Antigenic and Polypeptide Structure of Turkey Enteric Coronaviruses as Defined by Monoclonal Antibodies

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SUMMARY

Twenty-nine hybridoma cell lines, producing monoclonal antibodies (MAbs) to the Minnesota strain of turkey enteric coronavirus (TCV), have been established by fusion of Sp2/0 myeloma cells with spleen cells from BALB/c mice immunized with purified preparations of the egg-adapted or tissue culture-adapted virus. The hybridomas produced mainly IgG2a or IgG1 antibodies. Western immunoblotting experiments with purified virus, and immunoprecipitation tests with [^{35}S]methionine-labelled infected cell extracts, allowed assessment of the polypeptide specificity of the MAbs. Sixteen hybridomas secreted antibodies directed to the peplomeric protein (E2, gp200/gp100) and putative intracellular precursors of apparent M_r 170K to 180K and 90K. Four hybridomas produced antibodies that selectively reacted with a glycoprotein with an M_r of 140K (E3). This polypeptide species corresponded to the major structural component of small granular projections, located near the base of the larger bulbous peplomers, and was found to be responsible for haemagglutination. The major neutralization-mediating determinants were found to be carried by both E2 and E3 glycoproteins. Eight hybridomas produced MAbs directed to the major nucleocapsid protein (N, 52K), and only one MAb reacted with a low M_r structural glycoprotein (24K), corresponding to the matrix (E1) protein. By indirect immunofluorescence, MAbs of different specificity also revealed distinct patterns of distribution of the viral antigens within the cells. The location on the virion of the antigenic determinants recognized by MAbs of different specificity was determined by the use of an immunogold electron microscopy technique. Comparison of nine TCV Quebec strains, using MAbs directed to peplomer and haemagglutinin proteins of the prototype Minnesota strain, confirmed their close antigenic relationship, but also revealed the occurrence of at least two distinct antigenic groups.

INTRODUCTION

Coronaviruses are a group of enveloped RNA-containing viruses which replicate in the cytoplasm of vertebrate cells and are associated with diseases of great economic importance in man and domestic animals (Siddell *et al.*, 1983). Turkey enteric coronavirus (TCV) is one of the major causative agents of epidemic diarrhoea in turkey poults (Pomeroy, 1984; Dea & Tijssen, 1988*a*). In the natural host, it infects and destroys the mature absorptive epithelial cells of the small and large intestines leading to an often severe diarrhoea in poults under 6 weeks of age, but only mild clinical signs in adults (Deshmukh *et al.*, 1974; Pomeroy *et al.*, 1978). The morphological and physicochemical characteristics of TCV resemble those of other members of the *Coronaviridae* (Ritchie *et al.*, 1973; Panigrahy *et al.*, 1973; Dea *et al.*, 1986; Dea & Tijssen, 1988*b*). Little is known with respect to the molecular and antigenic structure of the virion, because of the difficulties in propagating TCV strains in conventional tissue culture systems and the lack of highly specific immunological probes (Deshmukh *et al.*, 1973; Pomeroy, 1984). Ritchie *et al.* (1973) did not observe cross-reactivity, in immunoelectron microscopy studies,

between the prototype Minnesota strain of TCV and coronaviruses from other species, using hyperimmune serum obtained by experimental inoculation of turkeys with coronavirus-positive caecal suspensions. Different isolates of TCV are considered to be antigenically identical or closely related (Pomeroy et al., 1975; Patel et al., 1977). Field isolates can be propagated by oral inoculation and intestinal infections of young turkey poults, or by inoculation into embryonating turkey eggs (Deshmukh et al., 1973; Ritchie et al., 1973). Our previous studies on the morphology and some of the biological activities of egg-adapted TCV strains have revealed that the virus possesses a haemagglutinating activity which may be associated with short granular projections located near the base of the characteristic large bulbous peplomers (Dea & Tijssen, 1988b). Recently, the prototype Minnesota strain was serially propagated in a continuous cell line derived from a human rectum adenocarcinoma (Dea et al., 1989).

Coronaviruses have three major structural proteins in common (Siddell et al., 1983; Sturman & Holmes, 1983). A predominant phosphorylated nucleocapsid (N) protein with an M_r ranging from 45K to 60K is associated with a non-segmented, single-stranded RNA genome of positive polarity. There are also two major virus-encoded envelope proteins: a peplomeric glycoprotein (S or E2), with an M_r of 170K to 200K, that makes up the large surface projections of the virion, and a transmembrane glycoprotein (matrix protein M or E1) with an approximate M_r of 20K to 30K. The peplomeric E2 glycoprotein is often cleaved post-translationally by host cell proteases into two 85K to 100K subunits (Sturman et al., 1985; Cavanagh et al., 1986b). The latter also is known to be responsible for virus attachment and cell membrane fusion (Collins et al., 1982), and elicits the production of virus-neutralizing antibodies (Fleming et al., 1983; Laude et al., 1986; Niesters et al., 1987; Deregt & Babiuk, 1987) which may be protective (Buchmeier et al., 1984; Wege et al., 1984; Cavanagh et al., 1986a). An additional glycoprotein of 130K to 140K, a disulphide-linked dimer of 65K subunits, is associated with the haemagglutinin (E3) of viruses belonging to the subgroup of haemagglutinating mammalian coronaviruses (King et al., 1985; Hogue & Brian, 1986: Sugiyama et al., 1986). Antigenic determinants on the E3 protein also elicit the production of virus-neutralizing antibodies (Deregt & Babiuk, 1987).

We have produced a set of anti-TCV monoclonal antibodies (MAbs) with the aim of obtaining information about the viral components involved in the immune response, and to characterize further the antigenic relationships among TCV isolates which have been associated with outbreaks of diarrhoea in turkey poults in eastern Canada.

METHODS

Cells. The human rectal tumour cell line HRT-18 (Tompkins et al., 1974) and the non-secreting mouse (BALB/c) myeloma cell line SP2/0-Ag14 (Shulman et al., 1978) were provided by Dr J. Laporte (Institut de Recherches Agronomiques, Thiverval-Grignon, France) and Dr B. R. Brodeur (Laboratory Centre for Disease Control, Health and Welfare Canada, Ottawa, Ontario, Canada) respectively. The cells were cultivated in RPMI 1640 medium (Flow Laboratories) containing 1% glutamine, 1% Eagle's non-essential amino acids, 1% sodium pyruvate, gentamicin (50 µg/ml) and 15% heat-inactivated foetal bovine serum (FBS).

Viruses. The prototype egg-adapted Minnesota strain (Ritchie et al., 1973) of TCV (obtained from Dr B. S. Pomeroy, College of Veterinary Medicine, St Paul, Mn., U.S.A.) was serially propagated on HRT-18 cells and purified from the supernatants of infected cultures by differential and isopycnic centrifugation on sucrose gradients (Dea et al., 1989). The protein concentration in purified viral suspensions was determined by the method of Bradford (1976). The origin of the nine tissue culture-adapted Quebec strains of TCV, listed in Fig. 9, has been reported (Dea et al., 1986; Dea & Tijssen, 1988a).

Reference antiserum. An anti-TCV hyperimmune serum was obtained after immunization of rabbits with the density gradient-purified egg-adapted Minnesota strain. The specificity of the antiserum was confirmed by immunoelectron microscopy and haemagglutination inhibition (HI) tests (Dea & Tijssen, 1988a).

Immunization of mice. Six- to 8-week-old BALB/c mice (Charles River Breeding Laboratories) devoid of anti-mouse hepatitis virus type 3 (MHV-3) immunity, were immunized either with purified whole virus or 1% SDS-denatured virus preparations. They were first injected intraperitoneally with 50 µg virus in 0·5 ml complete Freund's adjuvant (Difco) and then boosted twice, at 2-week intervals, with the same amount of antigen in incomplete Freund's adjuvant. At least 6 to 8 weeks after the initial immunization and 4 days before the mice were killed for the preparation of hybridomas, they received a final intravenous injection of 15 to 25 µg purified whole virus in phosphate-buffered saline (PBS).

Production of mouse hybridoma cell lines. Sensitized spleen cells were mixed at a ratio of 10:1 with Sp2/0 myeloma cells and were fused using 50% polyethylene glycol 1450 (Eastman Kodak), according to the method of Fazekas de St Groth & Scheidegger (1980). The hybrid cells were cultured in HAT medium (RPMI 1640, supplemented with 20% FBS, 1% glutamine, 25 µg/ml of gentamicin, 0·1 mM-hypoxanthine, 0·0004 mM-aminopterin and 0·02 mM-thymidine) in 96-well microtitre plates (1×10^5 myeloma cells/well) containing feeder layers of Swiss–Webster mouse macrophages (2×10^4 cells/well). The cells were incubated at 37 °C and HAT medium was changed twice a week. The supernatants of growing hybridomas were screened for production of TCV-specific antibodies by ELISA. Cells in positive wells were subcloned twice in microtitre plates by the limiting dilution method and subsequently expanded and reassayed.

Ascitic fluids were obtained by intraperitoneal injection of 5×10^5 to 5×10^6 specific antibody-producing hybridoma cells into BALB/c mice that had been primed with pristane (Sigma) 2 to 3 weeks previously. After centrifugation for 5 min at 15000 *g*, ascitic fluids were stored in aliquots at -20 °C. Immunoglobulins (IgG) from 1·5 to 3·0 ml of ascitic fluid were purified by affinity chromatography, using Protein A–Sepharose CL-4B (Pharmacia) or a DEAE–Affi-Gel MAPS kit (Bio-Rad). The protein concentration in IgG fractions was determined by absorbance at 280 nm using an extinction coefficient of 1·4 cm²/mg.

Screening ELISA procedure. Hybridoma culture media and mouse body fluids were screened for the presence of anti-TCV antibodies by ELISA, according to Wege *et al.* (1984). Density gradient-purified virus in 0·05 M-sodium carbonate buffer pH 9·6, was used to coat flat-bottomed microtitre plates (Flow Laboratories). The optimal virus antigen concentration was determined by chequer-board titration, using rabbit anti-TCV hyperimmune serum (diluted 1:1000), and corresponded to a range of 0·1 to 0·5 µg of protein/well. After incubation for 16 h at 4 °C, antigen-coated plates were treated for 1 h at 37 °C with 1% bovine serum albumin (BSA grade V; Sigma) in 0·05 M-Tris-buffered saline pH 8·0 (TBS). Then, the plates were washed twice with TBS containing 0·05% Tween 20 (TBS-T), dried, and stored under refrigeration.

All subsequent steps were performed at 37 °C. Fifty µl of dilution buffer (TBS-T) and 50 µl hybridoma supernatant or diluted ascitic fluid were added to each well and incubated for 2 h. The plates were washed and received 100 µl/well of a 1:1000 dilution of peroxidase-labelled goat anti-mouse IgG (heavy and light chains; Boehringer Mannheim). Then, following a 1 h incubation, the plates were washed and the enzyme substrate solution, containing 1 µg/ml of 5-amino-salicylic acid (Sigma) and 0·005% hydrogen peroxide (pH 6·0), was added. After the reaction had proceeded for 30 to 45 min at room temperature, the absorbance value of the fluid was measured at 474 nm using a multichannel photometer (Titertek Multiskan; Flow). The titres of the different clones were expressed as the highest 10-fold dilution giving absorbance values at least three times that of the TBS controls.

Indirect immunofluorescence (IIF) tests. HRT-18 cell monolayers, established in Lab-Tek eight-chamber cell culture slides (Miles Laboratories), were infected at an m.o.i. of 1·0 to 5·0 $TCID_{50}$ of virus/cell in RPMI supplemented with 10 units (U)/ml of trypsin (bovine crystallized grade IX, Sigma). After a 12 to 18 h incubation period at 37 °C, the cultures were rinsed twice with PBS and fixed in cold acetone. The slides were air-dried and processed for indirect staining (Dea *et al.*, 1989) using serial twofold dilutions of clarified ascitic fluids, and goat anti-mouse IgG–fluorescein isothiocyanate conjugate diluted 1:40 (Cappel Laboratories). For membrane fluorescence examination, cell monolayers were treated with 4% paraformaldehyde for 30 min at 4 °C.

Isotype determination. The immunoglobulin class of antibodies secreted by the established hybridoma cell lines was determined by double diffusion, using affinity-purified goat antisera to mouse IgG, IgA and IgM (Kirkegaard & Perry Laboratories) and goat antisera to mouse IgG1, IgG2a and IgG2b (Tago).

Virus neutralization and haemagglutination inhibition. The tests were done as described elsewhere (Dea *et al.*, 1979, 1986). The serum neutralization (SN) titre was expressed as the reciprocal of the highest serum or ascitic fluid dilution neutralizing 100 $TCID_{50}$ of the virus. The HI titre was expressed as the reciprocal of the highest serum or ascitic fluid dilution neutralizing 8 haemagglutinating units of the virus, when tested with rat erythrocytes.

Labelling of intracellular proteins. Confluent monolayers of HRT-18 cells in 150 cm² tissue culture flasks (Falcon) were infected (or mock-infected) at an m.o.i. of 0·1 or 1·0 $TCID_{50}$ of virus/cell. After 1·5 h adsorption at room temperature, the inoculum was discarded and the cultures received 15 ml of maintenance medium containing 10 U/ml of trypsin, and were incubated at 41 °C. After 6 to 9 h, infected cultures were rinsed and incubated in methionine-deficient medium for 30 min, after which 50 µCi/ml [³⁵S]methionine (Amersham; sp. act. > 800 Ci/mmol) was added. The cultures were reincubated at 41 °C until 18 to 24 h post-infection. The cell sheets were rinsed twice in PBS, and scraped into ice-cold lysis buffer made of 20 mM-Tris–HCl pH 6·8, 150 mM-NaCl, 1 mM-EDTA, 600 mM-KCl, 500 mM-MgCl₂, 1% Triton X-100, 0·1% SDS, 1% NP40, 10³ units aprotinin/ml and 200 µg PMSF/ml. The cell lysates were immediately passed through a 26-gauge needle 10 times in order to reduce their viscosity, and clarified by centrifugation at 15000 *g* for 20 min. The supernatants were stored at -70 °C.

For pulse–chase labelling of intracellular polypeptides, cell monolayers established in 25 cm² culture flasks (Falcon) were infected as above. The cells were starved of methionine for 1 h and then labelled with 100 µCi/ml of

[35S]methionine in methionine-free medium beginning at 6 to 9 h for different experiments. After a 30 min pulse, the cells were washed twice in complete RPMI medium and then further incubated, with the same medium, for 15 min to 4 h before harvesting.

Radioimmunoprecipitation. Immunoprecipitation experiments were performed essentially as described by Laude *et al.* (1986). Briefly, samples of 50 to 100 µl of clarified radiolabelled cytosol extracts (1 × 10⁶ c.p.m.) were mixed with 10 µl of either undiluted hybridoma ascitic fluid, or reference anti-TCV serum. After incubation at 4 °C overnight, the immune complexes were adsorbed to Protein A–Sepharose beads, washed, and dissolved in electrophoresis sample buffer.

Western immunoblotting. Viral proteins separated by SDS–PAGE were transferred to nitrocellulose membranes (0·45 µm, Schleicher & Schüll), as previously described (Dea & Tijssen, 1988*b*). After saturation with BSA, blots were cut into strips that were incubated for 2 h at room temperature in ascitic fluid (1 :100 dilution) or rabbit anti-TCV polyclonal serum (1 :500 dilution) diluted in TBS-T. The nitrocellulose strips were subsequently washed three times for 10 min in TBS-T and then incubated with 1 :1000 dilutions of peroxidase-labelled goat anti-mouse IgG or goat anti-rabbit IgG conjugates in TBS-T for 90 min at room temperature. Strips were again washed and immune reactions were visible after incubation for 30 to 45 min in the enzyme substrate solution consisting of 0·05% 4-chloro-1-naphthol (Sigma) in TBS containing 20% (v/v) methanol.

Polyacrylamide gel electrophoresis. Samples were mixed with equal volumes of double strength sample buffer with or without 2-mercaptoethanol, boiled for 3 min, clarified at 10000 *g* for 15 min, and analysed by electrophoresis (SDS–PAGE) on 8·5 or 10% SDS–polyacrylamide slab gels, as previously described (Dea & Tijssen, 1988*b*). Gels were stained either with Coomassie Brilliant Blue G-250 or silver (Morrissey, 1981). For fluorography, gels were soaked in Amplify (Amersham) for 45 min, dried, and autoradiographed with Kodak X-OMAT RP film at −70 °C. High and low *M*ᵣ marker proteins (Bio-Rad) or ¹⁴C-methylated marker proteins (Amersham) were run on each gel to allow *M*ᵣ estimates of viral proteins.

Electron microscopy and Protein A–gold immunolabelling. The purified viral preparation was adjusted to approximately 10⁷ particles/ml, as determined by electron microscope calibration with latex spheres. Aliquots (100 µl) were poured into nitrocellulose microtubes and centrifuged at 120000 *g* (Beckman Airfuge, rotor model A-100) for 5 min onto 400-mesh naked nickel grids, according to Alain *et al.* (1987). For immunogold labelling, virus-coated grids were first washed three times in TBS, partly dried, and floated for 5 min on a drop of TBS-T. The grids were then incubated for 10 min at room temperature on a drop of rabbit anti-TCV serum (diluted 1 :250) or clarified ascitic fluid (diluted 1 :100) in TBS-T. Following another washing step, grids were incubated on a drop of TBS-diluted Protein A–gold (PAG) complex (Garzon *et al.*, 1982). The colloidal gold particles in the PAG complex were 8 nm in diameter and prepared according to the method of Frens (1973), with the modifications suggested by De Mey (1983). A commercial anti-mouse IgG colloidal gold conjugate (Janssen) was used as an alternative. The grids were finally washed with TBS, rinsed three times in distilled water, and counterstained with 2% sodium phosphotungstate pH 7·0 (Dea *et al.*, 1979). Specificity of labelling was demonstrated by controls including non-immune sera and incubation with PAG complex alone.

The larger surface projections could be selectively removed by bromelain digestion (S. Dea & P. Tijssen, unpublished data), in order to distinguish MAb reactivity towards small and large surface projections. For this purpose, virus preparations were incubated with 1·3 mg/ml of bromelain (Boehringer-Mannheim) in TMEM (50 mᴍ-Tris–maleate, 1 mᴍ-EDTA, 150 mᴍ-NaCl, pH 6·0) buffer for 1 to 3 h at 37 °C. The virus was subjected to isopycnic centrifugation before immunogold labelling.

RESULTS

Production of hybridomas

Administration of purified preparations of the egg-adapted Minnesota strain of TCV to BALB/c mice induced a poor specific humoral response. Titres of sera from mice providing the immune cells ranged from 80 to 320 by ELISA, while titres lower than 64 were obtained by HI tests. The small amounts of purified virus prepared from the intestinal contents of TCV-infected turkey embryos and contamination by host proteins were probably responsible for inadequate immunization against TCV. Furthermore, electron microscopy of the virus preparation showed that most virions had lost their surface projections (data not shown). Nevertheless, from 112 clones obtained from two fusions, 32 reacted positively by ELISA, when tested with antigen prepared from the intestinal contents of TCV-infected embryos, but not with normal intestinal contents. Only three viable positive hybridoma cell lines were established from those clones and used to produce ascitic fluids in BALB/c mice.

Other immunizations were carried out with either purified whole virus or SDS-denatured virus prepared from the supernatant fluids of TCV-infected HRT-18 cells. Most of the viral
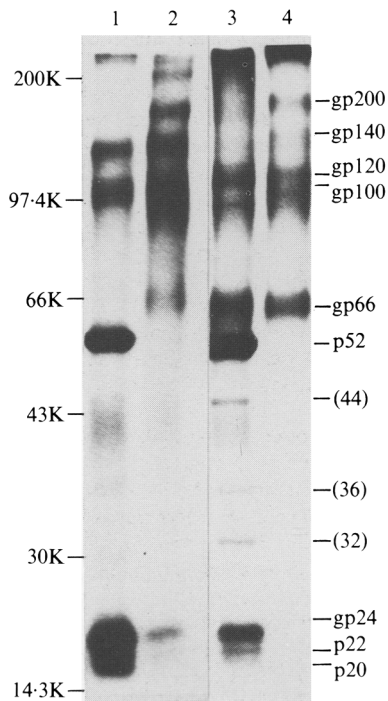
Fig. 1. TCV structural proteins. Sucrose gradient-purified TCV labelled with either [³⁵S]methionine (lanes 1 and 3) or [³H]gluco-samine (lanes 2 and 4) was electrophoresed after solubilization with sample buffer in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of 2-mercaptoethanol. The acrylamide concentration in the gel was 10%. Positions of M_r standards are shown on the left.

particles purified from supernatant fluids of infected cell cultures possessed the double fringe of surface projections (Dea *et al.*, 1989). Of 252 hybridoma cell lines initially secreting anti-TCV MAbs, as determined by ELISA, 26 could be subcloned, serially propagated, and used to produce ascitic fluids.

Polypeptide specificity of TCV MAbs

To determine the polypeptide specificity of these antibodies, purified tissue culture-adapted TCV was dissolved in electrophoresis sample buffer in the absence or the presence of 2-mercaptoethanol, fractionated by electrophoresis, and viral proteins were transferred to nitrocellulose. Individual strips were used for immunoblot analysis with the various antibodies.

Under non-reducing conditions, the purified preparations of [³⁵S]methionine-labelled TCV were resolved into four major (apparent M_r 140K, 100K, 52K and 24K) and two minor (apparent M_r 200K and 120K) polypeptide species (Fig. 1, lanes 1 and 2). Polypeptide 24K was usually resolved as a group of two or three closely migrating bands. Labelling with [³H]glucosamine revealed that the 200K, 140K, 100K and 24K species were glycosylated. A glycoprotein species with an M_r in excess of 200K (species 'a') was also identified with [³H]glucosamine-labelled virus (Fig. 1, lane 2). Under reducing conditions, a decrease in the intensity of the gp140 species was concomitant with the appearance of a new gp66 species in the gel (Fig. 1, lanes 3 and 4). The gp140 was thus suggested to be a disulphide-linked dimer of two gp66 molecules. Occasionally, three additional polypeptide species with approximate M_r values of 44K, 36K and 32K were also observed (Fig. 1, lane 3).

Representative immunoblot patterns obtained with anti-TCV hyperimmune serum and MAbs to purified TCV are illustrated in Fig. 2. From the polypeptide species described above, only the 32K species was not identified by the anti-TCV hyperimmune serum, suggesting its non-structural nature (Fig. 2*a*, *b*, *c*, lanes 1). This technique also allowed assessment of the polypeptide specificity of the 26 anti-TCV MAbs (Table 1). The polypeptide specificity of MAbs M11, M29 and M31 could not be determined with certainty using this technique. Under
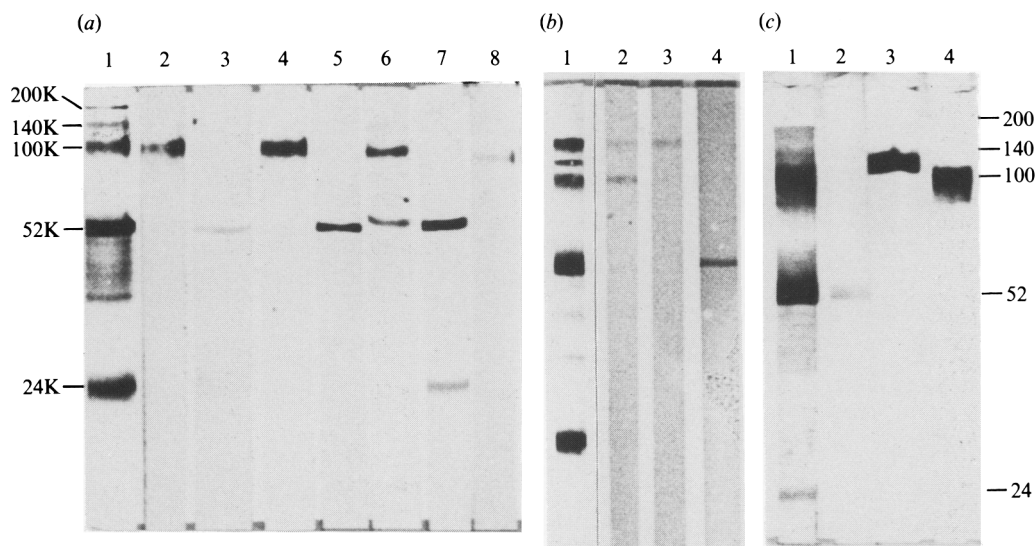
Fig. 2. Protein specificity of representative anti-TCV MAbs as determined by Western immunoblotting of purified TCV. Sucrose gradient-purified TCV was solubilized in sample buffer in the absence of 2-mercaptoethanol, fractionated in 9·5% polyacrylamide gels, and transferred to nitrocellulose. Individual nitrocellulose strips were incubated with rabbit anti-TCV serum (a, b, c, lanes 1), and MAbs of different specificities. Anti-N MAbs are represented in (a) lanes 3 (316C) and 5 (M21), (b) lane 4 (1F3) and (c) lane 2 (43C3); anti-E2 MAbs are represented in (a) lanes 2 (M1), 4 (M12), 6 (M20) and 8 (M29), (b) lanes 2 (M10) and 3 (6E5), and (c) lane 4 (12H7); an anti-E3 MAb is represented in (c) lane 3 (8B8) and the anti-E1 MAb is represented in (a) lane 7 (4B10). Immune reactions were revealed as described in the text. The positions of M_r markers are shown on the left.

the conditions used, 15 hybridomas produced MAbs that recognized either gp200 or gp100, or both protein species. MAbs of two other hybridomas reacted only with gp140. In addition, eight MAbs were specific for the major unglycosylated p52 protein, and only one hybridoma produced antibodies that reacted with gp24. The three clones (316C, 60D3 and 43C3) obtained from immunization schedules with the egg-adapted TCV recognized p52. To be consistent with the nomenclature used by several authors to designate coronavirus proteins (Siddell et al., 1983), the glycoproteins gp200 and gp100 were designated E2 (for peplomeric glycoproteins), and gp24 was designated E1 (for matrix glycoprotein). The major unglycosylated p52 was considered to correspond to N (nucleocapsid protein). The disulphide-linked dimer gp140 was designated E3 to be consistent with the nomenclature used by Deregt & Babiuk (1987) for the haemagglutinating protein of bovine enteric coronavirus.

Relationship among peplomeric polypeptides as defined by MAbs

Fig. 3 (a) shows the results obtained by pulse labelling of HRT-18 cells with [^{35}S]methionine at different times after infection. At an m.o.i. of 5 TCID$_{50}$/cell, five major (M_r 140K, 100K, 52K, 36K and 24K) and two minor (M_r 200K and 32K) polypeptides were detected approx. 12 h after infection. All these TCV-induced intracellular polypeptides, except the 32K and 36K species, comigrated with the viral polypeptides (lane V). The predominant N protein was revealed as soon as 6 h after infection.

Pulse–chase experiments were conducted at 8 h after infection (Fig. 3b). No processing of the N species was apparent during the 3 h chase period, but significant variation was noted in the intensity of the 200K, 140K and 100K protein bands. New immunoprecipitable protein species with apparent M_r values of 170K to 180K, 130K, 120K and 90K were also revealed. The N protein was initially revealed as a group of three closely migrating bands. The 90K protein at progressively longer chase times gave rise to the gp100 species, while only an increased intensity

Table 1. *Characteristics of anti-TCV (Minnesota strain) MAbs*

Clone no.	Ig isotype	IF assay*	ELISA titre†	Neutralization titre‡	HI titre‡	Polypeptide specificity
M1	G2a	+	5·0	<20	<20	E2
M3	G1	+	3·8	<20	<20	E2
M10	G2a	+	4·1	1280	<20	E2
M11	G2a	+	5·6	5120–10240	1280	E3
M12	G1	+	4·7	<20	<20	E2
M20	G1	+	5·3	<20	<20	E2
M21	G2a	+	3·2	<20	<20	N
M22	G1	+	4·4	<20	<20	E2
M23	G1	+	5·6	<20	<20	E2
M28	G2a	+	3·8	<20	<20	N
M29	G1	+	5·6	10240	<20	E2
M30	G1	+	5·0	<20	<20	E2
M31	G1	+	5·0	5120	1280	E3
M33	G2b	+++	6·2	5120–10240	<20	E2
316C	G2a	+	2·6	<20	<20	N
60D3	G2a	+	3·2	<20	<20	N
43C3	G1	−	4·1	<20	<20	N
1B12	G1	+	3·2	<20	<20	N
1F3	G2b	+	2·6	<20	<20	N
12H7	G§	+	3·2	<20	<20	E2
8B8	G2a	++	3·8	320	80	E3
15E10	G2a	+	>4·1	<20	20	E2
6E5	G2a	+++	3·8	<20	<20	E2
6C5	G2a	++	3·8	80	<20	E2
1F6	G2a	++	>4·1	<20	<20	N
8E6	G1	+++	>4·1	<20	80	E3
10H11	G2a	++	4·1	<20	<20	E2
6C7	G2a	++	4·1	320	<20	E2
4B10	G2b	+	5·7	<20	<20	E1

* Immunofluorescence assay, IFA, titres: +, <160; ++, >160 < 1280; +++, >1280.
† Log_{10} of the highest dilution of ascitic fluid giving an A_{492} value > 2·5 × A_{492} value of a buffer control.
‡ SN and HI titres are expressed as described in methods.
§ Subtype not determined.

was observed for the 120K species (Fig. 3b, arrowheads). Later in the infection, the 170K to 180K species became more evident, and at the end of the chase period it was replaced by the 200K species, concomitant with an apparent increase in the amount of the 100K and 120K species. Processing also appeared to occur among the 36K species. The 32K intracellular species was not identified by immunoprecipitation with the radiolabelled purified virus.

MAbs directed against E2 usually immunoprecipitated TCV-induced intracellular proteins 200K, 170K to 180K, and polypeptide species between 90K and 120K, as suggested by the diffuse band observed at this level (Fig. 4, lanes 5 and 7 to 11). These results thus suggested an antigenic relationship between these TCV-induced intracellular polypeptides. The MAbs M1, M12, M29 and 12H7 reacted with the 100K polypeptide only (Fig. 4, lane 4). The anti-E3 MAbs, including MAbs M11 and M31 for which the polypeptide specificity could not be determined by Western immunoblotting, immunoprecipitated the 130K polypeptide species only (Fig. 4, lane 6).

In immunoprecipitation experiments with [^{35}S]methionine-labelled purified TCV, only anti-N MAb M21 could resolve the N protein as a group of two or three closely migrating bands with M_r values ranging from 46K to 52K (Fig. 5).
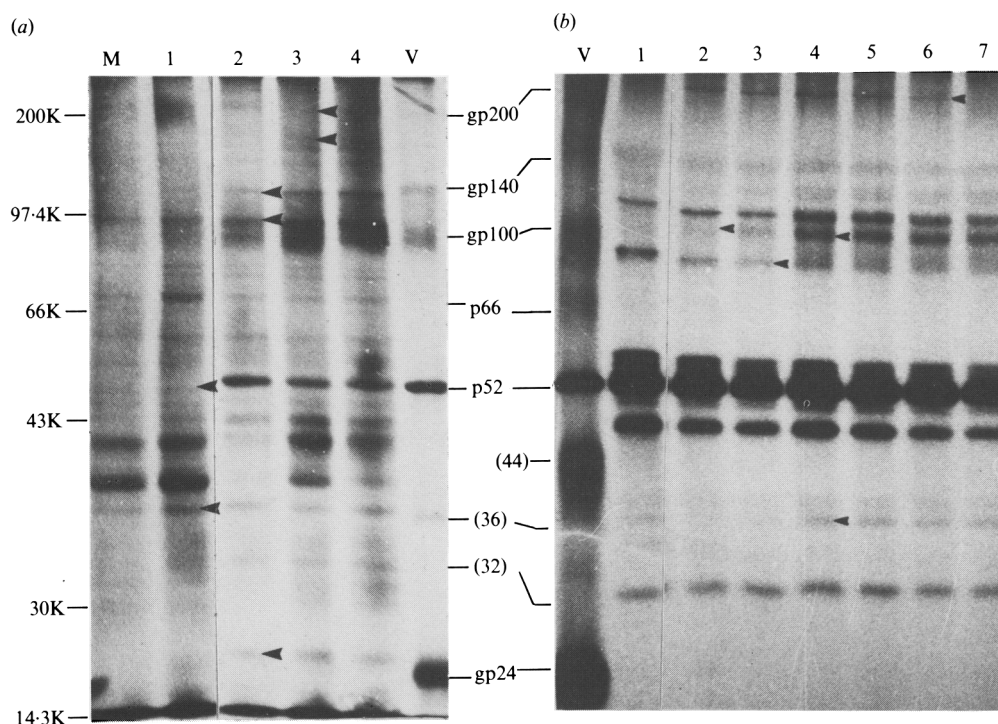
Fig. 3. Synthesis and processing of TCV-induced intracellular proteins. TCV-infected and mock-infected (M) HRT-18 cells were pulse-labelled with [^{35}S]methionine for 30 min at 6, 9, 12 or 18 h (lanes 1 to 4, respectively) and chased in RPMI for 1 h (a), or pulse-labelled with the same radioisotope for 30 min at 8 h post-infection and chased in RPMI for 30, 45, 60, 90, 120, 150 and 180 min (lanes 1 to 7, respectively) (b). Cell lysates were prepared as described in the text and analysed immediately by SDS–PAGE (a) or after immunoprecipitation with rabbit anti-TCV hyperimmune serum (b). The specimens were solubilized in sample buffer in the absence of 2-mercaptoethanol. Analysis was done in 9·5% gels. Positions of M_r standards are on the left. Chasing of the 90K intracellular protein into the virion 100K glycoprotein, and replacement of the 180K intracellular protein by the virion 200K glycoprotein are indicated by arrowheads. The 36K intracellular protein is also indicated. Lanes V, electrophoretic or immunoprecipitation profiles of purified, radiolabelled virus.

Reactivities of hybridomas

In order to correlate biological functions with polypeptides, MAbs were studied for their ability to neutralize the virus and to inhibit its haemagglutinating activity. The specificities of the 29 TCV hybridomas are summarized in Table 1. Characterization of the isotypes of the MAbs showed that most antibodies belonged to the IgG2A or IgG1 subclass. Neutralization studies showed that eight MAbs had SN titres between 80 and 10 240; three (M11, M31 and 8B8) were specific to E3, while the five other (M10, M29, M33, 6C5 and 6C7) were specific to E2. Only MAbs to E3 inhibited the haemagglutinating activity of TCV.

Anti-N MAbs had lower ELISA titres than MAbs directed to other viral polypeptides. Immunofluorescence titres of ascitic fluids varied from low (< 160) to strong (> 1280). Five out of eight anti-N MAbs had IIF titres under 320. As shown in Fig. 6, MAbs of a given specificity also induced characteristic patterns of fluorescence. Anti-N MAbs gave a weak fluorescence that was evenly distributed in the cytoplasm of TCV-infected cells (Fig. 6a). With the anti-E1 MAb, the fluorescence appeared as fine granulation, essentially limited to the perinuclear area (Fig. 6b). With anti-E2 and anti-E3 MAbs, the fluorescence was diffuse throughout the
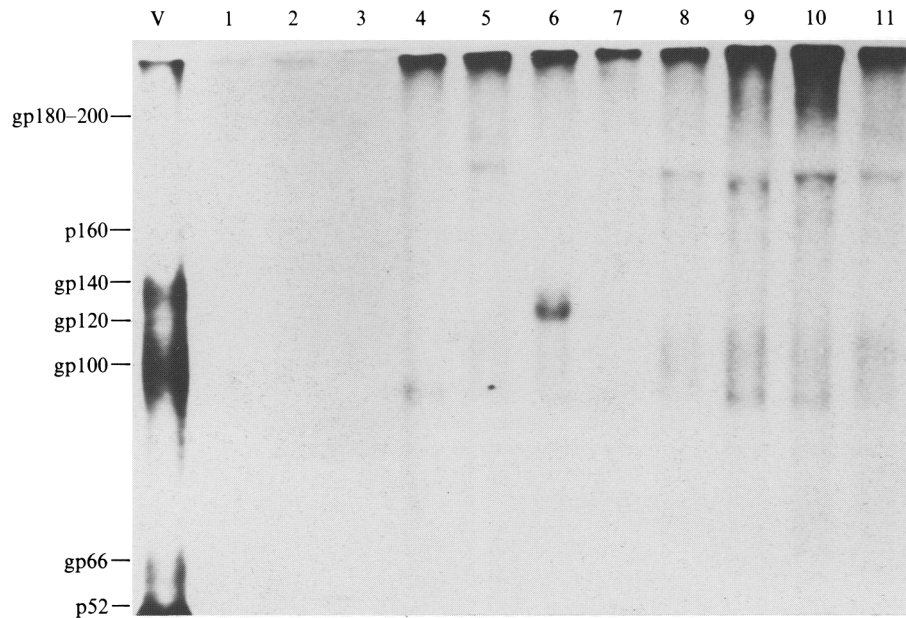
Fig. 4. Immunoprecipitation of virus-induced polypeptides from cytosol of TCV-infected HRT-18 cells by MAbs to E3 and E2 glycoproteins. Lanes 4 to 11, intracellular proteins precipitated by anti-E2 MAbs M29 (lane 4), M1 (lane 5), M22 (lane 7), 10H11 (lane 8), 6C7 (lane 9), 6C5 (lane 10), M30 (lane 11), and anti-E3 MAb M31 (lane 6). In this gel, the specificity of MAb M22 (lane 7) could hardly be defined Lane V, immunoprecipitation of [^{35}S]methionine-labelled TCV-infected cells by the rabbit anti-TCV serum. Intracellular proteins precipitated by non-immune rabbit (lane 2), and mouse (lane 3) sera. Immunoprecipitates were solubilized in sample buffer without 2-mercaptoethanol and analysis was done in a 9·0% polyacrylamide gel. Specificity controls included precipitation of mock-infected cell lysates by a pool of anti-E2 and anti-E3 MAbs (lane 1).
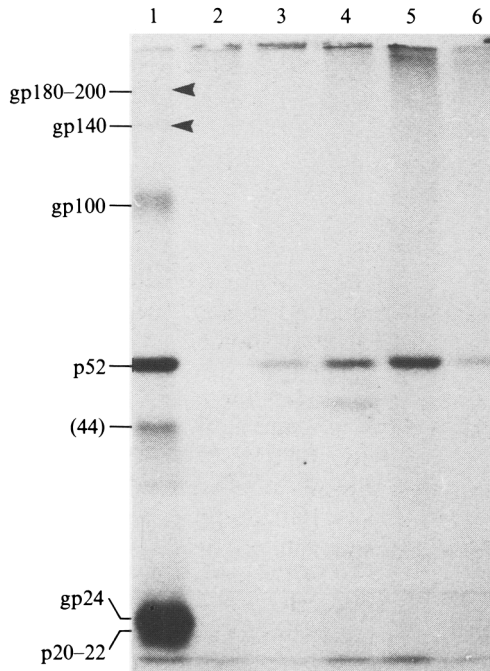

Fig. 5. Immunoprecipitation of [^{35}S]methionine-labelled purified TCV by rabbit hyperimmune serum (lane 1) or by MAbs directed against the N protein. Lanes 3 to 6 represent immunoprecipitation patterns obtained with MAbs 1F3, M21, 1F6 and 1B12, respectively. Immunoprecipitates were solubilized in sample buffer without 2-mercaptoethanol and analysed in 10·0% polyacrylamide gels. Incubation with serum from mock-infected mouse (lane 2).
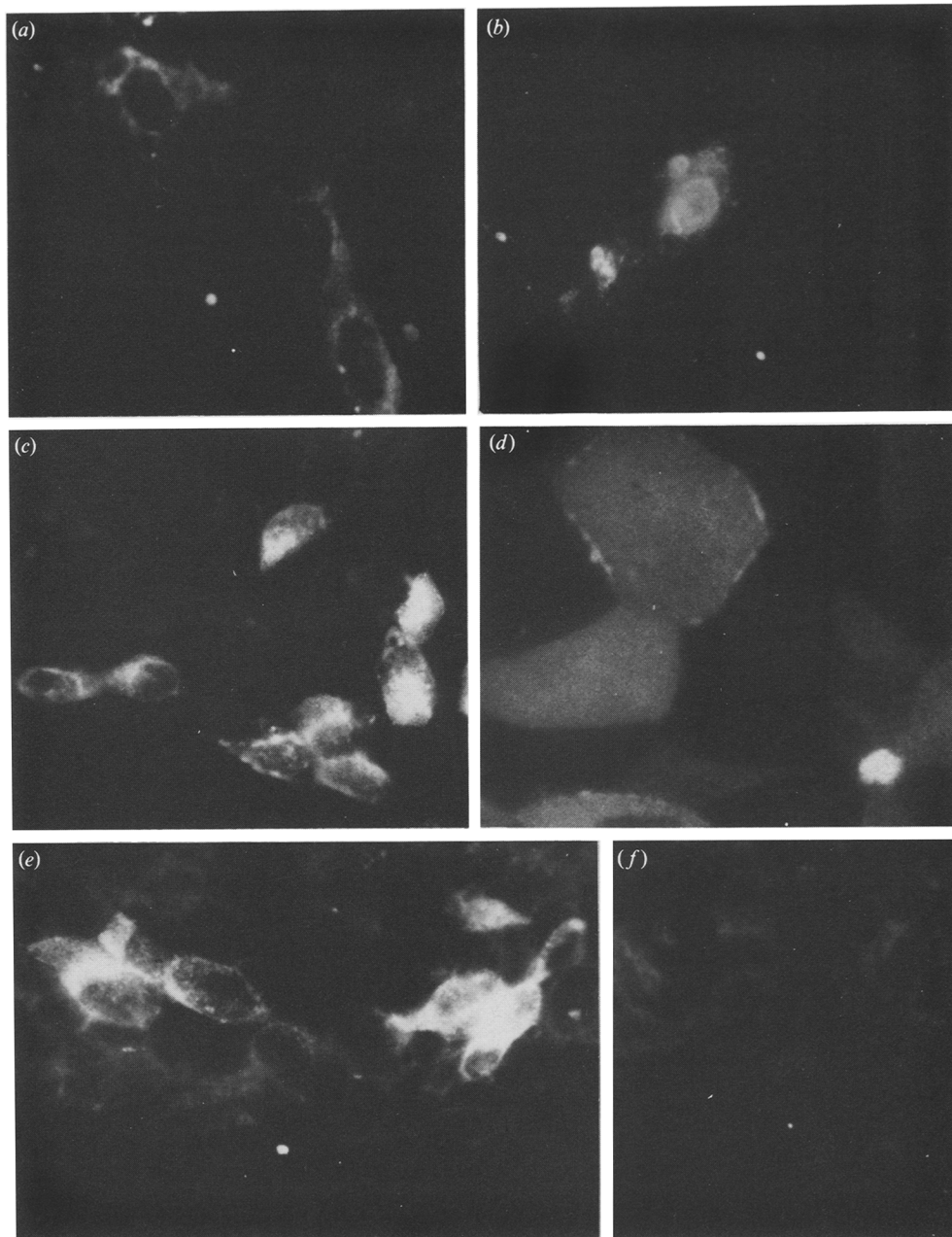
Fig. 6. Immunofluorescence staining of viral antigens by anti-TCV MAbs at 18 h after infection with TCV at an m.o.i. of 5 $TCID_{50}$/cell. Immunofluorescence in fixed cells with the MAbs anti-N 1F6 (a), anti-E1 4B10 (b), anti-E2 10H11 (c) and anti-E3 8E6 (e); membrane fluorescence was observed in paraformaldehyde-treated cells stained with MAbs anti-E2 and -E3 (d). Mock-infected cells treated with rabbit anti-TCV serum (f).

cytoplasm, but appeared more intense around the nucleus (Fig. 6c and e). Both anti-E2 and anti-E3 MAbs were able to induce a cell surface fluorescence, as suggested by the bright fluorescence observed around the periphery of the infected cells which were fixed with paraformaldehyde (Fig. 6d).
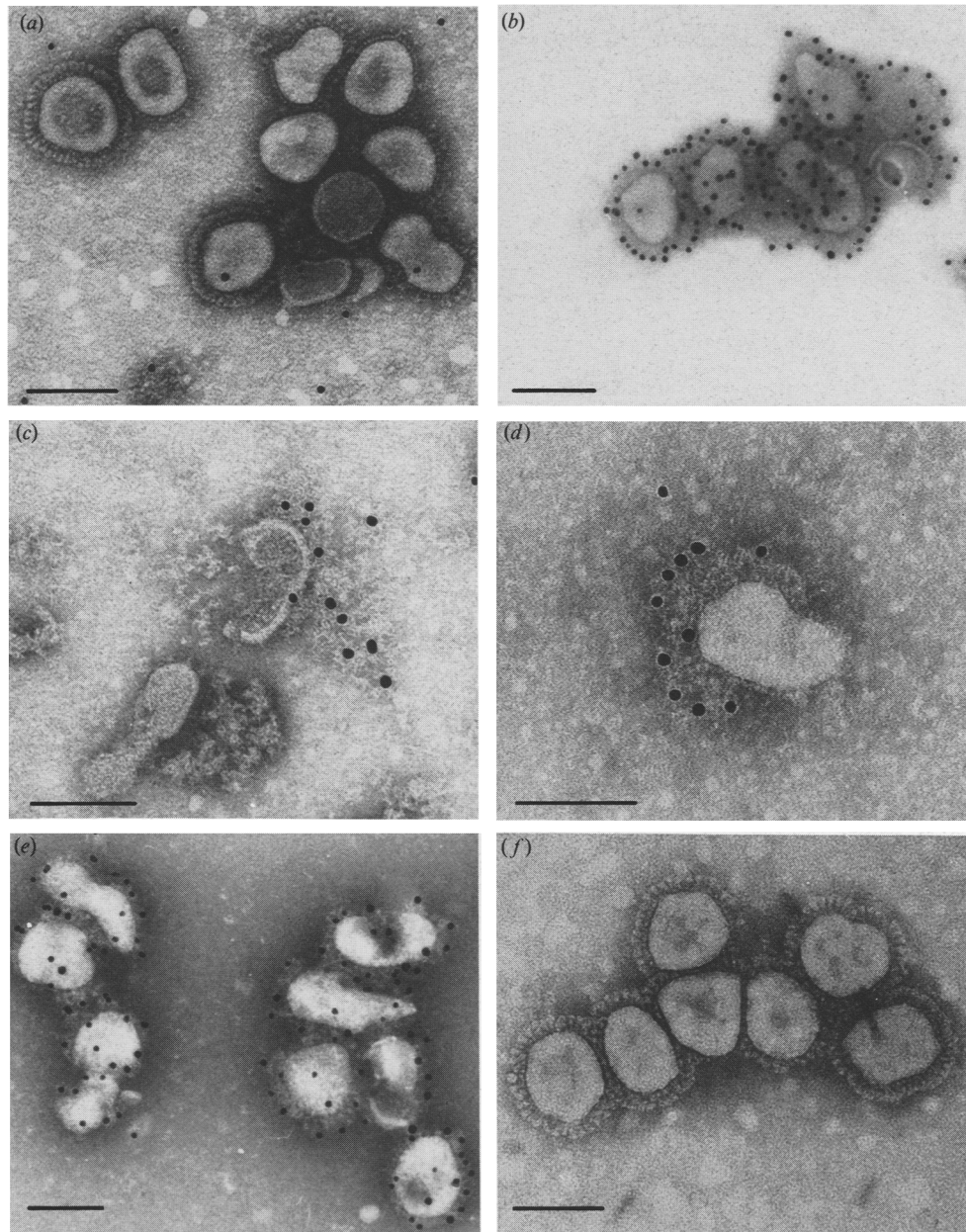
Fig. 7. Protein A–gold immunolabelling of TCV. Aliquots of purified TCV were processed for immunogold labelling using non-immune (*a*) and anti-TCV hyperimmune (*b*) rabbit sera, or the MAbs anti-N 1F6 (*c*) and anti-E2 10H11 (*e*). After treatment with bromelain, immunolabelling was obtained with the MAb anti-E2 10H11 only on residual large bulbous projections (*d*). The presence of a double fringe of surface projections is clearly seen on negatively stained untreated viral particles (*f*). Bar markers represent 100 nm.

Location of the antigenic determinants on the virion

The indirect immunogold electron microscopy technique was used in an attempt to determine the location on the virion of polypeptides recognized by anti-TCV MAbs. Following incubation with rabbit anti-TCV hyperimmune serum, gold granules appeared closely associated with the
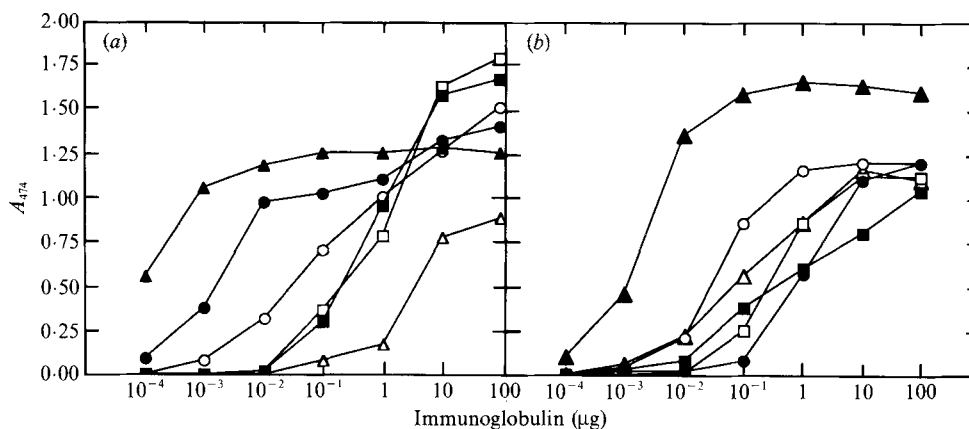
Fig. 8. Comparative avidity of MAbs directed against the TCV proteins E2 and E3 (a: △, 6C7; ▲,
8B8; ○, 10H11; ●, 8E6; □, 6C5; ■, 15E10A; b: △, M10; ▲, M33; ○, M29; ●, M3; □, M31; ■,
M11). Serial 10-fold dilutions of purified MAbs were incubated in the wells of microtitre plates coated
with viral antigen (0·5 μg of protein) prepared from the purified tissue culture-adapted Minnesota strain
of TCV. After incubation for 90 min at room temperature, binding to the viral antigen was detected by
the addition of peroxidase-labelled goat anti-mouse IgG. The resulting absorbance was read and used to
rate the MAbs for avidity.

virus particles, with minimal gold background staining. Frequently, two rows of gold granules
were present on the surface of viral particles; the gold granules were located near to, or on, the
tip of both types of surface projections (Fig. 7b). Only a few gold granules were observed around
particles that had lost their surface projections (data not shown). Control experiments where
preimmune serum was used or where the anti-TCV serum was omitted, showed that the viral
particles in the test were specifically labelled (Fig. 7a).

Distinct gold labelling patterns of the purified virus particles were obtained using MAbs of
different specificity. With anti-N MAbs, no labelling of intact virus particles was observed.
However, gold granules were usually found near filament-like structures which appeared to exit
from the damaged viral particles, possibly corresponding to the helicoidal nucleocapsid (Fig.
7c). With anti-E2 MAbs, the gold granules were usually located near the tips of the large bulbous
projections (Fig. 7e). Bromelain-treated virus particles were found to be labelled only on the few
large projections still remaining on their envelopes (Fig. 7d). Only one out of four anti-E3 MAbs
(M11) permitted gold labelling of the viral particles, but a specific pattern could not be defined
with certainty (data not shown). Particles devoid of both types of surface projections were
unlabelled by MAbs to E2 and E3.

Antigenic relationship among different TCV isolates

A direct relationship exists between the avidity and the maximal amount of MAb bound to a
given amount of antigen (Frankel & Gerhard, 1979; Tijssen, 1985; Niesters et al., 1987). Before
testing against various strains of TCV, MAbs directed against the E2 and E3 glycoproteins of
the Minnesota strain were titrated by an indirect ELISA (0·5 μg sucrose gradient-purified TCV
coated in the wells of the microtitrate plates), and avidities were estimated using the absorbance
values of the plateau level. The maximum values varied between 0·8 and 1·8 (Fig. 8). At a
concentration of 0·01 μg of gamma globulin per well, only MAbs 8E6, 8B8 and M33 were able to
saturate those viral antigens coated. MAbs M11, M3 and 10H11 did not reach a plateau level
within the range studied.

The MAbs were then tested for their cross-reactivity with nine Quebec isolates of TCV using
the same serological test. These isolates have been propagated in HRT-18 cells for less than five
passages. Twice the amount of MAb giving maximal binding to the Minnesota strain was used.
As illustrated in Fig. 9, binding to the heterologous virus was expressed as a percentage of the

MAb

	M10	M29	M33	M31	15E10	8E6	8B8	6C5	M3	10H11
TCQ.1713										
TCQ.2										
TCQ.12										
TCQ.15										
TCQ.13										
TCQ.11										
TCQ.19										
TCQ.1										
TCQ.17										
USA										

Virus strain

Fig. 9. Cross-reactivity of MAbs to the surface glycoproteins of the Minnesota strain (USA) of TCV with TCV Quebec isolates. The cross-reactivity between the various strains was tested by an indirect ELISA. Twice the amount of MAb giving maximal binding to the Minnesota strain was used. Binding to the heterologous virus is expressed as a percentage of the A_{474} value obtained with TCV Minnesota. Empty squares, 50 to 100%; crossed squares, 25 to 50%; filled squares, less than 25%. The results were expressed as in Niesters *et al.* (1987).

absorbance values obtained with the reference virus. Two Quebec isolates (TCQ15 and TCQ17) reacted similarly to the Minnesota strain with the 10 MAbs tested. One anti-E3 MAb (M31), and two anti-E2 MAbs (M3 and M29), recognized at least two antigenic groups among the TCV isolates studied. These MAbs did not differentiate between the reference Minnesota strain and the Quebec isolates TCQ15, TCQ17 and TCQ1713. In contrast, a weak reaction was obtained by testing these three MAbs against the other six Quebec isolates. The MAbs 6C5 and 15E10 did not identify antigenic variability among the TCV isolates studied, including isolate TCQ19 which reacted only moderately with the other MAbs.

DISCUSSION

The four unique major structural proteins (E1, E2, E3 and N) of TCV (Dea & Tijssen, 1988*b*) all elicited the production of MAbs. Immunogold labelling using MAbs reacting to the 200K protein confirmed its association with the large bulbous projections. Major glycoprotein species of 100K and 120K were more often revealed by SDS-PAGE than the 200K component, but were antigenically related. They may have been derived from the 200K species by proteolytic cleavage, as reported for other coronaviruses (Sturman *et al.*, 1985; Cavanagh *et al.*, 1986*a*). The reactivity of anti-gp200/100 MAbs with intracellular polypeptides corresponding to an approximate M_r of 170K to 180K, 120K and 90K also suggested a close relationship among these components. Some of these products may correspond to intracellular precursors or degradation products of the same polypeptide. For murine and bovine coronaviruses, it has been suggested that intracellular non-glycosylated species must first undergo glycosylation to yield 150K or 170K intracellular glycoproteins, before further glycosylation to yield the final virion peplomer protein (Rottier *et al.*, 1981; Deregt & Babiuk, 1987). After incorporation into the virion, a host-dependent proteolytic cleavage yields the gp90–100 species (Sturman *et al.*, 1985;

Deregt *et al.*, 1987). A few anti-TCV MAbs precipitated either the gp100 or the gp120 polypeptide species, suggesting that additional epitopes may result from the dimerization of gp100 to yield gp200, or are located near the proteolytic cleavage site. The M_r values assigned to the TCV-induced intracellular polypeptides are only indicative since considerable post-translational modifications of the proteins affect the relative mobility in polyacrylamide gels and thus the apparent M_r.

An intracellular polypeptide species with an apparent M_r of 130K was the only component precipitated by anti-TCV MAbs that inhibited the haemagglutinating activity of the virus. Thus, the haemagglutinin and peplomeric glycoproteins of TCV appeared to be derived from different intracellular precursors. None of the four anti-E3 MAbs reacted with the 66K polypeptide resulting from gp140 by reduction with 2-mercaptoethanol. On the basis of MAb reactivity, Deregt & Babiuk (1987) identified a 59K (monomer) and a 118K (disulphide-linked dimer) glycoprotein, as precursors of the bovine coronavirus haemagglutinin glycoprotein (gp124/gp62). They reported that among the anti-haemagglutinin MAbs reacting with gp118, only a few immunoprecipitated the gp59 monomer. The exact nature of the 36K protein immunoprecipitated from extracts of TCV-infected cells was not determined in the present study. It may correspond to a degradation product of a larger glycoprotein, possibly the haemagglutinating protein, as previously suggested for bovine coronavirus (Deregt & Babiuk, 1987). The lack of reactivity of the anti-TCV MAbs to the monomeric form of the haemagglutinating glycoprotein could, for MAbs M11 and M31, also be due to their lower avidities. Immunogold labelling did not permit the detection of gp140 on the TCV virion. However, the persistence of the small granular projections and gp140 in the electrophoretic gels but loss of gp200/100 (unpublished data) following bromelain digestion, the non-reactivity of anti-gp200/100 MAbs with particles that lost their larger projections, and because bromelain-treated viral particles can haemagglutinate strongly suggested a relationship between the haemagglutinin and the short granular projections. Thus, with respect to its morphological and molecular characteristics, TCV differs considerably from infectious bronchitis virus (IBV), an antigenically unrelated avian coronavirus (Bingham & Almeida, 1977; Stern *et al.*, 1982; Cavanagh *et al.*, 1986*b*) but resembles viruses belonging to the group of mammalian haemagglutinating coronaviruses (Hogue *et al.*, 1984; King *et al.*, 1985; Hogue & Brian, 1986). The antigenic relationships between TCV and members of this group remain to be determined.

In pulse–chase experiments, the N protein of TCV was seen to be a group of three closely migrating bands, which were all immunoprecipitated by anti-N MAb M21 (Fig. 5). The smaller bands which migrated slightly ahead of the N protein may correspond to degradation products, as previously described with some other coronaviruses (Siddell *et al.*, 1981; Robbins *et al.*, 1986; Deregt *et al.*, 1987). With purified viral preparations, a non-specific background of N protein was occasionally observed when anti-E2 and anti-E3 MAbs were tested, both by Western immunoblotting and immunoprecipitation experiments. Non-specific binding to N protein was also obtained in studies with other coronaviruses (Talbot *et al.*, 1984; Laude *et al.*, 1986; Deregt & Babiuk, 1987). The N protein is the most abundant structural component of the coronavirus virion (Siddell *et al.*, 1983). Furthermore, this non-specific interaction could be due to the particular charge distribution on these proteins. Published sequences of N proteins show that the non-phosphorylated precursors have very high isoelectric points (about 10·5), whereas serine residues (which are subsequently phosphorylated) occur primarily in clusters (Boursnell *et al.*, 1985; Lapps *et al.*, 1987; Skinner & Siddell, 1983).

The 30K to 32K polypeptide species which was regularly observed in SDS–PAGE analysis of extracts from TCV-infected cells, and occasionally with purified viral preparations (Fig. 1, lane 3), was considered to be a putative non-structural viral protein, as it was not identified by the anti-TCV hyperimmune serum either by Western immunoblotting or by immunoprecipitation (Fig. 3). A non-structural protein having a similar M_r has also been identified in cells infected with murine or bovine coronaviruses (Siddell *et al.*, 1981; Deregt *et al.*, 1987). Major polypeptide species with M_r values ranging from 41K to 46K have also been reported for murine and haemagglutinating mammalian coronaviruses, and were shown to correspond to aggregated forms of E1 (Sturman & Holmes, 1983; Hogue & Brian, 1986; Deregt & Babiuk, 1987). The only

anti-TCV MAb which was found to be directed against the E1 glycoprotein failed to react with the 44K protein, and thus we were not able to confirm their antigenic relationship.

Antigenic determinants located on both E2 and E3 reacted with MAbs involved in TCV neutralization, as previously described for bovine enteric coronavirus (Deregt & Babiuk, 1987). However, our results did not permit us to establish whether the sterically blocked critical areas are on both proteins. Anti-gp200/100 MAbs recognized at least one other functional domain not involved in neutralization. The anti-E2 MAbs reacted with gp200/100 in both Western blotting and immunoprecipitation assays. In contrast, two anti-E3 MAbs, M11 and M31, reacted with gp140 only by immunoprecipitation of cell extracts, whereas MAbs 8B8 and 8E6, which demonstrated lower HI titres, reacted with gp140 in both techniques. These data suggest that the critical antigenic determinants are probably conformation-dependent, as reported with other coronaviruses. Talbot *et al.* (1984) found that three neutralization epitopes in MHV-JHM are sensitive to SDS denaturation, whereas in the case of transmissible gastroenteritis virus (TGEV), all six critical determinants are sensitive to SDS denaturation and 2-mercaptoethanol (Jiménez *et al.*, 1986).

Distribution of viral antigens in TCV-infected HRT-18 cells appeared similar to those described for TGEV and MHV (Laude *et al.*, 1986; Collins *et al.*, 1982). Viral envelope proteins (E1, E2 and E3) were found in the perinuclear area which could correspond to the rough endoplasmic reticulum and Golgi apparatus. Both haemagglutinin and peplomer glycoproteins are expressed at the cell surface and may thus represent potential targets for specific antibody-dependent or cell-mediated cytotoxicity.

Studies of the heterologous activity of anti-E2 and anti-E3 MAbs indicated that a high degree of serological relatedness exists among Quebec strains, and that they appear closely related to the prototype Minnesota strain. Nevertheless, a distinct antigenic variability was evident at the peplomer level and at least two antigenic groups (represented by TCQ.12 and TCQ.15, respectively) could be distinguished among these strains. In preliminary studies, conventional antisera did not distinguish these strains in neutralization or HI tests (unpublished data). However, previous Western immunoblotting studies of egg-adapted TCV isolates, suggesting the existence of antigenic variants of TCV (Dea & Tijssen, 1988*b*), are in agreement with the present findings. It is noteworthy that this distinct antigenic variability was found on both E2 and E3. This variability of both envelope glycoproteins was linked for the isolates tested, and was independent of the fusion experiments (e.g. MAbs M31 and M33 were obtained from the same fusion). A larger collection of TCV-positive hybridomas and TCV isolates is needed to cover more epitopes and to obtain more representative data. Competition binding assays allowed the definition of four to six distinct antigenic sites on the peplomeric glycoproteins of MHV, IBV, TGEV and bovine coronavirus (BCV) of which only a few determinants are highly conserved among various strains (Wege *et al.*, 1984; Delmas *et al.*, 1986; Deregt & Babiuk, 1987; Niesters *et al.*, 1987). A comparative study of nine TGEV strains, using a panel of neutralizing MAbs that recognized four major antigenic sites and at least nine possible epitopes, distinguished two variants by seroneutralization, which could not be distinguished using conventional sera (Laude *et al.*, 1986). Nevertheless, distinct antigenic strains as defined for murine coronaviruses and IBV, where each isolate has a unique pattern of reactivity with anti-glycoprotein MAbs (Fleming *et al.*, 1983), was not reported for TGEV. Four epitopes were defined on the haemagglutinin of BCV (Deregt & Babiuk, 1987). Results presented in Fig. 9 suggested that anti-TCV MAbs were obtained against at least three epitopes on both E2 and E3. Studies are in progress to map the anti-TCV MAbs on the peplomeric and haemagglutinin proteins of the virus and to investigate the existence of more than one antigenic strain of TCV.

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