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 infectivity were directed against the peplomeric proteins. The immunoprecipitation and MAb studies suggest that TGEV and CCV are more closely related than A59V and BCV.
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
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December 1984
APPROVAL

of a thesis submitted by

Susan Herbert Goss

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

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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 hyperimmune 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 infectivity were directed against the peplomeric proteins. The immunoprecipitation and MAAb 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

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

<table>
<thead>
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<th>Group 1</th>
<th>Group 2</th>
<th>Unclassified</th>
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<td>OC43V</td>
<td>CV-777</td>
<td></td>
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<tr>
<td>TGEV</td>
<td>MHV</td>
<td>FECV</td>
<td></td>
</tr>
<tr>
<td>CCV</td>
<td>RCV</td>
<td>HECV</td>
<td></td>
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<tr>
<td>PIPV</td>
<td>SDAV</td>
<td>RbCV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCV</td>
<td>RbECV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HEV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD, SK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| 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 immunoelectron 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. 229E 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 coronavirus 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 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 electrophorograms 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-aminoethanesulfonic acid (TES), and 10 mM N-2-hydroxyethylpiperazine-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°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°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°C.

Plaque Assay

Virus stocks were cloned twice by plaque selection and titered by plaque assay on the appropriate cell monolayer. Cells (1 x 10^6 in DME-10) were seeded into plastic six-well dishes and incubated at 37°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$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$·6H$_2$O, 1 mM Na$_3$O$_4$, I 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 x 10^7 cells) were fused with 5 x 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 immunofluorescence. 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 \times 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 \times 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\(^\circ\)C for 8 hr (TGEV, A59V) or 18-24 hr (BCV, CCV), washed and fixed with methanol as described above and stored at -70\(^\circ\)C. 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 μl 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 activity 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 pH 7.4, 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°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 Radioimmunoprecipitation (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°C. Immune complexes were precipitated with 50 ul of 10% Staphylococcus aureus (Cowan) prepared by the method of Kessler (32) for 1 hr at 0°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°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-PO₄
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 $3 \times 10^6$ 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 intracellular 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 (○) 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 µl 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).
Figure 2. Comparison of unabsorbed and absorbed hyperimmune ascitic fluids. HRT-18 or 17CL-1 cells were infected with BCV, A59V, or mock-infected. Immunofluorescence assays using homologous absorbed and unabsorbed anti-viral ascitic fluids were conducted as described in the text. A phase contrast micrograph of the same field of cells is shown beside each immunofluorescence micrograph.
To further test the effectiveness of the hyperimmune ascitic fluids, virus neutralizing titers were determined and compared to those of serum from immunized mice. Virus neutralization (VN) curves using serum and ascitic fluid against each of the four viruses are shown in Figures 3 and 4. The VN titers of the corresponding serum and ascitic fluid were similar (less than a two-fold dilution apart) for A59V, BCV and CCV. The anti-TGEV serum was obtained from a different mouse than the ascitic fluid and had a much lower anti-viral activity. The ascitic fluids also compared favorably to serum in immunofluorescence assays (not shown).

**Intracellular Virus-specific Proteins**

Radiolabeled intracellular proteins from virus-infected cell lysates were immunoprecipitated using hyperimmune ascitic fluid. The results are shown in Figure 5. Seven to nine intracellular viral proteins were precipitated from A59V-infected 17CL-1 cells (lane 1). The apparent molecular weights of these polypeptides were 155, 113, 60, 56, 50, 45, and 23-26 kd. The 23-26 kd bands were heterogeneous. A smaller 18 kd protein was seen in some preparations. BCV-infected HRT-18 cells contained seven viral proteins with molecular weights of 141-152k, 106-115k, 100k, 60k, 48k, 33k and 26k (lane 3). The anti-CCV ascitic fluid precipitated four viral
Figure 3. Comparison of virus-neutralizing activity of anti-A59V and BCV serum and ascitic fluid. Serial dilutions of homologous anti-viral serum (+) or ascitic fluid (x) were tested for neutralizing activity against A59V and BCV in plaque reduction tests as described in Materials and Methods. The reciprocal of the antibody dilution was plotted against percent plaque reduction.
Figure 4. Comparison of virus-neutralizing activity of anti-CCV and TGEV serum and ascitic fluid. Serial dilutions of homologous anti-viral serum (+) or ascitic fluid (x) were tested for neutralizing activity against CCV and TGEV in plaque reduction tests as described in Materials and Methods. The reciprocal of the antibody dilution was plotted against percent plaque reduction.
Figure 5. Intracellular virus-specific proteins of coronaviruses A59V, BCV, CCV and TGEV. Virus and mock infected cells were labeled with $^{35}$S-methionine as described in the text. Cell lysates were immuno-precipitated with homologous anti-viral ascitic fluid and analyzed by SDS-PAGE on 8% slab gels. Viral proteins are indicated with their approximate sizes in kd. The figure shows (1) A59V or (2) mock-infected 17CL-1 cells, (3) BCV or (4) mock-infected HRT-18 cells, (5) CCV or (6) mock-infected A-72 cells, and (7) TGEV or (8) mock-infected ST cells. The lower portion of Lanes 5 and 6 was overexposed to visualize the smaller proteins.
polypeptides of 185, 92, 47-50 and 30 kd (lane 5). A 40 kd protein was sometimes detected. The intracellular proteins of TGEV were 186, 135, 92, 81, 77, 48-50, 41 and 29-30 kd (lane 7).

These intracellular proteins were reproducibly detected in several experiments using different cell lysates and in some cases different conditions of immunoprecipitation including heating and alkylation of samples. The polypeptide profiles were essentially the same in each experiment.

Serological Interrelationships

The four mammalian coronaviruses used in these studies are from two different antigenic groups. CCV and TGEV belong to Group 1, and A59V and BCV are members of Group 2 (Table 2; 68,76). These relationships were confirmed by reciprocal cross-immunofluorescence, neutralization and immunoprecipitation tests.

The results of an IFA using homologous and heterologous ascitic fluids are shown in Figure 6. It is clear that A59V and BCV shared antigenic determinants, as did TGEV and CCV. The results of an IFA are not quantitative and do not give information about the degree of cross-reaction between related viruses or the viral components responsible for the relationship.
Figure 6. Antigenic cross-reactions among coronaviruses by immunofluorescence. Cells infected with A59V, BCV, CCV or TGEV were analyzed by immunofluorescence assay using homologous and heterologous anti-viral ascitic fluids as described in the text.
Table 3 contains the VN titers for homologous and heterologous hyperimmune ascitic fluids against all four viruses. Homologous titers were higher in all cases; but cross-neutralization was seen between A59V and BCV, and between CCV and TGEV. The data suggest that the antigenic relationship between TGEV and CCV may be closer than that of BCV and A59V. In the latter case the cross-neutralization titers were much lower than the VN titers of the homologous antibody.

Table 3. VN\textsuperscript{a} titers of hyperimmune ascitic fluids.

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<th>anti-CCV</th>
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\textsuperscript{a}Reciprocal cross-neutralizations were conducted with homologous and heterologous ascitic fluids as described in Materials and Methods. The numbers given are the reciprocals of the dilutions resulting in 50% plaque reduction.

\textsuperscript{b}No detectable neutralizing activity.

Cross-immunoprecipitations of virus-infected cell lysates with homologous and heterologous ascitic fluids were done in order to examine the antigenic relationships among the viruses at the molecular level. The results of SDS-PAGE of the immunoprecipitates are shown in Figures 7-10. TGEV and CCV shared antigenic determinants on all
Figure 7. Immunoprecipitation of intracellular MHV-A59 proteins by homologous and heterologous hyperimmune ascitic fluids. $^{35}$S-methionine-labeled virus or mock-infected cell lysates were prepared as described in Materials and Methods and immunoprecipitated with anti-A59V, BCV, CCV or TGEV ascitic fluid. The immunoprecipitates were analyzed by SDS-PAGE. Viral proteins are indicated with their apparent molecular weights ($\times 10^3$). Lane 1(MOCK) shows mock infected 17CL-1 cells with anti-A59V. Lanes 2-5 show A59V-infected cells with anti-A59V, anti-BCV, anti-CCV, or anti-TGEV. Lane 6(CONT) shows A59V-infected cells, no antibody. Lane 7(NMA) shows A59V-infected cells with normal mouse ascitic fluid.
Figure 8. Immunoprecipitation of intracellular BCV proteins by homologous and heterologous ascitic fluids. Immunoprecipitations were carried out as described in the legend to Figure 6. Lane 1 shows mock-infected HRT-18 cells with anti-BCV. Lanes 2-5 show BCV-infected HRT-18 cells with anti-BCV, anti-A59V, anti-CCV or anti-TGEV.
Figure 9. Immunoprecipitation of intracellular proteins of CCV by homologous and heterologous ascitic fluids. Immunoprecipitations were carried out as described in the legend to Figure 6. Lane 1 shows mock-infected A-72 cells with anti-CCV. Lanes 2-5 show CCV-infected A-72 cells with anti-CCV, anti-TGEV, anti-A59V or anti-BCV. Lanes 6 and 7 show CCV-infected A-72 cells with (6) no antibody, or (7) normal mouse ascitic fluid.
Figure 10. Immunoprecipitation of intracellular TGEV proteins by homologous and heterologous ascitic fluids. Immunoprecipitations were carried out as described in the legend to Figure 6. Lane 1 shows mock-infected ST cells with anti-TGEV. Lanes 2-5 show TGEV-infected ST cells with anti-TGEV, anti-CCV, anti-A59V or anti-BCV. Lanes 6 and 7 show TGEV-infected ST cells with (6) no antibody, or (7) normal mouse ascitic fluid.
major viral proteins (Figures 9 and 10). The 30 kd protein of TGEV was precipitated by anti-CCV ascitic fluid. The band was visible on the original film although it cannot be seen in the photograph in Figure 10 (all four bands are shown in the cross-immunoprecipitation in Figure 12A, lane 1). The films were sometimes overexposed to visualize the low molecular weight polypeptides, which resulted in poor resolution of the large proteins (e.g. the P proteins, Figures 7, 9, 10). The apparent recognition of the 50 kd nucleocapsid protein of TGEV by all four hyperimmune ascitic fluids (Figure 10) was probably due to a non-immune binding of proteins to *S. aureus* (Cowan) bacteria during immunoprecipitation. Only the anti-TGEV and anti-CCV fluids reproducibly precipitated this protein. The non-specific binding of proteins to *S. aureus* has been a recurrent problem in immunoprecipitation studies (Control lanes on Figures 6, 8, 9; Bond et. al., unpublished data; J. Leibowitz, personal communication).

Homologous antigens of BCV and A59V also showed cross-reactions (Figures 7 and 8). The 115 kd protein of BCV was not precipitated by anti-A59V, and some of the intracellular polypeptides were poorly recognized by the heterologous antibody. The low molecular weight proteins were usually precipitated by anti-BCV and anti-A59V ascitic fluids, although they could not be seen on this
gel. No reproducible cross-reactions were seen between either A59V or BCV and CCV or TGEV.

**Antigenic Analysis using Monoclonal Antibodies**

Monoclonal antibodies (MAbs) were prepared against A59V, BCV, CCV and TGEV using hybridoma technology. A total of thirty-nine positive (by IFA) cultures was detected among the four fusions: eleven from A59V, eight from CCV, three from BCV and seventeen from TGEV. More than eighty positive clones were isolated from these original cultures. The clones were tested for virus specificity and cross-reactivity by IFA, and eighteen stable antibody-producing hybridoma lines were selected for further examination. The polypeptide specificity of the MAbs was determined by immuno-precipitation, and each was tested for VN activity by the plaque reduction test. A summary of the results is shown in Table 4.

Representative results of the immunoprecipitations using monoclonal antibodies are shown in Figure 11. Several of the MAbs precipitated more than one polypeptide (see Table 4). Each MAb was tested in two or three independent immunoprecipitations. Some of the minor polypeptides evident in the original data are not visible in the figure. None of the MAbs showed any activity against mock-infected cells by IFA or by immunoprecipitation.
Table 4. Characteristics of monoclonal antibodies.

<table>
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<tr>
<th>MAba</th>
<th>Polypeptide specificityb</th>
<th>Structural Intracellular</th>
<th>Cross-reactionsc</th>
<th>VNd</th>
<th>XVNe</th>
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<tr>
<td>A24</td>
<td>N (60)</td>
<td>50,57,160</td>
<td>-</td>
<td>-</td>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>P (155)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>40,47</td>
<td>CCV</td>
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aMonoclonal antibodies were designated with a number preceded by a letter identifying the immunizing virus. A-A59V, B-BCV, C-CCV, T-TGEV.
bPolypeptides are listed by size in kilodaltons.

As determined by immunofluorescence assay.

dVirus neutralization activity as determined by plaque reduction.

eCross-neutralization by viruses other than the immunizing virus as determined by plaque reduction.

fNot done.

Six MAbs with specificities for A59V polypeptides were isolated. Four of these were directed against the peplomeric glycoprotein (Figure 11, lanes 2 and 5), and
Figure 11. Analysis of monoclonal antibody specificities by immunoprecipitation. Radiolabeled virus-infected cell lysates were immunoprecipitated with monoclonal antibodies (MAbs) as described in Materials and Methods and the polypeptides separated by SDS-PAGE in 8% slab gels. Major viral proteins are indicated with their apparent sizes in kd. Lanes 1-5 show A59V-infected cells immunoprecipitated with (1) anti-A59V polyvalent ascitic fluid, (2) A59V MAb A8, (3) A24, (4) A27 or (5) A74. Lanes 6-7 show BCV-infected cells with (6) anti-BCV polyvalent ascitic fluid or (7) BCV MAb B24. Lanes 8-13 show TGEV-infected cells with (8) anti-TGEV polyvalent ascitic fluid, (9) TGEV MAb T41, (10) T22, (11) T20, (12) T39 or (13) T40.
the other two recognized the nucleocapsid protein (lanes 3 and 4). Several of the MAbs precipitated more than one protein, but none of the six cross-reacted with the antigenically related BCV. Three of the anti-peplomer antibodies neutralized the infectivity of A59V, but not of BCV. The other anti-P MAb, A74, had no detectable neutralizing activity against A59V or BCV. Only one stable hybridoma line was isolated which produced antibodies against BCV. This MAb (B24) precipitated the nucleocapsid proteins of both BCV and A59V. The four anti-CCV MAbs were all directed against the nucleocapsid protein, and all four cross-reacted with TGEV. Six MAbs were isolated which recognized the 50 kDa nucleocapsid protein of TGEV. Five of these cross-reacted strongly with CCV by immunofluorescence. Only one hybridoma cell line produced antibodies against the TGEV peplomer glycoprotein, MAb T22 (Figure 11, lane 10). These antibodies also cross-reacted with CCV and had neutralizing activity against both TGEV and CCV. None of the antibodies directed against the nucleocapsid protein showed neutralizing activity. Most of the anti-N immunoprecipitations showed a high molecular weight band which probably represents aggregation or complexing of smaller polypeptides, since it did not co-migrate with any of the intracellular viral proteins. No monoclonal antibodies recognizing the membrane glycoprotein(s) were isolated.
Cross-reactions of the monoclonal antibodies were assessed by immunofluorescence. Positive reactions were verified by immunoprecipitation. Cross-reacting MAbs precipitated homologous polypeptides from infected cell lysates of the related virus. The results for TGEV are shown in Figure 12A. The four anti-N MAbs of CCV precipitated various combinations of intracellular proteins from TGEV-infected cells. The TGEV hybridoma T6 was the only one isolated against CCV or TGEV which failed to cross-react with the related virus. It precipitated the 50 kd N protein and 4 other intracellular polypeptides from TGEV-infected cell lysates (Figure 12B, lane 1), but none from CCV-infected cells (lane 3). TGEV MAb T7 reacted with the 50 kd proteins of TGEV and CCV, and the 40 kd protein of TGEV (lanes 2 and 4).
Figure 12. Cross-immunoprecipitation of viral proteins by monoclonal antibodies. $^{35}$S-methionine labeled polypeptides from TGEV or CCV-infected cells were immunoprecipitated with heterologous monoclonal antibodies as described in Materials and Methods and the polypeptides separated by SDS-PAGE in 8% slab gels. Viral proteins are indicated with their sizes in kd. Figure 12A shows TGEV-infected cell lysates immunoprecipitated with (1) anti-CCV polyvalent ascitic fluid, (2) CCV-MAb C45, (3) C39, (4) C115, or (5) C49. Figure 12B shows TGEV-infected cell lysates immunoprecipitated with (1) TGEV MAb T6 or (2) T7, and CCV-infected cell lysates immunoprecipitated with (3) TGEV MAb T6 or (4) T7.
DISCUSSION

This project had two objectives. These were to develop a reproducible laboratory method for comparing antigens of diverse virus species, and to use the system in a comprehensive investigation of the molecular interrelationships among mammalian coronaviruses. This was accomplished by preparing murine polyvalent and monoclonal antibodies against four coronaviruses from two different antigenic groups, and using them to compare the intracellular proteins of the viruses.

Four different species of coronavirus were chosen for study: murine hepatitis virus A59 (A59V), bovine coronavirus (BCV), canine coronavirus (CCV) and porcine transmissible gastroenteritis virus (TGEV). Cell culture systems were established and optimized for each virus. The use of continuous cell lines made serial passage of virus and maintenance of cells relatively simple. Propagation of virus in cells with marked cytopathic effect (CPE) allowed cloning by plaque selection and quantitation of virus by plaque titration.

Previous studies have placed most of the known mammalian coronaviruses into one of two antigenic groups based on serological cross-reactions (reviewed in 68).
Most of these studies were conducted using heterotypic serum, and there was considerable ambiguity in the results. The use of heterotypic serum in interspecies neutralization tests gives misleading results because of endogenous antibodies directed against non-viral antigens in the sample. Antibodies which neutralized the infectivity of TGEV in a complement-dependent reaction were detected in serum from a number of animal species (19). It was suggested that the neutralization was due to heterophilic antibodies directed against porcine glycolipids in the viral envelope. The widespread occurrence of BCV-neutralizing antibodies in "normal" human and animal sera (21,53) precludes the use of such samples in studies of antigenic interrelationships. These antibodies could be the result of infection with BCV or a related coronavirus, or the cross-neutralization may be due to heterophilic antibodies such as those described above. I used anti-viral ascitic fluids prepared in inbred mice as a homogeneous source of antibody against A59V, BCV, CCV and TGEV. Infected-cell lysates were used as immunogen, eliminating the need for virus purification. Specific high-titer antibody was obtained in as little as four weeks. Large volumes of fluid accumulated in individual mice, so that a single immunization schedule yielded enough antibody for a complete battery of serological tests. The ascitic fluids detected the
intracellular polypeptides of the immunizing viruses as well as the structural proteins (Figure 5). The titers of the fluids compared favorably to serum in homologous virus neutralization tests (Figure 3). Although immunization with cell lysates produced antibodies against cellular as well as viral proteins, the non-viral antibodies were successfully removed by absorbing the ascitic fluids with uninfected cells (Figure 2). Monoclonal antibodies against all four viruses were produced by fusion of splenocytes from the immunized mice with myeloma cells, providing a bank of specific reagents for precise investigation of viral antigens at the level of the individual polypeptides. Thus this protocol fulfilled each of the criteria set down for establishment of a standardized system for studying antigenic interrelationships among viruses of different species.

Serological Comparisons

The absorbed hyperimmune ascitic fluids were used to investigate the antigenic interrelationships among the four coronaviruses. The IFA confirmed the cross-reactions described previously between A59V and BCV (26) and between CCV and TGEV (18, 29, 49). No cross-reactions were seen among viruses from different antigenic groups (Figure 6). Reciprocal VN tests showed the same relationships,
although the data seemed to suggest a closer relationship between CCV and TGEV than between A59V and BCV (Table 3).

Molecular Comparisons

Reciprocal cross-immunoprecipitations of intracellular viral proteins showed that the cross-reactions between related coronaviruses were due to common antigenic determinants on each of the major viral antigens: the peplomer, nucleocapsid and membrane proteins (Figures 7-10). The immunoprecipitation and monoclonal antibody studies confirmed that TGEV and CCV are more closely related than BCV and A59V. Of the ten anti-N MAbs isolated for TGEV and CCV, only T6 did not show cross-reactivity with the related virus, indicating that the N-proteins of CCV and TGEV are very closely related (Table 4, Figure 12B). The anti-P MAb, T22, neutralized the infectivity of both CCV and TGEV. In contrast, none of the A59V MAbs cross-reacted with BCV by IFA, immunoprecipitation or VN.

Intracellular Virus-Specific Proteins

The intracellular proteins of each virus were identified by immunoprecipitation of radiolabeled polypeptides from infected cell lysates with hyperimmune ascitic fluid. The intracellular proteins found in A59V-cells were in general agreement with those reported in the
literature (3,4,20,38,57,67). The 26, 60 and 155 kd proteins represent the major M, N and P antigens of the virus. The variable 45-57 kd proteins are probably nucleocapsid degradation products (57). However, an intracellular protein about 10 kd smaller than the N protein was reproducibly precipitated from infected cell lysates of all four viruses by polyvalent ascitic fluid and anti-N monoclonal antibodies, suggesting that this protein may be an intracellular precursor of the N protein.

Non-structural proteins of BCV, CCV and TGEV have not been described previously. Four of the polypeptides precipitated from BCV-infected cells had the same approximate molecular weights as the structural proteins reported by others (26,33,36). Additional polypeptides of 26 and 50 kd may be processing intermediates, analogous to the 18 and 50 kd intracellular proteins of A59V.

Major polypeptides of 185, 50 and 30 kd were immunoprecipitated from lysates of CCV-infected A-72 cells. These correspond to the three structural proteins described by Garwes and Reynolds (18). They also reported the presence in virions of a 23 kd glycoprotein which I did not find in infected cell lysates. However, the M proteins of coronaviruses are known to be heterogeneous, and the migration characteristics and number of bands seen
in SDS-PAGE vary widely in different laboratories (3,4,57,65,67).

The intracellular proteins of TGEV proved to be quite interesting. In addition to the proteins homologous to those described for CCV, three polypeptides of 77-92 kDa were repeatedly precipitated from infected-cell lysates. An obvious hypothesis is that they represent variously glycosylated monomers of the peplomeric protein, since the P proteins of A59V and BCV reportedly consist of dimers (20,48), and intracellular proteins of 90-100 kDa were often seen in gels of A59V, BCV and CCV. However, the recognition of these proteins by several of the anti-N MAbs (Figure 12) suggests that they are related to the 50 kDa nucleocapsid protein and not to the peplomeric glycoprotein.

**Monoclonal Antibodies**

Most of the MAbs isolated were directed against antigenic determinants on the nucleocapsid (N) protein. This was not unexpected, since this polypeptide is synthesized in the greatest amounts in virus-infected cells (57). Vautherot et al. (73,74) used purified virions to immunize mice for production of MAbs. Most of their antibodies had specificities for the peplomeric glycoproteins, presumably because these antigens are exposed on the viral surface.
Several of the MAbs precipitated more than one viral polypeptide. This could be due to (i) failure of the cloning procedure to separate hybridomas with different specificities, (ii) shared epitopes on the polypeptides because of primary sequence homologies, (iii) breakdown or aggregation of polypeptides to form antigens which migrate differently in SDS-PAGE gels but maintain the same specificity, or (iv) non-specific binding of antibody or S. aureus bacteria to viral proteins. All MAbs were cloned at least twice by limiting dilution, and only single colonies were selected for testing and subcloning. Therefore the presence of mixed clones in the final population is unlikely. Also, several hybridomas were recloned to maintain stability and showed no changes in antibody specificity upon repeat testing.

Preparation and treatment of samples during immunoprecipitation and gel electrophoresis can lead to anomalous migration patterns of coronavirus glycoproteins (65,67), giving multiple bands which in fact represent only one protein. I have done immunoprecipitations of TGEV, CCV and MHV-infected cell lysates under a variety of reducing and denaturing conditions. There were no substantial variations in the electrophoretic profiles when sample treatments were changed.

Non-specific reactions can be a problem in immunoprecipitations using S. aureus. However, these are usually
unpredictable and irreproducible. The intracellular proteins precipitated by MAbs in these experiments were seen in gels of two or more independent immunoprecipitations. Also, control experiments including the immunoprecipitation of infected-cell lysates with normal mouse ascitic fluid, heterologous non-reactive antibodies, and S. aureus in the absence of antibody were done to detect the presence of artifacts. Therefore it seems likely that the proteins precipitated by the MAbs are related because of primary sequence homologies.

Conclusions

The antigens of four coronaviruses were compared using several serological and molecular techniques. The serological comparisons were facilitated by the development of a homogeneous system for production of hyperimmune ascitic fluids in mice immunized with virus-infected cell lysates. Intracellular proteins of BCV, CCV and TGEV, which have not been described previously, were identified using these anti-viral ascitic fluids.

Monoclonal antibodies were isolated against nucleocapsid and peplomeric proteins of A59V and TGEV, and against the N proteins of BCV and CCV. These MAbs provided a powerful tool for analyzing antigenic interrelationships among viruses and for studying the biological activity of viral polypeptides (e.g. eliciting
of neutralizing antibodies by the peplomeric glycoprotein. MAbs are also valuable for investigation of intracellular protein processing events taking place during virus infection, and determining the relationships between intracellular and structural proteins.

The coronaviruses TGEV and CCV were found to be very closely related, with common epitopes on all major viral antigens. Although no MAbs were isolated which recognized the M proteins, polyvalent anti-viral ascitic fluids precipitated this protein in reciprocal cross-immunoprecipitation tests. Only one of eleven MAbs failed to show cross-reaction between CCV and TGEV. This hybridoma could be a useful diagnostic reagent for differentiation of the two viruses in clinical samples.

In conclusion, this research has introduced a standardized and comprehensive system for studying viral antigens and has illustrated the applications of this method in increasing the knowledge of coronavirus interrelationships.
LITERATURE CITED


D378   Goss, S. H.
G694    Serological and mole-
cop.2    cular comparisons...