Québec) and received water *ad lib*. The room access was limited to people directly involved with the project.

Three samples of fecal material were collected from each rabbit at intervals of two days and evaluated by IEM for the presence of RECV particles (15).

The infected material for inoculation was obtained from the cecum of a sick rabbit previously inoculated with RECV. The cecal content was homogenized in PBS (1:5) and centrifuged at 3,000 x *g* for 30 min at 4°C. An aliquot was examined by IEM to confirm the presence of RECV particles. The material was also evaluated by IEM for the presence of rotavirus particles using an antiserum to simian rotavirus SA 11 (obtained from Dr P. Payment, Virology Research Center, Institut Armand-Frappier, Laval, Quebec) and by electronmicroscopy (EM) for the presence of other viral contaminants. Although the material had been filtered (0.22 μm filter) it was also cultured on blood agar, McConkey, SS and Blaser agar. The cultures were incubated at 37°C for 72 h in aerobic, anaerobic and microaerobic conditions. Finally the material was centrifuged at 3,000 x *g* for 10 min and the precipitate was examined by light microscopy for the presence of coccidia.

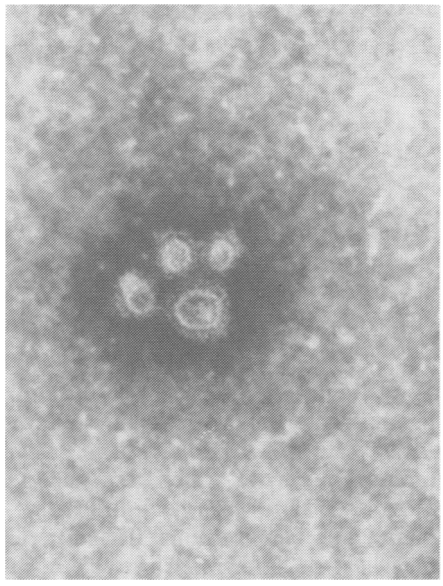

Fig. 1. Rabbit enteric coronavirus particles used to inoculate rabbits. They were obtained from the cecum of a rabbit with clinical signs of diarrhea. Negative staining.

Thirty rabbits were inoculated orally with 3 mL of the infected material and five were used as controls and received orally 3 mL of PBS. The animals were observed daily for signs of diarrhea. Inoculated animals were euthanized 2, 4, 6, 8, 12, 18 and 24 h postinoculation (PI). Thereafter they were killed two, three and six days PI. The controls were euthanized at the beginning of the study (0 h).

A postmortem examination was performed on each animal. Contents were collected from the cecum and evaluated by IEM for the presence of coronavirus particles as previously described. Specimens collected from the gut associated lymphoid tissue (GALT) and from the small intestine were fixed in Perfix, (Fisher Scientific, Montréal, Québec), prepared for light microscopic examination and stained with hematoxylin and eosin (H & E).

RESULTS

The cecal material used as the inoculum was free of bacteria. It was also free of coccidia and viral contaminants other than RECV particles. The particles observed were similar to those reported in the literature. They were pleomorphic, measured 70-100 nm in diameter with a fringe of surface projections approximately 10 nm long (Fig. 1). No other viral particles were observed.

The rabbits used were free of RECV particles on arrival in our facility. The IEM procedure failed to show any viral particles in the three samples tested from each animal.

Table I shows the results of the clinical observations following inoculation, the results of the IEM examination of cecal material collected postmortem as well as the number of animals evaluated at specific times PI. Eighteen animals were killed before the appearance of diarrhea, five showed signs of a watery diarrhea three days PI and seven remained free from clinical signs.

Following oral inoculation, viral particles were observed in the cecal material collected at necropsy from 6 h to 29 days PI. The immune aggregates contained RECV particles surrounded by immunoglobulins (Fig. 2).

The animals sacrificed at 2 and 4 h PI were free from gross lesions. Those evaluated between 6 h and 3 days PI had congested small intestines and the cecal contents were watery compared to noninoculated animals. Microspically the first lesions were observed 6 h PI. They were characterized by a loss of the brush border of mature enterocytes and by necrosis of enterocytes located at the tips of the intestinal villi. The corresponding crypts were normal. Enterocytes lining

TABLE I. Clinical observations following oral inoculation of RECV particles and IEM examination of fecal material collected from inoculated rabbits and control animals (0 h)

Periods PI	Number of animals evaluated	Number of positive animals	Clinical signs
0 h	5	0	0
2 h	4	0	0
4 h	2	0	0
6 h	2	2	0
8 h	2	2	0
18 h	2	2	0
24 h	2	2	0
48 h	4	4	0
3 d	5	5	5
6 d	1	1	0
14 d	2	2	0
23 d	2	2	0
29 d	2	2	0

PI = Postinoculation
RECV = Rabbit enteric coronavirus
IEM = Immune electron microscopy

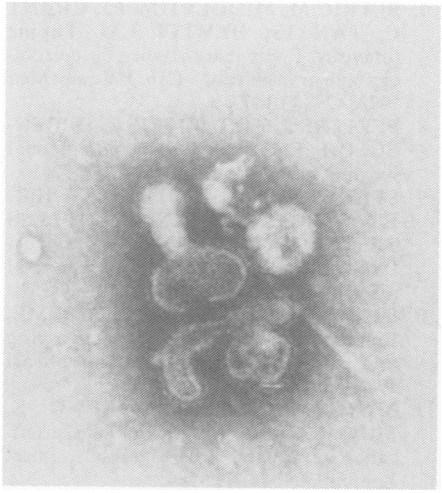

Fig. 2. Aggregates containing RECV particles and immunoglobulins observed by IEM in fecal samples collected from infected rabbits. Negative staining.

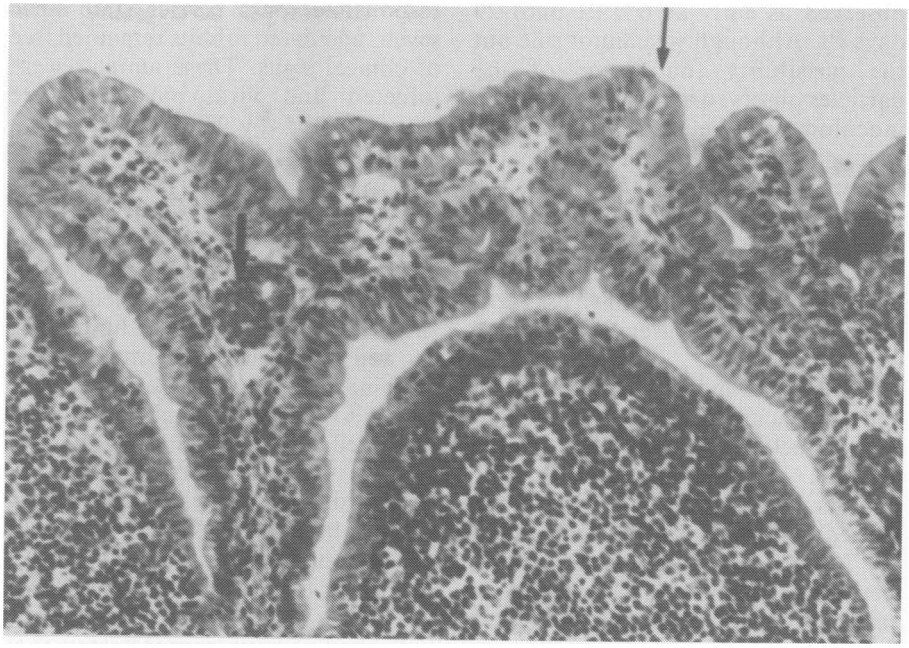

Fig. 3. Section from the appendix of an infected rabbit 6 h PI. The mature enterocytes appeared vacuolated and necrosis of epithelial cells is observed (→). Some crypts appears more cellular (→). H&E.

the villi overlying the GALT contained cytoplasmic vacuoles, the crypts were hypertrophic and the epithelial cells covering the lymphoid follicules showed signs of necrosis (Fig. 3).

Villous atrophy was present in all animals at 48 h PI. The intestinal villi were denuded. Increased numbers of mitotic figures as well as an increase in cellularity were seen in intestinal crypts of infected animals compared to controls. At three days PI, the villous epithelial cells of infected animals were cuboidal with cytoplasmic basophilia (Fig. 4). In the GALT areas, villous atrophy and an increased cellularity of the crypts were observed. Necrosis of the epithelium covering the lymphoid follicules was evident.

Inoculated animals evaluated between 6 and 29 days PI were free from microscopic lesions.

DISCUSSION

Since it is difficult to cultivate the RECV *in vitro* (18), it was decided to use viral particles obtained from the cecum of a sick rabbit as inoculum for the animals used in this study. Tests on this inoculum indicated that it was free from demonstrable viral particles other than the RECV. It was also free of bacteria and parasites.

The RECV particles observed showed morphological characteristics of *Coronaviridae* (19), with relatively short surface projections. Although short projections seem to be a characteristic of the RECV (13-14), we have observed particles with 20 nm surface projections (unpublished data) which suggests that the shorter projections may result from damage during the preparation procedure.

The rabbits used in this study were free of RECV particles on arrival in our facility. The virus is highly infectious for the rabbit since most of the inoculated animals became infected. Viral particles were observed by IEM in the content of the cecum. The particles were similar to those seen in the inoculum and they were

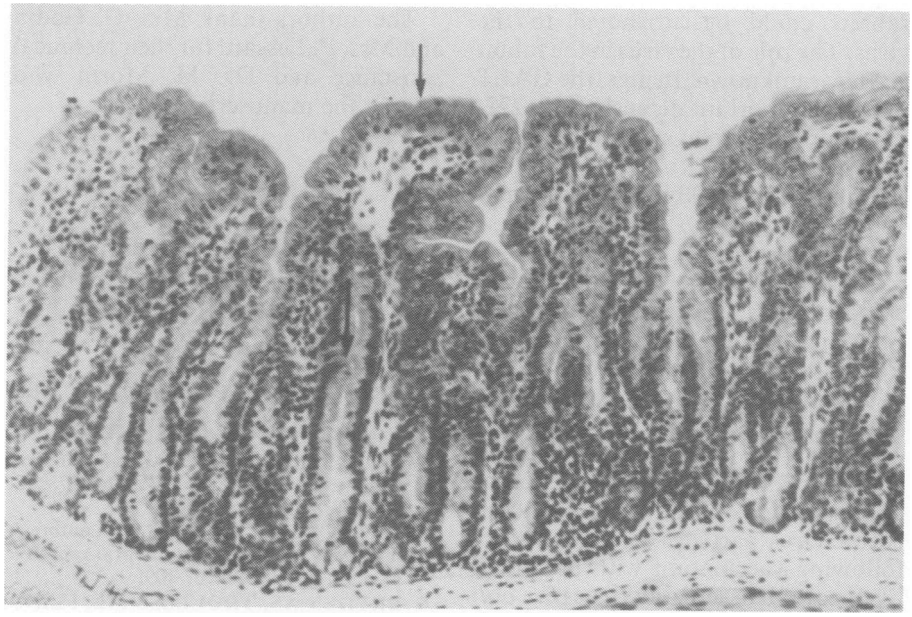

Fig. 4. Section from the small intestine of an infected rabbit showing clinical signs of diarrhea. Hypertrophy of the intestinal crypts are observed (→). Immature enterocytes lining the intestinal villi are cuboidal or low columnar with basophilic cytoplasm (→). H&E. X530.

observed as early as 6 h PI until 29 days PI. Although we cannot rule out the possibility that some of the particles observed at 6 h were from the inoculum, we believe that most of them were produced by infected intestinal cells.

The IEM procedure appeared to be a reliable procedure for the detection of infected animals. The use of specific antiserum enables coronavirus particles to be readily differentiated from debris.

Our results indicate that the virus can cause acute infections and subclinical infections in which infected animals remain carriers for long periods. The acute form was characterized by watery diarrhea three days PI. Lesions attributed to the RECV were observed in the small intestine as early as 6 h PI. At that time, they were difficult to assess. However, later in the infection they were more severe and were similar to those reported in piglets infected with the transmissible gastroenteritis virus (20).

Microscopic lesions were reported in the GALT of piglets infected with transmissible gastroenteritis virus and viral particles were observed in the cytoplasm of the epithelial cells covering the lymphoid follicles. They were also observed in the cytoplasm of adjacent lymphocytes (21). Although we did not study the presence of the virus in those cells, the lesions observed in the appendix and in the Peyer's patches of RECV infected rabbits could be attributed to the virus. The role of the virus in the rabbit GALT is unknown. In pigs, the GALT plays an important defensive role (22) and it probably plays a similar role in the rabbit.

Only five of the 30 inoculated rabbits showed diarrhea. Although these observations might suggest a low pathogenicity of the RECV, we must emphasize that 18 rabbits were killed before diarrhea was observed. The severity of the intestinal lesions observed in some of those animals suggests that some would have been sick.

Electron microscopic examination of feces from adult dairy cows demonstrated shedding of enteric coronavirus particles for at least 3 yr following vaccination with modified-live rotavirus-coronavirus-*E. coli* vaccine (23). As previously mentioned, the RECV is also probably responsible for a carrier state since seven inoculated rabbits remained free of clinical signs. These animals were infected and viral particles were demonstrated by IEM in the cecal contents, but the animals were free of microscopic lesions. These results indicate that cells lining the intestinal mucosa or in the GALT might be chronically infected and be responsible for the production and shedding of the virus in carriers. Although we showed that the inoculated rabbits remained carriers for at least one month, we suspect that the rabbits remained carriers for a longer period, because we have observed RECV paticles in feces of clinically normal adult rabbits (unpublished data). In the present study, serology on the PI sera from the carriers was not done. Indeed results from a previous study (not shown) have demonstrated that those animals did not seroconvert suggesting that local immunity is involved in their protection.

We have not yet identified the cells responsible for viral replication in chronically infected animals but the results of preliminary studies (not shown) suggest that these cells are quite likely located in the GALT. The presence of the virus in the GALT cells could contribute to immunity thus preventing a possible reinfection.

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