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

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.

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Susan Marie Williams

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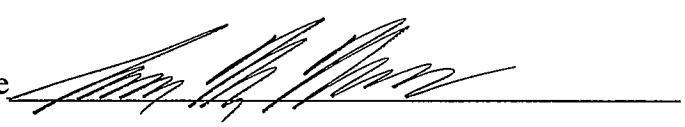
Signature 

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TABLE OF CONTENTS

Page

1. INTRODUCTION 1

The Coronaviruses 2
Classification of Coronaviruses 2
Virions of Coronaviruses 3
Molecular Biology of Coronaviruses 4
Genome of Coronaviruses 4
Subgenomic Viral RNAs 6
Coronavirus Proteins 9
Pathway of Intracellular Replication 13
Porcine Transmissible Gastroenteritis 16
History 16
Course of Disease 18
Epizootiology 21
Viral Persistence 24
Mechanisms of Viral Persistence 27
Noncytopathic Viruses 30
Genetic Restriction 32
Virus Mutants 33
Attenuated Virus Mutants 34
Extracellular Modulators 39
Coronavirus Persistence 41
Outline of Research 44

2. CHARACTERIZATION OF TGEV PERSISTENT CULTURES 46

Introduction 46
Materials and Methods 49
Cell Lines 49
Virus 49
Establishment of cultures 50
Antibodies 52
Viral Antigen Production by Indirect FITC 52

Test for Temperature Sensitive Virus 53
Defective Interference 53
Interferon Production 54
Superinfection 54
Results 55
Appearance and Stability of Cultures 55
TGEV Plaque Forming Units Produced 55
TGEV Specific Fluorescence 61
Test for Temperature Sensitive Conditions 61
Interferon Production 63
Defective Interfering Particles 63
Resistance to Superinfection 64
Discussion 65
Cell variants 66
Attenuated Virus 67
Interferon 67
Defective Interfering Particles 68
Extracellular Virus 70
Co-adaption of Virus and Cells 71

3. VIRUS SPECIFIC MACROMOLECULES SYNTHESIZED IN PERSISTENTLY INFECTED SWINE TESTICLE CELLS 72

Introduction 72
Materials and Methods 76
Immunoprecipitation of Intracellular TGEV Specific Proteins 76
Analysis of TGEV Specific Nucleic Acids 77
Preparation of Strand-Specific Probes 77
Preparation of Intracellular RNA 78
Northern Blot Analysis 78
DNA-Southern Blot 79
Results 80
TGEV Specific Proteins 80
TGEV Specific RNAs 83
TGEV Specific Nucleic Acid in DNA Form 86
Discussion 86
TGEV Viral Specific Proteins 86
TGEV RNA 92
Genomic 92
Subgenomic Positive-Sense RNA 93
Subgenomic Negative-Sense RNA 93
Unusual Viral Nucleic Acids 93
Conclusion 94

4. SUMMARY 96

Cell Line Characteristics 96
Cytopathic Virus 98
Interfering Effect 99
TGEV Proteins 100
Intracellular RNA 101
Interferon Production 102

LITERATURE CITED 104

LIST OF TABLES

Table Page

1.1. Classification of the Coronaviridae. 3

2.1. Conditions of establishment of persistently infected swine testicle cell lines. 51

2.2. Efficiency of plaque formation in mixed infection with TGEV Miller (titers are expressed as plaque forming units/ml). 64

LIST OF FIGURES

Figure Page

1.1. Subgenomic and genomic RNAs of coronaviruses (116). 6

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). 56

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. 57

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 monolclonal 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). 59

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. 60

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. ITC 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. 62

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. 82

3.2. Northern blot analysis of glyoxylated intracellular TGEV-RNAs probed with M13mp19-S2/^{32}P to detect positive-sense RNA. Frame A: Autoradiogram exposed 48 hours; lane*: hybridization and marker control lane pAL 141 plasmid digested with *Hind*III; 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/^{32}P to detect negative-sense RNA. The autoradiogram was exposed 48 hours. 84

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 *Hind*III; lane 1: 18 μg of RNA; lane 2: 10 μg of RNA; and lane 3: 5 μg of RNA. Frame A: Probed with M13mp19-S2/^{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/^{32}P to detect negative-sense RNA. 85

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/^{32}P 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 piI, RNase treated. Frame B: DNAs were applied to the membrane using a slot blot apparatus and probed with M13mp19-S2/^{32}P 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 *Hind*III -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. 87

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.

CHAPTER 1

INTRODUCTION

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.

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

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).

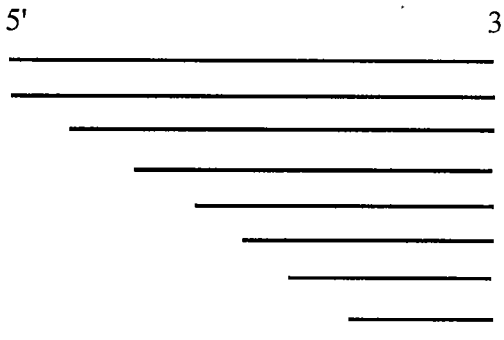
The virions are sensitive to ether and chloroform, and can be inactivated by incubation at 56°C for 30 minutes. Virions inactivate slowly at 25 to 37°C and are relatively stable at 4°C. Coronaviruses have variable sensitivity to acid pH but are unstable under alkaline conditions. Density gradient analysis in sucrose yields a density of approximately 1.18 g/cm^3 (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.



RNAs are not drawn to scale.
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 accepter 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 some times 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 amoung 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 identfied 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 villious 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 consitent 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 diahrea 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 demylinating 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 (C57BLx A/J) mice which could be ameleiorated 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 substitiutions 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 suceptible BALB/c mice (254). The resistence or suceptibility 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 *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 presure on virus population and on the cells themselves (112). Resistence 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 resistent to their anti-proliferative effects (138).

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 *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

pressure of persistent culture conditions formed the basis for early work on RNA virus mutability.

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 *in vivo* and *in vitro*. Poliovirus from the enterovirus group may persist *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 resistent 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 *in vivo* and *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 VP0 (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 resistent 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.