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Pathogenesis of a new porcine serotype of group A rotavirus in neonatal gnotobiotic and weaned conventional pigs

Gregory Wayne Stevenson
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Pathogenesis of a new porcine serotype of group A rotavirus in neonatal gnotobiotic and weaned conventional pigs

Stevenson, Gregory Wayne, Ph.D.
Iowa State University, 1990
Pathogenesis of a new porcine serotype of group A rotavirus in neonatal gnotobiotic and weaned conventional pigs

by

Gregory Wayne Stevenson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

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Iowa State University
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1990
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>Historical</td>
<td>4</td>
</tr>
<tr>
<td>Virion structure</td>
<td>6</td>
</tr>
<tr>
<td>Virus replication</td>
<td>7</td>
</tr>
<tr>
<td>Virus classification</td>
<td>9</td>
</tr>
<tr>
<td>Immunity and protection</td>
<td>11</td>
</tr>
<tr>
<td>Naturally occurring disease and epidemiology</td>
<td>14</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>15</td>
</tr>
<tr>
<td>Virus affinity and cell permissiveness</td>
<td>20</td>
</tr>
<tr>
<td>Variation in rotavirus virulence</td>
<td>21</td>
</tr>
<tr>
<td>Pathophysiology of diarrhea</td>
<td>22</td>
</tr>
<tr>
<td>Models for the study of rotavirus in pigs</td>
<td>23</td>
</tr>
<tr>
<td><strong>PART I. PATHOGENESIS OF A NEW PORCINE SEROTYPE OF GROUP A ROTAVIRUS IN NEONATAL GNTOBIOTIC PIGS</strong></td>
<td>26</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>28</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>29</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>35</td>
</tr>
<tr>
<td>Clinical disease and gross lesions</td>
<td>35</td>
</tr>
<tr>
<td>Virology and bacteriology</td>
<td>36</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>36</td>
</tr>
<tr>
<td>Light microscopy and morphometry</td>
<td>37</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Rotaviruses replicate almost exclusively in villous enterocytes of mammalian and avian species. Rotaviruses are commonly associated with diarrhea in 2-3 week old suckling pigs and in recently weaned pigs. Three antigenically distinct groups of porcine rotavirus (A, B, and C) have been reported in the United States. Group A is the most common rotavirus group reported in swine herds and is the most common group associated with diarrhea in pigs in the U.S. Two serotypes of group A rotavirus, serotype 4 (Gottfried) and 5 (OSU), have been commonly recognized and are well characterized as causes of diarrhea in suckling pigs in the United States. Two new serotypes of porcine group A rotavirus (strains ISU64 and ISU65) were recently isolated from diarrheic weaned pigs. One of these strains (ISU64) has been shown to be closely related to serotype 9 group A rotavirus (WI61) (Paul, unpublished), which was previously reported only in humans. The pathogenesis of new porcine serotypes of group A rotavirus has not been investigated.

It has been suggested that rotavirus-induced disease in pigs is the most severe and lethal during the immediate postweaning period; however, the role of rotavirus in postweaning diarrhea in pigs is unclear. Although rotavirus is commonly demonstrated in recently weaned diarrheic pigs, it is often found in combination with other infectious agents such as hemolytic strains of E. coli or TGE virus. Clinically normal pigs may also shed rotavirus during the postweaning period. The pathogenesis of rotavirus has not been investigated in weaned pigs.

Inoculation studies in neonatal gnotobiotic pigs have been the usual model used to investigate the pathogenesis of different rotavirus serotypes in pigs. The use of
gnotobiotic pigs has the important advantage of allowing the study of rotavirus-induced disease without interference from other infectious agents; however, there are major differences in neonatal gnotobiotic and weaned conventional pigs that could potentially alter rotavirus-induced disease. Small intestine villous enterocytes differ in turnover rate and average age, in surface glycoprotein and protein composition and in morphology.\textsuperscript{69,114,117,118} Diets differ in the type of constituents and percent dry matter. Colonic digestive and absorptive functions also differ substantially.\textsuperscript{6} Therefore, the pathogenesis of new rotavirus serotypes or other agents that replicate within or upon villous enterocytes need to be investigated in conventional pigs more typical of those in which naturally occurring disease is observed as well as in gnotobiotic pigs.

Factors determining the nearly exclusive replication of rotavirus within villous enterocytes are poorly understood. Rotavirus inoculation studies in mice and TGE virus inoculation studies in pigs have demonstrated an innate nonimmunologic age-related resistance to virus infection which may be related to villous enterocyte age.\textsuperscript{116,137} It is not known whether there is an innate age-related resistance to rotavirus infection in swine; however, the younger average enterocyte age in weaned pigs compared to that in neonatal gnotobiotic pigs might cause weaned pigs to be more resistant to rotavirus infection and/or disease.

The objectives of these studies were i) to determine the pathogenicity of the ISU 64 strain of porcine group A rotavirus in gnotobiotic pigs ii) to develop a rotavirus disease model in recently weaned conventional pigs iii) to determine the pathogenicity of ISU64 strain of porcine group A rotavirus in weaned conventional pigs and iv) to compare the severity and distribution of lesions between neonatal
gnotobiotic and weaned conventional pigs with attention to the relationship between enterocyte age and susceptibility to virus infection.

This dissertation is presented in the alternate thesis format and is composed of two manuscripts in the style of the Veterinary Pathology journal. The manuscripts are preceded by a general introduction and a literature review and are followed by a general discussion and summary and a literature cited section. Each manuscript ends with a list of references. References cited in the remainder of the dissertation are listed in the literature cited section.

The Ph.D. candidate, Gregory W. Stevenson, was the principal investigator for each study.
LITERATURE REVIEW

Rotaviruses cause acute diarrheal disease primarily in young humans, animals and birds by replication within and destruction of small intestine villous epithelial cells. Disease in pigs is usually within the suckling or immediate post weaning period and is often complicated by concurrent infection with other enteric viral, bacterial or protozoal agents.

Historical

In 1943, Light and Hodes induced diarrhea in calves by oral inoculation with a fecal filtrate from human infants with infantile diarrhea. Likewise in 1947, Cheever and Mueller transmitted epidemic diarrheal disease of suckling mice (EDIM) with fecal filtrates. The development of high resolution thin section transmission electron microscopy allowed Adams and Kraft in 1963 to observe virus-like particles in the intestinal tissue of mice affected with EDIM. In 1969, Mebus et al. demonstrated by negative stain contrast electron microscopy 70 nm virus particles in the stools of diarrheic neonatal calves and were subsequently able to serially pass the virus in calves and reproduce the diarrheal disease. Shortly thereafter in 1971, Mebus et al. reported successful cultivation of Nebraska calf diarrhea virus (NCDV) in primary fetal bovine cells and in 1972 Fernelius et al. reported that NCDV resembled reoviruses morphologically but was distinct antigenically.

Similar 70 nm viruses were soon identified in the intestines and feces of diarrheic neonates in many mammalian species. Notably, in 1973, Bishop et al. demonstrated virus particles in biopsies of duodenal mucosa taken from children
with infantile diarrhea and shortly thereafter several researchers demonstrated 70 nm particles in the feces of children with diarrhea.\textsuperscript{19,56,86,113} The morphology of the 70 nm particles, when viewed by negative stain contrast electron microscopy, could be differentiated from that of orbiviruses and reoviruses by a sharply defined outer capsid.\textsuperscript{56,113} The distinctive morphology of the 70 nm particles resembled a spoked wheel and in 1974 led to the suggested name of rotavirus taken from the Latin word rota which means wheel.\textsuperscript{57} Rotaviruses were first identified in naturally occurring outbreaks of diarrhea in pigs in 1975 and 1976 by Rodger et al.\textsuperscript{138} in Australia, by Lecce et al.\textsuperscript{95} in the United States and by McNulty et al.\textsuperscript{110} and Woode et al.\textsuperscript{168} in the United Kingdom. The NCDV strain of bovine rotavirus was used as a standard for comparison and for many years all rotaviruses were thought to be morphologically identical and antigenically related. However in 1979 and 1980, Debouck and Pensaert,\textsuperscript{65} Bridger,\textsuperscript{25} and Saif et al.\textsuperscript{141} reported rotaviruses in pigs in Belgium, the United Kingdom and the United States, respectively, which were morphologically identical but antigenically distinct from previously reported rotaviruses. Subsequently, 7 antigenically distinct groups of rotaviruses have been described in various species of mammals and birds.\textsuperscript{26}

Initially, the group A rotaviruses represented by NCDV proved to be fastidious and were difficult to isolate and maintain in continuous cell cultures. In 1977, Theil et al. reported success in culture of porcine group A strains aided by the inclusion of trypsin in the cell culture media.\textsuperscript{158} Soon thereafter, similar methods evolved for the successful propagation of most group A rotaviruses.\textsuperscript{62,144,163} Characterization and study of nongroup A rotaviruses have been hampered by an inability to propagate most in vitro. One nongroup A rotavirus of porcine origin has been successfully cultivated.\textsuperscript{142,157}
Virion structure

Rotaviruses and rotavirus gene structure and function are subjects of recent reviews. Rotaviruses comprise a genus within the family Reoviridae, and rotaviruses share common structural and functional characteristics. Rotavirus virions are 70 nm in diameter and are composed of a protein core which contains the genome surrounded by a double layered icosahedral protein capsid. Somewhere within the inner capsid and core there is an RNA dependent RNA polymerase and other enzyme activities capable of producing capped RNA transcripts. The genome consists of 11 segments of double stranded RNA (dsRNA) which are capable of genetic reassortment within viruses of the same group. Polyacrylamide gel electrophoresis (PAGE) of gene segments produces characteristic patterns or electropherotypes for virus groups and for some viruses within groups allowing a rapid method for diagnosis, differentiation, and epidemiological study of rotaviruses. Genes are named in order of migration on gels with the slowest migrating gene numbered as 1 and the fastest as 11. Current evidence suggests that all genes are monocistronic, except possibly gene 9. Gene products from genes 5, 7, 8, 10 and 11 are nonstructural. Gene products from genes 1, 2 and 3 are structural proteins of the virus core and together comprise approximately 17.5% of the virion protein. The gene 6 product (VP6) is the only protein of the inner capsid. It functions structurally as a trimer and comprises approximately 51% of the virion protein. The gene 4 and gene 7 products (VP4 and VP7) together form the outer capsid. VP7 is a glycoprotein and comprises approximately 30% of the virion protein. It is the major neutralization antigen and functions in cell attachment by interaction with a putative cell membrane virus receptor. VP4 is nonglycosylated and
comprises approximately 1.5% of the virion protein. It functions in hemagglutination in some strains, is a neutralization antigen, is involved in protease enhanced infectivity and has been implicated as a virulence factor in mice and humans.\textsuperscript{7,49,50,52,58,64,74,81,101,102,107,121} \(\text{VP4}\) is partially exposed on the outer virion surface and is cleaved by proteolysis into a larger (\(\text{VP5}\)) and a smaller (\(\text{VP8}\)) fragment. Cleavage of \(\text{VP4}\) enhances cell penetration but not binding of virus to cells and is associated with restriction of growth of certain rotavirus strains in tissue culture cells.\textsuperscript{38,60,83} The mechanism(s) by which \(\text{VP4}\) enhances infectivity is not known however there is a conserved sequence in \(\text{VP4}\) which shares deduced amino acid homology with internal fusion sites in Semliki Forest and Sindbis virus proteins.\textsuperscript{100} If \(\text{VP4}\) has a fusion function, it could mediate rotavirus entry into cells.

\textbf{Virus replication}

Rotavirus replication is the subject of a recent review.\textsuperscript{50} Virus replication occurs exclusively in the cytoplasm of infected cells by a unique morphogenic pathway. Virus attachment to cells occurs via \(\text{VP7}\) and is not dependent upon \(\text{VP7}\) glycosylation.\textsuperscript{60,103,131,139} The cell membrane receptor is not known. Virus binding to a continuous cell line derived from rhesus monkey kidney cells (\textit{MA104} cells) is sodium dependent, pH insensitive between pH 5.5 and 8 and is dependent upon sialic acid residues in the cell membrane.\textsuperscript{12,88,132} Sialic acid containing compounds such as mucin inhibit virus binding to cells.\textsuperscript{88,100} Whether sialic acid functions as an integral component of the virus binding site or whether it is necessary for maintenance of the configuration of a distinct binding site is unclear.\textsuperscript{50} The mechanism of rotavirus penetration into cells is unresolved and more than one mechanism is possible.
Porcine OSU strain uptake following VP4 cleavage is ultrastructurally typical of receptor-mediated endocytosis; however, acidification of endosomes is not needed for viral entry and/or uncoating.\textsuperscript{50,61,83,87,99} Studies with trypsin treated human strains of rotavirus provide convincing evidence that direct penetration of the host cell membrane occurs.\textsuperscript{83,156} Transcription is asymmetric, results in full length positive strand transcripts made from the negative dsRNA stand as template and is thought to be initiated by loss of the outer capsid (uncoating) resulting in the formation of subviral "transcription" particles.\textsuperscript{39,106} Interference with normal cell DNA, RNA and protein synthesis occurs early in the viral replication cycle resulting in progressive cell degeneration.\textsuperscript{30,47,105} Translation of most mRNA species occurs in association with ribosomes within the cell cytoplasm and results in homogeneous granular viroplasmic inclusions.\textsuperscript{133,136} VP7 and one nonstructural protein (NS28) are translated on the membrane associated ribosomes of the RER, are cotranslationally glycosylated and are inserted into the ER membrane by leader sequences to become integral membrane glycoproteins.\textsuperscript{7,24,47,48,80} The site and mechanism of dsRNA synthesis is unknown, but is assumed to occur within viroplasmic inclusions.\textsuperscript{134,136} Assembly is calcium dependent.\textsuperscript{145} Subviral particles form at the periphery of dense viroplasmic inclusions and the outer capsid is assembled through a unique process of budding into the RER where a transient envelope forms and is quickly lost.\textsuperscript{46} NS28 functions in assembly as a receptor for single shelled particles thus mediating budding through the RER and is also somehow involved in envelope removal during virion maturation within the ER.\textsuperscript{9,48,90,131} Mature virions accumulate within the ER and are released from cells by cell lysis.\textsuperscript{4,33,109}
Virus classification

Rotaviruses are classified serologically first into antigenically distinct groups then groups are subdivided into serotypes based upon reaction in neutralization assays. Six and possibly seven rotavirus groups (A–F or G) have been described. Group antigenic determinants are present on most if not all of the structural proteins of virions; however, VP6 is the primary group specific antigen. Group specific epitopes recognized by commercially available group A rotavirus antigen capture ELISA tests as well as a subgroup epitope (subgroups I and II) which was used in early epidemiological studies are located on VP6. Group A rotaviruses are common in many mammalian and avian species, group B is reported in humans, pigs, cattle, sheep and rats, group C is reported in humans and pigs, group E is reported in pigs, and groups D, F and G are reported in avian species. Groups A, B and C are reported in pigs in the United States; however, group A rotaviruses are much more commonly associated with suckling and weanling pig diarrhea than are groups B and C. Limited information is available regarding groups B and C in pigs. Unless noted otherwise, the remainder of this literature review focuses on group A rotaviruses.

Group A rotaviruses are further classified into serotypes based upon reactivity of viruses in plaque reduction or fluorescent foci reduction neutralization assays using hyperimmune serum. This method primarily measures reactivity of a single exposed conformational domain of VP7 containing multiple overlapping epitopes coded by two discrete nucleotide regions (A and C) of gene 9. Presumably, hyperimmunization procedures favor neutralization antigens of VP7 relative to VP4 owing to increased antigenic mass and/or increased immunogenicity of glycoprotein epitopes. VP7
serotypes can also be accurately determined by ELISA using
serotype specific monoclonal antibody panels directed against
serotype specific epitopes or by nucleotide sequence analysis
of the A and C region of gene 9. Some rotavirus
strains do not react clearly in reciprocal neutralization
assays. This is usually because the two viruses being
compared possess distinct VP4 neutralization epitopes.
Limited information is available regarding VP4 subtypes
because of a lack of VP4 specific monoclonal antibodies.
Comparison of gene 4 between group A rotavirus strains using
hybridization and nucleic acid sequencing suggests that there
are at least 8 types of gene 4. The porcine type strains of
VP7 serotypes 4 (Gottfried) and 5 (OSU) have distinct types of
gene 4 by hybridization. A binary system for serotype
classification in which both VP7 and VP4 subtypes are named
has been proposed; however, there is currently insufficient
information regarding VP4 subtypes and too few VP4 serotype
specific monoclonal antibodies for such a system to be
instituted.

Nine group A rotavirus serotypes have been recognized
thus far in animals and man. Serotypes 1-4, 8 and 9 are
reported in humans and serotypes 5, 6 and 7 are reported only
in animals. Serotypes 4 (Gottfried) and 5 (OSU) were the
first reported in pigs in the United States and have also been
reported in pigs in Australia. Two new serotypes (ISU64
and ISU65) were recently reported in the United States. One
of these strains (ISU64) is serotypically distinct from
serotypes 1-6 by 2-way neutralization and is related by 1-way
neutralization to serotype 9 (Paul, unpublished). Recently,
serotype 3 was reported in pigs in Australia and other
putative new porcine serotypes have also been reported in pigs
in Mexico and Argentina.
Immunity and protection

Molecular determinant of rotavirus neutralization and protection is the subject of a recent review. Immunity to rotavirus-induced disease occurs; however, what constitutes the immune response and exactly how it can be induced have not been well defined. Recent studies have demonstrated a potential role of cell mediated immunity (CMI) in the clearance of viral infection; however, the relative importance of CMI in protective immunity is not known. Considerable evidence acquired in a variety of animal models, studying passive or active immunity and utilizing a variety of rotavirus strains indicates that protection against disease is significantly dependent on the presence of locally produced or passively acquired (colostrum or milk) neutralizing antibody within the lumen of the intestine. Data regarding the serotypic specificity of the immune response are confusing. Some studies suggest heterotypic immunity may be protective. Other studies suggest an absence of heterotypic immunity. The majority of evidence suggests that active infection usually results in homotypic immunity and that when heterotypic immunity occurs, it is less protective and more transient than homotypic immunity. Two cross protection studies in pigs resulted in marked homotypic and little or no heterotypic protection. Oral immunization of gnotobiotic pigs with serotypically distinct bovine, canine-simian, and human strains of group A rotavirus did not prevent diarrhea with subsequent serotype 5 (OSU) porcine rotavirus inoculation whereas immunization with homologous virus conferred protection. Likewise, oral immunization with cell passage attenuated serotype 4 (Gottfried) or 5 (OSU) rotavirus protected pigs from diarrhea following subsequent inoculation.
with homologous but not heterologous virus. Cross protection studies have not been reported for porcine rotavirus serotypes other than 4 and 5.

Recent studies using single gene reassortants as immunogens have clearly demonstrated that antigens of either VP7 or VP4 will confer protection following inoculation with virus which carries homologous VP7 or VP4. One study in pigs further suggested that VP7 may stimulate immunity that is slightly more effective in prevention of infection and/or illness than that produced by VP4.

The efficacy of the immune response in the prevention of infection and/or disease in pigs depends upon the level of oral rotavirus challenge relative to the presence of continuous and adequate levels of specific neutralizing antibodies within the small intestine. The level of rotavirus challenge is presumably affected by sanitation, husbandry and management practices. Pigs are essentially born agammaglobulinaemic and therefore are without mucosal antirotavirus secretory antibodies and rotavirus primed lymphocytes. Protection against rotavirus enteric infection in the first days of life is passive and is conferred by lactogenic immunity, i.e., the presence of specific neutralizing secretory antibodies in the colostrum and milk of the dam. Protection presumes adequate milk production by dams and adequate suckling by pigs. The immunoglobulin fraction of sow colostrum at farrowing is primarily of serum origin and is composed of 80% IgG, 13% IgA and 7% IgM. During the first week of lactation the levels of IgG in milk decline 30 fold while the levels of IgA decline 3 fold, so that by one week after farrowing dimeric IgA of mammary gland origin is the primary immunoglobulin in milk. Neutralizing rotavirus antibody titers in colostrum at farrowing are comparable to those in sow serum but titers steadily decline
in milk during lactation with a half-life of approximately 10 days. Rotavirus antibody titers in serum, colostrum and milk are typically higher in sows than gilts. Rotavirus replication occurs in the intestinal mucosa of the pig when lactogenic immunity declines during the latter part of the suckling period or shortly following the abrupt removal of lactogenic immunity at weaning. The factors that determine whether virus replication results in subclinical or clinical disease are poorly understood. The duration of protective immunity conferred by an active secretory mucosal immune response following viral replication is not known and is probably dependent upon a variety of factors. In rotavirus-inoculated 5-day-old gnotobiotic calves, rotavirus specific IgM appeared in feces 5 days after inoculation and specific IgA first appeared in feces 10-14 days, peaked at 15-25 days and fell to low levels at 28 days after inoculation. The relative contribution and duration of mucosal CMI in protective immunity is not known.

Attempts to prevent rotavirus associated disease by vaccination have not been very successful. Milk rotavirus neutralizing antibody titers can be elevated and prolonged by oral or parenteral immunization of dams that have been previously immunologically primed. The net effect of vaccine enhanced lactogenic immunity in pigs may be to delay the onset of viral replication from the suckling period to the immediate postweaning period when all lactogenic immunity is removed and there is potential for severe clinical disease. Likewise, attempts to induce active mucosal immunity in neonates with oral administration of modified live attenuated rotavirus vaccines has not proved successful. The most likely cause of this failure is inadequate viral replication caused by too little virus per vaccine dose, excessive virus attenuation, or neutralization of the vaccine virus by
The recent discovery of new group A serotypes as well as groups B and C rotaviruses in pigs further complicates the potential development of efficacious vaccines.

Naturally occurring disease and epidemiology

Rotaviruses are extremely common in swine herds. Serological surveys have demonstrated that nearly all swine herds are seropositive for group A rotavirus and 70-90% are positive for groups B and C. \(^{27,140}\) Rotavirus likely circulates continually as a mild subclinical infection among adults in large swine herds. \(^{167}\) One study demonstrated that 42% of peripartum sows shed low numbers of group A rotavirus in the feces thus providing a source of virus to neonatal pigs. \(^{17}\) Nearly all pigs shed group A rotavirus (and presumably other groups) in feces between 1 and 8 weeks of age; however, most infections are subclinical. \(^{8,164,167}\) Gilt litters tend to be infected at a younger age and have a higher proportion of pigs with clinical disease than do sow litters. \(^{8,59}\) Rotavirus shedding in pigs may be intermittent and lasts for 1-12 days. \(^{8,59}\) Rotavirus infection is most often reported as a clinical disease in 2-3 week old suckling pigs where it is associated with a white pasty to watery diarrhea of 2-4 day duration. \(^{8,59,164,167}\) The age of onset, morbidity and mortality varies between herds. In one report of 6 swine herds where rotavirus was the sole determined etiologic agent in association with suckling pig diarrhea, the earliest age of onset ranged from 5-20 days and the mortality ranged from 7-20%. \(^{21}\) Rotavirus has also been implicated as the sole etiologic agent in postweaning diarrhea affecting 3-4 week old recently weaned pigs. \(^{94,168}\) More commonly rotavirus has been reported in combination with hemolytic strains of *E. coli* as a
cause of severe protracted postweaning diarrhea in pigs.\textsuperscript{21,92,93,162} It has been suggested that rotavirus may create an environment in the gastrointestinal tract that favors hemolytic \textit{E. coli} colonization and/or growth.\textsuperscript{92,93} The role of rotavirus in postweaning diarrhea is difficult to deduce from reports of naturally occurring disease. Examination for other viral, bacterial or protozoal etiologic agents was often not done or rotavirus infection was complicated by concurrent infection with other potentially pathogenic agents.

**Pathogenesis**

The pathogenesis of serotypes 4 and 5 group A rotavirus-induced enteric disease in suckling pigs has been studied in a variety of in vivo inoculation models. In most studies, 1–14 day old presumably seronegative gnotobiotic pigs fed a variety of liquid diets were inoculated orally or intranasally with filtered fecal suspensions containing an undetermined amount of virus (10^5.5 TCID\textsubscript{50} /dose in one study).\textsuperscript{16,40,42,104,159,161,168} In other studies, presumably seronegative colostrum deprived 1–28 day old pigs were maintained in clean but nonsterile environments on liquid diets and inoculated orally with 10^6.5 – 10^9 TCID\textsubscript{50} of virus (undetermined amount in one study).\textsuperscript{78,92,128} In one study, seropositive conventional pigs which were removed from their dams to clean but nonsterile cardboard isolettes, were maintained on a liquid diet and were orally inoculated at 21 days of age with 10^6.5 TCID\textsubscript{50} of virus.\textsuperscript{146} No rotavirus inoculation studies have been reported in susceptible weaned conventional pigs fed a typical commercial pelleted weaning diet.

Clinical disease is similar in all inoculation models, but tends to be less severe in older conventional pigs. Some pigs become depressed, lethargic and inappetent or exhibit
vomition beginning 3-4 hours prior to the onset of diarrhea. Sixteen to 48 hours following inoculation, nearly all pigs experience acute onset of a profuse diarrhea which is characterized by a watery consistency with floccules of partially digested unabsorbed food. Duration of diarrhea varies between 3-6 days, but was only 1 day in 28-day-old seronegative conventional pigs. Few pigs die unless also infected with concurrent E. coli or other pathogens. In most cases, appetite returns within 3 days after inoculation. In one study where pig weights were monitored, pigs lost 15% of body weight by 24-36 hours after inoculation and returned to preinoculation weights by 3-5 days after inoculation.

Gross lesions were similar for all pigs inoculated at 1-14 days of age. The stomachs of control pigs contained some food and the small intestines were of normal thickness with segmental differences in diameter caused by normal motility. The upper small intestine contained watery translucent yellow fluid with slimy bits of curd. The lymphatics within the intestinal wall and mesentery of the upper 1/2 of the small intestine contained chyle and the respective mesenteric lymph nodes were white, glistening and turgid. The cecal and colonic contents were of thick and pasty or solid consistency. In virus inoculated pigs gross lesions were observable at the onset of diarrhea. The stomachs usually contained food and the distal 1/2 to 2/3 of the small intestine was thin walled, flaccid and dilated with a large volume of watery flocculent yellow colored fluid. The lacteals in the midportion of the intestine contained no chyle and the respective mesenteric lymph nodes were small and tan. The caecum and colon were dilated and filled with similar contents. There were no significant gross lesions reported in 3-week-old conventional suckling pigs.

Immunofluorescence studies made on frozen tissue sections
or mucosal scrapings of small intestine revealed similar trends in viral replication. Fine granular evenly dispersed cytoplasmic fluorescence was first visible 12 hours after inoculation in villous enterocytes usually on the distal 1/2 of villi, but occasionally in a patchy distribution over an entire villous. In most studies, the greatest proportion of villous enterocytes fluoresced 24-48 hours after inoculation and the majority of these were in the jejunum and ileum. Occasionally, fluorescence continued longer after inoculation (1-4 days) and the villous enterocytes of the duodenum were infected to a relatively greater extent. Fluorescence was reported in a low number of surface epithelial cells of the caecum in one study.

Similar light microscopic lesions and small intestine mucosal morphometrics have been described in numerous rotavirus inoculation studies in suckling pigs. Villous atrophy caused by villous mucosal epithelial cell degeneration was the primary lesion and was similar in character but different in severity at different small intestine levels. Typically, villous atrophy was first detected 16-24 hours following inoculation, was most severe 24-72 hours following inoculation, and was most severe in the jejunum and ileum where villi are 1/10 to 1/4 normal length. Crypt hyperplasia resulted in significantly deeper crypts within 48-72 hours following inoculation. Lateral fusion of villi was variably present 24-168 hours following inoculation. Light microscopic lesions were first visible 16-18 hours after inoculation as degenerative changes in mucosal epithelial cells primarily on villous tips and occasionally in groups on lateral villi. Degenerative cells were characterized by cloudy swelling, cytoplasmic vacuolation, nuclear swelling, separation from adjacent cells and irregularity of brush borders. Sloughing of degenerative
cells, retraction and edema of the lamina propria and occasional villous tip erosion soon followed and resulted in villous atrophy. By 36-72 hours after inoculation, squamous to cuboidal epithelial cells covered the tips of short villi. There was usually some lateral villous fusion, and the lamina propria contained pyknotic nuclear debris. At 72-128 hours after inoculation, the villi were covered by differentiated columnar epithelial cells and the lamina propria was normal. The amount of time needed for complete villous repair depended on the age of pig. Morphometry has not been done in rotavirus-inoculated conventional pigs that are greater than 2 weeks old.

Lesions observed by scanning electron microscopy have been similar in several studies and follow the same time progression as do light microscopic lesions. Villous atrophy was the primary lesion and was characterized by swelling of mucosal epithelial cells, shortening and fragmentation of microvilli, decreased cell to cell adherence, occlusion of goblet cell openings, desquamation of mucosal epithelial cells with exposure of villous tip lamina propria and lateral villous fusion.

Ultrastructural studies of rotavirus-infected villous enterocytes in pigs have revealed similar lesions. Virus infected cells contained within their cytoplasm multifocal variably sized electron dense nonmembrane bounded granular viroplasms that occasionally had 31-38 nm electron dense circular core particles on the periphery. In addition, core particles budded into the cisternae of the RER which was dilated and contained large numbers of 75-78 nm diameter double shelled and fewer 50-55 nm diameter single shelled virus particles. Double shelled particles were composed of a dense central core enveloped by a well defined membrane and single shelled particles were round with moderate electron
density and had indistinct margins. Occasionally the dilated RER contained convoluted smooth membranous material that contained virus particles. In a study of serotype 5 (OSU) rotavirus morphogenesis in 10-day-old gnotobiotic pigs, approximately 5% of villous enterocytes in the jejunum were infected with virus. Infected cells were scattered among normal cells and there was little synchrony among virus infected cells. Infected cells often had few degenerative changes, some had rarified cytoplasm and dilated ER, some had swollen nuclei and others occasionally had shortened and irregular microvilli. In jejunal mucosal tissue sections, few cells were filled with large amounts of virus or were seen in the process of lysis while high numbers of degenerate cells containing large amounts of virus were observed in cell scrapings made from jejunal mucosa. These findings suggest that infected cells were rapidly desquamated into the intestinal lumen, possibly prior to release of virus.

Ultrastructural studies of rotavirus replication in cultured cells revealed virus morphogenesis similar to that reported in porcine enterocytes with some notable additions. Large irregularly shaped lysosome-like bodies containing crystalline arrays of 52 nm virus particles were present in the cytoplasm of MA104 cells that did or did not contain other evidence of viral infection. Double shelled virus particles were present within mitochondria of MA104 cells. Intranuclear tubules of 50 nm diameter were present in primary porcine kidney cells and intracytoplasmic and intranuclear tubules of 15-20 nm diameter were occasionally present on MA104 cells. Virus release from swollen cells occurred through disruption of the cell plasma membrane.
Virus affinity and cell permissiveness

Factors determining the nearly exclusive replication of rotavirus within subpopulations of villous enterocytes are poorly understood. In suckling pigs, replication occurs predominately in the jejunum and ileum. In mice, replication is limited to villous tips and is further limited by the age of mouse; virus will replicate in seronegative suckling but not adult mice.\(^ 1\) Average villous enterocyte age is much younger in adult mice relative to suckling mice and may have bearing on resistance to virus infection.\(^ 68,91\) Studies have demonstrated an innate age-related resistance to TGE virus infection in swine which was thought to be related to differences in villous enterocyte age.\(^ 116\) It is not known whether there is an innate age-related resistance to rotavirus replication in swine.

Virus binding studies done on villous enterocytes which were harvested from mice suggested that nonpermissive villous enterocytes in adult mice may lack virus receptors.\(^ 137\) Virus binding studies have not been done in pigs, but might be helpful in determining whether there are segmental or age-related differences in rotavirus binding to villous enterocytes. Distribution of rotavirus receptors is an attractive hypothesis to explain variations in virus-cell tropism because there are known differences in the glycoprotein and protein composition of the glycocalyx between subpopulations of villous enterocytes. Differences in binding of lectins in the small intestine of mice have suggested heterogeneity of glycocalyx glycoproteins relative to position on the villus (cell maturity), between different small intestine segments, and between the same small intestine segments in mice of different ages.\(^ 53,54\) Similar studies have not been reported in pigs. Well known differences in
glycocalyx distribution of diaccharidases and peptidases between segments of the pig small intestine and between different ages of pigs are also an indication of the variable composition of the enterocyte glycocalyx.\(^8\)

Virus binding to cell receptors occurs via outer capsid protein VP7; however, recent studies demonstrated that outer capsid protein VP4 is involved in cell penetration, in restriction of growth of some virus stains in tissue culture cells and in lowered virulence for some virus strains in humans and mice (see above). These findings suggest that factors other than receptor distribution may contribute to cell permissiveness in virus replication. Inability to maintain differentiated villous enterocytes in in vitro culture systems has interfered with direct investigation of these questions.

**Variation in rotavirus virulence**

Differences in virulence between group A rotavirus strains have been reported in humans, mice and calves.\(^{29,64,124}\) VP4 has been implicated as a virulence determinant in human and murine rotavirus strains, although there is continuing controversy whether host and/or virus factors are involved.\(^{50,51,86}\) Routine VP4 serotyping of virus strains may prove helpful in recognition of trends in serotype virulence. Differences in virulence among VP7 serotypes have not been reported. In a single study with 3 day old gnotobiotic pigs, no difference in virulence was detected between two strains representing VP7 serotypes 4 and 5.\(^{40}\) The virulence of strains representing new VP7 serotypes of group A rotavirus in pigs is not known.
Pathophysiology of diarrhea

Several mechanisms are involved in rotavirus-induced diarrhea. Loss of villous mucosal epithelial cells causes maldigestion and malabsorption which results in abnormally high numbers of osmotically active particles in the lumen of the small intestine. In 5–7 day old rotavirus-inoculated suckling pigs, diarrheic feces were hyperosmolar as a result of excess lactose and Na⁺. In rotavirus-inoculated pigs, enzyme assays done on mucosal epithelial cells harvested from the jejunum and ileum demonstrated reduced activities of disaccharidases (lactase) and (Na⁺,K⁺)ATPase. Decreased lactase activity results in lactose maldigestion with excessive lactose and water in the terminal small intestine, however studies done in TGE inoculated pigs suggest that age dependent differences in colonic microbial fermentive capacity may determine whether excessive lactose is lost in the feces, whether the colon can efficiently absorb excess fluid and whether diarrhea occurs. The role of colonic function in rotavirus-induced disease relative to pig age and/or diet has not been investigated. In vivo perfusion studies in jejunum and ileum of rotavirus-inoculated pigs demonstrated net Na⁺ flux toward the lumen. Net luminal Na⁺ flux is thought to be due to continued normal secretion of Cl⁻ and Na⁺ from crypts with decreased villous epithelial absorption of Na⁺ and Cl⁻ caused by reduced glucose mediated Na⁺ transport and (Na⁺,K⁺)ATPase activity. Recent studies in rotavirus infected children suggested that increased levels of PGE₂ and PGF₂ may also contribute to diarrhea. The contribution of prostaglandins or other inflammatory mediators to rotavirus-induced diarrhea in pigs is unknown.
Models for the study of rotavirus in pigs

Rotavirus associated diarrheal disease is most commonly reported in suckling and postweaned pigs yet nearly all inoculation studies have involved neonatal gnotobiotic or colostrum deprived conventionalized pigs and all have been in pigs on liquid diets. There are significant differences in the gastrointestinal tract between neonatal gnotobiotic or conventional pigs and weaned conventional pigs. The age of the pig, presence and character of microflora and type of diet could all potentially alter the pathogenesis of rotavirus infection.

The epithelium of the small intestine is a dynamic population of cells that are spatially segregated with respect to their degree of maturation. The cells divide in the crypts, and then move up the villi until they are extruded from the villous tips. The rate of cell migration, the average length of villi, and the average age of villous enterocytes vary between pigs of different ages. The average time required for complete turnover of villous epithelial cells is 7-10 days in newborn and 2-4 days in 3-week-old conventional pigs. Average villous length is approximately 800-1200 um in newborn and 300-680 um in 3 week old conventional pigs. The increased rate of cell turnover in older pigs is thought to be due, in part, to the role of microflora in cell death. Consistent with this hypothesis, the turnover rate remains slow and the villi remain long in 3 week old gnotobiotic pigs relative to 3 week old conventional pigs. Diet change associated with weaning may cause a further transient increase in the rate of cell turnover and decrease in average villus height in the postweaning period.

The morphology of porcine villous epithelial cells changes with age; cells older than approximately 4 days have a
prominent system of vesicles and vacuoles in the apical portion of the cell cytoplasm.\textsuperscript{117} This results in nearly all vacuolated villous epithelial cells in newborn pigs and no vacuolated villous epithelial cells in 3-week-old pigs.

Colonic fermentation of carbohydrates and colonic absorption of water are dependent upon colonic microbial flora.\textsuperscript{6} The capacity for colonic fermentation and water absorption increases with age in conventional pigs. Weaned conventional pigs should have a greater capacity to compensate for rotavirus-induced small intestine dysfunction by colonic fermentation and water absorption as compared to gnotobiotic or neonatal conventional pigs.

The difference in milk or liquid diet in suckling pigs and pelleted dry diet in weaned pigs might alter rotavirus-induced disease. Milk or liquid diets are typically 15-20\% dry matter while pelleted diets are 80-85\% dry matter thus requiring greater fluid absorptive capacity in suckling pigs in order to prevent diarrhea. Differences in diet also affect the composition of goblet cell and villous enterocyte glycocalyx glycoproteins.\textsuperscript{118} These diet related changes in the glycocalyx might have an effect on virus-cell interactions. Pelleted weanling diets have higher crude fiber than does milk. Increased fiber may stimulate increased colonic fermentation (see above).

The use of gnotobiotic pigs has the important advantage of allowing the study of rotavirus-induced disease without interference from any other infectious agents. Study of the pathogenesis of the ISU64 strain of swine rotavirus should include inoculation studies in neonatal gnotobiotic pigs in order to determine the potential pathogenicity for susceptible conventional pigs and to allow comparison with similar published inoculation studies using other serotypes of group A rotavirus in pigs. Study of the pathogenesis of ISU64 should
also include weaned conventional pigs since it was originally isolated from weaned pigs and because the role of rotaviruses in postweaning diarrhea in pigs is unclear. The development of a reliable postweaning rotavirus inoculation model in pigs would also allow the future investigation of the role of rotavirus in multifactorial postweaning diarrheal syndromes in pigs.
PART I. PATHOGENESIS OF A NEW PORCINE SEROTYPE OF GROUP A ROTAVIRUS IN NEONATAL GNOTOBIOTIC PIGS
PATHOGENESIS OF A NEW PORCINE SEROTYPE
OF GROUP A ROTAVIRUS IN NEONATAL GNOTOBIOTIC PIGS

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ABSTRACT

Eight neonatal gnotobiotic pigs were inoculated orally with a new serotype (strain ISU64) of porcine group A rotavirus and three additional sham inoculated pigs were held as controls. Pigs were killed at various times post inoculation (PI) and pathogenesis of infection was studied at 5 small intestine sample sites. Virus inoculated pigs developed severe diarrhea within 16-18 hours PI and some became depressed and inappetent. Viral antigen was detected by immunofluorescence in the cytoplasm of enterocytes primarily in the midjejunum through the ileum 18-48 hours PI. Villous atrophy was severe in the ileum by 18 hours PI and progressed to similar severity in the midjejunum by 24 hours PI. The distribution and kinetics of villous atrophy suggested that villous enterocytes in the midjejunum were less susceptible to virus infection than those in the ileum. Lesions demonstrated by light, scanning electron and transmission electron microscopy were typical of those described for other porcine serotypes of group A rotavirus. Ultrastructural examination of virus infected villous enterocytes demonstrated a subpopulation of cells that did not contain mature double shelled virus particles, but instead contained intracytoplasmic membrane bounded or nonmembrane bounded electron dense aggregates of viroplasm composed of crystalline arrays of target-like dense subviral particles. It was concluded that strain ISU64 of porcine group A rotavirus is pathogenic for neonatal gnotobiotic pigs and causes lesions and disease which are severe and indistinguishable from those reported for serotypes 4 and 5.
INTRODUCTION

Rotaviruses are commonly associated with diarrhea in suckling and recently weaned pigs. There are six distinct serogroups of rotaviruses (A to F) in animals and man. Groups A, B and C have been reported in pigs in the United States and group E is reported only in pigs in Great Britain. Group A rotaviruses are more often demonstrated in stools of diarrheic pigs in the U.S. than is either groups B or C.

Group A rotaviruses are further divided into serotypes based upon reactivity in plaque reduction or fluorescent focus-reduction neutralization assays using hyperimmune serum. This method primarily differentiates between neutralization epitopes of the major outer capsid constituent, glycoprotein VP7. Outer capsid protein VP4 comprises the remaining portion of the outer capsid and also contains less dominant neutralization epitopes. Some strains with distinct VP7 serotypes share common VP4 neutralization epitopes which may confer some cross reactivity and/or heterotypic immunity. A binary system for serotype classification in which both VP7 and VP4 subtypes are named has been proposed for group A rotaviruses, however there is currently insufficient information regarding VP4 subtypes for such a system to be instituted.

Nine distinct group A rotavirus serotypes have been recognized thus far in animals and man. Serotypes 4 and 5 have been reported in pigs in the United States and Australia. More recently, serotype 3 was reported in pigs in Australia and additional putative swine serotypes have been reported from pigs in Mexico and Argentina. Two new serotypes distinct from serotypes 4 and 5 were also recently reported in the United States. One of these strains (ISU64)
is serotypically distinct from serotypes 1-6 by 2-way neutralization, is related by 1-way neutralization to serotype 9 (strain WI61), and shows highest nucleotide sequence homology (85%) and deduced amino acid sequence homology (92%) with serotype 9 (Paul, unpublished). The prevalence of this new serotype in pigs is not known.

Immunity following active infection or passive immunization is based primarily upon the presence of neutralizing antibody in the intestinal tract and is usually homotypic. Oral immunization of gnotobiotic pigs with serotypically distinct bovine, canine-simian, and human strains of group A rotavirus did not prevent diarrhea with subsequent serotype 5 rotavirus challenge. Likewise, oral immunization with cell passage attenuated serotype 4 or 5 rotavirus protected pigs from diarrhea following subsequent challenge with homologous but not heterologous virus. Cross protection between serotypes 4 or 5 and new serotypes of porcine group A rotavirus has not been studied in pigs.

Differences in virulence between strains of group A rotavirus have been reported in calves and human infants. The serotypes of the bovine strains were not reported. Asymptomatic (avirulent or low virulence) human strains share a common or similar VP4 protein and are found within serotypes 1, 2, 3 and 4. A single study did not demonstrate differences in virulence between a serotype 4 and serotype 5 strain of porcine group A rotavirus in neonatal gnotobiotic pigs.

The pathogenesis of group A rotaviruses in the small intestines of gnotobiotic and conventional pigs of various ages has been studied. The serotype of the group A rotavirus inoculation strain has been determined in only a limited number of studies. Serotype 5 has been studied in gnotobiotic neonates, in colostrum deprived and colostrum fed conventional neonates and in 3-week-old conventional suckling
Serotype 4 has been studied in neonatal gnotobiotic pigs. The pathogenesis of other porcine group A serotypes has not been studied in detail.

The purpose of this study was to characterize the pathogenesis of a new serotype of porcine group A rotavirus (ISU64) in neonatal gnotobiotic pigs and to compare the findings with those previously reported for serotypes 4 and 5 porcine group A rotaviruses.
MATERIALS AND METHODS

Eleven gnotobiotic pigs were obtained by closed hysterotomy from a single sow and were randomly allocated to 1 of 3 stainless steel isolators where they were separated within isolators by partitions. Pigs were maintained germ-free by methods previously described. All pigs were fed steam sterilized SPF-Lac (Borden Inc., Norfolk, Virginia) 4 times daily. Clinical condition and fecal consistency for each pig were observed and recorded at each feeding time. Rectal swab samples were taken daily from each pig for virus isolation. At 2 days of age 8 pigs in 2 isolators were inoculated orally with 2.0 mls of virus inoculum (V) while the remaining 3 control pigs (C) were inoculated with 2.0 mls of virus free cell culture media. All pigs were fed within 30 minutes following inoculation. After inoculation, pigs were removed from the isolators and killed according to the following schedule: 2 V and 1 C pigs at 12 hours, 2 V pigs at 18 hours, 2 V and 1 C pigs at 24 hours, 1 V pig at 36 hours and 1 V and 1 C pig at 48 hours.

The ISU64 strain of rotavirus was plaque purified 3 times, passed 12 times in MA-104 roller cell cultures and passed 1 time in a gnotobiotic pig. The small intestine of the gnotobiotic pig was removed aseptically and a 10% homogenate was prepared in cell culture media. The inoculum was clarified by centrifugation, filtered through a 0.22 micron filter and the titer was determined by fluorescent focus assay to be 10^5.7 FFU/ml.

Rectal swab samples were taken from each pig for aerobic and anaerobic bacterial culture immediately prior to removal from the isolator. Pigs were anesthetized by intravenous administration of a mixture of 4 mg/kg xylazine and 0.5 mg/kg ketamine, the ileal-cecal junction was exposed via a ventral
midline incision and samples were sequentially removed from 5 locations as the small intestine was dissected free from the mesentery. The 5 sample locations were equidistant from one another with the first commencing 5 cm from the ileocecal junction and the last ending 5 to 10 cm from the pylorus. At each sample location three 4 cm long segments were harvested. One segment was frozen by immersion in liquid nitrogen. Two segments were ligated and fixed by intraluminal infusion and immersion in either B-5 mercurial fixative at 21 C or 3% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 at 4 C. Small intestine and colon contents were pooled and frozen at -70 C. Pigs were euthanatized by exsanguination.

Nucleic acid was extracted from intestinal content samples from each pig and polyacrylamide gel electrophoresis (PAGE) was performed. In addition, rotavirus isolation (VI) in MA-104 roller cell cultures and antigen capture enzyme linked immunoadsorbent assay (ELISA) for group A rotavirus (Rotazyme II*, Abbott Laboratories, North Chicago, Illinois) was attempted on each intestinal content sample. Aerobic and anaerobic bacterial culture on sheep blood agar plates was completed on rectal swab samples from each pig.

Frozen small intestine segments were embedded in OCT Compound (Miles Laboratories, Inc., Elkhart, Indiana) and sections were cut to a thickness of 6 um with a freezing microtome. Indirect fluorescent antibody methods were used to specifically stain rotavirus antigens using hyperimmune antisera to ISU-64 produced in a guinea pig as primary antibody and FITC conjugated anti-guinea pig sera produced in a goat (Organon Teknika-Cappel, West Chester, Pennsylvania) as secondary antibody.

B-5 fixed tissues were processed by routine paraffin techniques, microsectioned, and stained with hematoxylin and eosin. Villous height and crypt depth were measured for each
small intestine section with an ocular micrometer as previously described. Mean villous height and mean crypt depth were analyzed statistically using one-way analysis of variance. Differences between V and C pigs were determined significant at the level of $P < 0.05$. In addition, the interface between vacuolated and nonvacuolated enterocytes was measured for each villus.

Specimens were prepared for transmission and scanning electron microscopy from each glutaraldehyde fixed small intestine segment. Small 1 X 1 millimeter cubes of mucosa were trimmed from each segment within 24 hours of necropsy and fixed for an additional 24 hours in fresh glutaraldehyde fixative. Tissue cubes were processed and embedded in Embed 812 (Electron Microscopy Sciences, Ft Washington, Pennsylvania) by routine methods. Thin sections were stained with lead citrate and uranyl acetate and were viewed and photographed with a Hitachi H500 transmission electron microscope (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 75kV. Small intestine segments were incised along the mesenteric attachment and pinned open on a flat surface. The mucosa was gently rinsed with a stream of 0.01 M phosphate buffered saline. Three millimeter square portions were trimmed from the antimesenteric side of each segment and were processed using a modified thiocarbohydrazine method to crosslink osmium tetroxide. Specimens were dehydrated in graded ethanol and dried with a critical point drying apparatus in liquid carbon dioxide. Dried specimens were attached to aluminum stubs with metallic conducting paste and sputter coated with gold-palladium. Samples were viewed and photographed with a Cambridge Stereoscan S200 scanning electron microscope (Cambridge Instruments, Deerfield, Illinois) at an accelerating voltage of 10kV.
RESULTS

Clinical disease and gross lesions

All pigs were alert, consumed all liquid diet at each feeding and had semi-formed to formed feces at the time of inoculation and all C pigs continued similarly throughout the study. All V pigs developed severe diarrhea 14-18 hours following inoculation. Feces were watery and clear to slightly yellow in color and flocculent. By 20-24 hours following the onset of diarrhea, 2 of the 4 remaining V pigs were inactive, depressed and consumed little food until killed at 24 and 36 hours PI. The pig killed at 36 hours PI was moribund. No vomiting was observed in any of the pigs.

Control pigs and V pigs killed 12 hours PI did not have significant lesions. The stomachs contained food and there was chyle in the lacteals in the entire length of the small intestines. There were small to moderate amounts of dark brown homogeneous creamy small intestinal content and the colons contained moderate amounts of dark brown pasty to solid content. All V pigs killed at 18 hours and 24 hours PI had similar lesions. The hair coat was uniformly wet and soiled with diarrheic feces. The stomachs contained food and the distal 2/3 of the small intestines were flaccid and thin walled and were dilated with abundant watery yellow to brown colored fluid which contained suspended flocculent bits of food and flecks of blood. There was chyle in the lacteals in the proximal 1/3 to 1/2 of the small intestine and the colon was similarly dilated with watery fluid. The stomach was empty in the V pig killed at 36 hours PI and there was no chyle in lacteals of the small intestine. The small intestines were very motile and were devoid of contents. The colon contained a small amount of watery yellow colored
content. The V pig killed 48 hours PI had similar lesions with the exception that the stomach was full and the lacteals in the proximal 1/3 of the small intestine contained chyle.

**Virology and bacteriology**

No virus was demonstrated in intestinal contents from each of the C pigs by PAGE, VI or ELISA. Group A rotavirus was demonstrated in intestinal contents from each V pig by VI and ELISA and an RNA electrophoretic pattern characteristic of ISU64 was demonstrated by PAGE in all V pigs killed at 18 or more hours PI. No bacteria were isolated from rectal swab samples from any of the pigs.

**Immunofluorescence**

No specific fluorescence was observed in the small intestinal mucosa in any of the C or V pigs killed at 12 hours PI. By 18 hours PI the villi were shortened in the ileum and distal jejunum and nearly all remaining mucosal epithelial cells had bright granular evenly dispersed cytoplasmic fluorescence. There was fluorescent cellular debris present in the lumen. In the mid-jejunum the villi were slightly shortened and 1/2 to 2/3 of the cells on the distal 2/3 of the villi were fluorescent; however, the fluorescence within each cell was noticeably less intense than in the distal jejunum and ileum. Only occasional single or small groups of epithelial cells were fluorescent near the villous tips in the proximal jejunum and no cells were fluorescent in the duodenum. At 24 hours PI there was still strong granular intracytoplasmic fluorescence in most of the remaining epithelial cells near the tips of the short villi in the distal jejunum and ileum. There was also particulate
fluorescence of variable size and uneven distribution in the villous lamina propria. The villi in the mid-jejunum were still longer than those in the distal jejunum and ileum and approximately 1/5 of the epithelial cells distributed singly or in packets on the distal 1/2 of villi were strongly fluorescent. Fewer epithelial cells were fluorescent in a similar distribution on the villi of the proximal jejunum and again there was no fluorescence in the duodenum. At 36 and 48 hours PI there were occasional scattered fluorescent epithelial cells near villous tips and there was particulate fluorescence in the lamina propria of the distal jejunum and ileum. There was no fluorescence in the more proximal small intestine sampling sites.

**Light microscopy and morphometry**

Results of villous and crypt morphometry are graphically represented in Figure 1. There were no significant differences in villous height or crypt depth between C pigs or between C pigs and V pigs killed 12 hours PI. Villous atrophy was severe in the ileum by 18 hours PI and progressed to similar severity in the midjejunum by 24 hours PI. Villi were 1/10 to 1/5 the length of those in control pigs. Villus to crypt ratios were consistently less than 2.0. Crypts became progressively deeper at all sample locations and were all significantly different than those of controls by 48 hours PI.

The villi in all C pigs and V pigs 12 hours PI were long and finger-like (Fig. 2a); however, there were morphologic differences in villous enterocytes between small intestine sampling sites. In the duodenum the epithelial cells on the upper 72-80% of each villus had large clear apical cytoplasmic vacuoles, in the proximal jejunum the epithelial cells on the upper 82-94% of each villus were vacuolated and in the mid-
jejenum, distal jejenum and ileum all villous epithelial cells were vacuolated. The mucosal epithelial cell nuclei were all located in the cell apex in the duodenum and were all in a basal cellular location in the ileum. In the mid-jejenum the nuclei were in the basal portion of the epithelial cells on the lower half of each villus and were located in the apical, middle or basal portion of cells on the upper half of each villus.

Degenerative villous enterocytes were characterized by swollen, pyknotic or karyorrhectic nuclei, swollen rarified cytoplasm, multiple 0.3-1.0 um round refractile homogeneous eosinophilic intracytoplasmic inclusions, and detachment from adjacent cells and/or the basement membrane (Fig. 2b). The number and location of degenerate cells determined the severity of the villous lesions. At 18 hours PI the villi in the midjejunum were nearly normal with occasional degenerate enterocytes near the villous tips. In the distal jejenum and ileum, nearly all enterocytes were degenerate resulting in detached villous shaped shells of degenerate enterocytes and exposed shortened and condensed villous lamina propria (Figs. 2c and 2d). The base of villi in the ileum were populated by nonvacuolated enterocytes with slightly basophilic cytoplasm. By 24 hours PI, the villi in the proximal jejenum were identical to those in the midjejunum at 18 hours PI and the villi in the midjejunum at 24 hours PI were identical to those in the ileum at 18 hours PI. The villi in the distal jejenum and ileum at 24 hours PI were covered by a continuous layer of columnar nonvacuolated enterocytes with amphophilic cytoplasm. Numerous mitotic figures were present in epithelial cells in the crypts, between villi and on the bases of villi. There were groups of degenerate enterocytes on villus tips and occasionally single degenerate enterocytes on lateral villus surfaces. The lamina propria contained a variable amount of
eosinophilic exudate, nuclear debris and occasional small aggregates of macrophages and neutrophils. Villous atrophy was atypically severe in the pig killed at 36 hours PI and was consistent with the aforementioned more severe clinical disease (Fig. 2e). By 48 hours PI, villi were covered by a continuous layer of nonvacuolated columnar enterocytes with an occasional isolated degenerate cell on the distal 1/2 of the villus.

**Scanning electron microscopy**

Lesions visible by scanning electron microscopy were similar in character but followed a different time course (see above) in the midjejunum through the ileum. In all C pigs and V pigs 12 hours PI (Fig. 3a), villi were long and slender, enterocytes were uniform and formed a cobblestone-like surface, goblet cell openings were easily visualized, microvilli were dense and uniform and the extrusion zones contained few degenerate enterocytes. In the ileum at 18 hours PI (Fig. 3b), nearly all visible enterocytes were degenerate and were swollen and rounded or collapsed, had sparse microvilli and were at least partially detached from each other and the villus lamina propria. The lamina propria was exposed and retracted in a cone shape. By 24 hours PI, the atrophic villi in the ileum were again covered by a continuous layer of enterocytes, but there were no goblet cell openings visible (Fig. 3c). The enterocytes on the proximal 2/3 of each villus had somewhat irregular cell margins, were predominantly uniform in appearance and had dense and uniform microvilli. The distal 1/3 of nearly all villi was covered by swollen or collapsed, partially detached enterocytes with sparse irregular microvilli. At 48 hours PI, shortened villi in the ileum were tongue- or club-shaped and covered by a
nearly uniform layer of normal enterocytes with an occasional isolated partially detached degenerate enterocyte (Fig. 3d).

**Transmission electron microscopy**

Ultrastructural examination of the small intestinal mucosa did not reveal any difference in the morphology of virus-infected villous enterocytes between different sampling sites, but did demonstrate differences in the number of virus infected villous enterocytes.

Degenerative changes in virus infected villous enterocytes did not correlate with the stage of virus replication or with the amount of viroplasm and/or the number of virus particles within an infected cell. Cytoplasmic rarification was the most consistent and apparently earliest degenerative change followed by nuclear swelling and peripheral condensation of nucleoplasm, cell swelling with protrusion of the apical cell membrane, dilatation of the cytocavitary network, mitochondrial swelling, fragmentation of microvilli, the appearance of autophagosomes containing membranous and/or viral debris and progressive degeneration of cell membranes. Both productive and nonproductive virus infection of cells seemed to occur, with productive infection predominating. Productive infection was characterized by changes typically described for rotavirus including multifocal variably sized granular cytoplasmic viroplasms with variable numbers of peripheral electron dense core particles. Core particles were budding into the cisternae of the ER which contained variable numbers of double shelled particles and fewer single shelled particles (Figs. 4 and 5a). Less frequently, degenerative cells without double or single shelled virus particles in the ER, contained cytoplasmic membrane bounded or nonmembrane bounded electron dense
aggregates of viroplasm composed of crystalline arrays of target-like dense subviral particles (Figs. 4 and 5b). Those surrounded by a membrane were presumed to be within autophagosomes.

In areas with many virus infected cells, entire groups of virus laden swollen cells appeared to detach en masse. In contrast where there were fewer virus infected cells, collapsed virus containing degenerative cells with irregular cell margins projected into the lumen while retaining attachment via desmosomes with normal appearing subjacent enterocytes (Fig. 4). Collapsed cells containing virus were also occasionally trapped between the basement membrane and basal portions of adjacent normal enterocytes. Basal cytoplasmic extensions of enterocytes which normally project through the basement membrane into the lamina propria frequently contained virus. Macrophages in the lamina propria often had phagosomes containing virus and cell debris presumably from phagocytosis of infected enterocyte cytoplasmic extensions (Fig. 5c). In areas of villous atrophy where a high proportion of villous enterocytes were virus infected, there were often groups of degenerate enterocytes that had no morphologic evidence of virus infection (Fig. 5d).
Fig. 1: Villous height and crypt depth in control and virus inoculated pigs at 12, 18, 24, 36 and 48 hours PI. Error bars = S.E. of mean
Significantly different than the control at the 0.05 level.
Fig. 2a: Long slender villi. Ileum, C pig killed 12 hours PI. Bar = 100 um. HE

Fig. 2b: Swollen and degenerate villous enterocytes with multiple variably sized intracytoplasmic inclusions. Ileum, V pig killed 18 hours PI. Bar = 20 um. HE

Fig. 2c: Nearly all villous enterocytes are degenerate resulting in severe villous atrophy and erosions on villous tips. Ileum, V pig killed 18 hours PI. Bar = 100 um. HE

Fig. 2d: Nearly all villous enterocytes are degenerate resulting in severe villous atrophy and erosions on villous tips. Ileum, V pig killed 18 hours PI. Bar = 100 um. HE

Fig. 2e: Atypically severe villous atrophy. Ileum, V pig killed 36 hours PI. Bar = 100 um. HE
Fig. 3a: Long slender villi are characterized by uniform enterocytes forming a cobblestone-like surface, easily visualized goblet cell openings and extrusion zones that contain few degenerate enterocytes. Ileum, C pig killed 12 hours PI. Bar = 100 um

Fig. 3b: Nearly all villous enterocytes are degenerate resulting in severe villous atrophy and erosion of villous tips. Ileum, V pig killed 18 hours PI. Bar = 25 um

Fig. 3c: Atrophic villi are covered with a continuous layer of enterocytes. Enterocytes on villous tips are swollen and degenerate. Goblet cell openings are not evident. Ileum, V pig killed 24 hours PI. Bar = 25 um

Fig. 3d: Tongue or club shaped villi are partially regenerate and are covered by a nearly uniform layer of normal enterocytes. Ileum, V pig killed 48 hours PI. Bar = 50 um
Fig. 4: Partially collapsed nearly extruded degenerative rotavirus infected villous enterocyte is attached to subjacent normal enterocytes by tight junctions. Note the rotavirus within the ER, granular viroplasm within the cytoplasm, rarified cytoplasm, swollen and degenerate nucleus and sparse and degenerate microvilli. A virus infected enterocyte (lower right) which is in an earlier stage of degeneration is characterized by rarification of the cytoplasm, swelling and protrusion into the gut lumen and relatively normal microvilli. A degenerate cell (upper right) contains dense viroplasm composed of crystalline arrays of subviral particles (arrow). Ileum, V pig killed 18 hours PI. Bar = 1.0 um
Fig. 5a: Core virus particles are budding from the periphery of granular cytoplasmic viroplasm into the lumen of the ER which contains many double shelled virus particles. Villous enterocyte, ileum, V pig 18 hours PI. Bar = 500 nm

Fig. 5b: Dense viroplasm is composed of crystalline arrays of target-like subviral particles. No double shelled or single shelled virus particles are present. Villous enterocyte, ileum, V pig killed 18 hours PI. Bar = 150 nm

Fig. 5c: Macrophage in the lamina propria of an atrophic villus contains virus particles and cellular debris within a phagosome. Ileum, V pig killed 24 hours PI. Bar = 500 nm

Fig. 5d: Degenerate villous enterocytes. At higher magnification there is no evidence of virus infection. There are many degenerate enterocytes that contain virus in adjacent fields. Ileum, V pig killed 18 hours PI. Bar = 5.0 um
DISCUSSION

The clinical disease induced by ISU64 strain of group A rotavirus in neonatal gnotobiotic pigs was typical of that reported for serotypes 4 or 5 with the exception of a lack of vomition which is reported sporadically. Likewise, antigen distribution and the severity and distribution of villous atrophy was similar to that reported in most studies for serotypes 4 and 5.

It is interesting that there was a gradient of increased enterocyte susceptibility to virus infection from the proximal to distal small intestine. Villous enterocytes in the duodenum were resistant to virus infection whereas there were kinetic differences in enterocyte susceptibility between the midjejunum and ileum. The fluorescence of virus infected cells in the midjejunum at 18 hours PI was less intense than that of virus infected cells in the ileum whereas villous atrophy did not occur as rapidly in the midjejunum as in the ileum. These findings suggest that there was less viral antigen in the cytoplasm at 18 hours PI and slower cell degeneration of virus infected enterocytes in the midjejunum than in the ileum. The time between maximal atrophy in the midjejunum and in the ileum was only 6 hours which would not be adequate for a second round of viral replication in the jejunum. There were no ultrastructural differences noted between virus infected cells in the midjejunum and ileum at 18 hours PI that might explain the difference in kinetics, however there may have been subtle differences in the number of virus particles per cell that were not detected.

Virus binding studies done on villous enterocytes which were harvested from mice suggested that cell susceptibility to rotavirus infection may be related to the expression of virus receptors on the cell surface. The cell receptor for
rotavirus is not known, but it is attractive to speculate that variable distribution of receptors between villous enterocyte phenotypes may be responsible for the distribution of lesions and the kinetics of lesion development. The presence or absence of receptors may determine absolute cell permissiveness for viral infection while the number of receptors per cell might determine the average number of virus particles that infect a cell and thus determine the speed of cell degeneration.

Enterocyte phenotype differs between small intestine segments and between locations on villi and is influenced by complex and incompletely understood interactions between endocrine, paracrine and luminal factors. Differences were noted in morphologic phenotype of villous enterocytes in C pigs relative to the degree of cytoplasmic vacuolation and nuclear location between small intestine segments and between locations on the villi. Previous studies have demonstrated that apical vacuolation of villous enterocytes in pigs is related to cell age and indicates a minimal cell age of 3.8 days. The difference in percent of vacuolated enterocytes between small intestine segments in C pigs in this study suggests that the average villous enterocyte age was slightly younger in the more proximal portions of the small intestine relative to the more distal. When compared to the lesion distribution, these findings suggest that virus affinity may be somehow related to cell age. Previous studies in the kinetics of villous epithelial cell migration in day old conventional pigs demonstrated little difference in enterocyte migration rates between duodenum and ileum. This suggests that cell age alone is probably not responsible for the differences in virus affinity between intestinal segments. It is more likely that cell age (cell maturity) is related to the expression of permissive enterocyte phenotype within each
small intestine segment thus explaining the affinity of rotavirus for enterocytes on villous tips. There are apparently other factors independent of cell age that are unique to different small intestine segments which influence permissive enterocyte phenotypes.

Virus binding to cells is thought to be mediated through outer capsid glycoprotein VP7 while outer capsid protein VP4 is thought to be somehow involved in cell penetration.\textsuperscript{16} Low virulence strains of human group A rotavirus which cause asymptomatic infection in neonates share closely related VP4 but do consistently share similar VP7.\textsuperscript{28} It is not known whether there is a difference in lesion distribution between infants infected with low virulence strains relative to high virulence strains of rotavirus, however differences are likely. These findings suggest that virus affinity may not be related to virus binding but could alternatively be mediated through interaction of rotavirus VP4 and other cell molecules which vary between enterocyte phenotypes. Virus binding studies have not been done to determine whether rotavirus binding to enterocytes in different small intestine segments correlates to lesion distribution. Such studies would be helpful in determining what factors are involved in enterocyte susceptibility to rotavirus infection.

Light microscopy demonstrated prominent eosinophilic intracytoplasmic inclusions in degenerate enterocytes of virus infected pigs but no inclusions were observed in villous enterocytes of control pigs. No corresponding large aggregates were observed in the cytoplasm of immunofluorescent cells. These findings suggest that the inclusions were either aggregates of nonviral protein or alternatively were the result of fixation with mercury containing B-5 fixative. Since acidophilic intracytoplasmic inclusions are not commonly reported in rotavirus infection in pigs, they are most likely
related to the choice of fixative.

In general, the lesions in this study demonstrated with light, scanning electron and transmission electron microscopy were similar to those reported in serotype 4 and 5 group A rotavirus inoculation studies. Ultrastructural studies demonstrated virus infected cells that appeared to not produce mature virus particles. Similar dense crystalline viroplasms in lysosome-like bodies have not been reported in rotavirus infected pigs but have been described in simian rotavirus (SA11) infected MA104 cells. The most likely explanation would be cell infection by defective virus, however intrinsic cell associated factors cannot be ruled out.

There were moderate numbers of degenerate enterocytes with no morphologic evidence of virus infection in locations where there were high numbers of virus infected cells. A recent study in rotavirus infected mice suggested that edema resulting in ischemia may be important as a cause of cell damage in rotavirus-induced disease. Edema was variably present in the villous lamina propria of V pigs in this study, but was not a consistent lesion prior to evidence of virus replication as was reported in mice. Disruption of the mucous layer, changes in permeability caused by mechanical disruption of terminal bars by degeneration of adjacent virus infected cells, exposure of lateral cell membranes to luminal contents and edema and/or vasoconstriction resulting in ischemia are all possible causes of passive enterocyte death. It appears that a villus may compensate for the loss of a limited number of virus infected enterocytes by lateral movement of noninfected enterocytes. However, it is possible that when the number of virus infected cells reaches a critical threshold, compensatory mechanisms fail resulting in the degeneration of uninfected cells and disproportionately greater villus damage.
In conclusion, strain ISU64 group A rotavirus is pathogenic for neonatal gnotobiotic pigs and causes disease and lesions which are severe and indistinguishable from those reported for serotypes 4 and 5. The distribution and kinetics of villous atrophy suggested that villous enterocytes in the midjejunum were less susceptible to virus infection than those in the ileum.
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PART II. PATHOGENESIS OF A NEW PORCINE SEROTYPE
OF GROUP A ROTAVIRUS IN WEANED CONVENTIONAL PIGS
PATHOGENESIS OF A NEW PORCINE SEROTYPE
OF GROUP A ROTAVIRUS IN WEANED CONVENTIONAL PIGS

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ABSTRACT

A model for rotavirus inoculation in weaned conventional pigs fed a commercial pelleted grain based diet was developed to study the lesions and disease caused by the new serotype of porcine group A rotavirus represented by strain ISU64. Oral inoculation of susceptible weaned 24-day-old conventional pigs with ISU64 resulted in viral replication within villous enterocytes primarily on the distal 1/2 of villi in the duodenum through the distal jejunum. Villous atrophy was significant by 3 days after inoculation and resulted in villi that were approximately 1/3 the length of those in controls. Increased watery flocculent fluid was present in the small intestine of inoculated pigs, but no diarrhea resulted. Pigs remained alert and active with aggressive appetites throughout the experiment. In a previous study, inoculation of 2-day-old gnotobiotic pigs with an identical oral dose of ISU64 resulted in severe clinical disease. These findings suggest an innate age-related resistance to rotavirus-induced disease in pigs.
INTRODUCTION

Rotaviruses replicate nearly exclusively in villous enterocytes of mammalian and avian species. Rotaviruses are commonly associated with diarrhea in 2-3 week old suckling pigs and in recently weaned pigs. Three antigenically distinct groups of porcine rotavirus (A, B, and C) have been reported in the United States. Group A is the most common rotavirus group reported in swine herds and is the most common group associated with diarrhea in pigs in the U.S. Two serotypes of group A rotavirus, serotypes 4 (Gottfried) and 5 (OSU), have been commonly recognized and are well characterized as causes of diarrhea in suckling pigs in the United States. Two new serotypes of porcine group A rotavirus (strains ISU64 and ISU65) were recently isolated from diarrheic weaned pigs. One of these strains (ISU64) has been shown to be closely related to serotype 9 group A rotavirus (WI61) which was previously reported only in humans (Paul, unpublished). Studies in Neonatal gnotobiotic pigs demonstrated that the new porcine serotype represented by strain ISU64 causes lesions and disease which are severe and indistinguishable from those reported for serotypes 4 and 5 (Part I herein).

It has been suggested that rotavirus-induced disease in pigs is the most severe and lethal during the immediate postweaning period; however, the role of rotaviruses in postweaning diarrhea in pigs is unclear. Although rotavirus is commonly demonstrated in recently weaned diarrheic pigs, it is often found in combination with other enteric pathogens such as hemolytic strains of E. coli or TGE virus. Clinically normal pigs may also shed rotavirus during the postweaning period. The pathogenesis of rotavirus has not been investigated in weaned pigs fed a typical pelleted
Inoculation studies in neonatal gnotobiotic pigs have been the usual model used to investigate the pathogenesis of different rotavirus serotypes in pigs. The use of gnotobiotic pigs has the important advantage of allowing the study of rotavirus-induced disease without interference from other infectious agents; however, there are major differences between neonatal gnotobiotic and weaned conventional pigs that could potentially alter rotavirus-induced disease. Small intestine villous enterocytes differ in turnover rate, average age, surface glycoproteins, and morphology. Diets differ in the type of constituents and percent dry matter. Colonic digestive and absorptive functions differ substantially.

Factors determining the nearly exclusive replication of rotavirus within subpopulations of villous enterocytes are poorly understood. Virus receptor distribution or other intrinsic differences between permissive and nonpermissive cells may be involved in virus binding and/or replication. Rotavirus inoculation studies in mice and TGE virus inoculation studies in pigs suggest that villous enterocyte susceptibility to virus infection may be related to cell age. It is not known whether there is an innate age-related resistance to rotavirus infection in swine, however the younger average enterocyte age in weaned pigs compared to that in neonatal gnotobiotic pigs might cause weaned pigs to be more resistant to rotavirus infection and/or disease.

The objectives of this study were to develop a rotavirus inoculation model in recently weaned conventional pigs and to determine the pathogenicity of a new serotype of porcine group A rotavirus (ISU64) in recently weaned conventional pigs.
MATERIALS AND METHODS

A swine herd with mixed breed stock that was seronegative for TGE, had no history of clinical coccidiosis and farrowed in raised crates on plastic coated wire floors was used as a source of pigs. At 3 days of age, 16 pigs were removed from 2 litters, scrubbed thoroughly with an iodinated disinfectant solution (Weladol™, Pitman Moore Inc., Washington Crossing, New Jersey) and randomly assigned to 1 of 4 stainless steel gnotobiotic isolators where they were kept in communal groups. Isolator temperatures were slowly decreased from 35 to 27 C throughout the experiment. Pigs were fed a commercial milk substitute (SPF-Lac™, Borden Inc., Norfolk, Virginia) twice daily until they were 21 days of age, then they were weaned to an ad libitum diet of water and a commercial pelleted weaning ration (High Octane Baby Pig Chow™, Purina Mills, Inc., St. Louis, Missouri). At 24 days of age, 8 pigs were inoculated orally with 2.0 mls of either virus inoculum (V) or virus free cell culture media (C). Feed was withheld for 12 hours prior to inoculation and all pigs were fed immediately following inoculation. Two each V and C pigs were killed on days 3, 4, 5 and 14 post inoculation (PI). Clinical condition and fecal consistency were observed and recorded twice daily and every 4 hours after inoculation. Amprolium (Corid™, Merck & Co. Inc., Rathway, New Jersey) was added to the milk substitute or water twice daily to maintain an estimated intake of 10-15 mg/kg body weight/day. Sows were bled at farrowing and pigs were bled at 3, 7, 14, 21, 28* and 35* (*4 survivors only) days of age. Fecal samples were collected from the sows at farrowing and 3 days after farrowing and 2 rectal swabs and fecal samples (when available) were taken from each pig at 3, 7, 14 and 21 days of age. Fecal samples were collected daily from the 4 surviving pigs from 3-14 days PI.
Virus inoculum (ISU64 strain) was the same lot prepared and used for the gnotobiotic pig inoculation study (see Part I herein). Inoculum was stored at -70 C, slowly thawed in ice water and was retitrated by fluorescent focus assay to be $10^{6.2}$ FFU/ml.

At the appropriate sampling times pigs were removed from isolators, were anesthetized by intramuscular administration of a mixture of 0.5 mg/kg acepromazine, 5 mg/kg xylazine and 10 mg/kg ketamine and small intestine samples were removed and preserved in the same manner as for the gnotobiotic pigs in Part I herein except that 10% neutral buffered formalin was used instead of B-5 fixative for light microscopy samples. Contents from the small intestines and caecum from each pig were pooled and frozen at -70 C. Swabs of the intestinal mucosa were taken from the duodenum, jejunum and ileum of each pig for bacterial culture. Pigs were euthanatized by exsanguination.

Antigen capture enzyme linked immunoadsorbent assay (ELISA) for group A rotavirus (Rotazyme II™, Abbott Laboratories, North Chicago, Illinois) was performed on all fecal samples (or rectal swab washings if fecal samples were not available) from sows and pigs and on pooled gut contents from pigs. Virus was pelleted by high speed centrifugation from pooled intestinal content samples from each pig, nucleic acid was extracted and polyacrylamide gel electrophoresis (PAGE) was performed. Negative stain contrast electron microscopy (NCEM), using phosphotungstic acid at pH 7.2 as stain, was performed on intestinal content samples from each pig. Intestinal content samples were pooled from each 2 C pigs which were killed on the same day and rotavirus isolation (VI) was attempted in MA-104 roller cell cultures.

Fecal flotation and examination for coccidial oocysts was performed on all pig fecal samples. Aerobic bacterial culture
on sheep blood agar plates was completed on small intestine mucosal swab samples from each pig. Direct fluorescent antibody procedures for TGE virus were completed on frozen sections of jejunum and ileum from each pig.

Indirect fluorescent antibody serum titers for group A rotavirus were determined for the sera from the 4 surviving pigs collected at 3, 21, 28 and 35 days of age. Two-fold dilutions of pig serum were used as primary antibody and a 1:50 dilution of FITC conjugated anti-pig sera produced in a goat (Kirkegaard-Perry Laboratories Inc., Gaithezburg, Maryland) was used as secondary antibody to stain rotavirus (ISU64) infected MA104 cell monolayers in 24 well microtiter plates. End points were interpreted as the highest serum dilution that provided strong foci fluorescence when compared to a positive reference serum.

Samples were prepared for indirect immunofluorescent microscopy, light microscopy, scanning electron microscopy and transmission electron microscopy and each procedure was performed in a manner identical to Part I herein. Small intestine mucosal morphometry and statistical analysis were also completed as in Part I herein.
RESULTS

Clinical disease and gross lesions

All pigs acclimated to milk replacer within 24 hours and maintained aggressive appetites throughout the remainder of the experiment. Feces were gray and of a pasty to solid consistency. Pigs were clinically healthy throughout the experiment, gained weight consistently and attained weights of 5.5-6.8 kg at 21 days of age. No changes in attitude, appetite or fecal consistency were noted in any pigs following virus inoculation.

Differences in gross appearance of the intestines were noted between V and C pigs killed on days 3 and 4, but not on days 5 and 15 PI. The gross appearance of all C pigs and of V pigs killed on days 5 and 15 PI was the same. The stomachs contained abundant feed with a yellow ground grain appearance and the small intestines contained variable amounts of dark green grainy homogeneous content. There was chyle in the lacteals in the proximal 2/3 of the small intestine and there was prominent segmental motility in the small intestines. The ceca contained dark green homogeneous thick yet fluid contents; the spiral colons contained dark green homogeneous pasty to semisolid contents and there were formed feces in the rectums.

There were similar gross lesions noted in both V pigs killed on day 3 PI. The stomach contents were as described above. The small intestines were flaccid and thin walled in appearance and were dilated with a large volume of watery content. The content was light green and flocculent and quickly sedimented into stratified layers within the collection containers which was in contrast to the content from control pigs which remained homogeneous. There was chyle
in lacteals in the proximal 2/3 of the small intestine. The caeca contained dark green watery content and the proximal loops of the spiral colon contained green homogeneous thick liquid content. The content of the distal loops of the spiral colon and the rectums was indistinguishable from that in C pigs. No gross lesion were noted in other organs.

Both V pigs killed on day 4 PI were similar to the V pigs killed on day 3 PI, but the changes were not as dramatic. The small intestines were more motile, were not as dilated and did not contain as high a volume of content. The content was intermediate in consistency between the V pigs killed on day 3 PI and the C pigs. The content was fluid and somewhat flocculent and sedimented into layers; however, it was not as watery as in the V pigs from day 3 PI. The colonic content was the same as in the C pigs.

Virology

Group A rotavirus was not demonstrated by ELISA in any fecal samples from sows or from pigs prior to virus inoculation. Analysis of pooled intestinal contents from each C pig did not demonstrate group A rotavirus by ELISA or VI. Virus was not demonstrated by NCEM and virus nucleic acid bands were not demonstrated by PAGE.

Intestinal contents from V pigs killed on days 3, 4 and 5 PI contained group A rotavirus as demonstrated by ELISA and contained 11 RNA bands in a pattern typical of strain ISU64 rotavirus by PAGE (Fig. 1). NCEM demonstrated high numbers of single and double shelled rotavirus particles in contents from V pigs killed 3 days PI, moderate numbers in V pigs 4 days PI and low numbers in V pigs 5 days PI. ELISA examination of daily PI rectal swab samples from the 2 V pigs killed on day 14 PI demonstrated consistent group A rotavirus shedding on
days 3-7 PI and intermittent shedding on days 8-11 PI. No rotavirus was demonstrated in the intestinal contents from the same 2 V pigs killed on day 14 PI by ELISA, negative stain contrast electron microscopy or PAGE.

Fecal flotations, bacteriology and serology

No coccidial oocysts were demonstrated in fecal samples collected on 3, 7, 14 or 21 days of age from any pig. Bacterial culture of mucosal swab samples from the duodenum, jejunum and ileum of each pig did not demonstrate differences between gut segments or between pigs. Low numbers of nonhemolytic \textit{E. coli} of mixed colony types, alpha hemolytic \textit{Streptococcus} sp., \textit{Klebsiella} sp., \textit{Pseudomonas} sp., and \textit{Proteus} sp. were variably present. No hemolytic strains of \textit{E. coli} were isolated. Serology results suggested seroconversion in V pigs but not C pigs following virus inoculation (Fig. 2).

Immunofluorescence

Direct fluorescent antibody procedures did not demonstrate TGE virus in the jejunum or ileum of any pigs. Indirect fluorescent antibody procedures demonstrated group A rotavirus specific antigen in the small intestinal mucosa of V pigs killed on days 3 and 4 PI but not in V pigs killed on days 5 and 14 PI or in any C pigs. There was bright granular evenly dispersed fluorescence in the cytoplasm of villous enterocytes and less intense scattered particulate fluorescence in the villous lamina propria. Most fluorescent enterocytes were on the distal 1/2 of atrophic villi although occasionally fluorescent cells extended the entire length of a villus (Figs. 3a and 3b). The distribution and proportion of fluorescent cells varied between pigs (Table 1).
Table 1. Distribution and proportion of villous enterocytes with group A rotavirus specific fluorescence in the small intestinal mucosa of virus-inoculated pigs

<table>
<thead>
<tr>
<th>Pig</th>
<th>Days PI</th>
<th>Duodenum</th>
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Light microscopy and morphometry

Results of villous and crypt morphometry are graphically represented in Fig. 4. The most severe villous atrophy was present on day 3 PI in the duodenum through the middle jejunum where villi were 1/3 normal length. There was significant villous atrophy in nearly all V pigs in the duodenum through the distal jejunum on days 3-5 PI. There was no villous atrophy in the ileum of any V pigs. Crypts in most segments from the duodenum through the distal jejunum in V pigs were significantly deeper than in C pigs.

Examination of the small intestinal mucosa in all C pigs and V pigs killed 14 days PI revealed long slender villi in the duodenum progressing to shorter villi with broad bases in the ileum (Fig. 3c). The villous enterocytes in all locations had nonvacuolated cytoplasm and basal nuclei. There were progressively more goblet cells in crypts and on villi from the duodenum through the ileum. There were few neutrophils in
Light microscopic lesions in the small intestinal mucosa of V pigs on day 3 and 4 PI varied in severity (Fig. 4), but were similar in nature between segments. Villi were short with broad bases and were often fused laterally. Most villi had variegated margins on the distal 1/2 caused by scattered degenerate enterocytes that were in various stages of detachment (Fig. 3d). There were fewer degenerate enterocytes on villi of V pigs from day 4 PI relative to day 3 PI. Degenerate enterocytes were characterized by cloudy swelling, detachment from adjacent enterocytes, nuclear swelling with peripheral condensation of chromatin, pyknosis and karyorrhexis. Degenerate swollen detached enterocytes and cellular debris was often in the lumen between villi. There were inconsistent changes in the villous lamina propria including serous edema, dilatation of lymphatics, small to moderate aggregates of neutrophils, increased numbers of macrophages and pyknotic nuclear debris. The crypts were deep and lined by immature basophilic epithelial cells that often contained mitotic figures. There were fewer goblet cells in crypts and on villi than in C pigs. By day 5 PI, villi were less atrophic and were covered by a continuous layer of uniform columnar enterocytes. Some enterocytes on villous tips had prominent apical cytoplasmic vacuoles.

**Scanning electron microscopy**

The villi in all C pigs and in V pigs from 14 days PI were covered by a uniform layer of enterocytes with dense microvilli, goblet cell openings were easily visualized and villous extrusion zones contained few degenerate cells (Fig. 5a). Occasional bacteria were adherent to the villous surface. Villi were long and slim in the duodenum, were of
moderate length and conical in the midjejunum and shorter and club- or leaf-shaped in the ileum.

The lesions in the duodenum through the distal jejunum were similar for all V pigs on days 3 and 4 PI. The villi were short and laterally adherent in groups sometimes forming crests composed of multiple villi (Fig. 5b). Villous enterocytes on the distal portions of villi were degenerate and partially detached. Degenerate enterocytes were characterized by swelling and round or collapsed outlines and sparse and shortened microvilli. Some enterocytes were attenuated and formed bridges between villi. The only changes noted in the ileums of all V pigs from days 3 and 4 PI were slightly increased numbers of degenerate enterocytes in the extrusion zones on villous tips. By day 5 PI in the duodenum through the distal jejunum, there were few degenerate enterocytes on villous tips and conical apical extensions protruded from fused bases creating longer partially regenerate villi. The ileum was normal in V pigs on day 5 PI.

Transmission electron microscopy

Ultrastructural examination revealed differences in the number of virus infected enterocytes between small intestine segments consistent with the differences seen by immunofluorescence microscopy; however, there were no other significant differences noted between segments. Characteristics of virus infected cells were identical to those described in gnotobiotic pigs in Part I herein (Fig. 6a). Although there were enterocytes that contained large numbers of viral particles, a high proportion of degenerate enterocytes had few viral particles. There were also a large number of degenerate virus infected enterocytes that had cellular and viral debris within autophagosomes. On days 3
and 4 PI, there were very high numbers of macrophages in the lamina propria which contained virus and cellular debris within phagosomes (Fig. 6b). There were few degenerate villous enterocytes that did not have morphologic evidence of viral infection.
Fig. 1: Polyacrylamide gel electrophoresis of rotavirus RNA extracts. Lanes 1-3 contain standards. Lanes 4-11 contain intestinal content RNA extracts from V and C pigs. Note that the pattern in all V pigs is typical of strain ISU64

Lane 1 = Serotype 4 type strain (Gottfried)
Lane 2 = Serotype 5 type strain (OSU)
Lane 3 = ISU64
Lane 4 = Pig 2, virus inoculated, 3 days PI
Lane 5 = Pig 3, virus inoculated, 3 days PI
Lane 6 = Pig 6, virus inoculated, 4 days PI
Lane 7 = Pig 7, virus inoculated, 4 days PI
Lane 8 = Pig 10, virus inoculated, 5 days PI
Lane 9 = Pig 11, virus inoculated, 5 days PI
Lane 10 = Pig 15, virus inoculated, 14 days PI
Lane 11 = Pig 1, control, 3 days PI
Fig. 2: Indirect fluorescent antibody titers for group A rotavirus in 2 V and 2 C pigs suggest seroconversion in only V pigs. Titers are expressed as the inverse of the serum dilution.
Fig. 3a: Indirect immunofluorescence demonstrates group A rotavirus specific intracytoplasmic fluorescence in the cytoplasm of enterocytes on villous tips. Midjejunum, V pig killed on day 3 PI. Bar = 100 um

Fig. 3b: Indirect immunofluorescence demonstrates virus infected enterocytes sometimes extending along the lateral surfaces of villi. Midjejunum, V pig killed 3 days PI. Bar = 100 um

Fig. 3c: Moderately tall villi with broad bases have nonvacuolated cytoplasm and nuclei in the basal portion of enterocytes. Midjejunum, C pig on day 3 PI. Bar = 100 um. HE

Fig. 3d: Short, often fused villi have variegated margins caused by swollen degenerate partially detached villous enterocytes dispersed among normal enterocytes. Midjejunum, V pig killed on day 3 PI. Bar = 100 um. HE
Fig. 4: Villous height and crypt depth in control and virus inoculated pigs on days 3, 4 and 5 PI. Error bars = S.E. of mean
Significantly different than the control at the 0.05 level.
Fig. 5a: Moderately tall villi with broad bases are characterized by a uniform layer of enterocytes, easily visualized goblet cell openings and villous extrusion zones with few degenerate cells. Midjejenum, C pig killed on day 3 PI. Bar = 100 um

Fig. 5b: Fused groups of atrophic villi have large numbers of degenerate swollen partially detached enterocytes on their tips. Midjejenum, V pig killed 3 days PI. Bar = 100 um
Fig. 6a: Subviral particles form at the periphery of granular viroplasm and bud into the RER resulting in low to moderate numbers of double shelled virions in the cisternae of the ER. Villous enterocyte, midjejunum, V pig killed 3 days PI. Bar = 300 nm

Fig. 6b: Many macrophages in the villous lamina propria contain viral debris (left 2 arrows) and membranous cellular debris (upper right arrow) within phagosomes. Midjejunum, V pig killed 3 days PI. Bar = 1.0 um
DISCUSSION

The methods employed in this study resulted in a model of rotavirus infection in weaned conventional (WC) pigs. No pigs shed rotavirus prior to inoculation and C pigs remained free from virus infection. In virus inoculated pigs, rotavirus consistently replicated within enterocytes on the apical 1/2 of villi in the proximal 2/3 of the small intestine resulting in villous atrophy and accumulation of excess fluid in the small intestinal lumen. Pigs shed virus in feces consistently for 7 days PI and intermittently until day 11 PI. No other known enteropathic etiologic agents were demonstrated in either C or V pigs. The environmental conditions within the gnotobiotic isolators used in this study were nearly ideal and arguably not representative of more stressful conditions found in typical hot nurseries used for early weaning on intensively managed swine farms. Our goal was to study the pathogenesis of ISU64 strain of group A rotavirus in WC pigs in the same environment in which we previously studied the pathogenesis of ISU64 inoculation in neonatal gnotobiotic (NG) pigs so that valid comparisons could be made. Stressors such as reduced or variable ambient temperature, inconsistent feeding times, increased population density or mixing of communal groups at weaning time might alter the severity of rotavirus-induced disease. In addition, factors such as the level of sanitation and type of diet might also have an influence. This type of model offers the advantage of allowing careful control of the type and degree of stress applied to pigs during rotavirus inoculation and also allows concurrent or sequential inoculation with additional enteropathogens.

This is the first reported study of the pathogenesis of group A rotavirus in weaned conventional pigs. Although the clinical disease and lesions that were produced by ISU64 in NG
pigs (Part I herein) were as severe as those reported for serotypes 4 and 5, it is not known what the virulence of either serotype 4 or 5 compared to ISU64 is in WC pigs. Differences in virulence between strains of rotavirus have been reported in humans, mice and calves; however, a single study did not demonstrate differences in virulence between a serotype 4 and serotype 5 strain of porcine group A rotavirus in NG pigs.\textsuperscript{6,8,13,16,25} It is possible that other strains or serotypes of group A rotavirus may cause more or less severe disease in WC pigs.

Although inoculation of 2-day-old gnotobiotic pigs with an identical oral dose of ISU64 strain group A rotavirus resulted in severe clinical disease (Part I herein), no clinical disease was observed in weaned 24-day-old conventional pigs. These findings suggest an innate age-related resistance to rotavirus-induced disease in pigs. There are several possible explanations for the less severe disease in WC pigs compared to NG pigs.

It is possible that the inoculation dose was inadequate in WC pigs. The pH in the stomach of pigs decreases with age following birth. Low stomach pH in the WC pigs may have resulted in less virus reaching the small intestines when compared to the NG pigs; however, this is unlikely since studies have demonstrated that the stomach pH of weaned 3-4 week old pigs ranges from 3.5-4.5 and rotavirions are stable in a pH range of 3-7.\textsuperscript{10,17} Use of a buffer with the virus inoculum in weaned pigs might be advantageous. The dose of virus was not adjusted for weight differences between the 2 kg NG pigs and the 6.5 kg WC pigs; however, it is unlikely that such a small difference in weight adjusted viral dose would alter the outcome of the challenge study. A virus dose titration study in WC pigs would be interesting to determine whether more severe lesions or disease would occur at higher
Villus atrophy was not as severe in the WC pigs compared to NG pigs and probably resulted in less severe maldigestion and malabsorption. There was an increased volume and altered consistency of small intestinal contents in the virus inoculated WC pigs which suggests some degree of small intestine mucosal dysfunction. The distribution of villous atrophy also differed between the groups of pigs; the most severe villous atrophy was in the proximal small intestine in the WC pigs and in the distal small intestine in the NG pigs. This could be significant since the relatively normal ileum in the WC pigs would have substantial digestive and absorptive capacity and could compensate for some of the maldigestion and malabsorption in the more proximal small intestine.

The cecal and proximal colonic contents of virus inoculated WC pigs were more fluid than in control pigs suggesting that the ileum alone could not completely compensate for the rotavirus-induced upper small intestinal dysfunction. Colonic absorption of excess fluid from the small intestine appeared to be important in the prevention of diarrhea in the virus inoculated WC pigs. Differences in large intestine capacity for fluid absorption between WC and NG pigs is also a likely factor that affected the severity of rotavirus-induced disease. Studies in TGE virus inoculated 3-day-old and 21-day-old pigs demonstrated a greater colonic capacity to absorb excess fluid in 21-day-old pigs that was related to increased fermentation and volatile fatty acid (VFA) production. It was suggested that colonic absorption of water is dependent upon VFA absorption. As such, the sterile colon of a NC pig would be unable to ferment excess lactose (resulting from malabsorption in the small intestine) to VFA's and would therefore be unable to absorb fluid.

The difference in diet between NG and WC pigs might also
affect the severity of rotavirus-induced disease in several ways. The difference in the proportion of fluid in the diet (approximately 80% for the NG and 15% for the WC pigs) could potentially place a greater absorptive burden on the gastrointestinal tract of the NG pigs. Rotavirus inoculation studies done in 21 to 28-day-old colostrum deprived pigs which were fed liquid diets resulted in mild to moderate diarrhea. These findings support the contention that the relatively dry diet in the WC pigs helped prevent diarrhea. The diet may also have an effect on the efficiency of colonic fermentation and fluid absorption. The dry pelleted diet fed the WC pigs contained 4.5% crude fiber compared to no crude fiber in the liquid diet fed to the NG pigs. Crude fiber is known to promote microbial fermentation.

The reason for the difference in lesion distribution between NG and WC pigs is not clear. Possible factors involved in individual enterocyte susceptibility to viral infection were discussed in Part I herein. The turnover time for villous enterocytes is 7-10 days in NG pigs and is 2-4 days in WC pigs. Since ISU64 replication occurred primarily within enterocytes on the distal 1/2 of villi in susceptible segments of the small intestine in WC pigs, cell age may be one factor which influences cell susceptibility. Enterocytes may need to be 3-4 days old to be susceptible to ISU64 infection. The greater proportion of enterocytes older than 2 days on the villi of NG pigs may be the cause of high cell susceptibility and severe villous atrophy. Differential distribution of rotavirus receptors is one possible explanation for spatial differences in enterocyte susceptibility to rotavirus infection. Rotavirus binding studies using enterocytes harvested from different locations in the small intestine of both NG and WC pigs would be helpful in determining whether lesion distribution is related to a
difference in the number or distribution of rotavirus receptors.

Virus yield per enterocyte might be different between NG and WC pigs. Ultrastructural examination of rotavirus infected enterocytes in WC pigs suggested that there may be less virus produced per cell than in NG pigs. If this were substantiated by virus titration studies, less virus per round of virus replication in WC pigs might result in less virus shedding, lower oral doses and less severe disease in WC pigs.

This study demonstrates that ISU64 strain of group A rotavirus will replicate in, cause significant lesions in and cause dysfunction of the small intestine mucosa of weaned conventional pigs. It is not clear whether it causes economically significant disease. It is possible that in uncomplicated rotavirus infection in WC pigs, the colon may be able to completely compensate for small intestinal dysfunction. It is also possible that increased stress in rotavirus-inoculated WC pigs might result in more severe clinical disease. Rotavirus infection may also be significant as an initiator in bacterial postweaning diarrheic disease. Hemolytic strains of *E. coli* are often associated with severe postweaning diarrheic syndromes and studies have suggested that rotavirus infection in weaned pigs may create an enteroenvironment which favors the selection and growth of enteropathogenic *E. coli*. Studies have not been done to clarify the role of rotavirus in hemolytic *E. coli* associated postweaning diarrhea of pigs. This model of rotavirus infection in postweaning pigs should enable further investigation into the role of rotaviruses in postweaning diarrheic syndromes in pigs.
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GENERAL DISCUSSION AND SUMMARY

These studies demonstrated that the new porcine serotype of group A rotavirus represented by strain ISU64 is pathogenic for neonatal gnotobiotic (NG) and weaned conventional (WC) pigs. The lesions and disease produced by ISU64 in NG pigs were severe and were comparable to those reported for serotypes 4 and 5 in NG pigs.\textsuperscript{14,40,78,147,159} Since these studies represent the first report of rotavirus inoculation in weaned conventional pigs fed a typical pelleted weaning diet, the lesions and disease caused by ISU64 in WC pigs could not be compared to other porcine serotypes.

There were marked differences in the pathogenesis of ISU64 between NG and WC pigs which included differences in the kinetics of lesion development, differences in lesion distribution, differences in the severity of lesions and differences in clinical disease. These findings highlight the need to study the pathogenesis of rotavirus-induced disease in conventional pigs more typical of those in which naturally occurring disease is observed. In general, lesions and disease were more severe in NG than in WC pigs. The results of these studies suggested several potential mechanisms for the differences in ISU64-induced disease between NG and WC pigs.

The first and most obvious mechanism was that there were proportionately fewer virus susceptible villous enterocytes in the WC pigs than in the NG pigs. Further, the distribution and morphology of susceptible enterocytes suggested that enterocyte susceptibility to viral infection may be related to cell age.

A second potential mechanism suggested in these studies for the less severe disease in WC pigs was the difference in lesion distribution in the small intestines. There was severe
villous atrophy in the distal small intestines of virus inoculated NG pigs; however, the distal small intestines in the virus inoculated WC pigs was not severely damaged by virus replication. The normal digestive and absorptive functions in the distal small intestine of the WC pigs could have partially compensated for the virus induced maldigestion and malabsorption in the more proximal portions.

A third reason suggested in these studies for the less severe disease in WC pigs was an apparent increased capacity of the colon to absorb additional fluid originating from the damaged small intestine. This difference may be related to the presence of microbial fermentation and volatile fatty acids in the colon of WC pigs.6

A fourth reason suggested in these studies for the less severe disease in WC pigs was the difference in diet fed to the WC and NG pigs at the time of virus inoculation. The NG diet contained 85% water while the WC diet contained 15% water. Increased water in the diet of the NG pigs likely places a greater absorptive burden on the gastrointestinal tract of NG pigs compared to WC pigs.

These studies also provided new insights into the pathogenesis of group A rotavirus disease in pigs. The difference in the kinetics of villous atrophy between small intestine segments in NG pigs suggested that there may be degrees of villous enterocyte susceptibility to viral infection. This possibility was further supported by ultrastructural examination of virus infected enterocytes from both NG and WC pigs where it appeared that there was less virus being produced per cell in WC pigs. This might influence the time required for individual cell degeneration and also influence the time needed for villous atrophy. The interaction between the kinetics of villous atrophy and the kinetics of villous regeneration may also have significant
bearing on whether clinical disease occurs. Ultrastructural examination of virus infected villous enterocytes in NG and WC pigs also demonstrated a subpopulation of cells that did not contain mature double shelled viral particles, but instead contained intracytoplasmic electron dense aggregates of viroplasm which were composed of crystalline arrays of dense target-like subviral particles. These cells were all in stages of degeneration suggesting that nonproductive viral replication within cells is lethal.

Lastly, these studies resulted in the development of a model for rotavirus infection in weaned conventional pigs which allowed the first reported study of group A rotavirus pathogenesis in WC pigs. Although ISU64 strain of group A rotavirus replicated in, caused significant lesions in and caused dysfunction of the small intestinal mucosa of weaned conventional pigs, it is not clear whether it causes economically significant disease. These findings in WC pigs suggest that group A rotavirus may not be as virulent of a pathogen in WC pigs as had been previously suggested.167

There are many questions that remain unanswered regarding the role of group A rotaviruses in postweaning diarrheic syndromes. Would an increased dose of ISU64 produce more severe disease in WC pigs? Are there differences in virulence between group A rotavirus strains or serotypes in WC pigs? Can a normally functioning colon always compensate for small intestine dysfunction in group A rotavirus infection in WC pigs? Would increased environmental stress at the time of virus inoculation result in more severe disease? Does the composition of weaning diets affect rotavirus-induced disease? Does group A rotavirus infection predispose to intestinal colonization by hemolytic strains of E. coli as some have
suggested? This model of rotavirus infection in postweaning pigs should lead to future investigations which are designed to answer these and other important questions.
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