CHARACTERIZATION AND LOCATION OF THE STRUCTURAL POLYPEPTIDES OF TURKEY
ENTERIC CORONAVIRUS USING MONOCLONAL ANTIBODIES AND ENZYMATIC TREATMENTS

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INTRODUCTION

Turkey enteric coronavirus (TCV) is one of the major causes of epidemic diarrhoea in turkey poults (1, 2). The morphological and physicochemical characteristics of TCV resemble those of other members of the family Coronaviridae (3, 4). However, little is known with respect to the molecular and antigenic structure of the TCV virion, due to difficulties in propagating TCV strains in tissue cultures and lack of highly specific immunological probes (1, 4). Field isolates can be propagated by oral inoculation and intestinal infections of young turkey poults, or by inoculation into embryonating turkey eggs (4, 5). TCV possesses a hemagglutinating (HA) activity which may be associated to short granular projections located near the base of the characteristic larger bulbous peplomers (6). Recently, we adapted TCV isolates in HRT-18 cells, an established cell line derived from human rectum adenocarcinoma (7). In these cells, TCV induces cytopathic changes, including polykaryocytosis, which depended on trypsin in the culture medium (8).

We describe here the polypeptide structure of tissue culture-adapted TCV. We also begun to explore the antigenic features of the virus by producing monoclonal antibodies (MAbs) to its structural proteins.

RESULTS

Viral purification

The prototype egg-adapted Minnesota strain (4) of TCV was obtained from Dr B.S. Pomeroy, College of Veterinary Medicine, St-Paul, Minn., USA. The virus was serially propagated on HRT-18 cells in the presence of 10 U/ml bovine trypsin (Grade X, Sigma). The extracellular virus was purified by isopycnic ultracentrifugation on sucrose gradients (6).

High yields of viral infectivity, ranging from 10^8 to 10^{10} TCID₅₀/mL were recovered after less than 3 to 4 successive passages in HRT-18 cells. Maximal infectivity and HA activity, with rat erythrocytes, were recovered in sucrose-gradient fractions corresponding to a buoyant density of 1.18-1.20 g/ml. These fractions contained viral particles with a morphology consistent with that of coronaviruses, and possessed surface projections of two distinct sizes (Fig. 1).

An anti-TCV hyperimmune serum was obtained after immunization of rabbits with density gradient-purified egg-adapted Minnesota strain. The presence of anti-TCV antibodies was confirmed by IEM and HAI tests (6), and by the use of an indirect protein A-colloidal gold immunolabelling technique (PAG-IEM) (9). After incubation with antiserum, gold granules were usually located near to, or on, the tip of both types of surface projections (Fig. 1b). Control experiments, where pre-immune serum was used or where the anti-TCV serum was omitted, showed that the viral particles in the test were specifically labelled.

Structural polypeptides of TCV

Analysis by SDS-PAGE under non-reducing conditions of purified [³⁵S]methionine-labelled TCV revealed consistently four major and two minor polypeptide species (Fig. 2). The major components were estimated to possess Mr of 140K, 100K, 52K, and 24K, while two minor components had Mr of 200K and 120K. Polypeptide 24K was resolved as a group of 2 to 3 closely migrating bands. Labelling with [³H]glucosamine revealed that the 200K, 140K, 100K, and 24K species were glycosylated. Under reducing conditions, the gp140 species decreased in intensity concomitant with the appearance of a new gp66 species in the gel. The gp140 was thus suggested to be a disulphide-linked dimer of two gp65-70 molecules.

Fig. 1. Morphology and polypeptide structure of TCV. (a) Negatively stained TCV particles with a double fringe of surface peplomers; (b) Protein A-gold immunolabelling of TCV after incubation with rabbit anti-TCV hyperimmune serum. Bars= 50 nm. (c) Sucrose-gradient purified TCV labelled with either [³⁵S]methionine (lanes 1 and 3) or [³H]glucosamine (lanes 2 and 4) was electrophoresed after solubilization with sample buffer in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of 5% 2-mercaptoethanol. Positions of molecular weight standards are shown on the left.

Table 1. Characterization of monoclonal antibodies to TCV.

No. of MAb's directed against				Biological properties		
gp200/100	gp140	p52	gp24	SN	HAI	IFA
-	3	-	-	+	+	Cyto + Mb
-	1	-	-	-	+	Cyto + Mb
5	-	-	-	+	-	Cyto + Mb
11	-	-	-	-	-	Cyto + Mb
-	-	8	-	-	-	Cyto
-	-	-	1	-	-	Perinuclear

Characterization of TCV-specific MABs

For the production of anti-TCV MABs, BALB/c mice were immunized either with purified whole virus or SDS-denatured virus. Splenocytes from these mice were fused with SP2/O-Ag14, as described (9). Hybridoma supernates were screened against TCV virions in a solid phase ELISA (10) and subcloned twice by a limiting dilution method. Working preparations of antibodies were obtained by production of ascitic fluids in BALB/c mice primed with pristane. The polypeptide specificities of anti-TCV MABs were determined by Western immunoblotting with purified virus or by immunoprecipitations with radiolabelled TCV-infected cell lysates, according to Laude et al. (11) and Dereg and Babiuk (12). SN, HAI, and IFA tests were done as previously described (8, 9).

Twenty-nine hybridoma cell lines, producing MABs directed to TCV were obtained (Table 1). Sixteen hybridomas produced MABs that recognized either gp200 or gp100, or both protein species. MABs of 4 other hybridomas reacted only with gp140. In addition, 8 MABs were specific to the major unglycosylated p52 protein, and only one hybridoma produced antibodies that reacted with gp24. MABs directed against gp200/100 immunoprecipitated TCV-induced intracellular proteins 200K, 170 to 180K, and polypeptide species between 90 and 120K, whereas, anti-gp140 MABs immunoprecipitated a 130K polypeptide species, only (data not shown).

Neutralization studies showed that eight anti-TCV MABs had SN titres between 80 and 10,240; three were specific to gp140, while the five others were specific to gp200/100 (Table 1). Only MABs to gp140 inhibited HA activity of TCV. MABs of a given specificity also induced characteristic patterns of fluorescence. Anti-p52 MABs gave a weak fluorescence that was evenly distributed in the cytoplasm of TCV-infected cells. With anti-gp24 MAB, the fluorescence appeared as fine granulation, essentially limited to the perinuclear area. With MABs directed to gp200/100 and gp140, the fluorescence was diffuse throughout the cytoplasm, but appeared more intense around the nucleus. Both anti-gp200/100 and anti-gp140 MABs induced a cell surface fluorescence.

Location and biological properties of TCV structural proteins

To determine the location on the TCV virion of the various proteins identified by SDS-PAGE, aliquots of purified virus were mock-digested or digested with 1 mg/ml of pronase, trypsin, α -chymotrypsin or bromelain, repurified by isopycnic sedimentation, and analysed by EM and SDS-PAGE. Protease-treated TCV was tested also for residual HA activity (Table 2).

Virions treated with pronase lost their HA activity after 45 min, coincidentally with the disappearance of the high mol. wt. glycoproteins from the gel and loss of both types of surface projections. Incubation for 1 hour with either trypsin or α -chymotrypsin did not alter the HA activity of TCV. However, complete or nearly complete disappearance of the gp200 species from the gel was observed and the viral particles apparently lost the bulbous part of their large peplomers. Digestion of TCV with bromelain for two hours did not alter the HA activity of the virus, but caused the loss of the high mol.wt. glycoproteins, except gp140 or gp66, when virus was electrophoresed under reducing conditions. This treatment also left the internal p52 protein intact, but reduced the amounts of gp24. Bromelain-treated viral particles progressively lost their large bulbous peplomers, but their small granular projections persisted even after a 3 hour-exposure period.

Effects of glycosylation inhibitors on the synthesis of TCV glycoproteins

Extracellular virions purified from the supernatant fluids of TCV-infected cells, cultivated in the presence of 0.5 to 1.0 μ g/ml of tunicamycin, lacked both infectivity and HA activity (Table 2). The extracellular viral particles were devoid of both types of surface projections. By SDS-PAGE, high mol.wt. structural glycoproteins (gp200/100 and gp140) could no longer be detected with TM-treated virion. Furthermore, the gp24 species was reduced to its unglycosylated form p20, and it was no longer detectable by Western immunoblotting or immunoprecipitation with polyclonal antiserum. Similar results were obtained by immunoprecipitation with TM-treated TCV-infected cells (13).

Cultivation in the presence of sodium monensin (SM) at concentrations up to 2.5 μ M neither affected the virulence (cytopathogenicity) of the virus, nor changed the morphology of the extracellular progeny viral particles (data not shown). The virus envelope-glycoproteins, including gp24, could be immunoprecipitated from extracts of SM-treated TCV-infected cells and strongly incorporated [3 H]glucosamine (13).

Table 2. Hemagglutinating activity and TCV polypeptides detected by Western immunoblotting after treatment with proteases and tunicamycin (TM).

TCV polypeptides	Treatments				
	Trypsin 1000 μ g/ml	Chymotrypsin 1000 μ g/ml	Pronase 1mg/ml	Bromelain 1.3mg/ml	TM ^a 1 μ g/ml
gp180-200	-	-	-	-	-
gp140	++	++	-	++	-
gp120	+	+	-	-	-
gp100	++	++	-	-	-
gp66	++	++	-	++	-
p52	++	++	++	++	++
gp24-26	++	++	+/-	+/-	-
p20	++	++	++	+	+
HA	++	++	-	++	-

^a TM was added to the culture medium of TCV-infected cells after the adsorption period and extracellular virus was harvested at 24 h p.i.

Fig. 2 Protein A-gold immunolabelling of TCV by anti-TCV monoclonal antibodies (see legend in the text).

Location of the antigenic determinants on the virion

Distinct immunogold-labelling patterns of TCV particles were obtained by MABs (Fig. 2). With anti-p52 MABs, no labelling of intact virions was observed. However, gold granules were found near filamentous-like structures (helicoidal nucleocapsid) which appeared to exit the damaged viral particles (Fig. 2b). With anti-gp200/100 MABs, the gold granules were located near the tips of the large bulbous projections (Fig. 2d). Bromelain-treated viral particles were found to be labelled by anti-gp200/100 MABs only on a few large projections still remaining on their envelope (Fig. 2c). With anti-gp140 MABs, a specific labelling pattern could not be defined with certainty (data not shown). No labelling was obtained using pre-immune mouse serum (Fig. 2a).

DISCUSSION

The structural nature of the proteins resolved by SDS-PAGE of purified virus was confirmed by Western immunoblotting and immunoprecipitation, using antiserum to the original egg-adapted virus. Furthermore, 29 anti-TCV MABs were characterized with respect to polypeptide specificity, capacity to neutralize virus, and ability to inhibit its HA activity.

Four major viral protein species were identified and their location on the TCV virion was deduced from digestion studies with proteolytic enzymes and by immunogold-labelling with MABs. The TCV structural proteins consist of three glycoproteins of 180-200,000, 140,000 and 24,000 which were shown to correspond to the peplomer (E2 or S), hemagglutinin (E3) and matrix (E1 or M) proteins, and a predominant unglycosylated 52,000 species that apparently represents the nucleocapsid (N) protein. A similar polypeptide pattern has been described previously with virus purified from the intestinal contents of turkey embryos (7).

The largest virion glycoproteins of TCV thus appear to differ fundamentally from the largest glycoproteins of the avian infectious bronchitis virus (IBV) (14, 15). The presence of additional short granular projections on the surface of the TCV virion, and a hemagglutinin glycoprotein that appeared as a dimer of two smaller 65-70,000 molecules, are features that have been described so far only for viruses belonging to the group of mammalian hemagglutinating coronaviruses (16, 17, 18). The observation that bromelain treatment removed all glycoproteins from the virion except gp140, and that only small granular projections were retained on the particles, further suggested gp140 and small granular projections to correspond to the virion hemagglutinin.

In the presence of TM, non-infectious TCV virions lacking both types of surface peplomers were produced and released by infected cells. Biochemical data confirmed the absence of the high mol. wt. glycoproteins (gp200/100, gp140/66) consistent with a glycosylation process that involves attachment of oligosaccharide side chains via N-glycosidic bonds to asparagine residues, as demonstrated for other coronaviruses (19, 20). The matrix protein of TCV is apparently glycosylated by a similar process. In this respect, TCV resembles the avian infectious bronchitis virus (14, 21), but differs from the mammalian coronaviruses MHV-A59 and BCV. The matrix glycoprotein of these mammalian coronaviruses undergoes O-linked glycosylation by a process which is resistant to TM, but sensitive to SM, and involves attachment of oligosaccharides to serine residues (18, 22, 23). The envelope glycoproteins of TCV, including the matrix protein, were normally synthesized in the presence of SM. As previously reported for IBV and MHV (14, 22, 23), E1 appeared to be the only glycoprotein required for the formation of TCV virions, and its glycosylation is apparently not essential.

Antigenic determinants located on both E2 and E3 reacted with MAbs involved in TCV neutralization, as previously described for bovine enteric coronavirus (12). Both hemagglutinin and peplomer glycoproteins also were expressed at the cell surface and may thus represent potential targets for specific antibody-dependent or cell-mediated cytotoxicity.

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REFERENCES

1. B. S. Pomeroy. Coronaviral enteritis of turkeys, in: Disease of poultry, 8th ed. M. S. Holstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. W. Yoder, ed., Iowa State Univ. Press, Ames (1984).
2. S. Dea and P. Tijssen. Viral agents associated with outbreaks of diarrhea in turkey flocks in Quebec. Can. J. Vet. Res. 52:53 (1988)
3. D. R. Deshmukh and B. S. Pomeroy. Physicochemical characterization of a bluecomb coronavirus of turkeys. Am. J. Vet. Res. 35:1549 (1977).
4. A. E. Ritchie, D. R. Deshmukh, C. T. Larsen, and B. S. Pomeroy. Electron microscopy of coronavirus-like particles characteristic of turkey bluecomb disease. Avian Dis. 17:546 (1973).
5. D. R. Deshmukh, C. T. Larsen, and B. S. Pomeroy. Survival of Bluecomb agent in embryonating turkey eggs and cell cultures. Am. J. Vet. Res. 34:673 (1973).

6. S. Dea and P. Tijssen. Identification of the structural proteins of turkey enteric coronavirus. Arch. Virol. 99:173 (1988).
7. W. A. F. Tompkins, A. W. Watrach, J.D. Schmale, R.M. Schultz, and J.A. Harris. Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. J. Natl. Cancer Inst. 52:101 (1974).
8. S. Dea, S. Garzon, and P. Tijssen. Isolation and trypsin-enhanced propagation of turkey enteric (Bluecomb) coronaviruses in a continuous human rectal tumor (HRT-18) cell line. Am. J. Vet. Res. (In press).
9. S. Dea and P. Tijssen. Antigenic and polypeptide structure of turkey enteric coronaviruses as defined by monoclonal antibodies. J. Gen. Virol. (In press).
10. S. Dea and P. Tijssen. Detection of turkey enteric coronavirus by enzyme-linked immunosorbent assay and differentiation from other coronaviruses. Am. J. Vet. Res. 50:226 (1989).
11. H. Laude, J.M. Chapsal, J. Gelfi, S. Labiau, and J. GrosClaude. Antigenic structure of transmissible gastroenteritis virus. I. Properties of monoclonal antibodies directed against virion proteins. J. Gen. Virol. 67:119 (1986).
12. D. Deregt and L. A. Babiuk. Monoclonal antibodies to bovine coronavirus: characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. Virology 161:410 (1987).
13. S. Dea, S. Garzon, and P. Tijssen. Intracellular synthesis and processing of the structural glycoproteins of turkey enteric coronavirus. Arch. Virol. (In press).
14. D. F. Stern and B.M. Sefton. Coronavirus proteins: structure and functions of the oligosaccharides of the avian infectious bronchitis virus glycoproteins. J. Virol. 44:804 (1982).
15. D. Cavanagh, P. J. Davis, D. J. C. Pappin, M. M. Binns, M. E. G. Boursnell, and T. D. K. Brown. Coronavirus IBV: partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor polypeptide of IBV strains Beaudette and M41. Virus Res. 4:133 (1986).
16. B. G. Hogue, B. King, and D. A. Brian. Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC 43, and mouse hepatitis coronavirus A59. J. Virol. 51:384 (1984).
17. B. King, B. J. Potts, and D.A. Brian. Bovine coronavirus hemagglutinin protein. Virus Res. 2:53 (1985).
18. W. Lapps, B. G. Hogue, and D. A. Brian. Sequence analysis of the bovine coronavirus nucleocapsid and matrix protein genes. Virology 157:47 (1987).
19. W. Luytjes, L. S. Sturman, P. J. Bredenbeek, J. Charite, B. A. M. Van der Zeijst, M. C. Horzinek, and W. J. M. Spaan. Primary structure of the glycoprotein E2 of coronavirus MHV-A59 and identification of the trypsin cleavage site. Virology 161:479 (1987).
20. L. S. Sturman and K. V. Holmes. The molecular biology of coronaviruses. Adv. Virus Res. 56:904 (1983).
21. M. E. G. Boursnell, T. D. K. Brown, and M. M. Binns. Sequence of the membrane protein gene from avian coronavirus IBV. Virus Res. 1:303 (1984).
22. P. J. M. Rottier, M. C. Horzinek, and B. A. M. Van Der Zeijst. Viral protein synthesis in mouse hepatitis virus strain A59-infected cells: effect of tunicamycin. J. Virol. 40:350 (1981).
23. H. Nieman and H. D. Klenk. Coronavirus glycoprotein E1, a new type of viral glycoprotein. J. Mol. Biol. 153:993 (1981).