Synthesis and processing of structural and intracellular proteins of two enteric coronaviruses
by Lisa Marie Sardinia

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
The synthesis and processing of virus -specific proteins of two economically important enteric coronaviruses, bovine enteric coronavirus (BCV) and transmissible gastroenteritis virus (TGEV), were studied at the molecular level. To determine the time of appearance of virus-specific proteins, virus-infected cells were labeled with 35S-methionine at various times during infection, immunoprecipitated with specific hyperimmune ascitic fluid, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The peak of BCV protein synthesis was found to be at 12 hours postinfection (hpi). The appearance of all virus-specific proteins was coordinated. In contrast, the peak of TGEV protein synthesis was at 8 hpi, but the nucleocapsid protein was present as early as 4 hpi. Virus-infected cells were treated with tunicamycin to ascertain the types of glycosidic linkages of the glycoproteins. The peplomer proteins of both viruses were sensitive to inhibition by tunicamycin indicating that they possessed N-linked carbohydrates. The matrix protein of TGEV was similarly affected. The matrix protein of BCV, however, was resistant to tunicamycin treatment and therefore has CD-linked carbohydrates. Only the nucleocapsid protein of both viruses is phosphorylated as detected by radiolabeling with 32P-orthophosphate. Pulse-chase studies and comparison of intracellular and virion proteins were done to detect precursor-product relationships. The nucleocapsid protein of BCV may be the product of phosphorylation of a protein which migrates slightly faster in SDS-PAGE, while the TGEV nucleocapsid protein appears to be the cleavage product of a slightly larger species. Both the peplomer and matrix proteins of BCV and TGEV are produced by the glycosylation of polypeptide precursors. A panel of monoclonal antibodies (MAbs) against each virus was prepared using hybridoma technology. These MAbs were used to precipitate intracellular and virion proteins. A number of virus-specific proteins which do not show any precursor-product relationship do appear to share common determinants. Limited digest peptide mapping to compare structural relationships among virus-specific proteins indicated that each virus coded for a set of two proteins related to the nucleocapsid protein. The peplomer proteins of each virus produced a set of peptides that were identical to a smaller protein found in virions. All other structural proteins were found to have unique peptide maps.

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of

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ABSTRACT

The synthesis and processing of virus-specific proteins of two economically important enteric coronaviruses, bovine enteric coronavirus (BCV) and transmissible gastroenteritis virus (TGEV), were studied at the molecular level. To determine the time of appearance of virus-specific proteins, virus-infected cells were labeled with ^{35}S-methionine at various times during infection, immunoprecipitated with specific hyperimmune ascitic fluid, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The peak of BCV protein synthesis was found to be at 12 hours post-infection (hpi). The appearance of all virus-specific proteins was coordinated. In contrast, the peak of TGEV protein synthesis was at 8 hpi, but the nucleocapsid protein was present as early as 4 hpi. Virus-infected cells were treated with tunicamycin to ascertain the types of glycosidic linkages of the glycoproteins. The peplomer proteins of both viruses were sensitive to inhibition by tunicamycin indicating that they possessed N-linked carbohydrates. The matrix protein of TGEV was similarly affected. The matrix protein of BCV, however, was resistant to tunicamycin treatment and therefore has O-linked carbohydrates. Only the nucleocapsid protein of both viruses is phosphorylated as detected by radiolabeling with ^{32}P-orthophosphate. Pulse-chase studies and comparison of intracellular and virion proteins were done to detect precursor-product relationships. The nucleocapsid protein of BCV may be the product of phosphorylation of a protein which migrates slightly faster in SDS-PAGE, while the TGEV nucleocapsid protein appears to be the cleavage product of a slightly larger species. Both the peplomer and matrix proteins of BCV and TGEV are produced by the glycosylation of polypeptide precursors. A panel of monoclonal antibodies (MAbs) against each virus was prepared using hybridoma technology. These MAbs were used to precipitate intracellular and virion proteins. A number of virus-specific proteins which do not show any precursor-product relationship do appear to share common determinants. Limited digest peptide mapping to compare structural relationships among virus-specific proteins indicated that each virus coded for a set of two proteins related to the nucleocapsid protein. The peplomer proteins of each virus produced a set of peptides that were identical to a smaller protein found in virions. All other structural proteins were found to have unique peptide maps.

INTRODUCTION

The development of vaccines which provide protection against viral diseases is an ongoing research concern. Preparation of monoclonal antibodies with prophylactic and therapeutic value against these diseases is also an important goal. In order to develop truly effective and safe products, the basic biology and biochemistry of the virus must be studied. Since it is the virus-specific proteins which are immunogenic, the study of the synthesis and processing of these proteins will provide valuable information that can then be used in the development of anti-viral vaccines and treatments. The purpose of my research was to study at the molecular level the synthesis and processing of virus-specific proteins of two economically important enteric coronaviruses, bovine enteric coronavirus (BCV) and transmissible gastroenteritis virus (TGEV).

Biology and Pathogenesis of Coronaviruses

The Coronaviridae are a heterogeneous family of pathogenic viruses which naturally infect many animal species including humans and cause a variety of diseases involving a number of different organ systems. Coronavirions are spherical, moderately pleomorphic particles 60-220 nanometers in diameter which bear characteristic widely-spaced club-shaped surface projections. This "corona" of peplomers which is clearly visible in electron micrographs prompted the Internationl Committee on the Taxonomy of Viruses to approve the creation of a new family, Coronaviridae, in 1975 (63). The genome is a large (6-8 megadaltons) single-stranded, poly-adenylated RNA molecule which is infectious and of positive polarity. The outer shell of the virus consists of a cell-derived lipid bilayer which is necessary for infectivity. All coronavirus species multiply exclusively in the cytoplasm of infected cells. They bud internally into vesicles derived from the endoplasmic reticulum and the Golgi apparatus. Virions are released from intact cells by fusion of post-Golgi vesicles with the plasma membrane (39,49,59,63,68,70).

These viruses are widespread in nature and are associated with a wide variety of diseases with an acute or subacute disease process, as well as subclinical infections. For most coronaviruses causing enteric diseases, the disease is caused by virus multiplication and cytopathogenicity. The target cells for these infections are the epithelial cells of the intestines.

The mammalian coronaviruses can be divided into two groups on the basis of cross-reactivity in serological tests. BCV and TGEV do not cross-react with one another serologically although they cause very similar diseases in their respective hosts.

Bovine enteric coronavirus was first characterized in 1972 by Mebus and co-workers (40,51) as a coronavirus-like agent causing diarrhea in calves. Since it caused diarrhea in gnotobiotic calves, it is considered to be a primary pathogen. Symptoms begin 24-30 hours after inoculation, last 4-5 days, and can be lethal in newborn calves. The most severe lesions develop in the small intestine where destruction of the intestinal epithelium leads to pathophysiological changes followed by an extensive loss of water and electrolytes (68). The finding by Storz et al. (56) that trypsin treatment in vitro of BCV accelerated cytopathic functions and improved infectivity yields suggests that the initiation of infection might be promoted by the action of proteolytic enzymes in the intestinal tract. Maternal antibodies (IgA and IgM) are transmitted via the colostrum to calves and reduce the severeness of the disease (68).

The biochemical and molecular characterization of BCV has been hindered in the past by the difficulty of propagating and assaying the virus in vitro. Most of the earlier work was done in primary or non-continuous cell lines. Laporte et al. (34) reported in 1980 the use of HRT-18 cells, a human adenocarcinoma cell line, for cultivation of high titers of BCV. The next year, Vautherot (64) developed a plaque assay using this same cell line. These developments have greatly facilitated BCV studies.

Transmissible gastroenteritis is an acute disease affecting pigs of all ages. In a herd, morbidity approaches 100%. Mortality is usually resticted to piglets under 3 weeks of age but often reaches 100% in this age group. The disease is characterized by vomiting, diarrhea, and dehydration within 24 hours of infection. In addition, there is congestion of mesenteric lymph nodes and blood vessels and occasionally hemorrhages in the kidneys. TGEV replicates rapidly in the jejunum, duodenum, and ileum, but not in the stomach or colon. The virus is resistant to low pH and trypsin and is moderately resistant to bile. These factors contribute to its ability to survive passage in the alimentary tract. Replication of virus takes place only in the columnar cells of the villi with cell desquamation accompanied by shortening of the villi. Replacement of the villous epithelial cells begins 18-72 h post-infection by migration of undifferentiated cells from the crypts. No reinfection occurs although released virus is present. This may be due either to the presence of IgA in the alimentary tract or to the production of a new population of epithelial cells that are resistant to the virus.

Passive resistance to the virus in the form of neutralizing antibody is transferred to piglets through the colostrum (3,8,23,68,71). There has been much research into the pathogenesis of TGEV and a number of vaccines have been introduced, but these have been only partially successful (19).

Coronavirus Proteins

The majority of the studies on coronavirus proteins have involved different strains of infectious bronchitis virus (IBV), which is an avian virus, and murine hepatitis virus (MHV). In general, coronavirus virions contain from 3 to 7 structural proteins which seem to fall into three classes. The nucleocapsid (N) protein is a phosphorylated protein of 50-60 kilodaltons (kd) that is associated with the viral genome (9,37,49,59,69). N protein in intact virions is resistant to treatment with bromelain (9) and pronase (69) indicating that it is located internally. Siddell et al. (48) have detected a protein kinase activity associated with the virion which, in the absence of exogenous protein substrates, specifically phosphorylates the virion nucleocapsid protein *in vitro*. The same protein is the only virion protein that is significantly phosphorylated in infected cells.

The second species of structural proteins is a large (125-200 kd) glycoprotein, P, which appears to comprise the peplomers (5,9,11,19,30,37,67,69). Treatment with bromelain removes this protein from the virion (12). Smaller glycopeptides (65-120 kd) which are often detected may be the result of cleavage of the larger glycoprotein by proteases (55,59,97). In vitro cleavage of the P protein by trypsin is often associated with a twofold increase in infectivity (58) and a ten-fold increase in yield of infectious virus (56). MHV usually replicates and produces plaques better in transformed cells. This may be due to the fact that transformed cells show an increased level of protease (72). Biological activities associated with this protein include binding of virions to membrane receptors of susceptible cells (27,44,59), induction of neutralizing antibody (22,25,44), and cell fusion (15,27,60).

Some coronaviruses may have more than one peplomeric glycoprotein. BCV virions possess another smaller glycoprotein which is also associated with the surface projections and is about 100 to 120 kd (24,26,30,66). It appears that this protein is a dimer of lower molecular weight (65 kd) glycopeptides held together by disulfide linkages (26,30). This structural protein elicits neutralizing antibodies. Monoclonal antibodies to this glycoprotein suggest that it is responsible for the hemagglutinating activity of BCV (65,66).

The third family of structural proteins is a heterogeneous glycoprotein species, M, (matrix) of 20 to 30 kd which often appears as several bands on SDS-polyacrylamide gels. The bands generally reflect differing degrees of glycosylation (2,5,9,37,49,67,69). The matrix glycoprotein appears to possess three domains: a glycosylated hydrophilic region which projects outside the viral envelope, a hydrophobic portion which extends across the membrane, and a third domain which is probably associated with the viral RNA on the inner side of the viral envelope. Treatment of coronavirus virions with proteolytic enzymes such as pronase or bromelain produces a 20% reduction in the apparent size of the M polypeptide which corresponds to about 5 kd (2,58). This segment which is degraded contains all of the carbohydrate on the molecule. A hydrophobic domain resides within the lipid bilayer. Heating at 100°C in the presence of reducing agents induces aggregation of the M protein (57) indicating that reduction of disulfide bonds exposes a highly hydrophobic region (59). The third domain of M resides on the inner surface of the envelope and is presumably associated with the nucleocapsid. The M proteins can be separated from the nucleocapsid on gradients after solubilization of the viral envelope with NP-40 at 4°C, but will reassociate with the nucleocapsid at 37°C (61).

Most viruses that have been studied share a common pathway of glycosylation. This cotranslational event is dolichol-mediated and is sensitive to tunicamycin. Tunicamycin is an analog of UDP-N-acetylglucosamine which interferes with the formation of dolichol-linked intermediates that transfer oligosaccharides to asparagine residues on glycoproteins. These carbohydrate moieties are termed N-linked sugars. In contrast, the matrix protein of MHV has an O-glycosidic linkage. Glycosylation is a post-translational event and is resistant to tunicamycin (27,41). Not all coronaviruses, however, possess this unique glycoprotein. Glycosylation of the M protein of IBV is inhibited by tunicamycin (52).

The M protein appears to be the only protein required for coronavirus budding. In MHV-infected, tunicamycin-treated cells, P protein is made in markedly reduced amounts. MHV virions are nevertheless formed and released normally (59). Apparently glycosylation is not required for particle formation as virions are released from IBV-infected cells treated with tunicamycin (52). In either case, however, the particles are not infectious indicating that the P protein is indispensable for viral infectivity (49,52).

In MHV-infected cells, two additional virus-specific proteins are often found (4,5,47). These proteins are 14 kd and 35 kd. They are not found in virions and are therefore designated as non-structural proteins. They have been shown to be primary gene products by cell-free translation (36,47). Tryptic peptide maps indicate that these proteins are not related to each other or to any of the other MHV-specific proteins (4). The functions of these proteins are not known.

Bovine Enteric Coronavirus Proteins

There has been some disagreement as to the number and character of the structural proteins of BCV. Workers from different labs have reported from 4 to 7 polypeptides in purified virions. The molecular weights of these proteins range from 23 to 190 kd (24,26,30,33,55). The peplomer glycoprotein appears to be a 190 kd protein that is normally present as two smaller subunits of 120 and 100 kd (26,30). A second peplomer protein of 105 kd (65,66) or 140 (26,30) is responsible for the hemagglutinating activity of the virus. The two groups who reported on this second peplomer protein, Vautherot et al. (65,66) and the workers in Brian's laboratory (26,30), each work with a different strain of BCV which may account for the striking difference in size of this protein. 140 kd glycoprotein is apparently a disulfide-linked dimer of 2-65 kd subunits. Antibodies against the 105 kd glycoprotein defined at least two functional domains, one outlined by neutralizing monoclonal antibodies and the other recognized by monoclonal antibodies with neutralizing and hemagglutination inhibition activity (65,66). Trypsin treatment produces BCV virions with distinctly shorter, more regularly arranged surface projections. Cytopathic functions and cell fusion are accelerated and hemagglutination activity is improved when trypsin is added (56).

The matrix protein is a heterogeneous group of closely migrating glycoproteins of 23 to 26 kd (26,30,55). These bands may represent different degrees of glycosylation. In contrast to the peplomer protein, the M protein could not be metabolically labeled with mannose or fucose indicating that it represents an O-linked glycoprotein (55).

An internal phosphoprotein of 50 to 52 kd, corresponding to the nucleocapsid protein has been reported (26,30,33,55). The intracellular non-structural proteins of BCV have not been described.

Transmissible Gastroenteritis Virus Proteins

Purified particles of TGEV are reported to have three major polypeptide species. The largest polypeptide contains carbohydrate and is located in the virus surface projections (19,21). This 195-200 kd glycoprotein elicits a neutralizing antibody response (22). Only one serotype of TGEV is known so far, and this is determined by the peplomer protein. When *in vivo* glycosylation of the P protein is inhibited by tunicamycin, synthesis of the protein is either prevented or greatly reduced (21). Cleavage of most of the sugar moiety by endoglycosidase H generates a 145 kd protein (28). The nucleocapsid protein is rich in arginine residues (21) and has a molecular weight of 47,000 to 50,000 (19,21). This protein may be partially degraded to a 42 kd protein (19). The matrix glycoprotein is 28 to 30 kd. Glycosylation of the 26 kd precursor is prevented by tunicamycin (19,21). TGEV-specified intracellular proteins have not been described.

Synthesis and Processing of Virus-Specific Proteins

Almost all of the studies on the biogenesis of virus-specific proteins and their subsequent processing have been done using either IBV or strains of MHV. A number of techniques including pulse-chase labeling, analysis of glycosylation, *in vitro* translation, and tryptic peptide mapping have been used in these studies. The results obtained will be discussed below.

Radiolabeling of virus-specific proteins at various times during infection indicates that, for MHV, protein synthesis is moderately coordinated. Virus-specific proteins begin to appear by 3 to 4 hours after infection (49). In some cases, a large (150 kd) protein which is probably the peplomer precursor is detected 1 hour before other virus-specific proteins (5), although in cells which show minimum cytopathic effect, the synthesis of N was detected much earlier than that of P or M (59).

The nucleocapsid protein has been shown to be a phosphorylated protein of 50 to 60 kd. Since this protein can be detected after a very short (2 min) pulse-labeling period (13), and is produced in an in vitro translation system (36), it has been assumed that it is a primary translation product and is not post-translationally modified. Recently, however, Stohlman et al. (54) detected a slightly smaller (57 kd) protein after a 2 min pulse-labeling period. After a 10 min chase, the larger (60 kd) phosphoprotein was found. As the chase continued, the amount of 57 kd protein decreased and the amount of 60 kd protein increased. The 57 kd protein was not phosphorylated. It was detected by two monoclonal antibodies that precipitate the 60 kd protein but was not recognized by two other monoclonal antibodies to the 60 kd protein. The investigators theorized that additional determinants may be present on the 60 kd protein due to conformational changes induced by phosphorylation.

It has been noted by a number of workers (1,13,52) that after chases of long duration (1-2 h), a protein that is smaller than N appears. This protein, which is smaller by 6 to 10 kd, has a tryptic peptide map which is identical to that of N (4,13,52). This smaller protein has been presumed to be the product of proteolytic cleavage of N. Its appearance is variable according to the cell type and virus strain. It is also found as a product of in vitro translation (36). Premature termination of translation or, less likely, internal initiation, have been hypothesized to account for its presence in the translation system.

Both the intracellular and virion forms of the matrix protein are often seen as a broad band or group of bands on SDS-polyacrylamide gel electrophoresis. All of these proteins have identical tryptic peptide maps (4,13,46,52). The smallest of these proteins ranges in size from 18 to 23 kd and is not glycosylated (1,10,13,46,50,52). In MHV, glycosylation increases the size of the M protein by about 2 kd (13,46,50) whereas in IBV, the size is increased by 5 to 11 kd (10,52). The MHV M protein is unique among viral glycoproteins in that it contains only O-glycosidic linkages (27,41,42). In contrast, the M protein of IBV possesses the more common N-linked oligosaccarhides (52). Glycosylated forms of IBV M protein can be detected after only a 5 min pulse (52) which indicates that glycosylation is a cotranslational event. This is consistent with the concept of dolichol-mediated, tunicamycin-sensitive glycosylation (31). M glycoproteins of MHV are not labeled during a short pulse and appear only after a longer chase (13,41,50). This suggests that the glycosylation of M is a post-translational event.

The pathway of synthesis and processing of the peplomer protein has been much debated. The difficulty in sizing large glycoproteins has contributed to the confusion. Workers disagree as to the sizes of precursors and products, but several patterns of processing have been hypothesized.

The peplomers of IBV appear to be composed of two glycoproteins, 90 kd and 84 kd (9,11,12,52). A model by Cavanaugh (11,12) suggests that there are two molecules of each size in the peplomer. In this "ice cream cone" model the smaller glycoproteins are embedded in the membrane with a dimer of the larger glycoprotein forming the bulb at the end of the peplomer.

In IBV-infected cells, a large glycoprotein of 155 kd is detected which does not comigrate with virion proteins. This protein is sensitive to digestion with endoglycosidase H and is not detectable in cells treated with tunicamycin It is therefore an N-linked glycoprotein. The tryptic peptide map of this glycoprotein contains all of the peptides in the maps of both the 84 kd protein and the 90 kd protein, which are different from each other. In pulse-labeled cells, the 155 kd glycoprotein was evident but neither the 84 kd nor the 90 kd glycoprotein could be detected. These proteins appeared only after a

60 min chase. This indicated that these two proteins were derived from a precursor. Since the 155 kd glycoprotein includes the sequences of the smaller glycoproteins and is the only virus-specific protein larger than these two proteins, it must be the precursor (52).

The synthetic pathway of MHV P protein is somewhat more obscure. MHV virion peplomers have two glycoproteins associated with them. One is 180 kd (1,6,13,58) and one is 90 kd (5,50,58,60). These two proteins have identical tryptic peptide maps (4,46,58) and the large glycoprotein can be converted to the 90 kd glycoprotein by treatment of virions with trypsin *in vitro* (58). *In vitro* translation studies have shown that the primary translation product which is precipitated by antiserum specific for the P protein is 120 kd (36). This corresponds to the polypeptide core of P glycoprotein which has been found to be 120 kd (46). Pulse-chase studies, however, reveal a 150 kd glycoprotein after a short pulse, which is then chased into a 180 kd glycoprotein (6,50). The tryptic peptides of the 150 kd glycoprotein are also identical to those of the 180 kd and 90 kd glycoprotein (4,46). Recently, Sturman and Holmes (60) showed that there are actually two species of 90 kd protein. These have been designated 90A and 90B and were isolated by exploiting the fact that 90A is acylated. Taking all the information into consideration, the following pathway has been proposed by Siddell (46). The primary translation product *in vivo* is a 150 kd glycoprotein which has a 120 kd polypeptide core and is cotranslationally glycosylated. The conversion of this primary product to the larger virion peplomer protein possibly involves further modification of the carbohydrate side chains. The 90 kd glycoprotein has not been found by all workers. Trypsin mediated cleavage of the 180 kd glycoprotein may not occur in all cell lines used for propagating MHV. The evidence for the role of proteolytic cleavage of P in activation of cell fusion suggests, however, that this step may have importance in natural coronavirus infection and pathogenesis.

Goals and Experimental Design

The goal of my research was to analyze the synthesis and processing of virus-specific proteins of two economically important enteric coronaviruses. Although these viruses cause a similar disease in their respective hosts, they belong to different serological groups and appear to have differences in their structural proteins.

A number of methods were used in the analysis of proteins of BCV and TGEV. To determine the time of appearance of virus-specific proteins, virus-infected cells were radiolabeled with ^{35}S-methionine at various times during infection, radioimmunoprecipitated with specific hyperimmune ascitic fluid, and analyzed by SDS-polyacrylamide gel electrophoresis. Pulse-chase studies and comparison of intracellular and virion proteins were done to detect precursor-product relationships. Virus-infected cells were treated with tunicamycin to ascertain the type of glycosidic linkages of the glycoproteins. Phosphorylation of virus-specific proteins was detected by radiolabeling with ^{32}P-orthophosphate. A panel of monoclonal antibodies prepared against each virus was used to precipitate proteins and discover common determinants on viral proteins. Limited digest mapping was performed on intracellular and virion proteins and the patterns compared. Finally, a model was prepared describing the pathway of synthesis and processing of BCV- and TGEV-specific proteins.

MATERIALS AND METHODS

Chemicals and Media

Reagent grade liquid organic chemicals were obtained from J. T. Baker Chemical Co. Other chemicals and reagents were obtained from Sigma Chemical Co. unless otherwise stated in the text. Radioisotopes were obtained from New England Nuclear Corp. Cell culture media were purchased from Irvine Scientific (Santa Ana, CA), and sera were purchased from Sterile Systems (Logan, UT).

Most cell cultures were maintained in Dulbecco's Modified Eagle's (DME) medium that was supplemented with 200 Units/ml penicillin G, 25 ug/ml streptomycin, and 10% (vol/vol) calf serum. DME 20 was prepared as above except that 20% (vol/vol) fetal bovine serum was added instead of calf serum. Infection of cells with virus was done in DME 2. Instead of calf serum, 2% (vol/vol) fetal bovine serum was added to DME and the medium was supplemented with 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 10 mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES).

Hybridomas were maintained in DME 20 supplemented with 1 ug/ml amphotericin B, and 1% (vol/vol) of 10 mM hypoxanthine, 1.6 mM thymidine (HT) and 0.04 mM aminopterin (A) and was designated as HAT medium.

Virus Strains and Cell Lines

Bovine enteric coronavirus (BCV) was obtained from ATCC (ATCC VR-874) as was the Miller strain of transmissible gastroenteritis virus (TGEV) (ATCC VR-743). BCV was cloned by plaque purification and propagated in a continuous human adenocarcinoma cell line (HRT-18) (34) obtained from Dr. David Brian. A continuous swine testicle cell line, ST, established by McClurkin and Norman (38) and obtained from Dr. Brian, was used for the multiplication of TGEV. TGEV was also cloned by plaque purification. BCV was passaged 3 to 5 times in HRT 18 cells before use in experiments. TGEV was passaged 11 to 13 times in ST cells. These cell lines were maintained in DME 10.

A murine sarcoma cell line (S180) (ATCC TIB-66) was derived from Swiss Webster mouse ascitic tumor cells. NS-1 cells are a BALB/c myeloma line (P3/NS1/1-Ag4-1) that is a non-secreting clone of P3X63Ag8. NS-1 cells were obtained from the Salk Institute. Baby hamster kidney (BHK) cells were obtained from Dr. John Holland. S180 cells and BHK cells were maintained in DME 10, while DME 20 was used for NS-1 cells.

Virus Stocks

Monolayers of cells in 10 cm plastic dishes (Nunc) were infected with virus at a multiplicity of infection (MOI) of 1 in DME 2. The virus was allowed to adsorb at room temperature for 1 h. The inoculum was removed and replaced with 7 ml DME 2. The infected cells were incubated at 37°C until greater than 75% of the cells were lysed. The plates were frozen at -70°C, and freeze-thawed twice and the resulting lysates were scraped with a rubber policeman and sonicated for 105 s in a Heat Systems Sonicator (model W-225R) using a cup probe at 70% power. The lysates were clarified by centrifugation (1200 x g) for 5 min, aliquoted and stored at -70°C.

Plaque Assay

Virus stocks were titered by plaque assay on the appropriate cell monolayers. Cells were seeded into plastic six-well dishes (Nunc) in DME 10 and incubated at 37°C overnight. Monolayers were infected with 0.5 ml of serial 10-fold dilutions of virus in DME 2. Dilutions of virus were allowed to adsorb at room temperature for 1 h. The inoculum was removed and the monolayers were overlaid with 3 ml of DME 2 containing 0.75% (wt/vol) agarose (type II, Sigma), and incubated at 37°C for 3 days. Cells were fixed by adding 0.5 ml of 2% (vol/vol) glutaraldehyde to each well and incubating at room temperature for several hours. The agarose overlays were removed, the plates dried at room temperature and the plaques counted. Data were expressed as plaque-forming units per ml (PFU/ml).

Infection and Purification

Monolayers of cells were infected with virus at an MOI of 5 in DME 2. The virus was allowed to adsorb at room temperature for 1 h. The inoculum was then removed and replaced with DME 2. The virus-infected cells were incubated at 37°C for 18 h (TGEV) or 56 h (BCV) and then frozen at -70°C.

To purify virus, an adaptation of the method of Leibowitz, et al. (35) was used. The cell-associated virus was released by two cycles of freeze-thawing and the resulting lysate clarified by centrifugation in an HB-4 rotor at 10,000 x g for 30 min at 4°C. Virus was concentrated by centrifugation at 35,000 rpm for 60 min in an SW 41 rotor through a pad of 15% (w/w) potassium tartrate (KT) in MSE buffer (0.01 M morpholinopropanesulfonic acid, 0.15 M NaCl, 0.001 M EDTA, pH 6.8) onto a pad of 40% (w/w) KT in MSE buffer. The visible band was removed, diluted to less than 10% KT in MSE buffer, layered onto a 9 ml 10-40% KT gradient, and centrifuged in an SW 41 rotor at 37,000 rpm for 4 h at 4°C. The visible band was removed, diluted in MSE buffer and pelleted at

45,000 rpm for 1 h at 4°C in an SW 50.1 rotor. The pellet was resuspended in 0.1-0.2 ml B10 [50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.02% (wt/vol) sodium azide, 0.05% (vol/vol) NP40, 1% (vol/vol) Aprotinin, 0.1% (wt/vol) bovine serum albumin], transferred to a 1.5 ml Eppendorf tube and stored at -20°C.

Immunization of Mice

Eight-to-twelve week old BALB/c mice were inoculated intraperitoneally (ip) three times at weekly intervals with 0.15 ml of mock- or virus-infected cell lysates emulsified with an equal volume of complete Freund's adjuvant. A final booster inoculation of 0.3 ml cell lysate without adjuvant was given one week later. Each inoculation contained 10^5 to 10^8 PFU of virus.

Production of Hyperimmune Ascitic Fluid

Ascitic fluid was induced by ip inoculation of immunized mice with 10^6 S180 cells in 0.3 ml phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 9.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ pH 7.2) one day after the final booster inoculation (62). Ascitic fluid was harvested by abdominal paracentesis using an 18-gauge needle, stored at 4°C overnight and centrifuged at 800 x g for 5 min to remove cells and debris.

Prior to use, the ascitic fluid was absorbed twice at 4°C for 24 h with methanol-fixed cell monolayers to remove antibodies directed against non-viral components. The absorbed ascitic fluid was stored at -70°C and was used in the detection of virus-specified proteins by immunoprecipitation.

Preparation of Monoclonal Antibodies

Mice were immunized as described above. Spleens were removed from immunized mice 4 days after the final booster injection. Immune spleen cells (3×10^7) were fused with 5×10^7 NS-1 cells using 50% polyethylene glycol (mol. wt. 1000, Sigma cat. no. P3515) in DME 0. Fused cells were diluted in HAT medium and seeded into 96-well plates (Nunc) containing mouse peritoneal macrophages and irradiated baby hamster kidney cells as feeder cells. Incubation and maintenance of the hybridomas was carried out according to the microculture protocol of de St. Groth and Scheidegger (16). Culture fluids from growing colonies were screened for anti-viral antibodies by immunofluorescence as described below. Cells from positive wells were cloned twice by limiting dilution in 96-well plates. Ascitic fluids containing high concentrations of anti-viral monoclonal antibody were prepared by injecting 1.5×10^6 hybridoma cells into Pristane (2,6,10,14-tetramethylpentadecane) treated mice.

The mice were injected ip with 0.5 ml of Pristane at least one week prior to the injection of hybridoma cells.

Immunofluorescence Assay

Mock- and virus-infected cells were seeded (10 ul/ well) into 60-well Terasaki plates (Nunc), incubated at 37°C for 8 h (TGEV) or 12 h (BCV), washed with PBS and fixed with methanol as previously described (43). For indirect immunofluorescence staining, the plates were rinsed once with PBS and 30 ul of hybridoma supernatant fluid was added to each well. After 30 min at 37°C the plates were washed 4 times with PBS and 10 ul of fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobin (Antibodies Inc. cat. no. 2146) was added to each well. The plates were incubated at 37°C for an additional 30 min and washed 4 times with PBS. Immunofluorescence was observed with an Olympus IMT inverted microscope equipped with reflected fluorescence accessories.

Plaque Reduction Test

A plaque reduction test was used to determine the virus neutralizing activity of the hyperimmune ascitic fluid. Virus suspensions were diluted to approximately 300 PFU/ml and mixed with an equal volume of ascitic fluid containing monoclonal antibodies. After incubation for 30 min at 37^{o}C, 0.5 ml of the virus/antibody mixture was inoculated onto cell monolayers and the plaque assay was completed as described. Monoclonal antibodies were considered to be neutralizing if the plaque reduction was greater than 90%.

Determination of Isotype of Monoclonal Antibodies

The Ouchterlony method of gel diffusion (18) was used to determine the isotypes of monoclonal antibodies. One percent agarose was prepared in 0.15 M NaCl, 0.05% NaN_3 and poured into 50 mm plastic dishes. Wells were punched into the agarose. The center well was filled with 18 ul of class-specific goat anti-mouse immunoglobulin. The outer wells were filled with 18 ul of one of the following: supernatant fluid from hybridomas, ascitic fluid containing monoclonal antibodies, normal mouse serum, or normal mouse ascitic fluid. The plates were incubated at room temperature in a humidified chamber for 24-48 h until precipitin bands formed.

Radiolabeling of Virus-Specified Proteins

Purified virions radiolabeled with L-[^{35}S]-methionine (NEG-009A) were prepared as follows. Monolayers of cells in 10 cm plastic dishes were infected with virus at an MOI of 5 in DME 2 and incubated at 37^{o}C. At 6 h post-infection (hpi) for TGEV and 8 hpi for BCV, the medium was removed and replaced with labeling medium (80% methionine-free DME 2, 20% DME 2, 20-100 uCi/ml ^{35}S-methionine). The virus-infected cells were incubated at 37^{o}C and harvested at 18 hpi for TGEV and 56 hpi for BCV. The virions were purified from infected cell lysates as described above.

Virus-specified intracellular proteins were labeled with L-[^{35}S]-methionine using the following procedure. Confluent cell monolayers in 35 mm plastic dishes (Nunc) were mock-infected or inoculated with stock virus at an MOI of 5 in DME 2. After 1 h at room temperature, the inoculum was removed and replaced with 1 ml DME 2. At various times post-infection the medium was removed and replaced with 0.3 ml of methionine-deficient DME 2. Twenty min later, ^{35}S-methionine was added to a final concentration of 200-400 uCi/ml. After a 2-4 h labeling period, the medium was removed and the cells were lysed with 0.1 ml B10 for 5 min on ice. The cytoplasmic lysates were harvested and stored at -20^{o}C.

The same method was used to label infected cells with ^{32}P-orthophosphate (NEX-054) except that phosphate-deficient medium was used during the labeling period.

Immunoprecipitation of Virus-Specified Proteins

Cytoplasmic lysates were immunoprecipitated with hyperimmune ascitic fluid by a modification of the procedure of Bond et al. (6). Fifteen ul samples of cell lysates were diluted ten-fold in RIP buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.2% (vol/vol) NP40, 0.05% (vol/vol) SDS, 1% (vol/vol) Aprotinin, 0.02% (wt/vol) sodium azide]. Five ul of hyperimmune ascitic fluid were added and incubated at 0°C for 1 h. Immune complexes were precipitated with 50 ul of 10% (vol/vol) formalin-fixed Staphylococcus aureus (Cowan) (29) by incubation at 0°C for 1 h and pelleted by centrifugation at 6,500 x g for 15 s. The pellets were washed 4 times with RIP buffer. The proteins were eluted with 30 ul of 20 mM dithiothreitol (DTT), 1% (wt/vol) SDS for 15 min at room temperature and 5 min at 60°C. Bacteria were removed by centrifugation at 6,500 x g for 5 min. The supernatant fluids containing virus-specified proteins were removed, mixed with an equal volume of 1-D PAGE diluent [120 mM Tris-PO_4 (pH 6.7), 1% (wt/vol) SDS, 40% (vol/vol) glycerol, 0.02% (wt/vol) phenol red] and stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis

Virion proteins were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by adjusting the lysate to 0.02 M DTT, 1% (wt/vol) SDS, heating for 5 min at 60°C and then adding an equal volume of 1-D PAGE diluent. Immunoprecipitated proteins were used directly.

Proteins were electrophoresed on 8%, 10%, or 12% (wt/vol) polyacrylamide slab gels as described by Laemmli and Favre (32). Preparative gels were dried directly onto Whatman 3 MM paper and exposed to preflashed Kodak XAR-2 x-ray film. Analytical gels were fixed overnight in 5% trichloroacetic acid (TCA).

Standard proteins were detected by staining with Coomassie brilliant blue G (17). Labeled proteins were detected by impregnating the gels with 10% (wt/vol) 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide (DMSO) followed by drying and exposure to preflashed x-ray film at -70^{o}C (7).

The molecular weights of virus-specified proteins were determined from their distance of migration in slab gels relative to those of standard proteins of known molecular weight (45). The following proteins were used as standards in molecular weight determinations: thyroglobulin (200 kd), beta-galactosidase (115 kd), phosphorylase B (97.4 kd), bovine serum albumin (66 kd), ovalbumin (45 kd), carbonic anhydrase (29 kd).

Limited Digest Peptide Mapping

Virus-specific intracellular and virion proteins labeled with ^{35}S-methioninewere electrophoresed on a 10% polyacrylamide gel. Bands corresponding to virus-specified proteins were identified by exposure of dried preparative gels to x-ray film. Using the x-ray film as a template, the appropriate bands were excised and rehydrated in enzyme buffer [125 mM Tris (pH 6.8), 0.1% SDS, 1 mM EDTA, 10% glycerol]. A 16% acrylamide gel with a 3% stacking gel of 4 cm in height was prepared. Enzyme buffer with enzyme (5 ug of Staphylococcus aureus V8 protease or TLCK-chymotrypsin or 10 ug of TPCK-trypsin) and indicator dye was added to each well. Gel pieces were transferred to the wells. Electrophoresis was carried out at 120 V until the dye front was 1 cm above the resolving gel. The current was then turned off for 30 min to allow for digestion of the proteins by the enzyme. Electrophoresis was continued at 120 V until the dye front reached the bottom of the gel (14). The gel was then treated in the same manner as other SDS-polyacrylamide gels as described above.

RESULTS

Time Course of Virus Multiplication

The kinetics of BCV and TGEV multiplication at 37°C were determined (Figure 1). Cultures infected with BCV began yielding virus by 8 hpi and reached their maximum titer by 56 hpi. The maximum yield of BCV was approximately 9 x 10^7 PFU/ml. BCV-infected HRT 18 cells did not begin to lyse until 40 to 48 hpi. The concomitant rise of cell-associated and released virus may be due to released virus readsorbing to cell membranes.

The course of infection of TGEV was markedly faster than that observed for BCV. Cultures infected with TGEV reached their maximum yield of virus by 18 hpi. The maximum yield of TGEV was approximately 6 x 10^6 PFU/ml. TGEV-infected ST cells were almost completely lysed by 24 hpi, although cell remnants remained attached to the dish. The relatively low titers of released virus late in infection may be due to the instability of the virus for prolonged periods at 37°C.

Time Course of Virus-Specific Protein Synthesis

The kinetics of virus-specific protein synthesis in BCV- and TGEV-infected cells were determined. HRT 18 and

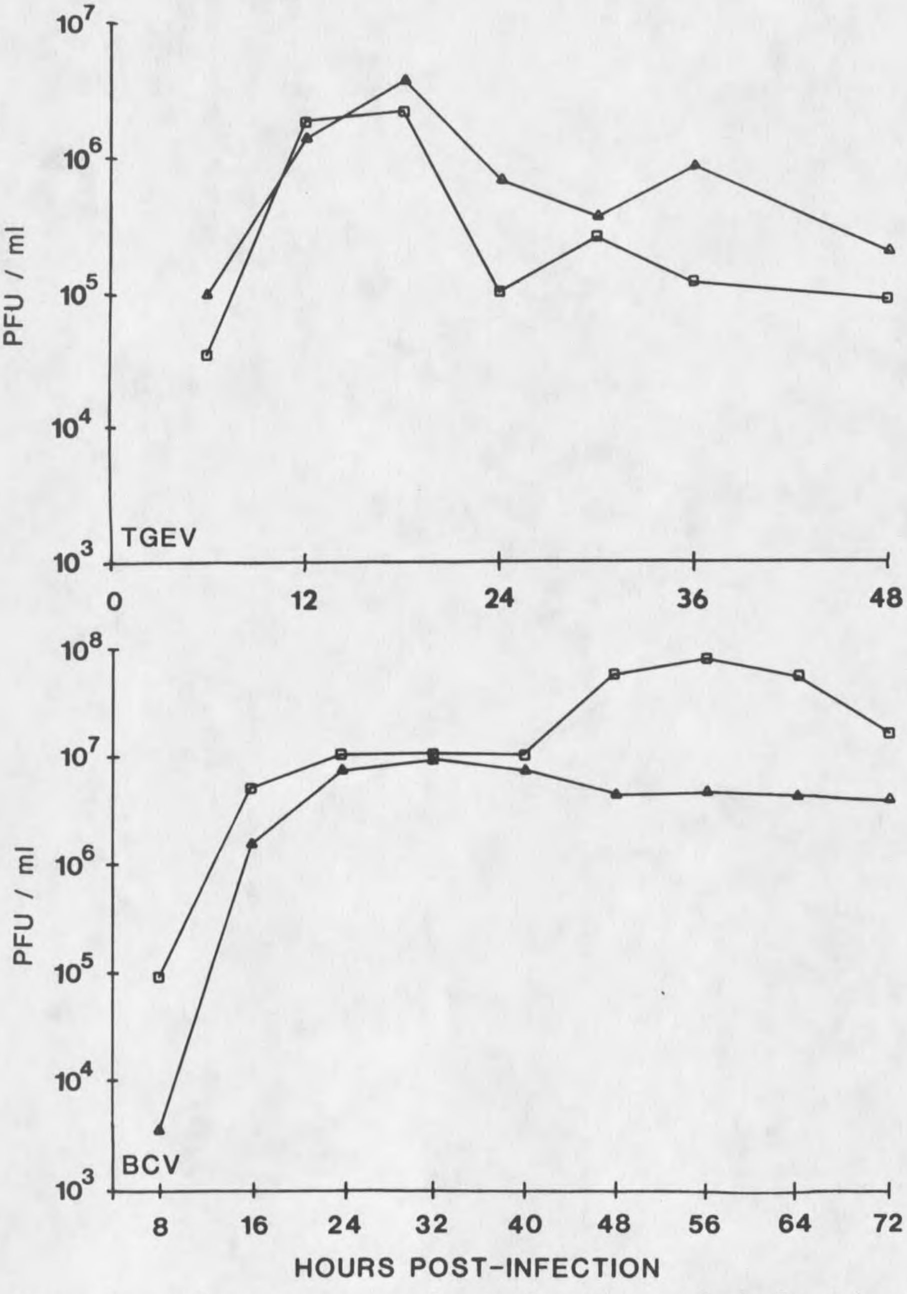

Figure 1. Time course of production of infectious virus from infected cells. Cells were infected at an MOI of 1.0. At the indicated times, the supernatant fluids from monolayers of infected cells were removed and the amount of infectious virus in the supernatant fluid was determined by plaque assay [released virus (□)]. DME 2 was added to the monolayers which were then frozen at -70°C. The lysates were clarified as described in Materials and Methods and titered by plaque assay [cell-associated virus (△)]. Data are expressed as plaque-forming units (PFU) per ml.

ST cells were infected with BCV and TGEV, respectively, at an MOI of 5.0. They were labeled for 2 h (TGEV) or 4 h (BCV) with ^{35}S-methionine at various times post-infection. At the times indicated in Figure 2, the labeling medium was removed and the cells were lysed with B10. The cell lysates were immunoprecipitated and a 2 ul sample was removed for liquid scintillation counting. TGEV-specific protein synthesis reached a peak at 6 to 8 hpi and dropped off sharply by 12 hpi. In contrast, BCV-specific protein synthesis peaked at 12 hpi but remained relatively high throughout infection. These results should be expected considering that BCV-infected HRT 18 cells retain their integrity and therefore their ability to support virus-specific protein synthesis for a much longer time than TGEV-infected ST cells.

The immunoprecipitated lysates of virus- and mock-infected cells were analyzed by SDS-PAGE. The results are shown in Figure 3 (TGEV) and Figure 4 (BCV). The positions and molecular weights of virus-specific polypeptides are indicated in the figures.

The first TGEV-specific protein to be detected was the nucleocapsid protein N (48 kd). This protein appeared faintly as early as 4 hpi. The majority of TGEV-specific proteins were first apparent by 6 hpi and the maximum rate of synthesis was between 6 and 8 hpi. Proteins corresponding to P (190 kd), N, and M (29 kd) were