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Alexander Dale Hall

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pathology

Glenn L Waxler

Major professor

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STUDIES OF THE EFFECTS OF AN ATTENUATED TRANSMISSIBLE GASTROENTERITIS VIRUS ON GNOTOBIOTIC PIGS

By

Alexander Dale Hall

A DISSERTATION

Submitted to
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ABSTRACT

STUDIES OF THE EFFECTS OF AN ATTENUATED TRANSMISSIBLE GASTROENTERITIS VIRUS ON GNOTOBIOTIC PIGS

By

Alexander Dale Hall

Nine litters of gnotobiotic pigs (n=86) and 1 litter of conventional pigs (n=8) were used in studies designed to determine: (1) the effect of variable ambient temperature on pigs orally exposed to a commercially available attenuated transmissible gastroenteritis (TGE) vaccine virus, (2) the lethal dose 50 (LD_{50}) of the virus in pigs, and (3) the immune responses following oral exposure to the virus.

When pigs from 4 litters (n=37) were exposed to the virus at 2 different ambient temperatures (16 C and 32 C), lesions of villus atrophy and lymph node hyperplasia appeared more severe in pigs kept in the cold environment than in those kept in the warm environment. Both infected and control pigs kept at 16 C had extensive subcutaneous edema. Hepatocellular vacuolization was present throughout the livers of the pigs kept at 32 C but not in those kept at 16 C.

The LD_{50} in a litter of conventional pigs (n=8) was determined to be 1 ml of a 1/100 dilution of the vaccine, but in a litter of gnotobiotic pigs (n=11) the LD_{50} could not be determined since all pigs survived.

Four litters (n=38) were used to determine the humoral and cell-mediated immunity elicited after oral exposure to the vaccine virus. Serum virus neutralization titers were detected in 13 of 24 exposed pigs killed at 2 or 3 weeks following exposure, with the titers ranging from 4 to 128. No titers were detected in the remaining 11 pigs. Lymphocytes from the peripheral blood and the mesenteric lymph nodes were obtained at necropsy and used to determine cell-mediated immunity. An attenuated TGE virus was used to stimulate lymphocyte replication *in vitro*. Although a wide range of results was obtained, no consistent levels of cell-mediated immunity were detected. Statistical analysis of the lymphocyte stimulation data revealed no significant differences between the control and exposed pigs.

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To all of my fellow graduate students, residents and friends in the Department of Pathology, I owe the deepest debt of gratitude of all. Their sacrifice of time and energy became the backbone of this project.

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LITERATURE REVIEW

Introduction

To be alive today, an individual must exist while under a constant barrage of physical forces, chemical reactions, and microbial invasions. The complexities of "living" are infinite, yet each infinitesimal interaction could become the force that separates life from death. For centuries man has undertaken the limitless quest to understand life by attempting to isolate one event or interaction and then methodically dismantling that interaction into its individual components.

Disease is but one aspect of life that depends upon a multitude of variables for its inception, progression and conclusion. In order to define and understand a diseae process, man must first eliminate as many of the variables as possible. To accomplish this elimination, gnotobiology was conceived.

Gnotobiotics in Research

The term "gnotobiotic" was derived from two Greek words, *gnotos*, meaning known, and *bios*, meaning life. When the term gnotobiotic is used in research, it means an individual that has a known microbial population (Reyniers, 1959). In many cases these individuals have *no* microbes either in their environment or within their tissues (and therefore they could also be designated as "germfree"). Thus,

gnotobiotic animals must be derived and maintained under strict aseptic conditions.

Although many species of animals have been used in gnotobiotic research, the pig has proven to be one of the most useful and practical large animal species to deal with (Landy et al., 1961). The sows tend to be quite consistent on their farrowing times, so that the pigs can be successfully derived by cesarean section at 112-113 days of the gestation period when accurate breeding records are kept. Another advantage to porcine gnotobiotics is that within 24 hours after birth, the pigs can be trained to eat by themselves from trays or pans. The usually large litter size provides the researcher with ample numbers of experimental subjects.

In dealing with gnotobiotic animals, it must be recognized that, because of their sterile environment, they are not completely "normal" in their physical development. It is now known that antigenic stimuli play an important role in developing tissues, especially within the gastrointestinal tract. Generally speaking, germfree or gnotobiotic animals have poorly developed lymphoid tissue and low levels of serum immunoglobulins (Dubos et al., 1963). Due to the 6-layered epitheliochorial placenta of swine, gnotobiotic pigs are born without any detectable serum immunoglobulins (Kim et al., 1966). Kim et al. (1966) did demonstrate immunologic competence in gnotobiotic pigs by the presence of serum antibodies within 48 hours of antigenic stimulation. Krüml et al. (1969) demonstrated that, although there was no developmental difference of the thymus between gnotobiotic and conventional pigs, the other lymphoid tissues, including the spleen, mesenteric lymph nodes and Peyer's patches, developed much more slowly in the gnotobiotic pigs. Krüml reported that the lymph nodes lacked the presence of germinal

centers even after 9 weeks of life. Waxler (1979a) noted the presence of primary nodules within lymph nodes of gnotobiotic pigs but that these nodules were composed of small, mature lymphocytes and only a few germinal centers were seen.

Miniats and Valli (1973) studied the gastrointestinal tracts of gnotobiotic pigs and found that the intestines were lower in weight when compared to conventional pigs of the same age. This weight difference could at least be partly attributed to the gnotobiotic intestinal wall being thinner with less cellularity in the lamina propria and muscularis. Also, the associated lymphoid tissue appeared to be less well developed (Miniats and Valli, 1973).

In conventional pigs, intestinal epithelial vacuolization is related to the age of the animal and the location along the intestinal tract (Moon, 1972). Moon found no vacuolization at any age in the porcine duodenum; jejunal vacuolization was present for the first 11 days of life and ileal vacuolization persisted for 2 to 3 weeks after birth. Moon et al. (1973) also found this cellular vacuolization to be a function of cell age (approximately 4 days post-DNA synthesis). When compared to conventional pigs of the same age, 4-week-old gnotobiotic pigs have longer villi, shallower crypts, and distal villus epithelial vacuolization (Moon et al., 1973; Heneghan et al., 1979). Moon described these vacuoles as membrane-bound, supranuclear, non-lipid material.

One peculiarity of gnotobiotic laboratory animals (rats, mice, rabbits and guinea pigs) that is not seen in pigs is the presence of a distended cecum. This problem tends to be associated with animals that are coprophagic and has been incriminated as the cause of death of animals in several gnotobiotic colonies (Miyakawa and Luckey, 1968).

Porcine Viral Enteritis

According to Moon (1978), there are 4 basic mechanisms in the pathogenesis of diarrhea: hypermotility, increased permeability, hypersecretion, and malabsorption. Moon goes on to state that diarrhea resulting from an infectious disease is never the result of *hypermotility*, although hypermotility might occur secondarily in some diseases.

Certain inflammatory processes of the intestinal tract might cause increased hydraulic pressure and *increased permeability* (i.e., increased pore size), permitting a greater flow of fluids and ions through the membrane down the pressure gradient from the vascular system to the gut lumen. This mechanism is often associated with protein-losing enteropathies.

Hypersecretion is usually associated with bacterial enteritides that are the result of enterotoxin release by pathogenic bacteria. In these cases it is usually the crypt cells that are primarily involved, since crypt cells secrete more than they absorb and villus cells absorb more than they secrete. The bacterial enterotoxin causes hypersecretion of the epithelial cells by stimulating an intracellular increase in cyclic AMP.

The viral enteric diseases of swine all cause diarrhea because of *malabsorption*. Both the coronavirus of transmissible gastroenteritis (TGE) and the porcine rotavirus penetrate and destroy the absorptive epithelial cells of the intestinal villus. With the destruction of these cells, the unabsorbed food particles that remain in the intestinal lumen act as an osmotic gradient to draw fluid out of the remaining enteric tissue. The process is even further enhanced by the fermentation of some of this ingesta by enteric bacteria. In an attempt to replace

the lost epithelium of the villus stump, undifferentiated crypt cells migrate onto the villus without undergoing their natural maturation process. This leads to increased numbers of undifferentiated cells within the intestine which, in turn, could produce a slight to moderate secondary hypersecretory effect. Similar viral diseases of other species act by the same mechanism.

There are basically only 2 pathogenic enteric viruses of swine other than TGE virus. Porcine rotavirus typically affects young pigs 10 to 14 days of age (Theil et al., 1978). The morbidity is usually high (>80%), yet the mortality is generally low (7-20%) (Bohl et al., 1978). It is now believed that rotavirus was also the cause of the previously described enteric infections of young pigs known as "milk scours" or "white scours" (Bohl et al., 1978). In natural outbreaks, the incubation period is generally 16 to 24 hours, with recovery occurring in approximately 3 days (Theil et al., 1978). This infection in young pigs could easily be confused with TGE due to the similar type of epithelial cell destruction and resulting villus atrophy (Pearson and McNulty, 1977).

Another coronavirus-like agent (CVLA) causing enteric disease in any age swine was recently reported in England (Chasey and Cartwright, 1978) and Belgium (Debouck and Pensaert, 1980). Again, the primary clinical sign is diarrhea, and the major lesion of the intestine is villus atrophy. Recovery is the usual outcome and occurs within 4 to 5 days. At least one study has shown that there is no cross protection between this viral agent and TGE virus (Debouck and Pensaert, 1980).

All of the enteric viral diseases of swine are influenced by the age of the animal, the immune status of the pig or its dam and the resulting colostral and milk antibody concentrations, ambient

temperature, degree of viral exposure, other stress factors (such as weaning, shipment, etc.), and the presence of other pathogenic microbes, such as *Escherichia coli* (Woode, 1978). Bohl (1977) maintains that the increased frequency of enteric infections encountered in U.S. swine operations is due to (1) rearing of large numbers of swine in crowded and often unsanitary conditions and (2) continuous farrowing which maintains a constant susceptible population on the farm.

Porcine Immunology

As previously stated, the 6-layered placentation of the sow serves as a formidable barrier against the entry of maternal antibodies into the developing fetus. Several studies have shown that the porcine fetus is capable of producing its own antibodies while *in utero*. Schultz et al. (1971) detected levels of IgM in the fetus that had been inoculated with sheep erythrocytes at the 74th day of gestation. Later, Curtis et al. (1974) discovered that the fetus was capable of antibody production by the 55th day of gestation. While working with gnotobiotic pigs, Yabiki et al. (1974) were able to detect very slight amounts of IgG in the serum of 2-day-old animals. These antibodies then disappeared at 5 days of age. Immunoglobin G was again present in the serum at 12 days after birth. Serum levels of IgM and IgA were not observed until ages 9 and 16 days, respectively. All classes of antibodies gradually increased in amount over the succeeding weeks of life (Yabiki et al., 1974).

As is the case with most mammals, newborn pigs can only absorb maternal antibodies from the colostrum for approximately the first 24 hours of life (Miller et al., 1962). In 1973, Baintner reported the presence of a colostral trypsin inhibitor (CTI) in the sow's colostrum.

This CTI is itself resistant to pepsin digestion, although the digestive tract of a newborn pig contains little or no pepsin and no hydrochloric acid. Thus, the colostrum passes unharmed through the stomach and duodenum and the CTI then protects the immunoglobulins from trypsin breakdown. Within approximately 24 hours of farrowing, the colostral concentration of CTI declines and the levels of proteases in the intestine of the young pig increase. Therefore, it appears that the cessation of antibody absorption by the young pig is due more to the onset of protein digestion by pancreatic enzymes than to a loss of ability to absorb these glycoproteins (Baintner, 1973). During this period of antibody absorption, IgG, IgM, and IgA are all absorbed nonspecifically. Thus, the serum levels of various antibodies in the 1-day-old pig are usually just a reflection of the colostral levels of these same antibodies (Bourne, 1973).

In 1976 Porter compared relative immunoglobulin levels in colostrum and milk from sows with those of women and cows. His findings were as follows:

	colostrum	milk
human	90% IgA	87% IgA
porcine	80% IgG (14% IgA)	62% IgA
bovine	82% IgG_1	66% IgG_1

Of these 3 species, the sow maintains the highest levels of antibody in the milk throughout the lactation. Ninety percent of the IgA in the sow's colostrum and milk is produced locally in the mammary tissue (Porter, 1976). In comparison, the cow does not produce significant amounts of immunoglobulin in the mammary gland. The origin of the IgA-producing plasma cells found within the sow's mammary tissue is believed to be the intestinal tract (Porter, 1976).

Svendsen and Brown (1973) calculated that a 2-day-old pig consuming approximately 250 ml of milk per day receives 1.1 grams of IgA. This amount of IgA is about 3 times the level that is usually present in the serum of a pig of that age. At 3 weeks of age, this same pig would consume 500 ml of milk that would contain a total of 1.6 g of IgA, which is 30 times greater than its serum level of IgA.

Secretory IgA is a very important immunoglobulin for the baby pig since the secretory piece on this antibody molecule prevents it from being degraded by enzymes (Porter, 1976). Immunoglobulin A can also spread through epithelial mucus more efficiently than either IgG or IgM (Porter and Allen, 1972). Because of these features, IgA plays a major role in protecting the young pig from pathogens which gain entrance to the body via the gastrointestinal tract. The constant bathing of the neonatal intestinal mucosa by maternal IgA that arrives via the colostrum and milk has been termed "lactogenic immunity" (Bohl et al., 1972a). Lactogenic immunity plays a major role in protecting the nursing pig against TGE.

Transmissible Gastroenteritis

Etiology

The causative agent of TGE belongs to the coronaviridae family (Bohl et al., 1969). Coronaviruses have an enveloped, helical nucleocapsid with an RNA genome. The capsids have surface projections that have been described as club-shaped or pear-shaped, which add slightly to the overall diameter of approximately 110 nm. Because of the lipid envelope surrounding this virus, it is both ether and chloroform labile. Cartwright et al. (1965) also noted this virus to be both thermo- and photosensitive. However, the TGE virus does appear to be relatively

stable at a low pH (Cartwright et al., 1965) and is able to withstand low concentrations of bile (Harada et al., 1968).

Pathogenesis

In 1946, Doyle and Hutchings were the first to describe "a transmissible gastroenteritis of pigs." Since then this same disease has been recognized in Japan, England, and throughout much of the European continent (Haelterman, 1971). In the years that followed these initial reports, TGE has been the subject of various exhaustive studies.

Haelterman (1971) defined TGE as "an acute diarrheal disease of swine caused by a virus which replicates in and destroys columnar epithelium of the small intestine resulting in an acute malabsorptive state." Under conditions of natural exposure, the TGE virus gains access to the susceptible mucosal epithelium either by ingestion or inhalation (Haelterman, 1971). This virus appears to have a tropism for the mucosa of the jejunum and ileum. Haelterman (1972) was able to detect the presence of virus by the immunofluorescence of individual mucosal cells at 5 hours postexposure (PE). At 9 to 12 hours PE, rows of cells fluoresced, possibly indicating a second generation of virus. By 16 to 18 hours PE, the majority of mucosal epithelial cells displayed varying amounts of fluorescence. Desquamation of degenerated villus epithelium began at 12 hours PE and shortening of villi was observed before 18 hours had elapsed following exposure. By 24 hours PE, villus atrophy was noted throughout the jejunum and ileum, and the sloughed columnar epithelium had been replaced by low cuboidal epithelium (Haelterman, 1972; Pensaert et al., 1970). Normally, the villi of a young pig measure about 795 μ in length, and the crypts are 110 μ deep, resulting in a villus to crypt ratio of 7:1. Yet at the time of

widespread villus atrophy, the villi average 180 μ in height and the crypts have elongated to a depth of 157 μ. Thus, the new villus to crypt (v:c) ratio becomes approximately 1:1 (Hooper and Haelterman, 1966; Haelterman and Hooper, 1967). If the young pig survives, the villi begin to lengthen and the covering epithelium returns to a columnar morphology at 3 days PE. By 6 days PE, the majority of villi have attained the pre-exposure height, and immunofluorescence is usually absent by PE day 7 (Haelterman, 1972).

The malabsorption syndrome seen with TGE is a consequence of 2 factors: (1) decreased surface area in the intestine and (2) decreased enzymatic activity of the enterocytes (Hooper and Haelterman, 1966). The epithelial cells covering the tips of the villi of newborn pigs are replaced much more slowly than those same epithelial cells of a 3-week-old pig. Moon (1971) observed that the jejunal villi of a 1-day-old pig are approximately 1029 μ long and the crypts are 110 μ, giving a v:c ratio of 9:1. The corresponding villi of a 3-week-old pig are 494 μ long, while the crypts are 186 μ, resulting in a v:c ratio of 3:1. Moon (1971) also noted that the villus absorptive cells of a newborn pig are replaced very 9 to 10 days, wehreas the enterocyte turnover rate of a 3-week-old pig is 3 to 4 days. It is possible that the prolonged enterocyte replacement rate is 3 times longer in the newborn pig because the v:c ratio is 3 times greater (Moon, 197; Moon et al., 1975; Mouwen, 1970). In any event, it is the prolonged enterocyte replacement rate that makes TGE so devastating in very young pigs.

The nearly complete loss of mature absorptive cells results in a vastly decreased amount of surface area. To compensate for the lost cells, the crypts become hyperplastic and deepen. The increased mitotic activity in the crypts produces abundant immature and undifferentiated

enterocytes which rapidly migrate over the shortened villi to protect the denuded lamina propria (Thake et al., 1973; Moon et al., 1973). This covering of the villi by undifferentiated cells is both advantageous and detrimental to the pig. On the plus side, these immature cells appear to be resistant to further viral (TGE) infection, possibly due to the lack of the necessary receptor (Shepherd et al., 1979). However, since the majority of the small intestine is now lined by undifferentiated crypt cells, which lack the essential enzymes needed for nutrient absorption, malabsorption occurs. The presence of crypt-like cells covering the villi was demonstrated enzymatically by Kerzner et al. (1977). They found increased thymidine kinase activity as well as decreased sucrase activity at this point in the progression of TGE lesions. Thymidine kinase is an enzyme associated with crypt cells and sucrase is associated with absorptive cells. Therefore, their respective increases and declines would indicate the types of cells present in the intestine. Maronpot and Whitehair (1967) detected decreased enteric levels of acid phosphatase,alkaline phosphatase, leucine aminopeptidase, and malic and succinic dehydrogenase. Cross and Bohl (1969) observed a complete loss of lactase activity at the onset of diarrhea. Thus, the decreased presence of these various enzymes would easily account for the malabsorption.

Swine of all ages are susceptible to TGE, yet the severity of lesions and the recovery rate are both age-related phenomena. In one study, Kemeny and Woods (1977) reported that sows which have been exposed to the TGE virus by various routes (intranasal, intravenous, and intramammary) all became clinically ill for a period of 4 to 5 days and then recovered. These sows were found to be shedding the TGE virus through their milk, nasal secretions, and feces for 2 to 5 days. All

of the pigs that were nursing these sows also became ill and 67% of the young pigs died. In a reverse study situation, Kemeny et al. (1975) inoculated nursing pigs with the TGE virus and then returned them to their dams. All sows became clinically ill within several days of the initial exposure to their suckling offspring.

Morin et al. (1974), working with 4- to 6-month-old feeder pigs, found them to be equally susceptible to the TGE virus and to exhibit the same clinical signs of illness after exposure. Recovery was usually complete within a week, but virus was present in the feces for the entire 7 days following exposure. Morin et al. (1978) also demonstrated that movement of feeder pigs from one farm to another could be one of the most important mechanisms of spread for the TGE virus. Morin and his associates monitored susceptible feeder pigs that were taken to areas of high pig concentration such as sale barns, auctions and buyer holding areas. These pigs apparently contracted the TGE virus at the various concentration centers and then were shipped to the various buyers' farms, where they broke with the clinical signs of TGE and began shedding the virus, thus exposing susceptible pigs at the new farm to TGE.

Larson et al. (1979) also investigated various possibilities of viral transfer, including studying the dog as a carrier. They orally exposed 14 puppies to the TGE virus and found that, even though none of the pups exhibited clinical signs of illness related to TGE, they continued to shed the virus for as long as 10 days PE. Therefore, loose-running dogs could also serve as a farm-to-farm transfer mechanism.

Clinical Signs

The clinical signs associated with transmissible gastroenteritis have not changed since the first reported cases in 1946 (Doyle and Hutchings). As mentioned earlier, the severity of the clinical illness and the subsequent mortality rate are functions of the age of the susceptible pig. Mortality rates of up to 100% are not uncommon in pigs under 1 week of age who are nursing nonimmune dams (Haelterman and Hooper, 1967). Following an incubation period of 14 to 20 hours, the sudden onset of clinical illness is usually marked by transient vomiting and profuse watery diarrhea (Haelterman and Hooper, 1967; Cornelius et al., 1968). The feces are typically yellow in color, have an offensive odor, and may contain small milk curds. In the very young suckling pigs (under 10 days of age), rapid dehydration is a prominent sequel to the diarrhea. These youngsters may lose up to 25% of their body weight in 24 to 48 hours. Pigs of this age appear to be more susceptible to drastic dehydration because they have a higher percentage of fluid comprising their total weight (Cornelius et al., 1968). Clinical pathologic parameters of these pigs reveal increased packed cell volume (PCV) due to dehydration, decreased plasma bicarbonate (probably lost in the feces) and a metabolic acidosis as a combined effect of the lost bicarbonate and increased plasma ketones and other acidic cell waste products. Although plasma sodium levels remain normal, plasma chloride and potassium concentrations are increased to compensate for the lost bicarbonate in the case of chloride and because of the shift from intracellular fluid to extracellular fluid for potassium (Cornelius et al., 1968). Terminal renal damage (Bay et al., 1951) exacerbates the metabolic acidosis, maintains the hyperkalemia and produces an absolute increase in blood urea nitrogen (BUN) (a

relative increase in BUN is seen as a result of the dehydration) (Cornelius et al., 1968). Death usually occurs in the very young pig in 3 to 5 days following exposure to the TGE virus (Haelterman and Hooper, 1967) and is attributed to a combination of the severe dehydration, metabolic acidosis, hyperkalemia, and cardiac dysfunction (which is a direct consequence of the hyperkalemia) (Cornelius et al., 1968; Kent and Moon, 1973).

Suckling pigs over 3 weeks of age, feeder pigs, and adult breeding swine are generally much less severely affected by the TGE virus (Moon et al., 1973; Olson, 1971; Norman et al., 1973). The marked reduction in severity of clinical signs and mortality in older swine is directly attributable to the increased rate of replacement of villus enterocytes in the small intestine (Moon, 1971; Moon et al., 1973; Moon et al., 1975). When clinical illness related to TGE occurs in older pigs, it is marked by occasional vomiting, inappetence, and transient diarrhea following a slightly prolonged incubation period of 24 to 72 hours (Norman et al., 1973). Lactating sows may experience temporary agalactia during the course of the illness. Hematologic studies indicate an initial pronounced leukopenia (Olson, 1971). Complete recovery occurs within 5 to 7 days after exposure.

Transmissible gastroenteritis appears to have a definite seasonal occurrence, since the majority of outbreaks in the United States occur in the colder months of November through April (Haelterman, 1971). This could be due in part to the physical properties of the virus which were previously mentioned--thermo- and photosensitivity. Transmissible gastroenteritis is also a very highly contagious disease because *all* susceptible swine on a farm will usually exhibit clinical signs of illness within 2 to 3 days. Finally, TGE seems to be more common or

enzootic on those farms that have less than adequate sanitary conditions and a continual new source of susceptible pigs (Haelterman, 1971).

Gross Lesions

There have been numerous descriptions of the lesions associated with TGE in the scientific literature over the last 34 years. With few exceptions, these lesions have been extremely consistent, no matter whether they arose from natural field cases or from experimental infections. Likewise, these descriptions have been nearly identical even though the origin of the paper may have been Japan, England, Belgium, or the United States.

Since the fatalities are nearly always restricted to pigs under 2 weeks of age, the lesions reported here are those seen in this age group. External evaluations of young pigs that have succumbed to TGE indicate dehydration and weight loss. Often the rear quarters are wet and stained with yellow fluid feces (Trapp et al., 1966; Bay et al., 1951). Internally, the stomach is usually filled with partially curdled milk, while the entire small intestine is distended by a combination of gas and fluid ingesta (Trapp et al., 1966; Cross and Bohl, 1969; Hooper and Haelterman, 1969). Due to the expansion of these organs, their walls appear very thin and nearly transparent. Hooper and Haelterman (1969) also noted small foci of hemorrhage in the submucosa of the stomach which could be a result of ruptured vasculature during vomition. Cross and Bohl (1969) reported an absence of chyle in the mesenteric lymphatics, which would be indicative of a lack of fat digestion and absorption in the intestine. In many cases of uncomplicated TGE, villus atrophy of the jejunum and ileum can be seen either grossly or with the aid of a dissecting microscope, especially when sections of

these areas are compared to the relatively normal duodenum (Hooper and Haelterman, 1969; Trapp et al., 1966).

Histopathologic Lesions

Histologic sections of jejunum and ileum from pigs infected with the TGE virus often contain markedly shortened or atrophied villi as well as deepened, hyperplastic crypts of Lieberkühn (Trapp et al., 1966; Hooper and Haelterman, 1969; Kent and Moon, 1973). As previously stated, the normal v:c ratio of 7:1 is often reduced to a v:c ratio of 1:1 in TGE (Trapp et al., 1966; Hooper and Haelterman, 1969). The affected villi may be partially fused to adjacent villi, but this does not lead to cystic crypts (Trapp et al., 1966). In some areas, the villi may be denuded of their epithelium (Bay et al., 1951). However, the majority of the villi are covered by viable, low cuboidal epithelium which may or may not be vacuolated (Hooper and Haelterman, 1969). The cytoplasmic borders of the epithelial cells may be indistinct, and the brush border of microvilli may be shortened or absent (Hooper and Haelterman, 1969). The nuclei of these epithelial cells appear immature with less dense chromatin and enlarged or multiple nucleoli (Trapp et al., 1966; Hooper and Haelterman, 1969).

The glandular epithelial cells lining the crypts, whose proliferation is thought to be regulated by the villus epithelial cells through a negative feedback mechanism (Kent and Moon, 1973), are often crowded and appear to be pseudostratified (Trapp et al., 1966). The mitotic activity of these cells is notably increased (Hooper and Haelterman, 1969).

While working with virus-infected gnotobiotic pigs, Trapp et al. (1966) remarked that the lamina propria was free of edema, hemorrhage and inflammatory cell infiltrate.

Bay et al. (1951) described degeneration and cloudy swelling of renal tubular epithelium to the point of luminal occlusion. Collecting ducts filled with cellular debris were also observed in these pigs.

Electron Microscopy

Intestinal tissues from pigs infected with TGE virus have also been studied with the aid of both the transmission and scanning electron microscopes. Thake (1968) was one of the first researchers to report the ultrastructural lesions associated with TGE. When he compared jejunal epithelial cells from control pigs to the same cells from pigs infected with TGE, he found that the infected cells had (1) sparse, short microvilli in the brush borders, in contrast to the numerous long microvilli of uninfected cells; (2) the presence of annulate lamellae in the cytoplasm of the villus epithelial cells (normally these lamellae are only present in immature, rapidly differentiating cells); (3) large fat globules in the cytoplasm, which might indicate the cells' inability to transport this fat to the lymphatics; and (4) a lack of distinct differences between villus cells and crypt cells as exemplified by the presence of numerous free polyribosomes in the villus epithelial cells (normally these vast numbers of polyribosomes are seen only in crypt cells).

Wagner et al. (1973) also studied the lesions of TGE with the transmission electron microscope. They observed microcanaliculi projecting into the epithelial cells as extensions of the plasmalemma. Coronaviral particles that were present in the cytoplasm of absorptive

epithelial cells were surrounded by a trilaminar membrane similar to that of the microcanaliculi. It was postulated that these microcanaliculi may be the route of entrance into the cell for this coronavirus. Generally duodenal epithelial cells lose these structures during the first 24 hours after birth, thus explaining why these cells are generally spared during TGE viral infection. However, microcanaliculi persist for up to 3 weeks in the epithelial cells of the jejunum and ileum. These microcanaliculi are believed to normally function in the absorption of macromolecules.

Scanning electron microscopy was employed by both Olson et al. (1973) and Waxler (1972). Waxler's SEM studies confirmed the light microscopic findings of unaltered duodenal villi, atrophied jejunal villi and fusion of villi above the levels of the crypts. Because of the villus fusion and atrophy, the bases of the villi and the crypt openings were more readily observable in the virus-exposed pigs. Olson et al. (1973) reported the presence of increased amounts of mucus and slightly shortened jejunal villi at 15 hours postexposure (PE), more severe villus shortening, villus fusion and exposure of crypts by 24 hours PE, and nearly complete villus atrophy by 48 hours PE.

Immunity to TGE

While pondering various methods of immunization against TGE, there are 2 major considerations to keep in mind. First, since the newborn pig experiences the most devastating effects of TGE, it is this age group that needs the greatest protection. Second, because the fetus cannot obtain maternal antibodies while *in utero* and because of the high risk of exposure to the TGE virus anytime after birth, the primary mode of protection for the porcine neonate must be via the colostrum and milk.

Thus, a vaccination procedure must be devised that will protect the suckling pig by providing high titers of viral neutralizing antibodies throughout the sow's lactation. It is now painfully evident that this is much more easily said than done.

Following natural exposure to and infection with the TGE virus, an active immunity to TGE arises that is based upon the production of IgA (Bohl et al., 1972b; Abou-Youssef and Ristic, 1972). The young pig experiences optimal protection when sufficient levels of the secretory form of IgA are present in the colostrum and milk. Secretory IgA is unique among the immunoglobulins because of the presence of a complex nonglobulin component called the "secretory piece" (Abou-Youssef and Ristic, 1972). This secretory piece is credited with protecting the IgA molecule from enzymatic breakdown. At the same time, IgA has a natural affinity for mucosal surfaces, persists in the sow's milk throughout lactation, and has a direct inhibitory role upon the TGE virus by preventing this pathogen from adhering to the intestinal mucosa (Bohl et al., 1974). Abou-Youssef and Ristic (1975) conducted studies which demonstrated that the *in vitro* neutralizing abilities of IgG and IgA were equal in preventing the cytopathic effects of the TGE virus propagated in cell cultures. However, this equality does not exist *in vivo*, for only IgA can adequately neutralize the TGE virus in the living pig (Bohl and Saif, 1975).

With this information at hand, several different routes of vaccination were attempted. Although intramuscular injection of the TGE virus stimulated high serum and colostrum levels of anti-TGE antibody, it was primarily IgG. So, not only was this IgG ineffective in protecting the suckling pig, but the levels in the milk rapidly declined during the first week of lactation (Bohl et al., 1972b; Bohl et al., 1975).

Intramammary (IMM) inoculation with the TGE virus also stimulates the production of high levels of anti-TGE antibodies which are, in turn, secreted in the milk. However, just as with IM vaccination, IMM vaccination primarily induces IgG production (Saif et al., 1972; Bohl et al., 1974). From these studies and others like them, Saif et al. (1972) hypothesized that oral-intestinal infection stimulates intestinal lymphoid tissue and mesenteric lymph nodes to produce anti-TGE antibody which is primarily IgA. This IgA then enters the serum and is deposited in the mammary secretions and/or anti-TGE, IgA-producing plasma cells migrate from the intestine and relocate in the mammary gland. On the other hand, parenteral infection (IM or IMM) stimulates peripheral lymph nodes to produce anti-TGE antibody that is primarily of the IgG class of immunoglobulin which offers no significant protection to the nursing pig (Bohl et al., 1972a; Saif et al., 1972). The use of intramammary injections of TGE was also explored by Thorsen and Djurickovic (1971) and Shibley et al. (1973). They found that 3 IMM vaccinations produced levels of immunoglobulins that were adequate to protect the nursing pigs. These researchers also demonstrated that all of the mammary glands secreted equal amounts of antibodies, even though only a few had been inoculated.

Stone et al. (1974) performed several quantitative studies on antibody levels. They found that the antibody titers in the colostrum were higher than those in that sow's serum. These levels gradually decreased so that, by the 11th day after farrowing, the levels of the antibodies in the milk were below the serum levels of the sow.

Saif and Bohl (1977, 1979) reported several studies comparing intranasal inoculation with oral inoculation. They concluded that oral and intranasal inoculations that ultimately led to intestinal infection

with the TGE virus would lead to the production of antibodies by the sow that would, in turn, be protective to the nursing pigs. If, however, there was intransal infection only, the resulting antibodies were not protective for the suckling neonates.

Redman et al. (1978) attempted intrafetal inoculation of pigs with an attenuated TGE virus. This procedure was performed at 74, 77 and 95 days of gestation. All inoculated piglets produced virus-neutralizing antibodies to TGE, and there was evidence of villus atrophy and repair in their intestines. Most of the uninoculated littermates were sero-negative and succumbed to challenge with a virulent TGE.

In summary, it is the local "lactogenic effect", i.e., the constant bathing of the nursing pig's intestinal mucosa by the titers of secretory IgA in the sow's milk, that offers the most effective protection against a potentially lethal exposure to the TGE virus.

Vaccines

There are currently 3 modified live virus vaccines against TGE available in the United States (TGE-VAC, Diamond Labs, Des Moines, Iowa; TGE-Guard, Syntex Agribusiness, Inc., Des Moines, Iowa; and TGE Vaccine, Ambico, Inc., Dallas Center, Iowa). Both the Diamond and Syntex companies recommend 2 intramuscular vaccinations for the sow during the last trimester of pregnancy. Ambico recommends that the first 2 doses of its vaccine be administered to the sow orally followed by a third dose given intramuscularly. Furuuchi et al. (1975) made a comparison between the Japanese attenuated virus and their virulent virus. They found that the virulent virus was resistant to trypsin and pepsin while the attenuated virus was inactivated by these enzymes. Both viruses were able to withstand a pH of 3. Both viruses would replicate at

ambient temperatures of 37 and 30 C. The attenuated virus would continue to replicate at a temperature of 28 C while the virulent virus would not.

Furuuchi et al. (1978) orally administered 10^7 Tissue Culture Infective Doses$_{50}$ ($TCID_{50}$) of an attenuated TGE virus to 3-day-old pigs. These pigs exhibited no undesirable postvaccinal reactions and produced good levels of neutralizing antibodies. The pigs in the northern climates produced more antibodies than those in the southern provinces. Approximately 25% of the nonvaccinated penmates also produced antibody against this virus.

Welter (1980) orally exposed 1-day-old pigs who were nursing non-immunized sows to the Ambico modified live vaccine virus and demonstrated antibody titers at 3 to 5 days of age. Graham (1980) used the same Ambico vaccine orally on 5-day-old pigs that were nursing immunized sows and concluded that these pigs were able to produce an active immune response to TGE without interfering with the passive immunity provided by the dam.

In 1966, while working with an attenuated TGE virus grown on canine kidney cells, Welter reported that oral administration of this vaccine caused no ill effects in baby pigs. However, Larson et al. (1980) described a study in which young pigs that had been orally inoculated with a similar attenuated TGE vaccine virus became clinically ill and died. In this study, the incubation period and the course of the disease were longer than what is seen with a field strain of the virus. Waxler (1979b) reported on a similar study using gnotobiotic pigs. He also produced clinical illness and lesions consistent with TGE after orally exposing young gnotobiotes to a modified live vaccine virus. Waxler also indicated a prolonged incubation period and length of disease due to this strain of virus. In yet another experiment, Waxler and Miskena

(1979) made 3 serial passages of the TGE vaccine through gnotobiotic pigs and found that this attenuated virus retained its ability to produce lesions but did not show any evidence of increasing virulence due to the pig-to-pig passage.

Furuuchi et al. (1976) orally administered an attenuated TGE virus to newborn pigs that had received colostrum. They found that this virus underwent diminished replication if the pigs were kept at 21 to 22 C yet replicated well when the pigs were kept in an ambient temperature of 10 to 11 C. Those pigs kept at 31 to 34 C exhibited little to no attenuated viral replication and rapidly died when challenged with a virulent strain of TGE. Fifty percent of the pigs kept at 18 to 22 C withstood a virulent challenge 4 days after oral inoculation with the attenuated virus. Furuuchi hypothesized that since the protection to challenge had arisen in 4 days, cell-mediated immunity must play an important role in the defense mechanism against the TGE virus.

Garwes et al. (1979) compared the antigenicity of 3 vaccine preparations: (1) whole virus, (2) purified viral surface projections, and (3) purified subviral protein. Only the first 2 stimulated viral neutralizing antibody production in the sow after intramammary inoculation. However, only IgG was detected either in the serum or in the colostrum.

Morilla et al. (1976) compared a field strain of the TGE virus (the Illinois strain) with the Miller-high passage (M-HP) tissue culture-adapted strain. Although both strains caused antibody production in exposed sows, the Illinois strain induced higher levels of IgA than the M-HP strain (which induced predominantly IgG), and only the anti-Illinois IgA antibodies prevented viral replication in the suckling pigs when challenged. The Illinois strain caused marked intestinal

lesions, while the M-HP strain did not. This difference in tissue damage may account for the difference in immunoglobulin stimulation. Additional studies by Morilla et al. (1977) proved that these same 2 viruses were the same size, had the same general characteristics, and had identical antigenicity.

Finally, Woods and Pedersen (1979) conducted a series of cross protection studies between the TGE virus and the coronavirus that has been incriminated in feline infectious peritonitis (FIP). Pigs vaccinated with the FIP virus had no detectable antibodies to TGE.

Detection of Immunity

As has been briefly alluded to in previous sections of this dissertation, there are 2 aspects of the immune system which apparently play a role in the protection of the pig against the TGE virus. These are humoral immunity and cell-mediated immunity. The most reliable and accurate means of detecting and quantitating antibodies against the TGE virus is the virus neutralization procedure. This technique requires a susceptible cell culture population and a cell culture-adapted TGE virus. Obtaining or producing a strain of TGE virus adapted to tissue culture survival is probably the most difficult obstacle to overcome in establishing this procedure in a laboratory. Many researchers working with TGE rely on this procedure (Woods, 1979; Morilla et al., 1976; Bohl et al., 1972a; Furuuchi et al., 1978). A description of this process will be detailed under Materials and Methods.

Cell-mediated immunity (CMI) against the TGE virus can be observed by several methods. Woods (1977, 1979) used leukocyte migration-inhibition in one study to measure CMI against the TGE virus. He first observed the migration inhibition 7 days after exposing the pigs to

the TGE virus. This inhibition lasted for at least 35 days (Woods, 1977).

Shimizu and Shimizu (1979a) worked with 2 procedures to delineate CMI with respect to TGE. The first is lymphocyte proliferation. Since this procedure was employed in this author's experiment, the techniques involved can be found under Materials and Methods. In their experiment, Shimizu and Shimizu (1979a) found positive stimulation 7 days after exposure to TGE in 2- to 3-month-old pigs. Maximum stimulation occurred at 14 days PE. Lymphocytes from Peyer's patches and mesenteric lymph nodes stayed reactive for 110 days after exposure, while lymphocytes from the spleen and peripheral blood lost their reactivity in 20 to 30 days PE. Shimizu and Shimizu (1979b) also demonstrated the presence of cytotoxic lymphocytes by using virus-infected autochthonous swine testicular cell cultures as target cells. Again, they collected lymphocytes from orally exposed 2-month-old pigs and found that cytotoxicity then peaked at 21 days PE, and the effect was slightly greater in lymphocytes from peripheral blood and the spleen. These results all indicate that the most effective protection against the infection produced by the TGE virus is a combination of humoral and cell-mediated immunity.

Probably one of the most reliable and rapidly performed diagnostic tests for the detection of the TGE virus is the fluorescent antibody technique (Roberts et al., 1973; Solorzano et al., 1978). Since this procedure was also employed by this author to confirm the presence of the TGE virus in these studies, a further explanation of the technique can be found under Materials and Methods.

Ambient Temperature

There appear to be several poorly understood interactions of age and ambient temperature on intestinal transit time in neonatal mammals. It has been shown in rats and mice that intestinal transit time accelerates with age. Ambient temperature also can have a direct effect on transit time in that higher temperatures can increase intestinal motility. Conversely, low temperatures can decrease motility (Moon et al., 1979). Although neonatal pigs are not as susceptible to temperature changes as infant mice, the very young pig is more sensitive to low ambient temperatures than calves or lambs (Moon et al., 1979). In contrast to neonatal mice, which are essentially poikilothermic, other mammalian neonates (including pigs) maintain a relatively constant body temperature by a combination of behavioral practices, biological adaptations (i.e., subcutaneous fat and/or pelage), and the expenditure of metabolic energy. Since the newborn pig is lacking in both large stores of subcutaneous fat and dense hair coat, the pig must rely extensively on increased metabolism to maintain its homeothermic state (Curtis, 1970).

Pigs less than 3 days of age are the most temperature sensitive. If the ambient temperature should fall below 34 C, these young pigs are unable to sustain thermoregulation (Curtis, 1970). The neonatal pig depends upon carbohydrate reserves (which appear to be higher in young pigs than in other mammals) to metabolize into energy for thermoregulation. Pigs respond to decreased ambient temperatures by sympathetic pathways, as evidenced by vasoconstriction, glycogenolysis, and adipokinesis. These are all regulated by the release of catecholamines. Catecholamines, however, antagonize insulin secretion, and the decreased insulin levels lead to decreased carbohydrate utilization. Thus, even with more than adequate carbohydrate stores, the infant pig may succumb

to cold stress because of an inability to utilize carbohydrates. Older pigs overcome this problem by sympathetically induced lipid catabolism, which replaces glucose oxidation to meet their energy needs (Curtis, 1970). Assuming that young pigs respond to decreased ambient temperature with decreased intestinal motility, as is seen in other species, slow transit time could predispose the intestine to colonization by pathogenic organisms (Moon et al., 1979).

Several studies have been undertaken comparing the effects of ambient temperature on the replication of the TGE virus *in vivo* and the immune response elicited in the infected pig. Furuuchi and Shimizu (1976) orally exposed young colostrum-deprived pigs to an attenuated TGE virus and kept groups of these pigs at 3 ambient temperatures: (1) 8 to 12 C, (2) 20 to 23 C, and (3) 35 to 37 C. Sixty-six percent of the pigs died in group 1 after 48 hours, while all other pigs survived. Viral titers in the tissues of the pigs kept at 8 to 12 C were markedly higher than in the other 2 groups.

Shimizu et al. (1978) exposed 2- to 3-month-old feeder pigs to a virulent TGE virus. If they maintained these pigs at an ambient temperature of 30 C, no clinical illness was noted. Pigs at an ambient temperature of 4 C did exhibit clinical illness consistent with TGE. Pigs kept in a continually fluctuating temperature displayed the most severe TGE-related clinical signs. Antibody titers were higher in those pigs that were clinically ill. This increased antibody response may have been the result of a greater antigenic mass (i.e., more extensive viral replication in the intestinal tract).

Shimizu and Shimizu (1979c) also orally exposed 2- to 3-month-old feeder pigs with a virulent TGE virus. Again, those pigs kept at 30 C developed no clinical illness, but lymphocytic blastogenic responses

to TGE antigen could be observed at 3 days PE. The pigs that were kept in an ambient temperature of 4 C began to vomit and have diarrhea after a 2-day incubation period. They did not develop a lymphocytic blastogenic response to the TGE antigen until 7 days PE. These results suggest that the decreased ambient temperature may have led to a reduced or delayed cell-mediated response and thus the onset of clinical illness occurred.

A Carrier State for TGE

At least 2 separate studies have shown that a possible carrier state for the TGE virus does exist (Underdahl et al., 1974; Underdahl et al., 1975). Viable, infective TGE virus was isolated from the lungs of market-weight swine which had gross lung lesions consistent with a mycoplasmal infection (Underdahl et al., 1974). In a separate study, Underdahl et al. (1975) isolated infective TGE virus from lung tissue and intestine of pigs which had been experimentally exposed to the TGE virus 30 to 104 days earlier. Both studies confirm the hypothesis that a carrier state can exist in enough pigs to keep a constant source of virus available on contaminated farms to later infect susceptible additions to the herd.

As mentioned earlier, Morin et al. (1978) monitored the movement of susceptible swine in and out of high pig concentration areas such as sale barns. Many of these pigs later developed clinical illness consistent with TGE, even though they had been in contact with clinically normal pigs. Therefore, pigs which were shedding the TGE virus must have been encountered during this transfer stage.

MATERIALS AND METHODS

I. Effects of Variable Ambient Temperature on the Clinical Signs and Lesions Caused by Oral Exposure of Gnotobiotic Pigs to the TGE Vaccine Virus

Experimental Animals

Thirty-seven crossbred, gnotobiotic pigs were derived by hysterotomy from 4 sows on the 112th or 113th day of gestation. Each sow received 20 to 25 ml of 2.5% procaine hydrochloride[a] by epidural injection at the lumbosacral articulation (Getty, 1963). Just prior to complete rear limb desensitization, the sow was placed in right lateral recumbency on the surgical table, and 5 ml of a tranquilizer[b] was administered intramuscularly.

Following surgical preparation of the sow's left flank, a plastic surgical isolator[c] was positioned and adhered to her skin with the use of an aerosol surgical adhesive.[d] The skin was incised by electrocauterization through the sealed isolator-port:skin interface. The lateral abdominal muscles and adipose tissue were dissected and separated, and the peritoneum was incised. The uterus was exteriorized from the abdomen into the surgical isolator, and the pigs were removed

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[a] Epidural, Haver-Lockhart, Kansas City, MO.

[b] Sparine, Wyeth Laboratories, Philadelphia, PA.

[c] G-F Supply Div., Standard Safety Equipment Co., Palatine, IL.

[d] Vi-Drape, Parke-Davis, Detroit, MI.

by hysterotomy (Waxler et al., 1966). The pigs were aseptically passed into maintenance isolators[c] via a transfer unit and kept in individual stainless steel cages. Their diet consisted of a commercially prepared sterile sow's milk replacer[e] fed 3 times daily according to the manufacturer's recommendations. In all phases of this research, unexposed pigs were kept in separate isolators from the virus-exposed pigs. Extra food and other materials were passed into the isolators via a sealed entry port that was decontaminated with an aerosol mist of 2% peracetic acid.[f]

Bacteriologic Procedures

The gnotobiotic status of the experimental animals was monitored by routine, periodic bacterial culturing of the isolators. Rectal and cage swabs were obtained prior to virus exposure and at weekly intervals thereafter. The material from these swabs was plated onto tryptose blood agar[g] and inoculated into thioglycollate broth.[g] All media were incubated aerobically and anaerobically at 25 C, 37 C, and 56 C. Additional swab material was inoculated into PPLO broth[g] and incubated aerobically for 5 days at 37 C. From this broth, 1 blind passage was made onto a PPLO plate[g] and incubated in the same manner for an additional 5 days. All blood plates were maintained for 5 days, while the thioglycollate broth was incubated for 30 days.

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[e]SPF-Lac, Borden, Inc., Norfolk, VA.

[f]FMC Corp., Buffalo, NY.

[g]Difco Laboratories, Detroit, MI.

Ambient Temperature

The maintenance isolators were kept in 2 separate rooms, with each room having its own thermostatic controls. For the first 4 days, the ambient temperature of both rooms was kept at 32 C. On day 5, the temperature in the cold room was lowered to 27 C. On days 6 and 7, the cold room's temperature was again lowered to 21 C and 16 C, respectively. Thus, for the remainder of the experiment the ambient temperature in the cold room remained at 16 to 18 C, while the temperature of the warm room was maintained at 32 to 34 C.

Virus Preparation

A modified live TGE vaccine virus[h] of porcine tissue culture origin was rehydrated with 20 ml of sterile diluent and passed through a 0.45 μ filter.[i] (According to the product insert, this vaccine contained approximately 10^4 $TCID_{50}$ of TGE virus per 2 ml of solution.) The vaccine was divided into 5 ml aliquots and sealed in glass ampules for transfer into the isolators. Each pig in the "exposed" isolators received 1 ml of this inoculum orally at 7 days of age. Each control pig was sham-exposed by oral administration of 1 ml of Hank's balanced salt solution (HBSS).[j]

Tissue Collection

In the first 2 litters, the experimental protocol had called for the euthanasia and necropsy of half of the litter at 3 and 7 days post-exposure (PE). However, at 5 days PE, 2 of the exposed pigs kept at 16 C in litter 2 became comatose and died. Therefore, in order to

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[h] TGE Vac, Diamond Laboratories, Inc., Des Moines, IA.

[i] Nalge, Sybron Corp., Rochester, NY.

[j] Grand Island Biological Co., Grand Island, NY.

attempt to obtain better tissue samples during the lethal stage of this disease, the euthanasia and necropsy dates were changed to 3 and 5 days PE for litters 3 and 4 (see Table 1).

Euthanasia was accomplished by the injection of 3 ml of sodium pentobarbital[k] in the anterior vena cava. The necropsy was performed while the pig was in dorsal recumbency. All thoracic and abdominal organs were exteriorized and examined. Sections of lung, heart, liver, spleen, kidney, pancreas, and mesenteric lymph node were placed in 10% neutral buffered formalin.

Seven sections of the small intestine were collected and formalin-fixed for both light microscopy and gross examination. Section 1 was taken approximately 5 cm from the pylorus, while section 7 was obtained from an area which was 5 cm from the ileocecal junction. The other 5 sections (2 through 6) were spaced equidistant along the small intestine between sections 1 and 7.

Intestinal loops approximately 2 mm long were also collected from 4 positions and flash frozen onto cork discs with O.C.T.[l] cryostat embedding medium. These discs were stored at -70 C until the tissues could be sectioned for fluorescent antibody procedures.

Histologic Preparation

The formalin-fixed tissues were trimmed to appropriate size, automatically processed,[m] and paraffin embedded for sectioning at 5 to 7 μm. Tissue sections were then stained with hematoxylin-eosin.

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[k]Fort Dodge Laboratories, Inc., Fort Dodge, IA.

[l]Lab-Tek Products, Miles Laboratories, Naperville, IL.

[m]Histomatic, Fisher Scientific Co., Pittsburgh, PA.

Table 1. Experimental design I: effects of variable ambient temperature on the clinical signs and lesions caused by oral exposure of gnotobiotic pigs to the TGE vaccine virus

Litter No.	16 C Ambient Temperature				32 C Ambient Temperature			
	No. Control Pigs Killed at 3 Days	No. Control Pigs Killed at 5 or 7 Days	No. Exposed Pigs Killed at 3 Days	No. Exposed Pigs Killed at 5 or 7 Days	No. Control Pigs Killed at 3 Days	No. Control Pigs Killed at 5 or 7 Days	No. Exposed Pigs Killed at 3 Days	No. Exposed Pigs Killed at 5 or 7 Days
I	1	1	1	2				
II	1	1	2	2*	1	1	2	2
III	1	1	2	2	-	1	1	1
IV	1	1	2	1* 1	1	1	1	2

*Died as a result of the viral infection

Immunofluorescence

The previously mounted frozen intestinal segments were cross sectioned[n] at a thickness of 8 μm, mounted on glass slides, and air dried. These tissues were fixed in 100% acetone for 10 minutes and air dried once again. A hyperimmune TGE antiglobulin that had been conjugated with fluorescein isothiocyanate[o] was spread over the tissue surface, and the slides were incubated at 37 C in a moist chamber for 30 minutes. The conjugate was removed by rinsing with phosphate-buffered saline (PBS) at pH 7.2, washed in clean PBS for 30 minutes, and rinsed in distilled water. After air drying, the slides were coverslipped with a 1:9 PBS:glycerol mounting medium.

The mounted specimens were examined on a Zeiss photomicroscope III[p] equipped with 10X eyepiece lenses, and using 10X and 25X objectives. An FITC primary filter was placed over the field diaphragm along with a heat filter and a red suppressor filter (BG 32). A secondary in-line filter (530) was also employed.

II. Lethal Dose$_{50}$ Determination

Experimental Animals

One litter of 8 pigs was farrowed normally and allowed to nurse the dam for 3 days. On day 4, they were removed from the dam, placed in individual stainless steel cages within plastic isolators, and taught to eat from pans. At 6 days of age, the pigs were orally exposed to 1 ml of a TGE vaccine virus solution varying from full-strength vaccine

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[n] Pearse Cryostat, SLEE Co., London, England.

[o] TGE conjugate, N.A.D.C., Ames, IA.

[p] Carl Zeiss, Oberkochen, West Germany.

through successive 10-fold dilutions (in sterile HBSS) to a final dilution of 1/1000. One pig served as a control and received 1 ml of sterile HBSS (Table 2).

One litter of 11 pigs was derived gnotobiotically (see Materials and Methods Section I). At 5 days of age the pigs were orally exposed to 1 ml of the vaccine virus solution either at full strength or at 1 of 3 serial 10-fold dilutions. Two pigs served as controls and orally received 1 ml of HBSS (Table 2).

All pigs were maintained until death or until 17 days after exposure, at which point all pigs were completely recovered from the clinical signs of illness.

Table 2. Experimental design II: LD_{50} of TGE vaccine virus

Types of Pigs	No. of Control Pigs (HBSS)	No. of Pigs Receiving Oral Vaccine At:			
		full strength	1/10	1/100	1/1000
conventional	1	2	2	2	1
gnotobiotic	2	2	3	3	1

Tissue Collection

All pigs that died during this phase of the research were necropsied as soon after death as possible. All surviving pigs were euthanatized and tissues were collected for light microscopic examination and fluorescent antibody procedures (see Materials and Methods Section I).

III. Studies of the Immune Response

Experimental Animals

Four litters, totaling 38 pigs, were taken aseptically by hysterotomy to procure the gnotobiotes for this portion of the research (see Materials and Methods Section I for the procedure used) (Table 3).

Bacteriologic Procedures

Bacteriologic monitoring of the isolators was performed in the same manner as described in Materials and Methods Section I.

Ambient Temperature

For the first 2 weeks of life these gnotobiotic pigs were kept in rooms with an ambient temperature of 32 to 34 C. For the remainder of the study the ambient temperature was reduced to 26 to 28 C and maintained at that level.

Table 3. Experimental design III: studies of the immune response to TGE vaccine virus

Litter No.	No. Control Pigs Euthanatized at 2 Weeks PE	No. Control Pigs Euthanatized at 3 Weeks PE	No. Exposed Pigs Euthanatized at 2 Weeks PE	No. Exposed Pigs Euthanatized at 3 Weeks PE
VII	4	-	6	-
VIII	2	1	3	3
IX	1	2	3	3
X	2	2	3	3

Virus Preparation

Although there appeared to be no LD_{50} for this particular modified live TGE vaccine virus when administered to gnotobiotic pigs (see Results - LD_{50}), the LD_{50} for this same virus in conventional pigs was chosen to be the level of virus exposure for this portion of the project. Thus, all exposed pigs received orally 1 ml of a 1/100 dilution of unfiltered vaccine virus in sterile HBSS. As in the first portion of this research, the viral inoculum was divided into 5 ml aliquots and aseptically passed into the isolators. The control pigs received 1 ml of HBSS orally. The pigs were 2 weeks of age at the time of oral exposure to the virus.

Tissue Collection

The pigs in this portion of the research were euthanatized at either 2 or 3 weeks PE. Twenty milliliters of venous blood was collected in glass tubes by venipuncture of the anterior vena cava. After clotting, this blood was centrifuged, and the serum was removed and frozen for later determinations of virus neutralization titers. Ten milliliters of peripheral blood was also collected by venipuncture of the anterior vena cava for lymphocyte harvesting. These 10 ml aliquots of whole blood were collected in heparinized syringes and stored at room temperature in covered, sterile, plastic test tubes. From this point on, euthanasia and tissue collection were the same as previously described in Materials and Methods Section I, with 2 exceptions. Sections of lung and mesenteric lymph node were collected and processed for future fluorescent antibody techniques. They were embedded in O.C.T. on cork discs and otherwise handled in the same manner as the intestinal sections for this procedure. Portions of mesenteric lymph

node were also harvested and placed in sterile vials containing cold Eagle's minimum essential medium (EMEM).[q] These vials were kept refrigerated until the lymphocytes could be harvested from the nodes.

Histopathologic Preparation

All formalin-fixed tissues were processed by the same method as reported in Materials and Methods Section I.

Immunofluorescence

All sections of intestine, lung and mesenteric lymph node were handled in the same manner as the intestine in Materials and Methods Section I.

Lymphocyte Stimulation

Lymphocyte cultures were initiated within 4 hours of necropsy. Ten milliliters of heparinized peripheral blood was mixed with 20 ml of sterile PBS and carefully layered onto 10 ml of ficoll/hypaque (24 parts of 9% ficoll[r] to 10 parts of 33.9% hypaque[s]) in a 50 ml sterile plastic centrifuge tube. After centrifugation at 1500 rpm for 30 minutes, the mononuclear cell layer was pipetted off and washed 3 times with 10 ml of sterile PBS, centrifuging at 750 rpm for 10 minutes each time. These cells were washed once in 2 ml of cell culture medium[t] (contining 10% fetal calf serum, 10,000 units penicillin, 10,000 µg streptomycin, and 20 mM glutamine[u]), centrifuged, and

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[q]Kansas City Biologicals, Inc., Lenexa, KS.

[r]Sigma Chemical Co., St. Louis, MO.

[s]Winthrop Laboratories, New York, NY.

[t]RPMI 1640, Flow Laboratories, Dublin, VA.

[u]Grand Island Biological Co., Grand Island, NY.

resuspended in 1 ml of RPMI. An aliquot of cells was diluted 1:10 with trypan blue and counted on a hemacytometer. The trypan blue also demonstrated the percent viability of the resulting cell culture. The cells were further diluted with RPMI to achieve a final concentration of 1 to 2 x 10^6 cells/ml.

Culturing was performed in covered sterile microtiter plates.[v] Twenty-four wells were used for each pig. Two antigens, each at 3 different concentrations, were used in this study. The first 3 wells of each antigenic row were control wells containing 0.1 ml of RPMI medium and 0.1 ml of the final cell concentration. The remaining 9 wells in each row contained 0.1 ml of the cell suspension, 0.05 ml of medium, and 0.05 ml of the antigen suspension. Concanavalin A[r] (ConA) served as a nonspecific antigenic stimulus. The ConA was diluted with RPMI to 3 concentrations (2 µg/ml, 5 µg/ml, and 10 µg/ml), with each concentration being represented by 3 wells. Likewise, 3 concentrations of a modified live tissue-culture-adapted TGE virus were placed into 3 wells each. This virus was grown on ST cells. The initial concentration of this virus was approximately 1 x 10^5 $TCID_{50}$. Two 10-fold dilutions were made to produce the other 2 concentrations of this antigen (i.e., 1 x 10^4 $TCID_{50}$ and 1 x 10^3 $TCID_{50}$). After incubating the covered plates at 37 C in 5% CO_2 for 48 hours, 0.5 µCi of [^{3}H]-thymidine in aqueous solution[w] was added to each well and the incubation was continued for an additional 18 hours.

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[v] Linbro Div., Flow Laboratories, Inc, Hamden, CT.

[w] New England Nuclear, Boston, MA.

The cells were harvested on glass fiber filters[x] by the use of an automatic cell harvestor.[y] The labeled cells were placed into scintillation fluid (Spectrofluor[z] in toluene) and were counted in a Beckman LS-9000 counter.[aa] The data were expressed as mean counts per minute ± 2 standard deviations. A stimulation index was defined as the proportion of mean counts per minute of the cultures stimulated with the antigens to counts from the control cultures.

The mesenteric lymph nodes were teased apart with needles and passed through an 80-mesh stainless steel screen in cold Eagle's Minimal Essential Medium (EMEM). This suspension was in turn filtered through a glass-wool packed column (Shimizu and Shimizu, 1979a). The resulting mononuclear cell suspension was treated the same as peripheral blood mononuclear cells after ficoll/hypaque separation.

Serum Virus Neutralization Titers

Viral neutralization tests were performed at Purdue University by Dr. C. L. Kanitz, who used a constant virus-varying serum dilution procedure in 96-well flat-bottomed microtiter plates.[v] The serum samples were initially diluted 1:2 in EMEM and were then inactivated at 56 C for 30 minutes. This was followed by serial 2-fold dilutions prepared with serumless MEM using a 25 µl microdiluter.

Stock TGE virus (113th or 114th passage of the Purdue strain in tissue culture) was diluted in MEM containing 2% fetal calf serum

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[x]Mash II filters, M.A. Bioproducts, Walkersville, MD.

[y]Model M12V, Brandel, Rockville, MD.

[z]PPO-POPOP, Amersham Corp., Arlington Heights, IL.

[aa]Beckman Instruments, Inc., Novi, MI.

(FCS) to a concentration of 100 $TCID_{50}$/25 µl. Each well received 25 µl of the viral suspension and the serum dilutions. The virus-serum mixtures were incubated at room temperature for 1 hours. A suspension of porcine turbinate cells containing 10^4 cells per 50 µl was prepared in MEM with 10% FCS and was added to each well in 50 µl quantities. Controls included the lowest serum dilution to which no virus was added, known positive and negative serums, as well as virus and diluent controls.

The plates were incubated at 37 C in a 5% CO_2 chamber and were examined at 1 to 2 days for serum toxicity. Endpoints were based on the presence or absence of cytopathic effect after 4 to 5 days. Titers were expressed as the reciprocal of the highest serum dilution neutralizing 100 $TCID_{50}$ of the Purdue strain tissue-culture-adapted TGE virus.

Porcine Parvovirus Titers

Serum titers for porcine parvovirus were determined at Michigan State University's diagnostic virology laboratory using the microtitration hemagglutination inhibition (H.I.) test. The following is a brief summary of the procedure.

The serum was inactivated at 56 C for 30 minutes. A volume of 0.3 ml of the serum was mixed with an equal volume of 50% washed guinea pig erythrocytes in Dulbecco's phosphate buffered saline (DPBS). Approximately 20 mg of Kaolin was added to the suspension and it was incubated at room temperature for 30 to 45 minutes. The tubes were then centrifuged at 1500 rpm for 10 minutes at 4 C. The supernatant was removed and diluted 1:16 with DPBS.

Serums were tested using paired wells for each dilution. Each of the paired wells of the microtiter plate received 25 µl of DPBS. The

paired wells in the first row served as controls and received 25 µl of the 1:16 dilution. The paired wells in row 2 received 50 µl of the 1:16 dilution. Serum titration was initiated in row 2 using 25 µl diluting loops, resulting in serum titrations of 1:16 to 1:16,384.

Twenty-five microliters of a viral suspension containing 4 to 8 hemagglutinating units per 25 µl was added to each well beginning with row 2. The plates were left at room temperature for 2 hours to allow the antibody to react with the virus.

Fifty microliters of 0.5% guinea pig erythrocytes was added to each well. The plates were stored at room temperature for 2 hours and then overnight at 5 C. The HI activity was then examined if all control systems appeared appropriate. The presence of a "button" of agglutinated erythrocytes indicated a negative result. A lack of agglutination indicated that the virus had serum antibody bound to it. The highest serum dilution inhibiting agglutination became the titer level for that pig.

Three other types of controls were run along with the unknown serums. These controls included: (1) positive and negative serums; (2) back titration of the virus to verify the initial concentration of the viral suspension; and (3) a diluent control to estimate the erythrocyte sedimentation rate in the diluent.

Serum Total Protein

The amount of total protein in each pig's serum was measured using a hand-held refractometer.[bb] The total protein value was read directly from the scale within this instrument.

[bb] A.O.T.S. Meter 10400A, American Optical, Buffalo, NY.

Statistical Analysis

The data were analyzed using least squares analysis of variance. The original model consisted of treatment, litter, and the treatment by litter interaction. The interaction was found to be poolable in all cases (test at $p \geq .25$) and was pooled with error. The tests for both treatment and litter were made with the pooled error (Steel and Torrie, 1960).

RESULTS

I. Effects of Variable Ambient Temperature on the Clinical Signs and Lesions Caused by Oral Exposure of Gnotobiotic Pigs to the TGE Vaccine Virus

Variations in Body Temperatures

Tables 4 through 7 contain the results of monitoring the rectal temperature of the pigs in this portion of the study. The pigs kept in the cold room maintained their body temperatures at a slightly lower level (1 to 2 C lower) than those pigs kept in the warmer ambient temperature. It appeared that whenever a pig's body temperature dropped below 36 C, death usually occurred within 24 hours. The only pigs to succumb to the TGE viral infection (see Tables 5 and 7) were from the groups kept in the cold ambient temperature. All of the control pigs in both environments, as well as all of the exposed pigs in the warm ambient temperature, survived until the termination of the experimental procedure.

Clinical Signs

The exposed pigs began to show evidence of diarrhea at approximately 48 hours after oral exposure to the modified live TGE virus. The feces were characteristically yellow in color with occasional undigested milk curds present within the fluid.

The activity and appetite of the pigs remained normal throughout the study, with the exception of those pigs which succumbed to the viral

Table 4. Effects of ambient temperature on body temperature (C) of gnotobiotic pigs exposed to a modified live TGE vaccine virus: litter I

Day	1*	2	3	4	5	6	7
Cold Room Ambient Temperature (C)	23	18	17	18	18	17	17
Pig No.							
1 C	39.0	37.0	36.5	36.5	---	---	---
2 C	39.0	37.0	37.0	38.5	39.0	39.0	38.5
3 E	39.0	38.5	38.5	39.0	---	---	---
4 E	39.0	39.0	38.5	38.5	39.0	38.5	38.5
5 E	39.0	38.5	38.5	39.0	38.0	36.0	38.5

*Day of viral exposure

C = control pig

E = exposed pig

---Pigs euthanatized on day 4

Table 5. Effects of ambient temperature on body temperature (C) of gnotobiotic pigs exposed to a modified live TGE vaccine virus: litter II

Day	1*	2	3	4	5	6	7
Warm Room Ambient Temperature (C)	32	32	32	32	32	32	32
Pig No.							
12 C	39.5	39.5	39.5	39.5	---	---	---
13 C	40.0	40.0	40.0	40.0	40.0	40.0	40.0
14 E	40.0	40.0	39.5	40.0	---	---	---
15 E	40.0	39.5	39.5	39.5	39.5	39.5	40.0
16 E	40.0	40.0	40.0	40.0	---	---	---
17 E	40.0	39.5	40.0	40.0	40.0	40.0	40.0
Cold Room Ambient Temperature (C)	19	19	19	19	18	18	17
Pig No.							
6 C	39.0	38.5	38.5	38.5	---	---	---
7 C	39.0	39.0	39.0	39.0	39.0	38.5	38.0
8 E	39.0	39.0	39.5	40.0	---	---	---
9 E	39.0	39.5	39.0	38.5	38.5	36.0	**
10 E	38.5	38.0	38.0	38.5	---	---	---
11 E	39.0	38.5	38.0	38.0	37.0	**	---

*Day of viral exposure; **Pig found dead; C = control pig; E = exposed pig; ---Pigs euthanatized on day 4.

Table 6. Effects of ambient temperature on body temperature (C) of gnotobiotic pigs exposed to a modified live TGE vaccine virus: litter III

Day	1*	2	3	4	5
Warm Room Ambient Temperature (C)	31	31	31	31	32
Pig No.					
24 C	40.0	39.0	39.5	39.5	40.0
25 E	39.5	39.0	39.5	---	---
26 E	40.0	39.5	40.0	40.0	40.0
Cold Room Ambient Temperature (C)	15	15	16	16	17
Pig No.					
18 C	38.0	38.0	37.0	---	---
19 C	38.0	38.5	37.5	37.5	38.0
20 E	39.5	38.5	38.5	---	---
21 E	39.0	40.0	39.5	---	---
22 E	40.0	39.5	39.5	39.0	39.0
23 E	39.0	38.5	39.0	39.0	40.0

*Day of viral exposure; C = control pig; E = exposed pig; --- Pig euthanatized on day 3.

Table 7. Effects of ambient temperature on body temperature (C) of gnotobiotic pigs exposed to a modified live TGE vaccine virus: litter IV

Day	1*	2	3	4	5	6
Warm Room Ambient Temperature (C)	32	32	32	32	32	33
Pig No.						
33 C	39.0	39.5	39.5	40.0	---	---
34 C	39.5	39.5	39.5	39.5	39.0	39.5
35 E	39.5	39.5	39.5	40.0	---	---
36 E	39.5	39.5	40.0	40.0	39.5	39.5
37 E	39.5	39.5	40.0	40.0	39.5	39.5
Cold Room Ambient Temperature (C)	16	17	16	16	17	17
Pig No.						
27 C	38.0	38.0	38.0	38.0	---	---
28 C	38.5	38.0	38.0	38.0	37.0	36.5
29 E	38.5	39.0	39.5	38.0	---	---
30 E	38.5	38.5	39.5	39.5	36.0	**
31 E	38.5	38.5	38.0	39.0	---	---
32 E	38.5	38.0	38.0	38.0	36.0	36.5

*Day of viral exposure; **Pig found dead; C = control pig; E = exposed pig; ---Pig euthanatized on day 4.

infection. Those 3 pigs were depressed, dehydrated, and anorectic approximately 6 hours prior to death. As they progressively weakened, they became comatose within the last 2 hours before death.

Gross Lesions

Severe villus atrophy was observed throughout the jejunum and ileum of the 3 pigs that died during the experiment. Their stomachs contained undigested milk, even though they had not eaten for 4 to 6 hours, and the samll intestine was distended with gas. The contents of the entire tract were composed of a yellow, fluid ingesta and some undigested milk curds. A fourth pig from the cold room also had extensive villus atrophy throughout the small intestine. Several other pigs from the cold room had shortened intestinal villi in the distal jejunum and in most of the ileum. Three exposed pigs kept in the warm room had moderate to severe villus atrophy within the small intestine. Only those pigs that survived for at least 5 days after exposure had intestinal lesions. All other pigs had very slight if any intestinal changes.

Various degrees of subcutaneous edema were noted in the animals from the cold room, both in the control as well as the exposed pigs. This edema had a tendency to be more extensive in the ventral abdominal area (Figure 1). In the more severe cases, the edema extended along fascial planes into the musculature of the rear limbs. Two pigs also had edematous superficial inguinal lymph nodes. The only pigs from the cold room that did not have the subcutaneous edema were the 3 that died after viral exposure.

Serous atrophy of fat in the coronary groove and in the bone marrow was also observed in the pigs from the cold room. This lesion did not appear to be related to viral exposure, since the control pigs were

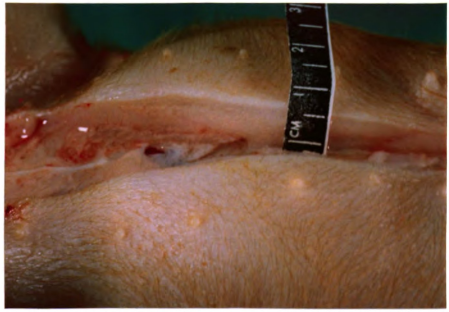

Figure 1. A ventral midline incision of this gnotobiotic pig reared in an ambient temperature of 16 to 18 C reveals the presence of extensive subcutaneous edema.

equally affected. All of the pigs kept in the warm room had evidence of normal fat in the coronary groove.

Histopathologic Lesions

Sections of the small intestine taken from the control pigs consistently contained structural differences that were characteristic of the area of the intestine from which they were obtained. The duodenum contained long, fingerlike villi that were covered by a layer of simple columnar epithelial cells that were nonvacuolated (Figures 2 and 3).

Examination of jejunal segments revealed slightly longer villi that were covered by a moderately vacuolated columnar epithelium (Figures 4 through 7). The ileum was comprised of equally long villi with a highly vacuolated epithelium which covered the entire surface of the villus (Figures 8 and 9). The lamina propria in all areas of the intestine of these gnotobiotic pigs was only sparsely populated with mononuclear cells.

Microscopic examination of the various tissues from the exposed pigs revealed a number of interesting changes. Seven of the 15 virus-exposed pigs that were kept in the cold room had jejunal and/or ileal lesions that were consistent with TGE. These lesions were characterized by shortened, and often fused, villi that were covered by a low cuboidal, nonvacuolated epithelium (Figures 10 and 11). Focal areas of mucosal erosion were also noted in a few of the more severely affected pigs. In some areas of the jejunum, the tips of the remaining villi were markedly hyperemic. These intestinal lesions were only found in those pigs that were permitted to live beyond 3 days after exposure. (None of the 7 exposed pigs from the cold room which were euthanatized at 3 days PE had any identifiable intestinal lesions.) Therefore, the 7 pigs with intestinal lesions from the cold room were all exposed pigs that lived beyond 3 days of age.

Figure 2. This section of duodenum was taken from a control gnotobiotic pig at 12 days of age. Notice the numerous long, fingerlike villi. H&E stain, 120X.

Figure 3. A higher magnification of a duodenal villus reveals the presence of nonvacuolated, columnar epithelial cells. H&E stain, 750X.

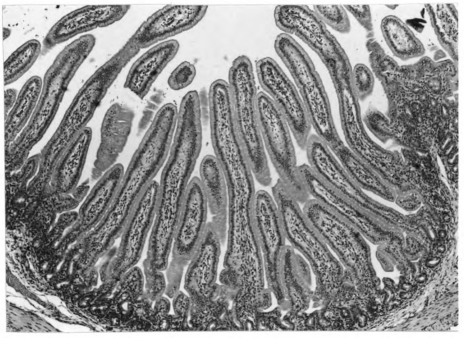

Figure 2

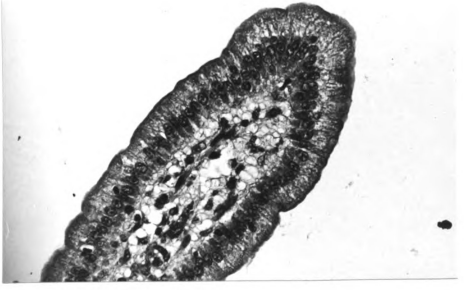

Figure 3

Figure 4. A section from the proximal jejunum of a 12-day-old, gnotobiotic pig contains multiple long villi. H&E stain, 120X.

Figure 5. At an increased magnification, the villi from the proximal jejunum are seen to be covered by a partially vacuolated, columnar epithelium. H&E stain, 750X.

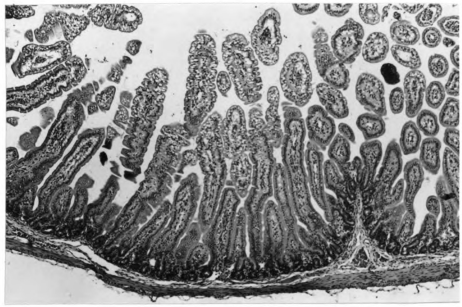

Figure 4

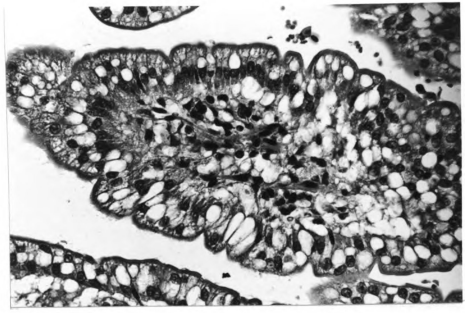

Figure 5

Figure 6. This section of distal jejunum from a 12-day-old, control, gnotobiotic pig also has many long slender villi. H&E stain, 120X.

Figure 7. Observation of the distal jejunum at a higher magnification reveals the presence of a moderately vacuolated columnar epithelium. H&E stain, 750X.

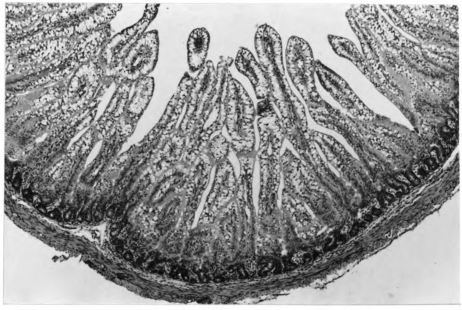

Figure 6

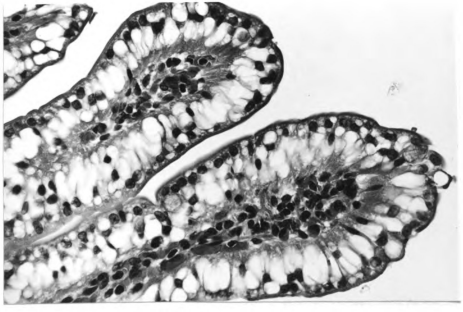

Figure 7

Figure 8. This section of distal ileum was taken from a control, gnotobiotic pig at 12 days of age. Again, note the numerous long villi. H&E stain, 120X.

Figure 9. With a higher magnification of the ileal section, a heavily vacuolated columnar epithelium can be seen. H&E stain, 750X.

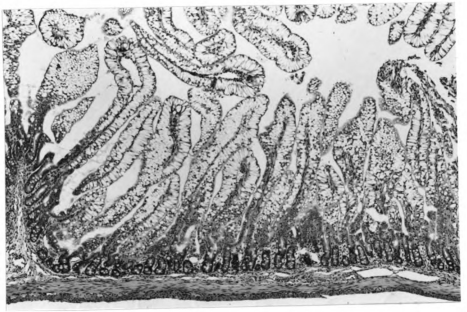

Figure 8

Figure 9

Figure 10. This section of distal jejunum was taken from a 12-day-old, gnotobiotic pig that had been orally exposed to a TGE vaccine virus 5 days earlier. The villi are blunt and severely shortened. H&E stain, 120X.

Figure 11. At an increased magnification the atrophic villi can be seen to be covered by a low cuboidal epithelium. H&E stain, 750X.

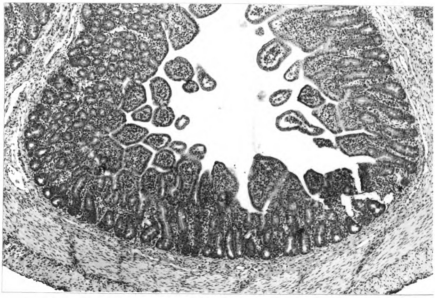

Figure 10

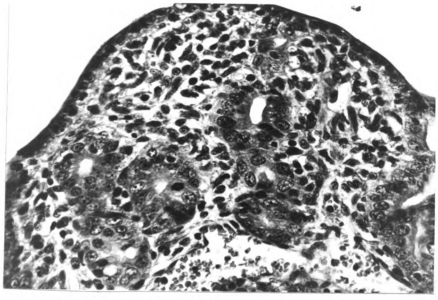

Figure 11

Of the 9 exposed pigs kept in the warm room, 4 had microscopic intestinal lesions which included short, blunt villi, fusion of villi, and an apparent deepening of the crypts. The mucosal lining was composed of cuboidal epithelium with several scattered foci of degenerating epithelium. As in the cold room,only those pigs that were allowed to survive until 5 to 7 days after viral exposure had any intestinal lesions. Therefore, of the 5 pigs in this group, 4 had lesions of TGE.

Hyperplasia of the mesenteric lymph nodes was observed by light microscopy (Figures 12 through 15). Not only were these lymph nodes hypercellular, but secondary follicles composed of lymphoblasts were present within the primary follicles. Hyperplastic lymph nodes were only observed in exposed pigs and never in control pigs. Of the 15 exposed pigs in the cold room, 6 of the pigs euthanatized at 5 to 7 days PE had lymph node hyperplasia. Only 1 pig that was euthanatized at 3 days PE from the cold room had this change. Of the pigs reared in the warm environment, a total of 5 had hyperplastic mesenteric lymph nodes--4 from the 5 to 7 day PE group and 1 from the 3 day PE group.

Hepatic changes were evident with the aid of the light microscope. All of the pigs reared in the warm ambient temperature had highly vacuolated hepatocytes (Figure 16). These vacuoles were most prominent in the centrilobular area and became slightly less intense towards the periphery of the lobule. In many of the cells, the vacuolization seemed to fill the entire cytoplasmic space (Figure 17). The nuclei of these hepatocytes appeared viable and undamaged. Oil red O stains of these vacuolated hepatic sections were negative for the presence of fat. In contrast to this diffuse hepatocellular vacuolization seen in the pigs from the warm room, all of the pigs reared in the cold room had

Figure 12. This mesenteric lymph node from a control, gnotobiotic pig appears to be sparsely cellular and has scattered primary follicles (arrows). H&E stain, 120X.

Figure 13. Examination of a primary follicle from the above mesenteric lymph node reveals the presence of small, mature lymphocytes and few lymphoblastic cells. H&E stain, 750X.

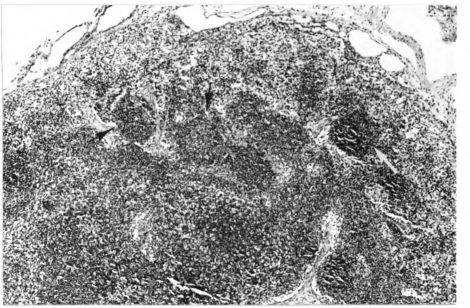

Figure 12

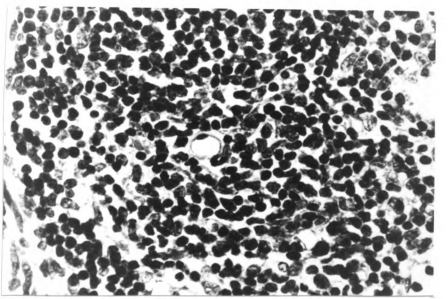

Figure 13

Figure 14. This mesenteric lymph node was removed from a gnotobiotic pig that had been orally exposed to a TGE vaccine virus 5 days earlier. The dense cellularity and enlarged follicles indicate hyperplasia. H&E stain, 120X.

Figure 15. By focusing on a primary follicle in the above lymph node with a higher magnification, large lymphoblastic cells can be identified (arrows). These cells constitute the presence of a germinal center (Gc). H&E stain, 750X.

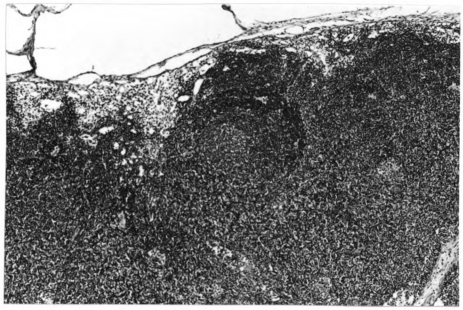

Figure 14

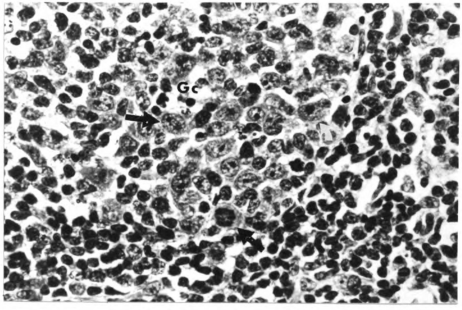

Figure 15

Figure 16. This photomicrograph was taken of hepatic tissue from a gnotobiotic pig which had been reared in a warm ambient temperature. Notice the diffuse hepatocellular vacuolization. H&E stain, 120X.

Figure 17. The extensive hepatocellular vacuolization can be better observed with this increased magnification of the above hepatic section. H&E stain, 750X.

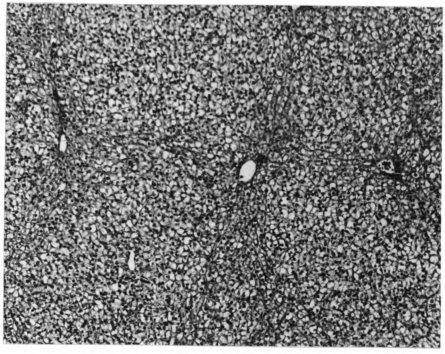

Figure 16

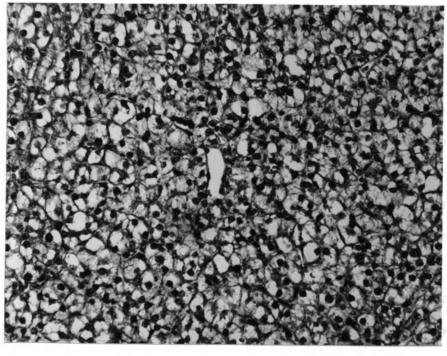

Figure 17

livers containing nonvacuolated hepatocytes (Figure 18). Not only were these hepatic cells devoid of vacuoles, but their cytoplasm was dense, homogeneous, and agranular in appearance (Figure 19). Thus, the non-lipid vacuoles of the hepatocytes from the pigs in the warm room probably represent large glycogen stores. In contrast, there was very little evidence of the presence of glycogen in the hepatocytes from the cold room reared pigs.

Foci of hepatocellular degeneration and necrosis were seen in hepatic sections from 7 of the 15 exposed pigs kept in the cold environment (Figures 20 and 21). These small multifocal areas were composed of a homogeneous proteinaceous material with karyorrhexis and karyolysis of hepatocellular nuclei. These lesions were primarily centrilobular and occasionally contained infiltrating lymphocytes. Two of the 9 exposed pigs from the warm room had multiple foci of lymphocytic accumulation without the hepatocellular destruction seen in the pigs from the cold room.

Foci of extramedullary hematopoiesis appeared to be more numerous in the sections of liver from pigs kept in the cold room when compared to their littermates that were reared in a warmer ambient temperature. This was true of both the control and virus-exposed pigs (Figure 18).

As in the hepatic sections, the spleen from the pigs in the cold room appeared to be more actively involved in extramedullary hematopoiesis. Again, this was true for both the control pigs and the virus-exposed pigs.

Sections of skin and subcutis taken from the areas of subcutaneous edema in the pigs from the cold room contained diffusely separated collagen fibers and fibroblasts, giving this portion of the dermis a mucoid appearance. Many of the mononuclear cells in this tissue had

Figure 18. In contrast to the preceding photomicrographs, this section of liver was taken from a gnotobiotic pig reared in a cold ambient temperature. Note the nonvacuolated hepatocytes. A focus of extramedullary hematopoiesis is clearly visible (arrow). H&E stain, 120X.

Figure 19. Increased magnification of the above hepatic section clearly illustrates the presence of hepatocytes with a dense, non-vacuolated cytoplasm. H&E stain, 750X.

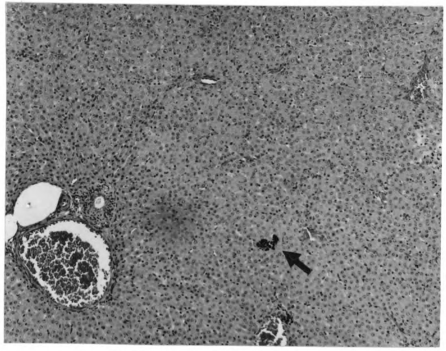

Figure 18

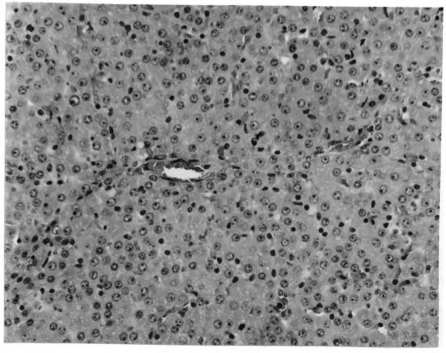

Figure 19

Figure 20. This section of liver contains a focal area of hepatocellular necrosis and an infiltration of mononuclear inflammatory cells. H&E stain, 120X.

Figure 21. A higher magnification of the necrotic focus in Figure 20 reveals karyolysis and karyorrhexis of hepatic nuclei along with the presence of infiltrating lymphocytes. H&E stain, 750X.

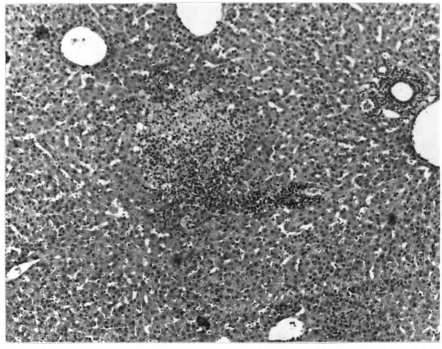

Figure 20

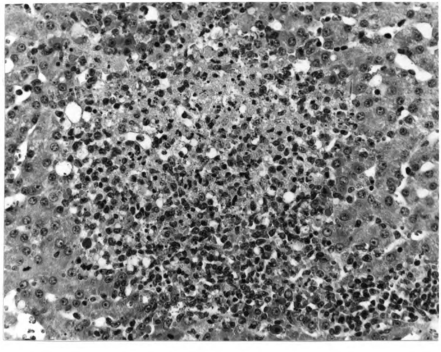

Figure 21

highly vacuolated cytoplasm, and the apocrine glands and ducts were noticeably dilated.

Sections of lung from all of the pigs contained varying degrees of atelectasis. No correlations between this finding and viral exposure or ambient temperature could be made. Three exposed pigs from different litters that were reared in the cold room had lesions of mild subacute alveolar and peribronchiolar pneumonia. These foci contained peribronchiolar lymphoid hyperplasia and accumulations of neutrophils and macrophages in surrounding alveoli. The cytoplasm of the alveolar macrophages was vacuolated and foamy.

All other tissues from these pigs appeared relatively normal for their age and stage of development.

Immunofluorescence

Of the 24 pigs that were exposed to the vaccine virus in this aspect of the study, 13 were positive for immunofluorescence for TGE virus in intestinal epithelium (see Table 8). In the cold group, intestinal sections from 9 of 15 exposed pigs fluoresced, whereas sections from only 4 of 9 in the warm group fluoresced. Figure 22 is a typical example of a positive reaction in the fluorescent antibody technique. Since the coronavirus that causes TGE is an RNA virus, the fluorescence occurs in the cytoplasm of the infected intestinal epithelial cell. As expected, immunofluorescence against the TGE viral antigen could not be demonstrated in the control pigs.

Serum Total Protein

Table 9 contains the results of the serum total protein assay. These values ranged from less than 2.5 g/dl to 4.5 g/dl, with the majority of values being between 2.8 and 3.8 g/dl. An examination of

Table 8. Fluorescent antibody detection of TGE virus in intestinal cells of gnotobiotic pigs exposed to a modified live TGE vaccine virus at two different ambient temperatures

Litter No.	Ambient Temperature 16-18 C		Ambient Temperature 32-34 C	
	Exposed Pigs, 3 da PE	Exposed Pigs, 5-7 da PE	Exposed Pigs, 3 da PE	Exposed Pigs, 5-7 da PE
I	1/1*	2/2	---	---
II	1/2	0/2**	1/2	0/2
III	2/2	2/2	1/1	1/1
IV	1/2	0/2***	0/1	1/2

*No. positive/no. tested

**Both pigs died as a result of viral infection

***One pig died as a result of viral infection

---No pigs from litter I in the warm room

(All control pigs had negative FA results.)

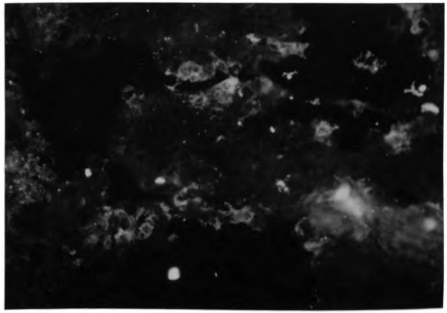

Figure 22. This photomicrograph illustrates the typical immunofluorescence associated with the presence of the TGE virus in jejunal epithelial cells. Immunofluorescent stain, 750X.

Table 9. Measurements of serum total protein (g/dl) in gnotobiotic pigs kept at varying ambient temperatures

Litter I		Litter II		Litter III		Litter IV	
Pig No.	Cold Room Total Protein	Pig No.	Cold Room Total Protein	Pig No.	Cold Room Total Protein	Pig No.	Cold Room Total Protein
1 C	3.6	6 C	3.5	18 C	3.3	27 C	3.7
2 C	4.5	7 C	3.2	19 C	4.0	28 C	4.0
3 E	3.6	8 E	3.6	20 E	3.3	29 E	3.5
4 E	3.7	9 E	*	21 E	3.0	30 E	*
5 E	<2.5	10 E	3.4	22 E	3.8	31 E	3.3
		11 E	*	23 E	3.7	32 E	4.0
Mean	3.6	Mean	3.4	Mean	3.5	Mean	3.7
			Warm Room		Warm Room		Warm Room
		12 C	3.3	24 C	3.1	33 C	2.8
		13 C	2.8	25 E	2.8	34 C	3.1
		14 E	3.3	26 E	4.1	35 E	3.2
		15 E	2.9			36 E	3.1
		16 E	2.8			37 E	3.2
		17 E	3.6				
		Mean	3.1	Mean	3.3	Mean	3.1

*Died as a result of viral infection; C = control pig; E = exposed pig.

the mean values reveals consistently higher total protein levels in the pigs from the cold room.

II. Lethal Dose$_{50}$ Determinations

Conventional Pigs

Clinical signs. The first clinical signs of illness related to the oral exposure to the vaccine virus in the conventional pigs were moderate depression and partial anorexia in one of the pigs at 48 hours PE. A second pig in the same exposure group (1/100) exhibited similar behavior at 72 hours PE. Diarrhea was first apparent at 4 days PE in 2 exposure groups (1/10 and full strength [F.S.]) simultaneously. Although the pigs in the 1/100 group were still experiencing a loss of appetite, their feces remained normal. By the fifth day PE the pigs in the 3 higher exposure groups all had diarrhea. At 6 days PE the pigs in the 2 highest exposure groups (1/10 and F.S.) were becoming weak, dehydrated and partially anorectic. By day 7 PE those 4 pigs (1/10 and F.S.) were recumbent and partially comatose. All 4 were dead by the end of day 7. On day 8 PE the weaker of the 2 pigs in the 1/100 group was comatose and died by mid-day. The remaining pig in the 1/100 group gradually regained her strength as the diarrhea dissipated over the following 3 days. The pigs in both the control group and the 1/1000 group did not experience any diarrhea or anorexia.

Gross lesions. Gross examination of the pigs dying from the oral exposure to the TGE vaccine virus revealed their stomachs to contain undigested liquid formula. The small intestines were distended with gas and contained only a yellow, fluid ingesta. Shortened villi were evident upon gross examination of the jejunum and ileum of these pigs.

The 3 surviving pigs were euthanatized at 15 days PE, and no gross lesions were observed.

Histopathologic lesions. The tissues of the 5 pigs that died as a result of exposure to the vaccine virus had various changes due to postmortem autolysis. Sections of jejunum and ileum from these pigs contained shortened and often fused villi. The covering epithelium, when present, appeared to be cuboidal rather than columnar. Two pigs had small focal accumulations of lymphocytes in the hepatic parenchyma.

No histologic lesions were observed in the 3 surviving pigs.

Immunofluorescence. Immunofluorescence was not detected in any of the intestinal sections from the 8 conventional pigs used for LD_{50} determination.

The LD_{50} for conventional pigs orally exposed to this vaccine virus was thus determined to be 1 ml of a 1/100 dilution of the vaccine.

Gnotobiotic Pigs

Clinical signs. The same dilutions of the vaccine virus were used in an attempt to determine the LD_{50} for this virus in gnotobiotic pigs. Diarrhea was the first sign of clinical illness observed in these pigs. The diarrhea began on day 5 PE in the 2 highest exposure groups (1/10 and F.S.). On day 6 PE, the pigs in the F.S. group appeared slightly weakened and less active. This weakness continued through day 10 PE. The diarrhea persisted for 8 days before the consistency of the feces gradually returned to normal. The pigs in the 1/100 group experienced diarrhea beginning on day 7 PE and lasting through day 13 PE. All pigs maintained a relatively normal appetite throughout their illness.

Gross lesions. Since all 11 pigs survived, postmortem examinations were performed following euthanasia of the pigs on day 18 PE. No gross lesions were observed, and no abnormal accumulations of gas were present in the intestinal tract. Subgross observations of the jejunum and ileum did reveal some mild villus shortening in 2 pigs.

Histopathologic lesions. Only 2 pigs were observed to have any intestinal lesions attributable to TGE. The jejunal villi were shortened, blunt and covered by low columnar epithelium. The mesenteric lymph nodes of these 2 pigs also appeared to be moderately hyperplastic. All other tissues appeared relatively normal.

Immunofluorescence. Immunofluorescence for the TGE virus was not observed in the intestinal sections of the 11 pigs used for the LD_{50} determination of this vaccine virus in gnotobiotic pigs.

Since all of the gnotobiotic pigs survived their exposure to the vaccine virus at various dilutions, no LD_{50} was established for gnotobiotic pigs.

III. Immune Response

Clinical Signs

Most of the exposed pigs in these 4 litters developed some degree of diarrhea by 48 hours PE. The diarrhea never became excessive, and the feces were normal in all but one pig by 7 days PE. One pig did have diarrhea intermittently for 10 days. This same pig was the only one to also display weakness and partial anorexia. These clinical signs had disappeared by the time of euthanasia 2 weeks PE.

In the pigs in this portion of the experiment, the feces were more of a brown color than yellow and were not as fluid as seen in the variable ambient temperature studies.

Gross Lesions

Since all pigs had recovered fully from their illness, there were no observable gross lesions at the time of euthanasia.

Histopathologic Lesions

Only 3 of the 24 pigs that were orally exposed to the vaccine virus had microscopic lesions of TGE. These lesions included shortened, partially fused villi that were covered by a cuboidal to low columnar epithelium. The crypts in these sections of jejunum and ileum appeared deeper and mildly hyperplastic. The mesenteric lymph nodes from these same 3 pigs seemed hypercellular, and secondary follicles were present.

Four of the 6 exposed pigs in the last litter had multifocal accumulations of lymphocytes either near or around small blood vessels of the myocardium (Figures 23 and 24). Scattered areas of very early muscle fiber degeneration were also seen in these same myocardial sections. One of these pigs had centrilobular hepatocellular degeneration and hemorrhage in the liver. These hepatic lesions were sporadic in the liver parenchyma and did not affect all lobules.

All other tissues appeared relatively normal.

Immunofluorescence

Of the 16 exposed pigs that were euthanatized at 2 weeks PE, only one pig still had TGE viral antigen present in the intestinal tract as detected by fluorescent antibody technique. No fluorescence was observed in the intestinal sections of the 8 exposed pigs that were euthanatized at 3 weeks PE.

Figure 23. This section of myocardium was taken from a 28-day-old gnotobiotic pig which had been orally exposed to an attenuated TGE virus 14 days earlier. Note the accumulations of lymphocytes near this artery. H&E stain, 300X.

Figure 24. A higher magnification of the above myocardial section confirms the presence of small, mature lymphocytes, as well as scattered plasma cells. H&E stain, 1200X.

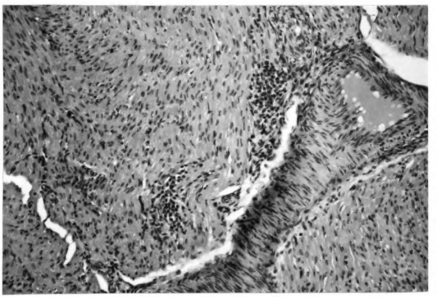

Figure 23

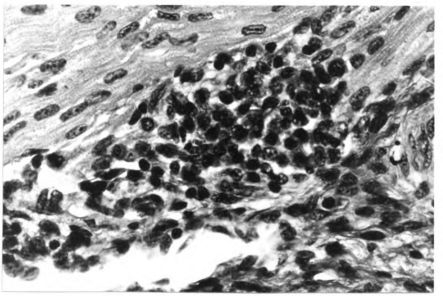

Figure 24

TGE Antibody Titers

The serum titers of anti-TGE antibody which were detected in these pigs are listed in Table 10. The titers in the exposed pigs ranged from <2 to 128. However, these titers were inconsistent. No correlations could be drawn between serum antibody levels and length of time after exposure, nor could any relationships be found between antibody levels and clinical signs.

Lymphocyte Stimulation

The results of the lymphocyte stimulations are listed in Tables 11 through 16. Concanavalin A, which specifically induces T-lymphocytes to replicate, was used as a control to demonstrate the cultured lymphocytes' capability to be stimulated. The stimulation index was used to illustrate the relative effect each antigen had on these cultures. The low stimulation indices present in Tables 13, 15 and 16 indicate that the ConA had lost its ability to stimulate lymphocyte replication. This may have occurred as a result of repetitive freezing and thawing of the ConA solution. When these results were obtained, a fresh ConA solution was made and used to complete the study.

The same loss of stimulatory effect occurred with the viral antigenic solution, as seen in Tables 14, 15 and 16. This solution was also subjected to several freeze-thaw sequences, which may have destroyed its antigenic properties.

A stimulation index of 10 or greater was considered a significant result. Although several indices were well above 10, there appeared to be no significant difference between the control and exposed pigs. These results were statistically analyzed on an individual litter basis as well as by grouping all of the control pigs together and comparing

Table 10. Antibody responses of gnotobiotic pigs orally exposed to a modified live TGE vaccine virus

Litter VII		Litter VIII		Litter IX		Litter X	
2 wks PE		2 wks PE		2 wks PE		2 wks PE	
Pig No.	SVN Titer	Pig No.	SVN Titer	Pig No.	SVN Titer	Pig No.	SVN Titer
57 C	<2*	67 C	<2	76 C	<2	87 C	<2
58 C	<2	68 C	<2	82 E	32	88 C	<2
59 C	<2	71 E	<2	83 E	128	92 E	<2
60 C	<2	73 E	128	84 E	8	93 E	<2
61 E	4	74 E	64			94 E	8
62 E	32	75 E	64				
63 E	16						
64 E	<2						
65 E	<2	3 wks PE		3 wks PE		3 wks PE	
66 E	<2						
		69 C	<2	77 C	<2	85 C	<2
		70 E	16	78 C	<2	86 C	<2
		72 E	<2	79 E	16	89 E	<2
				80 E	32	90 E	<2
				81 E	<2	91 E	<2

*Titers are expressed as the reciprocal of the highest serum dilution completely neutralizing 100 $TCID_{50}$ of TGE virus.

SVN = serum virus neutralization

C = control pig

E = exposed pig

Table 11. Proliferative responses of peripheral blood lymphocyte cultures from gnotobiotic pigs to ConA: litter VII

Pig No.	Stimulus	Concentration of Stimulus						
		Unstimulated Cultures	Concentration of Stimulus					
			2 µg/ml		5 µg/ml		10 µg/ml	
		Mean Count*	Mean Count	Stim. Index**	Mean Count	Stim. Index	Mean Count	Stim. Index
57 C	ConA	399	51279	128	85373	214	76542	192
58 C		193	2244	12	3517	18	3996	21
59 C		501	14647	29	33737	67	69902	140
60 C		1393	19774	14	72733	52	69926	50
61 E		1939	81953	42	86995	45	78805	41
62 E		1629	44724	27	86688	53	75959	47
63 E		84	387	5	440	5	460	5
64 E		1351	80967	60	83179	62	76270	56
65 E		396	948	2	7103	18	91944	232
66 E		1640	102468	62	94996	58	91255	56

*Mean of triplicate determinations (counts per minute)

**Stimulation index = mean count of stim. cultures/mean count of unstimulated cultures

C = control pig

E = exposed pig

Table 12. Proliferative responses of peripheral blood lymphocyte cultures from gnotobiotic pigs to ConA and TGE vaccine virus: litter VIII

Pig No.	Stimulus	Thymidine Incorporation					
			Concentration of Stimulus				
		Unstimulated Cultures Mean Count*	2 µg/ml		5 µg/ml		10 µg/ml	
			Mean Count	Stim. Index**	Mean Count	Stim. Index	Mean Count	Stim. Index
67 C	ConA	301	610	2	819	3	652	2
68 C		322	2062	6	6245	19	17670	55
69 C		121	186	2	371	3	324	3
70 E		400	3757	9	15489	38	50784	127
71 E		1448	39505	27	163825	113	244468	168
72 E		202	775	4	2451	12	8133	40
73 E		328	2965	9	7697	23	12905	39
74 E		663	2582	4	6434	10	11333	17
75 E		473	1941	4	4636	10	9291	20
			1/100 Dilution		1/10 Dilution		Full Strength	
67 C	TGE Virus	256	2942	11	2613	10	2183	9
68 C		1156	3582	3	4510	4	4895	4
69 C		322	2781	9	2611	8	2357	7
70 E		347	3106	9	4528	13	4751	14
71 E		2577	6208	2	7180	3	7990	3

Table 12 (continued)

		1/100 Dilution		1/10 Dilution		Full Strength	
72 E	165	4043	24	4677	28	4250	26
73 E	118	2558	22	2888	24	3206	27
74 E	328	3011	9	2796	9	3337	10
75 E	497	2977	6	4015	8	4386	9

*Mean of triplicate determinations (counts per minute)

**Stimulation index = mean count of stim. cultures/mean count of unstim. cultures

C = control pig

E = exposed pig

Table 13. Proliferative responses of peripheral blood lymphocyte cultures from gnotobiotic pigs to ConA and TGE vaccine virus: litter IX

Pig No.	Stimulus	Thymidine Incorporation					
			Concentration of Stimulus				
		Unstimulated Cultures Mean Count*	2 µg/ml		5 µg/ml		10 µg/ml	
			Mean Count	Stim. Index**	Mean Count	Stim. Index	Mean Count	Stim. Index
76 C	ConA	460	1068	2	3738	8	12268	27
77 C		204	171	1	241	1	342	2
78 C		247	270	1	250	1	323	1
79 E		176	144	1	247	1	274	2
80 E		333	293	1	351	1	581	2
81 E		321	275	1	307	1	407	1
82 E		548	381	1	503	1	2293	4
83 E		729	2118	3	8982	12	30179	41
84 E		255	533	2	2110	8	11403	45
			1/100 Dilution		1/10 Dilution		Full Strength	
76 C	TGE Virus	537	4827	9	5444	10	5475	10
77 C		187	351	2	2198	12	1656	9
78 C		288	421	1	1911	7	2081	7
79 E		161	285	2	2324	14	1326	8
80 E		391	321	1	1371	4	1222	3

Table 13 (continued)

		1/100 Dilution		1/10 Dilution		Full Strength	
81 E	264	444	2	1784	7	2023	8
82 E	545	3544	7	4592	8	8118	15
83 E	722	2316	3	4003	6	4496	6
84 E	238	2523	11	3987	17	4244	18

*Mean of triplicate determinations (counts per minute)

**Stimulation index = mean counts of stim. cultures/mean counts of unstim. cultures

C = control pig

E = exposed pig

Table 14. Proliferative responses of peripheral blood lymphocyte cultures from gnotobiotic pigs to ConA and TGE vaccine virus: litter X

Pig No.	Stimulus	Thymidine Incorporation: Unstimulated Cultures Mean Count*	Concentration of Stimulus: 2 µg/ml Mean Count	2 µg/ml Stim. Index**	5 µg/ml Mean Count	5 µg/ml Stim. Index	10 µg/ml Mean Count	10 µg/ml Stim. Index
85 C	ConA	207	310	1	496	2	1101	5
86 C		355	487	1	781	2	1612	5
87 C		495	1042	2	1367	3	2188	4
88 C		258	400	1	489	2	619	2
89 E		314	1353	4	2796	9	6935	22
90 E		215	1259	6	3630	17	6978	32
91 E		350	717	2	883	2	1226	3
92 E		623	862	1	997	1	1286	2
93 E		391	1058	2	1278	3	1771	4
94 E		394	464	1	420	1	505	1
			1/100 Dilution		1/10 Dilution		Full Strength	
85 C	TGE Virus	206	178	1	177	1	192	1
86 C		319	340	1	281	1	346	1
87 C		451	474	1	424	1	509	1
88 C		276	296	1	267	1	301	1
89 E		331	243	1	247	1	322	1
90 E		233	173	1	186	1	210	1

Table 14 (continued)

		1/100 Dilution		1/10 Dilution		Full Strength	
91 E	357	229	1	237	1	212	1
92 E	589	573	1	497	1	530	1
93 E	445	384	1	364	1	422	1
94 E	393	116	0	99	0	133	0

*Mean of triplicate determinations (counts per minute)

**Stimulation index = mean count of stim. cultures/mean count of unstim. cultures

C = control pig

E = exposed pig

Table 15. Proliferative responses of mesenteric lymph node lymphocyte cultures from gnotobiotic pigs to ConA and TGE vaccine virus: litter IX

Pig No.	Stimulus	Thymidine Incorporation					
			Concentration of Stimulus				
		Unstimulated Cultures	2 µg/ml		5 µg/ml		10 µg/ml	
		Mean Count*	Mean Count	Stim. Index**	Mean Count	Stim. Index	Mean Count	Stim. Index
76 C	ConA	73	94	1	103	1	84	1
77 C		80	67	1	70	1	59	1
78 C		69	98	1	92	1	72	1
79 E		67	76	1	82	1	65	1
80 E		121	83	1	123	1	106	1
81 E		76	81	1	92	1	74	1
82 E		76	89	1	103	1	108	1
83 E		148	207	1	735	5	2534	17
84 E		77	134	2	169	2	522	7
			1/100 Dilution		1/10 Dilution		Full Strength	
76 C	TGE Virus	75	734	10	2075	28	2405	32
77 C		67	75	1	78	1	67	1
78 C		64	70	1	72	1	71	1
79 E		69	102	1	86	1	66	1
80 E		119	158	1	153	1	129	1

Table 15 (continued)

		1/100 Dilution		1/10 Dilution		Full Strength	
81 E	67	65	1	78	1	66	1
82 E	71	2065	29	2295	32	2029	29
83 E	106	750	7	1976	19	2666	25
84 E	76	2507	33	3611	48	3058	40

*Mean of triplicate determinations (counts per minute)

**Stimulation index = mean count of stim. cultures/mean count of unstim. cultures

C = control pig

E = exposed pig

Table 16. Proliferative responses of mesenteric lymph node lymphocyte cultures from gnotobiotic pigs to ConA and TGE vaccine virus: litter X

Pig No.	Stimulus	Thymidine Incorporation					
			Concentration of Stimulus				
		Unstimulated Cultures Mean Count*	2 µg/ml		5 µg/ml		10 µg/ml	
			Mean Count	Stim. Index**	Mean Count	Stim. Index	Mean Count	Stim. Index
85 C	ConA	107	5885	55	8499	79	11334	106
86 C		86	251	3	264	3	364	4
87 C		81	91	1	117	1	74	1
88 C		78	77	1	77	1	70	1
89 E		169	7053	42	11215	66	20326	120
90 E		84	1128	13	2091	25	3612	43
91 E		424	13453	32	18803	44	22074	52
92 E		101	94	1	127	1	85	1
93 E		118	129	1	178	1	118	1
94 E		77	64	1	69	1	67	1
			1/100 Dilution		1/10 Dilution		Full Strength	
85 C	TGE Virus	108	94	1	147	1	116	1
86 C		81	91	1	83	1	87	1
87 C		87	130	1	144	1	133	1
88 C		95	159	2	208	2	254	3
89 E		139	115	1	132	1	131	1
90 E		126	66	1	90	1	80	1

Table 16 (continued)

		1/100 Dilution		1/10 Dilution		Full Strength	
91 E	432	137	0	164	0	209	0
92 E	123	188	1	239	2	262	2
93 E	81	180	2	189	2	244	3
94 E	95	147	1	137	1	157	2

*Mean of triplicate determinations (counts per minute)

**Stimulation index = mean count of stim. cultures/mean count of unstim. cultures

C = control pig

E = exposed pig

them to all of the exposed pigs. This lack of statistical significance resulted in spite of the wide difference between litters in their lymphocyte responsiveness.

DISCUSSION

Gnotobiotic Pigs Orally Exposed to a Vaccine Virus

As was repeatedly observed in the 10 litters studied during the course of this research, the modified live TGE vaccine virus is capable of causing clinical illness when susceptible pigs are orally exposed to it. Intestinal lesions occur in the jejunum and ileum similar to those seen resulting from a natural infection caused by the TGE virus. Occasionally, death does occur as a result of oral exposure to the vaccine virus.

This study was not the only investigation to demonstrate the possible danger of using a modified live TGE virus in an area where young pigs might become orally exposed. Larson et al. (1980) reported that clinical illness, consistent with TGE, and death resulted from the oral exposure of young pigs to an attenuated TGE virus. They observed a prolonged incubation period as well as a protracted course of illness when compared to naturally occurring cases of TGE. This longer-than-usual incubation time could actually make the attenuated strain of TGE more dangerous, in that pigs that had orally encountered the attenuated virus and were transported to another farm during the prolonged incubation period could then become ill and thus transmit this virus to a new susceptible swine population. Waxler (1979b) demonstrated the ability of the modified live TGE virus to cause disease in young gnotobiotic pigs also. Here, too, both the incubation period

and the course of the disease were prolonged. The present study had similar findings since the average incubation period appeared to be approximately 48 hours as opposed to 14 to 20 hours for the virulent strain of TGE as reported by Hooper and Haelterman (1967) and Cornelius et al. (1968).

The lesions produced by the oral administration of the vaccine virus were similar to those seen after exposure to the virulent virus. Wet rear quarters that were stained with yellow, fluid feces were observed in nearly all of the exposed pigs. This same observation had been repeatedly reported after exposure to the virulent TGE virus (Bay et al., 1958; Trapp et al., 1966). Internal gross lesions included stomachs that contained partially digested milk curds, gas-distended small intestine, and the presence of fluid ingesta throughout the intestinal tract. These same lesions have been noted by Trapp et al. (1966), Cross and Bohl (1969) and Hooper and Haelterman (1969). Sub-gross examination of various segments of the small intestine revealed obviously shortened villi in the jejunum and ileum. Again, this is consistent with cases of TGE caused by the virulent field virus (Trapp et al., 1966; Hooper and Haelterman, 1969).

Microscopic examination of the various areas of the intestinal tract also revealed many similarities between the disease caused by the vaccine virus and the lesions seen with the virulent virus. Markedly shortened or "atrophic" villi were seen in the jejunum and ileum along with deepened or hyperplastic crypts. Numerous reports of villus atrophy have been made when describing the histopathologic changes that occur with TGE (Trapp et al., 1966; Hooper and Haelterman, 1969; Kent and Moon, 1973). Often these short, blunt villi were fused to neighboring villi, as Trapp et al. (1966) had noted. Hooper and

Haelterman's (1969) finding of a low cuboidal epithelial covering was also seen in these pigs. Occasionally, the epithelium had been eroded away, leaving only an exposed lamina propria that was denuded of its protected mucosal lining, just as Bay et al. (1951) had reported. Particular attention was also paid to the lamina propria, but no evidence of edema, hemorrhage or inflammatory cell infiltration could be found. This lack of involvement in the lamina propria was also recognized by Trapp et al. (1966).

Unexposed pigs were used as normal reference animals in each litter to further illustrate the virus-related lesions in the exposed pigs. A normal progression from the unvacuolated columnar epithelium in the duodenum to the moderately vacuolated epithelium of the jejunum to the heavily vacuolated epithelium of the ileum was observed in all control pigs. These observations are in agreement with Moon et al. (1973), who found that the presence of these vacuoles was a function of age. As the pig grew older, the epithelial cell turnover rate increased and the presence of vacuoles decreased. This loss of vacuolization also progressed from anterior to posterior through the small intestine. The vacuolization appeared to remain in gnotobiotic pigs longer than in conventional pigs (Heneghan et al., 1979).

Of the 15 exposed pigs that were kept in the cold ambient temperature, 7 had visible lesions of TGE in the intestinal tract. Similarly, 4 of the 9 exposed pigs reared in the warm environment had TGE-related intestinal lesions. However, further scrutiny of these figures reveals that all of these pigs with lesions were euthanatized at 5 to 7 days postexposure, irrespective of the ambient temperature. Thus, 11 out of the 13 pigs that lived to at least day 5 PE had lesions of TGE. It appears that the observation of intestinal lesions in this aspect of

the study was more a consequence of time after viral exposure than the various ambient temperatures. This finding is consistent with the fact that an attenuated TGE virus was used which typically has a longer incubation period (Haelterman and Hooper, 1967; Cornelius et al., 1968). Therefore, it is possible that, had the pigs that were euthanatized at 3 days PE been allowed to survive until 5 to 7 days PE, they too would have developed lesions of TGE.

Variable Ambient Temperature: Its Effect on the Gnotobiote and the Virus

It is known from previous reports that the attenuated TGE viruses are more capable of replicating in pigs kept at low ambient temperatures (10 to 12 C) than the virulent TGE virus is (Furuuchi et al., 1975). On the other hand, it appears that these same attenuated viruses have difficulty replicating at high ambient temperatures (31 to 34 C) (Furuuchi et al., 1966). Furuuchi and Shimizu (1976) noted that the highest tissue levels of virus occurred in pigs kept at an environmental temperature of 8 to 12 C after exposure to an attenuated TGE virus. These researchers also reported that pigs kept at temperatures of 20 to 23 C and 34 to 37 C had much lower tissue levels of the attenuated virus.

It would appear that some of the results of the present study substantiate Furuuchi and Shimizu's findings. As previously mentioned, the only deaths (3 pigs) during this portion of the research occurred in the cold room. A review of the immunofluorescence results (Table 17) reveals that 60% (9/15) of the exposed pigs in the cold room had intestinal fluorescence for TGE antigen, whereas only 44% (4/9) of the exposed pigs in the warm room had a similar fluorescence. These 2

Table 17. Correlation between the presence of immunofluorescence for the TGE virus and intestinal lesions in gnotobiotic pigs orally exposed to a TGE vaccine virus and kept at two different ambient temperatures

Litter No.	Ambient Temperature 16-18 C				Ambient Temperature 32-34 C			
	Pig No.	Day PE Euthanatized	FA	Intestinal Lesions	Pig No.	Day PE Euthanatized	FA	Intestinal Lesions
I	3	3	+	-	**			
	4	5	+	-				
	5	5	+	+				
II	8	3	-	-	14	3	-	-
	9	5*	-	+	15	5	-	+
	10	3	+	-	16	3	+	-
	11	5*	-	+	17	5	-	+
III	20	3	+	-	25	3	+	-
	21	3	+	-	26	5	+	+
	22	5	+	+				
	23	5	+	+				
IV	29	3	+	-	35	3	-	-
	30	5	-	+	36	5	-	-
	31	3	-	-	37	5	+	+
	32	5*	-	+				

*Pigs died from viral infection

**No pigs kept in warm room

findings might be correlated to a possible increase in virus propagation in the exposed pigs kept in a cold ambient temperature.

There appeared to be no correlation between the cold environment and the formation of intestinal lesions related to TGE. Only 46% (7/15) of the exposed pigs in the cold room had intestinal lesions of TGE. Similarly, 44% (4/9) of the exposed pigs in the warm room had virus-induced intestinal lesions, nor was there any correlation between positive immunofluorescence and the presence of intestinal lesions. Only 20% (3/15) of the exposed pigs from the cold room had both immunofluorescence for the TGE virus and intestinal lesions. Of the exposed pigs kept in the warm environment, 22% (2/9) had positive fluorescence combined with villus atrophy. This lack of correlation between immunofluorescence and intestinal changes is not unusual. To obtain fluorescence for the TGE virus, the viral-infected epithelial cells must still be present in the intestine. After these infected cells slough off, the villus shortens and thus produces the atrophy that we associate with TGE. But by losing the cells, the intestinal sections also lose the ability to bind the fluorescein-labeled TGE antibody.

Another possible influence on the pig's ability to survive an exposure to the TGE virus was examined by Shimizu and Shimizu (1979c). In this study they reported that exposed pigs kept at 30 C had a lymphocyte blastogenic response against the TGE virus at 3 days PE. Those pigs that were kept at 4 C did not have a lymphocyte blastogenic response against the TGE virus until 7 days PE. Therefore, it appeared that the decreased ambient temperature either delayed or reduced the cell-mediated response to the TGE virus and, thus, left the virus to replicate unimpaired by the body's defense mechanism.

The cold environment also represents an added stress factor on the pig itself, as evidenced by the extensive subcutaneous edema which developed in all of the pigs kept in the cold room. The pooling of the body's fluids in the subcutis in an animal that is infected with an agent that has the potential to lethally dehydrate the animal can only decrease the animal's chance of survival.

The consistent finding of subcutaneous edema in the pigs from the cold room was probably due to the low serum total protein which was aggravated by the stress of the cold environment. These low serum protein levels in the pigs were not completely unexpected. Because of the placental structure, maternal antibodies could not cross over into the developing fetus. The fact that these were cesarean-derived, colostrum-deprived pigs meant that they were unable to receive any immunoglobulin from the sow after birth. The resulting serum protein deficit was then complicated by the cold ambient temperature, which could have initiated vasoconstriction in the subcutaneous tissues. The vasoconstriction would then lead to venous stasis which could, in turn, deprive the endothelial cells at the venous ends of the capillaries of oxygen and nutrients, thus weakening the endothelial walls and allowing an increased permeability resulting in the loss of plasma fluid (and protein) to the extracellular spaces.

The total protein data listed in Table 9 indicate slightly higher levels in the pigs from the cold room. These values are probably misleading and represent only relative increases in total protein due to the loss of plasma fluid into the extracellular spaces of the subcutaneous tissue. The exposed pigs' total protein could potentially be even lower, because these pigs are not only losing vascular fluid to the subcutaneous edema but also through the diarrhea resulting from the intestinal

lesions. There were no correlations between serum total protein levels and other parameters, such as exposure, clinical signs, etc.

It was interesting to note that the 3 pigs that died in the cold room did not have the subcutaneous edema that the rest of the pigs did in that same environment. This probably is an indication of the dehydration effect that the virus causes through the severe intestinal lesions it produces.

Hyperplasia of the mesenteric lymph nodes was seen most frequently in the virus-exposed pigs that were maintained beyond 3 days PE. Of the 7 pigs with this change from the exposed group in the cold room, 6 were euthanatized at 5 to 7 days PE. Similarly, 4 of the 5 exposed pigs from the warm room that had this lymph node change were euthanatized at 5 to 7 days PE. It appears that this lymphoid hyperplasia was a result of the viral infection and was not significantly influenced by the ambient temperature. This appears to be the first report of hyperplastic mesenteric lymph nodes related to exposure to the TGE virus in gnotobiotic pigs.

The ambient temperature of the pigs' environment did affect the liver. Those pigs kept in the cold room all had livers that contained nonvacuolated hepatocytes. The pigs that were reared in the warm room all had extensively vacuolated hepatocytes, especially in the centrilobular regions of the parenchyma. Since oil red O stains of these vacuolated hepatocytes did not reveal the presence of fat, the vacuoles most probably represent hepatic glycogen stores. The glycogen was probably depleted in those pigs kept in the cold environment because of their use of the glycogen to produce energy to maintain homeothermia (Curtis, 1970).

The increased hepatic extramedullary hematopoiesis observed in the pigs from the cold room is probably another result of the pigs' attempts to effectively control thermoregulation. The increased metabolic rate of these pigs would necessitate an increased oxygen consumption. In order for these pigs to meet their tissues' demands for more oxygen, more erythrocytes would need to be produced to carry the oxygen to the tissues.

The presence of focal hepatocellular necrosis in some of the exposed pigs from the cold room may be a result of the combined effect of the stress of a cold environment and the release of toxic products from dead tissue into the blood stream as a result of viral infection of the intestinal tract. The center of the lobule might be affected first because those hepatocytes could be slightly hypoxic, since they are at the end of the flow of oxygenated blood. Those hepatocytes would be more susceptible to a weak tissue toxin than the hepatocytes at the periphery of the lobule which receive an adequate supply of oxygen. It does not seem likely that these focal hepatic lesions represent a direct effect of this coronavirus on the hepatocyte. None of the previous reports correlate a hepatocellular lesion with TGE.

The focal lesions in the lung tissue from several of the pigs kept in the cold room could possibly be related to a mild form of aspiration pneumonia complicated by the additional stress of the cold environment.

LD_{50} Determinations

In the LD_{50} studies, 1 ml of a 1/100 dilution of the TGE vaccine was established as the oral LD_{50} in conventional pigs. The inability to determine an LD_{50} in the gnotobiotic pigs could mean that the lethal effects of TGE are actually due to a combined infection of the coronavirus

and an enteric bacterium, such as *Escherichia coli*. This would explain why the gnotobiotic pigs, which lack even the normal intestinal flora, did not succumb to the vaccine virus.

A second possibility for the inconsistent results between the 2 LD_{50} litters was a possible change in virulence of the attenuated virus. Two different lots of vaccine were used, one for each litter. Therefore, there is a possibility that one lot of vaccine, i.e., the lot used in the conventional pigs, may have been more virulent than the vaccine used in the gnotobiotic pigs.

Immune Response

The lack of significant lymphocyte stimulation in the previously exposed pigs and their inconsistent antibody production were both disappointing results in this experiment. It might be thought that possibly the virus had been inactivated by either the low gastric pH or intestinal enzymes, as Furuuchi et al. (1975) had observed in a portion of their research. But this could not have been the case here, since all but 3 of the pigs exhibited clinical signs of illness following the oral administration of the virus.

Part of the problem with the lymphocyte stimulation results may have been with the viral antigen that was used to activate the lymphocyte cultures. This viral antigen was very crude at best, due to the difficulty experienced in trying to reproduce the virus in porcine cell cultures. Therefore, possibly a more refined antigen may have yielded more dynamic results.

The loss of the stimulatory effect of this virus suspension in the last few lymphocyte stimulation studies was probably due to a loss of the peplomers which protrude from the outer surface of the coronavirus.

Several sequential freeze-thaw manipulations of the viral suspension could damage these peplomers to the point that the lymphocytes can no longer recognize the viral antigen and therefore do not respond to the presence of the virus.

Problems were also encountered in preparing the lymphocyte cultures from the mesenteric lymph nodes. The percentage of viable cells that were used to initiate the cultures was very low (less than 50% in some cases), either due to improper handling of the tissue, inappropriate media for transport of the tissue, or bacterial contamination of the cell cultures during preparation. Fortunately, these same problems were not experienced during the preparation of the peripheral blood lymphocyte cultures.

Despite all of the problems encountered with the process of detecting the cell-mediated response that should have arisen during the initial exposure to the vaccine virus, several pigs in litter VII (Table 12) did have peripheral blood lymphocytes that reacted strongly to the presence of the TGE virus in their cultures. Results such as these indicate that there definitely is a cell-mediated component to the immunologic response mounted against the TGE virus.

The anti-TGE antibody titers that were detected in the serum samples of these pigs following oral exposure to the attenuated virus were inconsistent both between litters and among members of the same litter. Since 2 pigs from different litters each had a SVN titer of 128, it appears that these pigs were immunologically competent. Slightly more than half of the exposed pigs (13/24) developed some level of humoral immunity against the TGE virus.

Both Woods (1979) and Furuuchi et al. (1978) found consistently high levels of SN antibodies against TGE in orally exposed pigs.

However, these researchers used much higher doses of an attenuated virus than was used in the present experiment. Woods administered 10 ml of a suspension that had 10^6 PFU of an attenuated TGE virus. Furuuchi and his co-workers inoculated their pigs with 4 ml of a 10^7 $TCID_{50}$ viral suspension. Considering that the pigs in this study only received 1 ml of a 5×10^2 $TCID_{50}$ viral suspension, this low oral dose may have accounted for the inconsistent antibody results.

Furuuchi et al. (1975) concluded that attenuated strains of TGE virus do not propagate as well in pigs maintained in a warm ambient temperature. Thus, even though these pigs did show clinical illness, i.e., diarrhea, which would indicate some degree of virus replication in the intestine, perhaps the titers produced would have been greater if the pigs had been reared in a colder environment.

The appearance of immunofluorescence in the jejunum of one of the pigs that was euthanatized 2 weeks after virus exposure probably represents a carrier state for the TGE virus. This adds further evidence to the hypothesis expressed by Morin et al. (1978) and Underdahl et al. (1974) that some pigs continue to shed the TGE virus for an unspecified length of time after the clinical signs of illness have disappeared and the pig seems "normal." This carrier-shedder pig would then act as a source of infection for any susceptible animals with which it had contact and, thus, perpetuate the disease.

The myocardial and hepatic lesions that were observed in several of the pigs in the last litter were consistent with a possible viral infection. Since these lesions have not been previously reported for TGE, and since none of the other litters exhibited the same lesions, other viral diseases were considered. Porcine parvovirus was ruled out because of the lack of antibody titers against this virus in the serum

of these pigs. Another possibility was the virus that causes porcine encephalomyocarditis. Since diagnostic tests for this virus were unavailable, this virus could not be completely dismissed. How and when these pigs could have come in contact with any other virus except the TGE virus cannot be easily answered. The 2 most logical times of exposure (although unlikely) would be either *in utero* or possibly as a contaminant of the TGE vaccine.

Inconsistent results seemed to plague the entire research effort. In the cold studies, some pigs would survive while others would die following exposure to this attenuated virus. A definitive LD_{50} was attained in conventional pigs, while no LD_{50} could be determined in gnotobiotic pigs. Several pigs produced high levels of antibodies after oral exposure to this virus, while other pigs apparently developed no antibody titer to the TGE virus at all.

From the positive results that were encountered, it appears that oral vaccination with an attenuated TGE virus could become a usable means of immunization, *if* the inoculum would consistently and safely induce immunity. This study, however, also demonstrated the possible dangers of using a modified live TGE vaccine, for the vaccine virus that was used still had the capability to produce clinical illness in young, susceptible pigs and, in a few instances, this illness resulted in death.

SUMMARY

Effects of Variable Ambient Temperature on the Gnotobiotic Pig Orally Exposed to a TGE Vaccine Virus

The pigs that were reared in a low ambient temperature (16 to 18 C) maintained a slightly lower body temperature (1 to 2 C lower) than their littermates that were kept in a warm ambient temperature (32 to 34 C). The incubation period for this portion of the research was approximately 48 hours. Three pigs in the cold room died as a result of their oral exposure to the attenuated TGE virus on day 5 PE. These 3 pigs were anorectic, depressed, and severely dehydrated prior to death. Gross lesions included: gas-filled stomach and intestine; yellow, fluid ingesta within the small intestine; and shortened villi in the jejunum and ileum. All of the pigs reared in the cold room developed extensive subcutaneous edema. Of the exposed pigs, 7/15 from the cold room and 4/9 from the warm room had histopathologic changes which included atrophic and fused villi covered by cuboidal epithelial cells and hyperplastic mesenteric lymph nodes. Foci of centrilobular hepatocellular necrosis with lymphocytic infiltration was seen in 7/15 pigs from the cold room only. All of the pigs kept in the warm room had highly vacuolated hepatocytes, whereas all of the pigs reared in the cold room had nonvacuolated hepatocytes. All pigs had very low serum total protein levels, which might partially account for the subcutaneous edema in the pigs from the cold room.

LD_{50} Determinations

Anorexia and diarrhea were seen in the pigs of the conventional litter after an incubation period of 2 to 4 days following oral exposure to the vaccine virus. By day 7 PE the 4 pigs in the 2 highest dose groups (F.S. + 1/10) were dead, and a fifth pig from the 1/100 dilution of the vaccine died on day 8 PE. Lesions of atrophic villi covered by cuboidal epithelium were present in these pigs.

The gnotobiotic pigs had a slightly longer incubation period (4 to 5 days) before diarrhea was first noted. The soft feces persisted for about 4 days, followed by a complete recovery by all pigs. No gross lesions were present at the time of necropsy on day 18 PE, and only 2 pigs had mild villus atrophy.

None of the 19 pigs from the 2 LD_{50} litters displayed any immunofluorescence in the intestinal epithelium.

Thus, 1 ml of a 1/100 dilution of the vaccine was the LD_{50} in conventional pigs. No LD_{50} was determined for gnotobiotic pigs.

Immune Response

The average incubation period for the pigs that were orally exposed to the TGE vaccine virus in this portion of the experiment was 48 hours. At the time of necropsy 2 to 3 weeks after exposure, no gross lesions were observed. Only 3 of these 24 exposed pigs had mild shortening of the jejunal villi and slight hyperplasia of the mesenteric lymph nodes. The jejunum of 1 pig still contained viral antigen at 2 weeks PE, as indicated by immunofluorescence.

The serum virus-neutralization titers for TGE ranged from <2 to 128 in the exposed pigs. Only 13/24 exposed pigs had detectable antibody titers for TGE.

Equally inconsistent were the results of the lymphocyte stimulation tests. Several cultures of peripheral blood lymphocytes reacted strongly to the presence of the TGE virus, yet many cultures only reacted weakly or not at all. Thus, no significant results were obtained on the extent of immunity induced in these pigs by their oral exposure to the TGE vaccine virus.

From these results, it appears that a cold ambient temperature can complicate or enhance the disease process when a pig is orally exposed to the TGE virus. Gnotobiotic pigs are also more susceptible to a decreased ambient temperature even without being exposed to an infectious agent. When working with gnotobiotic pigs, changes in the mesenteric lymph nodes may be a consistent outcome of infection with the TGE virus, as well as the usual jejunal and ileal lesions.

Finally, it appears that this particular attenuated virus would not be safe to use orally to induce immunity to TGE in young pigs. The humoral and cell-mediated immune systems appear to be only partially or inconsistently evoked by this strain of virus, and yet the virus still has the potential to produce a lethal illness in the young pig.

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VITA

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