Serological and molecular comparisons of coronaviruses
by Susan Herbert Goss

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 antigenic interrelationships among viruses of the family Coronaviridae were examined. Four mammalian coronaviruses from two different antigenic groups were compared using serological and molecular techniques. The viruses selected were murine hepatitis virus A59 (A59V), bovine coronavirus (BCV), canine coronavirus (CCV) and transmissible gastroenteritis virus (TGEV). Hyperimmune ascitic fluids were prepared in mice to provide a homogeneous source of antibody. Virus-infected cell lysates were used as immunogens to generate antibodies against intracellular as well as structural proteins. Antibodies directed against cellular proteins were removed by absorption with uninfected cells. The ascitic fluids compared favorably to hyper immune serum in immunofluorescence and virus-neutralization tests. The intracellular, proteins of A59V, BCV, CCV and TGEV were identified by immunoprecipitation of infected-cell lysates with hyperimmune ascitic fluids. Reciprocal cross-immunofluorescence and neutralization tests showed two-way cross-reactions between A59V and BCV, and between CCV and TGEV. Immunoprecipitation of virus-infected cell lysates showed that the serological relatedness was based on common determinants on each of the major viral antigens: the peplomer, nucleocapsid and membrane. Monoclonal antibodies (MAbs) against each virus were isolated using hybridoma technology. The MAbs were tested for virus specificity by immunofluorescence, polypeptide specificity by immunoprecipitation, and virus neutralization activity by a plaque reduction test. They were also tested for cross-reaction with the related virus. Most of the MAbs isolated recognized the nucleocapsid protein, which is the major polypeptide synthesized in infected cells. MAbs which neutralized virus infactivity were directed against the peplomeric proteins. The immunoprecipitation and MAb studies suggest that TGEV and CCV are more closely related than AS9V and BCV.

SEROLOGICAL AND MOLECULAR COMPARISONS

OF CORONAVIRUSES

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Susan Herbert Goss

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Susan Herbert Goss

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ABSTRACT

The antigenic interrelationships among viruses of the
family Coronaviridae were examined. Four mammalian
coronaviruses from two different antigenic groups were.
compared using serological and molecular techniques. The
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the peplomeric proteins. The immunoprecipitation and MAb
studies suggest that TGEV and CCV are more closely related
than A59V and BCV.

INTRODUCTION

The power of immunological techniques in research and clinical virology is well established. The serological characterization of viruses is useful for diagnosis and epidemiological studies of viral diseases. In fact, the identification of specific antibody is frequently the only available method of diagnosis, as many pathogenic viruses are difficult to isolate and characterize in vitro. A knowledge of antigenic interrelationships is valuable when identifying and classifying viral isolates and for investigation of evolutionary patterns within virus families. In spite of recent advances in viral immunology made possible by molecular cloning and monoclonal antibody technology, much remains to be done in clarifying the antigenic relationships within families of human and animal viruses. The purpose of my research was to establish a simple and effective method for studying antigenic groups of viruses and to use this system to investigate at the molecular level the immunological interrelationships among several mammalian coronaviruses.

Properties of Coronaviruses

The Coronaviridae are a heterogeneous family of
pathogenic viruses which cause a wide variety of diseases
in many animals including humans. Coronavirions are
pleomorphic particles 60-220 nanometers in diameter
surrounded by a lipid bilayer envelope studded with club-
shaped spikes or peplomers. It is this "corona" of
surface projections as seen in electron micrographs which
led to the naming of the group by an international
committee in 1968 (72). The genome is a large (6-8
megadaltons) single-stranded RNA molecule of positive
polarity. Virus replication takes place in the cytoplasm
and virions are released by budding through the membranes
of the endoplasmic reticulum, unlike other RNA viruses
which bud from the plasma membrane (51,57,68,72).

Virions contain from 3 to 7 structural proteins which
seem to fall into three classes. A phosphorylated
protein (pp), N, of 50-60 kilodaltons (kd) is located
internally and is associated with the viral genome
(51,57,76,79). The second family of structural proteins
is a heterogeneous glycoprotein (gp) species, M, of 20-30
kd which often appears as several bands on SDS-
polyacrylamide gels (65,66,67). This protein is mostly
embedded in a lipid bilayer and forms part of the viral
envelope. A short glycosylated portion (5 kd) protrudes

from the envelope and can be removed by treatment with
bromelain (66,68). The M protein determines the location
of viral budding and interacts with the nucleocapsid (68).
Antibodies directed against the M protein can neutralize
virus infectivity in the presence of complement (10). A
large (125-200 kd) glycoprotein, P, is associated with the
virion peplomers (17,66,68). Biological activities
associated with this protein include binding of virions to
cell membrane receptors (28,68,75), induction of
neutralizing antibody (19,24,27,55) and cell fusion
(10,27,28). Some coronaviruses may have more than one
peplomeric glycoprotein. Monoclonal antibodies to bovine
coronavirus (BCV) define another, smaller glycoprotein
which is also associated with the virion surface
projections and elicits neutralizing antibody (26,74,75).
This structural protein is probably responsible for the
hemagglutinating activity of BCV (26,75).

The current members of the Coronaviridae are listed in
Table 1. Several of the coronaviruses were originally
placed into the group on the basis of morphology alone.
The list has undergone frequent revision with the improved
sensitivity and specificity of available serological and
molecular techniques for the analysis of coronaviruses.

Table 1. Members of the Coronaviridae

Virus	Natural Host	Predominant Type of disease
Avian infectious bronchitis virus (IBV)	Chicken	Respiratory
Bluecomb disease virus (TCV)	Turkey	Enteric
Bovine coronavirus (BCV)	Cow	Enteric
Canine coronavirus (CCV)	Dog	Enteric
Feline enteric coronavirus (FECV)	Cat	Enteric
Feline infectious peritonitis virus (FIPV)	Cat	Peritonitis
Hemagglutinating encephalo-myelitis virus (HEV)	Pig	Encephalo-myelitis
Human coronavirus (HCV)	Human	Respiratory
Human enteric coronavirus (HECV)	Human	Enteric
Isolates SD and SK	Human	Demyelinating
Murine hepatitis virus (MHV)	Mouse	Hepatitis
Parrot coronavirus (PCV)	Parrot	Enteric
Pleural effusion disease virus (RbCV)	Rabbit	Pleuritis
Porcine virus CV-777	Pig	Enteric
Rabbit enteric coronavirus (RbECV)	Rabbit	Enteric
Rat coronavirus (RCV)	Rat	Respiratory
Sialodacryoadenitis virus (SDAV)	Rat	Adenitis
Transmissible gastroenteritis virus (TGEV)	Pig	Enteric

Antigenic Interrelationships

Although data on the antigenic interrelationships among
the Coronaviridae are not yet complete, the viruses have
been tentatively placed into groups on the basis of cross-
reactivity in serological tests (Table 2). The mammalian
coronaviruses which have been classified so far fall into
two groups. The avian coronaviruses, infectious brochitis

Table 2. Antigenic cross-reactions among coronaviruses

MAMMALIAN

Group 1	Group 2	Unclassified
229EV	OC43V	CV-777
TGEV	MHV	FECV
CCV	RCV	HECV
FIPV	SDAV	RbCV
	BCV	RbECV
	HEV	
	SD, SK	

AVIAN

IBV	TCV	PCV

virus (IBV) and turkey coronavirus (TCV), are apparently
unrelated to each other and to the other coronaviruses
(7,40,76). The human coronaviruses (HCV) have been placed
into separate antigenic groups based on the results of
enzyme-linked immunosorbent assay (ELISA) (37),
polyacrylamide gel electrophoresis (PAGE) (55), and
immunoelectrophoresis (IEP) (54). Group 1 contains HCV-
229E (229EV) and strains which can be propagated in cell
culture. The organ culture virus OC43V and other strains
that could not be cultivated in vitro are found in Group
2. Viruses of the OC43 group cross-react with murine
hepatitis viruses A59 and JHM (A59V, JHMV) in ELISA (37).
These results are consistent with earlier studies

in which virus neutralization (VN) (40), complement
fixation (CF) and gel diffusion (7) tests suggest a
relationship between the MHV's and several human
coronaviruses. They also report a complete lack of cross-
reactivity between IBV and any of the other known
coronaviruses. The rabbit coronavirus RbCV has been
reported to show cross-reaction with both 229EV and
OC43V (59). A coronavirus-like agent isolated from
swine and designated CV-777 (44) was compared by immuno-
electron microscopy and immunofluorescence to a number of
classified coronaviruses (12,45). No cross reaction was
seen between CV-777 and IBV, TGEV, CCV, HEV, BCV or FIPV.
The morphological appearance is so far the only criterion
for inclusion of this isolate in the coronavirus family.

The hemagglutinating encephalomyelitis virus of swine
(HEV), previously placed in the coronavirus family on the
basis of its characteristic morphology, was shown to be
related to the OC43V group of human coronaviruses in
hemagglutination inhibition (HAI), CF and VN tests (30).
A two-way cross-reaction between HEV and OC43V was
demonstrated by all three methods. Another interesting
addition to this antigenic group was recently reported by
Gerdes et al. (5,20). Coronaviruses SD and SK isolated
from the brains of two multiple sclerosis patients are
serologically related to A59V, JHMV, and OC43V.

The bovine coronavirus (BCV) cross-reacts in VN and
HAI tests with OC43V (21) and with HEV (53). Also,
neutralizing antibodies against BCV were detected in sera
from humans (61) and a number of animal species (52),
suggesting the possible existence of related viruses in
these species.

The final members of this antigenic group (Group 2) at
present are the rat coronavirus (RCV) and
sialodacryoadenitis virus (SDAV) which are closely related
to the murine hepatitis viruses (76).

Group 1 of the mammalian coronaviruses contains the
transmissible gastroenteritis virus of swine (TGEV),
canine coronavirus (CCV), feline infectious peritonitis
virus (FIPV) and the human coronavirus 229E and related
strains. 229EV cross-reacts with the other three viruses
in immunofluorescence assay (IFA), although the reactions
are weak (43). The existence of a canine coronavirus
(CCV) was first suspected when TGEV antibodies were found
in dogs that had never had contact with pigs (18). Later
the CCV was isolated from dogs (1) and the isolate indeed
proved to be closely related to TGEV in various
serological tests. The two viruses can be differentiated
by reciprocal VN experiments in which homologous titers
are significantly higher than heterologous antibody levels
(49,78). The FIPV also has a high degree of cross-
reactivity with TGEV and CCV in VN (47,48,49,77,78) and

IFA (43,48,77) tests. In cross-protection studies conducted by Woods and Pedersen (77) pigs immunized with FIPV did not develop detectable TGEV-neutralizing antibodies, but seemed to be protected to a certain extent against challenge with TGEV. On the other hand, cats vaccinated with TGEV produced cross-reacting antibodies to TGEV and FIPV, but were not protected against FIPV challenge. Cats given TGEV orally showed no signs of clinical disease, but shed infectious virus and developed VN antibodies (48,77).

Molecular Immunology

There are few data available on the cross-reactivity among the coronaviruses at the molecular level. Several investigators have reported on the antigenicity of subviral components or individual polypeptides. Virions contain three major antigens: the peplomeric glycoprotein P, the nucleocapsid protein N and the envelope or membrane glycoprotein(s) M (19,22,24,29,55,79). The peplomeric protein is apparently responsible for generation of neutralizing antibodies. Animals immunized with TGEV surface projections or intact virions develop VN antibodies, while subviral particles do not elicit neutralizing antibodies (19,24,49,55), suggesting that neutralizing antibodies raised during TGEV infection are

directed against virus peplomers. Horzinek et al. (29)
investigated antigenic relationships among homologous
structural proteins of TGEV, CCV and FIPV using ELISA, VN,
immunoprecipitation and immunoblotting techniques. These
studies suggest reciprocal cross-reactivity of all three
major viral antigens. The strongest antibody response was
directed at the envelope proteins, in contrast to the
previous work in which the response seemed to be toward
the peplomeric protein.

The polypeptides of 229EV and OC43 were compared by
PAGE (55) and quantitative IEP (54). Patterns were
similar for the two viruses but there was no cross-
reaction between them. Once again the neutralizing
antibodies were directed against the peplomeric
glycoprotein (37,54,55).

Antigenic interrelationships among murine corona-
viruses were investigated by Fleming et al. (16) using a
panel of monoclonal antibodies to JHMV. The patterns of
cross-reactivity were different for each strain,
confirming that they are closely related but in fact
separate strains. Bond et al. (3) and Cheley et al. (8)
also demonstrated a high degree of relatedness among MHV
strains by tryptic peptide mapping of proteins and RNA
blotting.

The polypeptides of coronaviruses SD and SK were
compared by radioimmunoprecipitation to proteins of

OC43V and A59V (20). Reciprocal cross-reactions were seen among the major antigens of all four viruses. Brian et al. (26) used immunoblotting studies to determine the cross-reactivity among homologous structural polypeptides of BCV, OC43V and A59V. Monospecific antiserum prepared against each of the three major antigens of BCV reacts with the corresponding proteins of OC43V and A59V. An additional antigen found in BCV and OC43 virions is not detectable in A59V. This protein is apparently the hemagglutinin and has no homolog in A59V, a non-hemagglutinating virus. Vautherot et al. (74,75) investigated the antigenicity of BCV using a panel of monoclonal antibodies. They reported finding two peplomeric antigens, with the smaller one associated with hemagglutinating activity of the virus (75). These studies also detected common antigenic determinants among BCV, MHV, OC43V and HEV.

I chose four mammalian coronaviruses for this study, two from each antigenic group. They are murine hepatitis virus A59, bovine coronavirus, canine coronavirus and transmissible gastroenteritis virus.

Murine Hepatitis Virus A59

The murine hepatitis viruses are a group of closely related virus strains with variable pathogenicity for mice (8,9,16). A59V causes an acute fatal hepatitis in

mice. It is readily propagated in a number of tissue culture cell lines and has been extensively studied. Together with the other MHV´s it is a problem in mouse breeding colonies. Part of the importance of these viruses is in their use as models for studying the pathogenesis of hepatitis, encephalitis and more recently, demyelinating diseases such as multiple sclerosis (20,76).

A59V has been well-characterized biochemically. Virions contain three major structural proteins: a peplomeric glycoprotein of approximately 180 kd, a 50-60 kd nucleocapsid phosphoprotein and a heterogeneous membrane glycoprotein of 23-26 kd (3,4,38,65). When virus particles are treated with trypsin, the P protein is cleaved by trypsin to form two 90 kd polypeptides (28,38,64,66). The tryptic peptide maps for the 90 kd and 180 kd proteins appear to be identical (66). The intracellular proteins of A59V and the closely related JHMV have been characterized in a number of laboratories (3,4,51,58). In addition to the structural proteins found in the virion, there are several virus-specific polypeptides found in infected cells with apparent molecular weights of 57k, 54k, 39k and 37k. Compared to other coronaviruses, the MHV´s are relatively easy to cultivate and assay and have been adapted to a number of continuous cell lines.

Bovine Coronavirus

Bovine coronavirus was first characterized about ten years ago by Mebus and co-workers (42,60) as a coronavirus-like etiologic agent of neonatal calf diarrhea. Symptoms begin 24-30 hours after inoculation, and last 4-5 days. BCV infection can be fatal in newborn calves (76). Like the parvo- and rotaviruses, BCV is a major cause of neonatal calf diarrhea and is of economic importance in the United States. One of the problems with BCV studies is the difficulty of propagating and assaying the virus in vitro. Most of the earlier work was done in primary or other non-continuous cell lines. Laporte et al. (35) reported in 1980 the use of a human adenocarcinoma cell line, HRT-18, for cultivation of high titers of BCV. Vautherot (73) later developed a plaque assay using this same cell line. These developments have greatly facilitated the biochemical and antigenic characterization of BCV.

There is so far only one serotype of BCV, although Dea et al. (11) recently reported differences in counter-immunoelectrophoresis and immunodiffusion patterns among five BCV isolates. These results are awaiting confirmation, since the same five strains cross-reacted in reciprocal VN experiments done earlier by the same group. Only two precipitating antigens are detected by these

methods, in contrast with the four antigens identified in the LY-138 strain of BCV by Hajer and Storz (23). The monoclonal antibodies prepared by Vautherot et al. (74,75) detected only minor antigenic variation among BCV isolates from various sources. Two of the monoclonal antibodies directed against a peplomeric glycoprotein of the immunizing (French) BCV strain failed to react with isolates from the United States and Great Britain. A bovine respiratory isolate tested had the same reactions as BCV with all of the monoclonal antibodies.

There is some disagreement as to the number and character of BCV structural proteins. Various workers report from four to nine polypeptides in purified virions, with molecular weights ranging from 23 to 190 kd (23,26,33,36,62,74). The most recent studies indicate that the peplomers are composed of a large (125-190 kd) glycoprotein which is normally present as two smaller subunits (33,74), and another glycoprotein of 105 kd (74,75) or 140 kd (26) which is responsible for the hemagglutinating activity of the virus. There is general agreement that the nucleocapsid protein is a phosphorylated polypeptide of about 50 kd and a heterogeneous glycoprotein of 23-25 kd is found in the envelope matrix (23,33,36). The intracellular non-structural proteins of BCV have not been described.

Transmissible Gastroenteritis Virus

Transmissible gastroenteritis is an infectious disease of pigs which is of major economic importance. It was first described by Doyle and Hutching in 1946 (14) and was later shown to be caused by a member of the coronavirus family (41). TGE is highly infectious and has an 18-24 hour incubation period followed by diarrhea and vomiting. The symptoms are much less severe in adults than in newborn animals, where the mortality rate can approach 100% (41,76). There is probably only one serotype of TGEV. A number of strains have been isolated in various parts of the world, but all are serologically identical as tested so far (41). CV-777, a coronavirus-like agent isolated recently from pigs with enteric disease (12,44,45), does not cross react with TGEV (or any other established member of the Coronaviridae) and is probably not a serotype of TGEV (45). There has been much research concerning the pathogenesis and prevention of TGE. A number of vaccines have been introduced, but these are only partially successful (17).

TGE virions contain three major structural proteins of 160-200, 50 and 28-30 kd (17). The large glycoprotein is associated with the peplomers and elicits the production of neutralizing antibodies (19). TGEV-specified intracellular proteins have not been described.

Canine Coronavirus

The canine coronavirus is closely related to TGEV;
indeed it is has been suggested that it, together with
FIPV, is a host-range mutant of TGEV rather than a
separate species (29). CCV was first described by Binn
et al. (1). This isolate, designated 1-71, can be
experimentally transmitted to puppies, resulting in a
self-limiting course of gastroenteritis and dehydration
(31). Adult dogs show no clinical signs of disease, but
develop neutralizing antibodies to CCV. From 62-87% of
kennel dogs are seropositive for CCV (76).

Canine coronaviruses is fastidious and has usually
been cultivated in primary or secondary cells (1,18,29,31,
49). These cell lines vary in their susceptibility to CCV
strains and cytopathic effects (CPE) are not always seen.
Woods (78) reported the development of a feline cell line
(FC) suitable for growth of CCV as well as for FIPV and
TGEV. All three viruses cause CPE and form plaques in FC
cells. A continuous canine cell line, A-72, was
established by Binn et al. (2) for propagation of a number
of viruses including CCV.

The biochemistry of CCV has been less studied than
that of other coronaviruses, perhaps because of the
difficulties in propagating the virus in vitro. Garwes
and Reynolds (18) have determined the polypeptide

structure of purified virions. There are four major structural proteins: gp204, pp50, gp32 and gp22. The first three correspond to the proteins of TGEV. The 22 kd glycoprotein apparently has no homolog in TGEV. The authors suggest that CCV may contain two membrane glycoproteins. However, the membrane glycoprotein is known to be heterogeneous in many of the studied coronaviruses (65,68), so separate bands seen in gel electrophoretograms may represent different stages of glycosylation rather than different polypeptide species. There are as yet no reports of non-structural proteins coded for by CCV.

Methods for Investigating Antigenic Relationships

One objective of my research was to use these four coronaviruses in a variety of immunological tests to further the previous studies of their antigenic relatedness and to extend the findings to the level of individual structural and non-structural polypeptides. In addition to verifying antigenic relationships, these studies would be helpful in increasing our understanding of intracellular processing of virus-specific proteins.

The antigenic groups shown in Table 2 are based primarily on inter-species serological tests. The experiments used serum from one host animal to test virus from another species. The use of heterotypic serum can

yield misleading results due to endogenous antibodies directed against non-viral substances present in the sample. Also, there may be significant variations in sample quality when using immune serum from infected animals. I wanted to conduct a study using a homogeneous system with antibody from a single source. Comprehensive serological studies of this type require substantial quantities of poly- and monospecific antibodies. Large volumes of hyperimmune ascitic fluid can be induced in individual immunized mice by injection of Freund's adjuvant (71) or sarcoma-180 tumor cells (25,70). In these procedures mice are generally immunized with purified virus particles to generate highly specific antibody preparations. However, in this case antibodies are produced only against external structural components of the immunizing virus. A comprehensive investigation of antigenic relationships at the molecular level should include comparisons of virus-specified intracellular polypeptides and internal virion proteins as well. I wanted to develop a protocol for production of anti-viral ascitic fluid in mice using infected-cell lysates as immunogen. Five criteria were established for development of this system.

1. The method should produce high-titer hyperimmune fluids containing antibodies directed against intracellular as well as virion proteins.

2. The system should be homogeneous, with all
   antibodies from a single source, e.g. the same
   inbred mouse strain. The methods should be
   applicable to a wide variety of cell-culture
   adapted viruses.

3. Large quantities of hyperimmune fluids should be
   obtainable from individual mice.

4. There should be a simplified protocol for
   preparation of viral immunogen to obviate the
   need for extensive purification.

5. Mice immunized in this manner could also be used
   for production of monoclonal antibodies using
   hybridoma technology.

A panel of monoclonal and polyvalent antibodies
recognizing both structural and intracellular proteins
can be generated for use in molecular studies of antigenic
relationships and protein processing.

Experimental Design

I conducted this research in three stages. The first
was the establishment and characterization of virus-cell
systems for each of the four coronaviruses chosen for the
study. Continuous cell lines were tested for cytopathic
effect (CPE), virus yield, and ability to quantify virus
by plaque titration. The second phase of this project was
the preparation of polyvalent and monoclonal antibodies

against each virus. Anti-viral hyperimmune ascitic fluids
were prepared in mice and monoclonal antibodies produced
by cell fusion techniques. Finally, serological and
molecular immunology methods were used to assess antigenic
cross-reactions among homologous intracellular
polypeptides of the four viruses. Anti-viral ascitic
fluids and monoclonal antibodies were used in reciprocal
cross-neutralization, immunofluorescence and
immunoprecipitation tests to define and compare the
antigens of the viruses.

MATERIALS AND METHODS

Chemicals and Media

Chemicals and reagents were obtained from Sigma
Chemical Co. unless otherwise stated in the text.
Radioisotopes were obtained from New England Nuclear
Corporation. Plastic dishes for cell culture were
obtained from Nunc, Denmark. Media used for cell culture
were purchased in powder form from Irvine Scientific
(Santa Ana, CA). Sera were obtained from Sterile Systems
(Logan, UT). DME-0 consisted of Dulbecco's Modified
Eagle's medium supplemented with 200 Units/ml of
penicillin G and 25 ug/ml of streptomycin. DME-10 was
prepared by adding 1 volume of calf serum to 10 volumes of
DME-0. DME-20 was prepared by adding 2 volumes of fetal
bovine serum and 1 ug/ml of amphotericin B to 10 volumes
of DME-0. DME-2 contained 2% fetal bovine serum and was
supplemented with 10 mM 3-(N-morpholino)propanesulfonic
acid (MOPS), 10 mM N-tris-(hydroxymethyl)methyl-2-amino-
ethanesulfonic acid (TES), and 10 mM N-2-hydroxyethyl-
piperazine-N'-2-ethanesulfonic acid (HEPES). HAT medium
for hybridoma cultures was prepared by adding 1% of 100X

stock solutions of HT (10 mM hypoxanthine, 1.6 mM thymidine) and A (0.04 mM aminopterin) to DME-20.

Virus Strains and Cell Lines

The murine coronavirus A59V was obtained from Dr. Lawrence Sturman. Bovine coronavirus (BCV) was obtained from the American Type Culture Collection (ATCC VR-874), as were the canine coronavirus (ATCC VR-809) and the transmissible gastroenteritis virus (ATCC VR-743).

A59V was propagated in murine 17CL-1 cells, a spontaneously transformed continuous cell line cloned from BALB/c-derived 3T3 fibroblasts and obtained from Dr. Sturman (63). A continuous human adenocarcinoma cell line obtained from Dr. David Brian, HRT-18 (35,69), was used for cultivation of BCV. TGEV was propagated in swine testicle (ST) cells, a continuous cell line established by McClurkin and Norman (39) and obtained from Dr. Brian. Canine coronavirus was adapted to the continuous A-72 cell line (ATCC CRL-1542) established by Binn. et al (2). The murine sarcoma cell line S180 (ATCC TIB-66) was used for induction of ascites in immunized mice. The BALB/c myeloma line P3/NS1/1-Ag4-1 (NS-1) is a non-secreting clone of P3X63Ag8. NS-1 cells were obtained from the Salk Institute. All cell lines were maintained in DME-10 except for NS-1 cells, which were passaged in DME-20.

Preparation of Cell Lysates

Virus-infected cell lysates were used as stock virus and to immunize mice for antibody production. Cell monolayers in 10 cm plastic tissue culture dishes were infected with virus at a multiplicity of infection (MOI) of 1-5. After an adsorption period of 1 hr at room temperature the inoculum was removed and 5 ml of DME-2 was added. The cultures were incubated at $37^{\circ}C$ and harvested when extensive cytopathic effect was seen. TGEV and A59V were harvested at 18-24 hr post-infection (HPI); BCV and CCV at approximately 48 HPI. Mock-infected cell cultures were prepared in the same manner, using an inoculum of DME-2 instead of virus. Cells were disrupted by one cycle of freeze-thawing at $-70^{\circ}C$. The resulting lysate was scraped with a rubber policeman and sonicated 1-2 min in a Heat Systems Sonicator (model W-225R) using a cup probe at 70% power. The lysates were clarified by centrifugation at 1500 x g for 5 min and stored at $-70^{\circ}C$.

Plaque Assay

Virus stocks were cloned twice by plaque selection and titered by plaque assay on the appropriate cell monolayer. Cells (1×10^6 in DME-10) were seeded into plastic six-well dishes and incubated at $37^{\circ}C$ overnight. Monolayers were washed with pre-warmed DME-0 and infected with 0.2 ml

of 10-fold dilutions of virus in DME-2. After an adsorption period of 1 hr at room temperature the inoculum was removed and 2 ml of an overlay consisting of 0.75% agarose (Sigma Type I) in DME-2 was added. Plates were incubated at 37°C for 2 days (TGEV, A59V) or 3-5 days (BCV,CCV). If staining was required for visualization of plaques, the cells were fixed with 2% glutaraldehyde. After fixation for 1 hr or more, the agarose overlay was removed. The fixed cells were stained with 1% aqueous crystal violet for 30 min, washed several times with water and air-dried at room temperature. Plaques were counted and the virus titers expressed as plaque-forming units per ml (PFU/ml).

Immunization of Mice

The immunogen preparation consisted of mock or virus-infected cell lysates emulsified with an equal volume of complete Freund's adjuvant (CFA). Adult (8-12 wk) Balb/c mice were given three intraperitoneal (ip) injections of immunogen, 0.3 ml each, at weekly intervals. A final booster injection consisting of 0.3 ml cell lysate without adjuvant was given one week later. Each inoculation contained 10^5 to 10^8 plaque-forming-units (PFU) of virus.

Harvesting of Serum and Ascitic Fluid

Ascites were induced in immunized mice by injection of Sarcoma-180 cells (25,70). Normal mouse ascitic fluid was prepared by injection of unimmunized mice with S-180 cells. One day after the final booster immunization, mice were injected ip with 0.3 ml of sterile 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 $MgCl_2-6H_2O$, 1 mM NaN_3) containing 5 X 10^6 S-180 cells. The abdomens became markedly distended within 10-15 days, at which time the accumulated fluid was removed by paracentesis using an 18-gauge needle. Surviving mice continued to accumulate ascitic fluid and were tapped again every 2-3 days. It was possible to obtain as much as 50 ml of fluid from an individual mouse treated in this manner. Sera were obtained from the same mice by tail bleeding. Three mice were immunized with each virus preparation and the fluids pooled. Ascitic fluids were refrigerated overnight and centrifuged at 800 x g for 5 min to remove cells and debris.

Absorption of Serum and Ascitic Fluid

Prior to use in serological studies the sera and ascites were absorbed with uninfected cells to remove antibodies directed against non-viral components.

Confluent monolayers of the cell lines used to propagate each virus were grown in 10 cm plastic dishes. The monolayers were washed carefully once with PBS and once with absolute methanol, fixed 2 min in methanol, dried with compressed air and stored in plastic bags at -70°C until needed. Immediately prior to use the plates were rehydrated by washing once with PBS. For absorption, 1 ml of serum or ascitic fluid was layered on a 10 cm dish containing the appropriate fixed cells and incubated overnight at 4°C. Fluids were absorbed twice in this manner and stored frozen (-70°C) until used.

Preparation of Monoclonal Antibodies

Mice were immunized as described above. Spleens were removed from immunized mice 3-4 days after the final booster injection. Immune spleen cells (3×10^7 cells) were fused with 5×10^7 NS-1 cells using 50% polyethylene glycol (mol. wt. 1000, Sigma cat. no. P3515). Fused cells were diluted in HAT medium and seeded into 96-well plates containing 10^3 mouse peritoneal macrophages per well (13). Incubation and maintenance of the hybrids was carried out according to the microculture protocol of de St. Groth and Scheidegger (13). Culture fluids from growing colonies were screened for anti-viral antibodies by immuno-fluorescence. Cells from positive wells were cloned twice by limiting dilution in 96-well plates. Supernatant

fluids from the cloned hybridoma cell lines were used in neutralization, immunofluorescence and immunoprecipitation tests. Alternatively, 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

A microculture immunofluorescence assay (IFA) developed by Robb (50) was used to assess antibody activity of ascitic fluids and sera and to screen hybridoma supernatant fluids for specificity. Cells were suspended in DME-2 at a concentration of 6×10^5 cells per ml and mixed with the corresponding virus in a ratio of 9 parts cells to 1 part virus or DME-2 (mock-infected). The infected and mock-infected cells were seeded (10 ul/well) into 60-well Terasaki plates using a Hamilton repeating dispenser such that each plate contained 30 wells of mock and 30 wells of virus-infected cells. The plates were incubated at 37^OC for 8 hr (TGEV, A59V) or 18-24 hr (BCV, CCV), washed and fixed with methanol as described above and stored at -70^OC. For indirect immunofluorescence staining the plates were thawed and rinsed once with PBS. Ten microliters of hybridoma culture medium, diluted serum

or ascitic fluid was added to each well. After 30 min at
room temperature the plates were washed 4 times with PBS
and 10 ul of fluorescent isothiocyanate (FITC)-conjugated
goat-anti-mouse immunoglobulin (Antibodies Inc. cat. no.
2146) was added to all wells. The plates were incubated
an additional 30 min, washed 4-5 times with PBS and
observed using an Olympus IMT inverted microscope with
reflected fluorescence accessories.

Plaque Reduction Test

A plaque reduction test was used to determine the
virus neutralizing (VN) activity of the hyperimmune sera
and ascitic fluid. Serial dilutions of antibody were made
in DME-2, mixed with an equal volume of virus suspension
(approximately 600 PFU/ml) and incubated 30 min at 37°C.
Confluent monolayers of the corresponding cell line in 6-
well dishes were inoculated with 0.5 ml of the virus-
antibody suspension and the plaque assay completed as
described above. The number of plaques in each antibody-
containing well was compared to the virus control wells to
determine the percent plaque reduction (PR). Computer
programs were used to plot PR vs the reciprocal of the
antibody dilution (BPS Business Graphics, Cambridge, MA)
and to calculate the dilution giving 50% plaque reduction
(Omicron Plotrax, Engineering Sciences, Atlanta, GA). The
VN titer of the antibody preparation was reported as the

dilution resulting in 50% plaque reduction. The percent PR of each virus by a 1:10 dilution of normal mouse ascitic fluid (NMA) was determined as a negative control. An antibody preparation was considered to have no detectable neutralizing acitivity against a virus if a 1:10 dilution resulted in a lower PR than the NMA.

Monoclonal antibody supernatants were tested for neutralizing activity by a modification of the plaque reduction assay. Virus suspensions were diluted to approximately 300 PFU/ml and mixed with an equal volume of undiluted hybridoma supernatant medium. After incubation for 30 min at 37°C, 0.5 ml of the virus/antibody mixture was inoculated onto washed cell monolayers and the plaque assay completed as described. HAT medium without antibody was used as a negative control. Monoclonal antibodies were considered to be neutralizing if the plaque reduction was greater than 90%.

Radiolabeling of Intracellular Proteins

Confluent cell monolayers in 6-cm plastic dishes were inoculated with stock virus at an MOI of 1 to 5 and incubated at 37°C. At various times post-infection the medium was removed and replaced with 0.7 ml of methionine-deficient DME-2 containing 200 uCi/ml of ^{35}S-methionine. Cells infected by A59V or TGEV were labeled at 8 hr post-infection (HPI), and BCV and CCV were labeled at 12 HPI.

After a 1 hr labeling period the medium was removed and
the cells were washed twice with DME-0. Cells were lysed
with 0.3 ml of buffer B10 (10mM Tris-HCl pH7.4, 5 mM
$MgCl_2$, 0.5% NP-40, 0.1% SDS, 1% Aprotinin, 50 ug/ml
ribonuclease A, 50 ug/ml deoxyribonuclease) for 5 min on
ice. The lysates were harvested and stored at $-20^{O}C$.

Immunoprecipitation of Virus-specific Proteins

Intracellular proteins were immunoprecipitated with
hyperimmune ascitic fluid as described previously (4),
with minor modifications. Radiolabeled virus and mock-
infected cytoplasmic lysates were prepared as described
above. Cell lysates (15 ul samples) were incubated with 5
ul of hyperimmune ascitic fluid or serum, or 20 ul of
hybridoma supernatant medium in 0.5 ml of Radioimmunpre-
cipitation (RIP) buffer (50 mM Tris-HCl pH 7.4, 150 mM
NaCl, 5 mM EDTA, 0.2% NP-40, 0.05% SDS, 1% Aprotinin,
0.02% sodium azide) for 1 hr at $0^{O}C$. Immune complexes
were precipitated with 50 ul of 10% Staphlylococcus aureus
(Cowan) prepared by the method of Kessler (32) for 1 hr at
$0^{O}C$. The bacteria were pelleted by centrifugation in an
Eppendorf microcentrifuge at 6500 x g for 15 sec and
washed 3 times in RIP buffer. The proteins were eluted
with 25 ul of 20 mM dithiothreitol, 1% SDS for 15 min at
room temperature and 5 min at $60^{O}C$. After centrifugation
at 6500 x g for 3 min the supernatants were removed, mixed

with an equal volume of SDS-PAGE diluent (120 mM Tris-PO$_4$

pH 6.7, 1% SDS, 40% glycerol, 0.02% phenol red) and stored

at -20°C. Controls consisting of lysates precipitated

with normal mouse ascitic fluid or S. aureus without

antibody were prepared in the same manner.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were electrophoresed on 8% polyacrylamide

slab gels as described by Laemmli and Favre (34), except

that the resolving gel was supplemented with 0.5% (wt/vol)

linear polyacrylamide (BDH Chemical Ltd.). Following

electrophoresis the gels were fixed overnight in 5%

trichloroacetic acid (TCA). Proteins were detected by

staining with brilliant blue G (15) or by impregnating the

gels with 10% (wt/vol) 2,5-diphenyloxazole (PPO) in

dimethyl sulfoxide (DMSO) followed by drying and exposure

to preflashed Kodak XAR-2 x-ray film at -70°C (6).

The molecular weights of virus-specific proteins were

determined from their distance of migration in slab gels

relative to those of standard proteins of known molecular

weight (56). Proteins used as markers were thyroglobulin

(200 kd), beta-galactosidase (115 kd), phosphorylase B

(97.4 kd), bovine serum albumin (66 kd), ovalbumin (45 kd)

and carbonic anhydrase (29 kd).

RESULTS

Establishment of Virus-Cell Systems

The four coronaviruses chosen for this study were adapted to cell culture as described in Materials and Methods. Cell lines were tested and selected on the basis of cytopathic effect (CPE), virus yield and plaque assay characteristics.

The murine hepatitis virus A59 was propagated in 17CL-1 cells, a spontaneously transformed derivative of the BALB/3T3 cell line. This virus-cell system had been previously established in our laboratory (4), and high titer (10^8 PFU/ml) virus-infected cell lysates were available. A59V caused extensive cytopathic effect in 17CL-1 cells, including syncytia formation and detachment of cells from the monolayer. Syncytia formation began at 5-6 hr post-infection (HPI), and cell destruction was usually complete by 24 HPI. Virus titers were determined by plaque assay, with plaques clearly visible at 48 HPI.

The bovine enteric coronavirus (BCV) was adapted for growth in a continuous human adenocarcinoma cell line, HRT-18 (35,69). Several blind passages were done before any evidence of CPE was seen. After adaptation to HRT-18 cells, CPE was observed beginning at about 24 HPI and

consisted of rounding up and vacuolization of the cells.
The CPE began in discrete foci but spread to the entire
monolayer by 48 HPI, although there was very little cell
detachment. Infected-cell lysates for stock virus were
harvested at 48 HPI or later, when CPE was complete. A
plaque assay developed by Vautherot (73) was used to assay
BCV in HRT-18 cells, and virus stocks with titers of at
least 10^7 PFU/ml were prepared.

The virulent Miller strain of transmissible
gastroenteritis virus (TGEV) was propagated in swine
testicle (ST) cells (39). After initial isolation and
plaque purification in ST cells, only low titer (10^5
PFU/ml) virus was produced, and CPE took 4-5 days to
develop. Further adaptation was carried out by Dr.
Andreas Luder. Repeated passages of virus at a high
multiplicity of infection (MOI) resulted in cell lysates
with titers of greater than 10^8 PFU/ml. Cells infected
with this stock virus began to round up and detach within
12 hr, and cell lysis was nearly complete by 24 HPI. A
plaque assay was established for TGEV in ST cells.
Plaques 2-3 mm in diameter were visible within 48 hr.

Canine coronavirus (CCV) was adapted to the continuous
A-72 cell line established by Binn et al. (2). Cytopathic
effects including syncytia formation and lysis of infected
cells were seen beginning with the third passage of CCV in
these cells. Syncytia formation began at about 12-15 HPI,

and cell lysis was usually complete in 48 hr. A plaque
assay was developed with some difficulty, since the A-72
cells were fragile and tended to lyse spontaneously after
several days incubation. Plaques appeared in 4-7 days and
usually required crystal violet staining for optimum
visualization. I was unable to produce CCV stocks with
titers greater than 3×10^6 PFU/ml despite repeated
adaptation passages. However, these titers were adequate
for my studies.

Once satisfactory stocks of the four coronaviruses
were prepared, a time course experiment was conducted to
determine the optimum time period for detection of intra-
cellular viral protein synthesis (Figure 1). The peak of
protein synthesis in A59V infected cells was from 6-9 HPI,
approximately the same as for TGEV-infected cells. CCV
protein synthesis reached a maximum at about 15 HPI.
Intracellular BCV protein synthesis continued over a
longer period (from about 12-21 HPI), probably because
there was very little cell destruction before 24 HPI.

Preparation and Testing of Anti-Viral Ascitic Fluids

Hyperimmune ascitic fluids were induced in mice
according to the protocol described in Materials and
Methods. Mice were immunized with virus-infected cell
lysates to produce antibodies against structural and
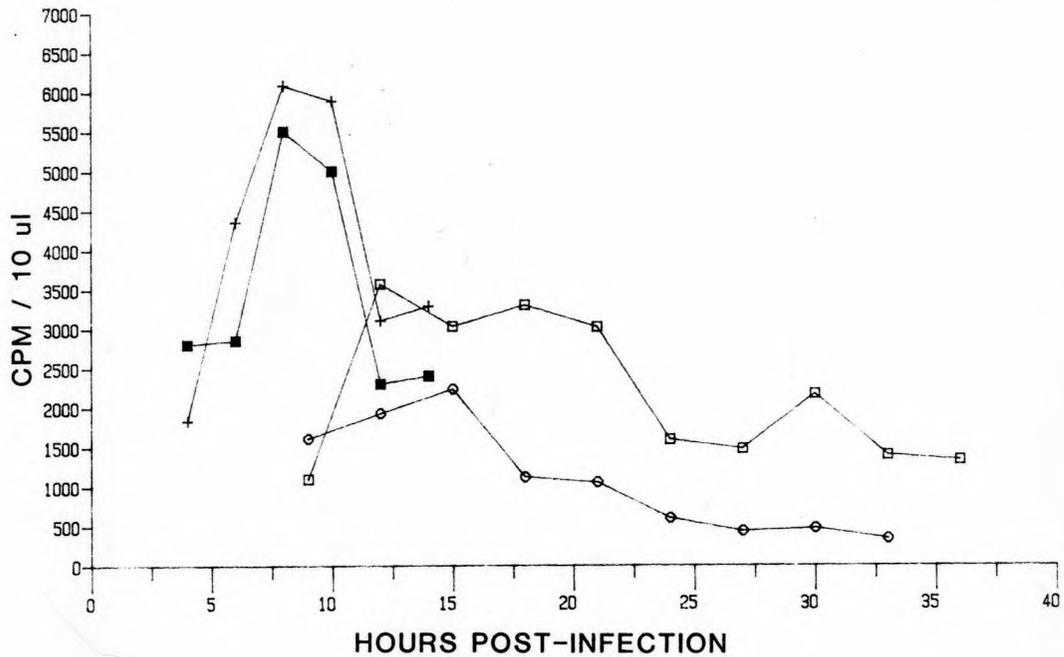intracellular proteins. Induction of ascites with

Figure 1. Time course for coronavirus protein synthesis in cells infected with A59V (+), BCV (☐), CCV (o) and TGEV(■). Virus and mock-infected cells were pulse-labeled with ^{35}S-methionine at various times post-infection as described in Materials and Methods. Cell lysates were immunoprecipitated with homologous anti-viral ascitic fluid, and the amount of virus-specific protein determined by liquid scintillation counting. Data are expressed as counts per minute (CPM) per 10 ul sample.

Sarcoma-180 cells resulted in substantial fluid
accumulation in individual mice. Up to 40 ml of fluid
could be obtained from a single mouse. Antibody-
containing fluids from three mice were pooled for each
virus to compensate for individual variations in immune
response. Fluids were absorbed with uninfected cells to
remove non-viral antibodies and tested for specificity
against the homologous virus by IFA. A comparison of
absorbed and unabsorbed anti-A59V and anti-BCV ascitic
fluids is shown in Figure 2. A59V was propagated in a
syngeneic cell line, so there should be no antibodies
against cellular antigens in the hyperimmune fluid. The
background (uninfected cell) fluorescence was about the
same for absorbed or unabsorbed anti-A59V (Figure
2A,2B,2C,2D). In the BCV IFA, it was impossible to
distinguish virus-infected from uninfected cells if
unabsorbed fluid was used (Figure 2E,2G). With absorbed
ascitic fluid the background fluorescence was greatly
reduced and the virus-infected cells were clearly visible
(Figure 2F,2H). The results for the other two viruses
were essentially the same as in the BCV assay. The anti-
CCV ascitic fluid had high background fluorescence and
required absorption to visualize infected cells. The
anti-TGEV fluid had a lower background, although
absorption improved contrast between infected and
uninfected cells (data not shown).