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Bovine Enteropathogenic Coronavirus: The Effect of the Host Cell and Trypsin Modification on the Virus Structure, Cytopathic Expression, and Infectivity.

Karen P. St. cyr-coats
Louisiana State University and Agricultural & Mechanical College

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Bovine enteropathogenic coronavirus: The effect of the host cell and trypsin modification on the virus structure, cytopathic expression, and infectivity

St. Cyr-Coats, Karen P., Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1987
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BOVINE ENTEROPATHOGENIC CORONAVIRUS:  
THE EFFECT OF THE HOST CELL AND 
TRYPsin MODIFICATION ON THE VIRUS 
STRUCTURE, CYTOPATHIC EXPRESSION, 
AND INFECTIVITY

A Dissertation

Submitted to the Graduate Faculty of the 
Louisiana State University and 
Agricultural and Mechanical College 
in partial fulfillment of the 
requirements for the degree of 
Doctor of Philosophy

in 
The Interdepartmental Program 
in 
Veterinary Medical Sciences

by 
Karen St. Cyr-Coats 
B.S., Southeastern Louisiana University 
M.S., Southeastern Louisiana University 
December 1987
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ABSTRACT

BOVINE ENTEROPATHOGENIC CORONAVIRUS: THE EFFECT OF THE HOST CELL AND TRYSIN MODIFICATION ON THE VIRUS STRUCTURE, CYTOPATHIC EXPRESSION, AND INFECTIVITY

by

Karen St. Cyr-Coats

Human rectal tumor-18 (HRT-18) cell clones 3F3, 3E3, D2, and 4B3 exhibited differences in cellular morphology in Giemsa-stained cultures and developing monolayers. Differences were evident in growth kinetics and plating efficiency of each clone. The clones produced colonies in soft agar, demonstrating anchorage independence. Cytopathic expression (CPE) including cytoplasmic vacuolization and cell fusion occurred in BCV-L9-infected clones 3F3, D2, and 3E3. Cell fusion was inapparent in clone 4B3.

Bovine coronavirus strain L9 (BCV-L9) and 5 wild-type isolates replicated in HRT-18 cells, inducing cell fusion. Strain L9, exclusively, replicated in D2BFS cells, requiring trypsin to induce cell fusion. Strain L9 produced plaques in the HRT-18 clones, but the ease of plaque formation and plaque morphology was host cell dependent. Host cell-dependent plaque formation was demonstrated by wild-type BCV strains, and plaque morphology was strain dependent. The intensity of
trypsin enhancement of CPE and plaque development depended on the virus strain and host cell.

Trypsin greatly enhanced the infectivity of BCV-L9 in D2BFS and HRT-18 cells, and to lesser extents in clones 3F3, 3E3, and D2. Trypsin-enhanced infectivity was not detected in clone 4B3. The infectivity of strain LY-138 in HRT-18 cells was slightly enhanced by trypsin.

Eleven structural proteins of BCV-L9 were detected by immunoblotting, including 185, 160, 140, 125, 110, 100, 52, 46, 37, 31-34, and 26-28 Kd species. Under reducing conditions 185, 140, and 100 Kd species migrated as 190, 65, and 95 Kd forms. Silver-stained proteins of 18, 20, and 23 Kd were reduced to a 20-23 Kd cluster.

Host cell-dependent differences in the protein profile of L9 were detected. A 62 Kd protein was specific to L9 (D2BFS with trypsin). L9 (4B3) and L9 (D2) lacked the 46 and 110 Kd bands, respectively.

Cleavage of the 185 Kd protein, an increase in the 100 Kd band, and conversion of the 31-34 Kd cluster to a 34 Kd band occurred when BCV-L9 was propagated in D2BFS cells with trypsin. Trypsin converted the reduced, 95 Kd protein found in L9 (HRT-18) to 90 Kd, and the 20-23 Kd cluster was altered to 19-23 Kd. In vitro trypsin treatment of L9 (3F3) resulted in cleavage of the 46 Kd molecule and the appearance of a 25 Kd species.
CHAPTER I. INTRODUCTION

Bovine enteropathogenic coronavirus (BCV), a member of the family Coronaviridae, is a major cause of gastroenteritis in newborn calves. The virus induces a severe diarrhea with dehydration and malabsorption resulting from destruction of villous enterocytes of the small intestine and colon (3,32,85). The high morbidity and mortality rate associated with this disease makes it of significant economic importance in the cattle industries (7,14).

Factors affecting the virulence of BCV are largely undefined. Study of the virus was hampered by the difficulty of propagating the virus in culture. The inclusion of trypsin in culture medium has aided in the propagation of virus. Trypsin enhances the replication, cell-fusing activity, and plaque development of many BCV strains (97,124,135). Some viral proteins are altered in their electrophoretic mobility following trypsin treatment (133).

The effect of trypsin on the infectivity of the virus has not been clearly defined. Trypsin and other proteases are normally found in the intestine - the site of coronavirus replication in the natural infection. Therefore, this enzyme may influence the virulence of the virus in the natural host.

In 1974 Tompkins and coworkers (122) established the human rectal tumor-18 (HRT-18) cell line from an adenocarcinoma of the lower gastrointestinal tract. These cells are permissive to many BCV strains, greatly improving in vitro culture methods for the virus. Immunofluorescent examination of plaques formed by BCV in HRT-18 monolayers reveals that not all of the cells express viral antigen.
This finding indicates that subpopulations of HRT-18 cells may exist which differ in susceptibility to BCV.

The replication and cytopathic effects of coronaviruses are highly dependent on both the host cell type and the virus strain. Host-dependent differences in proteolytic processing have been reported for mouse hepatitis virus strain A59 (MHV-A59) when the virus was propagated in different murine cell lines (37, 117). Cleavage of the 180 Kd peplomeric protein contributes to activation of cell fusion (37). Proteolytic processing is necessary for activating cell-fusing activity and infectivity of both orthomyxoviruses and paramyxoviruses, as well as a number of other viruses (24, 25, 39, 51-54, 92, 93, 98, 99, 106).

I.A. STATEMENT OF RESEARCH PROBLEM

HRT-18 cells consist of heterogeneous cell types which differ in BCV susceptibility. Four HRT-18 cell clones were established by limiting dilution in hopes of selecting cell types which were refractory or varied in susceptibility to the virus. To assess putative differences between the clones, it was necessary to characterize their susceptibility to BCV infection and the growth properties of each clone. Both host cell-dependent factors and the strain of virus affect coronavirus replication in culture (37, 47). The influence of the host cell, virus strain, and trypsin modification on BCV cytopathic expression was examined. Additionally, the effect of trypsin on BCV infectivity was explored. Activation of cell-fusing activity of MHV is dependent on cleavage of the E2 glycoprotein. The extent of protein processing varies between cell types (37, 117). Host cell-dependent
differences in the processing of structural proteins were suspected for BCV and were examined in this study.

I.B. OBJECTIVES

The following objectives were derived from the research problem.

1 - To characterize the HRT-18 clones
   a - Determine the cytopathogenicity of BCV in each clone.
   b - Characterize the clones based on morphology, growth kinetics, plating efficiency, and growth in soft agar.

2 - To determine the effect of virus strain, host cell, and trypsin on BCV-induced cytopathic expression and plaque formation.

3 - To assess the effect of trypsin on BCV infectivity.

4 - To analyze the polypeptide profiles of BCV propagated in different cell types.
   a - Assess differences in profiles.
   b - Examine the effect of trypsin on profiles.
   c - Correlate virus proteins to cell fusion.

The investigations designed to accomplish each objective were written as individual manuscripts suitable for submission as journal articles. They are entitled:


2. "The Influence of the Host Cell, Virus Strain, and Trypsin Modification on Bovine Coronavirus Cytopathic Expression and Plaque
Formation." Submitted to Journal of Veterinary Medicine.
Co-authored by J. Storz.

3. "The Influence of the Host Cell and Trypsin Treatment on Bovine
Coronavirus Infectivity". Submitted to Journal of Veterinary

4. "The Effect of the Host Cell and Trypsin Treatment on the Structural
Proteins of Bovine Coronavirus Strain L9." Submitted to Archives of
CHAPTER II. REVIEW OF PERTINENT LITERATURE

II.A. COMPARATIVE ASPECTS OF CORONAVIRUS PROPERTIES

II.A.1. Classification and Morphology

Coronaviruses are members of the family Coronaviridae, comprised of the single genus Coronavirus. Originally, this family was recognized based on the unique morphology of the virus—spikes or peplomers formed by surface glycoproteins extend from the virion, giving it the appearance of a "corona" (47,125). The family is now recognized for its distinct physical, chemical, and antigenic properties (83).

Coronaviruses are positive-stranded RNA viruses. The virions are spherical to pleomorphic with a diameter of 80-220 nm. These viruses are enveloped with a lipoprotein membrane which is obtained as the virus buds through intracellular membranes (47).

II.A.2. Structural Proteins

The number of structural proteins comprising the virion of different coronaviruses is shown in Table I.1. The best studied coronavirus is mouse hepatitis virus (MHV) strain A59. For many years this virus was thought to consist of three structural proteins: E1 (23 kilodaltons)—the matrix glycoprotein, E2 (180 Kd)—the peplomeric glycoprotein, and N (50 Kd)—a phosphoprotein forming the nucleocapsid (115). Recently, Sturman and coworkers (119) demonstrated that the 180 Kd protein consists of a dimer of two 90 Kd polypeptides, separable by hydroxyapatite chromatography and consisting of different peptides upon limited protease digestion. Robbins and coworkers (101) identified a 140 Kd protein from MHV virions and infected cells which is an RNA binding protein. This protein is reducible to 50 Kd products, and is
antigenically related to the nucleocapsid. The protein is probably a multimeric form of the nucleocapsid protein.

Six polypeptides comprise the MHV strain JHM virion. A group of three glycoproteins ranging in molecular weight from 97.5 Kd to 170 Kd form the peplomers. A 60.8 Kd protein most likely serves as nucleocapsid, and a 22.7 Kd protein and a 24.8 Kd glycoprotein are located in the lipid bilayer (128).

Diarrhea virus of infant mice (DVIM) is a newly isolated coronavirus. Sugiyama et al (120) have identified four structural proteins associated with this virus consisting of three glycoproteins - gp 180 Kd, gp 69 Kd, gp 25 Kd, and a 58 Kd protein. A 140 Kd glycoprotein formed by two identical molecules of gp 69 is evident under non-reducing conditions.

The polypeptide profiles of coronaviruses associated with respiratory and enteric infections of humans was determined. HCV-229E is a respiratory pathogen comprised of 3 major polypeptides: a 180 Kd gp, a 50 Kd phosphoprotein, and a 21-25 Kd gp family, as well as 3 minor proteins of 107 Kd, 92 Kd, and 39 Kd (62). The respiratory pathogen HCV OC43 is made up of 4 major proteins - gp 190 Kd, gp 130 Kd (a disulfide-linked dimer of 65 Kd), pp 55 Kd, and a glycoprotein cluster at 26 Kd (46). The newly identified human enteric coronaviruses (HECV) 24 and 35 show major protein bands at 62 Kd, 60 Kd, 34 Kd, and 32 Kd and 5 minor bands ranging from 56-110 Kd (5). Human enteric coronavirus 14 consists of 5 major proteins with molecular weights within the range of 190-23 Kd (100).

Of particular interest is the polypeptide composition of bovine coronaviruses. Discrepancies exist about the number of structural
proteins forming this virus, indicating that virus strain, cultural conditions, and/or purification schemes may affect the number of protein bands obtained by polyacrylamide gel electrophoresis. In 1979 Hajer and Storz (42) reported 7-10 structural proteins for BCV strain LY-138, a field isolate purified from diarrhea fluid. These proteins included gp 110, gp 100, p 82, p 70, gp 53, gp 45, and p 36. Williams and Storz (133) identified two forms of BCV strain L9 from isopycnic CsCl gradients. These virions had densities of 1.223 and 1.255 g/cm³ and exhibited different polypeptides profiles. Common to both types were polypeptides of 53 Kd, 47 Kd, 35 Kd, and a 22-26 Kd pentacluster. Light virions had additional bands at 182 Kd, 109 Kd, and 20 Kd, while dense virions had unique proteins of 172 Kd, 95 Kd, 21 Kd, and 15 Kd.

Other investigators reported fewer proteins associated with the virion of BCV-L9. King and Brian (63) described 5 proteins comprising the virus: glycoprotein 140 which is a disulfide-linked dimer of 65 Kd subunits, gps 120, 100, and 26 Kd and a 52 Kd phosphoprotein. Hogue, King and Brian (46) identified a 190 Kd glycoprotein which was normally present as 120 Kd and 100 Kd subunits. Using pronase and bromelain digestion, King and coworkers (64) determined that gp 140, 120, and 100 are surface proteins with gp 140 serving as viral hemagglutinin. Using monoclonal antibody reactivity to BCV G110 surface proteins, Vautherot and other investigators (126) showed that a 105 Kd glycoprotein was important in virus-cell interactions and hemagglutination. The profiles of a wild-type BCV isolate (BECV-WS) and a cell-adapted strain (BECV-F15) were compared by Laporte and Babulesco (71). They found very similar protein compositions for the two viruses with polypeptide bands at 125 Kd, 65 Kd, 50 Kd (major protein), 45 Kd, 36 Kd, and 28 Kd.
The structural proteins associated with other mammalian coronaviruses was evaluated. The composition of a rabbit enteric coronavirus was examined by Descoteaux and coworkers (31). They found 8 structural proteins - 7 associated with the envelope with molecular weights ranging from 100 Kd-34 Kd and a single nucleocapsid protein of 54 Kd.

The porcine coronavirus of transmissible gastroenteritis (TGEV) reportedly consists of 4 major proteins - gp 200 (surface protein), gp 30, gp 28.5, and vp 50. Two minor proteins of 105 and 80.5 Kd comprise the 200 Kd protein (41). Hemagglutinating encephalomyelitis virus (HEV), a cause of porcine vomiting and wasting disease, is composed of 8 structural proteins with molecular weights ranging from 180-31 Kd. The surface projections are formed by gps 180, 130, and 75, with gp 76 serving as viral hemagglutinin. Glycoproteins 31 Kd and 32 Kd are membrane associated. A 64 Kd protein is the only nonglycosylated protein (17).

Garwes (40) determined the polypeptide structure of canine coronavirus, an enteric pathogen antigenically and structurally related to TGEV. The virus has 4 structural proteins - gp 204, p 50, gp 32, and gp 22.

Coronaviruses causing feline peritonitis and gastroenteritis have been identified. Boyle and investigators (9) demonstrated three major proteins comprising these viruses. A 45 Kd protein forms the nucleocapsid; a 210 Kd protein is the peplomeric protein; and a 27-33 Kd cluster is the matrix protein. A minor protein of 42 Kd is also associated with feline coronavirus.
Infectious bronchitis virus (IBV), an avian coronavirus, was once considered to be more complex than mammalian coronaviruses with reports of 7-16 structural proteins (73,111). However, MacNaughton and Madge (78) discovered that additional protein bands on SDS-PAGE gels were a result of harsh treatment during virus preparation. Cavanagh (18,19,20) reported 3 major proteins for IBV. $S_0$ is a surface protein precursor which is cleaved to $S_1$ (gp 90 Kd) and $S_2$ (gp 84 Kd). The matrix protein is composed of glycosylation isomers of 23-34 Kd, and N (nucleocapsid) has a molecular weight of 50-54 Kd. Urea treatment of IBV virions revealed that $S_1$ is responsible for viral infectivity and hemagglutinating activity, but not attachment to susceptible cells. This molecule also elicits antibodies which cause virus neutralization and hemagglutination inhibition (21,22).

II.A.3. Non-structural Proteins

Very little is known about the structure and function of the coronavirus non-structural proteins. Most likely they function in regulation of replication and transcription of the virus. Messenger RNA 1, a genome length transcript, codes for the 200 Kd viral RNA-dependent RNA polymerase. Two other non-structural proteins of 30 Kd and 14-17 Kd, coded for by mRNAs 2 and 5, respectively, have been identified, but the function of these is unknown (66,80). Budzilowicz and Weiss (12) sequenced the nucleotides of MHV A59 mRNA 5 - a nonstructural gene. Two open reading frames (ORF) coding for two non-structural proteins were identified. One ORF codes for a 13 Kd protein which is rich in basic amino acids and possibly serves as an RNA binding protein. The other ORF codes for a 9.6 Kd protein containing a long stretch of hydrophobic
amino acids. This protein may function in the association of viral polymerase complexes with membranes.

II.A.4. The Glycosylation Process

The number and sizes of the glycoproteins associated with coronaviruses is different, but those associated with MHV are well characterized according to structure and function. A 20-30 Kd transmembrane glycoprotein (E1) forms the matrix of the MHV virion. Using dog pancreatic microsomes, Rottier and coworkers (103) determined that the protein is primarily located within the membrane with only small portions of the amine and carboxy termini protruding from the membrane. The protein has no cleavable membrane insertion sequence. Instead, three hydrophobic regions of approximately 20 amino acids each are located within the protein at positions 27-46, 57-77, and 82-102 (56). The hydrophobic sequence located closest to the carboxy terminus serves as the signal sequence. This internal signal sequence requires signal recognition particle for proper membrane insertion (102). Glycosylation of E1 of murine and bovine coronaviruses is resistant to tunicamycin, indicating that sugar residues are linked via O-glycosidic bonds to serine or threonine residues with no involvement of dolichol-linked N-acetylglucosamine intermediates found in N-linked glycosylation (50,95). However, N-linked oligosaccharides are found on the E1 glycoprotein of IBV (112). Glycosylation of E1 does not occur in the rough endoplasmic reticulum, rather, the protein is most likely glycosylated as completed virions pass through the Golgi (103). Studies indicate that E1 probably directs budding of the virion through intracellular membranes. For example, Tooze et al (123) demonstrated
the localization of E1 on membranes of the endoplasmic reticulum and Golgi, where budding occurs. Additionally, tunicamycin-treated cells which lacked E2 showed normal budding (49). E1 also interacts with the nucleocapsid, probably binding N to the viral envelope as it buds (118).

The number of glycoproteins comprising the coronavirus peplomer depends on the strain of virus. The peplomeric glycoprotein of MHV-A59 (E2) has a molecular weight of 180 Kd and is comprised of two 90 Kd species, 90A and 90B, which are noncovalently associated and are separable by host protease or by trypsin cleavage. 90A is acylated with palmitic acid and is probably associated with the lipid bilayer. 90B is not acylated (119). The native 180 Kd molecule has one trypsin cleavage site, and intrachain disulfide bonding contributes to its conformation (115,116). Unlike the matrix glycoprotein, glycosylation of E2 is tunicamycin sensitive, involving en bloc transfer of oligosaccharides from a dolichol-linked N-acetylglucosamine intermediate. The resulting linkage is N-glycosidic and occurs between N-acetylglucosamine and asparagine (95). This molecule can migrate to the plasma membrane rather than remaining localized at intracellular membranes (117). Several biological activities are directly attributed to the peplomer. This molecule is responsible for binding the virus to cell receptors. For example, decreased binding of virus to the cell occurred with virus which lacked peplomers (50). Monoclonal antibody directed against gp 105 of BCV neutralized the virus and inhibited hemagglutination (126). Limited enzymatic digestion with pronase or urea demonstrated hemagglutinating activity of surface proteins of bovine and avian coronaviruses (21,64). Immunologically, E2 is responsible for induction of neutralizing antibody to the virus, and its presence on the cell
surface elicits a cell-mediated response (26,43). Cell-fusing activity of MHV is attributed to E2. Using monoclonal antibody against E2 of MHV-JHM, Collins and others (26) inhibited cell fusion of L241 cells. Tunicamycin treatment of MHV-A59-infected 17C11 cells blocked glycosylation of E2 and prevented cell fusion (50). With MHV-A59, cell fusion is a result of cleavage of inactive 180 Kd protein into its 90 Kd subunits (37,119).

II.A.5. Antigenic Properties

Antigenically, coronaviruses can be divided into two main groups: 1 - avian strains and 2 - mammalian strains. The mammalian strains can be further subdivided (Table II.2) (77). Antigenic relatedness between strains was for the most part determined by immunofluorescence studies. Recent studies have focused on the cross reactivity of individual proteins. For example, Hogue et al (46) used antiserum against BCV structural proteins to assess cross reactivity of BCV-L9, MHV-A59, and HCV-OC43. HCV-OC43 was found to have four proteins which are antigenically homologous to BCV, while MHV-A59 had three antigenically-related proteins. Monoclonal antibodies were used by Snyder and Marquardt (108) to assess the antigenic relationships of IBV serotypes and Fleming et al (36) to assess antigenic relationships of murine coronaviruses.

II.B. UNIQUE FEATURES OF CORONAVIRUS REPLICATION

II.B.1. Attachment and Penetration

Attachment of coronaviruses to the receptors of susceptible cells occurs via the peplomers (21,50,64,126). The nature of the coronavirus receptor is unknown. Boyle, Weismiller, and Holmes are attempting to
clone the MHV receptor gene (Mhv-1) from susceptible mice (8). The internalization process used by this virus is undetermined, but must occur either by fusion of the viral envelope to the plasma membrane or by endocytosis followed by fusion with an endocytic vesicle (47).

II.B.2. Coronavirus Genetic System and Its Replication

The coronavirus genome consists of positive-stranded RNA which is single-stranded, linear, and unsegmented. The genome is capped, polyadenylated, and infectious, making it capable of serving as messenger. The RNA has a molecular weight of $5.5 \times 10^6$ daltons, consisting of 15,000-20,000 bases (47,110).

Following internalization and uncoating, genomic RNA serves as messenger for transcription of RNA-dependent RNA polymerase. This polymerase transcribes the genome into a full-length, negative-stranded replicative form, which serves as a template for mRNA transcription and synthesis of genomic RNA (83). Different polymerases are used for synthesis of mRNA and genome RNA. Replication of genomic RNA from the replicative form proceeds by a discontinuous or "stop and go" mechanism. This mechanism was suggested by the discovery of many small leader-containing RNA species whose size corresponded to lengths between the 5' end and probable hairpin loops of genome RNA. These small RNA species were thought to be pausing intermediates (66). The first demonstration of recombination between the genomes of murine coronaviruses A59 and JHM was reported in 1985 (68). Makino and coworkers (81) have since demonstrated high frequency recombination between different strains of murine viruses. Since the genome of coronaviruses is non-segmented, this finding suggests that segmented RNA intermediates might be
generated and supports the discontinuous model. The discontinuous mode of replication has also been shown to occur in QB, MS2, and T2 phages (61,87).

II.B.3. Transcription and mRNA Properties

Coronavirus mRNA consists of a "nested set" of six subgenomic and 1 genome-sized mRNA molecules. The mRNAs share common 3' ends, but differ in length toward the 5' end. The mRNAs are functional - each is capped, polyadenylated, and associated with polysomes. One protein is coded for by each mRNA - the translated sequence is found in the 5' region not contained in the next smaller mRNA species. The protein product translated from each mRNA has been defined as follows for MHV and TGEV:

mRNA 1 - genome length - polymerase
mRNA 2 - non-structural protein
mRNA 3 - E2
mRNA 4 - non-structural protein
mRNA 5 - non-structural protein
mRNA 6 - E1
mRNA 7 - N

These mRNAs are produced in non-equimolar amounts, but in constant proportions. The 5' ends of the genome and all mRNAs are identical, consisting of a cap structure and 50-70 identical nucleotides (58,64,69,110).

The fact that the leader and translated sequences of mRNAs are discontinuous on the genome has led to speculation about the mechanism of transcription. UV transcription mapping showed that target sizes for UV inactivation were identical to the physical sizes of mRNAs.
Therefore, transcription does not involve processing of larger RNAs (59). In 1983 Spaan and investigators (110) proposed a polymerase-jump mechanism involving synthesis of short RNA transcripts from the 3' end of the negative-stranded RNA and translocation of the polymerase-leader complex to internal positions on the template. More recently this mechanism has been termed "leader-primed transcription." Specifically, this model involves the transcription of leader RNA from the 3' end of negative-stranded template which dissociates from the template. This free leader RNA binds to intragenic regions with homologous sequences on the RNA template. Leader RNA is cleaved at mismatched points generating a primer for initiation of mRNA transcription. Therefore, the leader-body fusion site varies for each mRNA species, according to the location of mismatched nucleotides. A regulatory mechanism for controlling the synthesis of RNA in infected cells may be the number of homologous nucleotides found at the intragenic sites preceding each mRNA (66).

"Leader-primed transcription" is supported by several studies. a) Leader RNAs of 50-90 nucleotides have been found in the cytoplasm of MHV cells (4) b) Budzilowicz et al (13) found three intragenic regions of MHV genome RNA with sequences common to the 3' end of mRNA leader sequences. These sequences may contain a binding site for hybridization of leader RNA to negative-stranded viral RNA at the beginning of each gene to prime synthesis. c) Leader RNAs separate from the RNA template, and ts mutants which produce leader RNA but not mRNA indicate that transcription of leader and mRNA are separate events (67). d) A high frequency of reassortment of leader sequences between coinfecting murine coronaviruses indicates a potential trans-acting property of leader RNA.
(82). Additionally, evidence for leader-primed transcription was shown with avian coronaviruses (11).

II.B.4. Role of Cellular Organelles in Assembly

Assembly of mammalian coronaviruses occurs in the cytoplasm of infected cells. There is no involvement of the cell nucleus. Tooze and coworkers (123) examined the replication and assembly of MHV in Sac-cells using immunofluorescent analyses and electron microscopy. The uptake of $[^3]$H uridine in actinomycin D-treated cells revealed the synthesis of viral RNA within 4-5 hours post infection. Synthesis of the viral RNA occurs in the cytoplasm of infected cells (131). Immunofluorescent analyses using polyclonal antibodies against disrupted whole virions and viral nucleocapsid (N) protein detected the presence of N within 4 hours post infection. The N protein is translated from the most abundant viral mRNA on free polysomes (104,123). N protein and newly synthesized genomic RNA associate in the cytoplasm to form a helical ribonucleoprotein. Translation of E2 occurred on the rough endoplasmic reticulum within 6 hours post infection. E2 is cotranslationally glycosylated via N-glycosidic linkages at asparagine residues, and core oligosaccharides are trimmed as the molecule passes through the Golgi. This protein is capable of migration to the plasma membrane (117). Translation of E1 was detected by immunofluorescence within 6 hours post infection in the rough endoplasmic reticulum. Using its internal insertion signal, the molecule is inserted into the membrane of the rough endoplasmic reticulum and transported to a smooth membrane perinuclear compartment where it is incorporated into virions. Intact virions are transported to the Golgi, where O-glycosylation of E1
occurs (56,103,123). Glycosylation of this protein does not involve the dolichol-linked N-acetylglucosamine intermediate. Rather, sugar moieties are linked at serine or threonine residues (94). El is not transported to the plasma membrane. The protein is confined to the intracellular membranes of the rough endoplasmic reticulum and Golgi, and it only reaches the cell surface as a part of the mature virion. Budding occurs at sites where El accumulates, and is initiated when ribonucleoprotein recognizes the C-terminus of El projecting from intracytoplasmic membranes. Namely, budding in the smooth perinuclear vesicles and tubules located between the rough endoplasmic reticulum and Golgi was detected by EM within 6-7 hours post infection. At late stages of infection, El accumulated in the membranes of the rough endoplasmic reticulum, and budding occurred there (123). Doughri and coworkers (33) demonstrated that release of mature virions from the cell is a result of fusion of post-Golgi vesicles with the plasma membrane. This mechanism of release probably utilizes the host secretory apparatus.

Unlike mammalian coronaviruses, the replication of IBV is impaired in enucleated, UV irradiated, or α-amanitin treated cells, indicating the involvement of the cell nucleus and host transcriptional factors in virus replication (34). In addition, the El glycoprotein of IBV is glycosylated through N-glycosidic linkages (112). These differences between mammalian and avian coronaviruses suggest that the replication strategy of the two virus groups are different.
II.C. EVIDENCE FOR PROCESSING OF CORONAVIRUS PROTEINS AND ACTIVATION OF CELL FUSION

In some cell culture systems coronaviruses are able to induce cell fusion. The relationship between induction of cell fusion and cleavage of viral proteins is of particular interest. The prototype virus, MHV, has provided much of the current knowledge in this area of study.

Using four different murine cell lines, Frana and coworkers (37), demonstrated that processing of the 180 Kd E2 glycoprotein of MHV-A59 to its 90 Kd subunits was host dependent. Different cells cleaved the precursor molecule to different extents and the cell-fusing activity of the virus correlated in part to the degree of proteolytic cleavage. For example, virions obtained from Sac- cells contained E2 which was completely cleaved to the 90 Kd form. These virions induced rapid cell fusion in L2 cells without requiring virus replication. In contrast, virions obtained from 17C11 cells had partially cleaved E2 and required trypsin for activation of rapid cell fusion.

The role of protease in MHV-induced cell fusion was also studied by Yoshikura and Tejima (135). Using a cold-sensitive plaque mutant, they showed that treatment of infected cells with trypsin enabled the mutant virus to form plaques consisting of fused cells on otherwise resistant cells, and allowed a virus which usually produced plaques consisting of dead cells to form fusion plaques.

Storz and coworkers (114) found that cell fusion of BCV strain L9 was enhanced in the presence of trypsin when the virus was grown in bovine fetal brain or bovine fetal thyroid cells. Cell fusion occurred only minimally in thyroid cells and not at all in brain cells in the
absence of trypsin, but fusion was apparent within 12 to 18 h post infection when trypsin was present.

The protein(s) responsible for cell fusion are under investigation. In 1982, the 170 Kd glycoprotein of MHV-JHM was identified as the viral glycoprotein responsible for attachment and cell fusion. Sturman and investigators (119) demonstrated that the 180 Kd E2 glycoprotein of MHV-A59 could be cleaved by trypsin or cellular proteases to two different 90 Kd products, 90A and B, and consequently activate cell-fusing activity of the virions. It is unknown which of the two species mediates cell fusion, but the amino acid sequence at the new terminal domains of the proteins is currently under investigation.

Electron microscopy of negatively-stained particles revealed that BCV virions with shortened surface projections were produced in trypsin-treated cultures (114). Williams and Storz (133) resolved two BCV-L9 phenotypes in linear CsCl gradients. These had densities of 1.223 and 1.255 g/cm$^3$, but the relative proportion of dense virions increased in the presence of trypsin. The two virus phenotypes contained common polypeptides of 53, 47, 35, and 22-26 Kd pentamer. Light virions contained additional proteins of 182, 109, and 20 Kd. Trypsin-dependent bands of 172, 95, 21, and 15 Kd were present in dense virions. The protein(s) responsible for BCV-induced cell fusion are undetermined.

The effect of trypsin on plaque development of BCV-L9 in bovine fetal brain and bovine fetal thyroid cells was examined (114). Plaque diameter was increased more than 5-fold when the enzyme was present in the overlay, and plaques were less turbid and more distinct. Plaque
formation by many IBV strains in chicken embryo cell cultures required
the presence of trypsin (97).

Toth (124) treated BCV-infected bovine embryonic lung cells with
increasing concentrations of trypsin. He found that the viral
infectivity, as well as the rate of replication was enhanced by trypsin
in direct correlation with increases in trypsin concentration.

Indirectly, protease was shown to play a role in the production of
human coronavirus. Appleyard and Tisdale (2) treated HCV strain
229E-infected MRC-C cells with the protease inhibitor leupeptin. They
found that the drug prevented multiplication of the virus if added
within 2 h of infection, suggesting action at an early stage of
replication. Frana and coworkers (37) also found that leupeptin delayed
the onset of fusion in MHV-infected L2 cells by 4-6 hours and reduced
the size of giant cells.

II.D. CYTOCIDAL ACTION OF CORONAVIRUSES IN BOVINE GASTROENTERITIS

Gastroenteritis is a major cause of morbidity and mortality in
newborn animals. Numerous bacterial and viral agents cause this
syndrome, but coronaviruses are a major cause of enteric infections in
neonatal bovines. The high morbidity and mortality rate associated with
the disease makes it one of economic significance in the cattle
industries (7). Chronic shedding of coronavirus antigen-antibody
complexes was demonstrated by ELISA in clinically normal cows, with 85
of 121 cows (70%) responding positively. Shedding of the virus was
found to be seasonal, with detectable virus occurring during the winter
months (November to March), while no virus was detectable during summer
months (July to September). Subclinical infections of adult cattle may serve as a source of infection for the neonate (27,29,30).

The high stability of the virus contributes to spread of infection, which occurs by ingestion of the agent. The proximal part of the small intestine is the first site of infection, followed by progression to the jejunum, ileum, and colon. The virus primarily attacks the differentiated absorptive epithelial cells and goblet cells of the villi, but there is some involvement of undifferentiated cells of the Crypts of Lieberkuhn and fibroblasts and endothelial cells of small blood capillaries of the lamina propria mucosae. Histologically, the most severe cytopathic changes occur in the proximal jejunum of the small intestine. Villi become broadened and shortened, fusion between adjacent villi occurs, with some denuding of the villi. Absorptive epithelial cells may become vacuolated and necrotic; goblet cells are reduced in number; and crypt epithelium becomes hyperplastic. The lamina propria mucosae may be edematous and infiltrated with mononuclear and polymorphonuclear cells. Distention of the smooth and rough endoplasmic reticulum and vesiculation of the Golgi occurs, and mitochondria swell, dilate, and decrease in number. Severe degeneration and necrosis of cellular organelles occurs, including ribosome depletion, distortion of mitochondrial cristae, and vacuolation of the cytoplasm. Fragmentation of the plasmalemma is apparent, and nuclei become pyknotic and lyse. Viral inclusions are evident in the cytoplasm.

As a result of the cytocidal action of bovine coronavirus in the intestine, a profuse, watery diarrhea develops. Fluid loss is due to an osmotic imbalance caused by undigested lactose in the lumen - a consequence of the loss of cells possessing disaccharidases and
replacement of these mature cells by undifferentiated cells retaining secretory function. With the loss of mature enterocytes there is a decrease in the glucose, sodium carrier and (Na\(^+\)-K\(^+\)) ATPase causing the loss of sodium, potassium, chloride, bicarbonate, and water. Depletion of bicarbonate causes acidosis which in turn contributes to K\(^+\)-H\(^+\) ion exchange across cell membranes with a net loss of potassium from cells. Calves become hypoglycemic with inhibited gluconeogenesis and increased glycolysis. Death is often the result of severe diarrhea (3,32).

II.E. HOST CELL RANGE OF CORONAVIRUSES.

Most coronaviruses are highly species specific, causing disease only in the species from which the virus was isolated, and demonstrate a marked degree of tissue tropism. For example, IBV is restricted to diseases of the respiratory tract, the site of virus replication. TGEV and bovine, canine, and turkey coronavirus replicate in the intestinal epithelium, causing enteric infections. The human coronaviruses HCV-229E and HCV-OC43 cause respiratory infections, and the human enteric coronavirus (HECV) is a cause of infantile enteritis (5,76,100). Coronaviruses were isolated from the brains of patients with multiple sclerosis (15,109). Murine coronaviruses cause respiratory, enteric, and neurological diseases in mice (47). Two cases of cross species infection have been reported - a natural infection of dogs by TGEV, and an accidental transmission of BCV to man (72,113,127).

Typically, coronaviruses do not replicate well in cell culture, usually requiring organ cultures or differentiated cells from the host species (47). Some strains of BCV and HECV do not replicate in cultured cells and have been identified by electron microscopy of diarrhea fluids
adapted BCV-L9 to culture using primary cultures of bovine fetal kidney cells. Successful culture systems using established cell lines have been developed for several coronaviruses. Avian and bovine coronaviruses replicate well in Vero cell lines (1,121). The adenocarcinoma cell line, HRT-18, established by Tompkins et al (122), is used for propagation of BCV (63,133). Enhancement of the replication of coronavirus in culture was accomplished by including trypsin in the culture medium (37,97,114,124,133,135).

Depending on the host cell, coronavirus infections may range from highly cytocidal to inapparent (47). In cytocidal infections cells may fuse into syncytia or lyse. In contrast, feline, murine, human, and avian coronaviruses are able to establish persistent infections leading to carrier cultures in some cell culture systems (47,48,23,75). The mechanism of resistance of cells to coronavirus infection is mostly unknown. Boyle et al (8) demonstrated that genetic resistance of the inbred mouse strain SJL/J to MHV infection is due to the lack of a receptor which is present on susceptible mice.

II.F. PROTEOLYTIC ACTIVATION OF THE ENVELOPE PROTEINS OF PARAMYXOVIRUSES AND ORTHOMYXOVIRUSES

Proteolytic activation of infectivity and cell fusion was first observed in paramyxoviruses. Hosaka in 1967 (134) and Young and Ash in 1970 (136) reported a decrease in the cell-fusing activity of Sendai virus and Newcastle disease virus following a single passage in tissue culture. Classically, Homma (51,52) and Homma and Tamagawa (54) noted that Sendai virus propagated in chicken embryos was infectious and
induced cell fusion in L cells, and was hemolytic to guinea pig erythrocytes. After a single round of replication in L cells, Sendai virus was noninfectious, did not induce fusion, and was nonhemolytic. Trypsin treatment of purified Sendai virus restored all three biological activities. Cell-fusing activity, hemolysis, and infectivity are a result of cleavage of an inactive precursor molecule, Fo, to its active subunits, F1 and F2, by host cell proteases or by trypsin (51-54,93,99,106). Since its identification in paramyxoviruses, proteolytic activation of infectivity was identified in other viruses. Orthomyxoviruses, rotaviruses, and coronaviruses require cleavage of precursor proteins for activation of viral infectivity and/or cell fusing activity (35,37,39,97,114,124,133,135).

II.F.1. Paramyxoviruses.

Two surface glycoproteins are found on paramyxoviruses - hemagglutinin - neuraminidase (HN) and fusion (F). HN is a 72 Kd protein which is responsible for adsorption of the virus to host cell neuraminic acid receptors and cleavage of viral neuraminic acid residues to prevent self aggregation during release from the cell. The protein is a dimer of identical disulfide-linked molecules. Studies with Newcastle disease virus show that inactive HN0 is cleaved to yield the active HN molecule. Failure to cleave the precursor molecule results in noninfectious virus (93).

The 65 Kd F protein allows penetration into the host cell by lysing the host cell membrane and causing fusion of the viral envelope and cell membrane. The nucleocapsid can then be released into the cell
cytoplasm. The presence of F protein on the surface of infected cells also induces lysis of membranes and cell fusion (24).

An inactive precursor polypeptide, Fo, is initially produced in infected cells. Fo is cleaved by host cell protease to F1 and F2 subunits (52 Kd and 13 Kd, respectively). These subunits are disulfide linked to form the F protein. F1 contains the carboxy terminus of the precursor molecule and is anchored in the viral envelope, and F2 contains the original N terminus. Cleavage of Fo reveals a new N terminus on F1 that is very hydrophobic and contains 18 highly conserved amino acids (25). The hydrophobic sequence is important in fusion of virus-cell membranes and infected cell-uninfected cell membranes.

II.F.1. Orthomyxoviruses

Orthomyxoviruses have two surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA). HA functions in attachment of the virus to sialic acid-containing glycoproteins on the cell surface and in penetration into the cell during initial stages of infection. Outbreaks of influenza are attributed mainly to variation in HA protein. The function of NA is unclear, but its enzymatic activity may prevent aggregation of virus particles and promote release from infected cells by removing receptors from HA, itself, and the infected cell surface (92,98). The NA protein consists of a single polypeptide which undergoes no post-translational processing.

The HA protein is synthesized as a single polypeptide of 75 to 80 Kd (28). Following post-translational cleavage of the N terminal signal sequence and removal of one or more intervening amino acid residues, two polypeptides result. HA1 and HA2 (36 and 27 Kd, respectively) are
linked by a single disulfide bond, and a trimer of these molecules comprises the HA spike (92).

Garten and coworkers (39) identified the amino acid sequence at the cleavage site of the HA protein. In the active form, the C terminus of HA1 has the sequence gln-ser, and the N terminus of HA2 has the sequence gly-leu-phe-gly-ala-ile. Activation of the protein is accompanied by an acidic shift in the isoelectric point of HA, indicating that an intervening arginine residue is removed from the cleavage site. Two proteases are involved in the activation of HA: trypsin or a trypsin-like endoprotease of cellular origin to cleave the arg-gly bond; and an exopeptidase of the carboxypeptidase B type to remove the arginine. In vitro cleavage of HA in the presence of purified virus and trypsin resulted in removal of the arginine residue. Therefore, this enzyme is either a host component tightly associated with the virus particle or a virus-encoded protein. Interestingly, cleavage by chymotrypsin or thermolysin only slightly shifts the cleavage site, but these do not activate HA. Consequently, activation of HA involves a highly specific cleavage site. Cleavage of the precursor molecule into HA1 and HA2 is required for viral infectivity. Orthomyxoviruses exhibit low infectivity if grown in cells lacking the proper proteases, but the virus can be activated by treatment with trypsin (92).

II.G. PROPERTIES OF NEWLY RECOGNIZED GASTROENTERITIS AGENTS: THE TOROVIRUSES

The proposed family Toroviridae is composed of two recognized gastroenteritis agents – Berne virus (BEV) and Breda virus (BRV). Berne virus was isolated in 1972 from a rectal swab of a diarrheic horse in
Berne, Switzerland (130). Breda virus was obtained during an acute epizootic of neonatal calf diarrhea in Breda, Iowa in 1982 (134). A morphologically similar and antigenically related virus was detected in cattle from Lyon, France and named Lyon 4 virus (89,90). Virus particles resembling BEV and BRV were isolated from humans with gastroenteritis (6,55).

Morphologically, toroviruses are pleomorphic particles measuring 120-140 nm, and were described as spherical, oval, elongated, or kidney-shaped particles. These viruses are enveloped and surrounded by drumstick-shaped peplomers of 20 nm in length. The nucleocapsid is elongated, tubular, and probably helical. The name "Toroviridae" was derived from the Latin torus = a doughnut shaped ring. The RNA genome of these viruses is single-stranded with a molecular weight of $5.7 \times 10^6$ d. The genome is polyadenylated and infectious and probably of positive polarity (130,134).

BEV and BRV are structurally similar, consisting of proteins in the molecular weight range 75-100, 37, 22, and 20 Kd. The 20 and 37 Kd proteins of BEV are phosphorylated, and the 20 Kd species is the most prevalent protein. This protein has RNA binding properties and represents the major capsid protein. The 22 Kd protein probably serves as a constituent of the envelope, while the phosphorylated 37 Kd species may be a matrix protein. The 75-100 Kd glycoproteins are tunicamycin sensitive, probably containing N-linked oligosaccharides (129).

Toroviruses are extremely UV sensitive, but withstand pH extremes of 2.5 to 10.3. Infection of susceptible hosts is probably by ingestion (134). BRV was shown by immunofluorescence and electron microscopy to replicate in vivo in epithelial cells of the colon, the caudal part of
the jejunum, and the ileum. Crypt cells and villous cells of the ileum and jejunum are infected, as are most cells of the large intestine (129,134). BEV replicates in cell culture in equine cells, but attempts to propagate the virus in other mammalian cells were unsuccessful (130). BRV and human toroviruses do not replicate in any culture system attempted so far (6).

The replication of BEV was inhibited by treatment of infected cells with actinomycin D, α-amanitin, and UV irradiation, indicating some nuclear involvement in the replication of BEV. Within 6 hours post infection virus-specific proteins were evident in BEV infected equine dermal cells. A glycosylated, tunicamycin-sensitive species of 200 Kd was detected which was shown to be a precursor of the 75-100 Kd protein. Six or seven subgenomic and 1 genome-sized RNA species were extracted from BEV-infected equine dermal cells. These RNAs were polyadenylated and served as messenger. Tubular structures representing nucleocapsid were detected in the cytoplasm and nucleus of infected cells.

Assembly of the virions involves budding of a preformed rigid nucleocapsid through intracytoplasmic membranes. Budding occurs predominantly through the Golgi and to a lesser extent through the rough endoplasmic reticulum. Nucleocapsid attaches to the membrane and buds through the membrane, apparently acquiring its diameter and electron density. Enveloped, rod-shaped virus is found in the lumen of the cytoplasmic cisternae. Virus is released after virus-containing vesicles merge with the plasma membrane. The morphology of the virus changes from rod-like to the torus form during transmission from the intravesicular to the extracellular state (129).
The morphology, genetics, and replication of toroviruses closely resemble coronaviruses. Both viruses replicate in the intestinal epithelium, causing gastroenteritis. The identification of these disease agents has obvious implications from the standpoint of prevention or diagnosis of gastroenteritis infections. Further study of toroviruses on the molecular level is necessary to fully understand the replication of the viruses and their major differences from coronaviruses.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Source</th>
<th>No. Proteins</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Hepatitis virus</td>
<td>17Cl1, Sac-, L2 cells</td>
<td>5-6</td>
<td>(101,119,128)</td>
</tr>
<tr>
<td>Diarrhea virus of infant mice</td>
<td>DBT cells</td>
<td>4</td>
<td>(120)</td>
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<tr>
<td>Human coronavirus-229E</td>
<td>MRC5</td>
<td>6</td>
<td>(62)</td>
</tr>
<tr>
<td>Human coronavirus-OC43</td>
<td>HRT-18 cells</td>
<td>4</td>
<td>(46)</td>
</tr>
<tr>
<td>Human enteric coronavirus 24 and 25</td>
<td>diarrheic fluid</td>
<td>9</td>
<td>(5)</td>
</tr>
<tr>
<td>Human enteric coronavirus 14</td>
<td>human fetal intestinal organs</td>
<td>5</td>
<td>(100)</td>
</tr>
<tr>
<td>Bovine enteropathogenic coronavirus</td>
<td>diarrheic fluid, HRT-18 cells</td>
<td>7-10</td>
<td>(42,133)</td>
</tr>
<tr>
<td></td>
<td>BEK-1 cells, HRT-18 cells</td>
<td>5-6</td>
<td>(46,63,64,71,126)</td>
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<tr>
<td>Rabbit enteric coronavirus</td>
<td>diarrheic fluid</td>
<td>8</td>
<td>(31)</td>
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<tr>
<td>Transmissible gastroenteritis virus</td>
<td>primary pig kidney cells</td>
<td>4</td>
<td>(41)</td>
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<tr>
<td>Hemagglutinating encephalomyelitis virus</td>
<td>primary pig kidney cells</td>
<td>8</td>
<td>(17)</td>
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<tr>
<td>Canine coronavirus</td>
<td>dog kidney cells</td>
<td>4</td>
<td>(40)</td>
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<tr>
<td>Feline coronavirus</td>
<td>fetal cat whole fetus cells</td>
<td>4</td>
<td>(9)</td>
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<tr>
<td>Infectious bronchitis virus</td>
<td>chorioallantoic membrane of de-embryonated chicken eggs</td>
<td>3</td>
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<td>TABLE II.2. Antigenic Groups of Coronaviruses Determined by Immunofluorescence Analysis</td>
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<tr>
<td><strong>Group 1</strong></td>
<td><strong>Group 2</strong></td>
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<tr>
<td>Avian Subgroup 1</td>
<td>Mammalian Subgroup 2</td>
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<td>Human Coronavirus-229E</td>
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<td>Bovine Coronavirus</td>
<td>Transmissible Gastroenteritis Virus</td>
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<td>Mouse Hepatitis Virus</td>
<td>Virus</td>
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<td>Sialodacryoadenitis Virus</td>
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CHAPTER III. CHARACTERIZATION OF SPECIFIC PROPERTIES OF HRT-18 CELL CLONES: CELL MORPHOLOGY, GROWTH PROPERTIES, PLATING EFFICIENCIES, AND EXPRESSION OF BOVINE CORONAVIRUS

III.A. INTRODUCTION

Human rectal tumor-18 (HRT-18) cells are used routinely for the propagation of bovine coronavirus (46,63,64,133). Turbid plaques containing some apparently normal cells were produced by BCV-L9 in HRT-18 cells. This observation indicated that the HRT-18 cell population is heterogeneous, consisting of subpopulations of cells which may differ in susceptibility to infection or in production of infectious virus. In hopes of isolating cell types that were either refractory or highly susceptible to BCV infection, HRT-18 cells were cloned by limiting dilution. All of the clones obtained by this method were found to be permissive to BCV, but there was some variation in the way virus infection was expressed cytopathically.

The HRT-18 cell line was established from an adenocarcinoma of the lower gastrointestinal tract. These cells are epithelial, with large nuclei and scanty cytoplasm. By ultrastructural examination, numerous uniform, frequently parallel microvilli are evident. The microvilli cover the apical surface of the cells, closely resembling the brush border of intestinal epithelium. Cytogenetic analysis of HRT-18 at the thirty-second passage showed a mode of 48 chromosomes, but additional chromosomes appeared randomly, mostly in karyotype groups D and E. Chromosome loss was not evident (122).

The purpose of this investigation was to analyze putative differences between four HRT-18 cell clones, 3F3, D2, 3E3, and 4B3. The clones were characterized according to cell morphology, growth
properties, and plating efficiencies. Further, these cells were infected with BCV-L9 to assess the susceptibility of each clone to infection based on cytopathic expression of the virus.

III.B. MATERIALS AND METHODS

III.B.1. Cells and Media

HRT-18 clones 4B3, D2, 3E3, and 3F3 were isolated from HRT-18 cells at approximately the 30th passage level by limiting dilution, and stocks were prepared. All clones were at the 8th passage level for these experiments. HRT-18 parent cells were used at passage 55 for these investigations. Cells were grown in Dulbecco's Modified Minimum Essential Medium (DMEM) with 44 mM NaHCO₃ buffer. Penicillin (100 units/ml)-streptomycin (100 ug/ml) was added to the medium. Medium was supplemented with 5% fetal calf serum for routine cell maintenance, but no serum was used during virus propagation.

III.B.2. Screening for Contaminating Mycoplasma or Viruses

Culture supernatant was inoculated into Modified Hayflick broth (96) then streaked onto Modified Hayflick Agar containing 1000 IU/ml of Penicillin G to assay for contaminating Mycoplasma. Cells were also examined on the ultrastructural level for Mycoplasma or contaminating viruses using transmission and scanning electron microscopy.

III.B.3. Immunofluorescent Analysis of Plaques Formed by BCV-L9 in HRT-18 Cells

Confluent monolayers of HRT-18 cells in 6-well polystyrene plates were inoculated with 0.5 ml of 10-fold serially diluted BCV-L9. After a 1 h adsorption period, excess inoculum was aspirated from the wells,
cells were washed, and 3 ml of DMEM containing 0.6% agarose (Gibco, electrophoresis grade) was added to the wells. The plates were incubated at 37°C in 5% CO₂. Cells were fixed at 48 h post infection with 4% paraformaldehyde for 15 min, and the overlays were removed. Cells were rinsed with PBS, pH 7.4, then post-fixed with methanol for 5 min., rinsed with PBS, and dried. Serum 1745 (a bovine serum for tissue culture) was diluted 1:20 in PBS, added to the wells, and incubated at 37°C for 30 min. Cells were washed 3 times for 5 min. each with PBS and dried. Rabbit anti-bovine IgG (H and L chain) FITC-conjugated antiserum (U.S. Biochemical Corp.) was diluted 1:16 in PBS, added to wells, and incubated at 37°C for 30 min. Three 5 min. PBS washes were done as before, and cells were dried. A 0.002% Evan's blue solution was used to counterstain the cells for 2 min., then the cells were briefly rinsed with PBS and dried. A drop of buffered glycerol mounting medium was used to mount coverslips, and coverslips were sealed with nail polish. Plaques were examined microscopically using a Zeiss epifluorescence microscope equipped with a 40 X oil immersion lens.

### III.B.4. Virus

BCV strain L9 was adapted to cell cultures by Mebus et al (84) using bovine fetal kidney cells. The virus used in these investigations was passaged 42 times in bovine fetal kidney cells, 16 times in bovine fetal brain cells, 15 times in bovine fetal spleen cells, and 8 times in HRT-18 cells.

### III.B.5. Infection of Cells

Monolayers of each HRT-18 clone in 25 cm² tissue culture flasks were infected with BCV-L9 at a multiplicity of infection (MOI) of
approximately $3 \times 10^{-3}$ PFU/cell. Following an adsorption period of 1 h at 37°C, excess inoculum was removed, and DMEM was added to the cells. Infected flasks were incubated for three days, then cytopathic expression was monitored by phase-contrast microscopy and photographed.

### III.B.6. Analyses of Cell Morphology

Confluent flasks of each clone were trypsinized and viable cell counts were made using the trypan blue exclusion method and a hemacytometer. 60 mm Petri plates were seeded with $1 \times 10^6$ cells. After 24, 48, and 72 h incubation, cells were fixed with Bouin's solution and stained with Giemsa. Confluent monolayers (4 days after passage) of each clone were examined by phase-contrast microscopy and photographed.

### III.B.7. Evaluation of Growth Properties

Following viable cell counts (described above) cells were seeded into 25 cm$^2$ flasks at a density of $2 \times 10^5$ cells/ml in 5 ml medium. Flasks were incubated at 37°C. At 24 h intervals duplicate flasks of each cell type were trypsinized and viable cell counts were made. The average cell count at each time period was calculated, and growth curves were drawn by plotting viable cells versus time (h). From the growth curves the doubling time, generation time, population density, and saturation density for each clone was extrapolated. Doubling time was the number of hours required for viable cell count to reach $2 \times 10^6$ cells. Generation time was obtained by averaging 3 doubling times taken from the logarithmic phase of growth. The saturation density (maximum number of viable cells obtained) was the value where three successive
cell counts showed no increase in number. The population density was the saturation density/cm².

III.B.8 Evaluation of Plating Efficiency

Triplicate 60 mm Petri plates were seeded with 100, 500, 1000, or 10,000 viable cells in 5 ml medium. Plates were incubated at 37°C in 5% CO₂. After a 6 day incubation period, cells were fixed with Bouin's solution and stained with Giemsa. Colonies consisting of 4 or more cells were counted, and the plating efficiency was calculated as follows:

\[
\frac{\text{No. colonies formed}}{\text{No. cells seeded}} \times 100
\]

The plating efficiency reported was the average of the three plates counted.

III.B.9. Plating Efficiency in Soft Agar

The efficiency of colony formation in soft agar was assessed according to a modification of the method used by MacPherson and Montagnier (79). Base layers of 7 ml medium containing 0.5% agarose in 60 mm Petri dishes were allowed to solidify. A second layer seeded with 100, 500, 1000, or 10,000 cells in 1.5 ml medium plus 0.3% agarose was added. Plates were prepared in triplicate for each clone and incubated at 37°C in 5% CO₂. Cell counts were done after 9- and 21-day incubations, as follows: Plates were sectioned into quarters. All cells within microscopic fields at a fold magnification of 200 X were counted until a total of 25 cells per quarter had been counted. The number of colonies within this population which contained 4 or more cells was determined. The efficiency of colony formation was the average of the triplicate counts.
III.B.10. Statistical Methodology

The Statistical Analysis System (105) was used to analyze the data with the general linear models (GLM) procedure and Tukey's Studentized Range Test for analysis of individual mean differences. All individual mean separation tests were considered significant at a probability level of 0.05.

III.C. RESULTS

III.C.1. Screening for Contaminants

No contaminating Mycoplasma or viruses were evident in culture or by ultrastructural examination.

III.C.2. Immunofluorescent Analysis of Plaques Formed by BCV-L9 in HRT-18 Cells

Microscopic evaluation of the fluorescent antibody-stained plaques revealed fluorescing cells within the center of the plaques, indicating the presence of BCV-L9 antigen. Among the fluorescing cells within the plaque center were many cells which did not express viral antigens (Fig. III.1).

III.C.3. Analyses of the Morphology of HRT-18 Clones

HRT-18 clones, like the parent cells, were polygonal in shape. The nuclei were large and ovoid to pleomorphic and surrounded by a meager cytoplasm. Some differences in developmental morphology were seen in Giemsa-stained cultures. After 24 h growth, only a few single cells were present in clone 3F3 cultures. Well-defined, tightly-packed colonies of different sizes had developed (Fig. III.2.A). Giemsa-stained cultures of clone D2 consisted of a large number of single
cells. Colonies formed by this clone were poorly defined and loosely associated (Fig. III.2.B). Clone 3E3 produced small, discrete colonies of similar size within 24 h (Fig. III.2.C). Some large, densely-packed colonies were formed by 4B3 cells, but most colonies consisted of only a few cells (Fig. III.2.D). Within 72 h, clones 3F3, 3E3, 4B3 had coalesced into confluent monolayers but, growth was slower for clone D2.

Although difficult to demonstrate in a single micrograph, each clone exhibited characteristic patterns within the monolayer that were evident by phase-contrast microscopy. The patterns included a distinct surface topography and orientation of cells that was consistent and reproducible with subpassage. Vacuolation of cells occurred for each clone, but it consistently appeared earliest in clone D2, and to lesser extents in the other three clones (Fig. III.3.A-D).

III.C.4. Assessment of Growth Properties of HRT-18 Clones

The growth curve of each cell type showed the classical lag, logarithmic, stationary, and death phases of growth (Fig. III.4). The growth properties demonstrated by each clone are summarized in Table III.1. Each cell type exhibited a different doubling time. The shortest doubling time of 27 h was found for clone D2, while clone 4B3 required 52 h to double its cell population. Clone 4B3 was the only cell type to exhibit an initial decrease in viable cell count at the 24 h incubation period. This drop in viable cells also occurred with the HRT-18 parent cells, which had a 52 h doubling time. The generation times for clones 3F3, 4B3, D2, and parent cells were similar at 21, 21, 18, and 20 h, respectively, but it was 30 h for clone 3E3. The kinetics
of growth for clone 4B3 most closely resembled those of the HRT-18 parent cells.

Saturation densities for clones 3E3, 4B3, and D2 were approximately 8 million, while the saturation density was less than 7 million for clone 3F3. Population densities of approximately $3 \times 10^5$ cells/cm$^2$ were obtained. The maximum viable cell count for HRT-18 parent cells was 4 to 5 million cells higher than those of the clones, but these cells were assayed in a separate experiment. Therefore, the higher counts may be attributable to slightly different culture conditions.

### III.C.5. Plating Efficiency

The plating efficiencies were determined by counting the number of colonies of 4 or more cells per number of cells seeded. The set seeded with 100 cells was within the countable range, while the other three sets were too numerous to count. The plating efficiencies obtained for each clone are reported in Table III.2. Clones 3F3 and D2 plated with efficiencies of 100% and 77%, respectively. These values were significantly higher ($F = 30.76$, $p<0.0002$) than those obtained for clones 3E3 and 4B3 which had efficiencies of 33% and 50%, respectively.

Three basic colony types were apparent after Giemsa-staining: small colonies consisting of only a few cells; intermediate colonies of about 0.5 mm diameter; and large colonies of approximately 1 mm in diameter. The colony types produced by the clones are given in Figures III.5.A-D. All three types of colonies were produced by clone D2. Clones 3F3 and 3E3 formed small and intermediate-sized colonies. Mostly large colonies were produced by clone 4B3.
III.C.6. Growth in Soft Agar as an Indicator of Transformation

Table III.2. presents the efficiencies of colony formation in soft agar for each clone. The efficiencies of clones 3E3 and 4B3 were identical at 17%, and slightly lower at 15% for clone D2. The highest efficiency of 23% was produced by clone 3F3. Statistical analysis did not reveal significant differences between the clones.

III.C.7. Cytopathic Expression of BCV-L9 in each Clone.

All four HRT-18 clones were susceptible to BCV infection, but the cytopathic expression produced in each clone varied (Table III.3). Clone 3F3 appeared to be the most susceptible to BCV infection because it produced the most extensive CPE (Fig. III.6.A). The monolayer was heavily vacuolated, and areas of fused cells were observed. The cytopathic changes of infected clones D2 and 3E3 were less extensive than 3F3. Although milder, cytopathic expression also included vacuolated monolayers and areas of cell fusion (Figs. III.6.B and C). Based on cytopathic expression, clone 4B3 appeared the least affected by infection (Fig. III.6.D). Some vacuoles developed in the monolayer of this infected clone, and some cells rounded up and detached. Cell fusion was not seen in clone 4B3.

III.D. DISCUSSION

Turbid plaques are produced by BCV-L9 in HRT-18 cells. Immunofluorescent analysis of these plaques revealed that many of the cells within the plaques did not express viral antigen. Presumably, the non-fluorescing cells represent cells which are non-permissive to virus infection or incapable of replicating internalized virus. HRT-18 cell
clones were established in an attempt to isolate both refractory and susceptible cell types.

HRT-18 clones 3F3, D2, 3E3, and 4B3 were characterized to assess putative differences between the cell lines. The four clones exhibited differences in morphology both in Giemsa-stained cultures and in 4-day monolayers. The basis for these differences is unknown. The diversity in colony size and number of single cells observed after 24 h incubation probably reflects each clone's ability to readjust to culture following trypsinization or differences in rate of growth. Variation in the cultural morphology may indicate differences in cell size, the orientation of junctional complexes (desmosomes) between cells, and/or varied degrees of contact inhibition. These traits contribute to the proximity between cells and the way the cells pile up in culture. In combination, these characteristics could contribute to the unique cultural patterns displayed by each clone. Vacuolization of the cells was a morphological characteristic which probably reflects the aging process.

Studies of the growth properties of the cells were conducted to assess any variance between the clones in kinetics of growth. Each clone required a unique period of time to double in number, indicating that the log or adjustment phases were dissimilar. All clones except 3E3 showed a similar generation time. Therefore, 3 of 4 clones multiplied logarithmically at approximately equal rates, with clone 3E3 multiplying at a somewhat slower rate. Similar saturation densities were obtained by clones D2, 4B3, and 3E3, indicating that the growth rates leveled off and allowed nearly the same cell numbers to be achieved. The maximum cell count was over 1 million cells fewer for
clone 3F3. The viable cell counts were reduced to a different level for each clone at the final count of 260 h. For example, the count did not deviate from the saturation density for clone 3F3, but decreased by \(4 \times 10^6\) cells for clone 3E3.

The growth of the HRT-18 parent cells was assayed at a different time than the clones, and the higher saturation density may have resulted from slightly different culture conditions. Clone 3F3 formed colonies to the highest efficiency both under normal culture conditions and in soft agar. MacPherson and Montagnier (79) found that BHK-21/13 cells, transformed by polyoma virus, formed colonies in soft agar medium while uninfected cells did not. This procedure demonstrates anchorage independence and is an indicator of transformation. All the clones shared this transformed quality, but it was most apparent in clone 3F3. It is likely that the extent of transformation is greatest in this clone. Perhaps transformed traits such as cell membrane changes or increased metabolic activity contribute to the extreme susceptibility of clone 3F3 to BCV infection.

According to a study of HRT-18 cells at passage 6-12 done by Tompkins et al (122), the generation time for the cells was 31.1 h and the plating efficiency was 91.3%. These data most closely resemble those obtained for clone 3F3. This study demonstrated that HRT-18 at passage 55 had a doubling time and generation time nearly identical to clone 4B3. Perhaps the growth properties of the HRT-18 cells shifted from predominately 3F3-like characteristics at low passage to predominately 4B3-like characteristics at high passage.

All four clones were susceptible to BCV infection, but the cytopathic expression produced as a result of BCV infection differed
between the clones. Several plausible reasons may explain this result:

a) The clones may process the virus differently. Cellular proteases may be produced at different levels or not at all in some cells, allowing the virus to replicate more efficiently in a particular clone. Presumably, cell fusion is a consequence of proteolytic cleavage and activation of the cell-fusing ability of this virus. Frana and coworkers (37) demonstrated varied ability to induce cell fusion when four different murine cell lines were infected with MHV. These cell lines processed the 180 Kd glycoprotein differently.

b) The cell membrane plays a major role in cell fusion. Huang (57) showed that glycolipids are involved in membrane fusion. Glycolipids containing galactose at terminal positions inhibited hemolysis and fusion of erythrocytes by Sendai and influenza viruses. The investigator assumed that the lipids interfered with the fusion process by competing with viral glycoproteins responsible for fusion. In studies of liposome fusion by Sendai virus, the presence of gangliosides in the liposomes was essential for fusion (44,45). Miyake and other investigators (88) demonstrated the involvement of the cytoskeleton in cell fusion by disrupting microfilaments with cytochalasin D and inhibiting fusion. Therefore, it is quite possible that the appearance of cell fusion in clones 3F3, D2, and 3E3 but not 4B3 may reflect differences in the composition of the cell membranes.

c) The HRT-18 clones possibly have different receptor proteins on their surfaces, or the virus receptor may appear more frequently on certain clones. This would contribute to different levels of infection. The lack of a virus receptor on the target tissues of mice genetically resistant to MHV was demonstrated (8).

d) Perhaps the replication and/or morphogenesis of the virus
occurs with varied efficiency in the clones. Lytic infections with MHV showed coordinated synthesis of viral proteins throughout infection, while in non-cytocidal infections, synthesis of the N protein appeared earlier. Pulse-chase experiments demonstrated different rates of shut-down of host protein synthesis for MHV-infected cells (117). Any one of these phenomena or a combination of them may explain the differences that the clones exhibit in expression of virus infection.
### Table III.1. Growth Properties of HRT-18 Cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Doubling Time (h)</th>
<th>Generation Time (h)</th>
<th>Saturation Density (cells)</th>
<th>Population Density (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3F3</td>
<td>30</td>
<td>21</td>
<td>$6.7 \times 10^6$</td>
<td>$2.7 \times 10^5$</td>
</tr>
<tr>
<td>3E3</td>
<td>36</td>
<td>30</td>
<td>$8.1 \times 10^6$</td>
<td>$3.2 \times 10^5$</td>
</tr>
<tr>
<td>4B3</td>
<td>52</td>
<td>21</td>
<td>$7.6 \times 10^6$</td>
<td>$3.0 \times 10^5$</td>
</tr>
<tr>
<td>D2</td>
<td>27</td>
<td>18</td>
<td>$8.3 \times 10^6$</td>
<td>$3.3 \times 10^5$</td>
</tr>
<tr>
<td>HRT-18 parent</td>
<td>52</td>
<td>20</td>
<td>$11.9 \times 10^6$</td>
<td>$4.8 \times 10^5$</td>
</tr>
</tbody>
</table>
### Table III.2. Efficiencies of Colony Formation for HRT-18 Cell Clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Plating Efficiency</th>
<th>Efficiency of Colony Formation in Soft Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>3F3</td>
<td>100%</td>
<td>23%</td>
</tr>
<tr>
<td>3E3</td>
<td>33%</td>
<td>17%</td>
</tr>
<tr>
<td>4B3</td>
<td>50%</td>
<td>17%</td>
</tr>
<tr>
<td>D2</td>
<td>77%</td>
<td>15%</td>
</tr>
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</table>
Table III.3. Cytopathic Expression of BCV-L9 in HRT-18 Cell Clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Extent of Cytopathic Expression</th>
<th>Cytopathic Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>4B3</td>
<td>Minimal</td>
<td>Vacuoles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell rounding</td>
</tr>
<tr>
<td>D2</td>
<td>Moderate</td>
<td>Vacuoles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell fusion</td>
</tr>
<tr>
<td>3E3</td>
<td>Moderate</td>
<td>Vacuoles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell fusion</td>
</tr>
<tr>
<td>3F3</td>
<td>Extensive</td>
<td>Vacuoles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell fusion</td>
</tr>
</tbody>
</table>
Figure III. 1. Photomicrograph of a Plaque Produced by BCV-L9 in HRT-18 Cells and Examined by Immunofluorescent Analysis. Magnification - 400 X
Figure III. 2. Photomicrograph of Giemsa-Stained, 24 h Cultures of HRT-18 Clones. A. Clone 3F3  B. Clone D2
C. Clone 3E3  D. Clone 4B3  Magnification - 100 X
Figure III. 3. Phase-Contrast Photomicrograph of Confluent Monolayers of HRT-18 Clones. A. Clone 3F3  B. Clone D2  C. Clone 3E3  D. Clone 4B3
Magnification - 200 X
Figure III. 4. Growth Curves of HRT-18 Clones and Parent Cells.
Figure III. 5. Photomicrograph of Colonies Produced by HRT-18 Clones. Giemsa-Stained Cultures. A. Clone 3F3 B. Clone D2 C. Clone 3E3 D. Clone 4B3. Magnification - 100 X
Figure III. 6. Phase-Contrast Photomicrograph of BCV-L9-Infected HRT-18 Clones. A. Clone 3F3
B. Clone D2 C. Clone 3E3 D. Clone 4B3.
Arrows indicate fused cells.
Magnification - A,B - 200 X
C,D - 320 X
CHAPTER IV. INFLUENCE OF HOST CELL, VIRUS STRAIN, AND TRYPsin
MODIFICATION ON BCV CYTOPATHIC EXPRESSION AND PLAQUE
FORMATION.

IV.A. INTRODUCTION

An infectious form of Sendai virus was produced in chicken embryos. The virus was hemolytic and activated cell fusion. Propagation of chicken embryo-borne Sendai virus in L cells resulted in a single round of replication, and the L cell-borne Sendai virus was noninfectious, nonhemolytic, and did not induce fusion. Treatment of the L cell-borne virus with trypsin activated the three biological activities. Activation of the virus was a result of cleavage of the inactive precursor protein Fo to the subunits F1 and F2 which are disulfide linked to form the active F protein (51-54,93,99,106). Proteolytic activation of viral infectivity was also reported for Newcastle disease virus and orthomyxoviruses (24,39,99).

Coronaviruses appear to have a dependence on the host cell for replication in culture. The spectrum of cytopathic expression ranges from mild to highly cytocidal according to the virus strain and the host cell. Cytocidal infections may cause cell fusion and polykaryon development or cell lysis. In contrast, persistent coronavirus infections were reported in some cell lines (23,48,75). Many strains of coronavirus do not replicate in culture. Frana and coworkers (37) reported host-dependent processing of the structural proteins of MHV-A59. They found that activation of the viral fusion factor was due in part to cleavage of the 180 Kd peplomer to 90 Kd subunits. Trypsin enhanced the cell-fusing activity of this virus (37,119,135). A trypsin-dependent increase in cell-fusing activity and plaque
development of bovine coronavirus was demonstrated using bovine fetal brain and bovine fetal thyroid cells (114).

The purpose of this study was to determine the effect of the host cell, virus strain, and trypsin modification on the cytopathic expression and plaque development of bovine coronaviruses. For this investigation cells which varied in susceptibility to infection were used, and several wild-type virus isolates, as well as strain L9 were assayed.

IV.B MATERIALS AND METHODS

IV.B.1. Cells

HRT-18 parent cells and HRT-18 cell clones 3F3, D2, 3E3, and 4B3 were maintained as described in Chapter III. D2BFS cells were derived from bovine fetal spleen cells. They are a population of cells which survived precrisis and continue to multiply well past the thirtieth passage. D2BFS cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 25 mM Hepes and penicillin (100 units/ml)-streptomycin (100 ug/ml). MEM was supplemented with 10% fetal calf serum. No serum was used in the presence of virus.

IV.B.2. Virus

BCV-L9 and 5 wild-type strains (isolated from Colorado and Utah) were used. Wild-type isolates were designated Meeker, Miller, Fisher, Calf 50, and LY-138. These isolates were obtained from diarrhea fluids of sick calves and were passaged 2-3 times in HRT-18 cells.
IV.B.3. Propagation of Virus in Different Cells

Monolayers of HRT-18, clones, and D2BFS cells in 25 cm$^2$ flasks were infected in duplicate with BCV. BCV-L9 was inoculated at an MOI of 3 x 10$^{-3}$ PFU/cell. Stock wild-type strains were inoculated at approximate MOIs of 1 and 0.1 PFU/cell. Cells were infected as described in Chapter III. Following a 1 h adsorption period, excess inoculum was removed, and 5 ml of medium was added. One flask from each set received trypsin-free medium, the other set received medium + the maximum concentration of trypsin which did not produce damage to the cell monolayers. 5 ug/ml trypsin was used with HRT-18 cells; 7.5 ug/ml trypsin (Difco 1:250) was used with D2BFS. All flasks were incubated at 37°C and monitored daily for CPE.

IV.B.4. Plaque Formation

Confluent monolayers of HRT-18 cell clones 3F3, D2, 3E3, and 4B3 in 6-well polystyrene plates were infected with 10-fold serially diluted virus (0.5 ml/well in paired wells). The overlay medium consisted of DMEM + 0.6% agarose (Gibco, electrophoresis grade). Trypsin (Difco, 1:250, 2.0 ug/ml) was added to the overlay of half the wells. After a 3-4 day incubation period, cells were stained for 2 h with neutral red, fixed with formalin saline for 10 min., and the overlays were removed.

IV.C. RESULTS

IV.C.1. Cytopathic Expression of BCV in Different Cells

The cytopathic expression of five wild-type BCV strains and strain L9 were analyzed in the presence and absence of trypsin using HRT-18 parent cells, four HRT-18 cell clones, and D2BFS cells. The results of this investigation are given in Table IV.1. HRT-18 cells were
permissive to all strains of virus. These cells were initially used for isolation of wild-type strains from diarrhea fluids and adaptation of the viruses to culture. Each strain of virus induced cell fusion in HRT-18 cells, and trypsin enhanced the cytopathic expression. Extensive fusion occurred in HRT-18 cells infected with strains L9 and Meeker in the presence of trypsin, while moderate fusion was a result of infection with strains Miller, Fisher, Calf 50, and LY-138. The cytopathic expression resulting from infection of HRT-18 cells with BCV-L9 with and without trypsin is demonstrated in Figure IV.1. D2BFS cells were non-permissive to infection by all the field isolates even in the presence of trypsin. Strain L9 replicated in D2BFS cells, but cell fusion only occurred in the presence of trypsin (Fig. IV.2). Strain L9 replicated with varied intensity in the four HRT-18 cell clones. Trypsin increased the cytopathic expression in clones 3F3, D2, and 3E3, but not in clone 4B3. Cell fusion was observed in all clones except clone 4B3.

IV.C.2 Plaque Development

The production of plaques by BCV-L9 was monitored in HRT-18 clones 3F3, 3E3, D2, and 4B3 both with and without trypsin to assess any host-dependent differences in plaque formation (Fig. IV.3). The virus produced sharp, distinct plaques of 1-2 mm diameter in clone 3F3 within 4 days post infection, and the plaques developed within 2-3 days in the presence of trypsin. The plaque diameter more than tripled, the plaques were less turbid, and they were more sharply defined when trypsin was included in the overlay. At least 4 different plaque phenotypes of L9 were apparent. Small, turbid plaques of approximately 1-2 mm diameter
developed in clone 4B3. The plaques were more distinct in the presence of trypsin but the plaques remained turbid, and the diameter was unchanged. Distinct plaques of about 0.5-2 mm diameter were formed by L9 in clone D2. Trypsin did not increase the size of these plaques, but the plaques were slightly less turbid and easier to see. Plaques were not evident in the 3E3 cell monolayer in the absence of trypsin. Plaques remained indistinct, but they were apparent in wells containing trypsin.

Based on cytopathic changes, HRT-18 clones 3F3 and 4B3 represent highly susceptible and minimally susceptible cells to BCV infection, respectively. Consequently, the two clones were used to assess plaque formation by four wild-type strains of BCV. Plaques were not produced by any of the isolates in clone 4B3 either in the presence or absence of trypsin. In clone 3F3, plaques developed readily, and they were enhanced by trypsin (Fig. IV.4). Plaques ranging in size from 2-4 mm were produced by strain Miller. The number of detectable plaques increased with trypsin, but the plaques remained relatively turbid and of similar size to those produced without trypsin. The plaques formed by strain Meeker were 1 mm or 2 mm in diameter with fairly clear centers. These plaques were greatly enhanced by trypsin. Plaque diameters at least doubled, and the centers were very clear. Strain Calf 50 produced turbid plaques of 1-2 mm diameter in the absence of trypsin. Plaques that developed with trypsin in the overlay were less turbid, but the size of the plaques did not increase significantly. LY-138 formed mostly medium-sized, turbid plaques of about 2 mm diameter in the absence of trypsin. Some of the plaques were enhanced by trypsin, but other plaques within the monolayer exhibited no enhancement.
IV.D. DISCUSSION

Host-dependent differences in the cytopathic expression of bovine coronaviruses were demonstrated in this study. HRT-18 parent cells allowed the replication of all strains of BCV. The four HRT-18 clones were permissive to infection with strain L9, and the cytopathic changes were different in these cells. In contrast, D2BFS cells only allowed the replication of the cell-adapted virus strain, L9, while these cells were non-permissive to infection by the wild-type strains.

Cell fusion was induced by all strains of virus in HRT-18 parent cells, and strain L9 induced fusion in clones 3F3, 3E3, and D2. Trypsin enhanced the CPE and cell fusion in these cells. Fusion was not evident in L9-infected clone 4B3 either with or without trypsin. These cells may be incapable of fusion due to the composition of the cell membrane (44,45,57,88), or they may process the virus differently. Cell fusion occurred in L9-infected D2BFS cells only in the presence of trypsin. This finding indicates that these cells may lack a specific protease necessary for activating the cell-fusing activity of the virus. This situation is analogous to that found for Sendai virus propagated in L cells, which was reported by Homma and others (51,52,54).

Plaque formation was dependent upon the host cell line, the strain of virus, and the presence of trypsin in the overlay. Strain L9 produced plaques in the four HRT-18 cell clones, but the ease of plaque production and the morphology of the plaques varied between the cells. Differences in the rate of replication of the virus in the clones could produce this result. Within a given incubation period, larger plaques would form in cells which allow rapid replication of the virus, while smaller plaques would develop in cells in which the virus replicates
slowly. Trypsin dramatically enhanced the morphology of plaques produced in clone 3F3, but the morphology of the plaques formed in clones D2, 3E3, and 4B3 were less affected by trypsin. Enhanced plaque production of BCV-L9 by trypsin was also demonstrated in bovine fetal brain and thyroid cells (114).

Clone 4B3 was nonpermissive to plaque production by the wild-type BCV strains, but plaques developed readily in clone 3F3, again demonstrating the host-dependence of BCV replication. Each wild-type strain produced a number of distinct plaque phenotypes that were enhanced by trypsin. Since these virus stocks were not plaque purified, they probably consist of a variety of genotypically distinct viruses.

BCV-L9 is a highly cell-adapted strain of virus which replicates easily in culture. In contrast, the wild-type strains were isolated from diarrhea fluids and were passaged only 2-3 times in cultures of HRT-18 cells. The wild-type strains differ in cytopathogenicity in culture. Williams (132) reported differences in the polypeptide profiles of these viruses. Structural differences may affect the viruses' ability to adsorb to or replicate in a cell type, or affect the ease and extent of processing of viral proteins by the host cell or by trypsin. These factors probably contribute to the differences in cytopathic expression and plaque development observed in this study.
Table IV.1. Cytopathic Expression of BCV Strains in Different Cell Types.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Trypsin</th>
<th>L9</th>
<th>Miller</th>
<th>Meeker</th>
<th>Fisher</th>
<th>Calf 50</th>
<th>LY-138</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2BFS</td>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>++(ef)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRT-18</td>
<td>A</td>
<td>+(lf)</td>
<td>+(lf)</td>
<td>+(lf)</td>
<td>+(lf)</td>
<td>+(lf)</td>
<td>+</td>
</tr>
<tr>
<td>parent</td>
<td>P</td>
<td>++(ef)</td>
<td>++(mf)</td>
<td>++(mf)</td>
<td>++(mf)</td>
<td>++(mf)</td>
<td>++(mf)</td>
</tr>
<tr>
<td>Clone 4B3</td>
<td>A</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Clone D2</td>
<td>A</td>
<td>+(lf)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>++(mf)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Clone 3E3</td>
<td>A</td>
<td>+(lf)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>P</td>
<td>++(mf)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Clone 3F3</td>
<td>A</td>
<td>+(mf)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>++(ef)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

(lf) - light fusion  
(mf) - moderate fusion  
(ef) - extensive fusion  
A - trypsin absent  
P - trypsin present  
ND - not done
Figure IV. 1. Phase-Contrast Photomicrograph of BCV-L9-Infected HRT-18 Cells. A. (-) trypsin
B. (+) trypsin
Magnification - 100 X
Figure IV. 2. Photomicrograph of BCV-L9-Infected D2BFS Cells. Giemsa-Stained Cultures.

A. (−) trypsin  B. (−) trypsin

Magnification – 400 X
Figure IV. 3. Plaque Development of BCV-L9 in HRT-18 Clones

3F3, 4B3, D2, and 3E3. a - trypsin absent,
p - trypsin present.
Figure IV. 4. Plaque Development of Wild-Type BCV Strains in Clone 3F3. a - trypsin absent, p - trypsin present.
CHAPTER V. THE INFLUENCE OF THE HOST CELL AND TRYPsin TREATMENT ON BCV INFECTION.

V.A. INTRODUCTION

Proteolytic cleavage of surface proteins is a prerequisite for infectivity in a number of virus systems. Specifically, orthomyxovirus requires cleavage of the 75-80 Kd HA protein into the 36 Kd HA1 and the 27 Kd HA2 species for the virus to be infectious. Activation of the cell-fusing potential and infectivity of paramyxovirus is a result of cleavage of the F0 precursor protein into F1 and F2 (52 and 13 Kd, respectively) which are disulfide-linked subunits of the active F protein. Activation of the infectious potential of these two viruses is accomplished either by cellular proteases or by trypsin (25,39,51-54,92,99,106). The infectivity of rotaviruses can be increased if the outer capsid polypeptide VP3 is cleaved into VP5 and VP8 (60 Kd and 28 Kd, respectively). Cleavage by trypsin, elastase, or pancreatin causes an increase in infectivity of rotaviruses (35,74).

Proteolytic cleavage of the 180 Kd envelope protein of MHV into its 90 Kd subunits activates the cell-fusing activity of this virus, but this cleavage pattern has not been correlated to an increase in the infectivity of the virus. Cell-dependent differences in processing of MHV proteins have been demonstrated (37,119). Trypsin enhances the plaque development and the cell-fusing capacity of murine, bovine, and avian coronaviruses (97,114,135). The polypeptide profile of BCV is altered by trypsin treatment (133). Toth (124) found that the rate of replication and the infectivity of BCV was enhanced when the virus was propagated in bovine embryonic lung cells in the presence of trypsin.
In the previous chapter the effect of the host cell, virus strain, and trypsin modulation on the cytopathic expression and plaque development of bovine coronaviruses was examined. All three of these parameters affected the growth potential of BCV. The intent of this experimentation was to determine the influence of the host cell and trypsin treatment on the infectivity of the virus.

V.B. MATERIALS AND METHODS

V.B.1. Cells and Virus

HRT-18 parent cells, HRT-18 clones 3F3, D2, 3E3, and 4B3, and D2BFS cells were maintained as described in previous chapters. BCV strains L9 and LY-138 were used in these assays.

V.B.2. Propagation of Virus in the Presence and Absence of Trypsin

Cells were cultured in 25 cm² tissue culture flasks until confluent. Cells were washed with Dulbecco's PBS and infected in duplicate with BCV strain L9 or LY-138 diluted 10⁻² - 10⁻⁶. Using L9 the approximate MOI was 3 x 10⁻³ - 3 x 10⁻⁷ PFU/cell for HRT-18 and 3 x 10⁻² - 3 x 10⁻⁶ PFU/cell for D2BFS. The approximate MOI was 1.4 x 10⁻² to 1.4 x 10⁻⁶ PFU/cell for LY-138 in HRT-18 cells. Virus was allowed to adsorb for 1 h at 37°C. For each cell type, paired flasks received either medium or the same medium containing trypsin. HRT-18 parent cells and clones received 5 ug/ml trypsin; D2BFS cells received 7.5 ug/ml trypsin. All flasks were incubated at 37°C for 48 h with CPE monitored daily. Flasks were frozen at -70°C, thawed, sonicated for 15 sec, and aliquots were frozen. Samples were titrated for virus yields by hemagglutination assay and plaque assay.
V.B.3. Hemagglutination Assays

1% mouse erythrocytes were used for detection of BCV hemagglutinin. Two-fold serial dilutions of 25 ul virus samples were made in 96-well microtiter plates using PBS as diluent. Mouse red blood cells were added, and plates were agitated to assure mixing in wells. Plates were incubated at room temperature for 2 h or overnight at 4°C, then examined for agglutination of red blood cells.

V.B.4. Plaque Assays

Plaque assays were used to enumerate infectious virus. The assays were performed as described in the preceding chapter using a 0.5 ml inoculum.

V.B.5. Assessment of Infectious Units in the Virus Population

PFU:HA ratios were calculated from the titers obtained from plaque assays and hemagglutination assays. A 0.5 ml inoculum was used in plaque assays, and a 25 ul sample was tested by hemagglutination assay. These values represent the amount of infectious virus per hemagglutinating unit. Graphs were plotted as log_{10} PFU:HA versus inoculum virus dilution.

V.B.6. Propagation of BCV-L9 Using Different Trypsin-Treatment Schemes

Duplicate 25 cm² flasks of confluent D2BFS cells were treated as follows:

Set 1) Cells were washed three times with Dulbecco's PBS, then treated with 2 ml of MEM containing 1 ug/ml trypsin (Sigma, TPCK treated). Cells were incubated for 10 min at 37°C. Cells were washed to remove trypsin, then infected with L9 propagated in D2BFS cells without trypsin
(MOI = 6 x 10^-4 PFU/cell). After adsorption for 1 h at 37°C, excess inoculum was removed, and cells were washed. MEM without trypsin was added to the flasks.

Set 2 - Cells were washed and infected with virus as described above. After adsorption, MEM containing 1 ug/ml trypsin was added to flasks.

Set 3 - Virus was treated with 1 ug/ml trypsin for 30 min. at 37°C. Trypsin activity was stopped by adding 1 ug/ml soybean trypsin inhibitor (Sigma) and incubating for 30 min. at 4°C. Virus was diluted to an MOI of 6 x 10^-4 PFU/cell and used to infect cells. After adsorption, medium lacking trypsin was added to the flasks.

Set 4 - The virus was treated with trypsin as described above, then immediately diluted and used as inoculum. MEM containing 1 ug/ml trypsin was added after adsorption.

Set 5 - Cells were washed and infected with virus as described above. After adsorption MEM without trypsin was added.

All flasks were incubated for 24 h, then frozen at -70°C. Cells were thawed, sonicated, and aliquots were frozen. Yields were titrated from duplicate samples obtained from replicate flasks by plaque assay in HRT-18 cells. The four values were averaged to obtain the PFU titer.

V.B.7. Statistical Methodology

The Statistical Analysis System (SAS) (105) was used to analyze the data with the general linear model (GLM) procedure and Tukey's Studentized Range Test for analysis of individual mean differences. All individual mean separation tests were considered significant at a probability level of 0.05.
V.C. RESULTS

V.C.1. Effect of Trypsin on BCV Infectivity

Infectivity ratios are displayed graphically as \( \log_{10} \) PFU:HA versus inoculum virus dilution. An increase in PFU:HA occurred at dilutions \( 10^{-4} \) and \( 10^{-5} \) when BCV-L9 was propagated in D2BFS cells. At these dilutions there was no detectable infectious virus in the absence of trypsin, but a PFU:HA of greater than 5 log units occurred with trypsin. Enhanced infectivity was not apparent at lower dilutions of virus (Fig. V.1).

The infectivity ratios produced in HRT-18 parent cells were nearly identical with and without trypsin at BCV-L9 dilutions of \( 10^{-2} - 10^{-5} \). At the \( 10^{-6} \) dilution of inoculum, an increase in PFU:HA of 5 log units was obtained (Fig. V.2).

The infectivity of BCV-L9 propagated in the HRT-18 clones was less dramatically affected by trypsin, but definite trends in virus yields were observed. At the \( 10^{-6} \) dilution of L9 propagated in clone 3F3, an increase in PFU:HA of about 1 log unit occurred in the presence of trypsin. A consistent increase in the infectivity ratio did not occur at lower dilutions (Fig. V.3). Clone D2 produced a PFU:HA that was increased by 1/3 log with trypsin at the \( 10^{-4} \) dilution, and this slight increase persisted at \( 10^{-6} \) dilution (Fig. V.4). The infectivity ratios at the \( 10^{-2} \) and \( 10^{-3} \) dilutions were 1/2 to 1 log lower with trypsin in clone 3E3. However, continuing the trend shown by all other cell types, at \( 10^{-4} \) and \( 10^{-5} \) dilutions of inoculum, the trypsin-treated sets produced slightly elevated PFU:HA. No detectable virus was produced when the dilution was \( 10^{-6} \) (Fig. V.5). A reverse trend occurred when L9 was propagated in clone 4B3 with or without trypsin. At the highest
dilution, an infectious yield of nearly 1 log unit higher occurred in
the absence of trypsin (Fig. V.6).

The wild-type strain LY-138 propagated in HRT-18 parent cells showed
no enhanced infectivity at dilutions $10^{-2} - 10^{-5}$. A 1/2 log increase
was evident in the presence of trypsin at the $10^{-6}$ dilution (Fig. V.7).

Figure V.8. shows the PFU:HA ratios with trypsin:without trypsin
obtained in each cell type at the highest dilution producing detectable
virus - the limiting virus dilution. The ratios were 5:1, 2:1, and 3:1
for clones 3F3, D2, and 3E3 respectively, and less than 1 for 4B3.
Infinitely enhanced yields were produced by D2BFS and HRT-18 parent
cells in the presence of trypsin.

The PFU:HA ratios with:without trypsin were calculated for each cell
type at all dilutions of inoculum and are displayed graphically in
Figure V.9. The purpose of this graph is to simplify the comparison of
the effects of trypsin on viral infectivity between the different cell
types. Infinitely enhanced infectivity was apparent in D2BFS cells at
the $10^{-4}$ inoculum dilution. This dilution represents an initial MOI of
$3 \times 10^{-4}$ PFU/cell. HRT-18 cells produced infinitely enhanced viral
infectivity in the presence of trypsin at the $10^{-6}$ dilution (initial MOI
of $3 \times 10^{-7}$ PFU/cell). Therefore, enhanced viral infectivity occurred
at a 1000-fold higher MOI in D2BFS cells. When L9 was propagated in
HRT-18 clones D2 and 3E3, trypsin enhancement of viral infectivity was
evident at the $10^{-4}$ dilution. A detectable increase in infectious virus
yields was evident in clone 3F3 cells at $10^{-6}$ dilution, but at the same
dilution infectious yields were decreased by trypsin when L9 was assayed
from clone 4B3.
V.C.2. **Effect of Different Trypsin-Treatment Schemes on Virus Yields**

The yields of infectious virus produced in D2BFS cells under different trypsin-treatment conditions was titrated in quadruplicate by plaque assay in HRT-18 cells. These data are displayed as $\log_{10}$ PFU in Figure V.10. The PFU counts were essentially the same for pretreated cells (set 1) and the control system (set 5). Nearly identical infectious yields were obtained when trypsin was added to infected cells post infection (set 2) and when the inoculum was pretreated, then trypsin was added post infection (set 4). These values were significantly higher than those obtained from sets 1 and 5 ($F = 134.56$, $p<0.0001$). The lowest yield was obtained from set 3 (inoculum pretreated with trypsin, then soybean trypsin inhibitor). This set was significantly lower than all other treatments.

V.D. **DISCUSSION**

The effect of trypsin on BCV infectivity was quantitated by determining PFU:HA. This ratio represents the amount of infectious virus per hemagglutinating unit obtained from a given sample. This method of quantitation was chosen over a simple PFU titration because it depicts the effect of trypsin on the entire virus population. These data represent consistent trends and are reported as such.

Trypsin enhanced the infectivity of BCV-L9 propagated in HRT-18 parent and D2BFS cells to infinite proportions. The increase in infectivity was apparent at a 100-fold lower inoculum dilution (1000-fold higher MOI) in D2BFS cells. L9 propagated in the HRT-18 clones exhibited differences in the amount of trypsin enhancement and the dilution at which the increased yields appeared. A plausible
explanation for these phenomena is that bovine coronaviruses require proteolytic processing to activate their infectious potential. In a given cell type, the pool of progeny virus consists of two forms of virus – infectious and noninfectious, reflecting the cell's ability to process the virus to the infectious form by endogenous protease. With dilution the supply of infectious virus in the inoculum is minimized, emphasizing the cell's ability to produce infectious virus. Trypsin converts noninfectious progeny virus to the infectious form, thereby enhancing the infectivity of the virus yield. Therefore, cells with a high capacity for production of infectious progeny require a higher dilution of inoculum before trypsin enhancement is apparent. If the ratio of infectious virus exceeds noninfectious virus, then trypsin enhancement is not detectable. Such an event would explain the marginal trypsin effect in the HRT-18 clones. It is likely that HRT-18 parent cells are innately capable of producing high yields of infectious BCV, unlike D2BFS cells, which require the addition of trypsin for activation of the virus. It is evident from these investigations that trypsin-like proteases are required to activate the infectivity of BCV in some cultured cells.

Interestingly, the effect of trypsin on the infectivity of L9 correlates well with the results of the cytological studies described in the preceding chapters. The CPE produced in D2BFS and HRT-18 cells was enhanced by trypsin, and the four HRT-18 clones varied in response to trypsin treatment. Clone 4B3, which was only minimally affected by L9 with or without trypsin, was the only cell line to produce lower infectious yields in the presence of trypsin. The molecular basis for this finding is not fully understood.
LY-138 was slightly enhanced in infectivity when propagated in HRT-18 cells, as compared to the dramatic increase shown with L9. Williams (132) found differences in the protein profiles of trypsin-treated L9 and LY-138, although their untreated profiles were nearly identical. This indicated that the two strains are cleaved differently, a trait which may account for differences in the infectious potential of the viruses.

Different trypsin-treatment conditions of L9-infected D2BFS cells were employed to discern the target of trypsin action. It was found that pretreatment of cells with trypsin had no effect on the production of infectious virus. It was concluded that any putative effect that trypsin exerted on the host cell did not affect virus production. On the other hand, the presence of the enzyme in culture medium enhanced the virus titer. In this case, trypsin had access to the progeny virus and probably activated noninfectious particles to the infectious form as previously discussed. Trypsin alters the polypeptide profile of BCV (133), so it was considered likely that the effect would be exerted directly on the virus. However, pretreated inoculum produced the lowest yields of all 5 sets. Soybean trypsin inhibitor was not removed from the system in this set. Potential deleterious effects of the inhibitor on the host cells or virus were not examined, but may have contributed to the low yield.

From these studies it can be concluded that the infectivity of BCV is dependent upon the host cell and trypsin modification. Trypsin is a constituent enzyme of the intestinal tract, the site of BCV replication in the natural host. Therefore, it may play a role in activation of virulent virus in natural infections. Factors causing variance in BCV
replication between cell types are unknown and must be assessed to fully understand the mechanism of BCV infectivity in culture. Frana and coworkers (37) found the murine coronavirus MHV-A59 to be dependent on the host cell or trypsin treatment for activation of the cell-fusing activity of the virus. Proteolytic activation of infectivity has been well documented in paramyxoviruses, orthomyxoviruses, and rotaviruses as well (25,35,39,51-54,74,92,99,106).
Figure V. 1. Infectivity Ratios of BCV-L9 Propagated in D2BFS Cells With or Without Trypsin
Figure V. 2. Infectivity Ratios of BCV-L9 Propagated in HRT-18 Cells With or Without Trypsin
Figure V. 3. Infectivity Ratios of BCV-L9 Propagated in Clone 3F3 With or Without Trypsin
Figure V. 4. Infectivity Ratios of BCV-L9 Propagated in Clone D2 With or Without Trypsin
Figure V. 5. Infectivity Ratios of BCV-L9 Propagated in Clone 3E3 With or Without Trypsin
Figure V. 6. Infectivity Ratios of BCV-L9 Propagated in Clone 4B3 With or Without Trypsin
Figure V. 7. Infectivity Ratios of BCV Strain LY-138
Propagated in HRT-18 Cells With or Without Trypsin
Figure V. 8. Infectivity Ratios With to Without Trypsin for BCV-L9 Propagated in each Cell Type at the Limiting Dilution of Virus.
Figure V. 9. Infectivity Ratios With to Without Trypsin for BCV-L9 Propagated in each Cell Type.
Figure V. 10. Yields of Infectious Virus Produced in D2BFS Cells Using Different Trypsin-Treatment Schemes
CHAPTER VI. EFFECT OF THE HOST CELL AND TRYPsin TREATMENT ON THE
POLYPEPTIDE PROFILE OF BCV-L9

IV.A. INTRODUCTION

Proteolytic cleavage of inactive precursor polypeptides of assembled
virions into an active form is necessary for inducing the infectivity of
a number of viruses. Notably, orthomyxoviruses and paramyxoviruses
require cleavage of envelope proteins for the viruses to become
infectious (24,25,39,51,52,54,92,93). Such findings are emerging for
coronaviruses as well. Processing of the viral proteins is accomplished
either by cellular proteases or by exogenous proteases, such as trypsin.

Franca et al (37) found that processing of the 180 Kd glycoprotein of
MHV-A59 was host cell-dependent and affected the functional capacity of
the virus. Those cells which fully cleaved the 180 Kd protein to its 90
Kd subunits activated rapid cell fusion. Cells which cleaved the
protein incompletely required trypsin to activate rapid cell fusion.
Trypsin enhances the replication and cytopathic expression of bovine
coronaviruses. In Chapter IV it was shown that fusion of BCV-L9-
infected D2BFS cells required trypsin, but the protein responsible for
cell fusion has not been determined.

Williams and Storz (133) detected two phenotypes of virus when
BCV-L9 was purified from HRT-18 cells by isopycnic centrifugation in
sucrose or CsCl gradients. The dense and light forms of the virus had
densities of 1.255 and 1.223 g/cm³, respectively. The two virus forms
had distinct polypeptide profiles, and the light form was converted to
the dense form by in vitro trypsin treatment.

There were three objectives to this investigation. The first
objective was to assess putative cell-dependent differences in the
protein composition of BCV-L9. The virus was raised in HRT-18 parent cells, HRT-18 clones 3F3, D2, 3E3, and 4B3, and in D2BFS cells, since these cells display different cytopathic effects when infected with the virus. The second objective was to compare the protein profiles of in vitro trypsin-treated or untreated L9 propagated in HRT-18 cells. The profiles were examined under reducing and non-reducing conditions to assess the effect of the enzyme on the viral polypeptides. Assessment of the structural proteins of BCV-L9 propagated in D2BFS cells in the presence or absence of trypsin by Western blotting was the third objective. Cell fusion occurs with infected D2BFS cells only in the presence of trypsin. This technique was employed to identify the viral protein(s) responsible for cell fusion.

VI.B. MATERIALS AND METHODS

VI.B.1. Cells and Virus

HRT-18 parent cells and four HRT-18 clones, 3F3, D2, 3E3, and 4B3 were grown in 850 cm² Corning roller bottles or in 150 cm² flasks. When seeded in roller bottles, DMEM containing 25 mM Hepes and 12 mM NaHCO₃ was used to stabilize pH. After 1 day incubation at 37°C, this medium was replaced with standard DMEM (described in Chapter III). D2BFS cells were grown in MEM in 750 cm² glass roller bottles. BCV-L9 was the virus strain used in these studies.

VI.B.2. Virus Propagation and Purification

Cells were infected with BCV-L9 at an MOI of 0.01-0.1 PFU/cell. Following adsorption for 1 h at 37°C, excess inoculum was removed and cells were washed three times with Dulbecco's PBS. Medium was added, and cells were incubated at 37°C. MEM containing 5 µg/ml trypsin was
added to bottles of infected D2BFS cells. When CPE affected
approximately 80% of the monolayer, cells were frozen at -70°C. Bottles
were thawed; the infected material was pooled into 250 ml centrifuge
bottles, sonicated, and refrozen.

Virus was purified from thawed cell lysate according to a
modification of the procedure described by Wege et al (128). All steps
were done at 4°C. NaCl was added to the collected fluid to a final
concentration of 0.5 M. Culture fluid was clarified by centrifugation
at 10,000 x g for 20 min. 30% polyethylene glycol - 8000 was added to
the clarified supernatant to a final concentration of 10%, and mixed on
ice for 10 min. The resulting precipitate was sedimented by
centrifugation at 10,000 x g for 30 min. The precipitate was collected
in TNE buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.001 M EDTA) pH 7.4,
resuspended by sonication, and dispersed by Dounce homogenization. The
slurry was centrifuged at 1000 x g for 10 min. to remove aggregates.
The suspension was centrifuged through a continuous 5-20% sucrose-TNE
gradient onto a 4 ml 60% cushion at 70,000 x g for 2 h. The band at the
20-60% interface was collected, diluted to less than 20% with TNE
buffer, and layered onto a continuous 20-60% sucrose-TNE gradient.
Tubes were centrifuged at 55,000 x g for 16-18 h. Gradient fractions
were collected by puncturing the bottom of the centrifuge tubes.
Virus-containing bands were identified by hemagglutinating activity.
Fractions containing the highest HA titers were pooled and concentrated
by pelleting through a 5 ml 20% sucrose cushion for 2 h at 90,000 x g.
Virus was resuspended in TNE buffer. The virus suspension was layered
onto a preformed CsCl-TNE gradient (1.0606 - 1.2886 g/cm³) and
centrifuged at 55,000 x g for 20 h to further purify the virus. Bands were collected as described above, concentrated, and resuspended in TNE.

VI.B.3. Partial Purification or Concentration of Virus.

Partially purified or concentrated virus was of adequate purity for immunoblot analyses. Virus was propagated in HRT-18 clones grown in roller bottles or 150 cm² flasks. To partially purify the virus, centrifuge bottles of pooled cell lysate were thawed, then clarified at 10,000 x g for 20 min. at 4°C. Supernatant fluid was loaded onto continuous 5-20% sucrose-TNE gradients and centrifuged at 70,000 x g for 2 h. The resulting pellet was resuspended in TNE buffer, loaded onto continuous 20-60% sucrose-TNE gradients, and centrifuged at 55,000 x g for 16-20 h. Bands were collected, concentrated, and resuspended as described.

To minimize loss of virus, L9 was directly concentrated from D2BFS cells. D2BFS cells in 150 cm² flasks were infected with BCV-L9 at an MOI of approximately 0.1 PFU/cell. After adsorption, cells were washed well to remove unadsorbed virus. MEM was added to one set of flasks, and MEM with 5 μg/ml trypsin (Difco 1:250) was added to the other set. Flasks were incubated at 37°C for 3 days, then frozen, thawed, and the cell lysate was pooled. Fluid was clarified at 10,000 x g for 20 min. to remove cell material. The supernatant was centrifuged through a 5 ml 20% sucrose-TNE cushion for 2 h at 90,000 x g. The pellet was resuspended in TNE, concentrated, and resuspended as described.

VI.B.4. Preparation of Uninfected Cell Antigen for Western Blots

Washed cells in 150 cm² flasks were frozen at -70°C, then thawed, and cell material was collected in centrifuge tubes. Cell material was
sonicated for 1 min., then the suspension was centrifuged at 1000 x g for 15 min. to settle nuclei. The supernatant was centrifuged at 90,000 x g for 2 h, and the pellet was resuspended in TNE buffer.

VI.B.5. In Vitro Trypsin Treatment

Aliquots of partially purified virus preparations were treated with 1 ug/ml of highly purified trypsin (Sigma, TPCK treated) in Dulbecco's PBS at 37°C for 30 min. The action of trypsin was stopped by the addition of 1 ug/ml soybean trypsin inhibitor (Sigma) and incubation at 4°C for 30 min. The virus suspension was centrifuged through a 2 ml 20% sucrose-TNE cushion at 90,000 x g for 2 h. Pellets were resuspended to original volume in TNE.

VI.B.6. Protein Quantitation

Protein concentrations were initially determined using the protein-dye binding technique developed by Bradford (10). The BioRad Automated Protein Assay was employed in later experiments because this technique required less sample to quantitate the protein. In addition, the procedure was simpler to perform, and the results were read more rapidly.

VI.B.7. Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE of virus samples was done using the Laemmli buffer system (65). Samples were dissociated by diluting 1:2 or 1:3 in buffer containing β-mercaptoethanol, a reducing agent, or in the same buffer lacking the reducing agent, then boiling for 5 min. Gels containing 10% or 12% bis-acrylamide were prepared in 1 mm thicknesses. Gels were run at a constant power of 4 watts/gel (approximately 25 mA/gel) for 3-4 h.
BioRad low and high molecular weight standards were used: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase B (92,500), β-galactosidase (116,250), myosin (200,000).

Proteins were visualized using the BioRad silver stain kit developed according to the procedure of Merrill et al (86). The Coomassie blue protein stain was used in combination with the silver stain to identify proteins which did not stain with silver.

VI.B.8. Immunoblot Procedures

Immunoblots (Western blots) were done according to the procedure of Burnette (16). Briefly, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using Tris-glycine transfer buffer (20 mM Tris base, 150 mM glycine), pH 8.3. Transfer was essentially complete after 18-20 h at 35 volts at approximately 10-15°C. Transferred proteins were stained with Ponceau S (91), and molecular weight markers were cut off and saved for use in determining molecular weights of viral proteins. Viral proteins were detected as follows: Nitrocellulose membranes were immersed for 1-4 h in blocking solution consisting of 5% Blotto (60) in Tris buffered saline (TBS) consisting of 10 mM Tris base and 0.9% NaCl, pH 7.4. Primary antibody consisting of anticontrolavirus serum, obtained from an experimentally infected calf, in TBS with 1% Blotto was added and allowed to incubate 16-18 h. Excess antibody solution was removed, and nitrocellulose was washed three times for 5 min. each using TBS containing 0.05% Tween 20. Goat antiovine IgG (H + L chain), peroxidase conjugated, was obtained from Kirkegaard and Perry Laboratories and diluted 1:5 in ELISA PBS + 0.1% human
albumin. The conjugate was diluted to a final concentration of 1:1000 in TBS, added to the membrane, and incubated for 2 h. The membrane was washed as described, and substrate consisting of 15 mg 4-chloronapthol in 5 ml methanol, 25 ml 0.05 M Tris pH 6.8, 12 ul H₂O₂ was added. After optimal color development had occurred, the reaction was stopped by rinsing with distilled water.

VI.C. RESULTS

VI.C.1. Comparison of the Polypeptide Structure of BCV-L9 Propagated in Different Cells

The polypeptide composition of purified BCV-L9 propagated in D2BFS cells with trypsin or in HRT-18 cells is revealed in Figure VI.1. Proteins were visualized by double staining with silver followed by Coomassie blue. Coomassie blue was used to stain proteins which did not stain with the silver reagent. The profiles are compared in Table VI.1. Common proteins of 140, 100, 52, 37, 34, 26, and 18-23 Kd were present under non-reducing conditions, and a 62 Kd protein was present in D2BFS-propagated virus. Distinctly different profiles were obtained under reducing conditions. In addition to common proteins of 95, 65, 52, 37, 34, 26 and 18-23 Kd, L9(HRT-18) had a 46 Kd protein that was not observed in L9 (D2BFS). The reduced profile of L9 (D2BFS) had unique proteins of 93, 71, and 62 Kd.

The protein composition of partially purified BCV-L9 from HRT-18 cells and concentrated L9 from D2BFS cells without trypsin was detected by Western blots (Fig. VI.2). This technique allowed the detection of high molecular weight proteins not observed by silver staining, since higher concentrations of virus were used with the immunoblot procedure.
The structural proteins of L9 produced in the two cell lines are compared in Table VI.2. Common proteins of 185, 140, 100, 52, 37, and 31-34 Kd were observed. Notable differences included the following: A >200 Kd protein was found in D2BFS-produced virus, but not in virus obtained from HRT-18. This protein may be an uncleaved precursor or an aggregate of viral proteins. A 160 Kd protein was present in the L9 (D2BFS) profile but not L9 (HRT-18). However, this protein was present in the profile of L9 obtained from other HRT-18 parent and clone preparations, and probably was not detected due to the concentration of sample on the gel. Interestingly, the 185 Kd protein was present in high concentration in L9 (D2BFS), but in proportion to the other proteins comprising the profile, it was present in low concentration in L9 (HRT-18). The 46 Kd protein was not detected in the HRT-18 preparation in this instance.

L9 was propagated in the four HRT-18 clones, partially purified, and the proteins were separated by SDS-PAGE. Immunoblotting was the method used to detect these proteins (Fig. VI.3). Under non-reducing conditions common proteins of 185, 160, 140, 125, 100, 52, 46, 37, 31-34, and 26-28 Kd were found in virus produced in clones 3F3, D2, and 3E3 (Table VI.3). The 46 Kd protein was absent in L9 prepared from clone 4B3. Doublet bands of 110 and 100 Kd were found with L9 obtained from clones 3F3, 3E3, and 4B3, whereas only a single 100 Kd band was present in L9 from clone D2. The profiles of L9 propagated in clones D2 and 3F3 are compared in Figure VI.4. to better illustrate this finding.
VI.C.2. **Effect of Reducing Conditions on the Polypeptide Profile of BCV-L9**

Proteins of 140 and 100 Kd were consistently present in BCV-L9 preparations under non-reducing conditions. The silver staining technique revealed that these proteins disappeared under reducing conditions with the concomitant appearance of 95 and 65 Kd proteins (Figs. VI.1 and 6). Three distinct bands of 23, 20, and 18 Kd were converted to a cluster of bands ranging from 20-23 Kd by reducing agent (Fig. VI.6).

The effect of reducing agent on the L9 profile was also examined by Western blot, but this method failed to detect all reduced products accurately (Fig. VI.3). The proteins of 185, 160, 140, 125, 110, and 100 Kd were not seen under reducing conditions and proteins of 190 and 65 Kd were evident (Table VI.3).

VI.C.3. **Effect of Trypsin on BCV-L9 Polypeptides**

BCV-L9 was propagated in D2BFS cells in the presence and absence of trypsin. Cell lysates were clarified and concentrated, and the viral proteins were separated by SDS-PAGE under non-reducing conditions. Following their transfer to a nitrocellulose membrane, viral proteins were visualized by Western blotting (Fig. VI.5.). Virus propagated under trypsin-free conditions had the following profile: >200, 185, 140, 100, 52, 46, 37, and a 31-34 Kd cluster. The 185 Kd protein was only faintly visible, the 160 Kd protein was not apparent, and the 100 Kd protein was present in higher concentration in L9 produced in D2BFS in the presence of trypsin. An additional difference between the profiles was the absence of the 31-34 Kd cluster in trypsin-treated virus.
L9 was propagated in HRT-18 cells and purified, then treated in vitro with trypsin. The profiles of untreated and trypsin-treated L9 were examined under non-reducing and reducing conditions (Fig. VI.6). The structural proteins observed under these conditions are compared in Table VI.4. In the absence of reducing agent, the profiles were identical. Detectable bands had molecular weights of 140, 100, 52, 27, 23, 20, and 18 Kd. Differences in the electrophoretic migration of trypsin-treated and untreated viral proteins were evident only under reducing conditions. A 95 Kd protein was found in untreated virions, while a 90 Kd protein was found in trypsin-treated virions. Additionally, a cluster of 20-23 Kd proteins was found in untreated virions, but this cluster ranged from 19-23 Kd with a 17 Kd protein in trypsin-treated virions.

BCV-L9 propagated in clones 3F3 and 4B3 was treated in vitro with trypsin and the profiles of trypsin-treated and untreated virus were compared under non-reducing conditions (Fig. VI.7). The profile of L9 from clone 4B3 was unchanged with trypsin treatment. Trypsin-treated L9 from clone 3F3 lacked the 46 Kd protein present in untreated virus. In addition, a band of approximately 25 Kd appeared. The profiles of trypsin-treated and untreated L9 from clone 3F3 were also compared under reducing conditions (Fig. VI.8). Again the 46 Kd protein was absent from trypsin-treated virions, and the 25 Kd protein was evident. The effect of trypsin on the high molecular weight proteins was undetermined since these proteins were not readily detectable under reducing conditions. The effect of trypsin on the non-reduced profiles of L9 in D2BFS cells, clone 3F3, and clone 4B3 are compared in Table VI.5.
VI.D. DISCUSSION

Host cell-dependent differences in the polypeptide profile of BCV-L9 were demonstrated in this investigation. Notably, a 62 Kd protein was evident by silver stain in a preparation of L9 propagated in D2BFS cells with trypsin. Trypsin was used in the propagation to increase the yield of virus from D2BFS cells. The 62 Kd protein may represent a cell-specific viral protein or a trypsin cleavage product. Western blot analyses of L9 (D2BFS) preparations failed to detect this protein. A 110-100 Kd doublet band was detected in L9 propagated in clones 3F3, 3E3, and 4B2, but only the 100 Kd species was evident from clone D2. This difference may reflect a variation in processing or in glycosylation of the protein. A major protein of 46 Kd was evident in virus raised in all cells except clone 4B3, which lacked the protein. The function of this protein species is unknown, but the absence of the protein may be the reason that relatively mild cytopathic expression occurs in infected clone 4B3 (Chapters 3 and 4).

Storz and coworkers (unpublished results) detected host cell-dependent differences in the polypeptide profile of BCV-L9 by radioimmune precipitation. The virus was propagated in different cell types in medium containing \[^{14}C\] - labeled amino acids or \[^{35}S\] - methionine. The proteins detected when the virus was propagated in HRT-18 cells included 180, 82, 47, and 35 Kd species. Using \[^{14}C\] - labeled amino acids, BCV from bovine fetal thyroid cells had proteins of 140, 100, 70, 52, and 47 Kd, while viruses propagated in the same cells in the presence of \[^{35}S\] - methionine had an 80 Kd migrating protein species instead of the 70 Kd species. BCV propagated in bovine fetal...
brain cells in the presence of $[^{14}\text{C}]$-labeled amino acids or $[^{35}\text{S}]$-methionine had a composite profile of 140, 100, 60, 52, and 47 Kd.

Silver-stained gels revealed that the 140 and 100 Kd protein that were present under non-reducing conditions disappeared under reducing conditions. At the same time, proteins of 95 Kd and 65 Kd appeared in the presence of reducing agent. Hogue and coworkers (46) found that the 140 Kd species consists of disulfide-linked subunits of 65 Kd. They did not observe a change in the electrophoretic migration of the 100 Kd species under reducing conditions. The 18, 20, and 23 Kd group of proteins was converted to a 20-23 Kd cluster by reduction. Western blotting also revealed a change in the electrophoretic mobility of the 185 Kd protein to 190 Kd under reducing conditions. Rather than consisting of disulfide-linked subunits, the 185 and 100 Kd molecules and the 18-23 Kd group of proteins probably contain intrachain disulfide bonding. In this case, reduction may alter the migration pattern by breaking the intrachain bonds and allowing the proteins to be coated by SDS more efficiently. Consequently, the conformation of the intact proteins would be different under reducing and non-reducing conditions, which would influence the electrophoretic migration.

Silver staining was a superior method to Western blotting for the detection of reduced L9 proteins. An extended reaction time in the substrate was required to visualize reduced proteins by Western blotting. The long incubation in substrate led to a high background and made it difficult to discern the viral protein bands. It was impossible to determine the relationship between non-reduced and reduced proteins by this method. Since Western blotting depends on the antigenicity of
proteins, it is likely that the viral proteins were antigenically altered when their conformation was changed by reduction.

The non-reduced form of virus is the disease agent in natural infections of calves. Therefore, it was considered more important to examine the non-reduced profile of BCV. The use of antiserum obtained from an experimentally infected calf in Western blot procedures indicated the antigenically active components of the virus.

Interestingly, the action of trypsin on the virus structure was evident by silver stain only under reducing conditions. This finding indicates that trypsin-cleaved peptides were held intact by disulfide bonds. When reduced by B-mercaptoethanol, cleavage products were separable. Unique proteins of 93, 71, and 62 Kd were detected in preparations of L9 from D2BFS cells + trypsin. Trypsin-dependent bands of 90 Kd and 17 Kd were detected in L9 (HRT-18), and the 20-23 Kd cluster found in untreated virions was converted to a 19-23 Kd cluster by trypsin (Table VI.4). In vitro trypsin-treated and untreated L9 from clone 4B3 gave identical profiles. Trypsin-treated L9 from clone 3F3 differed from untreated virus - the 46 Kd protein was not present in treated virions, but a 25 Kd protein was found. The 46 Kd protein must be a surface component since it was accessible to trypsin. The relationship between the 46 and 25 Kd proteins has not been determined conclusively, but the 25 Kd protein may be a cleavage product of the 46 Kd species. Trypsin greatly enhances the cytopathic expression of L9 in clone 3F3, but has little effect on the cytopathic changes of infected 4B3 cells (Chapters III and IV). The cytopathic changes may be a reflection of the trypsin-induced changes in polypeptide structure observed here.
The effect of trypsin on the polypeptide structure of L9 propagated in D2BFS cells was examined because these cells fuse only in the presence of trypsin. The intent was to identify the viral protein(s) responsible for cell fusion. Under non-reducing conditions the 185 Kd protein was present in high concentration in the absence of trypsin, but only a faint band was present in virus produced with trypsin. The 160 Kd protein was barely evident without trypsin and not seen with trypsin. Concomitantly, the concentration of the 100 Kd species was much higher in the presence of trypsin. No additional proteins were found in virus propagated with trypsin, but trypsin cleavage may have affected the antigenicity of some proteins so that they were undetectable by Western blotting. For example, silver staining detected a 62 Kd protein when L9 was propagated in D2BFS cells with trypsin, but the protein was not evident by Western blot detection. These data strongly suggest that cleavage of the 185 Kd protein to the 100 Kd form and/or other undetected smaller species is required for activation of cell fusion.

In Fig. VI.2. the profiles of the virus raised in HRT-18 and D2BFS are compared. The 185 Kd species is present in proportionately low concentration in L9 (HRT-18) as compared to L9 (D2BFS). The protein was also found in low concentration in virus obtained from the four clones. Evidently, cleavage of the 185 Kd protein occurs by a cellular protease in HRT-18 cells, while cleavage is less efficient in D2BFS cells. These cells require trypsin to cleave this protein and activate fusion. This finding is analogous to the MHV model. With MHV-A59 a 180 Kd protein must be cleaved by a cellular protease or by trypsin to activate the fusing capacity of the virus (37,119).
It appears that clone 4B3 is capable of cleaving the 185 Kd protein (Fig. VI.2), but this cell line does not fuse. The lack of fusion in clone 4B3 may be a result of the composition of the plasma membrane of these cells or other cellular characteristics, rather than the manner in which these cells process the virus.

The 160 Kd protein may also be cleaved by trypsin since it was not evident in the presence of trypsin. However, the concentration of this protein was so low in the absence of trypsin that its cleavage would not have caused the dramatic increase in concentration of the 100 Kd species. It is possible that cleavage products of the 160 Kd protein were not detectable. In addition, a 31–34 Kd group of proteins was found in the absence of trypsin, while a single 34 Kd species was found in virus produced with trypsin. These proteins must represent external proteins since they were accessible to the enzyme.

Western blot detection allowed the identification of at least eleven BCV-L9 structural proteins from HRT-18 cells. The proteins include the following approximate molecular weight species: 185 Kd, 160 Kd, 140 Kd, 125 Kd, 110 Kd, 100 Kd, 52 Kd, 46 Kd, 37 Kd, a 31–34 Kd group, and a 26–28 Kd cluster. The 52 Kd species was the predominate protein. The 140 and 125 Kd proteins were closely migrating species, as were the 110 and 100 Kd proteins. When these proteins were present in high concentration, as with L9 (D2BFS) (Figs. VI.2. and 5), or when poor separation of high molecular weight proteins occurred, these proteins were not resolved as individual species. Rather, single, broad bands of approximately 140 and 100 Kd were detected.

A group of proteins of 18–23 Kd was apparent on silver-stained 12% gels under non-reducing conditions, while a concise cluster of 20–23 Kd
was detected on reduced gels. These proteins were not detected on 10% gels by Western blot. The 26-28 Kd proteins detected by Western blot and the 18-23 Kd proteins detected by silver stain may represent the same proteins with different migration patterns in 10 and 12% gels. Hogue and coworkers (46) used a 5-15% gradient gel to pinpoint the molecular weight of this cluster at 26 Kd.

The polypeptide profile of BCV-L9 propagated in HRT-18 cells was also examined by other investigators (46,63,64,133). The structural proteins reported by these investigators, as well as those identified in this study are compared in Table VI.6. David Brian's group detected high molecular weight species of 190, 140, 120, and 100 Kd. These proteins were identified as surface glycoproteins, with gp 140 serving as hemagglutinin (64). The investigators found that the 140 Kd species consisted of disulfide-linked subunits of 65 Kd, a finding supported by this investigation. Hogue and coworkers (46) observed a 160 Kd band which reacted with antiserum against pp 52. They suggest that this protein represents a trimer of the 52 Kd species. Williams (133) reported high molecular weight proteins of 182 and 109 Kd in light virions and 172 and 94.5 Kd in dense virions. Both profiles were obtained under reducing conditions, making it difficult to compare these profiles to those obtained in the present study.

A 52-53 Kd major protein was identified in all investigations of BCV structure (46,63,64,133). This protein is an internal phosphoprotein which probably serves as nucleocapsid. The nucleocapsid of nearly all coronaviruses is a phosphoprotein of 48-52 Kd.

A cluster of proteins of approximately 26 Kd was reported by the three groups of investigators. King et al (64) demonstrated that this
molecule is a surface glycoprotein, probably serving as a matrix protein. The clustering of the protein presumably indicates glycosylation isomers with slightly varying molecular weights. The matrix protein of most coronaviruses is a glycoprotein of 20-29 Kd.

Additional proteins of 46, 37, and 31-34 Kd were also observed. The functions of these protein are unknown. The 46 and 31-34 Kd proteins were cleaved by trypsin, indicating that they are external proteins. Williams (133) reported common proteins of 47 and 35 Kd in dense and light virions. A 20.5 Kd species was detected in light virions, and 21 and 15 Kd species were identified in dense virions. Similar proteins were detected in this study when the profiles were examined by silver stain. Dense and light forms of L9 were not separated in this investigation. Therefore, these analyses represent a composite of both the dense and light profiles, probably contributing to the greater complexity of the profiles detected in this study.

The MHV virion was thought to consist of only three structural proteins originally - a 180 Kd peplomeric glycoprotein, a 50 Kd nucleocapsid phosphoprotein, and a 23 Kd matrix glycoprotein. Recently, Sturman and coworkers (119) demonstrated that the 180 Kd protein is actually comprised of two 90 Kd subunits, 90A and 90B, that are distinctly different species. Obviously, MHV is a more complex virus than originally described, and further examination may reveal even greater complexity. In contrast, our investigation revealed at least eleven structural proteins for BCV-L9. The protein profiles depended on the host cell used to propagate the virus and the detection system applied. For example, proteins of greater than 140 Kd were not detected in silver-stained gels due to a low concentration of protein on the gel.
Conversely, the 140 and 125 Kd proteins appeared as a single band from D2BFS preparations since the high concentration of the proteins did not allow them to be resolved separately. There were differences in the BCV-L9 profile obtained in this investigation as compared to those reported by Williams (133), Hogue and coworkers (46), and King and coworkers (63,64). Obviously, the protein composition of this virus is still unsettled.
Table VI.1. Polypeptide Profile of BCV-L9 Purified from HRT-18 or D2BFS Cells as Detected by Silver Staining.

<table>
<thead>
<tr>
<th></th>
<th>L9 (HRT-18)</th>
<th></th>
<th>L9 (D2BFS + trypsin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>reduced</td>
<td>non-reduced</td>
<td>reduced</td>
</tr>
<tr>
<td>-</td>
<td>140</td>
<td>-</td>
<td>-</td>
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<tr>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>95</td>
<td>-</td>
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<td>-</td>
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<td></td>
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<td>18-23</td>
<td>18-23</td>
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Table VI.2. Comparison of the Structural Proteins of BCV-L9 Obtained from HRT-18 and D2BFS Cells under Non-reducing Conditions as Detected by Western Blotting.

<table>
<thead>
<tr>
<th>L9 (D2BFS)</th>
<th>L9 (HRT-18)</th>
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<td>52</td>
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<tr>
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<td></td>
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<td>37</td>
</tr>
<tr>
<td>31-34</td>
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</tbody>
</table>
Table VI.3.  Structural Proteins of BCV-L9 from HRT-18 Clones as Detected by Western Blotting.

<table>
<thead>
<tr>
<th></th>
<th>Non-reducing Conditions</th>
<th>Reducing Conditions</th>
</tr>
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<tbody>
<tr>
<td>D2 3E3 3F3 4B3</td>
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<td></td>
</tr>
<tr>
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<td>185 185 185 185</td>
<td>190 190 190 190</td>
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<td>160</td>
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<td>190 190 190 190</td>
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<td>110 110 110</td>
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<td>31-34 31-34 31-34 31-34</td>
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Table VI.4. Effect of Trypsin on the Polypeptide Profile of BCV-L9 Propagated in HRT-18 Cells as Detected by Silver Stain.

<table>
<thead>
<tr>
<th>Trypsin (-)</th>
<th>Trypsin (+)</th>
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<td>Non-reduced</td>
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<tr>
<td>-</td>
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Table VI.5. Effect of Trypsin on the Non-reduced Polypeptide Profile of BCV-L9 Propagated in Different Cells as Detected by Western Blotting.

<table>
<thead>
<tr>
<th></th>
<th>L9 (D2BFS)</th>
<th>L9 (3F3)</th>
<th>L9 (4B3)</th>
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</thead>
<tbody>
<tr>
<td>Trypsin(-)</td>
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<td>Trypsin(-)</td>
<td>Trypsin(+)</td>
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* Increased in concentration
Table VI.6. Comparison of Reported BCV-L9 Profiles from HRT-18 Cells.

<table>
<thead>
<tr>
<th></th>
<th>Williams (133)</th>
<th>Brian (46, 63, 64)</th>
<th>St. Cyr-Coats (immunoblot detection)</th>
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<td>dense virions</td>
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<td>reducing conditions</td>
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<td>22-26</td>
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<td>26-28</td>
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<tr>
<td>20.5</td>
<td>21</td>
<td>15</td>
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</table>
Figure VI.1. Polypeptide Profiles of BCV-L9 Propagated in D2BFS Cells (+ trypsin) or HRT-18 Cells.

Proteins were detected by silver and Coomassie staining. 12% acrylamide. Virus in lanes B and C was prepared with reducing agent. Lanes E and F contained non-reduced virus. Lanes A, D, G - molecular weight standards, Lanes B and E - L9 (HRT-18), lanes C and F - L9 (D2BFS). Arrows indicate additional proteins.
Figure VI. 2. Polypeptide Profiles of BCV-L9 Propagated in D2BFS Cells and HRT-18 Cells as Detected by Western Blotting. Non-reduced. 10% acrylamide.

Lane A - L9 (HRT-18), lane B - L9 (D2BFS), lane C - uninfected D2BFS cells. Arrows indicate differences in the profiles.
Figure VI. 3. Structural Proteins of BCV-L9 Propagated in HRT-18 Clones as Detected by Western Blotting.

10% acrylamide. Lanes A-E contained non-reduced virus. Lanes F-J contained reduced virus.

Figure VI. 4. Structural Proteins of BCV-L9 Propagated in HRT-18 Clones D2 and 3F3 as Detected by Western Blotting. Non-reduced. 10% acrylamide. Lane A - L9 (D2), lane B - L9 (3F3). Arrows indicate differences in the profiles.
Figure VI. 5. Polypeptide Profiles of BCV-L9 Propagated in D2BFS Cells in the Presence and Absence of Trypsin as Detected by Western Blotting. Non-reduced. 10% acrylamide. Lane A - (-) trypsin, lane B - (+) trypsin, lane C - uninfected D2BFS cells. Arrows indicate structural differences.
Figure VI. 6. Structural Profiles of In Vitro Trypsin-Treated and Untreated BCV-L9 Propagated in HRT-18 Cells as Detected by Silver Staining. 12% acrylamide. Lanes A and B contained non-reduced virus. Lanes C and D contained virus prepared with reducing agent. Lanes A and C - trypsin-treated virus, lanes B and D - untreated virus, lanes E and F - molecular weight standards.
Figure VI. 7. Western Blot-Detected Protein Profile of In Vitro Trypsin-Treated BCV-L9 Produced in Clones 3F3 and 4B3. Non-reduced. 10% acrylamide. Lane A - untreated L9 (4B3), lane B - trypsin-treated L9 (4B3), lane C - trypsin-treated L9 (3F3), lane D - untreated L9 (3F3). Arrows indicate structural differences.
Figure VI. 8. Western Blot-Detected Protein Profile of In Vitro Trypsin-Treated BCV-L9 Produced in Clone 3F3. Reducing Conditions. 10% acrylamide.
Lane A - uninfected 3F3 cells, lane B - untreated L9 (3F3), lane C - trypsin-treated L9 (3F3). Arrows indicate structural differences.
CHAPTER VII. SUMMARY AND PERSPECTIVES

Turbid plaques consisting of some apparently normal cells are produced by BCV-L9 in HRT-18 monolayers. This observation suggests that the HRT-18 cell population is heterogeneous and consists of subpopulations of cells with different susceptibility to BCV infection. Immunofluorescent analysis of these plaques revealed that many cells within the plaque do not express viral antigen, indicating that some of the cells within the plaque are not infected by virus.

Four HRT-18 cell clones, 3F3, D2, 3E3, and 4B3, were characterized according to morphology, growth kinetics, plating efficiency, and growth in soft agar. Cell monolayers examined by phase-contrast microscopy exhibited distinct cell orientations which produced unique surface topographies and patterns within the monolayers. These features were consistent and reproducible with subpassage. Giemsa-stained cultures revealed some variation in colony development at 24 h, with essentially confluent monolayers at 72 h.

The kinetics of growth were determined from the growth curve of each clone. The doubling times were distinct for each clone, but the generation times were similar for clones D2, 3F3, and 4B3. Clone 3E3 displayed a longer generation time. A saturation density of approximately 8 million cells was achieved by clones D2, 3E3, and 4B3, but was about 1 million cells fewer for clone 3F3. The plating efficiencies of clone 3F3 and D2, 100% and 77%, respectively, were significantly higher than those of clones 4B3 and 3E3, which were 50% and 33%, respectively. No significant differences were found in the cells' ability to form colonies in soft agar, and efficiencies of 23%, 17%, 17%, and 15% were found for clones 3F3, 4B3, 3E3, and D2,
respectively. The ability to form colonies in soft agar indicated that all clones had transformed characteristics.

The growth kinetics of clone 4B3 closely resembled those of the HRT-18 parent cells at 55th passage. However, clone 3F3 acted most like the HRT-18 parent cells at low passage (122). This finding may represent the dominant influence of one cell type over the others in the parent population at different passage levels.

HRT-18 cell clones were established by limiting dilution in an attempt to isolate cell types which were either refractory or highly susceptible to BCV infection. The clones displayed differences in the type and degree of cytopathic expression when infected with BCV strain L9. Clone 3F3 produced extensive CPE, while clones D2 and 3E3 produced moderate CPE. In all three cases cytopathic changes included cytoplasmic vacuolization and cell fusion. In contrast, only mild CPE occurred in infected clone 4B3, with no detectable cell fusion.

Based on these data it was concluded that the four HRT-18 clones are distinctly different cell populations. The basis for their differing susceptibility to BCV infection was not determined in this investigation.

The characterization of the HRT-18 cell clones could be expanded to include other parameters. For instance, the nucleic acid content of the clones at 24, 100, and 124 h after seeding is presently being examined by flow cytometry to assess putative differences between clones. The lack of cell fusion in BCV-infected clone 4B3 in the presence and absence of trypsin may reflect the cells' inability to fuse rather than the inactivity of the viral fusion factor. The components of the plasma membrane contribute to a cell's ability to fuse (109,110,111,112).
Therefore, analysis of the biochemical make-up of the plasma membrane of clone 4B3 in comparison to clone 3F3, which readily fuses when infected with BCV, would be useful in determining a possible influence of the cell membrane on the cells' ability to fuse when infected with BCV.

The effect of the virus strain, host cell, and trypsin modification on the cytopathic expression and plaque development of bovine coronavirus was analyzed. HRT-18 parent cells, HRT-18 clones 3F3, D2, 3E3, and 4B3, and D2BFS cells were used to assay the growth of five wild type BCV strains and strain L9. All virus strains replicated in HRT-18 cells, inducing cell fusion to different degrees. D2BFS cells were non-permissive to all wild-type BCV strains, but L9 replicated in these cells. Trypsin enhanced the cytopathic expression, activating cell fusion in D2BFS cells. The CPE varied in L9-infected HRT-18 clones. Trypsin enhanced CPE and cell fusion in clones 3F3, D2, and 3E3, but not in clone 4B3. The plaque development of strain L9 varied in the four clones. Plaques were readily produced in clone 3F3, and these were dramatically enhanced by trypsin. The diameter of the plaques increased significantly, plaques became less turbid and more distinct, and they developed more rapidly in the presence of trypsin. Plaques developed less efficiently in the other three clones, with trypsin enhancing plaque development only marginally. No plaques were produced in clone 4B3 cells infected with wild-type virus strains, but plaques developed in clone 3F3. Strains Meeker, Miller, Fisher, Calf 50, and LY-138 produced different plaque phenotypes in clone 3F3, and in all cases the plaques were enhanced by trypsin.

The effect of trypsin on the infectivity of BCV-L9 propagated in different host cells was analyzed. The virus was propagated in the
presence and absence of trypsin, and PFU:HA ratios at virus dilutions $10^{-2} - 10^{-6}$ were compared. The infectivity of the virus was enhanced to infinite proportions at high dilutions in both D2BFS cells and HRT-18 cells, but the increase was evident at a 100-fold lower dilution in D2BFS cells. Limited trypsin enhancement of infectivity occurred in clones D2, 3E3, and 3F3 which displayed PFU:HA ratios in the presence or absence of trypsin of 2:1, 3:1, and 5:1, respectively. Trypsin caused a decrease in infectivity of L9 propagated in clone 4B3. Additionally, strain LY-138 was assayed in HRT-18 parent cells. An infectivity increase of less than 1 log unit was obtained at the highest dilution of virus. These data indicate that the infectivity of BCV was affected by the strain of virus, host-dependent factors, and by trypsin.

Host cell-dependent differences in the non-reduced polypeptide profile of BCV-L9 were identified. A 62 Kd protein was evident by silver stain from L9 propagated in D2BFS cells with trypsin. This protein was not detected in L9 (HRT-18). The protein may be a cell-specific viral protein or a trypsin cleavage product. The 62 Kd protein was not detectable by Western blot. A 46 Kd protein was present in L9 produced in all cell types except clone 4B3. L9 propagated in clones 3F3, 3E3, and 4B3 had a doublet of 110 and 100 Kd proteins, but only a single 100 Kd band was detected in L9 (D2). The two species may represent glycosylation isomers, such as those found at 26 Kd, or different cleavage products, but their relationship is undetermined.

The 140 Kd protein was reduced to a 65 Kd species, an observation previously reported by Hogue and coworkers (46). Additionally, the 185 Kd protein migrated as a 190 Kd species, and the 100 Kd protein migrated as a 95 Kd species in the presence of reducing agent. Changes in the
electrophoretic migration of these proteins may reflect intrachain
disulfide bonding as discussed in the previous chapter. The 18, 20, and
23 Kd molecules were converted to a 20-23 Kd cluster under reducing
conditions. The relationship of reduced and non-reduced proteins was
not discernable by Western blot. The antigenic configuration was
probably altered by reducing conditions making the reduced products less
antigenic in the blotting procedure.

L9 propagated in D2BFS cells with and without trypsin were compared
under non-reducing conditions by Western blotting. Notable differences
in the two profiles included the following: The 185 Kd protein was
present as a broad band in virus produced without trypsin, but the
protein was nearly inapparent in L9 propagated with trypsin. Coincident
with the disappearance of the 185 Kd species was a significant increase
in the 100 Kd protein. The precursor-product relationship of the two
species must be examined either by tryptic peptide mapping or with
monospecific antiserum against the 185 Kd species. Since L9-infected
D2BFS cells fuse only in the presence of trypsin, cleavage of the 185 Kd
protein to the 100 Kd form probably activates the fusion factor, but
other undetected cleavage products, such as the 62 Kd protein, may be
involved in cell fusion. In addition, the 31-34 Kd cluster was present
as a single 34 Kd protein in the presence of trypsin, indicating that
these proteins are an external component since they were accessible to
trypsin.

Trypsin cleavage products of L9 propagated in HRT-18 cells or D2BFS
cells with trypsin were evident in silver-stained gels only under
reducing conditions. Evidently, the cleaved proteins were held intact
by disulfide bridges. Trypsin-dependent bands of 93, 71, and 62 Kd
were identified in the L9 (D2BFS + trypsin) preparations, and a 90 Kd trypsin-dependent band was obtained from L9 (HRT-18). The 93 and 90 Kd species are possibly the same protein which migrated differently on different gels. Trypsin also acted on the 20-23 Kd cluster seen in the L9 (HRT-18) preparation, converting it to the 19-23 Kd range.

In vitro trypsin-treated L9 obtained from clones 4B3 and 3F3 were compared to untreated virus. Changes in the polypeptide profile of L9 (4B3) were not detected. The 46 Kd protein was present in untreated L9 (3F3) but absent in trypsin-treated virions. Concomitantly, a 25 Kd protein appeared in the treated preparation. The precursor-product relationship of the 46 and 25 Kd proteins must be analyzed before a correlation between the two species can be determined.

At least eleven structural proteins of BCV-L9 were identified. These proteins had approximate molecular weights of 185, 160, 140, 125, 110, 100, 52, 46, 37, 31-34, and 26-28 Kd, but the profile of BCV-L9 showed differences depending on the host cell used for propagating the virus and the detection system employed. Hogue et al (46) and King and coworkers (63,64) identified high molecular weight glycoproteins of 190, 140, 120, and 100 Kd which are surface proteins. It was our experience that when the protein concentration was high on the gels or when poor separation of the proteins occurred, then the 160 Kd protein was not distinguishable from the 185 Kd protein; the 140 and 125 Kd proteins appeared as a single broad band that migrated at 140 Kd; and the 110 and 100 Kd proteins were not resolved and appeared as a single, broad 100 Kd band. Consequently, detection of all the viral proteins required an optimal protein load and running conditions.
The high molecular weight proteins identified by Williams (133) cannot be accurately compared to our profile since Williams performed his experiments under reducing conditions. The two virus phenotypes observed by Williams were not analyzed separately in our experimentation. Rather, our analysis represents a composite profile of the two BCV phenotypes, probably contributing to the greater complexity of our virus profile.

David Brian's group (46,63,64) identified the 140 Kd protein as the hemagglutinin and suggested that the 52 Kd and 26 Kd species are nucleocapsid and matrix proteins, respectively. Using anti-pp 52 antiserum in immunoblots, these investigators detected a 160 Kd species in some BCV preparations. They assumed that the protein represents a trimer of pp 52 (46). The functions of our additional proteins are undetermined. The location and functions of the constituent viral proteins could be determined by in vitro enzymatic iodination of intact and disrupted virions. With intact virions, only external proteins would be iodinated, while internal proteins would also be labeled using disrupted virions (63).

While it was important to examine the reduced profile of BCV to determine the subunit structure of the virus, the non-reduced virus is the form of virus which causes disease in the natural infection. Immunoblot detection of the non-reduced viral proteins was conducted using antiserum obtained from an experimentally infected calf. This technique allowed the identification of the immunogenic viral proteins, and provided information about the form of virus presented to and recognized by the host humoral immune system. It is possible that other viral proteins were not detected due to a loss of antigenicity through
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heat denaturation, SDS-treatment, transfer to nitrocellulose, or other procedures used to separate and analyze the proteins.

A hypothetical model of the structure of BCV-L9 is proposed based on the data obtained from these investigations and those previously reported (46,63,64,133) (Fig. VII.1). This model depicts the viral proteins identified under non-reducing conditions, since this form of virus is the infectious agent in nature.

In the infectious form the 185 Kd peplomeric protein is cleaved to 100 Kd. This study has revealed that processing of the 185 Kd protein occurs either by cellular proteases or by trypsin. HRT-18 cells and clones 3F3, 3E3, D2, and 4B3 were capable of efficient cleavage of the protein. Conversely, the 185 Kd protein was only partially cleaved in D2BFS cells, and trypsin was required for complete cleavage of the protein. As a result, cell fusion occurred in BCV-L9-infected D2BFS cells only in the presence of trypsin.

Brian's group (46,63,64) proposed that the 140, 120, and 100 Kd proteins were surface glycoproteins. Our 110 Kd molecule is probably a peplomeric protein as well. The protein may represent an additional cleavage product of the 185 Kd species, or the 110 and 100 Kd bands may be glycosylation isomers.

The nucleocapsid protein of BCV-L9 has a molecular weight of 52 Kd. Hogue and coworkers (46) suggested that the 160 Kd protein is a trimer of this species since it cross reacts with monospecific antiserum directed against pp 52. The 26-28 Kd cluster presumably represents glycosylation isomers of the matrix protein. The 31-34 Kd cluster may also comprise the matrix.
The 140 Kd protein consists of disulfide-linked subunits of 65 Kd (46,63,64). Our data indicates that intrachain disulfide linkages are present in the 185, 100, 26-28, and 31-34 Kd proteins.

Undefined proteins of 46 and 37 Kd were identified. The 46 Kd protein is probably an external protein since it was cleaved by trypsin in some preparations. Trypsin had no effect on the electrophoretic migration of the 37 Kd species. Although the data are insufficient to accurately position these proteins, they have been incorporated into the hypothetical model.

There is tremendous diversity in the recognized polypeptide composition of different coronaviruses, and the complexity of these viruses is emerging. For instance, three structural proteins were originally identified for MHV, but four proteins are now accepted. A number of coronaviruses are considered to consist of four proteins including DVIM, HCV-OC43, TGEV, and others (41,46,120), while up to nine proteins are found with HECV-24 and 35 (5). As demonstrated in these investigations, the structural composition of bovine coronaviruses is far from settled. Inevitably, the accepted profiles of many coronaviruses will change as experimentation progresses.

The data presented in this manuscript have significant implications for the continuing study of coronavirus-host relationships, virulence of BCV, and prevention of disease. Cell types with different susceptibility to BCV infection were identified. Varied cytopathic expression of the virus in the HRT-18 clones and D2BFS cells were correlated to differences in the structural profile of the virus. Proteins which potentially contributed to cytopathic expression and infectivity of the virus were identified.
The different cell types could be further examined to identify cellular factors, such as proteases, which contribute to the production of infectious virus. At the same time, cellular resistance factors could be determined.

Trypsin enhanced the CPE and infectivity of BCV and altered the polypeptide profile of the virus. Since trypsin is a constituent digestive enzyme of the intestinal tract, it may influence BCV virulence in natural infections. If this is the case, inhibition of trypsin action in the gut may be a useful method for prevention of infection in newborn calves. However, it would be necessary to devise such a treatment without disrupting the digestive process of the animal.

Wild-type BCV strains displayed differences in CPE and plaque formation with and without trypsin. These viruses also have some differences in their polypeptide profiles (132). These strains may vary in their ease of processing to the infectious form by trypsin or cellular proteases. This observation possibly reflects a viral virulence factor in cases of natural infection.

The structural profile of BCV-L9 was determined, revealing that BCV is a complex virus. Knowledge of the prevalence, stability, and antigenicity of the viral proteins is important in the development of viral vaccines - especially when developing inactivated, recombinant, or synthetic peptide vaccines.

Cleavage of the 185 Kd precursor protein to the 100 Kd form correlated to activation of cell fusion and enhanced viral infectivity. Therefore, the 100 Kd protein is most likely the viral fusion factor, the protein responsible for cell to cell spread of infection.
Figure VII. 1. Hypothetical Model of Bovine Coronavirus Strain L9
Chapter VIII. BIBLIOGRAPHY


CHAPTER IX. CURRICULUM VITAE

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- The Bdellovibrios: Graduate seminar at Southeastern Louisiana University - 1982.
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Major Field: Veterinary Medical Sciences

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