Antigenic Relationships Among Proteins of Bovine Coronavirus, Human Respiratory Coronavirus OC43, and Mouse Hepatitis Coronavirus A59

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Antisera prepared against each of three single and one pair of major structural proteins of the bovine coronavirus (Mebus strain) were used in immunoblotting studies to measure cross-reactivity with the structural proteins of the human coronavirus OC43 and the mouse hepatitis coronavirus A59. We conclude that the bovine coronavirus is comprised of four major structural proteins, gp190 (normally present as 120- and 100-kilodalton subunits), gp140, pp52, and gp26. The human coronavirus OC43 has an antigenically homologous counterpart of similar molecular mass to each of these proteins. The mouse hepatitis coronavirus A59 has an antigenically homologous counterpart to only three of these proteins: gp190, pp52, and gp26. There is no counterpart in the mouse virus to the 140-kilodalton glycoprotein, the apparent hemagglutinin of the bovine coronavirus.

Coronaviruses were initially grouped together on the basis of their electron microscopic appearance. Recent experiments to define the replicational strategy of some family members, namely, the avian infectious bronchitis virus, the mouse hepatitis virus, and the porcine transmissible gastroenteritis virus (1, 3, 13), have suggested a common scheme, establishing that coronaviruses are a unique family from the point of view of molecular biology. Antigenic studies on coronaviruses, however, demonstrate the existence of at least four distinct subgroups within the family (10, 11, 19). To date there is little or no evidence of shared antigens among the four groups, and this suggests that the subgroups are widely divergent evolutionarily.

In this study we investigated the antigenic relatedness among the proteins of the bovine coronavirus (BCV) and two other viruses in the same antigenic subgroup, the human respiratory coronavirus OC43 (HCV OC43) and the mouse hepatitis coronavirus A59 (MHV A59). Using antiserum prepared against each of three single and one closely migrating pair of major structural proteins of the BCV, we demonstrated by immunoblotting experiments that the BCV is comprised of four major structural proteins: gp190 (normally present as subunits of 120 and 100 kilodaltons [kd]), gp140, pp52, and gp26. The HCV OC43 was shown to have an antigenically homologous counterpart of similar molecular mass to each of the four proteins. The MHV A59 had antigenically homologous counterparts to only three of the proteins; a homolog to the 140-kd glycoprotein of BCV, the apparent hemagglutinin, was missing.

MATERIALS AND METHODS

Cells. The human adenocarcinoma cell line HRT-18 (17) and the mouse L2 cell line were grown in Dulbecco modified Eagle medium containing 50 µg of gentamicin per ml and 8% fetal bovine serum (Sterile Systems, Inc., Logan, Utah).

Virus. The Mebus strain of BCV was grown in HRT-18 cells, and stocks were prepared as previously described (6). HCV OC43 was obtained from S. Weiss, University of Pennsylvania, Philadelphia, Pa., and had been passaged 7 times in human embryonic tracheal organ culture, followed

by 15 passages in suckling mouse brain. HCV OC43 was cloned in our laboratory by two isolations from single plaques and passaged twice at a multiplicity of <0.1 PFU per cell. Viral stocks were prepared in HRT-18 cells from passages three through eight by infecting cells at a multiplicity of 0.8 PFU per cell. Viral titers ranging from 10^6 to 10^7 PFU/ml were obtained in stock preparations. Passage five of cloned MHV A59 was obtained from K. Holmes, Uniformed Services University, Bethesda, Md. Viral stocks were prepared from passages six through nine in L2 cells.

Growth and purification of virus. Confluent monolayers of cells in 150-cm² flasks were infected with virus at a multiplicity of ca. 1 PFU per cell. After 1.5 h of adsorption at 37°C, the inoculum was removed and replaced with 15 ml of medium containing 1% fetal bovine serum. Supernatant fluids were removed at 24 h post-infection for MHV A59 or 72 to 96 h post-infection for BCV and HCV OC43 and clarified by centrifugation at $7,500 \times g$ for 10 min. Virus was concentrated by centrifugation at $90,000 \times g$ for 2 h onto cushions of 60% (wt/wt) sucrose in TMEN (50 mM Tris-maleate [pH 6], 0.1 M NaCl, 1 mM EDTA). Concentrated virus was then isopycnicly centrifuged on an 18-ml continuous 60 to 20% (wt/wt) sucrose gradient in TMEN for 4.5 h at $90,000 \times g$ in a Sorvall AH-627 rotor. Gradients were fractionated by using an Isco gradient fractionator. The virus was diluted in TMEN and pelleted at $90,000 \times g$ for 2.5 h in a Beckman SW 27.1 rotor. All sucrose solutions were made in TMEN, and steps were carried out at 4°C.

Polyacrylamide gel electrophoresis. The discontinuous buffer gel system of Laemmli was used (7). Samples were dissolved in 0.125 M Tris-hydrochloride (pH 6.8)–4% sodium dodecyl sulfate–5 M urea and treated at 100°C for 2 min immediately before electrophoresis. Electrophoresis was performed in slab gels (0.75 by 100 by 160 mm) by using the concentrations of polyacrylamide indicated in the figure legends. A 1-cm stacking gel of 3% polyacrylamide was used. Electrophoresis was carried out by using a current of 15 mA per slab gel. For fluorography, gels were treated with En³Hance (New England Nuclear Corp.), dried, and exposed to preflashed Kodak X-Omat R film by the method of Laskey and Mills (9). ¹⁴C-labeled protein molecular weight markers were myosin (200 kd), phosphorylase b (97.4 kd),

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FIG. 1. Electrophoretic analysis of HCV OC43, BCV, and MHV A59 polypeptides. HCV OC43, BCV, and MHV A59 labeled with ^3H -amino acids were electrophoresed in a 5 to 15% polyacrylamide gradient gel. Virus was analyzed in the absence (lanes 1, 3, and 5) or presence (lanes 2 and 4) of 2% 2-mercaptoethanol. Positions of the ^{14}C -labeled protein molecular weight markers are indicated (lane 6). The arrow identifies a species of ca. 240 kd.

ovalbumin (46 kd), and carbonic anhydrase (30 kd) obtained from New England Nuclear Corp.

Antiserum preparation. Proteins from 1.2 mg of purified BCV were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a cylindrical tube after treatment with a mixture of 2% sodium dodecyl sulfate, 5 M urea, and 2% 2-mercaptoethanol. Protein positions were determined by calculating mobilities relative to bromophenol blue tracking dye and Coomassie blue-stained BCV proteins in a parallel gel. Polyacrylamide plugs containing the isolated proteins were minced and divided into three portions. One portion was emulsified with Freund incomplete adjuvant and 400 μg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Calbiochem-Behring) and delivered subcutaneously to a rabbit. This process was repeated at 2-week intervals with the remaining portions of the gel plug. The estimated total amount of each protein used was 144, 240, 552, and 264 μg for gp120-gp100 (combined), gp65, pp52, and gp26, respectively. Antiserum was prepared against the combination of gp120 and gp100 since these were difficult to separate (6).

Immunoblotting. The method of Towbin et al. (18) as modified by Burnette (2) was used, except that proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose paper for 2 h by using a voltage gradient of 100 V, and the nitrocellulose wash buffer consisted of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride (pH 7.4), 0.25% gelatin, 0.5% Triton X-100, and 0.1% sodium dodecyl sulfate. Nitrocellulose paper with bound proteins was incubated with either preimmune serum or antiserum, diluted 1:10 in buffer containing 50 mM Tris-hydrochloride (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.25% (wt/vol) gelatin, and 0.05% Nonidet P-40, and then reacted with ^{125}I -labeled staphylococcus A protein as described by Burnette (2). ^{14}C -

labeled protein molecular weight markers were included as a separate lane on the slab gels, and their positions were determined by autoradiography of the nitrocellulose paper after protein transfer.

RESULTS

Direct comparison of virion polypeptides reveals distinct differences between the viruses. The structural polypeptide profiles of BCV, HCV OC43, and MHV A59 were compared by electrophoresis under denaturing conditions (Fig. 1). The five major proteins described previously for BCV (6) are identified, including a 140-kd glycoprotein that behaves as a disulfide-linked dimer of 65-kd subunits, closely migrating proteins of 120 and 100 kd, a 52-kd internal phosphoprotein, and a group of closely migrating glycoproteins of ca. 26 kd. In addition, 190- and 240-kd species were observed that we described before as being highly variable in amount and not always present (6). We continued to find their presence variable. The 140-, 120-, and 100-kd glycoproteins more precisely migrated as 130-, 110-, and 90-kd species, respectively, but the original molecular weights assigned (6) will be used in this paper to describe BCV proteins and to identify antiserum made against them.

Four major proteins were observed for HCV OC43 (Fig. 1). They include a prominent 190-kd glycoprotein, a 130-kd glycoprotein that behaves as a disulfide-linked dimer of 65-kd subunits, a 55-kd phosphoprotein, and a group of closely migrating glycoproteins of ca. 26 kd. The glycosylated and phosphorylated states of these proteins have been identified (B. G. Hogue and D. A. Brian, submitted for publication). A protein migrating with a molecular mass of 240 kd that had a variable presence was also identified (Fig. 1).

FIG. 2. Immunoblotting of HCV OC43, BCV, and MHV A59 with antiserum against the BCV gp120 and gp100 proteins. Purified virus, ca. 10 μg of protein per lane, was electrophoresed in an 8% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper, and analyzed as described in the text. Lanes 1 through 5 were treated with anti-gp120-100 serum, and lane 6 was treated with preimmune serum. Virus in lanes 2 and 4 was treated with 2% 2-mercaptoethanol before electrophoresis. BCV was the virus used in lane 6. Positions of transferred molecular weight markers are indicated.

FIG. 3. Immunoblotting of HCV OC43, BCV, and MHV A59 with antiserum against the BCV gp65 protein. Purified virus was electrophoresed in a 9.5% polyacrylamide gel and immunoblotted as described in the legend to Fig. 2. Lanes 1 through 5 were treated with anti-gp65 serum, and lane 6 was treated with preimmune serum. Virus in lanes 2 and 4 was treated with 2% 2-mercaptoethanol before electrophoresis. BCV was the virus used in lane 6. Positions of transferred molecular weight markers are indicated.

Consistent with reports describing MHV A59, we found three major polypeptide species for this virus. These included a 180-kd protein (E2), shown to be a glycoprotein that is tryptically cleavable into 90-kd subunits, a 50-kd protein (N), shown to be an internal phosphoprotein, and a closely migrating group of proteins of ca. 23 kd (E1), shown to share a common polypeptide and to have different levels of glycosylation (15, 16).

Immunoblotting reveals four homologous proteins between BCV and HCV OC43 but only three between BCV and MHV A59. When purified BCV, HCV OC43, and MHV A59 were electrophoresed on the same gel and immunoblotted with rabbit antiserum prepared against the gp120 and gp100 proteins of BCV, homologous proteins were found in HCV OC43 and MHV A59 (Fig. 2). Antibody bound to the gp120 and gp100 proteins of BCV as expected, but it additionally bound to the 190-kd protein in BCV. This suggests that gp190 of BCV is an aggregate of the gp120 and gp100 molecules or, alternatively, that gp190 is an uncleaved precursor to the gp120 and gp100 molecules. Antibody bound only to the 190-kd glycoprotein of HCV OC43. We have learned that the gp190 molecule of HCV OC43 is converted by treatment with trypsin to molecules that comigrate with gp120 and gp100 of BCV (Hogue and Brian, submitted for publication) and therefore behaves in a way analogous to the gp180 (E2) protein of MHV A59 that is tryptically cleavable into 90-kd subunits (16). Antibody bound to the gp180 (E2) protein of MHV A59 and to its 90-kd subunit. The 190-kd glycoprotein of HCV OC43 and the 180-kd glycoprotein of MHV A59 therefore behave as homologous proteins antigenically and in their response to trypsin treatment. Because a 190-kd glycoprotein was found in BCV as a protein that varies in quantity from preparation to preparation, we hypothesize that it too is a precursor molecule to gp120 or gp100, or to both, and that it is more

proteolytically labile than the HCV OC43 or MHV A59 counterparts. At times some reactivity against the 65-kd subunit of gp140 was observed after electrophoresis of BCV with 2-mercaptoethanol (Fig. 2, lane 4). We interpret this to mean that some antibody to gp140 is present in the anti-gp120-100 serum since 2% 2-mercaptoethanol does not fully reduce large amounts of protein, and some gp140 may have been in the electrophoretically purified gp120-100 used to prepare antiserum. The converse is not true. Antiserum to gp65 did not react with the gp120-100 bands (see below). No gp65 in HCV OC43 was ever observed to react with the anti-gp120-100 serum, and this may have been due in part to the relative abundance of the gp140 protein of BCV and HCV OC43. For BCV, gp140 represents ca. 20% of viral protein (6), whereas for HCV OC43 it represents ca. 4% (Hogue and Brian, submitted for publication).

When the three viruses were immunoblotted with rabbit antiserum prepared against the gp65 protein of BCV, homologous proteins were found between BCV and HCV OC43 but not between BCV and MHV A59 (Fig. 3). Antibody bound to the 65-kd glycoprotein when BCV was electrophoresed in the presence of 2-mercaptoethanol and to the 140-kd glycoprotein when the reducing agent was not included. This confirms our earlier suggestion that gp65 is a disulfide-linked subunit of gp140 (6). A homologous, disulfide-linked protein of similar molecular weight was identified for HCV OC43, but none was observed for MHV A59, even when the immunoblot was overexposed.

Antibody prepared against the internal 52-kd phosphoprotein of BCV bound to the phosphoproteins of BCV, HCV OC43, and MHV A59 (Fig. 4). A narrow phosphoprotein band of ca. 160 kd is often observed when 2-mercaptoethanol is not included in the dissolution buffer. This phosphoprotein behaves as a possible trimer of pp52.

Antibody prepared against the gp26 protein of BCV bound

FIG. 4. Immunoblotting of HCV OC43, BCV, and MHV A59 with antiserum against the BCV pp52 protein. Purified virus was electrophoresed in a 9% polyacrylamide gel and immunoblotted as described in the legend to Fig. 2. Lanes 1 through 5 were treated with anti-pp52 serum, and lane 6 was treated with preimmune serum. Virus in lanes 2 and 4 was treated with 2% 2-mercaptoethanol before electrophoresis. BCV was the virus used in lane 6. Positions of transferred molecular weight markers are indicated.

to this protein in BCV and to homologous proteins of the same molecular weight in HCV OC43 and MHV A59 (Fig. 5). One striking feature of this protein is an apparent aggregation into multimeric structures.

Preimmune sera did not react with any protein in regions of interest when tested against BCV (Fig. 2, 3, 4, and 5, lanes 6) or against HCV OC43 or MHV A59 (data not shown).

DISCUSSION

Previous serological studies have demonstrated common antigens among BCV, HCV OC43, and MHV 3 (11). Although MHV 3 and MHV 59 are distinctly different strains of MHV, they are related antigenically (5, 8). Our studies confirm that there are shared antigens among BCV, HCV OC43, and MHV A59 and further identify which proteins among these viruses are antigenically homologous. Although we could not precisely measure the degree of relatedness among the three viruses by our studies, the overall polypeptide patterns would suggest that BCV and HCV OC43 are more closely related than BCV and MHV A59. BCV and HCV OC43 were distinguishable structurally by a difference in the behavior of the 190-kd glycoprotein, a difference in the abundance of the 140-kd glycoprotein, and a slight difference in the molecular weight of the internal phosphoprotein. These differences are consistent with a nucleotide sequence divergence of ca. 4% that we observed in two-dimensional T1 oligonucleotide fingerprints of the two viruses (W. Lapps and D. A. Brian, submitted for publication).

Our finding that the gp140 protein of BCV has no homologous protein on the nonhemagglutinating MHV A59 is consistent with the notion that gp140 functions as the hemagglutinin on BCV (B. King et al., submitted for publication). Since MHV A59 apparently has no homologous protein, the question arises as to the function of this protein in the replication of BCV and HCV OC43. Does gp140 represent a protein that was lost by MHV A59 or, alternatively, one that

was gained by BCV and HCV OC43? Preliminary studies in our laboratory demonstrate that the antigenically related hemagglutinating encephalomyelitis virus of swine, strain 67N, also possesses a reducible, disulfide-linked glycoprotein of around 140 kd (unpublished data). There appear therefore to be evolutionary pressures for the retention of this protein in the three viral species. A comparison of genome sizes, RNA messages, and sequence analyses among BCV, HCV OC43, and MHV A59 would be useful in determining the true extent of relatedness among these viruses.

A previous study describing the structural proteins of HCV OC43 did not identify gp140 (12). The apparent reason for this is that Coomassie blue staining was used to identify electrophoretically separated viral proteins, and a minor protein such as gp140 would not be readily visible by this method. In our studies, gp140 represented ca. 4% of the total viral protein on the basis of incorporated radiolabeled amino acids from a mixture (Hogue and Brian, submitted for publication).

Two reports have recently described the existence of neutralizing antibodies in cattle against both BCV and HCV OC43 and in humans against both HCV OC43 and BCV (4, 14). These results could be explained either by the presence of common epitopes which give rise to heterologous neutralizing antibodies or by a zoonosis by one or both viruses. Our data cannot be used to rule out either possibility. The notion that these are possibly zoonotic viruses is consistent with our finding that HCV OC43 and BCV (Mebus strain), as well as three other primary isolates of BCV (one from Tennessee and two from Colorado), grow readily in human rectal tumor (HRT-18) cells without requiring adaptation. Since these cells maintain many features of primary cells, i.e., microvilli (17), ready infection by BCV strains suggests there is no strict host tropism for BCV and leaves open the possibility of human infection by this virus. Our experiments would suggest that antigenic differences between BCV and HCV OC43 would not be useful in studying the epidemiology of these viruses serologically. On the other hand, distinct differences between the 190-kd glycoproteins, the internal phosphoproteins, or the genomic oligonucleotide fingerprint patterns may be useful in distinguishing the viruses during epidemiological studies.

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LITERATURE CITED

1. Baric, R. S., S. A. Stohlman, and M. M. C. Lai. 1983. Characterization of replicative intermediate RNA of mouse hepatitis virus: presence of leader RNA sequences on nascent chains. *J. Virol.* 48:633-640.
2. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
3. Dennis, D. E., and D. A. Brian. 1982. RNA-dependent RNA polymerase activity in coronavirus-infected cells. *J. Virol.* 42:153-164.
4. Gerna, G., P. M. Cereda, M. G. Revello, E. Cattaneo, M. Battaglia, and M. Torsellini Gerna. 1981. Antigenic and biological relationships between human coronavirus OC43 and neona-

FIG. 5. Immunoblotting of HCV OC43, BCV, and MHV A59 with antiserum against the BCV gp26 protein. Purified virus was electrophoresed in a 9% polyacrylamide gel and immunoblotted as described in the legend to Fig. 2. Lanes 1 through 5 were treated with anti-gp26 serum, and lane 6 was treated with preimmune serum. Virus in lanes 2 and 4 was treated with 2% 2-mercaptoethanol before electrophoresis. BCV was the virus used in lane 6. Positions of transferred molecular weight markers are indicated.

- tal calf diarrhea coronavirus. *J. Gen. Virol.* **54**:91–102.
5. **Hierholzer, J. C., J. R. Broderick, and F. A. Murphy.** 1979. New strain of mouse hepatitis virus as the cause of lethal enteritis in infant mice. *Infect. Immun.* **24**:508–522.
 6. **King, B., and D. A. Brian.** 1982. Bovine coronavirus structural proteins. *J. Virol.* **42**:700–707.
 7. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 8. **Lai, M. M. C., and S. A. Stohman.** 1981. Comparative analysis of RNA genomes of mouse hepatitis viruses. *J. Virol.* **38**:661–670.
 9. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335–341.
 10. **McIntosh, K.** 1974. Coronaviruses: a comparative review. *Curr. Top. Microbiol. Immunol.* **63**:85–129.
 11. **Pedersen, N. C., J. Ward, and W. L. Mengeling.** 1978. Antigenic relationship of the feline infectious peritonitis virus to coronaviruses of other species. *Arch. Virol.* **58**:45–53.
 12. **Schmidt, O. W., and G. E. Kenny.** 1982. Polypeptides and functions of antigens from human coronaviruses 229E and OC43. *Infect. Immun.* **35**:515–522.
 13. **Stern, D. F., and S. I. T. Kennedy.** 1980. Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. *J. Virol.* **36**:440–449.
 14. **Storz, J., and R. Rott.** 1981. Reactivity of antibodies in human serum with an enteropathogenic bovine coronavirus. *Med. Microbiol. Immunol.* **169**:169–178.
 15. **Sturman, L. S.** 1977. Characterization of a coronavirus. I. Structural proteins: effects of preparative conditions on the migration of protein in polyacrylamide gels. *Virology* **77**:637–649.
 16. **Sturman, L. S., and K. V. Holmes.** 1977. Characterization of a coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. *Virology* **77**:650–660.
 17. **Tompkins, W. A. F., A. M. Watrach, J. D. Schmale, R. M. Schulze, and J. A. Harris.** 1974. Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. *J. Natl. Cancer Inst.* **52**:101–106.
 18. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**:4350–4354.
 19. **Wege, H., S. Siddell, and V. ter Meulen.** 1982. The biology and pathogenesis of coronaviruses. *Curr. Top. Microbiol. Immunol.* **99**:165–200.