An Experimental Rotavirus Infection in Neonatal Gnotobiotic Dogs.

Charles Audrey Johnson

Louisiana State University and Agricultural & Mechanical College

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AN EXPERIMENTAL ROTAVIRUS INFECTION IN NEONATAL GNOTOBIOTIC DOGS

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AN EXPERIMENTAL ROTAVIRUS INFECTION IN
NEONATAL GNOSTOBIOTIC DOGS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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in
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in Veterinary Medical Sciences
Veterinary Pathology

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D.V.M., Texas A & M University, 1974
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May 1982
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ABSTRACT

A canine rotavirus isolate caused clinical disease, small intestinal lesions, serum antibody response, and shedding of rotavirus in the feces in newborn gnotobiotic dogs. The results of this study demonstrated this rotavirus was a pathogen in neonatal gnotobiotic dogs. The pathogenesis of the intestinal lesions was similar to the pathogenesis of rotaviral enteritis in other species.

In three consecutive experiments, twenty puppies were derived by caesarean section and transferred to sterile isolators. Gnotobiotic puppies were inoculated orally with a rotavirus isolated from a neonatal puppy with fatal diarrhea. Fecal samples from the puppies were examined for virus particles by negative contrast electron microscopy. Infected and control puppies were killed at 12, 18, 24, 48, 72, 96, 132, and 154 hours post-inoculation (PI). Tissue samples were collected for fluorescent antibody, light, scanning electron, and transmission electron microscopy. Serum samples were examined for rotaviral antibody by a fluorescent antibody test.

The rotavirus isolate did infect the small intestine of the inoculated puppies. Diarrhea was observed in the inoculated puppies at 20 to 24 hours PI, persisting throughout the experiment. Rotavirus particles were observed in fecal samples of inoculated puppies 12 through 154 hours PI.
Rotavirus group-specific immunofluorescence was seen in intestinal villus epithelial cells at 12 hours PI in the duodenum, jejunum, and ileum. Fluorescence persisted in the jejunum and ileum through 154 hours PI. Morphological lesions in the small intestines were confined to the jejunum and ileum. Light and scanning electron microscopy revealed swelling and necrosis of villus epithelial cells with loss of epithelium. Slight to moderate villus atrophy was observed and the villi were covered by flattened to cuboidal epithelial cells. Rotavirus particles, rotaviral precursor material, and necrotic villus epithelial cells were seen early in the infection by transmission electron microscopy. In the later stages of infection, villus epithelial cells were similar, ultrastructurally, to crypt epithelial cells. Serum rotavirus antibody was detected in infected puppies killed at 96, 132, and 154 hours PI.

This rotavirus isolate caused moderate enteritis in gnotobiotic puppies and could serve as a model for pathogenesis studies of rotavirus infection in other species.
CHAPTER I

Literature Review and Objectives

History

Since Iwanosky reported transmission of tobacco mosaic disease by sap filtered through bacteria-proof filters in 1892 and Loeffler and Frosch's serial transmission of foot-and-mouth disease using bacteria-free filtrates in 1898, researchers without the use of x-ray diffraction and high resolution electron microscopy could only define the etiologic agent of numerous infectious diseases as "filterable agents". The history of rotavirus infections in man and animal species followed this same course of events. In 1943, a "filterable agent" was associated with an outbreak of neonatal human diarrhea and caused diarrhea in experimentally inoculated calves. An epidemic diarrheal disease in infant mice in 1947 was attributed to a "filterable agent".2

Rotavirus was identified in the calf in 1969,3 man in 1973-1974,4 and in mice in 1972.5 It has since been shown that rotavirus was present in the material used in 1943 to inoculate calves.1 The description and pathologic findings were quite similar to Epizootic Diarrhea of Infant Mice caused by a rotavirus.5 The current studies of rotaviruses began in 1969 with the report of experimental production of diarrhea in colostrum deprived calves by
inoculation with fecal material from naturally occurring infections. In that study, bacteria cultured from the feces did not cause diarrhea, but the bacteria-free filtrates containing a reovirus-like agent did cause diarrhea.

The study in 1969 did not receive marked attention until electron microscopic examination of intestinal biopsies from children with acute non-bacterial gastroenteritis revealed the presence of orbivirus or reovirus-like particles within epithelial cells of the duodenal mucosa. Soon to follow, virions morphologically indistinguishable from the calf reovirus-like particles were found in the stools of children with gastroenteritis in England and Australia. Later, orbivirus or reovirus-like particles were detected in the feces of numerous animal species, and it was proposed that these viruses should be included as a new genus within the family reoviridae. The names "duovirus" and "rotavirus" were proposed; however, the generic name "rotavirus" was adopted by the Reoviridae Working Team established under the World Health Organization, Food and Agriculture Organization, Comparative Virology Program in 1975. In addition, this team selected the Nebraska calf diarrheal virus (NCDV) strain of bovine rotavirus as a reference virus.

Morphology Of Rotaviruses

Several different interpretations of the fine structure of rotavirus using negatively stained preparations have been suggested, and these varying opinions were primarily
concerned with differences in the number of and spatial arrangement of the capsomeres making up the capsids. The majority of researchers agreed that intact rotavirus particles have a double-layered capsid structure with a central icosahedral core. Rotavirus particles have been described as either double-shelled or smooth particles with an approximate diameter of 70 nm. A complete rotavirus virion has a nucleocapsid core about 38 nm in diameter surrounded by an inner capsid layer, and an outer capsid layer. The virions have the appearance of a wheel with a hub (the nucleocapsid), short spokes (the inner capsid layer), and a rim (the outer capsid layer), thus the name rotavirus. This unique morphology has been used to distinguish rotaviruses from reoviruses and orbiviruses. Without the outer capsid layer the rotaviruses cannot be differentiated from orbivirus particles. Rotaviruses from various mammalian species and birds are morphologically indistinguishable, although variations in size have been reported.

Morphogenesis Of Rotaviruses

The morphogenesis of rotaviruses in infected intestinal epithelial cells was studied in several species including human, calf, lamb, pig, and mouse. Also, in vitro studies with calf rotavirus, simian rotavirus, human rotavirus, and procine rotavirus have been reported.

Rotavirus particles have been observed by transmission


electron microscopy on the microvilli of absorptive epithelial cells in several experimental studies.\textsuperscript{21,22,25,26} However, the exact method for viral attachment is not known. It has been suggested that rotavirus gained entry into the villus epithelial cells by the pinocytotic activity of the cells.\textsuperscript{25}

Within the villus epithelial cells, rotavirus particles were located in the cytoplasm, usually in dilated cisternae of rough endoplasmic reticulum.\textsuperscript{16,21-30} Viral precursor material, called viroplasm, consisted of masses of granular or fibrillar material containing virus-like cores measuring 30-35 nm in diameter.\textsuperscript{21-30} In addition, viral associated single-membraned tubules in the nucleus, and both single and double-membraned tubules in the cytoplasm have been observed.\textsuperscript{26} An hypothesis of the maturation process of rotaviruses was that the virus particles form by the budding of single-layered virions from the viroplasm into the cisternae of rough endoplasmic reticulum thus acquiring an outer membrane.\textsuperscript{29} After completion of maturation, release of the virions was by disruption of infected cells.\textsuperscript{29}

**Biochemical and Biophysical Properties Of Rotaviruses**

It has been demonstrated that the genome of calf rotavirus consisted of 11 or 12 double-stranded segments of ribonucleic acid (RNA) ranging in molecular weight from 2.2 X 10\textsuperscript{6} to 0.2 X10\textsuperscript{6} daltons.\textsuperscript{30,31} The total molecular weight of the RNA segments was 11 to 12x10\textsuperscript{6} daltons. In addition, the analyses of the genomes of other rotaviruses
from different species were similar.\textsuperscript{15} Rotaviral RNA from
different animal species differed with respect to the
electrophoretic mobility of one or more genome segments on
polyacrylamide gels.\textsuperscript{32-34} Size variation of RNA segments
between different isolates of human \textsuperscript{33} and calf \textsuperscript{35}
rotaviruses has been observed. However, a relationship
between electropherotypes and serotypes has not been
established.

The analyses of the polypeptide composition of rota­
viruses from various species showed marked similarities
among the profiles obtained by different researchers.\textsuperscript{15}
Investigation of the polypeptides of purified single-shelled
rotaviruses revealed two major and three or four minor polypep­
tides in the inner capsid of the virus. Three or four
polypeptides were associated with the outer shell.\textsuperscript{30,31,37}
Therefore, eight or nine structural polypeptides have been
identified, but these need further investigation. The
polypeptide profiles of single-shelled Simian II virus
(rotavirus) and human and calf rotaviruses were
indistinguishable,\textsuperscript{31} but differences were observed
between the low molecular weight components of the outer
capsid layer.\textsuperscript{36} These findings were of interest, because
the proposed locations for the group- and species-specific
antigens of the rotaviruses are the inner and outer capsid
layers, respectively.\textsuperscript{37}

Investigation using immunoelectron microscopy, showed
that single-shelled rotavirus particles from different animal
species were agglutinated by all convalescent sera examined regardless of the species of origin of the sera. But, double-shelled human rotavirus particles were agglutinated only with antiserum to human virus indicating the group antigen is located on the inner capsid layer. Furthermore, the antigens reacting in the neutralization and immunofluorescent antibody tests were shown to be distinct.

Sera collected from different animal species having high immunofluorescence antibody titers to calf rotavirus were unable to neutralize the virus. Also, cross neutralization tests revealed the existence of a species-specific rotavirus antigen because each species of rotavirus tested was neutralized by at least a fourfold greater dilution of homologous convalescent serum than by a heterologous serum. In addition, the outer capsid layer was demonstrated to be necessary for infectivity.

Using cesium chloride gradient centrifugation, calf rotaviruses were separated with an intact outer capsid layer from virus particles without the outer layer. These were referred to as smooth and rough particles, respectively. The particle/infectivity ratio was about 1000-fold greater for the smooth than the rough particles. Similar results were reported using a human rotavirus isolate.

Additional reports have concerned the importance of the outer capsid layer. The resistance of rotaviruses to a wide variety of proteases have been attributed to the discovery that the major polypeptide component of the outer layer is
glycosalated. Intact double-shelled rotavirus particles have little detectable RNA-dependent RNA polymerase activity, but enzyme activity was readily demonstrated in single-shelled virions. A component on the outer capsid layer of calf rotavirus was responsible for agglutination of erythrocytes from a variety of animal species.

Clinical Disease, Pathology and Pathophysiology
Rotaviruses are one of the most common causes of non-bacterial gastroenteritis in young animals and human infants. Natural enteric infections caused by rotaviruses have been documented in calves, piglets, mice, foals, lambs, deer and puppies. In addition, rotaviruses have been detected in the feces of numerous other mammals and birds. The clinical disease was usually limited to diarrhea and dehydration. Detailed studies of experimentally infected calves, pigs, and mice have indicated that the rotavirus invades the absorptive epithelial cell on the upper one-third to two-thirds of the intestinal villus. Histopathologic changes observed with the light microscope included moderate loss of enterocytes, villus atrophy, blunting and fusion of the intestinal villi, and an increased number of cuboidal rather than columnar epithelial cells covering the villi. Either no inflammatory response or a mild to moderate increase in the number of lymphocytes and neutrophils or reticulum cells in the lamina propria was reported. Scanning electron
microscopic examinations demonstrated villus atrophy, rounded and fused villi, a decrease in the number of microvilli on the enterocytes, and a loss of several absorptive epithelial cells from the tip and sides of the villi. These changes were most prominent between 24 and 96 hours after inoculation.25,26,50-55

Rotaviruses are the most common cause of non-bacterial infantile gastroenteritis in children in many parts of the world.56 Children in the age range of six months to two years are the most commonly affected; however, less severe forms of gastroenteritis have been reported in older children, adults, and geriatric patients.56-59 Reports also indicate a seasonal incidence with the greater incidence of infection being reported in the winter months.60,61

The histopathology of human rotavirus infections has been achieved by biopsy studies of duodenum and upper jejunum, and revealed similar lesions to those observed in animals.61

The pathophysiologic changes associated with rotaviral infection in animals and humans had marked similarities to the alterations in pigs infected with transmissible gastroenteritis virus, a coronavirus.62-66 In summary, these studies revealed at least three significant causes for the diarrhea and electrolyte imbalance. First, since rotavirus and coronavirus selectively infect and destroy villus absorptive epithelial cells, the loss of significant numbers of absorptive cells, surface area, and intestinal enzymes resulted in a decreased absorptive capacity. As food was
ingested and accumulated in the small intestine where absorption was poor, the unabsorbed intestinal content was passed along to the colon. Diarrhea resulted when the amount of material present exceeded the absorptive capacity of the colon. Second, digestive functions of the intestinal villus absorptive cells were impaired causing maldigestion. As an example, destruction of the brush border disaccharidases in transmissible gastroenteritis in pigs caused a lactase deficiency and lactose was poorly digested. Depressed mucosal diasaccharidase levels were found in the intestine in 14 of 16 infants with rotaviral gastroenteritis. Third, hypersecretion contributed to diarrhea in pigs infected with human rotavirus or transmissible gastroenteritis virus. Rotaviruses, like transmissible gastroenteritis viruses, did not destroy the epithelial cells in the crypts of Lieberkühn, which are secretory cells and the secretory processes continued. Following destruction of the absorptive villus epithelium by rotavirus or transmissible gastroenteritis virus, an increased rate of crypt cell proliferation and accelerated migration of these cells out of the crypts has been detected. The atrophic villi were covered with these immature cuboidal cells which retain some of their secretory functions. The secretion of water, sodium, and chloride from the small intestine of pigs infected with transmissible gastroenteritis virus was greater when compared with uninfected pigs. It has been suggested that this mechanism was part of the
cause for a disordered electrolyte transport in the pathogenesis of rotaviral enteritis.\textsuperscript{68,70}

Other factors that have been suggested to contribute to diarrhea as a result of a rotavirus infection include increased osmotic activity of the undigested and unabsorbed intestinal contents and bacterial fermentation in the lumen of the small intestine and colon.\textsuperscript{68} In addition, impaired absorption of bile salts and fatty acids produced by microbial fermentation may stimulate colonic secretion.\textsuperscript{71}

**Diagnosis of Rotavirus Infection**

The diagnosis of rotavirus infections has been based primarily on the detection of virus or viral antigen in the feces. There are usually adequate numbers of virions in rotavirus infected feces to be visualized, allowing electron microscopic examination of fecal material to have become a standard diagnostic technique. It has been demonstrated that the virus is less difficult to see if it has been concentrated by ultracentrifugation\textsuperscript{7,8,72} or aggregated using specific antiserum.\textsuperscript{10,73}

As a result of the cost of equipment and the expertise necessary for electron microscopy, alternative diagnostic techniques have been developed. Several diagnostic techniques have been based on immunofluorescence using specific rotavirus antisera. These included immunofluorescent staining of fecal smears\textsuperscript{2,27} and cell cultures infected with fecal preparations.\textsuperscript{74,75} An increased sensitivity of the cell culture immunofluorescence method was
accomplished by low spread centrifugation of inoculum onto the cells. The sensitivity of this method was increased by infecting freshly trypsinized cells in suspension. Several established cell lines (LLC-MK2, PK15, and MA-104 cells) have been shown to be susceptible to infection by human and a variety of animal rotaviruses and served as useful cell lines for the cell culture immunofluorescence method. In addition, a "free" virus immunofluorescence assay using antibody and infected feces with detection of the antigen antibody complex by immunofluorescence was developed. The results obtained by these immunofluorescence methods were in good agreement with those obtained by electron microscopic examination.

The detection of rotavirus or rotavirus antigen in the feces by other methods have been shown to be useful, but had variable sensitivity when compared to negative contrast electron microscopy (NCEM). The complement fixation method has been found to be less sensitive than NCEM due to the anticomplementary activity present in stool samples. Counterimmunoelectrosphoresis has variable sensitivity when compared with NCEM. The use of a solid phase radioimmunoassay test on feces produced results similar to NCEM in routine diagnosis. An enzyme-linked immunosorbent assay for rotavirus was described which was potentially more sensitive than electron microscopy for detection of rotavirus. Since 1977, the enzyme
linked immunosorbent assay has been used in numerous laboratories and a commercial rotavirus diagnostic kit\textsuperscript{a} based on this technique has been developed.

Complement fixation,\textsuperscript{89-91} immunofluorescence,\textsuperscript{17} and neutralization tests\textsuperscript{92} are used for serologic diagnosis of rotavirus infection.

**Immunity in Animals**

In cows,\textsuperscript{93-95} sheep,\textsuperscript{96} and pigs,\textsuperscript{97} reports indicate that colostral rotaviral antibody and/or the presence of antibodies in the small intestine, especially secretory IgA, are the principal factors preventing rotaviral disease in these animals.

**Objectives**

The objectives of this study were:

1) To find out if a canine rotavirus isolated from a neonatal dog with fatal diarrhea would infect and cause clinical disease in newborn gnotobiotic dogs inoculated orally with this isolate.

2) To observe morphological changes between inoculated neonatal dogs and time-matched uninoculated neonatal dogs with light, scanning electron and transmission electron microscopy.

3) To compare the lesions observed in the inoculated gnotobiotic dogs with lesions in other species experimentally infected with rotaviruses.

\textsuperscript{a}Rotazyme\textsuperscript{tm}, Abbott Laboratories, North Chicago, Ill.
4) To propose the pathogenesis of canine rotavirus infection in neonatal gnotobiotic dogs and also determine if this experimental model could be proposed for use in pathogenetic studies of rotavirus infections in other species.
Experimental Infection of Neonatal Gnotobiotic Dogs with Canine Rotavirus

Introduction

Rotavirus particles have been observed in diarrheal feces from pigs, calves, mice, and many other species including man. Experimental studies have confirmed that rotaviruses are important etiologic agents of neonatal diarrhea in their natural hosts. In addition, some of the rotaviruses can infect and induce diarrhea in species other than their natural hosts.

Recent reports have implicated rotaviruses as a cause of diarrhea in dogs. In a 12-week-old puppy, with nonfatal diarrhea, viral particles with morphological features of rotavirus were observed. Rotavirus-like particles were found in the feces of young puppies having died as a result of severe enteritis. A rotavirus, was identified by negative contrast electron microscopy (NCEM) in the intestinal contents of a 3-day-old dog with fatal diarrhea, and this rotavirus infected trypsin-treated Madin Darby canine kidney cell cultures. Serologic evidence of infection in dogs, based on the presence of serum antibodies, has been reported.

In dogs, experimental inoculation has been reported using a human rotavirus isolate, a bovine rotavirus
isolate,106 and a canine rotavirus isolate.47 The dogs inoculated with the human and bovine rotavirus isolates did shed rotavirus in their feces, but the dogs inoculated with the canine isolate did not shed rotavirus in the feces. None of the dogs had clinical signs of disease.

An outbreak of fatal diarrhea in several litters of newborn puppies occurred in a kennel in Baton Rouge, Louisiana, and rotavirus-like particles were observed in the intestinal contents from one of the puppies by NCEM.48 Subsequently, a rotavirus was isolated from a homogenate of the intestine and used to inoculate fetal rhesus monkey kidney (MA-104) cells. Polyacrylamide gel electrophoretic (PAGE) analysis of the ribonucleic acid (RNA) of this rotavirus determined this isolate to be unique when compared with PAGE analyses of RNA from bovine, porcine, and simian rotavirus isolates.a This rotavirus was shown by serum neutralization tests and cross-protection infections in pigs to be antigenically distinct from isolates of human, bovine, and porcine rotavirus.a This canine rotavirus isolate was designated LSU 79C-36.48

In this experiment, two-day-old gnotobiotic puppies were inoculated orally with a cell culture passage of the canine rotavirus isolate LSU 79C-36, and observed for evidence of infection.

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aGaul S, Woode GN: Personal communication, 1980. Department of Veterinary Microbiology and Preventive Medicine, Iowa State University; Ames, Iowa.
Materials and Methods

**Gnotobiotic Puppies**—In three sequential inoculation trials, twenty neonatal puppies were derived by caesarean section from three mongrel bitches. The techniques used in this experiment for obtaining and rearing the puppies have been described. Two modifications of the techniques were made. First, the caesarean section was performed outside of an isolator and the gravid uterus was transferred to a sterile, plastic isolator through a germicidal trap connected to the entry port of the isolator. Then the puppies were removed from the uterus. The second modification was sterilization of the maintenance diet. The powdered milk diet was sterilized by exposure to 8.5 megarads of ionizing radiation from a $^{60}$Co source. Twenty-four hours after delivery, puppies chosen at random were transferred to a second sterile, plastic isolator. Fecal samples were collected daily and cultured using aerobic microbiological methods.

**Virus**—A rotavirus isolate, designated LSU 79C-36, was isolated from a newborn puppy that died after having clinical signs of diarrhea. The canine rotavirus was inoculated into MA-104 monolayer cultures (fetal rhesus monkey kidney cell line). Sterile 1 ml aliquots of the 16th and 17th cell culture passages of the rotavirus were used as inoculum.

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Obtained from Dr. GN Woode, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa.
The virus titer was approximately $10^5$ tissue culture infective dose $50/\text{ml. (TCID}_{50})$.

**Experimental Animals**—A total of twenty puppies from three litters, 11 principals and 9 controls were used in this experiment (Table 1). At least one rotavirus infected puppy and one control puppy were sacrificed at 12, 18, 24, 48, 72, 96, 132 and 154 hours post-inoculation (PI). The principals were inoculated orally with $1 \text{ ml}$ of the rotavirus suspension at 48 hours of age. The control puppies were not inoculated. Intravenous blood samples were drawn once before inoculation and once post-inoculation (at the time of euthanasia) from each puppy for complete blood counts (CBC) and serology. All the puppies were examined several times daily for clinical signs of disease.

**Negative Contrast Electron Microscopy (NCEM)**—Fecal samples were collected daily from each puppy, suspended in distilled water and centrifuged at 3000 RPM for 15 minutes. The supernatant was collected and subjected to ultracentrifugation at 100,000 xg for 1 hour. The pellet was stained with neutralized phosphotungstic acid and sprayed onto a carbon-coated, collodian-filmed 200 mesh grid with an all-glass nebulizer following a previously described technique. The grids were examined for the presence of virus particles with a transmission electron microscope.

**Serologic Analysis**—An indirect fluorescent antibody

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\*Zeiss EM-10, Carl Zeiss, Inc., New York, NY.*
**TABLE 1**

Euthanasia Times After Rotavirus Inoculation
In Gnotobiotic Puppies

<table>
<thead>
<tr>
<th>EUTHANASIA TIME (HOURS)</th>
<th>INFECTED (I) PUPPY</th>
<th>CONTROLS (C) PUPPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1Ia</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1Ib</td>
<td>18</td>
</tr>
<tr>
<td>18</td>
<td>2I</td>
<td>24</td>
</tr>
<tr>
<td>24</td>
<td>3I</td>
<td>48</td>
</tr>
<tr>
<td>48</td>
<td>4Ia</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>4Ib</td>
<td>96</td>
</tr>
<tr>
<td>72</td>
<td>5Ia</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>5Ib</td>
<td>154</td>
</tr>
<tr>
<td>96</td>
<td>6I</td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>7I</td>
<td></td>
</tr>
<tr>
<td>154</td>
<td>8I</td>
<td></td>
</tr>
<tr>
<td><strong>TOTALS:</strong></td>
<td><strong>11</strong></td>
<td><strong>9</strong></td>
</tr>
</tbody>
</table>
test (IFAT) was used to examine serum samples from the puppies and three bitches. Cell monolayers (MA-104) on 8-well chamber slides were infected with the LSU 79C-36 rotavirus isolate, rinsed 3 times with 1% phosphate buffered saline solution (PBSS) and fixed in cold acetone. Two-fold serial dilutions of the serum samples were prepared using 1% PBSS. Serum dilutions of 1:10 through 1:160 were incubated in different wells of the rotavirus infected MA-104 cell monolayers for 30 minutes at 37°C. Afterwards, the slides were washed with 1% PBSS, air dried, counter-stained with a commercial fluorescein isothiocyanate (FITC) labeled rabbit anticanine globulin, and incubated at 37°C for 1 hour. After final rinsing in 1% PBSS, the slides were mounted with buffered glycerol, coverslipped, and examined by fluorescent microscopy. MA-104 cell monolayers were prepared using antiserum against bovine rotavirus from a gnotobiotic calf and treated with commercially prepared FITC labeled rabbit antibovine globulin to serve as a positive control. Uninfected cell cultures were reacted with bovine rotavirus antiserum or with canine serum containing rotavirus antibody as negative controls. Separate infected cell monolayers were reacted only with the two commercial

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eCappel Laboratories, Inc., New York, NY.
fOrthoplan® Universal Largefield Microscope with a Fluorescence Vertical Illuminator, Ernst Leitz Company, Midland, Ontario.
gObtained From Dr. A Torres-Medina, Department of Veterinary Science, University of Nebraska, Lincoln, Neb.
hAntibodies, Inc., Davis, Calif.
FITC labeled antiglobulins to check for non-specific fluorescence. Rotavirus positive immunofluorescence for antibody in the test serum was defined as the serum dilution which reacted with infected cells emitting specific fluorescence when compared to negative controls.

**Tissue Immunofluorescence (IF)**—An IFA test was used to detect common immunofluorescent antigen in sections of duodenum, jejunum, ileum, colon, mesenteric lymph node, and lung. During necropsy, sections of intestine were collected, filled with embedding medium, placed in glass vials, and frozen at -70°C. Mesenteric lymph node and lung sections were collected in glass vials and frozen, also. The tissue sections were cut 4-6 um thick in a cryostat microtome, mounted on glass slides, fixed in cold acetone, then stored at -20°C for 2-7 days. The IFA test was performed by reacting the tissue sections with antiserum against bovine rotavirus prepared in a gnotobiotic calf for 1 hour at 37°C. Afterwards, the sections were rinsed with 1% PBSS, air dried, counter-stained with a commercial FITC labeled rabbit antiovine globulin, and incubated for 1 hour at 37°C. Then the sections were again washed with 1% PBSS, coverslipped using buffered glycerol, and examined by fluorescent microscopy. Negative control sections were prepared by reacting tissue sections with gnotobiotic fetal calf serum free of rotavirus antibody and counter-staining

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1O.C.T. Compound, Lab-Tek Products, Naperville, Ill.
with FITC labeled rabbit antibovine globulin. In addition, tissue sections were observed for evidence of fluorescence using direct immunofluorescent antibody (DFA) tests for canine distemper virus, coronavirus, and parvovirus.

Results

Gnotobiotic Puppies—In inoculation trials 1 and 2 no bacterial contamination was detected. During inoculation trial 3, a single bacterial isolate, *Pseudomonas pseudomallei* was cultured from the feces of two four-day-old, control puppies. They were puppies 7C and 8C, the 132 and 154 hour PI control puppies, respectively. The source of the contaminate was identified as a bottle of water used to dissolve the powdered milk.

Clinical Signs—Diarrhea was observed in the inoculated puppies between 20 and 24 hours PI and persisted through 154 hours PI. The feces were light to dark green, and semi-liquid in consistency containing a moderate amount of mucus. No blood was observed in the feces. Except for puppy 8I at 154 hour PI, all the inoculated puppies were alert, active, and had good appetites until sacrificed. Puppy 8I was killed in a moribund condition. The infected puppies killed after 24 hours PI had rough haircoats and the hair around the perineal region was matted with feces. Mild

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[^1]Analytical Profile Index, Analytab Products, Plainville, NY.
dehydration was evident. These signs were marked in puppy 81, beginning at 132 hours PI. The feces from the control puppies varied from dark green, dry, and well formed to light green and semi-liquid. The two bacterially contaminated puppies did not show clinical signs of diarrhea. All the control puppies remained alert and active and nursed voraciously. Their haircoat was shiny and smooth and no clinical signs of dehydration were observed.

Hematologic values obtained from complete blood counts did not vary significantly between the rotavirus infected and control puppies.

**NCEM**—Examination of the feces from inoculated puppies revealed rotavirus particles at 12 hours PI through 154 hours PI. Both complete and incomplete viral particles were observed in the feces throughout the experiment. Incomplete viral particles lacked a nucleocapsid, represented by a round electron-dense center and/or did not have the outer capsid layer (Fig 1). Complete viral particles consisted of both outer and inner capsid layers and a nucleocapsid (Fig 2). No viral particles were observed in fecal samples collected from all puppies before inoculation or control puppies during the experiment.

**Serologic Analysis**—The results of serum rotavirus antibody detected by the IFA test in the rotavirus infected puppies have been summarized in Table 2. The first evidence of serum rotavirus antibody was observed at 96 hours PI at a level of 1:10. Increased serum rotavirus antibody levels
Fig 1-Negative contrast electron micrograph of fecal sample from a gnotobiotic puppy 24 hours after inoculation with canine rotavirus. Two incomplete viral particles are present. Phosphotungstic acid stain; X 120,000.

FIG 2-Negative contrast electron micrograph from the same fecal sample as Fig 1 showing a complete rotavirus particle. Phosphotungstic acid stain; X 180,000.
TABLE 2
Serum Rotavirus Antibody Titers in Rotavirus Infected Gnotobiotic Puppies Using an Indirect Fluorescent Antibody Test*

<table>
<thead>
<tr>
<th>HOURS POST-INOCULATION</th>
<th>1:10</th>
<th>1:20</th>
<th>1:40</th>
<th>1:80</th>
<th>1:160</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>18</td>
<td>-</td>
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<td>48</td>
<td>-</td>
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</tr>
<tr>
<td>72</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>96</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>132</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>154</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*All controls were negative.
were found in infected puppies killed at 132 and 154 hours PI at levels of 1:40 and 1:80, respectively. No serum rotavirus antibody was detected in pre-inoculation samples or in the control puppies.

Serum antibody levels for rotavirus were detected in the three pregnant mongrel bitches. The levels determined were 1:80, 1:40, and 1:60. Failure to receive passively transferred rotaviral antibody was indicated by the negative results of the pre-inoculation serum samples of the gnotobiotic puppies.

**IF In Tissue Sections**—The results of the IFA tests have been listed in Table 3. The intestinal sections of the inoculated puppies demonstrated fluorescence in moderate to marked numbers of villus epithelial cells in the jejunum and ileum from 12 hours PI through 96 hours PI. Between 12 and 96 hours PI, no or a slight number of villus epithelial cells in the duodenum had evidence of fluorescence. No fluorescence was seen in epithelial cells of the colon at any observation time. In sections of mesenteric lymph node, slight to moderate numbers of mononuclear cells in the cortex and paracortex had cytoplasmic fluorescence at 48, 96, 132, and 154 hours PI.

The majority of the fluorescent villus epithelial cells were on the upper one-half to one-third of the villus. The fluorescence was confined to the cytoplasm and had a granular pattern (Fig 3). Starting at 48 hours PI and persisting through 154 hours PI, small numbers of mononuclear cells in
TABLE 3

Indirect Fluorescent Antibody Test on Tissue from Rotavirus Infected Gnotobiotic Puppies*

<table>
<thead>
<tr>
<th>HOURS POST-INOCULATION</th>
<th>PUPPY</th>
<th>DUODENUM</th>
<th>JEJUNUM</th>
<th>ILEUM</th>
<th>COLON</th>
<th>MESENTERIC LYMPH NODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1Ia</td>
<td>1+**</td>
<td>2+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1Ib</td>
<td>1+</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>2I</td>
<td>1+</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>3I</td>
<td>-</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>4Ia</td>
<td>-</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>4Ib</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>5Ia</td>
<td>-</td>
<td>3+</td>
<td>2+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5Ib</td>
<td>2+</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>96</td>
<td>6I</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
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<td>1+</td>
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<td>132</td>
<td>7I</td>
<td>-</td>
<td>1+</td>
<td>1+</td>
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<tr>
<td>154</td>
<td>8I</td>
<td>-</td>
<td>1+</td>
<td>1+</td>
<td>-</td>
<td>2+</td>
</tr>
</tbody>
</table>

* All controls were negative.

** 1+ = slight, 2+ = moderate, 3+ = marked.
Fig 3-A section of ileum from an infected puppy 24 hours PI. The granular fluorescence is confined to the cytoplasm of villus epithelial cells on the upper one-half of the intestinal villi. Immunofluorescent stain; X 850.
the lamina propria of the intestinal villi contained intracytoplasmic fluorescence. No fluorescent cells were observed in sections of lung. Fluorescence was not observed in any tissue samples from the control puppies.

The sections of small intestine from the inoculated puppies showed no fluorescence when reacted with parvovirus, coronavirus, or canine distemper virus antibody conjugates.

Discussion

In this study, gnotobiotic, newborn puppies were infected by oral inoculation with an isolate of rotavirus from a newborn puppy and developed diarrhea. The results are similar to experimental infections with rotavirus in other young, germ-free animals. The interval of time for onset of diarrhea in the inoculated puppies, 20 to 24 hours PI, is similar to the times at which diarrhea was noted in experimental inoculations of porcine rotavirus in gnotobiotic piglets,52-55 bovine rotavirus in gnotobiotic calves,107 and rotavirus in suckling mice.108 In addition, infected puppies showed signs of mild dehydration 24 hours PI similar to that seen in gnotobiotic piglets.52 However, no vomiting was observed as reported in germ-free pigs.52,53

Rotavirus particles were observed in the feces of inoculated gnotobiotic pigs starting at 24 hours PI and persisting through 96 hours PI.55 Fifty to 75% of the viral particles were coreless (incomplete virus particles) from 24 through 72 hours PI and the majority of virus
particles seen 84 to 96 hours PI possessed an electron-lucent core and outer capsid layer (complete virus particles). In the present study, both incomplete and complete rotavirus particles were seen in the feces starting at 12 hours PI and persisting through 154 hour PI. No attempt was made to quantitate the virus in the feces.

The results of the indirect immunofluorescent antibody tests on frozen sections of small intestine and colon from experimental rotavirus inoculations of gnotobiotic pigs, gnotobiotic calves, and suckling mice closely paralleled the patterns of fluorescence in the intestinal sections of the infected puppies in this study. Fluorescence was confined to the cytoplasm of villus epithelial cells on the upper one-half to one-third of villi with the relative number of fluorescent cells variable or absent in the duodenum, moderate to marked in the jejunum and ileum, and variable or absent in the colon. In gnotobiotic piglets and calves with rotavirus infection, increased numbers of fluorescent epithelial cells were observed between 24 and 96 hours PI with a decrease in the number of fluorescent cells beginning at 96 hours PI. In the jejunum and ileum of the inoculated puppies in this study, moderate to marked numbers of villus epithelial cells had specific fluorescence starting at 12 hours PI persisting through 96 hours PI. A decrease in the relative number of cells with fluorescence was observed after 96 hours PI. Fluorescence in the villus lamina propria of the small
intestine in rotavirus infected gnotobiotic calves has been reported, but not regarded as specific because many eosinophils were present. Immunofluorescence within the cytoplasm of cells in the villus lamina propria of the jejunum and ileum of the inoculated puppies in this study was considered specific because no similar fluorescence was observed in the intestinal villi of control puppies. Fluorescence in the cytoplasm of cells in mesenteric lymph nodes of inoculated pigs was reported which was similar to the observations in the mesenteric lymph nodes of the infected puppies.

Experimental rotavirus inoculations in conventional dogs have been conducted using a human rotavirus isolate, a bovine rotavirus, and a rotavirus isolated from a 3-day-old puppy with fatal diarrhea. However, inoculation studies with rotavirus in germ-free puppies have not been reported.

In the dogs inoculated with the human rotavirus isolate, immunofluorescence was demonstrated in a small number of villus epithelial cells in sections from the small intestine, but no fluorescence was observed in the colon. Also, most of the puppies infected with this human rotavirus had increasing serum neutralizing and complement fixing antibody titers starting about 4 days PI. The infected, gnotobiotic puppies in the present study showed evidence of antibody against the group-specific rotaviral antigen about the same time (4 days PI) as serum neutralizing and complement fixing
antibody was demonstrated in puppies infected with human 
rotavirus.

A rotavirus from the diarrheic feces of a 3-day-old 
puppy was used to inoculate two conventional 6-month-old 
Beagle dogs. The inoculated dogs showed no clinical 
signs and did not shed virus in their feces. The reasons for 
failure to induce disease in this inoculation trial were 
discussed by the authors, and attributed to the age of the 
dogs, immunity against infection, and/or low multiplicity of 
infection.

In the present study, gnotobiotic puppies were used to 
provide a pathogen free, nonimmune animal. The time of 
inoculation (2 days of age) was based on reports that rota­
virus had been detected in dogs less than 1-week-old with 
diarrhea. The results indicated that newborn, 
gnotobiotic puppies develop enteritis and shed virus in their 
feces after inoculation with the canine rotavirus isolate, 
LSU 79C-36.
CHAPTER III

Gross and Light Microscopic Lesions in Neonatal Gnotobiotic Dogs Infected with a Canine Rotavirus

Introduction

The gross, light microscopic, and intestinal morphometric changes induced by rotavirus infection of the small intestines have been described in pigs, calves, and lambs. Gross lesions were slight to moderate characterized by dilated, thin-walled segments of the small intestine and/or colon with variable quantities of semi-liquid to liquid contents in the lumen. Histopathological changes included mild to moderate villus atrophy, cuboidal to squamous villus epithelial cells covering the villi, and an increased number of reticuloendothelial cells in the villus lamina propria. The most prominent histological lesions were observed in the caudal two-thirds of the small intestine and observed first at 24 to 60 hours post-inoculation (PI). Morphometric results demonstrated evidence of intestinal villus atrophy and suggested that there was an increase in the production of crypt cells when there was damage to the mature villus epithelial cells by the rotavirus. The increased number of crypt cells was interpreted as a response by the animal to replenish the damaged villus epithelial cells and cover the denuded villi.

Reports have implicated rotavirus as a cause of diarrhea.
However, experimental inoculations with rotavirus in dogs have not demonstrated significant clinical or morphological changes.47,102,106

In this study, canine rotavirus was used for oral inoculation of two-day-old gnotobiotic puppies to determine whether gross, light microscopic, and intestinal morphometric changes could be detected in the small intestine. The changes observed in the intestine were compared with lesions described for rotaviral enteritis in other species. The canine rotavirus isolate, designated LSU 79C-36,48 used in this study was shown to have unique ribonucleic acid segments and antigenic properties from bovine, porcine, simian, and human rotavirus isolates.a

Materials and Methods

Experimental Design—A total of twenty gnotobiotic puppies were derived by caesarean section from three mongrel bitches as described in Chapter II. Eleven two-day-old puppies were inoculated orally with 1 ml of a cell culture suspension containing the 16th and 17th passage of a canine rotavirus isolate.48 The virus titer was 10^5 TCID50/ml. Nine control gnotobiotic puppies were not inoculated.

At least one inoculated and one control puppy were

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aGaul S, Woode GN: A Personal Communication, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa.
sacrificed at 12, 18, 24, 48, 72, 96, 132 and 154 hours PI for necropsy and tissue collection.

**Necropsy Procedures**—Prior to necropsy, the animal was anesthetized, and three segments of small intestine and one section of colon were removed. A segment of the duodenum was removed at the beginning of the right lobe or head of the pancreas, then a section of small intestine was taken approximately 60 cm distal to the head of the pancreas, to represent the jejunum. A section of the ileum was excised 5 cm proximal to the ileocecal valve. A segment of colon was removed 5 cm distal to the ileocecal valve. These segments were incised along the mesenteric border, flattened and pinned to a piece of hard paper with the mucosal surface up and immersed in 10% buffered formalin. After the gut samples were collected the puppies were killed with an overdose of sodium pentobarbital. Sections of small intestine, colon, liver, and blood from the heart were collected aseptically for aerobic microbiological culture. Tissue sections of the following organs were placed in 10% buffered formalin: liver, gall bladder, pancreas, stomach, mesenteric lymph node, spleen, kidney, adrenal gland, urinary bladder, ureter, reproductive organs, heart, lung, thymus, trachea, esophagus, tongue, eyes, brain, skeletal muscle, sciatic nerve, femur and bone marrow from the midshaft of the femur.

**Microbiological Culture**—Blood agar plates were inoculated by impression smears with samples of small intestine, colon, and liver from each puppy. The same
culture medium was inoculated with the heart blood from each puppy. The blood agar plates were incubated at 37°C for a total of 96 hours and checked every 24 hours for bacterial growth.

**Histopathology**—Formalin-fixed tissues were embedded in paraffin, sectioned 6μm thick, and stained with hematoxylin and eosin by standard procedures.

**Intestinal Morphometry**—Mucosal measurements of the duodenum, jejunum, and ileum were made in all the gnotobiotic puppies with a digital analyzer\(^b\) and light microscope. The villus length (from the tip of the villus to the junction of the crypt) and the crypt depth (junction of the villus to base of the crypt) was measured on five properly oriented villi in the duodenum, jejunum, and ileum. The means of the villus (V) length, crypt (C) depth, and villus length to crypt depth (V:C) ratio from the three sections of small intestine from each puppy were evaluated by analysis of covariance.\(^c\)

**Results**

**Necropsy**—No gross lesions were observed in inoculated puppies at 12 and 18 hours PI. Inoculated puppies, killed at 24, 48, 72, 96, 132 and 154 hours PI had external lesions consisting of roughened, dry haircoats and the hair around

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\(^b\)Zeiss MOP-3 Digital Analyzer, Carl Zeiss, Inc., New York, NY.

\(^c\)Dr. N Keith, Dept. of Experimental Statistics, Louisiana State University, Baton Rouge, La.
the perineal region was matted by feces. The other gross lesions were limited to the small intestine. Moderate dilatation and thinning of the wall of the ileum with a moderate amount of semi-liquid to liquid greenish-yellow contents were observed in the lower one-third of the small intestine, starting at 24 hours PI. The same lesion was present in more than one-half the length of the small intestine beginning at 72 hours PI and persisting through 154 hours PI. Puppy 81, necropsied 154 hours PI, had moderate dilatation of the entire small intestine. Moderate to large quantities of dark green, semi-liquid stool were present in the colon from 24 through 154 hours PI. In addition, slight to moderate hyperemia of the small intestine was seen from 24 through 154 hours PI. All the infected puppies except puppy 81, had curdled milk in their stomachs at necropsy. Puppy 81 had an empty stomach. Other organs of the infected puppies appeared normal on gross inspection.

No gross lesions were found in the control puppies.

**Microbiological Culture**—The only aerobic bacterial isolate from tissue samples was from smears of the small intestine and colon from control puppies 7C and 8C, 132 and 154 hours PI, respectively. The organism was identified as *Pseudomonas pseudomallei*.d

**Histopathology**—In the control puppies, the villi of the small intestine were generally long and narrow (Fig 1). At

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dAnalytical Profile Index, Analytab Products, Plainville, NY.
Fig 1—Duodenum from a control puppy killed 24 hours PI. The villi are long and narrow. H&E stain; X 225.
12 hours PI, the intestinal villus epithelial cells in the control puppies were cuboidal to columnar and the nuclei occupied the apical, middle, or basal position of the cell in the duodenum, jejunum and ileum (Fig 2). After 12 hours PI, the majority of the villus epithelium consisted of tall columnar cells and the nuclei were in the basal portion of the cell. Vacuolation in the cytoplasm of intestinal villus epithelial cells varied in degree and distribution with time PI in the control puppies. Rarely, vacuoles were seen in the cytoplasm of the villus epithelial cells in the duodenum at 12 and 18 hours PI (Fig 2). At 24 hours PI and throughout the experiment, only a small number of small, clear vacuoles were present in the cytoplasm of the duodenal epithelium. As in the duodenum, no vacuoles were seen in the cytoplasm of intestinal villus epithelial cells in the jejunum to 18 hours PI. In the control puppies at 18 and 24 hours PI, the majority of the villus epithelial cells in the jejunum had small cytoplasmic vacuoles. From 48 hours PI throughout the experiment, large, clear vacuoles in the cytoplasm of the villus epithelium and in the villus lamina propria of the control puppies' jejunum were observed (Fig 3). In the intestinal villi of the ileum in control puppies, large, clear cytoplasmic vacuoles in the villus epithelium and villus lamina propria were present at 12 hours PI and persisted throughout the study (Fig 4). The villus lamina propria in all sections of the small intestine from control puppies contained a few mononuclear cells and eosinophils.
Fig 2—Duodenum from a control puppy killed at 12 hours PI. The villus epithelial cells are cuboidal to columnar with the nuclei of each cell in the apical, middle, or basal position of the cell. H&E stain; X 1000.
Fig 3—Jejunum from a control puppy killed at 48 hours PI. Large, clear cytoplasmic vacuoles are present in the villus epithelial cells and lamina propria. H&E stain; X 575.
Fig 4—Ileum from a control puppy killed at 12 hours PI. Large, clear cytoplasmic vacuoles are present in the villus epithelial cells and lamina propria. H&E stain; X 1000.
There was no loss of epithelial cells covering the intestinal villi. No morphological changes were observed in the colon, mesenteric lymph nodes, or other organs.

The microscopic lesions in the infected puppies were limited to the jejunum and ileum. The nature of the lesion varied with time after inoculation. Puppies killed at 12 hours PI had columnar epithelial cells covering the villi in all sections of the small intestine and were indistinguishable from the 12 hour control puppy. In the jejunum (Fig 5) and ileum (Fig 6) of infected puppies killed at 18 to 48 hours PI, large numbers of columnar villus epithelial cells on the upper one-third of the villus had a ground-glass appearance of the cytoplasm and a pyknotic nucleus in the center of the cell. Some of the villi in the jejunum and ileum had small, focal, denuded areas on the upper portion of the villus (Fig 7). The infected puppies sacrificed from 24 to 72 hours PI had villi in the jejunum and ileum which were covered with cuboidal to flat squamous-like epithelial cells (Fig 8). These epithelial cells did not contain cytoplasmic vacuoles as did the jejunal and ileal villus epithelium in the control puppies. In addition, fewer and shorter villi were observed in the infected puppies (Fig 9) than in the control puppies. The central lacteals in the lamina propria were slightly to moderately dilated (Fig 5 and 9). Sections of the jejunum and ileum from puppies killed 72 to 154 hours PI had slight to moderate villus atrophy and the villi were covered with cuboidal to low columnar epithelium (Fig 10). A
Fig 5—Jejunum from an infected puppy killed at 48 hours PI. Numerous villus epithelial cells have a ground-glass appearance of the cytoplasm and a pyknotic nucleus in the center of the cell. The central lacteal is moderately dilated. H&E stain; X 1000.
Fig 6—Ileum from an infected puppy killed at 48 hours PI. The majority of the villus epithelial cells have a ground-glass appearance of the cytoplasm and pyknotic nucleus. H&E stain; X 1000.
Fig 7—Ileum from an infected puppy killed at 48 hours PI. A denuded area is present on the upper portion of the intestinal villus. H&E stain; X 1000.
Fig 8—Ileum from an infected puppy killed at 24 hours PI. The intestinal villi are covered with flattened to cuboidal epithelial cells and no cytoplasmic vacuoles are present. H&E stain; X 875.
Fig 9—Ileum from an infected puppy killed at 72 hours PI. The villi are shorter, reduced in number and there is moderate dilation of the central lacteals. H&E stain; X 225.
Fig 10—Ileum from an infected puppy killed at 132 hours PI. The villi are covered with cuboidal to low columnar epithelial cells and there is marked absence of cytoplasmic vacuoles. H&E stains; X 850.
marked absence of the large clear vacuoles in the jejunal and ileal villus epithelial cells was still evident in the infected puppies when compared with the control puppies. At 18 to 48 hours PI, a slight increase in the number of neutrophils and mononuclear cells was present in the lamina propria of the infected puppies' jejunum and ileum. However, from 72 to 154 hours PI, no neutrophils were observed in the lamina propria, but a slight increase in the number of mononuclear cells was still evident in the infected puppies when compared with time-matched control puppies.

Small to moderate numbers of clear vacuoles were present in the lamina propria of the duodenum, jejunum, and ileum of the infected puppies and did not differ from the appearance of the small intestine of the age-matched control puppies.

No changes were observed by light microscopy in the other organs.

**Intestinal Morphometry**—The means of the villus (V) lengths, crypt (C) depths, and villus to crypt (V:C) ratios from the sections of duodenum, jejunum, and ileum from rotavirus infected and control puppies have been listed in Tables 1, 2, and 3, respectively.

In the duodenum, there was no significant (P>0.05) difference in the mean villus lengths between the infected and control puppies (Fig 11). The mean crypt depths in the duodenum were significantly (P<0.05) different between the two groups. An increase in the mean crypt depths occurred by
TABLE 1
Mean Duodenal Villus Lengths, Crypt Depths, and Villus to Crypt Ratios in Rotavirus Infected and Control Gnotobiotic Puppies

<table>
<thead>
<tr>
<th>HOURS POST-PUPPY INOCULATION</th>
<th>VILLUS(V) LENGTH(um)</th>
<th>CRYPT(C) DEPTH(um)</th>
<th>V:C RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROTAVIRUS INFECTED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>12</td>
<td>620.0±37.4*</td>
<td>138.6±25.0</td>
</tr>
<tr>
<td>1b</td>
<td>12</td>
<td>1180.0±58.3</td>
<td>98.1±01.8</td>
</tr>
<tr>
<td>2I</td>
<td>18</td>
<td>860.0±24.4</td>
<td>155.2±27.6</td>
</tr>
<tr>
<td>3I</td>
<td>24</td>
<td>820.0±37.4</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>4Ia</td>
<td>48</td>
<td>620.0±48.9</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>4Ib</td>
<td>48</td>
<td>1000.0±00.0</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>5Ia</td>
<td>72</td>
<td>600.0±44.7</td>
<td>180.0±20.0</td>
</tr>
<tr>
<td>5Ib</td>
<td>72</td>
<td>620.0±66.3</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>6I</td>
<td>96</td>
<td>640.0±40.0</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>7I</td>
<td>132</td>
<td>640.0±60.0</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>8I</td>
<td>154</td>
<td>540.0±24.4</td>
<td>180.0±20.0</td>
</tr>
<tr>
<td>CONTROLS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1C</td>
<td>12</td>
<td>620.0±20.0</td>
<td>91.0±04.2</td>
</tr>
<tr>
<td>2C</td>
<td>18</td>
<td>666.7±68.8</td>
<td>79.3±07.9</td>
</tr>
<tr>
<td>3C</td>
<td>24</td>
<td>1020.0±58.3</td>
<td>140.0±24.4</td>
</tr>
<tr>
<td>4Ca</td>
<td>48</td>
<td>500.0±63.2</td>
<td>74.7±07.4</td>
</tr>
<tr>
<td>4Cb</td>
<td>48</td>
<td>1020.0±73.4</td>
<td>120.0±20.0</td>
</tr>
<tr>
<td>5C</td>
<td>72</td>
<td>1020.0±58.3</td>
<td>139.1±24.8</td>
</tr>
<tr>
<td>6C</td>
<td>96</td>
<td>760.0±50.9</td>
<td>117.3±20.8</td>
</tr>
<tr>
<td>7C</td>
<td>132</td>
<td>620.0±20.0</td>
<td>86.8±07.7</td>
</tr>
<tr>
<td>8C</td>
<td>154</td>
<td>760.0±74.8</td>
<td>110.0±22.8</td>
</tr>
</tbody>
</table>

*Std. Error.
TABLE 2
Mean Jejunal Villus Lengths, Crypt Depths, and Villus to Crypt Ratios in Rotavirus Infected and Control Gnotobiotic Puppies

<p>| HOURS POST- | VILLUS(V)   | CRYPT(C)   | V:C |</p>
<table>
<thead>
<tr>
<th>PUPPY INOCULATION</th>
<th>LENGTH(um)</th>
<th>DEPTH(um)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ROTAVIRUS INFECTED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Ia 12</td>
<td>640.0±24.4*</td>
<td>97.4±02.6</td>
<td>6.6±0.4</td>
</tr>
<tr>
<td>1Ib 12</td>
<td>800.0±31.6</td>
<td>94.6±03.4</td>
<td>8.5±0.4</td>
</tr>
<tr>
<td>2I 18</td>
<td>420.0±20.0</td>
<td>97.2±01.7</td>
<td>4.3±0.2</td>
</tr>
<tr>
<td>3I 24</td>
<td>500.0±31.6</td>
<td>116.2±21.2</td>
<td>4.6±0.5</td>
</tr>
<tr>
<td>4Ia 48</td>
<td>480.0±20.0</td>
<td>129.9±28.9</td>
<td>4.5±0.9</td>
</tr>
<tr>
<td>4Ib 48</td>
<td>400.0±31.6</td>
<td>106.0±24.4</td>
<td>2.8±0.5</td>
</tr>
<tr>
<td>5Ia 72</td>
<td>380.0±37.4</td>
<td>118.4±20.4</td>
<td>3.5±0.6</td>
</tr>
<tr>
<td>5Ib 72</td>
<td>640.9±50.9</td>
<td>160.0±24.4</td>
<td>4.4±0.6</td>
</tr>
<tr>
<td>6I 96</td>
<td>480.0±20.0</td>
<td>260.0±24.4</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>7I 132</td>
<td>520.0±37.4</td>
<td>180.0±20.0</td>
<td>3.2±0.7</td>
</tr>
<tr>
<td>8I 154</td>
<td>480.0±20.0</td>
<td>157.2±26.3</td>
<td>3.5±0.7</td>
</tr>
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<td>CONTROLS</td>
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<tr>
<td>1C 12</td>
<td>620.0±37.4</td>
<td>86.0±05.5</td>
<td>7.2±0.3</td>
</tr>
<tr>
<td>2C 18</td>
<td>600.0±59.3</td>
<td>80.0±07.9</td>
<td>6.3±0.6</td>
</tr>
<tr>
<td>3C 24</td>
<td>900.0±77.4</td>
<td>139.7±24.6</td>
<td>7.0±1.0</td>
</tr>
<tr>
<td>4Ca 48</td>
<td>680.0±37.4</td>
<td>87.3±07.9</td>
<td>8.0±0.9</td>
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<tr>
<td>4Cb 48</td>
<td>1140.0±60.0</td>
<td>98.5±01.5</td>
<td>11.5±0.5</td>
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<td>5C 72</td>
<td>960.0±24.4</td>
<td>119.7±20.0</td>
<td>8.6±0.9</td>
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<td>6C 96</td>
<td>760.0±50.9</td>
<td>94.9±05.0</td>
<td>8.0±0.5</td>
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<td>7C 132</td>
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<td>8C 154</td>
<td>740.0±67.8</td>
<td>93.2±05.4</td>
<td>7.9±0.5</td>
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</table>

*Std. Error.
<p>| HOURS POST- | VILLUS(V) | CRYPT(C) | V:C |</p>
<table>
<thead>
<tr>
<th>INOCULATION</th>
<th>LENGTH(um)</th>
<th>DEPTH(um)</th>
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<td>1Ia</td>
<td>12</td>
<td>680.0±20.0*</td>
<td>76.4±05.8</td>
</tr>
<tr>
<td>1Ib</td>
<td>12</td>
<td>900.0±83.6</td>
<td>88.3±04.8</td>
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<td>2I</td>
<td>18</td>
<td>340.0±24.4</td>
<td>65.8±07.8</td>
</tr>
<tr>
<td>3I</td>
<td>24</td>
<td>460.0±50.9</td>
<td>140.0±40.0</td>
</tr>
<tr>
<td>4Ia</td>
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<td>440.0±40.0</td>
<td>115.3±21.6</td>
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<td>4Ib</td>
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<td>580.0±20.0</td>
<td>140.0±24.4</td>
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<td>5Ia</td>
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<td>260.0±40.0</td>
<td>73.6±06.5</td>
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<tr>
<td>5Ib</td>
<td>72</td>
<td>760.0±40.0</td>
<td>200.0±00.0</td>
</tr>
<tr>
<td>6I</td>
<td>96</td>
<td>320.0±48.9</td>
<td>179.1±20.8</td>
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<td>7I</td>
<td>132</td>
<td>400.0±31.6</td>
<td>84.6±06.9</td>
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<td>8I</td>
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<td>3C</td>
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<td>91.7±05.2</td>
</tr>
<tr>
<td>4Ca</td>
<td>48</td>
<td>440.0±24.4</td>
<td>61.8±06.7</td>
</tr>
<tr>
<td>4Cb</td>
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<td>6C</td>
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<td>8C</td>
<td>154</td>
<td>700.0±83.6</td>
<td>61.8±06.8</td>
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</table>

*Std. Error.*
Fig 11—Mean villus lengths (um) in the duodenum of control (---) and infected (----) puppies vs hours PI. There is no significant (P>0.05) difference between the mean villus lengths in the duodenum.
24 hours PI in the infected puppies and was present throughout (Fig 12). The mean V:C ratios in the duodenum were significantly (P<0.05) lower in the infected puppies (Fig 13).

In sections of jejunum from infected puppies, the mean villus lengths were significantly (P<0.05) shorter than in control puppies. The difference in the mean villus lengths was distinct at 24 hours PI (Fig 14). Analysis of the mean crypt depths in the jejunum demonstrated a significant (P<0.05) difference between the infected and control puppies. A distinct difference in the mean crypt depths was observed at 48 hours PI with an increase in the mean crypt depths in the infected puppies (Fig 15). The mean V:C ratios in the jejunum of the infected puppies were significantly (P<0.05) lower than the control puppies' jejunal sections (Fig 16).

Mean villus lengths in the ileum of infected and control puppies differed significantly (P<0.05) and was apparent at 18 hours PI, although not always evident (Fig 17). The mean crypt depths in the ileum of infected puppies were significantly (P<0.05) deeper than the mean crypt depths in the control puppies' ileal segments (Fig 18). The mean V:C ratios in the ileum differed significantly (P<0.05) between the infected and control puppies and was evident by 18 to 24 hours PI (Fig 19).

Discussion

Gross and microscopic lesions in newborn, gnotobiotic
Fig 12—Mean crypt depths (μm) in the duodenum of control (---) and infected (-----) puppies vs. hours PI. A significant (P<0.05) increase in the mean crypt depths of infected puppies is apparent by 24 hours PI.
Fig 13—Mean V:C ratios in the duodenum of control (---) and infected (-----) puppies vs hours PI. The mean V:C ratios in the duodenum of the infected puppies are significantly (P<0.05) lower than in the control puppies.
Fig 14—Mean villus lengths (μm) in the jejunum of control (---) and infected (-----) puppies vs. hours PI. Mean villus lengths are significantly (P<0.05) shorter in the infected puppies, appearing distinct by 24 hours PI.
Fig 15—Mean crypt depths (μm) in the jejunum of control (---) and infected (——) puppies vs. hours PI. A significant (P<0.05) increase in the mean crypt depths of the infected puppies was observed at 48 hours PI.
Fig 16—Mean V:C ratios in the jejunum of control (---) and infected (----) puppies vs. hours PI. The mean V:C ratios in the infected puppies are significantly (P<0.05) lower than in the control puppies.
Fig 17—Mean villus lengths (μm) in the ileum of control (---) and infected (——) puppies vs. hours PI. Significantly (P<0.05) shorter mean villus lengths in the infected puppies are evident by 18 hours PI.
Fig 18—Mean crypt depths (um) in the ileum of control (---) and infected (-----) puppies vs. hours PI. The mean crypt depths in the infected puppies are significantly (P<0.05) deeper than in control puppies.
Fig 19—Mean V:C ratios in the ileum of control (---) and infected (-----) puppies vs. hours PI. Significantly (P<0.05) lower V:C ratios were apparent by 18 to 24 hours PI in the infected puppies.
puppies infected with a canine rotavirus were confined to the small intestine. In addition, the onset of diarrhea, at 20 to 24 hours PI as previously described, correlated well with the time at which gross and histopathologic changes were observed. Gross lesions in the small intestine of dogs experimentally infected with rotavirus have not been reported, however, the gross lesions in the jejunum and ileum in this study are similar to the gross changes in experimentally inoculated gnotobiotic piglets and gnotobiotic calves.

The histopathological changes in the jejunum and ileum over the post-inoculation observation period in this study closely paralleled pathogenetic studies of rotaviral enteritis in gnotobiotic pigs, gnotobiotic calves, and suckling mice. The early lesions seen in the jejunum and ileum of the infected puppies in this study were characterized by moderate hydropic degeneration and necrosis of absorptive villus epithelial cells with denuded foci seen on the upper one-third of the intestinal villi. These changes represented the destruction of the mature villus epithelial cells as a result of rotavirus infection.

A second prominent early morphological change observed in the infected puppy intestine was slight to moderate villus atrophy with the villi covered by cuboidal and flattened epithelial cells. In addition, there was a marked absence of the large, clear, cytoplasmic vacuoles seen in the absorptive villus epithelium of the control puppies. The histological
histological changes that were present in the later stage of infection were slight to moderate villus atrophy with the villi covered by cuboidal to low columnar epithelial cells lacking the cytoplasmic vacuoles as seen in the intestinal epithelium of the control puppies. These morphological changes were interpreted to represent the replacement of desquamated mature villus epithelial cells by undifferentiated, cuboidal crypt epithelial cells as reported for rotaviral enteritis in other species\textsuperscript{51,52,54,109} and as established for rotaviral infection of lambs by measurement of crypt cell production rates.\textsuperscript{69}

The mucosal measurements of the jejunum and ileum provided data supporting the proposed pathogenesis of rotaviral enteritis in other species.\textsuperscript{51-53,69,109} The villus lengths of the jejunum and ileum in the infected puppies were decreased significantly from the control puppies. This change was distinct at 24 to 48 hours PI. There was a substantial increase in the depths of the crypts of the infected puppies observed at 48 hours PI when compared with control puppies. These changes in mucosal morphometry correlated well with the times at which villus atrophy and immature villus epithelial cells were observed in the rotavirus infected jejunum and ileum. This observation suggested that the increased depth of the intestinal crypts associated with villus atrophy represented increased numbers of immature epithelial cells in the crypts to replace the damaged or desquamated mature villus epithelial cells.
The distinct differences in the mean V:C ratios in the duodenum between the infected and control puppies was due to an increase in the mean crypt depths of the infected puppies with the mean lengths of the villi remaining approximately the same as in the control puppies. An explanation of this finding may be related to the infection of duodenal villus epithelial cells as reported in the indirect immunofluorescence study in Chapter II. Infection of these epithelial cells by the rotavirus was slight, but apparently did initiate crypt cell hyperplasia.

The marked dehydration and moribund condition of puppy 81 at 154 hours PI was unexpected. However, the comparison of microscopic sections of the small intestine from this puppy with those in the age-matched control puppy suggested that the epithelium covering the villi in the infected puppy was immature at this time. This would have accounted for the persistent diarrhea, and possibly malabsorption and/or hypersecretion with subsequent severe dehydration causing the moribund condition.

In animals, morphological changes in the small intestine similar to those changes described in this study have caused impairment of the digestive and absorptive processes that normally occur at the luminal surface of the intestinal villi. Consequently, an acute malabsorptive diarrhea persists until the villi are again covered by mature, absorptive villus epithelial cells.

The oral inoculation of neonatal, gnotobiotic puppies
with canine rotavirus isolate LSU 79C-36 resulted in slight to moderate enteritis. The gross and microscopic lesions were similar to enteric rotavirus infections in other species.\textsuperscript{25,51-55,61,109} Therefore, the dog could serve as an experimental model for the study of pathophysiologic mechanisms of rotaviral enteritis in other species.
A Scanning and Transmission Electron Microscopic Study
Of Rotavirus Induced Intestinal Lesions
In Neonatal Gnotobiotic Dogs

Introduction

The ultrastructural changes observed by scanning and transmission electron microscopy in rotavirus infected small intestines have been well characterized in gnotobiotic piglets,\(^54,55,111\) gnotobiotic calves,\(^21,107\) and suckling mice.\(^25,26,108\) Scanning electron microscopic (SEM) observations have shown swelling and loss of absorptive villus epithelial cells from the upper one-half of intestinal villi, denuded villi, villus atrophy, and covering of the denuded villi by epithelium in rotavirus infected gnotobiotic piglets,\(^54,55\) gnotobiotic calves,\(^107\) and suckling mice.\(^108\) Transmission electron microscopic (TEM) studies in these same species infected with rotavirus have demonstrated various replicative stages of the rotavirus within the villus epithelial cells, necrosis of villus epithelial cells, and the presence of cuboidal epithelial cells on the intestinal villi with similar ultrastructural morphology as the immature crypt epithelium.\(^21,25,26,111\)

The purpose of this study was to examine the SEM and TEM changes in the small intestine of newborn gnotobiotic puppies infected with a canine rotavirus isolated from a neonatal...
puppy with fatal diarrhea.48

Materials and Methods

Experimental Design—A total of twenty gnotobiotic puppies were derived by caesarean section from three mongrel bitches as previously described in Chapter II. Eleven two-day-old puppies were inoculated orally with 1 ml of a cell culture suspension containing the 16th and 17th passage of a canine rotavirus isolate.48 The virus titer was $10^5$ TCID50/ml. Nine control gnotobiotic puppies were not inoculated.

At least one inoculated and one control puppy were anesthetized at 12, 18, 24, 48, 72, 96, 132, and 154 hours post-inoculation (PI). Samples were removed from three sites on the small intestine and one site on the colon and immediately immersed in a 1.25% glutaraldehyde and 2% formaldehyde solution with 0.1M sodium cacodylate buffer (pH 7.4) for scanning and transmission electron microscopy. The puppies were subsequently sacrificed and a complete necropsy was performed as previously described in Chapter III.

Scanning Electron Microscopy (SEM)—Small (0.5 cm$^2$) portions of tissue were cut from the samples of gut previously fixed in glutaraldehyde-formaldehyde fixative. These specimens were fixed for 1.5 hours, then rinsed twice with cold 0.1M cacodylate buffer in 5% sucrose (pH 7.4) and stored in this buffer at 4°C. Glutaraldehyde, tannic acid, and osmium tetraoxide ($\text{Os}_2\text{O}_4$) were used to post-fix the
tissue samples using a method previously described. Briefly, the process involved post-fixing the tissue in a 3% glutaraldehyde-tannic acid-osmium solution in 0.1M sodium cacodylate buffer (pH 7.4) for 30 minutes. After 3 washes in buffered sucrose, the samples are placed in 1% O₅0₄ with sodium cacodylate buffer (pH 7.4) for 1 hour. Then washed in distilled water, and put in 5% tannic acid prepared with distilled water for 1 hour. Following this step, the samples were again washed in distilled water, then placed in 1% O₅0₄ prepared with distilled water for one hour. The specimens were then washed in distilled water and dehydrated through one change each of 50%, 70%, 80%, 90%, and 95% ethanol for 10 minute intervals. The tissues went through 3 changes of 100% ethanol at 10 minute intervals. The dehydrated specimens were placed in a critical point drying apparatus and dried from liquid carbon dioxide. Dried samples were attached with silver conducting paint to 12 mm aluminum SEM studs. The specimens were then sputter-coated with gold palladium alloy. Processed tissue samples were examined in a scanning electron microscope operated at an accelerating voltage of 20 KV and a working distance of 12-15 mm. Images were recorded on film with a scan of 2000 lines/frame.

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bLadd Research industries, Inc., Burlington, Vermont.
cHummer V®, Technics Corp., San Jose, Calif.
ePolaroid®, 4x5 Land Film, Polaroid Corp., Cambridge, Mass.
Transmission Electron Microscopy (TEM)—Small (1 mm$^3$) pieces of tissue were minced from the samples of gut. These specimens were fixed for 1.5 hours, then rinsed twice with cold 0.1M sodium cacodylate buffer in 5% sucrose (pH 7.4) and stored in this buffer at 4°C. The tissue samples were post-fixed in 1% OsO$_4$ in sodium cacodylate buffer (pH 7.4) for 1 hour, washed 3 times, 5 minutes each wash, in 5% buffered sucrose (pH 7.4). Then placed in 1% tannic acid for 1 hour, washed 3 times, 5 minutes each wash, in distilled water. The specimens were then dehydrated in graded concentrations of ethanol and embedded in an epon-araldite mixture. Sections were cut with a diamond knife on an ultramicrotome$^f$, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope$^g$.

Results

SEM—In all the sections of the small intestine from the control puppies, the majority of the villi were long and slender with numerous transverse grooves on the villus surfaces. The epithelial surface appeared smooth without distinct cell borders except on the tips of the villi (Fig 1 and 2).

The colon had a flattened, undulating surface with numerous goblet cells and the majority of the mucosal surface

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$^f$Sorval MT-2B, Dupont Company, Newton, Conn.
$^g$Zeiss EM-10, Carl Zeiss, Inc. New York, NY.
Fig 1—A scanning electron micrograph (SEM) of the duodenum from a control puppy killed at 12 hours PI. The majority of the intestinal villi are long and slender with numerous transverse grooves. X 125.

Fig 2—A higher magnification SEM of Fig 1 showing the uneven surface on the tips of some of the intestinal villi. X 332.
was covered by a thick layer of mucus.

Differences in the appearance of the small intestines between the control puppies and infected puppies were found only in the jejunum and ileum. At 12 and 18 hours PI, numerous single and small clusters of rounded epithelial cells were on the upper one-third of several intestinal villi and protruded from the surface (Fig 3 and 4). A few focal sites on the villi were denude of absorptive intestinal epithelium (Fig 4). By 24 and 48 hours PI, mild to moderate villus atrophy and broadened intestinal villi were seen in the jejunum (Fig 5). Some villi appear fused with only a few rounded epithelial cells and denuded areas observed (Fig 6). In the ileum, moderate villus atrophy, numerous broadened, and several fused villi were present (Fig 7). A few sites denude of epithelium and small numbers of protruding intestinal epithelial cells were also seen.

The jejunum from infected puppies killed from 72 to 154 hours PI had long, slender villi and did not appear different from the sections of control puppies sacrificed at these times (Fig 8). However, at 72 and 96 hours PI, mild villus atrophy was still evident in the ileum of the infected puppies (Fig 9). In addition, the epithelial surface of the intestinal villi appeared roughened and irregular (Fig 10). No differences were observed in the ileum between infected and control puppies at 132 and 154 hours PI.

**TEM**—In the small intestine of the uninfected puppies, the ultrastructural morphology of the villus epithelial cells
Fig 3—A SEM of the jejunum from an infected puppy killed at 12 hours PI. Numerous clumps of villus epithelial cells protrude from the upper portion of villi. X 114.

Fig 4—A higher magnification SEM of Fig 3 showing the clumps of rounded villus epithelial cells and an area denude of epithelial cells (arrows) on the villi. X 450.
Fig 5—A SEM of the jejunum in an infected puppy killed at 24 hours PI. Short and broadened intestinal villi are present. X 122.

Fig 6—A higher magnification SEM of Fig 5 showing short and fused (arrow) intestinal villi. Only a few epithelial cells protrude from the surface of the villi. X 227.
Fig 7—A SEM of the ileum from an infected puppy killed at 24 hours PI. The majority of the villi are short and broad, several appear fused together (arrows), epithelial cells protrude from the surface of the villi, and an area denude of villus epithelial cells is present (arrowhead). X 124.
Fig 8—A SEM of the jejeunum from an infected puppy killed at 132 hours PI. The intestinal villi are long and slender, appearing similar to jejunal sections from the time-matched control puppy. X 125.
Fig 9—A SEM of the ileum from an infected puppy killed at 72 hours PI. Villus atrophy is still evident. X 163.

Fig 10—A higher magnification SEM of Fig 9 illustrating the uneven epithelial surface of the intestinal villi. X 585.
in the duodenum did not change throughout the experiment. These cells were tall and columnar with many, long, uniform, regularly spaced microvilli, and a well-developed terminal web (Fig 11). Mitochondria were numerous and the majority were oriented in a plane perpendicular to the lumen. The nucleus was located at the base of the cell. A moderate amount of rough endoplasmic reticulum (RER) and numerous polyribosomes were present in the cytoplasm. There were numerous small to moderate-sized aggregates of electron-dense material in cisternae of vacuolated endoplasmic reticulum.

The morphology of the jejunal villus epithelial cells in the control puppies was similar to the duodenal epithelium through 18 hours PI. However, at 24 through 154 hours PI, the villus epithelium in the jejunum contained a single large or multiple moderate-sized, membrane-bound, supranuclear, cytoplasmic vacuoles (Fig 12). The majority of the vacuoles had a large electron-lucent space containing variable quantities of irregular-shaped, homogenous electron-dense material which was membrane-bound. In addition, small to moderate-sized lamellate bodies were present within the vacuoles. Numerous invaginations of the cell membrane on the luminal surface were observed. Several pinocytotic vesicles could be seen beneath the terminal web. There were dilatations of the lateral interdigital spaces which contained variable numbers of homogenous, electron-dense globules. The ultrastructural morphology of villus epithelial cells in the ileum of the control puppies was
Fig 11—A transmission electron micrograph (TEM) of villus epithelial cells in the duodenum of a control puppy killed at 12 hours PI. These are columnar cells with long, uniform microvilli, and a well-developed terminal web (1). There are numerous mitochondria, polyribosomes (arrow), aggregates of electron-dense material in cisternae of endoplasmic reticulum (double arrow), a moderate amount of rough endoplasmic reticulum (arrowhead), and electron-dense globules within intercellular spaces (2). Uranyl acetate and lead citrate stain; X 12,250.
Fig 12—A TEM of jejunal epithelial cells from a control puppy killed at 24 hours PI. Supranuclear, membrane-bound, cytoplasmic vacuoles contain variable quantities of electron-dense material and lamellate bodies (arrow). Uranyl acetate and lead citrate stain; X 12,250.
similar to the jejunum, except the supranuclear vacuoles were present at 12 hours PI and persisted through 154 hours PI. The epithelial cells of the crypts at the base of the jejunal and ileal mucosa were cuboidal to columnar with a reduced number of shorter microvilli than absorptive villus epithelial cells (Fig 13). In addition, no large, clear, membrane-bound vacuoles were present in the crypt epithelium and there appeared to be reduced numbers of mitochondria. Polyribosomes were abundant in the cytoplasm of the crypt cells.

The villus epithelial cells in the colon of control puppies were cuboidal to columnar with sparse, short, irregularly-spaced microvilli on the surface (Fig 14). The nuclei were located at the base of the cell, numerous polyribosomes were present throughout the cytoplasm, and the quantity of RER and the numbers of mitochondria appeared to be reduced when compared to the villus epithelium of the small intestine. A few of the villus epithelial cells in the colon contained moderate-sized, clear, membrane-bound cytoplasmic vacuoles. Numerous goblet cells were observed throughout the colonic mucosa.

In the infected puppies, ultrastructural changes were limited to the absorptive villus epithelial cells of the jejunum and ileum. The changes in the epithelium of the jejunum and ileum were quite similar. At 12 hours PI, a few villus epithelial cells had a less electron-dense cytoplasm than the majority of the others and virus particles could
Fig 13--A TEM of crypt epithelial cells in the ileum of a control puppy killed at 132 hours PI. The crypt epithelial cells are cuboidal to columnar with a reduced number of short microvilli when compared with absorptive villus epithelial cells. No large supranuclear, cytoplasmic vacuoles are seen and there are less numbers of mitochondria. Uranyl acetate and lead citrate stain; X 9300.
Fig 14—A TEM of villus epithelial and globet cells in the colon of a control puppy killed at 132 hours PI. Sparse, irregularly-spaced microvilli and less numbers of mitochondria are seen. Uranyl acetate and lead citrate stain; X 11,270.
Fig 15---A TEM of jejunal epithelial cells from an infected puppy killed at 12 hours PI. The infected cell (a) has a less electron-dense cytoplasm than the adjacent cell (b) in which no rotavirus particles were seen. A cluster of rotavirus particles are seen in cell a. Uranyl acetate and lead citrate stain; X 28,950.
usually be observed in these cells (Fig 15). In these cells, virus particles had a centrally-located, round, electron dense core about 25-30 nm in diameter, surrounded by one or two membranes. The virus particles were located in dilated cisternae of RER (Fig 16). At 12 and 18 hours PI, virions were readily observed on the luminal surface of villus epithelial cells in the jejunum and ileum (Fig 17). Some of the virions appeared to be associated with intracytoplasmic vacuoles in the region of the apical tubules (Fig 18).

From 18 through 48 hours PI, moderate numbers of necrotic villus epithelial cells were readily observed in the jejunum and ileum. Often, 2-4 necrotic cells were present at the same site and the changes in the ultrastructure of the cells were characterized by loss of microvilli, swollen mitochondria, a moderate amount of membrane-bound, lipid-like material in the cytoplasm, clumped chromatin around the periphery of the nucleus, indistinguishable cell boundaries between adjacent degenerative cells, and disruption of the cytoplasmic membrane with loss of cellular contents into the lumen (Fig 19). Also, from 18 to 48 hours PI, rotavirus-like particles were observed in dilated cisternae of RER and some of the virions appeared to be budding through the membrane into the dilated cistern (Fig 20). Two sizes of rotavirus-like particles were observed in these cisternae of RER, one approximately 75 to 80 nm in diameter, and the second, about 55 to 65 nm in diameter. In addition, small to moderate-sized, membrane-bound spaces in the cytoplasm were
Fig 16—A higher magnification TEM of Fig 15 showing the rotavirus particles about 60 nm in diameter in dilated cisternae of rough endoplasmic reticulum. The virus particles have an electron-dense core about 30 nm in diameter surrounded by one or two membranes (arrows). Uranyl acetate and lead citrate stain; X 113,400.
Fig 17—A TEM showing the surface of a villus epithelial cell in the ileum from an infected puppy killed at 18 hours PI. Two rotavirus particles (arrows) are on the luminal surface. Uranyl acetate and lead citrate stain; X 150,000.
Fig 18—A TEM of an ileal villus epithelial cell from an infected puppy killed at 18 hours PI. A rotavirus particle is associated with an intracytoplasmic vacuole. Uranyl acetate and lead citrate stain; X 125,000.
Fig 19—A TEM of the villus epithelial cells in the ileum from an infected puppy killed at 18 hours PI. Two degenerating villus epithelial cells (a & b) are present. A goblet cell (c) is also seen. Swollen mitochondria (arrows), lipid-like material (arrowheads), loss of the tight cellular junctions between adjacent degenerating epithelial cells (double arrows), and loss of cellular contents from cell (b) into the lumen are observed. An aggregate of rotavirus particles are present in cell a (v). Uranyl acetate and lead citrate stain; X 17,150.
Fig 20--A TEM of an ileal villus epithelial cell from an infected puppy killed at 48 hours PI. A rotavirus particle appears to be budding (arrows) into the dilated cistern of rough endoplasmic reticulum. Two sizes of rotavirus particles are present in the dilated spaces, one, 75-80 nm in diameter (arrowhead), the other 55-65 nm in diameter (double arrowheads). Swollen mitochondria are present (m). Uranyl acetate and lead citrate stain; X 56,000.
composed of varying quantities of a fine granular material and round, electron-dense particles approximately 25-30nm in diameter (Fig 21).

Large clear, membrane-bound cytoplasmic vacuoles similar to the vacuoles seen in the jejunal and ileal villus epithelial cells of the control puppies were observed in the jejunal and ileal villus epithelial cells of infected puppies from 12 to 18 hours PI. These cytoplasmic vacuoles were not seen in the villus epithelium of the infected puppies from 24 through 154 hours PI.

In the jejunum and ileum at 72 to 154 hours PI, there appeared to be fewer necrotic villus epithelial cells and fewer cells containing virus particles. The ultrastructural morphology consisted of cuboidal to columnar cells with tall, regularly-spaced microvilli, and a nucleus located at the base of the cell (Fig 22). There appeared to be fewer mitochondria and less RER than in the time-matched control puppies' jejunal and ileal villus epithelium.

Discussion

The ultrastructural changes, observed by SEM and TEM, in the small intestines of newborn gnotobiotic puppies demonstrated that the rotavirus infected the villus epithelial cells with subsequent propagation of the rotavirus and destruction of villus epithelial cells. These changes were similar to ultrastructural changes seen in gnotobiotic pigs,54,55,111 gnotobiotic calves,21,107 and suckling
Fig 21—A TEM of a jejunal villus epithelial cell from an infected puppy killed at 24 hours PI. Fine granular material (g) and numerous, small, round, electron-dense particles about 25-30 nm in diameter (arrows) are encompassed by a membrane within the cytoplasm. The nucleus (n) of the cell is located in the upper 1/3 of the photograph. Uranyl acetate and lead citrate stain; X 60,000.
Fig 22—A TEM of villus epithelial cells in the ileum from an infected puppy killed at 154 hours PI. The villus epithelial cells are cuboidal with long, uniform microvilli and several mitochondria in the apical position of the cell. There are no large, membrane-bound cytoplasmic vacuoles. Uranyl acetate and lead citrate stain; X 14,700.
mice\textsuperscript{25,26,108} which were each inoculated with rotaviruses isolated from their own species.

The early SEM observations in this study consisted of swollen villus epithelial cells, denuded foci on intestinal villi, and slight to moderate villus atrophy. Later changes were slight villus atrophy with no denuded intestinal villi. These changes closely paralleled the SEM observations in rotavirus infected gnotobiotic pigs\textsuperscript{54,55} Though the same sequence of lesions were observed by SEM in the small intestine of rotavirus infected gnotobiotic calves\textsuperscript{107} and suckling mice,\textsuperscript{108} the initial lesions were not seen until 20 to 40 hours PI. The changes in the infected puppies' intestine suggested that in the initial stages of infection, the rotavirus did infect villus epithelial cells on the upper portion of the intestinal villi with subsequent cell swelling, degeneration of infected epithelial cells, and detachment of these epithelial cells from the villus. The partially denuded intestinal villi contracted resulting in villus atrophy. Then in the later stages of the infection, the denuded intestinal villi were covered by epithelial cells and the length of the villi increased to near the same length as intestinal villi in the control puppies.

The TEM observations in this study have shown that the canine rotavirus replicated within villus epithelial cells and provided an insight into the pathogenesis of rotaviral enteritis in the neonatal dog.

The morphogenesis of the canine rotavirus isolate used
to inoculate newborn gnotobiotic puppies in this study was similar as reported for gnotobiotic piglets,\textsuperscript{55,111} gnotobiotic calves,\textsuperscript{21} and suckling mice.\textsuperscript{25,26,108}

The process by which the rotavirus enters the absorptive villus epithelial cells has not been clearly demonstrated. In this study rotavirus particles were apparently associated with intracytoplasmic vacuoles near the terminal web and apical tubules of villus epithelial cells in thin sections of the jejunum and ileum at 18 hours PI. It could have been possible that the virus gained entry into these villus epithelial cells by way of the pinocytotic activity of the cell. The presence of variable sizes of rotavirus particles within dilated cisternae of RER seen in this study has been documented in experimental inoculations in gnotobiotic piglets,\textsuperscript{55,111} gnotobiotic calves,\textsuperscript{21} and suckling mice.\textsuperscript{25,26,108} In addition, the budding of virions through the RER into the dilated space has also been observed in these three species. This observation has been interpreted as part of the maturation process of the rotavirus and represents acquisition of the outer capsid layer.\textsuperscript{25} However, the presence of these smaller rotavirus particles within these cisternae of RER has raised doubt about this hypothesis. In discussing the presence of these smaller virus particles, one author suggested that these virus particles were degenerating particles or the developmental process may have been altered.\textsuperscript{25} The dilated spaces filled with a fine granular material and
small, electron-dense spheres seen within the cytoplasm of some of the villus epithelial cells in infected puppies was interpreted to represent the viroplasm and nucleocapsids reported in ultrastructural studies in pigs, calves, and mice.21,25,26,108,111

In this study, virus-associated single or double-membraned tubules were not observed in the nucleus or cytoplasm of the intestinal villus epithelial cells of the infected puppies. The presence of intranuclear and intracytoplasmic tubules has been reported and associated with the morphogenesis of rotavirus in these other studies. However, the presence of the developmental stages of the rotavirus within infected villus epithelium coupled with the shedding of rotavirus particles in the feces of the infected puppies, as previously reported in Chapter II, indicated that the canine rotavirus did infect and replicate in the villus epithelial cells of the inoculated puppies.

Necrosis of rotavirus infected villus epithelial cells was observed in the small intestine of the infected puppies. The majority of the necrotic villus epithelial cells were observed between 18 and 48 hours PI similar to reports in gnotobiotic piglets111 and suckling mice.26 At 24 through 154 hours PI, villus epithelial cells in the jejunum and ileum of rotavirus infected puppies lacked the large, clear, membrane-bound cytoplasmic vacuoles seen in the time-matched control puppies. A similar finding was reported in gnotobiotic calves.21 Since age-matched infected and
control puppies were used in this study, the absence of these cytoplasmic vacuoles in the infected puppies was interpreted as a pathophysiologic change. This finding may have represented a decreased absorptive activity of the jejunal and ileal villus epithelium in the infected puppies.

At 72 through 154 hours PI, many of the villus epithelial cells in the jejunum and ileum of the infected puppies were cuboidal to low columnar and had ultrastructural morphology similar to the crypt epithelium. Thus, evidence was provided to suggest that the damaged villus epithelium was replaced by the immature crypt epithelium as reported in other species infected with rotavirus.21,25,26,108,111

The ultrastructural changes of the intestinal villi and villus epithelial cells of the small intestine from rotavirus infected puppies correlated well with the changes described in gnotobiotic piglets, gnotobiotic calves, and suckling mice. These findings demonstrated that the pathogenesis of canine rotavirus infection in newborn gnotobiotic puppies is similar.
CHAPTER V

Summary and Conclusions

The canine rotavirus isolate, LSU 79C-36, caused clinical disease, small intestinal lesions, serum antibody response and shedding of rotavirus in the feces of orally inoculated two-day-old, gnotobiotic puppies. The results of this study demonstrated that this rotavirus isolate is a pathogen in neonatal gnotobiotic dogs. Also, the pathogenesis of the intestinal lesions in the rotavirus infected puppies was shown to be similar to the pathogenesis of rotaviral enteritis in other species.

The clinical sign of diarrhea beginning at 20 hours post-inoculation in the infected puppies correlated well with the presence of degenerating and necrotic absorptive villus epithelial cells observed at 18 to 48 hours post-inoculation using light microscopy, scanning electron microscopy and transmission electron microscopy. The lesions were limited to the jejunum and ileum. During this time, rotavirus and/or rotaviral antigen was observed by an indirect fluorescent antibody test in the villus epithelial cells covering the upper one-half of the intestinal villi. In addition, rotavirus and rotavirus precursor material was readily observed in jejunal and ileal villus epithelial cells 18 to 48 hours after inoculation. After 24 to 72 hours of infection, slight to moderate villus atrophy was observed by
light and scanning electron microscopy. Significant
differences in intestinal mucosal morphology between the
rotavirus infected and control puppies provided additional
evidence of villus atrophy in the infected puppies' small
intestine. After 24 hours post-inoculation, the flattened to
cuboidal villus epithelial cells, especially from 72 to 154
hours post-inoculation, which covered the intestinal villi in
the jejunum and ileum had ultrastructural characteristics
quite similar to the crypt epithelial cells. The replacement
of rotavirus damaged mature absorptive villus epithelial
cells by undifferentiated epithelial cells suggested that
normal intestinal absorptive functions were not completely
restored at the termination of the experiment. This
observation correlated with the persistent diarrhea
throughout the experiment and clinical signs of dehydration
in the infected puppies.

Serum rotavirus antibody was detected by an indirect
fluorescent antibody test in the infected puppies during the
later stages of the infection. Also, specific
immunofluorescence was observed within the cytoplasm of
mononuclear cells in the lamina propria of the intestinal
villi and mesenteric lymph nodes in the later stages of
infection. This suggested that rotavirus and/or rotavirus
antigen was phagocytosed by macrophages within the intestinal
villus, reaching the local lymph nodes by way of the
lymphatics where the viral antigen could be processed for
subsequent antibody production.
Evidence of rotavirus replication within the villus epithelial cells of infected puppies was supported by the transmission electron microscopic observation of rotavirus and rotaviral precursor material within epithelial cells. In addition, rotavirus particles were readily detected by negative contrast electron microscopy in the feces of the infected puppies throughout the experiment.

In this study, the pathogenesis of a canine rotavirus after oral inoculation in neonatal gnotobiotic puppies was characterized by the infection and destruction of significant numbers of mature villus absorptive epithelial cells. This resulted in villus atrophy and loss of normal intestinal absorptive functions. The necrotic villus epithelial cells were replaced by partially undifferentiated epithelial cells as were found in the intestinal crypts. The immature epithelial cells lacked absorptive capabilities normally present in mature villus epithelial cells. Subsequent malabsorption resulted in the clinical signs of diarrhea and dehydration.

Since the same pathogenetic pattern for rotaviral enteritis has been demonstrated in other species, the neonatal gnotobiotic dog would be an excellent animal model for pathophysiological and immunological studies of rotavirus infection in other species.
BIBLIOGRAPHY


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ACADEMIC HONORS:

Phi Eta Sigma, Texas A & M University, 1970.
Phi Zeta, Louisiana State University, 1981.

LICENSE TO PRACTICE VETERINARY MEDICINE:

Texas

PROFESSIONAL ORGANIZATIONS:

American Veterinary Medical Association

RESEARCH ACTIVITIES:

An Experimental Rotavirus Infection in Neonatal Gnotobiotic Dogs - Rotavirus (Ph.D. Thesis). Department of Veterinary Pathology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana, 1979-1981.

A Technique for Reconstruction of the Anterior Curciate Ligament. Performed gross and histopathological examinations of the surgical sites. Louisiana State University, 1979-1980; Chief investigator: Dr. D. A. Hulse.

LD/50 test of Imidocarb in horses; Babesia research in cattle. Texas A & M University, Department of Veterinary Pathology, August, 1973 to August, 1974; Chief investigator: Dr. L. Gary Adams
**PUBLICATIONS:**


**ABSTRACTS AND SCIENTIFIC PRESENTATIONS:**


Candidate: Charles A. Johnson

Major Field: Veterinary Medical Sciences
Veterinary Pathology

Title of Thesis: An Experimental Rotavirus Infection in Neonatal Gnotobiotic Dogs

Approved:

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Date of Examination:

January 18, 1982