Persistence of porcine Coronavirus, transmissible gastroenteritis virus, in swine testicle cells
by Susan Marie Williams

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Microbiology
Montana State University
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Abstract:
Porcine transmissible gastroenteritis virus (TGEV) readily and reproducibly established persistent
infection in swine testicle (ST) cells. Swine testicle cells persistently infected with TGEV resisted
plaque formation by homologous virus and vesicular stomatitis virus infection, apparently by
producing interferon. In most cultures in our study, approximately 10^4-6 plaque forming units of
TGEV per milliliter were produced throughout the five year culture period. One cell line, 2B-S2,
produced virus only intermittently. In most persistently infected cultures, 20-30% of the cells were
positive for viral antigens by fluorescence. No temperature sensitive virus was produced. The persistent
infection could not be cured by neutralizing polyclonal antibody or by cloning. Interference with
plaque formation on naive ST cells by homologous TGEV Miller strain was demonstrated. Defective
interfering virus was a possible explanation. Transmissible gastroenteritis virus proteins were found in
quantities comparable to those seen in acutely infected ST cells. With the exception of the membrane
(M) protein, which appeared to form multimers the proteins migrated normally during denaturing
polyacrylamide gel electrophoresis. Persistently infected ST cells produced few TGEV specific RNA
containing nucleocapsid (N) gene sequences. Anti-sense RNAs containing the nucleocapsid gene
region were not found in persistently infected cells, although they could be detected in acutely infected
cells, The subgenomic message that codes for N protein was the predominant TGEV specific RNA in
persistently infected ST cells.
PERSISTENCE OF PORCINE CORONAVIRUS, TRANSMISSIBLE GASTROENTERITIS VIRUS, IN SWINE TESTICLE CELLS

by

Susan Marie Williams

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology

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APPROVAL

of a thesis submitted by

Susan Marie Williams

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Porcine transmissible gastroenteritis virus (TGEV) readily and reproducibly established persistent infection in swine testicle (ST) cells. Swine testicle cells persistently infected with TGEV resisted plaque formation by homologous virus and vesicular stomatitis virus infection, apparently by producing interferon. In most cultures in our study, approximately $10^{4.6}$ plaque forming units of TGEV per milliliter were produced throughout the five year culture period. One cell line, 2B-S2, produced virus only intermittently. In most persistently infected cultures, 20-30% of the cells were positive for viral antigens by fluorescence. No temperature sensitive virus was produced. The persistent infection could not be cured by neutralizing polyclonal antibody or by cloning. Interference with plaque formation on naive ST cells by homologous TGEV Miller strain was demonstrated. Defective interfering virus was a possible explanation. Transmissible gastroenteritis virus proteins were found in quantities comparable to those seen in acutely infected ST cells. With the exception of the membrane (M) protein, which appeared to form multimers, the proteins migrated normally during denaturing polyacrylamide gel electrophoresis. Persistently infected ST cells produced few TGEV specific RNA containing nucleocapsid (N) gene sequences. Anti-sense RNAs containing the nucleocapsid gene region were not found in persistently infected cells, although they could be detected in acutely infected cells. The subgenomic message that codes for N protein was the predominant TGEV specific RNA in persistently infected ST cells.
Transmissible gastroenteritis virus (TGEV) is a pathogen of swine that causes transient acute enteritis in adult animals (225). The symptoms include anorexia, profuse watery diarrhea, occasional vomiting, and agalactia in nursing sows. Newborn pigs and piglets under two weeks of age suffer from severe dehydration. Mortality in this age group averages 90% and surviving piglets grow poorly for some time (105,225).

Chronic virus production may contribute to the complex epidemiology of TGEV infection (270). Investigation of chronic infections in animals is complicated by the interaction of the virus with the immune system (1). *In vitro* cultivation of transformed cells with virus provides a model system for examining virus-cell interactions that may allow the virus to persist in intact animals.

Transmissible gastroenteritis virus is a large pleomorphic enveloped virus approximately 240 nanometer (nm) in diameter (264). The single stranded RNA genome of 25 to 28 kilobases has a positive polarity. The surface of the virion is covered with glycoprotein spikes or peplomers. These characteristics led to its classification as a member of the family *Coronaviridae* (264).
The Coronaviruses

Coronaviruses are pathogens of many species of mammals and birds (283). They cause acute and chronic infections of the liver, gastrointestinal tract, and respiratory and nervous systems. Individual viruses may exhibit a specific tissue tropism or infect all tissue types listed. The type species for the family, infectious bronchitis virus (IBV), causes respiratory disease in chickens (283).

Classification of Coronaviruses

Coronaviruses are divided into four antigenic groups based on immunofluorescence and cross neutralization studies using both polyclonal and monoclonal antisera (261). Avian and mammalian coronaviruses fall into two distinct groups. In addition to TGEV, porcine respiratory coronavirus (PRCV), porcine hemagglutinating encephalitis virus (HEV), and porcine epidemic diarrhea virus (PEDV) are other coronaviruses known to infect pigs. Porcine epidemic diarrhea virus is not yet firmly classified in one of the antigenic groups, although it can be antigenically distinguished from TGEV (104). Transmissible gastroenteritis virus, feline infectious peritonitis virus (FIPV), canine coronavirus (CCV), and PRCV are so closely related antigenically that some authors have suggested that they are host range variants of a single virus species (121,199). Coronaviruses have been isolated from human infants with necrotizing enterocolitis and propagated in organ culture. The relationship of these human enteric coronaviruses (HECVs) to enteric disease and other coronaviruses remains unclear.
(213). The members of the Coronaviridae are classified into three antigenic groups (Table 1.1) consisting of mammalian coronaviruses (groups I and II) and IBV (group III) (261,275).

Table 1.1. Classification of the Coronaviridae.

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus</th>
<th>Acronym</th>
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<tbody>
<tr>
<td>I.</td>
<td>transmissible gastroenteritis virus</td>
<td>TGEV</td>
</tr>
<tr>
<td></td>
<td>canine coronavirus</td>
<td>CCV</td>
</tr>
<tr>
<td></td>
<td>feline enteric coronavirus</td>
<td>FECV</td>
</tr>
<tr>
<td></td>
<td>feline infectious peritonitis</td>
<td>FIPV</td>
</tr>
<tr>
<td></td>
<td>human coronavirus-229E</td>
<td>HCV-229E</td>
</tr>
<tr>
<td></td>
<td>porcine respiratory coronavirus</td>
<td>PRCV</td>
</tr>
<tr>
<td>II.</td>
<td>mouse hepatitis</td>
<td>MHV</td>
</tr>
<tr>
<td></td>
<td>hemagglutinating encephalomyelitis of swine</td>
<td>HEV</td>
</tr>
<tr>
<td></td>
<td>bovine coronavirus</td>
<td>BCV</td>
</tr>
<tr>
<td></td>
<td>rabbit coronavirus</td>
<td>RbCV</td>
</tr>
<tr>
<td></td>
<td>human coronavirus OC43</td>
<td>HCV-OC43</td>
</tr>
<tr>
<td></td>
<td>turkey coronavirus</td>
<td>TCV</td>
</tr>
<tr>
<td>III.</td>
<td>infectious bronchitis virus</td>
<td>IBV</td>
</tr>
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</table>

**Virions of Coronaviruses**

In negatively stained preparations, coronavirus virions are spherical or pleomorphic and enveloped by a lipid bilayer surrounded with characteristic club shaped spikes "like the corona spinarum in religious art" (261). Virions have a diameter of approximately 80 to 160 nm. Large, widely spaced spikes 12 to 24 nm long cover the surface. The nucleocapsid is difficult to visualize in mature virions and is easily disrupted. It
appears to contain a helical arrangement of a thin ribonucleoprotein strand 9 to 11 nm in diameter. There is ultrastructural evidence of an internal membrane sac possibly continuous with the exterior membrane, although the relationship between the observed structure and the nucleocapsid is not known (73,215,268).

The virions are sensitive to ether and chloroform, and can be inactivated by incubation at 56°C for 30 minutes. Virions inactivate slowly at 25 to 37°C and are relatively stable at 4°C. Coronaviruses have variable sensitivity to acid pH but are unstable under alkaline conditions. Density gradient analysis in sucrose yields a density of approximately 1.18 g/cm³ (239).

**Molecular Biology of Coronaviruses**

Coronavirus structural proteins and mRNAs will be addressed using the nomenclature recommended by the Coronavirus Study Group of the Vertebrate Virus Subcommittee of the International Committee on Taxonomy of Viruses (36). Nomenclature for nonstructural proteins and their genes was not covered in the subcommittee's recommendations and has not been standardized for coronaviruses.

**Genome of Coronaviruses.** The genome of coronaviruses is an unsegmented single-stranded RNA of approximately 6 to 10 megadaltons and has the same polarity as messenger RNA (142). It is capped, polyadenylated and infectious (22,144,164,233,261,280). Infectious bronchitis virus has a genome 27.6 kilobases in length (20), while the genome of MHV is 31 kilobases in length, the largest RNA described to date (155). Because of the difficulty in accurately sizing such large
RNAs, each viral genome must be cloned and sequenced to determine its precise size. The genomic RNA for the Purdue strain of TGEV has been sequenced and is 28 kilobases in length (74). Each genome codes for three to four virion proteins and three to six nonstructural proteins (Figure 1.1). All the coronaviruses share approximately the same gene order although they differ in the total number of genes. At the 5' end of the genome are two large open reading frames (ORFs) which code for the putative viral RNA-dependent-RNA-polymerase(s). These two nonstructural genes utilize two thirds of the coding capacity of the genome and are probably translated directly from the positive sense genome (20,155). In MHV, BCV, and HEV the gene for the hemagglutinin/esterase protein follows. This gene is homologous to the hemagglutinin (HA) gene of influenza virus and may be the result of a relatively recent genetic exchange between these two unrelated virus groups. This gene is absent in many other coronaviruses. The spike protein (S) is encoded by the next gene. This protein, previously designated E2, is the major virion glycoprotein that forms the peplomers. Two to three ORFs for putative nonstructural proteins follow the S gene. The gene encoding the membrane (M) protein is 3' to the nonstructural genes. This unusual viral glycoprotein, previously called E1, functions like the matrix proteins of orthomyxo and paramyxoviruses, but is structurally different. A 3'-terminal gene codes for the nucleocapsid (N) protein, a phosphoprotein which interacts with the RNA genome to form the internal nucleocapsid. In the TGEV genome the nucleocapsid coding region is followed by an additional ORF that codes for a very small hydrophobic protein (Hb) of unknown function.
S is the preplomer protein.
M is the thematrix or membrane protein.
N is the nucleocapsid protein.
ns represents nonstructural proteins.
SM represents the small membrane protein.

Figure 1.1. Subgenomic and genomic RNAs of coronaviruses (116).

Subgenomic Viral RNAs. With the exception of the polymerase genes, viral proteins are translated from a nested set of subgenomic mRNAs which were first described in cells infected by the JHM strain of MHV (216). Coronaviruses express 6 to 8 subgenomic mRNAs depending on the species (142). Early analysis of these smaller RNAs by RNase T1 oligonucleotide mapping showed that they shared a common 3' end extending for various distances in the 5' direction along the genome (250). The subgenomic RNAs form a nested set in which each RNA contains all the information of those RNAs smaller in size with additional unique information at the 5' ends. All but the smallest mRNA are structurally polycistronic. In vitro translation experiments conducted on isolated mRNAs as well as similar studies in transfected
oocytes have shown that only the most 5' gene on a given RNA is usually translationally active making each RNA functionally monocistronic (124,241,251). Two ORFs have been described and translated in vitro from the 5' unique regions of RNA 1 (polymerase genes) and RNA 5 (MHV, nonstructural genes) (27,242,266). The nucleocapsid protein gene of BCV contains an internal ORF with a coding capacity for a 29 kiloDaltons (kDa) protein. This protein (I) of unknown function has been immunoprecipitated from infected cells suggesting that internal ORFs may be expressed (235). The intracellular abundance of subgenomic RNAs vary in a roughly negative relationship to their size. The smallest species, mRNA 7, is the most numerous while larger species are rare. The regulation of RNA frequency is not well understood.

Transcription of the subgenomic mRNAs from the positive sense genome requires a negative-sense RNA intermediate and a mechanism of transcription that allows messages of varying length to be expressed from the full length genome. The mechanism for transcription of the nested set of subgenomic messages from a large template is controversial (142,161). The simplest explanation would be independent initiation of transcription at a separate site along the template for each mRNA with termination at the 3' end of the genome. Alternative splicing of a large precursor RNA might be expected to yield the functional mRNAs with 5' leaders under the general model of eukaryotic mRNA synthesis. However, coronavirus RNA synthesis occurs exclusively in the cytoplasm separated by the nuclear envelope from the cell RNA splicing machinery. A discontinuous transcription process seems to be operational (142).
Several models have been proposed for this novel mechanism of mRNA transcription (246). The first model would allow large regions of the intact template to loop out during transcription. In the second model the leader RNA and the coding regions would self-splice using undetermined donor and acceptor sites. The third model calls for the synthesis of free leader RNAs that bind to the template at or near the consensus sequence priming transcription of the subgenomic RNA. The discovery of complete protection for genome length template in replicative intermediates after RNase A digestion seemed to rule out a looping out mechanism of editing in transcription (14,143). Splicing of separate leader and body sequences seemed unlikely as the leader was found attached to incomplete transcription products that were apparently nascent mRNA chains (14). Support for the leader primed model of discontinuous transcription has been provided by the finding of small leader RNAs in the cytoplasm of MHV infected cells. The putative free leaders were larger than the leaders found on mature mRNAs and would require trimming or editing at their 3' end before they could prime the template internally (15,142). Leader sequences have been found to recombine at high frequency during co-infection of cells with strains of MHV with different leader sequences (170). This may indicate that the leaders are transcribed as independent units. These three models assume that the full length negative template is the only species of negative polarity active in transcription. Coronavirus transcription is being re-examined in light of the discovery of subgenomic length negative stranded species first described for TGEV and later for MHV (231,237). A negative sense RNA (anti-message RNA) has been found by strand-
specific northern blot analysis for each of the major mRNA species in TGEV infected cells.

It is possible that the discontinuous transcription that produces and regulates coronavirus subgenomic RNA synthesis occurs at the level of the negative templates (163). RNA recombination takes place between negative and positive coronavirus sequences and the genome when they are transfected into infected cells. This reopens the possibility of looping out or splicing of sequences in the synthesis of template subgenomic RNAs. Alternatively, the anti-mRNAs could be copied from completely edited mRNAs and simply serve in subsequent rounds of transcription to amplify messenger RNAs (163). Sethna et al. (236) described the packaging of coronavirus mRNAs in virions suggesting that the mRNAs may act as semi-independent replicating elements relying on RNA polymerase supplied by the complete genome. This observation has not yet been confirmed by other workers.

**Coronavirus Proteins.** Coronavirus structural proteins are well characterized. The spike or S protein (formerly E2) is a large glycoprotein 180 to 200 kDa in size. It forms the spikes or peplomers of the virions. The S protein of MHV, BCV, OC43, and IBV is cleaved into two 90 to 110 kDa subunits during virus maturation (35, 51, 106, 263). S protein cleavage does not normally occur in other coronaviruses including TGEV. The S protein is responsible for virus attachment to cell receptors. The fusion of infected cells, a cytopathic effect evident in several coronavirus infections, is also mediated by S protein (43, 83). S protein plays a major role in the
immune response to the virus since neutralizing antibodies (polyclonal and monoclonal), passive antibody protection, and effective cell mediated immunity are all directed against S protein (43,89,119,265,282,286). Immunization with purified S protein from MHV-A59 provides protection against lethal encephalitis in mice, providing evidence that this glycoprotein is the major inducer of protective immunity (48).

The coronavirus spike protein is translated on the rough endoplasmic reticulum (RER) and subsequently modified during virion maturation. In TGEV the unmodified polypeptide chain has 1447 amino acids for a predicted molecular size of 158 kDa with the final protein having a size of 200 to 220 kDa after N-glycosylation.

A second protein, hemagglutinin-esterase (HE), is located in the peplomers of certain coronaviruses, but not TGEV (23). It may be responsible for the different peplomer morphology that can sometimes be visualized on a single virion by electron microscopy (135). This protein is a 65 kDa hemagglutinin, HE (formerly HA or E3). It is expressed in BCV, TCV, HEV, HCV, and the JHM strain of MHV. The protein carries N-linked oligosaccharides and can be isolated as a 140 kDa dimer from BCV (69,135). The biological significance of HE expression is not well understood. In MHV, HE is expressed in some strains and has been correlated with changes in tissue tropism and neurovirulence. The control of expression has been related to the number of UCUAA repeats at the 3'end of leader sequence encoded at the 5' end of the genome (145,238).
A new structural protein, the small membrane protein (SM) with a molecular weight of 12 kDa has been described for TGEV (86,90,289). It is found between the S and M protein coding regions on the TGEV genome. This protein of unknown function is also expressed by IBV, MHV, and BCV (240).

A fourth glycoprotein, designated M for membrane protein, is found in all coronaviruses and appears to be crucial for virion formation. It is a 23 to 34 kDa integral membrane glycoprotein formerly called E1 or gp23 and sometimes referred to as a matrix protein. Although M shares some functional similarities to the matrix proteins of othomyxo- and paramyxoviruses, its structure is substantially different. Sequence analysis has detected several long hydrophobic regions that apparently span the membrane three times (6). Only a small region on the C-terminus and another on the N-terminus are susceptible to proteolysis suggesting that most of the protein is buried in the membrane (260). The M protein of TGEV has a signal peptide of 17 residues which is not present in the virion form of the protein. The protein in its nonglycosylated form contains 245 amino acids after removal of the signal peptide and has a molecular size of 28 kDa (24,153). M accumulates inside infected cells in the golgi, particularly in the perinuclear area. M interacts with viral nucleocapsids in vitro and may serve as the primary effector of virus formation (261,262).

The fifth virion structural protein is a 50 kDa nucleocapsid phosphoprotein designated N. Together with the genomic RNA it forms the helical nucleocapsid (256). The N gene in TGEV is 1149 bp in length and encodes a 382 amino acid polypeptide with a predicted molecular size of 43 to 48 kDa (25,128). The protein is very basic
and phosphorylated at serines. The nucleotide sequences between groups do not exhibit significant homology and the nucleocapsid proteins do not cross react antigenically (7,25,148). In a RNA-overlay-protein-blot-assay (ROPA), bound N protein reacted in a non-sequence specific manner with several radiolabeled RNAs suggesting it is the major RNA binding protein in coronavirus virions. Anti-N antibodies specifically precipitate MHV RNAs containing the 5' leader sequence (253). This indicates that N protein is capable of both specific and nonspecific interactions with viral RNA and suggests N may regulate viral RNA synthesis and encapsidation (13). The protein is synthesized in the cytosol and after phosphorylation becomes associated with cellular membranes possibly through interactions with the integral membrane protein M (255,262).

The remaining viral proteins are non-structural and are not well characterized. They are synthesized in extremely small amounts in infected cells and are known from their nucleotide sequence rather than by their properties as proteins. Not all coronaviruses appear to synthesize all of these proteins and the location of their genes is not consistent between members of the group (116).

RNA-dependent-RNA-polymerase activity has been detected in TGEV infected cells but cannot be found associated with the virion (64). Because the coronavirus virion lacks polymerase activity, the 5' most gene in the coronavirus genome must code for the viral RNA-dependent-RNA-polymerase. Two separate polymerase activities have been detected in MHV infected cell at different phases of infection indicating substantial regulation of polymerase activity (21).
In TGEV several nonstructural genes are located between the spike and membrane genes. There are three ORFs (A, B, and C) in this region that may be expressed from as many as three subgenomic mRNAs. TGEV appears to be unique among the coronaviruses in expressing a small nonstructural hydrophobic protein of 9.1 kDa from its most 3' gene. A subgenomic mRNA and intracellular protein have been described for this 3' ORF (64,128,237).

Pathway of Intracellular Replication. Coronaviruses infection begins with binding to a host cell at a specific receptor. Coronaviruses are highly specific for both host species and tissue type. Much of this host restriction occurs at the receptor level (44,261). Infections have been initiated in nonpermissive cells by transfecting them directly with viral genomic RNA (142). However, other factors also clearly influence host cell susceptibility (11,191). The receptor for MHV has been identified as a member of the carcinoembryonic antigen family of glycoproteins (44,243). The carbohydrate moiety appears to be required for virion binding but whether it is directly or indirectly involved is not known (205). The virus receptor on ST cells has been determined to be porcine aminopeptidase N (APN) (57). ST cell lines engineered to overproduce APN could be infected by TGEV efficiently they produced fewer TGEV particles and gave rise to small plaques (59). Receptor binding is not species specific among TGEV and its close relatives, canine coronavirus (CCV), and feline infectious peritonitis virus (FIPV) (17,267). In addition, an accessory receptor which binds to a different domain of the viral S protein appears to be required for enteric tropism.
Antigenic sites B and C (nt 21-245) may be involved (10,228). These additional residues may bind a different receptor identified as a 200 kDa non-glycosylated protein isolated from ST cells and found on enterocytes from newborn piglets (285). A loss of sialic acid binding correlated with mutations and deletion of S protein residues 145-155 and a reduction in enteropathogenicity (137). The antireceptor for most of the coronaviruses is probably the spike protein, although there is some evidence that in viruses that express it, the HE protein may also act as a antireceptor (234). After interactions at the membrane the virions appear to be internalized by viroplexis and uncoated inside endosomes. Lysosomal acidity may be required for uncoating mediated by the spike protein (83). Subsequent steps in viral transcription, translation, replication, and assembly all take place in the cytoplasm. Coronaviruses are able to replicate efficiently in enucleated cells (291).

The translation of the uncoated viral genome to produce RNA-dependent RNA polymerase is required for transcription and translation of other coronavirus genes. Two peaks of polymerase activity occur during the infection cycle. The early peak probably coincides with full length or subgenomic negative stranded template production and the late one with synthesis of viral mRNAs (21). Whether negative or positive subgenomic RNAs are produced first in most coronaviruses has not yet been determined. For TGEV negative strand anti-mRNA, production peaks 2 hours before maximal mRNA synthesis (237). Temporal regulation of RNA synthesis varies among the coronaviruses. The rate of MHV mRNA synthesis has been characterized with a peak of activity at 5 to 6 hours after infection with the proportion of one RNA species
to another unchanged throughout the cycle. A gradual increase in genomic length RNA occurs late in the infection without a sharply defined switch (160). Bovine coronavirus has two clearly defined peaks of transcription with maximal mRNA synthesis occurring at 4 to 8 hours post infection (pi) and genome replication at 70 to 72 hours pi (132). Transmissible gastroenteritis virus mRNA synthesis occurs maximally at 6 to 8 or 8 to 10 hours pi (Dr. Andreas Luder, Montana State University, personal communication), but production of genome length RNA has not been temporally characterized (124).

Translation of coronavirus proteins from viral mRNAs utilizes host cell machinery. N protein is synthesized on free polysomes and on the rough endoplasmic reticulum (RER). Large amounts of N protein are synthesized during the infection and accumulate in the cytoplasm of infected cells where it may exercise a regulatory function (255,261). N is phosphorylated and binds genomic and subgenomic viral RNAs with some degree of specificity (13). Recognition signals in the leader RNA plays a role in this binding (253). Membrane protein is translated on RER and transported to the Golgi specifically in the perinuclear region where it remains and accumulates (261). There it forms a complex with the nucleocapsid and probably determines the budding site for the virus. Membrane protein also has RNA binding capabilities and may be active in interpreting encapsidation signals (262). Analysis of efficiently packaged defective interfering particles (DIPs) and artificial constructs has localized the encapsidation signal to a 347 nucleotide segment in the 3' portion of gene 1 (172,272). Spike protein is synthesized on the RER and transported through the Golgi to the plasma membrane where it can be found in excess late in infection. The
virus acquires S protein and possibly HE protein as it buds into the lumen of the golgi through the perinuclear membranes. Spike protein is not required for the release of virus although it is required for infectivity (73, 261). Virus is released by fusion of smooth walled vesicles containing virus with the plasma membrane. Efficient release of virus appears to depend on good condition of the host cells and the majority of virus is released by intact cells and not by host cell lysis (261). Cytopathic effects including the disruption of host cell protein synthesis occur in 6 to 24 hours for most coronavirus infections (97). The mechanism of cell destruction is not known except in the case of cell fusion that is mediated directly by mature S protein on the plasma membrane. Cell fusion is not observed in TGEV infected cells. Membrane changes induced by the S protein are not directly responsible for the other cytopathic effects observed (183).

Porcine Transmissible Gastroenteritis

History. The disease was first described in 1946 by Doyle and Hutchings working in Lafayette Indiana (72). The investigators described outbreaks of a highly contagious diarrheal disease which affected most animals in the herd but resulted in mortality only in the youngest. The disease could be transmitted experimentally by oral inoculation of filtered gastrointestinal contents and was unmodulated by treatment with sulfathalidine or penicillin. It was found to be serologically unrelated to hog cholera but of viral origin. The authors suggested naming the disease transmissible gastroenteritis temporarily to differentiate it from other types of scours until a better understanding of the causative agent was reached. They noted that gastroenteritis was a common but not
universal symptom (72). By 1958 the disease was reported in England and Japan and has since been recognized in most of the swine producing areas of the world (91,192,230). Its prevalence and history among wild swine is not known although a recent serological survey of feral pigs found no evidence of exposure (295). The virus was first propagated in tissue culture by Lee in 1954 and was identified as a coronavirus in 1970 (157,264).

There is only one known serotype of TGEV, although several laboratory strains have been isolated and described which can be differentiated using monoclonal antibodies (107,133). These vary in their tissue culture cytopathology and in their virulence for pigs. The Purdue strain obtained by Laude has been attenuated by repeated passage in tissue culture where it is highly cytolytic (151). Its pathogenicity for pigs is reduced and it has been used with limited success as a live vaccine. Purdue is also the most widely used strain for genetic and biochemical analysis because of its high replication rate in tissue culture systems. The basis for its attenuation is not known. The Miller strain of TGEV is a virulent tissue culture adapted strain, at least at low passage levels (18). Miller is less commonly used for laboratory analysis as it replicates less efficiently in culture. Several virulent isolates of TGEV are also used for genetic analysis without repeated passage in culture (26,107).

Transmissible gastroenteritis virus is not antigenically related to PEDV or HEV, (Table 1.1) two other swine coronaviruses, but it is very closely related to PRCV which is considered a separate virus species by some investigators and a strain of TGE with altered tissue tropism by others. Porcine respiratory coronavirus causes a mild
respiratory infection that is usually asymptomatic (204). Transmissible gastroenteritis virus can also replicate in lung tissue (150,269,270). Anti-PRCV serum can be differentiated from anti-TGEV only at carefully selected nonneutralizing epitopes (29). The overall sequence homology for the structural genes is 96% at both the nucleotide and amino acid level. Most of the genetic difference is accounted for by two large deletions (210). Several small deletions and base substitutions have been found in nonstructural genes (199). Transmissible gastroenteritis virus and PRCV leader sequences have been shown to be completely homologous (198).

**Course of Disease.** Transmissible gastroenteritis virus is transmitted by the fecal/oral route and probably by respiratory secretions (269). It can be produced experimentally by feeding susceptible animals contaminated intestinal contents (72,91). The virus survives exposure to gastric secretions and has been reported to cause gastric ulcers (9,91). The primary site of replication is the villous epithelial cells of the ileum and jejunum. Infection results in nearly complete villus atrophy. The wall of the small intestine appears thin and translucent. Electrolyte imbalance, diarrhea, and dehydration result from tissue destruction.. Replacement of enterocytes by migration of epithelial cells from the crypts of Lieberkuhn appears to be critical for recovery. The new cells are resistant to further TGEV replication although the mechanism of this resistance is not known. The severity of symptoms is increased by stress and by immune suppression with corticosteroids (225). Virus replication or pathological changes have also been reported in the lungs, kidneys, spleen, bladder, lymph nodes, larynx,
meninges, and cerebellum (91,270). Older animals may support replication in more diverse tissues (230).

Clinical symptoms include occasional vomiting and inappetence, with a profuse watery yellow foul smelling diarrhea. In weaned or adult pigs the symptoms are mild and last only a few days. More severely affected animals, often nursing sows, may develop fever and suffer from dehydration, agalactia, and weight loss. Morbidity may reach 100% but mortality is generally very low, usually less than 4% for pigs over three weeks of age. The incubation period lasts 18 hours to three days. Piglets less than seven days of age suffer from diarrhea with curds of undigested milk found throughout the gastrointestinal tract. They develop severe dehydration, accompanied by extreme thirst and die two to seven days after onset of symptoms. Adult pigs mount an immune response against TGEV with neutralizing antibodies present in serum seven to eight days after infection and detectable for at least 18 months (293). Serum antibody does not appear to affect the course of disease or subsequent resistance to reinfection. Mucosal immunity, specifically SIgA produced in the gut, is probably responsible for resistance to reinfection.

When passively transferred from immune sows to newborn piglets in colostrum and milk, IgA prevents infection (93,95,188,224). Antibodies directed against viral glycoproteins S and possibly M appear responsible for this protection (84,93,149). The major neutralizing epitopes have all been located as a cluster on the spike protein where they are designated site A which is comprised of amino acids from several regions in the S protein primary sequence. The spike protein also contains several
non-neutralizing epitopes designated B, C, and D (58,87,208,209). The antigenicity of A, B, and D sites were strongly modulated by glycosylation (60). Passive immunity against TGEV acquired by newborn pigs correlates with development of maternal antibody directed against epitope A on the S protein (50). Antibodies against M protein are neutralizing only in the presence of complement proteins (296).

The protective role of cell mediated immunity in TGEV infection is less clear. Such responses have been documented in infected animals (38,286). Interferon of the α-β type is produced at high levels in the gut in response to TGEV infection at least in part by leukocytes. The viral M protein induces cultured leukocytes to produce α interferon even in the absence of infectious virus. Infected epithelial cells also produced interferon probably of the β type (40,140,152). In vivo augmentation of INF before or after exposure to attenuated TGEV did not enhance the immune response of piglets as measured by serum antibody production. Interferons can be both immunostimulatory and immunosuppressive and are known to stimulate natural killer (NK) cell activity (61,68). TGEV replication is only moderately sensitive to the direct antiviral activity of interferon compared to many other porcine viruses (67). Beta interferon has a greater inhibitory effect than alpha interferon but even at high concentrations (100U/ml defined by VSV interference) neither could eliminate TGEV replication in vitro. Porcine beta interferon was also found to be cytotoxic to autologous cells (284).
Epizootiology. Serological studies conducted in the 1970s indicated that 19 to 54% of the adult swine in North America and Europe had been exposed to TGEV. A survey of swine in the southern United States found no evidence of TGEV exposure (225,295). Porcine respiratory coronavirus has been recently described in Europe and the United States, but probably existed before its recognition. It can not be differentiated from TGEV by neutralization of florescence assays and may be represented in estimates of TGEV exposure. Patterns of TGEV infections can be divided into epizootic and enzootic transmission.

Epizootic transmission occurs in herds in which few animals have resistance to TGEV, probably due to a lack of previous exposure. TGEV appears in such herds in winter between November and April (225). The importance of low temperatures for TGEV transmission is not clear, although the virus is less labile at low temperatures, perhaps enhancing infectivity. It is less common in warmer areas and rare during summer in temperate climates. In addition, stress on animals due to low temperatures and crowding may decrease their resistance to infection as well (105,192,225,295). TGEV spreads rapidly during an epizootic. Young pigs less than three weeks old develop diarrhea, become dehydrated and die. The symptoms are progressively less severe in older piglets with mortality also decreasing. Conditions for their litters may worsen when nursing sows become ill. Other pigs in the herd will also exhibit diarrhea and anorexia.

Enzootic transmission will occur in a herd with previous TGEV exposure. This pattern of transmission is seen most commonly in operations with frequent farrowing or
other introductions of susceptible animals. The breeding sows will be immune and able
to passively protect their litters during nursing. Once the animals are weaned they
become susceptible and develop TGEV. Overall mortality among the piglets will be
greatly reduced as will the severity of their symptoms. Other young swine introduced
since the previous outbreak will also develop mild TGEV symptoms. Diagnosis of
enzootic TGE can be difficult as the signs of the outbreak are not dramatic and may be
confused with other intestinal infections (225). Enzootic TGEV has much less serious
consequences for newborn pigs. Herds with enzootic TGEV may serve as a reservoir
for the disease.

Prevention of the disease is accomplished by animal management and vaccination.
Uninfected herds are isolated from contaminated material and animals. When the
disease appears in a herd, farrowing pregnant sows are exposed to virulent virus and
allowed to recover before delivery, thereby providing lactogenic immunity to litters in
colostrum and milk. This has the negative effect of increasing exposure in the herd
leading to clinical disease in the adults. Sows with previous exposure to TGEV can be
boosted with one of several commercially available live attenuated vaccines.
Transmissible gastroenteritis virus vaccine strains do not uniformly produce good
secretory immune responses in naive animals, but they do appear able to boost
previously infected animals reproducible (225). New animals can also be tested for
TGEV exposure before they are added to an unexposed herd.

Transmission between herds is not well understood. Contaminated objects or
personnel may serve to physically carry the virus especially during cold weather.
There is no evidence that humans can be actively infected by TGEV. Dogs and cats can carry two closely related viruses, CCV and FIPV, respectively. Experimentally, pigs can be infected with FIPV and possibly by CCV, though in the latter case without obvious clinical signs. Dogs and cats will shed TGEV after artificial exposure, although neither is susceptible to disease. House flies and starlings passively shed virus after feeding on contaminated material (225).

The role of previously infected animals as carriers has been investigated but without conclusive results. Most workers report detectable virus shedding from the gut for only two weeks following infection. Recently infected animals clearly act as carriers (225). Experimentally infected pigs have been reported to shed infectious virus for eight weeks (157) and to have it present in intestinal scrapings for 35 days (184). Experimentally infected animals produced infectious virus in intestinal and lung tissue for 104 days pi (post infection), even in the presence of neutralizing antibody (270). Information on naturally infected animals is limited. Transmissible gastroenteritis virus was isolated from lung lesions in market weight swine; although some animals in the group had respiratory symptoms, none displayed intestinal signs of TGEV infection (269). The animals were taken from a herd with a previous history of diarrhea among young pigs which had been ascribed to bacterial infection. Enzootic TGEV was subsequently found. Other researchers have described a long term carrier state in recovered piglets. The pigs did not continuously excrete virus but could be induced to by stress. This reactivation was sufficient to cause an anamnestic response in the persistently infected animals and disease in naive animals exposed to them (93,105).
Under controlled conditions unexposed pigs were added to an enzootically infected herd three, four, and five months after an outbreak and monitored for seroconversion. None of the animals showed evidence of TGEV exposure (67).

TGEV has been described as persisting for long periods in vitro culture systems. Roger Woods derived a stable small-plaque variant from the virulent Miller strain by growing the virus persistently in a leukocyte cell line. The culture was maintained for three years with consistent virus titers of $10^6$ plaque forming units per milliliter (PFU/ml) as detected on McClurkin swine testicle (ST) cells. Early virus production was low beginning with $10^3$ PFU/ml at three months post infection and building slowly for the first year. The virus was tested for virulence in adult and neonatal pigs and found to be nonvirulent but capable of inducing good passive protection for nursing piglets (292,294). Early attempts to culture TGEV were frustrated by its noncytopathic growth in several cell culture systems. TGEV was hypothesized to be caused by two different viruses (176). One noncytopathic could be recovered occasionally and one noncytopathic was consistently present. Improvements in virus isolation and characterization have led to a current consensus that TGEV is caused by only one virus that is usually cytopathic when isolated. Earlier descriptions of its noncytopathic chronic infection of tissue culture cells have not been pursued (156).

**Viral Persistence**

As a better understanding of the natural history of viruses emerges, it has become clear that chronic infection of host cells is an extremely common component.
Persistent infections can be symptomatic or virtually invisible making their discovery and elucidation challenging (2). Some virus groups rely on a chronic infection as an essential component of their life cycles (5). Among animal viruses some of the best studied examples include the Retrovirus family and the Herpes-virus family. Retroviruses include Rous sarcoma virus of chickens (RSV), visna virus of sheep, and human immunodeficiency virus (HIV), the causitive agent of AIDS. Some common human herpes viruses include herpes simplex virus types 1 and 2 (HSV1, HSV2), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), and Varicella-Zoster virus, the causitive agent of both chicken pox and shingles. Only Varicella-Zoster virus appears to spend a significant portion of its life cycle as an agent of acute disease. The *Adenoviridae* and *Arenaviridae* also rely on persistent infection for their maintenance and transmission. A large number of other viruses which have been characterized as causing acute disease will also frequently establish persistent infections depending on the virus strain and the condition of the host (81). Viruses in this group include parvoviruses, measles virus (Rubeola), rubella virus, bovine viral diarrhea virus (BVDV), hepatitis B virus, papilloma viruses, Theiler's virus of mice, and MHV and IBV from the coronavirus family.

In addition to chronic virus infections of known etiology, a number of progressive diseases may have a viral component. Multiple sclerosis, which is a progressive demyelinating disease of the central nervous system, may be initiated or maintained by a virus infection (45). Infection with measles virus, canine distemper virus, paramyxoviruses, CMV, and coronaviruses have all been considered as a contributing
factor. Rheumatoid arthritis, systemic lupus erythematosus, myocarditis, Graves disease, and juvenile onset diabetes are all known to have an autoimmune component. Some form of insult or injury is hypothesized to initiate the immune reaction and chronic viral infections are likely candidates (195). In addition to indirect injury by the host immune response, chronic virus infection may cause a cell to loose differentiated functions such as hormone secretion without affecting "house-keeping" function that the cell requires for survival (136). In mice persistently infected with an arenavirus, lymphocytic choriomeningitis virus (LCMV), diabetes-like disease results from chronic infection of beta cells in the pancreas and growth deficiencies from infection of the pituitary (195,271). These pathologies exist without obvious damage to the cells making the infection detectable only with virus specific nucleic acid probes or antibodies.

Chronic viral infections have been labeled using a variety of terms: chronic, persistent, quiescent, slow, latent, and inapparent (298). An attempt is often made to differentiate between those chronic productive infections in which virus is constantly produced and latent infection in which episodes of virus production or reactivation occur interspersed with long unproductive periods. This distinction is becoming less useful as the complex nature of host-virus interaction becomes more apparent. A number of viruses can cause both types of infection, depending on the type and developmental state of the host cell they are infecting (134). When determining virus production especially, in intact animals, the sensitivity of detection methods limits
designating an infection as productive or latent. Such a determination may be useful when studying the mechanics of carefully defined virus/cell systems.

All persistent infections are fundamentally different from acute infections. In acute infections typified by smallpox and influenza, virus presence and host survival are not compatible. The virus replicates in cells killing them and the host attempts to eliminate the virus by mounting an immune response. After a brief, well defined period of infection, the host is unable to respond effectively and dies or the virus is eliminated and the host is free of the infection. In a chronic infection, the host is able to coexist with the virus. Common examples in humans include AIDS, herpes simplex, and hepatitis B. The virus is maintained by replication or some other mechanism, often in the face of active host immunity and the host is able to survive the effects of infection. This relationship is relatively stable for a long period, usually as defined by the life span of the host. Viruses like varicella-zoster and rubella cause both acute and persistent infections. The establishment and maintenance of a persistent infection exert selective pressure on both the virus and host. The virus must maintain its genetic integrity in spite of the immune response of the host. In addition, the virus must limit its lytic effect to avoid destroying the host. The host must limit viral replication and its damaging effects without destroying debilitating amounts of its own tissues.

**Mechanisms of Viral Persistence.** Viruses that are successful in causing persistent infections use a variety of mechanisms to maintain themselves within the host (112,134). Some viruses cause infections which are nonlytic at the cellular level.
There is no tissue destruction and an associated lack of inflammation may limit the immune reaction of the host (196). Other viruses cause cell destruction when they replicate, but are able to restrict their gene expression and maintain their genome without producing progeny virus (31,214). This also limits the targets presented to the immune system.

RNA viruses that have inherently high mutation rates can also maintain themselves by producing a variety of mutants (111-113). Attenuated mutants may be produced that have a reduced lytic effect or replicate slowly limiting tissue destruction. The production of temperature sensitive mutants is a hallmark of persistent infection, although whether they are a by-product of persistence or important for its maintenance has not been established (5). Defective interfering mutants or defective interfering particles (DIPs) are also commonly produced during persistence (2,112). These mutants have the specific property of limiting the replication of homologous wild type virus in cells coinfected with the mutant and standard virus. They can effectively limit virus replication and are an important mechanism in virus persistence (81,114,122). Tissue restricted variants may also develop that cause different kinds of infection in different cell types. This allows the virus to maintain itself by colonizing areas like the central nervous system that are less susceptible to immune intervention (196).

Successful avoidance of host defenses is required to maintain persistent infection. Virus variants that can competitively replicate in the face of selective pressure from their hosts will predominated in persistent infection, (226). In whole animal systems it is well recognized that chronic viral infection can only be maintained if the antiviral
activities of the host's immune system can be avoided (1,96,125,195,201). Fatal chronic infection with MHV3 induces immune suppression in (C57BL x A/J) mice which could be ameliorated by adoptive transfer of syngeneic T cells and or B cells (147). Viral characterization of in vivo persistent coronavirus infections has concentrated on predicted heterogeneity of the spike (S) glycoprotein. MHV-JHM specific RNA isolated from the central nervous system (CNS) of persistently infected mice were found to contain deletions in the "S1 hypervariable region" of the spike (S) glycoprotein in 55% of the animals tested (222). MHV-JHM persistent infection of C57BL/6J mice produced viral RNAs with multiple mutations in the S1 and N regions, the only two segments investigated. No clustering of mutations was observed (79). A similar study of MHV-JHM-Pi, generated by in vitro persistent infection, and used to establish CNS persistent infection in Lewis rats, low levels of heterogeneity were found in S specific RNA sequences obtained by PCR from several regions in the ORF and adjacent sequences. In fact, the nucleotide substitution rate determined was lower than that predicted for Taq polymerase alone (0.5 substitutions per $10^3$) (258). As the S protein is the receptor binding protein for this and other coronaviruses and the major antigenic target for virus neutralization (149), this low rate of genetic change indicates that in vivo persistence requires complex interactions beyond the avoidance of antibody neutralization. Adoptive transfer of MHC class I restricted CTLs specifically responsive to an epitope on the MHV-JHM nucleocapsid (N) protein prevented the establishment of persistent CNS infection and chronic demyelination in otherwise susceptible BALB/c mice (254). The resistance or susceptibility to MHV-induced
disease of inbred strains of mice is determined by genetic variations in MHV receptor expression as well as macrophage and lymphocyte characteristics (28).

Even during \textit{in vitro} persistent infection the virus population is selected in part by pressure from host defenses. Beta or alpha interferons (IFN) can be made by non-lymphoid cells lines. They are often produced in persistently infected cells and undoubtedly exert some selective pressure on virus population and on the cells themselves (112). Resistance to the antiproliferative effects of exogenous INF was demonstrated in Vero cells persistently infected with Sendai virus or SSPE varients of measles virus (46). Exposure to interferon generates cell populations resistant to their anti-proliferative effects (138).

\textbf{Noncytopathic Viruses.} Nonlytic viruses are able to establish persistent infection in tissue culture. Their persistence in intact animals is determined by their interactions with the immune system. These viruses are often called noncytopathic because they are able to complete their life cycle including production of progeny virus without killing the infected cell. A persistent infection in mouse cell lines infected with LCMV has been characterized as a noncytopathic carrier culture (158). This virus persists without inflicting tissue damage in chronically infected mice. Changes in cell metabolism are subtle and nonlethal. Virus is shed continuously in urine and saliva. Mice become carriers when they are infected \textit{in utero} or as neonates. A significant antibody response to LCMV and immune-mediated glomerulonephritis may develop, but without detectable CTL activity against LCMV. Infection of adult mice with LCMV is
normally acute and self-limiting. Infection with some strains can result in fatal immune-mediated choriomeningitis (211). When grown on cell culture, LCMV will readily establish persistent infection. Some variants of the virus are cytopathic and can form plaques in cell monolayers while others will not. DIPs can be easily recovered from both cytopathic and noncytopathic infections. They are also produced in infected tissues from acute and chronic infections in vivo. The relationship between these interfering particles and conventional virus is not clear. Interference appears to occur through a novel mechanism rather than by competition for cellular machinery required for replication (279). Cells in infected cultures cycle through periods of infection with virus production, then refractivity, and finally a return to the uninfected state when they become susceptible to reinfection. The cell cycle has been correlated with these transitions, but the underlying mechanisms are not understood (76). Susceptibility of cells to infection by another arenavirus pichinde virus is clearly dependent on cell differentiation and activation (207).

Interactions between LCMV and the immune system during persistence are also complex. Among many other tissue targets, the virus infects cells of the thymic medulla and the T cell-dependent areas of the spleen and lymph nodes. Adoptive transfer H-2 matched CD8+ lymphocytes from LCMV immune mice to persistently infected mice allowed the infection to be cleared (76,196). Some strains of the virus which differ at as few as two amino acids from their parent strain are able to supress CTL responses even in immunocompetent mice and establish persistent infections (226).
Another nonlytic persistent infection occurs when BVDV infects its host, the cow, during fetal development. Strains able to persist in calves are noncytopathic and do not generally cause tissue damage although subtle changes in tissue growth patterns have been observed (194). Cytopathic strains of BVDV are also common. They cause a mild transient diarrhea in adult animals but do not persist. Persistently infected calves are immunotolerant. If they are subsequently infected by an antigenically similar cytopathic strain of the virus, a fatal infection develops known clinically as mucosal disease. These strains may evolve endogenously in the persistently infected animal or be contracted from acutely infected animals. The basis for cytopathic and noncytopathic relationships with the host cells are not understood. Sequence analysis of matched cytopathic and noncytopathic isolates from animals with mucosal disease shows a consistent pattern of inserted bovine sequences in the genomes of cytopathic isolates. These sequences are absent in the genetic material of noncytopathic strains (180).

Genetic Restriction. Viruses can restrict genetic expression while maintaining their genetic material in infected cells in some normally lytic virus infections. In order to produce progeny virus, this restriction must in turn be suppressed with fatal consequences for the host cell. True latent infection, which viral genetic material is found in a stable form and viral gene expression is restricted to a regulatory gene products, has been found only for virus groups with DNA genomes or intermediate forms such as the Papovaviridae (154,278), Herpesviridae
(56,123,178,219,220,252,277), the *Hepadnaviridae* (173,174) and *Retroviridae* (273) virus families. Chromosomal integration has been suggested as a possible mechanism for RNA virus persistence as well (300).

**Virus Mutants.** Most RNA viruses maintain a persistent relationship with a host using a strategy which relies on the generation of virus mutants (112,113). Such mutants are more efficiently generated in RNA virus infections because of the low fidelity of RNA polymerases. These enzymes apparently lack proofreading functions. RNA genomes are able to undergo rapid evolution. Nucleotide substitution rates have been assessed at $10^3$ to $10^5$ misincorporations per position for VSV (248). In carefully selected neutral mutations, error frequencies of $10^{-4}$ have been described for VSV and poliovirus in culture (55,109). Error frequencies appear to be at or near the maximum tolerable error rate. The mutation frequency cannot be increased substantially by chemical mutagenesis (110). RNA viruses are therefore more accurately described as heterogeneous populations or quasispecies even in clonally derived populations (71,108). Usually one variant dominates the population at any given time. Even virus variants more fit for the particular culture conditions may be unable to overwhelm the predominant variant due to its preponderance in the culture (54). It may be replaced by quasispecies with greater relative fitness if culture conditions change (108,249). Rapid virus evolution during persistent infections have been described (111,298). Rapid change in the genetic make-up of VSV populations caused by the altered selective
Attenuated Virus Mutants. Attenuated mutants have been described for many persistent virus infections. Several classes of such variants have been identified in persistently infected cultures. Whether these mutations make persistence possible or are its by-product is not known in most systems. Temperature sensitive mutants are able to replicate efficiently at a permissive temperature but cannot at a higher temperature. Such mutants are described in relation to the temperature requirements of the wildtype or parent strain of the virus. Defective interfering particles (DIP) are encapsulated defective genomes that are able to interfere with the replication of wildtype virus in a mixed infection (122). They are generally deletion mutants with intact encapsidation signals but without essential replication functions. Defective interfering particles can modulate the virulence of the overall population causing cycles of depressed virus replication as DIP numbers rise at the expense of competent virus. When titer virus drop to levels too low to allow co-infection DIP are no longer replicated. This allows the efficient replication of competent virus and a corresponding rise in titer (122). Mathematical models predict that the effects of DIPs can account for the fluctuating titer and long term stability seen in many persistent cultures (12). Attenuated mutants of other types are described by their phenotype. For example, plaque size mutants leave a smaller or larger area of disrupted cells on a monolayer after an infection initiated in a single cell. A plaque forming unit may consist as few as
one virus particle or as many as 100 particles. Small plaques are generally considered a sign of attenuation but plaque formation is a complex phenomenon involving interactions between cells, virus, and the overlying medium. The significance of these variants is not established in most cases.

Picornaviruses is one of the best studied families of RNA viruses. They cause classic acute viral diseases. They can, however, establish persistent infections \textit{in vivo} and \textit{in vitro}. Poliovirus from the enterovirus group may persist \textit{in vivo}. Hybridization studies of nucleic acids in the CNS have been positive for poliovirus sequences. Cases of reactivation or chronic infection in immunocompromised individuals have been reported (5,49). The Sabin vaccine strain can chronically infect cultures of human neuroblastoma cells. The cultures are dominated by viral mutants but the genetic requirements for chronic replication are not known (202). Long term culture with poliovirus also selects cells resistant to infection (129). Some of these cells are mutants with reduced expression of poliovirus receptors (130). Theiler's murine encephalomyelitis virus (TMEV) is also an enterovirus, although not closely related to poliovirus. It causes acute and chronic CNS disease depending on the strain of the virus and the genetic background of the host (297). The genetic determinants for persistent growth by TMEV in mice are located in the VP1 capsid protein coding region (175,266). Foot-and-mouth disease virus (FMDV), another picornavirus, can cause persistent infections \textit{in vivo} and \textit{in vitro}, although it is normally considered an acute pathogen. Virus isolated from persistently infected cultures showed decreased plaque size and temperature sensitive growth at 42°C. Cell lines derived from BHK-21
and IBRS-2 cultured with the virus in this persistent system developed resistance to superinfection co-evolving with the virus (53). Persistently cultured FMDV becomes increasingly cytopathic for parental BHK-21 when cultured persistently in BHK-21 derived lines but less virulent for cattle and mice (70). This is unusual since attenuated virus forms are generally selected for in chronic infections. Persistent infection in tissue culture by echovirus 6, a human picornavirus results in a nonproductive but chronic infection. An unusual association between the viral genome and the host cell results in the production of large numbers of defective virus particles. The particles lack mature virion proteins VP2 and VP4 and contain instead the unprocessed provirus form VPO (88). RNA extracted from these defective particles is unable to initiate lytic infection when transfected into uninfected cells pointing to a genetic defect in the persistently produced virus. The genome is apparently maintained solely by vertical transmission. There is no known mechanism for maintaining an RNA genomes in this situation (214). However, these cultures are not virus infected in the classic sense. There is no evidence that the virus has the ability for independent replication in uninfected cells and is a permanent parasite dependant on vertical transmission.

A more conventional persistent infection occurs in human erythroleukemic K562 cells cultured with the picornavirus encephalomyocarditis virus (EMC). These cells are marginally permissive for EMC virus infection and readily establish persistence. This low infectivity may be determined at the receptor level. Co-evolution of cells and virus apparently occurred with the cells becoming resistant to superinfection while the virus developed a small plaque phenotype (200). Hepatitis A virus, also a human
picornavirus, has not been reported to persist in nature but grows normally in tissue culture as a persistent infection. Cytopathic variants can develop during persistent growth with altered antigenicity (162). The determining factors in picornavirus persistence remain unknown although clearly genetic changes in the virus and cell populations are important.

The ability of the family *Paramyxoviridae* to persist in its natural hosts has been appreciated for some time. Measles virus causes two rare but fatal syndromes following acute infection. Persistent infection of the CNS results in subacute sclerosing panencephalitis (SSPE) or measles inclusion body encephalitis (MIBE). Measles virus recovered from SSPE patients is antigenically different from normal measles and has an altered matrix (M) protein (32). Lack of stability in the M protein inhibits virion maturation after mutation at multiple sites (34). Altered fusion and hemagglutinin proteins with different cell fusion properties have also been described (33). Persistent measles virus infection in culture generated DIPs (78) and displayed cyclic fluctuations in titer characteristic of DIP modulated cultures. However, their M proteins appeared normal (271). Transcription of measles virus genes can be modulated by extracellular antibodies in neuroblastoma cells during persistent infection (232). Human parainfluenza virus 3 also causes persistent infection in culture and probably in nature. Transcription and subsequent viral protein production was altered in persistently infected culture when compared with acutely infected cells (185). Defective interfering particles are associated with parainfluenza virus persistent infection and numerous transitions were found in viral genomes (186,189). Persistently infected cells lose the
ability to fuse with one another apparently due to a loss of neuraminic acid on the cell surface (186). Sendai virus persists in culture with dramatically lower virus production, although viral mRNAs and genomic RNA levels are only slightly reduced (120). Paramyxoviruses may have some undiscovered mechanism for reducing replication beyond the level of transcriptional control.

Influenza viruses do not persistently infect immunocompetent hosts. Tissue culture infections can be maintained chronically, however. Influenza A develops both temperature sensitive and a small plaque phenotype during persistent infection in baby hamster kidney (BHK) cells (82). Interference with VSV replication indicates that interferon production may be involved in maintaining the persistent infection. Interferon production in the lung correlates well with recovery from acute infection (80). Interferon has also been found in persistent infection with Newcastle disease virus, poliovirus, influenza virus, FMDV, parainfluenza virus, vaccinia virus, polyoma virus, and HSV (77). The role of interferon in maintaining persistence is not well established. A 10 month influenza virus infection of a child with severe combined immune deficiency syndrome produced a population shift from one dominant genotype to the next with $10^3$ substitutions per nucleotide site per year in two viral structural genes—a rate comparable with that seen in in vitro studies (218).

The genetic basis for viral attenuation during persistent culture is known in a few systems. Reovirus, a normally cytolytic virus, can establish persistent infection if it has been previously passaged at high multiplicity of infection allowing the replication of many components of the viral population, not simply the dominant variant. Many
variants in such a population are defective, exhibiting a temperature sensitive, attenuated, or DIP phenotype. The genetic basis for these characteristics are varied and complex (3). Determining which of these mutations is selected for by persistent conditions and which is genetic baggage is a laborious task. Mutations in the reovirus genes S4 and S1 have been shown to be biologically important (4,131). Some VSV persistent infections are established and maintained by DIPs present in the virus population (114,126). Dominant interfering particles develop and control virus replication until a resistant virus genome arises and is selected (193). This pattern of continuous evolution dominates VSV persistent infections (65).

**Extracellular Modulators.** Early explanations for viral persistence often relied on the effects of external modulating elements such as virus specific neutralizing antibodies or endogenously produced interferon. These cultures were called carrier cultures because infection occurred in only a small percentage of the cells the majority being protected by modulators in the medium.

Interferons are soluble cytokines produced by virally infected cells or cells exposed to some other microorganisms or any of several simple chemicals. Interferons interact with specific high affinity plasma membrane receptors on uninfected cells inducing the expression of genes for antiviral proteins (62). These proteins inhibit viral infection, replication, and transcription at different points. For example, herpes simplex virus is inhibited in the transactivation of its immediate early genes and again when virions are released. Reovirus mRNA translation is inhibited and the accumulation of VSV
primary viral RNA is prevented (247). A role for interferon has been postulated for VSV persistent infections (276). Parainfluenza, Sendai virus, Sindbis virus, and tick borne encephalitis virus persistent infections also exhibit some level of interferon involvement (81). Interferons, particularly interferons α and β inhibit cell growth and can be cytotoxic during long exposure. They also induce pathological changes in intact animals. Interferons may be responsible for some of the pathology seen in persistently infected animals. Noncytopathic viruses, like LCMV, induce interferon that causes kidney and liver damage, two pathologies often seen in LCMV persistent infections (61).

Antiviral antibodies can also modulate persistence. In culture, they are only present if purposefully added. Neutralizing antibody lowers the effective titer of extracellular virus and protects cells from infection. Intact animals produce neutralizing antibody and still carry viruses persistently. The interaction of antibody and infected cells is complex. Anti-VSV antibodies cause a loss of VSV surface antigens and inhibited the maturation of virions. Intracellular levels of viral proteins are also reduced (197). In murine neuroblastoma cells, measles virus transcription is dramatically reduce when measles specific antibodies were added to the culture (232). These antibody induced modulations may prolong virus infection by making virally infected cells less effective targets for cytotoxic T cells. They also effectively attenuate the infection enhancing the virus's ability to persist.
Coronavirus Persistence. Coronaviruses cause persistent infections in culture and in intact animals as well. The best studied persistent coronavirus infections are those caused by MHV. These infections may be inapparent, modulating immune responses to other microorganisms but producing no overt disease (63,139). A range of persistent infections can be established in mice depending on the route of infection, strain of virus, and genetic background of the animal. Those in the CNS have been studied as a model for demyelinating human diseases. Chronic hepatitis and immune deficiencies also occur (215).

Tissue culture persistence can also be readily established. Persistently infected cultures have displayed a variety of characteristics. Small plaque morphology developed in MHV A59 produced by chronically infected 17Cl-1 cells. Ten to twenty percent of the cells in culture were positive for MHV antigens by florescence. The persistently infected cells produced $10^3$ to $10^6$ PFU/ml virus and could not be cured with anti-MHV antibody. The cells showed no CPE in response to infection with parental virus, but could be infected with VSV and Semliki Forest virus. Some temperature sensitive mutants were produced by the culture, but no defective interfering particles (118). Another neurotropic MHV, JHM persists in tissue culture. It produces a heterologous population of temperature sensitive progeny virus with reduced cytopathic effect, but a good rate of virion production. Some change in the antigenicity of the peplomer protein was detected by monoclonal antibody, although neither INF production nor defective interfering particles were detected (16). In a similar system, a small plaque variant arose and was credited with stabilizing the
culture. This culture produced no detectable interferon, temperature sensitive mutants or DIPs but was resistant to superinfection with parental JHM (98). Small plaque variants with low virulence have also been isolated from and used to produce persistent infection in vivo (75). Neuroblastoma cells persistently infected with MHV-JHM produced $10^5$ to $10^7$ PFU/ml and did not produce a temperature sensitive population. The cells resisted superinfection with homologous virus but were sensitive to mengovirus and VSV infection (159). Oligonucleotide maps of virus released by the culture showed substantial change from the JHM progenitor but no difference in viral protein migration patterns could be detected. Cells could be cured of the infection by passage under neutralizing antibody. The virus was then "rescued" by fusing cured cells to uninfected cells (159). A cold sensitive MHV variant was also rescued from persistently infected cells after fusion (257). MHV infection of mouse fibroblasts could be cured by passage under hygromycin B that was found to inhibit viral, but not cell protein synthesis, probably because uninfected cells are not permeable to the antibiotic. This indicated that with the elimination of the carrier cells from culture, the remaining cells were truly uninfected and not sequestering the genome in a latent form (166).

Other studies have indicated that selection of cell variants with ability to resist cytopathic changes or virus infection is the crucial event in the establishment of coronavirus persistence. Cells resistant to MHV A59 induced cell fusion and therefore both CPE and infectability were found in mouse LM cells (182).

Although not implicated in studies of persistent cultures, MHV infection does produce DIPs (171,172). Those reported were derived from serial undiluted passage in
tissue culture, a process similar to persistent culture in that the inoculum is not diluted allowing coinfection and preventing the less numerous members of the virus population from being lost through dilution. In some cases, yields were reduced by 57% (12). The RNAs of these DIPs were deletion mutants of parental JHM of varying sizes most were not packaged efficiently (169). Several MHV DIPs have been shown to contain in frame deletions that allow them to code for fusion proteins (172). Disruption of one of these ORFs by nonsense and frame shift mutations decreased the fitness of DIP genome and, in fact, variants quickly took over the culture which had the fusion ORF restored. The proteins coded for by these ORFs are all quite different, suggesting that translation or interaction with ribosomes is required for RNA stability or for efficient packaging (52).

Persistent *in vitro* infections by other coronaviruses have also been characterized. Human cells infected with human coronavirus 229E produced $10^5$-$10^6$ PFU/ml for 300 passages. Plaque size increased during long term culture and culture conditions were temperature dependent, although it was not determined if the virus or the cells were the sensitive component. These cultures were unstable and could not be established reproducibly (39). Human coronavirus OC43 in a neuronally derived cell line produced $10^8$ PFU/ml and could be cured with polyclonal antisera. Temperature sensitive virus was recovered (43). Bovine coronavirus (BCV) also persists in culture (103) as well as in its intact host (47,127). All coronavirus RNAs including anti-mRNAs were demonstrable in infected cells for the 120 days the cultures were tracked. The culture produced $10^5$ PFU/ml on the two occasions monitored and 10%
of the cells were positive for antigen production by immunofluorescence. An aberrant RNA with viral specific sequence was detected for approximately 30 passages during the culture period. It was hypothesized to be a defective genome although no interference with viral genomic or mRNA production was observed (103).

TGEV has also been reported to persist in culture. Leukocytes infected with the Miller stain were maintained for three years. Virus titers were $10^5$ to $10^6$ for the 25 weeks reported. This culture gave rise to a small plaque TGEV variant that was subsequently used to vaccinate sows and produced lactogenic immunity to virulent TGEV. TGEV produced by serial passage in the same cell line was dominated by a large plaque virus variant that produced more CPE than the original inoculation (292).

**Outline of Research**

As our understanding of the life cycle of viruses grows, it is clear that persistence is an important strategy for many virus groups. TGEV persists in its natural host and persistence is probably important in the transmission and maintenance of the disease. In contrast to acute infections, persistently infected cells usually produce viral nucleic acids and proteins in small amounts. Viral specific macromolecules may become undetectable for extended periods. Studies of persistence in intact hosts with an intact immune system provides the best information about host virus interactions, but are difficult to interpret. *In vitro* culture systems provide a less complicated view of cell and viral interactions. Information about macromolecular synthesis, selection of attenuated mutants, or effects of extracellular modifiers can then be applied to the
natural host. The mutual survival of both cells and virus in these systems, although artificial, is interesting in its own right as it provides a miniaturized view of the ecological and evolutionary potential of viral pathogens.

The goal of this project was to determine which viral and cellular factors were important for establishing and maintaining TGEV persistent infections. McClurkin swine testical cells were infected with TGEV and maintained in culture for up to five years. The virus yield of each culture was assessed at intervals and the virus was monitored for signs of attenuation in uninfected ST cells. The growth and appearance of the cells were evaluated. Resistance of the cells to heterologous and homologous virus infection was assessed. Viral protein synthesis was determined by polyacrylamide gel electrophoresis of immunoprecipitated proteins. Cell surface expression of viral antigen was monitored by indirect immunofluorescence. TGEV-specific-RNA produced by the persistently infected cells was evaluated by slot blot and northern analysis. Southern blots of digested cellular DNA were examined for integrated TGEV sequences. The virus produced during persistent infection was assayed for temperature sensitive or defective interfering phenotypes. Interferon production by persistently infected cells was examined. The establishment of infections was modified by the addition of several specific monoclonal antibodies and attempts were made to cure the culture with the addition of neutralizing polyclonal antisera.
CHAPTER 2

CHARACTERIZATION OF TGEV PERSISTENT CULTURES

Introduction

Porcine transmissible gastroenteritis virus (TGEV) causes acute enteritis that is normally self-limiting in adult animals, results in poor growth of recovered neonates, and is often fatal in piglets less than two weeks old (224). This member of the coronavirus family can also infect the respiratory system and is closely related to porcine respiratory coronavirus (37, 203, 204, 274, 290). Enzootically infected herds exhibit disease regularly (105). Young animals are infected after weaning when passive protection from maternal antibodies wanes. Disease is mild and weanling piglets recover quickly. Persistently infected animals from such herds may be capable of spreading the infection to naive populations. Following an experimental TGEV infection, recovery occurred after 104 days (270). Porcine transmissible gastroenteritis virus has also been isolated from apparently healthy adult swine (157, 244).

McClurkin swine testicle (ST) cells have been shown to readily establish a persistent relationship with TGEV (292). In early reports of TGEV in vitro persistence, persistently infected ST cells produced $10^6$ plaque forming units (PFU) of
TGEV per milliliter (ml) of culture fluid when tested over three years. A small plaque mutant isolated from this system was determined to be nonvirulent in pigs.

Many viruses of humans and animals are known to persist in their hosts (116). Virus propagation in the infected individual as well as opportunities for transmission can be greatly enhanced by persistent infection. *In vitro* studies of virus persisting in tissue culture cells allow direct virus cell interaction to be studied in a simplified system. Mutant virus populations accumulate defects of several types during persistent infection. Temperature sensitive mutants, defective interfering particles (DIP), and attenuated mutants are frequently described in such populations (3,81). Cells derived from *in vitro* persistent infections may also have altered characteristics (117,130). Cultures persistently infected by RNA viruses may be partially protected by endogenously produced interferon (81).

Other members of the coronavirus family persist *in vitro* and *in vivo*. Mouse hepatitis virus infections are often chronic and inapparent in the natural murine host (63,139). Persistent infections have been established in a number of cell lines. The infected cells produce cytopathic virus and virus specific RNAs for long periods in culture (42). Like the MHV carrier culture described by Mizzen et al. (182), some persistent MHV infections could be cured by neutralizing antisera and other techniques (159,166) while others could not (117). Cured neuroblastoma cells were rendered productive again by cytoplasmic fusion with uninfected cells (159). Temperature sensitive mutants have been isolated from persistently infected lines (16,117). The development of small plaque mutants during persistent infection (75,98) may indicate a
general trend toward attenuation, although plaque formation is a complex phenomena. At least one group found virus with enhanced cytopathic abilities evolving during MHV persistence (42). Cells which survive persistent culture with MHV display resistance to cytopathic effects of infection by homologous virus (98,117,182). Persistently infected mouse LM cells were found to resist MHV induced cell fusion (182). The mechanism of resistance and the level at which infection or cytopathic effect is inhibited are not known for other cultures described. Interferon has only rarely been reported as a stabilizing influence in these systems (212).

Bovine coronavirus (BCV) establishes in vitro persistence readily (103). Viral specific RNAs were detected in this culture during the 120 day experiment. Both positive and antisense BCV mRNAs were found as well as an aberrant RNA containing BCV sequences. No interference with BCV replication was detected but this was hypothesized to be a defective genome. Cows can also be chronically infected by BCV (47,103,127). Defective interfering particles have generally not been described in coronavirus persistent infections (98,117,159), although coronavirus DIPs can be produced by serial undiluted passage (168,169,171,172).

Human coronaviruses have also been used to initiate persistent infections. Human coronavirus 229E established an unstable persistent infection in human cells. This culture was temperature sensitive and gave rise to population of large virus variants producing plaques (39). Human coronavirus OC43 produced temperature sensitive variants when cultured persistently in neuronally derived human cells. This culture could be cured of virus by polyclonal antisera (43).
Materials and Methods

Cell Lines

The McClurkin swine testicle cell line was acquired from Dr. David Brian (University of Tennessee) at passage 148. Baby hamster kidney (BHK-21) cells were provided by Dr. John Holland (University of California, San Diego). Maden Darby Bovine Kidney (MDBK) cells were obtained from Dr. Peter Roberts (Montana State University, Bozeman). Cells were passed in Delbecco's modified Eagles Media (DME) from Sigma with 10% bovine serum (Hyclone) incubated in 6% CO₂ at 37°C. Glass prescription bottles in a 95 ml (32 oz) size were used for routine cultures. Trypsin (Sigma) at 2 mg/ml in HEPES buffered saline solution with (0.1 g/500 ml) EDTA was used to remove cells from glass. Cells were passed at a 1:10 ratio every seven days under normal circumstances. Costar 25 cm² and 75 cm² plastic T-neck flasks were used to culture persistently infected cell lines.

Virus

TGEV of the Miller strain was obtained from American Type Culture Collection (ATCC 743). It was passed in ST cells to increase the titer and provide reference stocks. Confluent monolayers of ST cells in circular 10 cm² Costar plastic dishes were used for infections. Most infections used a multiplicity of infection (MOI) of 3 PFU/cell with a titer of approximately 10⁷ PFU/ml. Vesicular stomatitis virus (VSV) Indiana serotype was provided by Dr. John Holland (University of California, San Diego). Virus stocks were stored at -70°C.
Establishment of cultures

Cultures were established with ST cells between passage 158 and 160 (Table 2.1). Cultures were grown to confluence in 25 cm\(^2\) plastic flasks with the exception of culture 2B-S2, which was established in a 75 cm\(^2\) flask. The overlying medium was aspirated from the monolayer and replaced with a small volume of DME supplemented with 2% fetal bovine serum (Sigma) or calf serum. For cultures established under antiTGEV antibody, neutralizing and non-neutralizing monoclonal antibody against TGEV was present in the inoculating media. Neutralizing monoclonal was diluted to a level sufficient to neutralize the input virus. This rinse was aspirated and virus diluted in the same formulation of DME was applied at a multiplicity of infection (MOI) between 0.2 and 1.0 PFU/ml in a small volume, normally 1 to 2 ml. The inoculum remained on the cells for one hour at room temperature and was then aspirated. The infected monolayers were covered with fresh media and incubated at 37°C. Cells were maintained in DME with 10% bovine serum with fresh media every 48 hours. Flasks were passed when confluent. For stable persistently infected cells, confluence occurred approximately every seven days. Cultures experiencing extensive cytopathic effect (CPE) were passed as infrequently as once a month.

Cells were photographed using an Olympus IMT inverted phase microscope. Cells were normally photographed and observed by phase contrast microscopy. They were photographed using TriX ASA 60 or Tripan X film. Methanol fixation followed by Wright-Geimsa staining was used to examine persistently infected cells cultured on
sterile coverslips in six centimeter dishes. These persistently infected cells were photographed with Ektachrome ASA 100 daylight film.

The concentration of virus as PFU/ml was estimated by plaque assay in Costar 6 well dishes (159). The inoculate was aspirated and 0.75% type 2 Sigma agarose in DME with 2% serum was applied after a one hour infection period. Seaplaque GTG agarose (FMC Bioproducts) was later substituted for Sigma type 2 agarose after toxicity problems were experienced with some lots. Plaques appeared two to five days after infection. Monolayers were fixed in 2% glutaraldehyde and stained with 1% crystal violet (Sigma) in 20% ethanol.

Table 2.1. Conditions of establishment of persistently infected swine testicle cell lines.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Cell passage number</th>
<th>Multiplicity of infection</th>
<th>Antibody</th>
<th>Characteristicsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B-S2</td>
<td>158</td>
<td>0.7</td>
<td>none</td>
<td>no PFU, stable</td>
</tr>
<tr>
<td>C1-1s</td>
<td>160</td>
<td>0.2</td>
<td>none</td>
<td>PFU, stable</td>
</tr>
<tr>
<td>C1-2s</td>
<td>160</td>
<td>0.2</td>
<td>none</td>
<td>PFU, stable</td>
</tr>
<tr>
<td>2C-1</td>
<td>157</td>
<td>1.0</td>
<td>none</td>
<td>PFU, unstable</td>
</tr>
<tr>
<td>3NN1</td>
<td>157</td>
<td>1.0</td>
<td>TI</td>
<td>PFU, stable</td>
</tr>
<tr>
<td>4N1-1</td>
<td>157</td>
<td>1.0</td>
<td>T254</td>
<td>PFU, unstable</td>
</tr>
<tr>
<td>4N1-2</td>
<td>160</td>
<td>1.0</td>
<td>T254</td>
<td>PFU, unstable</td>
</tr>
<tr>
<td>11N6-2</td>
<td>subcloned from 4N1-2</td>
<td>1.0</td>
<td>T254</td>
<td>PFU, stable</td>
</tr>
</tbody>
</table>

a - PFU: plaque forming units consistently measurable; stable: monolayer grew without crisis after five post infection passages; unstable: monolayer in crisis periodically after post infection passages.
Antibodies

Polyclonal anti-TGEV ascitic fluid and monoclonals antibodies (MAb) T1 and T254 were kindly provided by Dr. Sue Goss, Montana State University. T1 is an anti-TGEV MAb which reacts with the nucleocapsid protein (N) during radioimmunoprecipitation. It is non-neutralizing. T254 is a neutralizing MAb which binds TGEV spike (S) glycoprotein. Hybridoma cells were used to establish ascites. Rabbit anti-mouse IgG conjugated to FITC was obtained from Sigma.

Viral Antigen Production by Indirect FITC

Trypsinized cells were washed and were seeded 800 cells per well in Terizaki plates. The cells were incubated at 37°C for twelve to twenty four hours to allow attachment. When exogenous infections were assayed the virus was applied at a MOI of three PFU per cell for a period of 24 hours in a 10 microliter volume of DME2. The plate was rinsed twice in phosphate buffered saline (PBS) and fixed with methanol for all assays. Dehydrated plates were dried with compressed air and stored at -70°C. Before being reacted with antibody, plates were rinsed with PBS. Monoclonal and polyclonal antibodies were applied in five microliters per well after dilution in PBS. Primary antibody was left in contact with the fixed cells for one hour at room temperature. Non-adhering antibody was removed by five PBS rinses. Goat anti-mouse IgG conjugated to FITC was diluted in PBS and five microliters were reacted per well. After one hour at room temperature the plates were rinsed five times in PBS. Fluorescent cells were viewed with an Olympus IMT inverted phase microscope fitted
with a mercury vapor lamp and JB 50 filters. Wells were scored as positive or negative for TGEV specific fluorescence or individual fluorescing cells were counted and compared with total cells counted under bright field illumination.

**Test for Temperature Sensitive Virus**

Duplicate cultures were established at 37°C in T25 flasks. After overnight incubation one set of dishes was incubated at 32°C while the other was returned to 37°C. Cells were observed for cytopathic effects and media was titered for plaque forming virus. Six-well dishes of ST cells were used to assess temperature sensitivity in virus produced by persistently infected cultures. Spent media from persistent cultures maintained at 37°C was assayed for plaques in duplicate. Following attachment at room temperature, one set was incubated at 37°C and the other was held at 32°C. Plates were fixed and PFUs counted as described previously.

**Defective Interference**

Direct interference was tested by mixing cell culture fluid from persistently infected cell lines with parental TGEV Miller and used to coinfect ST cells. TGEV Miller was used at an MOI of 0.1. Virus released was compared with TGEV Miller at the same MOI used alone in a parallel infection.

Interference with plaque formation titered parental TGEV by plaque assay in parallel with virus released by persistently infected cultures. Virus preparations were mixed and subjected to a plaque assay. PFU produced by these infections was compared with parental TGEV or persistent virus when assayed alone.
Interferon Production

Medium from cultures to be tested was serially diluted 1:2 across a 96 well dish in DME with 2% serum in duplicate. Maden Darby bovine kidney cells were seeded into wells with $10^8$ cells per well and incubated 10 to 16 hours at 37°C. Interferon containing medium was then removed and one row of each duplicate set was fed with DME with 2% serum as a control for cytolysis from the interferon. The other well of each duplicate was infected with $10^5$ PFU of VSV per well. The plates were incubated at 37°C and examined for cytopathic effect at 16 and 24 hours after VSV infection following the method of Friedman (81).

Superinfection

Established monolayers of persistently infected cells in T25 flasks were rinsed and super-infected with parental TGEV at an MOI of 0.25 or 6.7 PFU/cell. After an hour at room temperature for virus attachment, the inoculum was diluted approximately four times with medium. Samples of cell culture fluid were removed at various times after superinfection and compared with material released by parallel cultures that had not been super-infected. Cells were also observed comparatively for cytopathic effects.

The ability of persistently infected monolayers to support plaque formation by exogenous TGEV and by VSV was tested by using persistently infected monolayers as host cells for exogenous virus in a standard plaque assay. The highest effective MOI of TGEV applied was 80 PFU/cell. Vesicular stomatitis virus was used at MOIs of 0.1 and 100 PFU/cell.
Results

Appearance and Stability of Cultures

Physical changes in persistently infected cells include the formation of giant cells with very large nuclei (Figure 2.1). These cells may have been formed by the fusion of many normal sized cells or by abnormal growth and division. Repeated photographs of two large cells over a three hour period indicated that cells were fusing. Giant cells showed moderate to no fluorescence with TGEV specific antibodies. Cytopathic changes more similar to an acute infection were also seen in persistently infected cell lines. Vacuoles were observed in the cytoplasm of both normal and giant cells. Previously establish monolayers were also occasionally disrupted by widespread cytolysis. Cytopathic changes were seen at intervals in stable cell lines and continuously in unstable cultures. Generally, cell lines became stable and grew well approximately ten passages after they were infected.

TGEV Plaque Forming Units Produced

Persistently infected cell lines were checked for virus production every two weeks and produced between 0 and 10^7 PFU/ml. Virus production over time increased gradually from 10^4 to 10^6 PFU per ml as cultures became stable and the number of viable cells increased (Figure 2.2). Production of virus gradually increased in
Figure 2.1. Giant cells visible in persistently infected culture 2B-C at post-infection passage three. Persistently infected cells (cultured on sterile coverslips in 6 cm dishes) were fixed using methanol followed by Wright-Geimsa staining. Cells were photographed using an Olympus IMT inverted phase microscope and Ektachrome film (ASA 100).
Figure 2.2. Log$_{10}$ plaque forming virus produced per milliliter of tissue culture media plotted against cell monolayer passages post infection for cultures, 11N6-2, 2C-1, Cl1-S, and Cl2-S.
cultures maintained over 20 passages (approximately one year) to $10^{7-9}$ PFU/ml in culture media (Figure 2.3). Virus production as measured by PFU released into the culture media spontaneously disappeared in cultures 2B-S2 prior to passage 10 post infection (pi).

Because the assumption of uncorrelated (independent) error terms, generally required with regression methods, is often not appropriate for times series data, a Durbin-Watson test (190) was conducted for autocorrelation (serial correlation). For three of the four tests conducted (Cl1-1, 11N6-2, and 2B-S2) there was no statistical evidence ($P > 0.10$) of autocorrelation. The Durbin-Watson test involving culture Cl1-2 indicated error terms were positively correlated, which necessarily confounds interpretation of the results generated from the regression analysis for this culture (190).

In cultures initiated under TGEV specific neutralizing monoclonal antibody, cell monolayers were protected. The cells began to show cytopathic effect 100 hours after infection and produced $10^4$ PFU/ml virus that were not neutralized by the antibody seven days after infection. For the first 100 days pi, virus production averaged $1.8 \times 10^6$ PFU/ml compared with $1.2 \times 10^5$ PFU/ml for control cultures (Figure 2.3). Cells infected and maintained under a non-neutralizing anti-TGEV monoclonal produced less virus, an average of $3.8 \times 10^5$ PFU/ml. These culture and their derivatives stabilized more quickly than cells maintained without antibody. Over extended periods, no differences in extracellular virus production or stability were found between cell lines derived with and without antibody (Figure 2.4).
Figure 2.3. Log$_{10}$ plaque forming virus produced per milliliter of tissue culture media plotted against days post infection, for cultures established under anti-TGEV neutralizing monoclonal antibody (4N1-2, 6N2-1, and 11N6-2), non-neutralizing anti-TGEV monoclonal antibody (3NN1, 5NN-2), and a control established without antibody (2C-1).
Figure 2.4. $\log_{10}$ plaque forming virus produced per milliliter of tissue culture media plotted against cell monolayer passage post infection for cultures 2B-S2, 11N6-2, 2C-1, Cl1-S, Cl2-S, and 11N6-2.
The persistently infected cultures could not be cured by exogenous polyclonal antibody added to culture. After passage under neutralizing levels of antibody for 57 days, $4 \times 10^6$ to $2 \times 10^7$ PFU/ml were produced two days after the cultures were returned to normal media. Growth rate and stability of cell monolayers were not affected by the application of neutralizing antisera nor by its withdrawal.

**TGEV Specific Fluorescence**

Seventy to 80% of the cells in control cultures tested with polyclonal anti-TGEV as the primary antibody were positive for fluorescence by 12 hours pi. Persistently infected cells were 15 to 85% positive by indirect immunofluorescence for TGEV antigens (Figure 2.5). Cells were strongly to moderately fluorescent and varying over time for individual cultures. Like acutely infected ST cells, fluorescence was brightest with monoclonal antibody specific for the N protein.

**Test for Temperature Sensitive Conditions**

Parental TGEV Miller and culture media from persistently infected cultures Cl1-S and 2B-S2 were titered at 37°C and 32°C. Plaque formation was more efficient at 37°C for Miller and Cl1-S derived virus. Counts of PFUs were approximately 50% greater for both. No plaques were produced by 2B-S2 cell culture supernatant fraction at either temperature. Uninfected ST cells were best maintained at 37°C.
Figure 2.5. Indirect FITC labeled swine testicle (ST) cells. Part A: Uninfected ST cells labeled with polyclonal anti-TGEV antisera at 800X magnification. Part B: Acutely infected ST cells labeled with monoclonal T1 at 800X magnification. Part C: persistently infected cell line 5NN-2 labeled with monoclonal T1 at 800X magnification. Uninfected or persistently infected ST cells 800 per well were washed and seeded in Terizaki plates. The cells were incubated at 37°C for 12 to 24 hours to allow attachment. Acute TGEV infections with a multiplicity of infection (MOI) of three PFU per cell were carried out in the dish for 24 hours. The plates were rinsed twice in phosphate buffered saline (PBS) and fixed with methanol. Monoclonal and polyclonal antibodies were diluted in PBS. Non-adhering antibody was removed by five PBS rinses. FITC conjugated goat anti-mouse IgG was diluted in PBS and plates were rinsed five times in PBS. Fluorescent cells were photographed with an Olympus IMT inverted phase microscope fitted with a Mercury Vapor lamp and JB 50 filters and Tripan X film.
Interferon Production

Culture fluid from persistently infected ST cell protected MDBK cells against infection by VSV at an MOI of 0.01 PFU/cell. Spent media from acute infections and cultures CL1S, 2B-C, and 11N6-2 all protected MDBK cells. Media from acutely infected ST cells consistently offered protection when diluted to 1:8. Effective dilutions for media from persistently infected cultures ranged from 1:8 to 1:1. The degree of protection was not consistent for any persistently infected culture nor did it correlate with PFU of TGEV produced. Supernatant fraction from ultracentrifuged media (37,000 RPM SW41 Ti rotor) protected MDBK cells. Media from 2B-S2, which rarely produced plaque forming TGEV, could not protect MDBK cells at any of the time points tested. 2B-S2 monolayers were consistently susceptible to infection by VSV as were uninfected ST cells. Persistently infected cell lines 11N6-2 and 2C-B resisted infection by VSV at some passages.

Defective Interfering Particles

In mixed infections with TGEV Miller strain, media from persistently infected ST cells did not reduce the production of virus when compared to infection with TGEV Miller alone. Media from persistently infected cells also introduced additional competent virus making it difficult to determine whether both populations of virus were replicating efficiently in mixed infections. Cytolytic virus was not released by cell line 2B-S2 and media from this culture had no discernable effect on infection of ST cells by standard virus. When media from persistently infected cultures was titered in parallel
with parental TGEV and then plaqued together in a mixed assay, titers were reduced from the expected additive PFU concentration. Reductions were one to one-half log of the expected titer. Cultures 11N6-2 and 2B-S2 produced reductions in titer at some passage levels (Table 2.2).

Table 2.2. Efficiency of plaque formation in mixed infection with TGEV Miller (titers are expressed as plaque forming units/ml).

<table>
<thead>
<tr>
<th>Passage</th>
<th>Titer of culture fluid</th>
<th>Expected titer</th>
<th>Titer of mixed infection</th>
<th>Expected titer minus actual titer</th>
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<tr>
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<td>4.3 x 10^6</td>
<td>2.0 x 10^6</td>
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<td>8.0 x 10^6</td>
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<tr>
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<td>2.0 x 10^6</td>
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<td>2.5 x 10^5</td>
</tr>
</tbody>
</table>

**Resistance to Superinfection.** Cell monolayers of persistently infected ST cells consistently resisted cytopathic effects caused by exogenous TGEV Miller. There was no evidence of damage to the monolayer for cultures Cl1-S and 11N6-2 at an MOI of 6.7 PFU/cell. At an MOI of 0.25 PFU exogenous virus per cell, no changes were noted in Cl1-S and 11N6-2 monolayers. Both cultures were producing over 10^6 PFU/ml of endogenous virus when super-infected. Numbers of virus produced by these super-infected cultures were equal to or slightly below time matched samples.
from the same cell lines. Culture 2B-S2 did show substantial cytopathic effect and produced $10^{5.6}$ PFU/ml for four months following superinfection. No PFUs were detected in media from the parent culture that was not producing detectable plaque forming virus when super-infected.

When persistently infected monolayers were used in place of ST cells in plaque assays for TGEV Miller, cell lines producing endogenous virus were completely refractory to plaque formation. Cultures 11N6-2 and 2C-1 did not support plaque formation at any of the passage levels tested. Culture 2B-S2 permitted plaque formation nearly as efficiently as ST cells and produced no endogenous virus. Culture CL1-S supported plaque formation for one passage level in which it was not producing endogenous virus. Other passages tested did produce virus and would not support TGEV Miller plaque formation. A cell lines resistance to TGEV did not consistently correspond with resistance to VSV plaque formation.

Discussion

Stable characteristics of ST cells persistently infected by TGEV included rapid growth, consistent production of extracellular cytolytic virus, and resistance to cytolytic effects of exogenous virus. This resistance is a fundamental alteration of the normal relationship between the Miller strain of TGEV and ST cells. The mechanism of this resistance may involve a variety of host cell and virus modifications. Previous research with coronaviruses and other positive stranded RNA viruses has implicated
virus resistant cell variants (182), attenuated virus populations (16,75,98,117,222,258),
endogenous interferon, and interference from defective viral genomes (2,81,114).

Cell variants

The carrier model for persistent *in vitro* infections hinges on the overall resistance
of the cell population to virus infection. A small fraction of the population periodically
develops susceptibility to infection through mutation or variation in the cell cycle
phase. Productive virus infection is limited to these cells in which it proceeds in a
normal cytolytic fashion. As found by Holmes et al. (117), cells from such cultures
could be cured by cloning and subsequently resist infection by exogenous virus. When
tested for viral antigen production the majority were negative while a small proportion
was strongly positive. In contrast, ST cells persistently producing TGEV could not be
cured by passage under neutralizing polyclonal antibody nor by cloning. Although the
percentage of positively fluorescing cells varied greatly in persistently infected
monolayers, strongly fluorescent individual cells or foci were not observed. The usual
appearance, when stained with TGEV specific antibody, was an intact, morphologically
normal, moderately fluorescent monolayer. In cell lines where virus production had
stopped spontaneously cells were susceptible to cytolysis by exogenous virus. When
cytolytic TGEV production resumed in a previously quiescent cell line it reached $10^6$
PFU/ml and then disappeared after only five passages (four months). These cell lines
did not exhibit the expected characteristics of a carrier culture, although some evidence
for ST cell adaption to the conditions of persistent infection clearly existed.
**Attenuated Virus**

Virus populations can accumulate defects, becoming gradually less cytopathic under conditions of persistent infection. Evidence cited by others for attenuation includes reduction in plaque size, temperature sensitive phenotypes, and production of aberrant viral RNAs. All of these phenotypes have been described previously for coronavirus persistent infections (16,102,117,292). In the current study, plaque size was variable and heterogenous for TGEV Miller and virus produced by our persistent infections. No temperature sensitive component of the virus population was detected. Virus produced by persistently infected cultures consistently and efficiently lysed naive ST cells. Virus in described MHV persistent infectioned was also highly cytolytic (42).

**Interferon**

Persistently infected cells appeared to produce interferon. Monolayers of persistently infected cells were resistant to VSV infection at some passage levels. Resistance was not displayed consistently by any culture. Some samples of cell media from persistently infected ST cells protected MDBK cells from VSV infection.

Whether interferon is an important moderator of TGEV pathogenicity in persistence depends on its ability to interfere with TGEV infection, its cytopathic effect on ST cell, and the consistency of its production. In experimental systems with acute infections both *in vivo* and *in vitro*, TGEV has been shown to be only modestly inhibited by porcine interferon (67). Porcine interferon has been shown to have cytopathic effects on ST cells (284). Acutely infected ST cells produced more interfering capacity than
persistent cultures when culture media was tested for VSV interference on MDBK
cells. However, these cells suffer from cytopathic effects of TGEV even with low
inputs of virus. Persistently infected cultures sometimes produced no detectible
interfering effect on heterologous virus yet remained stable while producing
endogenous PFU and resisted infection by exogenous TGEV Miller. Interferon
production was clearly not a singular regulator of culture stability.

Defective Interfering Particles

Defective interfering particles have been implicated in the generation and
maintenance of persistently infected cell lines for a variety of RNA viruses. Vesicular
stomatitis virus persistent infections are thought to be directed almost exclusively by
the moderating influence of defective interfering variants controlling this otherwise
highly cytolytic virus (114). When VSV is passed sequentially at high multiplicity,
replication of competent VSV is depressed by interfering variants. This produces
resistant populations of competent virus leading to the further generation of new
defective interfering variants. The cyclic evolution of interfering and resistant
populations of VSV leads to mathematically predictable rise and decline in cytolytic
virus production (122). In such a system, cell variants do not occur since fresh
monolayers are used at each virus passage.

It has been shown that DIPs do occur in coronavirus populations as well
(168,169,171,172). They have served as model systems for the study of RNA
replication and packaging. However, interference from defective variants has not been
demonstrated to be biologically important for coronavirus persistence. They have not previously been demonstrated in these systems and the level of interference seen under high multiplicity passage is less dramatic than that observed for VSV.

Media from persistently infected ST cells consistently contained plaque forming virus for most cultures. When this medium was used in co-infections at low multiplicity with TGEV, interference occurred indicating that interfering units were present. In order to limit plaque formation in this system DIPs would need to be numerous as elimination of a single plaque would require co-infection of a single cell by a PFU and interfering unit under conditions of low multiplicity of infection. Whether this level of interference contributes to the maintenance of the persistent state is a more complex question. Efficiency of interference was clearly insufficient to protect naive cells when undiluted culture medium from established infections was used as an inoculum. Occasionally infection of ST monolayers by standard virus at low MOIs (0.1 to 0.5) left a few cell foci after destruction of the remaining monolayer. It is these cells that appeared to give rise to the replacement monolayer in newly established persistent infections. These cells may have survived infection due to co-infection by a defective genome. Cell foci have been used as a means to quantify interfering particles in other systems (122). Following destruction of the monolayer in the acute phase of the infection at 48 to 72 hours pi, the TGEV in the media drops to $10^4$ PFU/ml. Few healthy cells were present to produce virus and incubation at 37°C inactivates TGEV (Dr. Cliff Bond, Montana State University, Bozeman, personal
communication). Under such conditions, low levels of interference from defective genomes may be relevant.

**Extracellular Virus**

Once the persistent state is established, extra-cellular virus was not required to maintain the infection. Neutralizing polyclonal antisera that maintained extracellular virus below two PFU/ml over a one month period failed to cure two well established cultures. Virus may have been maintained by stable infection of individual cells or by direct transfer between adjacent cells. Non-neutralizing monoclonal antibody T1 accelerated the stabilization of persistently infected cell lines. This may have been due to a modification of cell to cell virus transmission. Alternative explanations include capping and endocytosis of viral antigen on the surface of infected cells or enhanced interferon production.

Neutralizing monoclonal antibody delayed but did not prevent destruction of the ST cell monolayer when applied during culture establishment. Antibody T254 interacts with the spike protein which is involved in membrane fusion of infected cells. S protein might logically play a role in cell to cell transfer of TGEV, although not necessarily at the epitope bound by monoclonal T254. Escape mutants quickly developed in cultures established under this monoclonal. Modification of the virus population by this selective pressure indicated that extracellular virus played an active role during culture establishment. This contrasts with its diminished role in established persistent infections.
Co-adaption of Virus and Cells

Long term persistent infection of ST cells by TGEV virus led to enhanced cell survival and increased output of extracellular virus. Cells intermittently produced interferon and consistently resisted homologous virus cytolysis. Adaption to cytotoxic effects of interferon may also have contributed to their survival. Cells also reached high passage levels, in several cases more than 250 passages. Adaption to culture conditions may have contributed to the rapid growth of established cell lines.

TGEV produced an interfering component during persistent infection of TGEV cells. In the presence of this interfering component, levels of extracellular cytolytic virus produced increased steadily after 100 cell passages for most cultures.

Historically, isolation and characterization of TGEV was hampered by a lack of cytolytic virus isolates and appropriately cells susceptible to cytopathic effect (156). The appearance of several TGEV variants with reduced pathogenicity and altered tropism indicates that the agent was highly plastic. This study, along with previous in vivo and in vitro findings of nonapparent and persistent infection, indicate that the well studied acute, cytopathic infection represents a single aspect of this virus's diverse capacity.
CHAPTER 3

VIRUS SPECIFIC MACROMOLECULES SYNTHESIZED IN PERSISTENTLY INFECTED SWINE TESTICLE CELLS

Introduction

The mechanisms of viral persistence are particularly relevant for members of the family Coronaviridae. The type species for the coronavirus family, mouse hepatitis virus (MHV), causes persistent, quiescent, subclinical, or symptomatic infections. Neurotrophic variants of MHV such as JHM and A59 produce chronic demyelinating disease that has been used as a model system for human diseases such as multiple sclerosis (75,140). Feline infectious peritonitis virus (FIPV) produces both rapid and chronic forms of infection with a clinical course that can last several months (287). Bovine coronavirus (BCV) produces an acute enteric disease that can be followed by long-term carriage in adults and young animals (47,98,128). Like BCV, transmissible gastroenteritis virus (TGEV) is associated with acute infection of the gastrointestinal tract, but one study found healthy market-weight swine shedding TGEV (272). TGEV is antigenically a member of the group I coronaviruses, and is genetically related to canine coronavirus (CCV), FIPV, and human coronavirus HCV-229 (124,206,263). Viral transmission by the fecal-oral route can be demonstrated and disease appears as
damaging epizootics on some farms or as enzootic disease with maternal immunity and low mortality on others (117). Chronically infected carrier animals may help to account for the obscure transmission of TGEV, although experimental addition of recovered animals to naive herds failed to produce detectable new infections (67).

Studies of *in vitro* persistence by coronaviruses have pointed to a number of potential mechanisms permitting long term infection. Mechanisms invoked for other viruses (5,40,81) have also been found in some coronavirus persistent infections. These mechanisms include temperature-sensitive virus variants and small plaque variants (16,121,254), interferon production (217), and carrier cultures in which the virus replicates in subsets of the overall cell population (149). Generally, cells from these persistent infections resist infection by homologous virus.

In other studies, no evidence for a temperature sensitive population of virus could be found nor were defective interfering particles or interferon present, however, changes were frequently noted in the virus population. Coronavirus MHV-JHM from a persistently infected murine neuroblastoma cell line displayed greater genetic heterogeneity than isolates from a wide geographic range that included Japan and the United States (146). Variants with reduced cytopathic properties such as small plaque mutants have been described for TGEV produced by persistent infection (299). Over a 120 day study period, viral subgenomic RNAs of both positive and negative sense were produced by BCV (105). Virus variants with reduced cytolysis properties and an accumulation of mutations in the viral leader sequence during BCV persistent infection
have been used to infer an autoregulatory role for this portion of the viral genome (103).

Infectious JHM virus produced by persistently infected cultures of 17Cl-1 cells lost the cytopathic property of inducing cell fusion. Oligonucleotide fingerprints indicated a genetic change in the population of MHV-JHM RNA (168). Virus produced by a persistent MHV-JHM infection no longer displayed cytopathic effects after 15 passages as a persistent culture. In addition, these cells were resistant to homologous, but not heterologous virus with no evidence for defective interference or temperature sensitive mutants and no change of tropism in vivo. The virus produced included small plaque variants with reduced virulence in vivo (99). Virus from persistently infected DBT cells was able to produce a chronic recurrent hepatitis in nude mice (94).

Investigations of MHV-A59 persistence in DBT cells found an overall reduction in viral RNA synthesis, although all mRNAs and their negative polarity counterparts were present (41). In contrast to BCV persistent virus, characterization of viral RNA from reverse transcriptase derived sequences revealed stability in the viral leader sequence during persistence but an increased number of mutations in the 5' - untranslated region of the genome (5'-UTR). An A to G point mutation at nucleotide 77 appeared to enhance transcription of a downstream non-structural open reading frame (ORF). The mutation was positively correlated with extended passage under persistent infection. This ORF is thought to encode part of the viral RNA polymerase (42). Unfortunately, these studies rely on individual clones obtained from a diverse population of viral genomes.
Among populations of viral RNAs, defective genomes of varying structure have been described. Some of these genomes in the form of defective interfering particles (DIPs) exert negative pressure on the synthesis of full-length RNA. Although these genomes have not previously been considered an important mechanism for viral persistence by coronavirus, they have been obtained and studied as models for virus RNA replication and packaging for several coronaviruses including MHV (52,169,171,172,177,281) and TGEV (179). Defective RNAs containing discontinuous sections of the IBV genome have also been described, but they did not appear to interfere with the replication of standard virus (206).

In Chapter 2, interference was demonstrated by a plaque reduction in mixed infections of virus from persistent infections and parent virus. In undiluted mixed infection no overall reduction in virus replication could be detected. However, as cytopathic TGEV capable of efficient plaque formation was also consistently released by persistently infected ST cells this would obscure subtle interference with parental virus. When both persistent and parental virus were diluted and used in a combined plaque assay, a reduction in plaque formation was noted compared with parental virus tested alone.

Continued virus replication in cells resistant to the cytopathic effect is the focus of this research. Transmissible gastroenteritis virus specific macromolecules from persistently infected cultures of ST cells were compared with those produced during the acute infection. The objectives of this investigation were to determine if TGEV proteins produced by independently derived persistent cultures were the same as those
produced by the Miller strain of TGEV during acute infection, to determine if viral RNAs of positive and negative polarity were produced during persistent infection, and to determine whether TGEV specific nucleic acids are found in other configurations such as a provirus in the cellular DNA of persistently infected ST cells.

Materials and Methods

Immunoprecipitation of Intracellular TGEV Specific Proteins

TGEV specific proteins were detected by immunoprecipitation as previously described (19). Briefly, monolayers were labeled with 200 μCi of S\textsuperscript{35} methionine (New England Nuclear) per ml of methionine deficient Dulbecco’s Modified Eagles Media (DME) supplemented with 2% (vol/vol) dialyzed fetal bovine sera from 10 to 14 hours post infection (pi). Mock infected ST cells were labeled in parallel. Swine testicle (ST) cell lines persistently infected with TGEV included 2B-S2 at passage 35, 11N6-2 at passage 49, 2B-C at passage 32, and Cl-1S at passage 90. These cells were labeled in like manner for a four hour period. All cell lines were growing at consistent rates. The radiolabeled monolayers were lysed and immunoprecipitated with polyclonal ascitic fluid kindly supplied by Dr. Susan Goss as described by Bond et al. (19). Immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis by the method of Laemmli (141). Molecular weight standards (Sigma SDS-7 and SDS-6H) were electrophoresed in parallel with all samples for reference. Gels were fixed, dried and exposed to Kodak X-O-Mat AR film for periods of time determined empirically and developed according to Kodak instructions.
Analysis of TGEV Specific Nucleic Acids

Preparation of Strand-Specific Probes. Plasmid pAL141 was obtained from Dr. Andreas Luder (Montana State University, Bozeman). The plasmid contained a 4.3 kilobase pair (kbp) insert in the PstI site of pBR322. The insert is representative of the 3'-end of the genome and subgenomic mRNAs of the Miller strain of TGEV. A 564 base pair (bp) Xba1/Kpn1 fragment from the 3' region was subcloned into the replicative forms of bacteriophage M13mp18 and M13mp19 using standard methods (183). The Xba1/Kpn1 fragment represents the 3'-end of the open reading frame coding for nucleocapsid (N) protein. The nucleocapsid ORF is found in all of the TGEV mRNAs and genome.

Single-stranded, M13 virion DNA, from the M13mp19 construct was designated M13mp19-S2 and contained TGEV specific sequence with the same polarity as the positive stranded virion RNA and TGEV mRNAs. The identity of the construct was confirmed by dideoxy sequence analysis (Sequenase kit, United States Biochemical).

M13mp18 clones containing inserts of the appropriate size were screened for complementarity to M13mp19-S2 by the C-test (181). The construct M13mp18-S28 was determined to contain a fragment complementary to the positive stranded virion RNA and TGEV mRNAs.

Single stranded DNA from M13mp19-S2 and M13mp18-S28 phage was purified and used as a template for synthesis of complementary single stranded DNA. Synthesis was primed with universal sequencing primer for M13 (United States Biochemical), extended with Klenow fragment of DNA polymerase I (Boehringer-Mannheim) and
radiolabeled with α-[32P]dCTP (New England Nuclear). To prime the synthesis of single stranded probe, 2 µl (0.5 µg) of single stranded template and 1 µl (0.5 pmol/µl) of the USB primer described previously were annealed in Klenow salts buffer provided by the enzyme supplier (Boehringer Mannheim). The radiolabeled M13mp19-S2 and the M13mp18-S28 double-stranded products were cut with XbaI and EcoRI, respectively, to recover the TGEV sequences. The digestions were separated by electrophoresis on a 7.5 % (wt/vol) denaturing polyacrylamide sequencing gel. The bands were detected by autoradiography and recovered by electroelution (245). The activity of the probes was determined by liquid scintillation spectroscopy. The radiolabeled probes were designated M13mp19-S2/32P (detects positive sense sequences) and M13mp18-S28/32P (detects negative strand sequences).

Preparation of Intracellular RNA. TGEV infected, mock-infected, and persistently infected cell monolayers were washed with ice cold sterile NET buffer (10 mM Tris, 1 mM EDTA (pH 7.4), 100 mM NaCl). Cells were then lysed in NET with 1% (vol/vol) NP40 (Nonidet P-40, Shell) and held on ice for 5 minutes. Cell lysis was confirmed microscopically. Lysates were digested for 30 minutes at 55°C with 100 µl proteinase K per ml in 1% (vol/vol) SDS, 0.4 M NaCl, 0.01 M EDTA. The RNAs were extracted with phenol-chloroform-isopentanol as previously described (91).

Northern Blot Analysis. RNAs were denatured with glyoxal and electrophoresed by the procedure of McMaster and Carmichael (177). Fractionated RNAs were transferred to Nytran nylon membranes (Schleicher and Schuell) following the protocol
specified by the manufacturer. Sterile 10X SSPE Buffer (1X SSPE: 0.18 M NaCl in 10 mM NaPO₄ (pH 7.7), 1 mM EDTA) was used for transfer and a germicidal UV lamp 30 cm from the wet filter for five minutes was used to cross-link RNAs to the filter. Filters were dried at 80°C for one hour. If membranes could not be processed immediately, they were stored at -20°C. Membranes were deglyoxlated using 50 mM TrisHCl 10 mM EDTA (pH of 8.0) at 45°C for 40 minutes. Blots were then prehybridized with 5X SSPE Buffer, 5X Denhardt’s solution (1X: 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone and 0.02% (wt/vol) bovine serum albumin), with 1% (wt/vol) SDS and 50 μg/ml carrier DNA. Carrier salmon sperm DNA was sheared by sonication and phenol-chloroform extracted. Prehybridization was carried out in sealed bags at 45°C overnight. Blots were hybridized in 50% (vol/vol) formamide with 4X SSPE, 1% (wt/vol) SDS, 50 μg/ml carrier DNA, 3X Denhardt’s solution and radiolabeled strand-specific probes M13MP19-S2⁳²P or M13mp18-S28⁳²P. Hybridization was carried out at 45°C overnight in the resealed bag. Following hybridization blots were washed twice for one hour each in 2X SSPE, 0.1% (wt/vol) SDS at room temperature followed two one hour washes in 1X SSPE, 1% (wt/vol) SDS at 37°C. Wet blots were wrapped in plastic film supported by 3M paper and exposed to Kodak X-O-Mat AR film with an intensifying screen at -70°C and developed as recommended by the manufacturer.

**DNA-Southern Blot.** Monolayers of TGEV infected, uninfected, and persistently infected cells were trypsinized and the DNA extracted as described by Sambrook (227).
DNAs were digested with \textit{HpaI} to fragment the genomic DNA. Samples (50 \( \mu \)g) were loaded after treatment with DNase-free-RNase (Sigma), RNase-free-DNase (Promega) or untreated. Samples were either blotted directly to Nytran filters using a slot-blot apparatus (BRL) or electrophoresed on a 0.8\% (wt/vol) agarose gel. Marker lanes containing \textit{HindIII} digested lambda DNA were stained with ethidium bromide and photographed along with a ruler. The rest of the gel was blotted directly onto a Nytran membrane with 6 X SSPE and 0.15 M NaOH. The blotted DNA was probed with radiolabeled strand-specific probe M13mp18-S28/\(^{32}\)P. Conditions for pre-hybridization and hybridization were the same as described for Northern analysis. After autoradiography, the blot was striped with 25\% (vol/vol) formamide and 1.5X SSPE at 65\(^\circ\)C overnight, rinsed with 2X SSPE and re-exposed to x-ray film to ensure that probe had been removed. The membrane was then hybridized under the same conditions with probe M13mp19-S2/\(^{32}\)P. As little as 30 ng of the hybridization control (plasmid pAL141 DNA) could be detected. The DNA extracted from approximately 10\(^6\) cells was fixed in each slot. Thirty nanograms of plasmid pAL 141 is the molar equivalent of a single TGEV specific DNA sequence per cell. DNA present at levels lower than this could not be detected.

\textbf{Results}

\textbf{TGEV Specific Proteins}

All persistently infected cultures producing detectable plaque forming units of TGEV expressed the TGEV specific proteins S and N, as determined by SDS-PAGE
analysis of immunoprecipitated radiolabeled proteins (Figure 3.1). However, M protein was present at low levels or non-detectable. During the period of analysis, persistently infected cultures C11-S, 11N2-6, and 2B-C were producing $10^6$ or greater PFU/ml of extra-cellular virus (data not shown). Spike protein (S) was detectable in acutely infected cells and in cultures C11-S, 11N2-6, and 2B-C (Figure 3.1). In culture 2B-s2 which was producing less than 10 PFU/ml at the time of analysis, a faint band was present slightly above the expected position of S at 220 kDal. A similar faint band was present in proteins precipitated from uninfected cells. Nucleocapsid (N) protein was present in persistently infected cultures Cl-1s, 11N2-6, and 2B-c. A faint band at slightly higher molecular weight was visible in the 2B-S2 culture, but a band was also present in the uninfected cell lysate, although very light. Membrane (M) protein was difficult to detect in any of the persistently infected cell lysates although it was detected as a strong band in the acutely infected cell lysate that was processed in parallel. Faint bands were detected at or slightly above the expected position for M protein at 29 kDa in cultures 11N2-6, and to a lesser extent in Cl-1S.

Two bands not known to be TGEV specific proteins were present in three of the persistently infected cultures. A band at 75 kDa was visible in the lysates from Cl-1S, 11N2-6, and 2B-C but was not present in the lysate from acutely infected cells. The 75 kDa band was most obvious in the lysate from culture 2B-C. A low molecular weight band smaller than 14 kDa was visible in three of the persistently infected cultures.
Figure 3.1. SDS-Page 10% (wt/vol) polyacrylamide gel of immunoprecipitated proteins from uninfected (mock), acutely infected (beginning at 10 hours post infection (pi)) and persistently infected ST cell lysates. TGEV specific proteins are labeled (S, N, M). Lane 1: mock infected; lane 2: acutely infected 10 to 14 hours pi. Lanes 3-6 persistently infected cells: lane 3: 2B-S2 at 35 passages pi; lane 4: 2B-C at 32 passages pi; lane 5: 11N2-6 at 49 passages pi; lane 6: Cl-1S at 90 passages pi.
Cl-1S, 11N2-6, and 2B-C. It matches a band seen on SDS-page analysis of S. aureus precipitated proteins. Although pre-absorption with S. aureus was used to minimize absorption by S. aureus, the band may represent non-specifically bound protein or a small TGEV specific protein.

**TGEV Specific RNAs**

The amount of TGEV specific RNA and plaque forming virus produced by persistently infected cell monolayers were both less than the amount produced by acutely infected monolayers. A comparison of RNA extracted from monolayers of the same surface area (Figure 3.2) indicated that cell lines producing less than $10^6$ PFU/ml expressed very low levels of TGEV specific RNAs. Only the smaller subgenomic RNAs 6 and 7 could be detected by hybridization. The numbering of Wesley (288) is used here for TGEV subgenomic RNAs. Cell lines producing at least $10^6$ PFU/ml produced RNAs of the appropriate size for RNA 6 (2.6 kb, M protein) and RNA 7 (1.9 kb, N protein) at detectable levels; but larger, typically less numerous, mRNAs for RNA 2 (8.4 kb, S protein), RNA3 (3.8, NS1 protein), RNA 4 (3.5, SM protein), and RNA 5 (2.8, NS2 protein) were not detected. Acute infections with parental virus typically produced $10^7$ PFU/ml and subgenomic RNAs 2, 3, 4, 5, 6, and 7 were detected by glyoxal agarose gel electrophoresis (Figure 3.3a). Antisense subgenomic messages could be detected only in very small amounts in acutely infected cells (Figure 3.3b).
Figure 3.2. Northern blot analysis of glyoxylated intracellular TGEV-RNAs probed with M13mp19-S2/32P to detect positive-sense RNA. Frame A: Autoradiogram exposed 48 hours; lane*: hybridization and marker control lane pAL 141 plasmid digested with HindIII; lane 1: mock infected ST cells; lane 2: TGEV infected ST cells at 14 hours pi; lanes 3-6: persistently infected cells; lane 3: 2B-S2 cells at 25 passages pi producing less than 10 PFU/ml; lane 4: 2B-C at 18 passages pi producing 10^6 PFU/ml; lane 5: Cl-1S at 77 passages pi producing 2x 10^6 PFU/ml; lane 6: 11N2-6 at 35 passages pi producing 10^5 PFU/ml. Frame B: Autoradiogram in Frame A exposed 2 hours to allow specific subgenomic bands to be visualized. Frame C: The Nytran membrane containing blotted RNA from Frame A was stripped as described in Methods and probed with M13mp18-S28/32P to detect negative-sense RNA. The autoradiogram was exposed 48 hours.
Figure 3.3. Northern blot analysis of glyoxylated intracellular TGEV-RNAs of ST cells acutely infected by TGEV at multiplicity of infection of 20. Intracellular RNA was extracted at 14 hours pi. Lane*: hybridization and marker control lane pAL 141 plasmid digested with HindIII; lane 1: 18 \mu g of RNA; lane 2: 10 \mu g of RNA; and lane 3: 5 \mu g of RNA. Frame A: Probed with M13mp19-S2/\textsuperscript{32}P probe to detect positive-sense RNA. Frame B: The Nytran membrane containing blotted RNA from Frame A was stripped as described in Methods and hybridized to single stranded probe derived from M13mp18-S28/\textsuperscript{32}P to detect negative-sense RNA.
TGEV Specific Nucleic Acid in DNA Form

One hypothesis to account for the persistence of RNA viruses is to sequester the viral information as a DNA provirus. DNA species containing TGEV specific sequences were not detected either in uninfected, persistently infected, or acutely infected cells (Figure 3.4). Southern blot hybridization failed to show the expected band at 1.2-kb following Hpa digestion for any of the cultures. Control experiments using dilutions of pAL141 indicated that a single TGEV specific DNA molecule per cell would be detectable with the DNA slot-blot. No signal was evident.

Discussion

TGEV Viral Specific Proteins

Structural proteins of the size expected for S (280 to 195 kDa), M (33 to 28 kDa) and N (48 kDa) proteins were readily detectible in persistently infected ST cell lysates. All the proteins were precipitated by polyclonal antisera raised against the parental Miller strain of TGEV. Thus, it was likely that the proteins retained their major antigenic determinants.
Figure 3.4. Autoradiogram of Southern blot analysis of intracellular DNA of mock, acutely and persistently infected cells. Frame A: Samples (50 µg) of DNA were treated with DNase-free-RNase, RNase-free-DNase or untreated, and electrophoresed on a 0.8% (wt/vol) agarose gel, transferred to a Nytran membrane and probed with M13mp19-S2/32P to detect positive-sense RNA. Lane 1: pAL 141 digested with Hind III; lane 2: 2B-S2 at 23 passages post infection (pi) untreated; lane 3: 2B-S2, DNase; lane 4: 2B-S2 RNase; lane 5: 2B-C at 29 passages pi untreated; lane 6: 2B-C DNase treated; lane 7: 2B-C RNase treated; lane 8: Cl-1S at 82 passages pi untreated; lane 9: Cl-1S DNase treated; lane 10: Cl-1S RNase treated; lane 11: 11N2-6 at 43 passages pi; lane 12: 11N2-6 DNase treated; lane 13: 11N2-6 RNase treated; lane 14: mock-infected ST cells, untreated; lane 15: mock-infected ST cells, DNase treated; lane 16: mock-infected ST cells, RNase treated; lane 17: TGEV-infected ST cells, 12 hours pi, untreated; lane 18: TGEV-infected ST cells, 12 hours pi, DNase treated; lane 19: TGEV-infected ST cells, 12 hours pi, RNase treated. Frame B: DNAs were applied to the membrane using a slot blot apparatus and probed with M13mp19-S2/32P to detect positive-sense RNA. Row A: 50 µg of DNA were applied per slot to a Nytran nylon membrane. Slot 1: uninfected ST cells; slot 3: TGEV infected ST cells at 18 hours pi, slot 5: 2B-S2, 11N6-2, Cl-1S, and slot 7: 2B-S2. Row B: Dilutions of HindIII-cut pAL141 plasmid DNA containing TGEV specific sequences were applied as a positive control and to determine the sensitivity of the assay. Slot 1: 30ug; slot 2: 3ug; slot 3: 300 ng; slot 4: 30 ng; slot 5: 3 ng; and slot 6: 0.3 ng.
Spike protein heterogeneity or altered antigenicity was not detected in virus from persistently infected cell lines that were not actively selected for neutralization escape mutants. Spike protein derived from \textit{in vivo} persistent coronavirus infections often displays marked variability, characterization has concentrated on predicted heterogeneity of the spike (S) glycoprotein (16,121,227). Coronavirus MHV-JHM specific RNA isolated from the central nervous system (CNS) of persistently infected mice was found to contain deletions in the “S1 hypervariable region” of the spike (S) glycoprotein in 55% of the animals tested (227). Coronavirus MHV-JHM persistent infection of C57BL/6J mice has been found to produce viral RNAs with multiple mutations in the S1 and N regions, the only two segments investigated, although no clustering of mutations was observed (79). In a similar study, MHV-JHM-Pi, generated by \textit{in vitro} persistent infection was used to establish CNS persistent infection in Lewis rats. Low levels of heterogeneity were found in S specific RNA sequences obtained by PCR from several regions in the ORF and adjacent sequences (259). In fact, the nucleotide substitution rate determined was lower than that predicted for Taq polymerase alone (0.5 substitutions per $10^3$). Particularly because the S protein is the receptor binding protein for this and other coronaviruses and the major antigenic target for virus neutralization (152), this low rate of genetic change indicates that \textit{in vivo} persistence requires complex interactions beyond the avoidance of antibody neutralization. Adoptive transfer of MHC class I restricted cytotoxic T-lymphocytes specifically responsive to an epitope on the MHV-JHM nucleocapsid (N) protein prevented the establishment of persistent CNS infection and chronic demyelination in
otherwise susceptible BALB/c mice (253). The resistance or susceptibility to MHV induced disease of inbred strains of mice is determined by genetic variations in MHV receptor expression as well as macrophage and lymphocyte characteristics (28). Transmissible gastroenteritis virus variants derived from acute infections, which have been attenuated or have an altered tissue tropism, have been reported to display changes in size and antigenicity of the S protein (212,284,298). In our study, only active selection of S variants using monoclonal antibodies directed against S produced virus with altered antigenic properties (Chapter 2). In the absence of selection, no S protein changes were seen.

Properly glycosylated M protein is believed to have an important role in coronavirus particle assembly (263) and in triggering interferon (INF) production during TGEV infection (40). Transmissible gastroenteritis virus displayed limited sensitivity to porcine interferon when compared with other porcine viruses (66), but some protection of cells could be obtained in \textit{in vitro} and in explant cultures. Porcine interferon was found to be highly and rapidly (18 hours) cytotoxic to ST cells (288). \textit{In vivo} interferon production may damage enterocytes in very young piglets or hinder the maturation of replacement cells from the crypts (68,141). In addition, lack of glycosylation of M protein near residues 17 to 19 greatly reduced the induction of interferon (153). Nonglycosylated M protein has an estimated size of 27.8 kDa (159). Viral protein in this size range was observed in several of our well established persistently infected cell lines.
Nucleocapsid protein was detected in the acute and persistently infected cell lines in this study. N protein is the predominant viral protein made in acutely infected ST cells. Nucleocapsid (N) protein is not known to have a central role in pathogenicity or regulation of coronavirus gene expression but may protect viral RNA from degradation (258). When La Cross virus, a negative stranded RNA virus of the Bunyaviridae, persistently infects its mosquito vector, N protein encapsidates viral mRNAs and is thought to down regulate viral gene expression (140). La Cross virus N protein has a relatively low affinity for its own mRNA. Coronavirus N protein has a high affinity for RNAs containing coronavirus leader sequence, including viral mRNAs. It is the predominant RNA binding protein detected in MHV infected cells (222,258,289).

Nucleocapsid protein associates with both positive and negative sense MHV RNAs and may play a role in the MHV transcription complex (13). In this study, a host protein near the appropriate size for N protein was detectable in uninfected ST cells complicating interpretation of the weak band observed in the 2B-S2 cell line. This cell line produced no cytopathic virus at the time of analysis but did produce TGEV nucleocapsid protein. Consistent detection of N indicates that viral gene expression occurs at high levels after several years of persistent infection and was demonstrated in a cell line which was not producing infectious virus.

A non-structural protein of 27.7 to 30 kDa has been predicted (26,290) from the unique region of mRNA 4. This protein would migrate near TGEV M protein. In our analysis, the 30 kDa protein may be represented in the second viral band near M rather than an altered glycosylation of the structural protein.
A small protein near the smallest (15 kDa) molecular weight marker was detected in acutely and persistently infected cells and is most likely the TGEV specific SM protein. Small membrane (SM) protein has been found by other investigators in small amount in TGEV virus particles (92). It is encoded by ORF 4, in the unique region of RNA 5 and has an approximate molecular size of ten kDa. With the SDS-PAGE gel system used, this protein would run very near the expected migration front and with the smallest of the molecular weight markers. Other sizes obtained for the product of ORF 4 are 14 kDa (90) and 17 kDa (292). This protein is antigenic for mice (92) and pigs (292). An additional small TGEV protein called the small hydrophobic protein (Hb) has been described for TGEV (139). Hydrophobic protein is encoded by the most 3' predicted ORF 7 (RNA 8), has a predicted size of 9.1 kDa and is not thought to be structural. The small TGEV specific band in our analysis could be either Hb or SM, although it is more likely SM, because of the antigenicity and structural role of this protein.

A dark band (approx mol wt 75-80 kDa) was present in all of the cell lysates from productively infected persistent cell lines and most likely represents a multimeric N or M protein or a breakdown product of S protein since the precipitating antibody recognizes viral structural proteins. The band was not present in uninfected ST cells. Two light bands migrating slightly slower, were observed in acutely infected cells (10 hours pi) and in the persistently infected cell lysates. Multimeric N and M proteins and cleaved S protein have been documented in coronavirus MHV infected cells. Nucleocapsid protein subunits in multimeric forms found in MHV infected cells were
trimers with an apparent molecular weight of 140 kDa and were linked by sulfhydryl bonds (222). Cleaved forms of S protein migrating at 90 kDa are well documented in other coronaviruses (19, 69, 272) and cleavage is thought to play a role in cell fusion mediated by MHV S protein and viral persistence (187). Spike protein cleavage and cell fusion do not ordinarily occur in TGEV in vitro infection (85), however, incomplete RNA 2 (S mRNA) translation products in the appropriate size range have been generated in vitro (127). Transmissible gastroenteritis S protein from different virus strains displays variable resistant to proteolysis (9). The M protein of MHV can form aggregates when boiled with 2-mercaptoethanol (261). Although bands produced by these aggregates were heterogenous, a band at approximately twice the molecular weight of M was present. A band attributed to multimeric M protein was detected in Neuro-2A cells persistently infected with JHM (168). As relatively little M protein of 29 kDa was detected in several of the persistently infected cell lines, multimeric forms may be preferentially formed by lysates of persistently infected cells.

TGEV RNA

Genomic. Intracellular TGEV genomic RNA (RNA 1) was observed only in lysates from acutely infected cells. In the cytoplasm of persistently infected ST cells, genome length RNA was not detectable. Detection of intracellular TGEV genomic RNA was unlikely since persistently infected cultures were not synchronized and full length RNA is known to be packaged and exported by cells during late infection. In
addition, large RNAs do not transfer efficiently to membranes in Northern blot
analysis.

**Subgenomic Positive-Sense RNA.** The most numerous TGEV specific RNA in
persistently infected ST cells was subgenomic RNA 7 (1.7 kb). It was the only
detectable TGEV RNA in most persistent cells assayed. Subgenomic RNA 7 serves as
the mRNA for viral N protein. With abundance three to ten times that of other
messages, it is the most numerous viral RNA in acutely infected cells (101). The
subgenomic RNA 6 (2.5 kb) from which M protein was translated was also detected in
some cell lines after extended passage. Even when the gels were overexposed larger
viral RNAs were not detected.

**Subgenomic Negative-Sense RNA.** Negative sense RNA, corresponding in size to
TGEV subgenomic messages, was present in lysates from acutely infected ST cells.
Negative polarity subgenomic RNAs were not detected in persistently infected cells.
Given the small amounts of viral specific RNA seen, this was not unexpected. It does
make unlikely the possibility that subgenomic negative sense RNAs in larger than
normal amounts caused down regulation of viral gene expression and a reduction in
cytopathic effects induced by virus (100,105).

**Unusual Viral Nucleic Acids.** Unusual forms of viral nucleic acid, such as double
stranded RNA, DNA, and large RNAs in the size range of previously described
coronavirus DIPs, were not detected by Northern blot analysis of TGEV persistently
infected cultures. DIPs characterized in other coronaviruses include large RNAs containing substantial, but discontinuous portions of the genome (52,169,171,172,177,231). Transmissible gastroenteritis DIP RNAs have been found to be 22 kb, 10.6 kb, and 9.7 kb in size. These defective RNAs contain much of the viral polymerase ORFs 1a and 1b and the 3'-untranslated region (UTR), but only small fragments of the viral structural protein genes (181). These DIPs would not be detected by our probe since they are missing the N gene ORF. Other than the expected subgenomic RNAs, the only TGEV specific RNA detected in persistently infected cells was heterogenous low molecular weight RNA, most likely degradation products.

Conclusion

Transmissible gastroenteritis virus specific viral proteins and infectious plaque forming virus were produced efficiently by persistently infected cell lines even after 90 passages (3 years) in continuous culture. In comparison, little intracellular viral RNA was found even in cultures producing significant cytopathic virus (10^6 PFU/ml). In contrast, all viral subgenomic RNAs were detected with BCV persistent infection characterized by Hofmann et al. (105). However, BCV cultures displayed only the smaller subgenomic mRNA and antisense RNA at most of the time points shown in their analysis. Only at the last time point (120 days) were all of the viral RNAs present (105). Time points chosen to evaluate monolayers during BCV persistence analysis were considerably earlier than those used in our study because TGEV persistent infection of ST cells requires 40 to 80 days to regenerate a monolayer. Only one time
point for one TGEV persistently infected cell line (11N6-2) produced detectable RNA2 (8.4 kb). Virus production and viral gene expression was variable over time in a single cell line and highly variable between cell lines. In general, viral RNAs were expressed at low levels although PFU production was often near that of acutely infected cultures ranging between $10^4$ to $10^7$ PFU/ml (Chapter 2).

In conclusion, M protein was the only viral structural protein or viral RNA that showed altered migration. Changes in concentration, glycosylation, or perhaps other structural changes modified the mobility of M protein favoring the formation of multimeric forms. Given the role of M in virion maturation (263) and induction of interferon (40), changes in M protein could have significant bearing on virus persistence.
CHAPTER 4

SUMMARY

Cell Line Characteristics

Porcine transmissible gastroenteritis (TGEV) readily and reproducible established persistent infection in ST cells. Following infection at multiplicities of infection between 0.1 and 1, viral-induced CPE destroyed the monolayer, leaving 1 to 5% of the cells. Surviving cells produced foci that regenerated a complete monolayer one to four months after the initial infection. With one exception, cells produced plaque forming virus 48 hours after the initial infection. The majority of persistently infected ST cells appeared normal, however, monolayers often contained some very large cells with multiple nuclei and vacuoles.

Persistently infected ST monolayers consistently resisted the development of CPE when exposed to the Miller strain of TGEV. Superinfection by TGEV Miller also failed to produce plaques or modify the number of TGEV specific fluorescent cells in persistently infected cell populations. Cells persistently infected by MHV have been described as resisting superinfection by homologous virus (39,117,212). Unfortunately it is unclear what criterion was used to designate the cell line as resistant. A distinction is required between resisting damage and resisting infection. Persistently infected cells that were actively producing cytopathic virus had clearly
developed resistance to the CPE that this virus population was capable of causing in naive or unselected cells. Resistance to homologous virus is not surprising. Resistance to superinfection on the other hand, means that newly introduced virus was unable to establish infection of any type including a noncytopathic interaction with the cells. This is much more difficult to address, given that coronavirus induced cell damage is neither universal nor understood.

Cells able to resist the cytopathic effect of coronaviruses have been reported. Persistent infection as an outcome of coronavirus MHV-JHM infection was reported to be dependent on host cell characteristics (165). Mizzen et al. (182) demonstrated that resistance to virus induced cell fusion by LM-K cells was a crucial parameter in the establishment of successful persistent infection. Later work in the same system found it to be a carrier culture with 1% of the cells infected. Infected cells displayed CPE in the form of altered permeability as determined by sensitivity to hygromycin, a protein synthesis inhibitor, which could be used to eliminate MHV infection from the culture (166). Leibowitz et al. (159) determined that MHV-JHMV persistently infected Neuro-2A cells resisted syncytia formation by MHV-JHM or MHV-A59. In addition, virus released by superinfected cells displayed the same inability to induce syncia as persisting virus populations. Some coronavirus, such as BCV, display such limited CPE under normal conditions that cell resistance receives little discussion (103).

Mechanisms that might allow ST cell to minimize CPE are likely to be distinct from those in MHV infected cultures since TGEV does not normally induce cell fusion (176). Possible changes in the cell population include modification of the APN virus
receptor. Failure to produce the APN receptor would logically enhance resistance to infection. Surprisingly, overproduction of APN in engineered cells also reduces the appearance of CPE in ST cells, particularly the formation of plaques (59). Resistance to the demonstrated cytotoxic effects of porcine interferon β might also allow for enhanced cell survival (138,284).

**Cytopathic Virus**

Cytopathic virus capable of inducing plaques was consistently produced at titers between $10^4$ and $10^8$ PFU/ml throughout the extended period of culture (up to five years). Virus titers increased by about one log after passage 10. We found evidence for cyclical fluctuation in PFU production such as that induced by DIPs (115). Cyclic production of plaque forming virus (2-3 logs) by persistent coronavirus infections has been described by numerous other investigators (117,159,165,212,245). The maintenance of TGEV in persistently infected ST cells was not dependent on extracellular virus, because even 45 days passage under neutralizing levels of polyclonal antisera failed to cure stable, persistently infected cultures. Virus with enhanced cytolytic properties has been produced, or selected, by some coronavirus persistent infections (39,42). In contrast, virus with reduced cytopathic effect or virulence has been reported by other investigators (16,102,117,146,292). No evidence was found in our persistently infected cultures for noncytopathic virus or for virus with reduced cytopathic characteristics.
Interfering Effect

Interference with TGEV plaque formation was demonstrated by mixing TGEV strain Miller with culture fluid from persistently infected ST cells. Undiluted cell culture fluid did not demonstrate any interference with virus production, however it itself contained cytolytic virus. Because there was no obvious phenotypic difference between persistent and parental virus, progeny produced by these mixed infections could have come from either source. TGEV Miller strain and culture fluid from persistent infections were diluted and mixed. Interference was demonstrated because actual plaque counts were consistently lower than would be predicted based on the plaque forming ability of either virus population assayed alone. DIPs would be one possible mechanism for interference, although in the mixed infection either virus stock could be responsible. Aberrant cytoplasmic TGEV RNA species containing N gene sequences have been observed in BCV persistent infection, however, they were not characterized as interfering with standard virus and there was no evidence that they were packaged as particles (103). We examined cytoplasmic RNA in TGEV persistently infected cells for evidence of similar defective TGEV RNA species but found none.

Defective interfering RNAs have been described for TGEV and although ample evidence has been provided of their requirement for helper virus, their ability to interfere with standard virus was not described (179). Like the true DIPs described for MHV, TGEV defective genomes contain complete or partial ORFs from the 5'
sections of the coronavirus genome and the untranslated region at the 3' terminus (52, 167-169, 171, 179, 272). Intervening structural genes including N are often absent. We could not detect these unusual RNAs as our probe contained only N gene coding sequences.

**TGEV Proteins**

Cells persistently infected with TGEV were positive for viral antigens by immunofluorescence throughout the culture period. The percentage of fluorescent cells varied widely as did the intensity of the fluorescence. Based on radioimmunoprecipitaion, TGEV proteins were found in quantities comparable to those seen in acutely infected monolayers. In addition, with the exception of M protein, they migrated normally. The M protein is known to form multimers under sample preparation protocols similar to those used in our study (215) and has been described in MHV persistently infected cells (159). A protein band migrating with a predicted size of 75-80 kDa band was seen in many of our persistently infected lysates and is probably composed of M dimers. A protein with the same migration is described in the original characterization of TGEV specific proteins by Garwes et al. (221). The recently described SM protein (56, 223, 231) appears to be present, although confirming its size was difficult with our gel system.

Attempts to modify the outcome of persistent infection using monoclonal murine antibodies produced no permanent change in the virus population. Antibody directed against the S protein neutralized extracellular virus and extended the initial survival
time of the cell monolayer by two to three days. Virus released by the culture could be neutralized again by the same monoclonal antibody two weeks after the antibody treatment was suspended. Surprisingly, in two separate experiments, a non-neutralizing monoclonal antibody included in the experiment as a control and directed against M protein, enhanced the recovery of the monolayer to a greater extent than did neutralizing monoclonal antibody. The monolayer regenerated four weeks earlier.

**Intracellular RNA**

In contrast with TGEV acute infection, persistently infected ST cells produced little TGEV specific RNA containing N gene sequences. A reduction of viral RNA production was also noted during MHV persistent infection (105). The N gene coding region is present on all of the TGEV messengers RNAs except the smallest and is of course present in the virus genome (130). Anti-sense RNAs containing the same region of the genome were not detected in our persistently infected cells, although they could be detected in acutely infected cells. This would indicate that antisense RNA does not suppress TGEV gene expression during persistent infection or if it does, it is no greater than its regulation of the virus during acute infection. The subgenomic message that codes for N protein (RNA 7) was the predominant TGEV specific RNA in persistently infected cells. During acute infection by TGEV, this RNA is three to 10 times more numerous than other TGEV RNAs, although this estimate may include antisense RNA (101). It has been suggested that this transcript as well as other subgenomic RNAs play a regulatory role during BCV persistence by acting as defective interfering
genomes (30). In addition the N protein may attenuate gene expression by binding messenger and genomic TGEV RNAs.

**Interferon Production**

Cell monolayers persistently infected by TGEV resisted CPE when superinfected with VSV. Cell culture fluid from the persistently infected cultures could protect naive ST and MDBK cells from VSV infection. When this apparent production of interferon was quantified by limiting dilution, it was discovered that ST cells undergoing acute infection produced, on average, eight times more interferon than persistently infected ST cell. Anti-VSV activity was resistant to sedimentation and acid treatment. Although TGEV is relatively insensitive to the antiviral effect of porcine interferon, it is known to induce interferon production both in vivo and in vitro (40,140). Interferon β which can be produced by fibroblasts and other non-leukocyte cell lines (81), is highly cytolytic to ST cells (284). When we pretreated naive ST cells with undiluted interferon-containing cell culture fluid (cleared of TGEV), the monolayer was destroyed within 18 hours. It was not until the fluid was diluted that the anti-viral effects on VSV could be quantified. Most investigators of MHV persistent infection have seen no evidence of interferon induction during persistent infection (16,39,98,117,212). In other cases the persistently infected cells resisted heterologous virus (often VSV), although the transfer of this resistance to naive cells was not described (165,212).

Populations of TGEV with altered M proteins may have a selective advantage
during persistent infection in ST cells by lessening induction of interferon and sparing themselves and the cells from the inhibitory effects of interferon β. Glycosylated M protein is known to play an important role in interferon induction (40,152). In persistently infected cultures changes in the M protein profile were noted. Single amino acid changes in M are sufficient to reduce the induction of interferon 100 to 1000 fold (152). Our persistently infected ST cells were also able to tolerate levels of endogenous interferon that induced damage in naive ST cells. Lower INF production and resistance to cytotoxic effects of interferon, may represent cell phenotypes most able to survive under conditions of persistent infection.
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