Journal of General Virology (1992), 73, 1725–1737. *Printed in Great Britain*

Bovine coronavirus peplomer glycoproteins: detailed antigenic analyses of S1, S2 and HE

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Forty-four monoclonal antibodies (MAbs) to the G_{110} isolate of bovine enteric coronavirus were used for the characterization of the peplomer proteins S and HE. Fourteen of these MAbs reacted with HE and the remaining 30 with the products of the S gene, S1 (19 MAbs), S2 (six MAbs) and gp200 (five MAbs). S1 and HE were found to carry major neutralization determinants, and S1 appeared to elicit the production of the MAbs displaying the highest neutralizing activity. The topography of the epitopes was assessed by means of a competitive binding assay; the 44 MAbs defined four independent antigenic domains on S1, two on S2, one on gp200 and two on HE. All the neutralizing anti-S1 MAbs mapped in antigenic sites A and B and all the neutralizing anti-HE MAbs in HE-B. Antigenic site S1-B was further subdivided into four subsites. Functional mapping was performed by testing a library of neutralization-resistant mutants against the neutralizing MAbs. Analysis of their reactivity in a neutralization test confirmed the overall distribution of epitopes in S1-B and HE-B.

Introduction

Bovine coronavirus (BCV) is an enteric virus which multiplies in the differentiated enterocytes of the small intestine and colon, causing acute enteritis in newborn calves (Stair *et al.*, 1972; Babiuk *et al.*, 1985) and chronic shedding (Crouch *et al.*, 1985) or diarrhoea (Takahashi *et al.*, 1980) in adult cows. BCV particles consist of large single-stranded positive RNA (30 kb) associated with a basic phosphonucleoprotein (N, M_r 52K) forming a helical ribonucleoprotein protected by a viral envelope containing three glycoproteins, M, S and HE (Laporte & Bobulesco, 1981; King & Brian, 1982; Deregt *et al.*, 1987).

The transmembrane glycoprotein M is deeply embedded in the lipid bilayer and migrates as a heterogeneous set of polypeptides (23K to 28K) reflecting various degrees of glycosylation (Deregt *et al.*, 1987). The other two glycoproteins, S and HE, form the two fringes of spikes which are characteristic of BCV and other haemagglutinating coronaviruses (Dea & Tijssen, 1989). The S glycoprotein, presumably forming the club-shaped peplomers, is a large (200K), highly glycosylated protein first synthesized as a precursor gp170 or pS. This is further glycosylated to yield a transient gp190 (Deregt *et al.*, 1987) which is proteolytically cleaved into two fragments, S1 and S2, respectively corresponding to the N- and C-terminal subunits of the S protein (Boireau *et al.*, 1990). The second spike-forming protein, HE, is a dimer of 125K to 140K, formed by the association of two 65K units by disulphide bridges (Laporte & Bobulesco, 1980; King *et al.*, 1985; Kienzle *et al.*, 1990). The role of HE in the agglutination of murine red blood cells was demonstrated by biochemical investigations (King *et al.*, 1985) and by the inhibiting activity of monoclonal antibodies (MAbs) to HE (Parker *et al.*, 1989). HE also has a receptor-binding and a receptor-destroying activity (RDE) similar to that of influenza C virus (Vlasak *et al.*, 1988*a*, *b*; Schultze *et al.*, 1991); the RDE is due to acetylesterase (AE) activity which appeared to be essential for viral infectivity (Vlasak *et al.*, 1988*b*).

Peplomer proteins have been shown to play a major role in the interaction between the coronaviruses and their target cells or the host immune system (for a review see Spaan *et al.*, 1988). Consequently considerable work has been done on the characterization of B cell epitopes on the S protein of mouse hepatis virus (MHV) (Wege *et al.*, 1984), transmissible gastroenteritis virus (TGEV) (Laude *et al.*, 1986; Delmas *et al.*, 1986, 1990) and infectious bronchitis virus (IBV) (Niesters *et al.*, 1987; Koch *et al.*, 1990), and their possible association with a biological function. The S and HE glycoproteins of BCV were shown to elicit the production of neutralizing MAbs (Vautherot & Laporte, 1983; Deregt *et al.*, 1987), which, for some (Deregt *et al.*, 1987), were used in a topographical study of S1 and HE, and tested for their protective

activity against BCV infections in calves (Deregt *et al.*, 1989). To characterize the peplomer glycoproteins further, and in particular the cleavage products of S, we selected a large panel of MAbs which allowed us (i) to characterize S1 and S2 on the basis of their reactivity with MAbs, (ii) to analyse the functional inhibitions exerted by MAbs on the virus and (iii) to study the distribution of B cell epitopes on each glycoprotein.

Methods

Viruses and cell lines. All BCV isolates (Laporte *et al.*, 1979; Vautherot & Laporte, 1983) as well as coronaviruses OC43 and haemagglutinating encephalitis virus (HEV) were plaque-purified three times and serially passaged in HRT-18 cells (Tompkins *et al.*, 1974), as described previously (Vautherot & Laporte, 1983). Infected cells were maintained in RPMI 1640 medium with 2% foetal calf serum and 10 μg/ml of Baytril (Bayer) [maintenance medium (MM)]. For biochemical analyses, viruses were purified according to a method described previously (Laporte & Bobulesco, 1981).

Plaque assay and virus neutralization tests. Viruses were titrated essentially as described (Vautherot, 1981) except that viral plaques were visualized 36 to 48 h post-infection by haemadsorption with rat red blood cells (RBCs). Neutralization assays were performed as described (Vautherot & Laporte, 1983) and neutralization titres were expressed as the reciprocal of the dilution giving a 50% reduction in the plaque number. Neutralization kinetics were measured according to the method described by Volk *et al.* (1982).

Production of MAbs. The production of mouse–mouse hybrids secreting anti-BCV MAbs has been described (Roseto *et al.*, 1982). Rat–rat hybridomas were generated by fusing IR983F myeloma cells (Bazin *et al.*, 1980) with splenocytes from a LOU rat which had been immunized intravenously three times with purified BCV strain G_{110}. The fusion was performed according to the protocol described by Bazin (1982). The supernatant from growing clones was screened for anti-BCV antibody production both by indirect immunofluorescence (IIF) and ELISA (Vautherot & Laporte, 1983). Cloning of hybridomas and immunoglobulin isotyping were performed as described (Roseto *et al.*, 1982). Ascites fluids containing MAbs were obtained by injecting intraperitoneally 10^7 hybrid cells/6-week-old LOU rat primed 15 days before with 1 ml of Pristane (2,6,10,14-tetramethylpentadecane; Sigma).

Isotopic labelling of intracellular viral polypeptides. HRT-18 monolayers were infected as described (Vautherot *et al.*, 1984) except that the m.o.i was raised to 20 p.f.u./cell, and, depending on the experiment, 1·85 MBq/ml of each of [^{3}H]valine and [^{3}H]leucine (CEA), or 0·185 MBq/ml of a ^{14}C-labelled amino acid mixture (CEA) were used. At the end of the labelling period, the cells were rinsed with cold PBS, lysed in radioimmunoprecipitation assay (RIPA) buffer [20 mM-Tris–HCl pH 7·5, 0·5 M-NaCl, 2% Triton X-100, 0·2 mM-PMSF and 2 μg/ml Aprotinin (Sigma)]. The lysates were then prepared as described (Delmas *et al.*, 1986).

Isotopic labelling of virus proteins. HRT-18 cells were infected with BCV at an m.o.i of 0·05 p.f.u./cell and incubated for 24 h. Infected monolayers were placed in MM lacking the labelling amino acid for 1 h, after which 1·48 MBq/ml of each of [^{3}H]valine and [^{3}H]leucine (Amersham) were added. Infection was stopped 48 to 50 h post-infection and the labelled virus was harvested and purified according to standard procedures. Spike proteins were solubilized by incubating the virus pellet for 30 min at 25 °C in a 60 mM-octylglucoside (Boehringer Mannheim) solution made in Tris–HCl buffer pH 7·5, containing 0·15 M-NaCl and 2 μg/ml Aprotinin (octyl buffer). Prior to immunoprecipitation, the detergent-disrupted virus was ultracentrifuged for 1 h at 45000 r.p.m. in a Beckman Ti 45 rotor.

Radioimmunoprecipitation. Immunoprecipitations were carried out as described (Vautherot *et al.*, 1984; Delmas *et al.*, 1986). Immune complexes were washed four times with RIPA buffer or octyl buffer, once in 20 mM-Tris–HCl buffer pH 7·5, dissolved in electrophoresis sample buffer (Laemmli, 1970), and heated at 65 °C for 5 min. Viral polypeptides were separated by electrophoresis on polyacrylamide gels, using the discontinuous buffer gel system of Laemmli (1970). Gels were dried, fluorographed and exposed to Fuji X-ray films (RX films, Fuji) at −80 °C.

Western blotting. Viral proteins separated by electrophoresis on 10% SDS–polyacrylamide gels were transferred to a nitrocellulose sheet (BAS 85, Schleicher & Schuell) by transverse electrophoresis (Towbin *et al.*, 1979) in a Milliblot SDE electroblotting apparatus (Millipore) at 2·5 mA/cm^2 for 30 min using a discontinuous buffer system (Khyse-Andersen, 1984). Blots were washed once with cold PBS and incubated for 1 h at 37 °C in a 3% gelatin solution made in Tris–saline buffer (10 mM-Tris–HCl pH 7·4, 150 mM-NaCl). To visualize the reaction of MAbs with viral proteins, blots were incubated with MAb dilution buffer (in Tris–saline buffer with 3% gelatin and 0·5% Triton X-100), washed four times (in PBS with 0·05% Tween 20) and incubated with an anti-rat or -mouse IgG–alkaline phosphatase conjugate (Biosys). Bound phosphatase was revealed after four additional washes by using 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine/nitroblue tetrazolium chloride (Gibco-BRL) under the conditions described by the manufacturers.

Haemagglutination inhibition (HI). Purified MAbs (see below) were tested for HI as described (Vautherot *et al.*, 1984).

AE inhibition assays. For inhibition of AE activity, serial twofold dilutions of purified MAbs, starting at a concentration of 1 mg/ml, were mixed in wells of microtitration plates with a fixed amount of BCV (2 μg/well) and incubated for 30 min at room temperature. Substrates for the AE were *p*-nitrophenyl acetate (pNPA) (Sigma) at 1 mM in PBS or 5-carboxyfluorescein diacetate (5-CFDA; Calbiochem) at 6 μM in HEPES buffer (50 mM-HEPES pH 7·4, 110 mM-NaCl, 5 mM-KCl and 1 mM-$MgCl_2$). Absorbances were read on a multichannel spectrophotometer (Titertek Multiskan MCC/340; Flow) at 405 nm for pNPA or 492 nm for 5-CFDA.

Purification and conjugation of MAbs. MAbs were purified from ascites fluids by ammonium sulphate precipitation and gel filtration on a Sephacryl S-300 (Pharmacia) column equilibrated in PBS. Purified MAbs I7, I9, B5, A20, J18a, A9, Z17 and P11 were conjugated to horseradish peroxidase (HRPO) by the two-step method of Avrameas & Terninck (1971). All other MAbs were biotinylated using biotin *N*-hydroxysuccinimide ester (Boehringer Mannheim), as described by Guesdon *et al.* (1979). The integrity of labelled neutralizing MAbs was assessed by comparing their neutralizing titres before and after conjugation.

Solid-phase immunoassays for binding and competition binding of MAbs. For binding of labelled and unlabelled MAb, wells in microtitration plates (ProBind plates, Falcon) were coated with 2 μg of purified virus (G_{110} isolate) in 100 μl of 50 mM-PIPES buffer pH 6·5, 150 mM-NaCl for 16 h at 37 °C, a temperature which was chosen for all subsequent incubations. Plates were washed four times in PBS containing 0·05% Tween 20 (PBST), and unreacted sites were blocked by a 1 h incubation in a 1% solution of gelatin in 50 mM-Tris–HCl buffer pH 7·4, 150 mM-NaCl (100 μl/well). After washing, the wells were filled with 100 μl of serially diluted MAbs in PBST and the plates were incubated for 1 h. Unlabelled MAbs were detected with a sheep

anti-mouse immunoglobulin conjugated to HRPO (Biosys) and biotinylated MAbs with HRPO-labelled streptavidin (Amersham) diluted 1/2000 in PBST (1 h incubation). Peroxidase activity was revealed with the TMB microwell peroxidase system (Kirkegaard & Perry Laboratories). Absorbances at 450 nm were read on a multichannel photometer (Titertek Multiskan MCC/340, Flow). Net absorbances (NA) were expressed as A_{450} in antigen-coated wells minus A_{450} in wells without antigen.

Competitive binding between labelled and non-labelled MAbs was assayed by incubating either sequentially or simultaneously the competitor and probe antibody dilutions, following the method described by Delmas *et al.* (1986). The concentration of the competitors was adjusted to yield a competitor/probe ratio of 100 or 500 in the first dilution. Concentrations of labelled MAbs were adjusted to give a NA of 1·5 to 1·8 (60 to 80 % saturation levels) in the direct binding assay. Results were expressed as percentage competition, calculated using the formula 100[1 − (B/A)], where A and B are the NA in the absence and in the presence of competitor antibody, respectively.

Selection of neutralization escape mutants. Spontaneously arising mutants resistant to neutralizing anti-S and anti-HE MAbs were selected from BCV strains G_{110} and F_{15}. Serial 10-fold dilutions of BCV ($2{\cdot}8 \times 10^8$ p.f.u./ml) in MM were mixed with an equal volume of ascites fluid diluted 1:50 in MM and incubated at 37 °C for 30 min. Sheep anti-mouse IgG (Biosys) diluted 1:50 was added to the virus–antibody mixture which was incubated at 37 °C for an additional 30 min period (Vautherot *et al.*, 1984). Virus dilutions were plated on HRT-18 monolayers in six-well plates and, after adsorption, the cells were overlaid with MM containing 0·6% agarose and the selecting antibody diluted 1:100. Well defined plaques were picked off, diluted in 2 ml of MM and frozen. Neutralization-resistant mutants were recloned twice, retested against the relevant antibody and, if resistant, tested in a neutralization assay against a panel of neutralizing MAbs.

Results

Production of MAbs and determination of their polypeptide and antigenic specificities

Five fusions using the splenocytes of four BALB/c mice and one LOU rat, each immunized with purified BCV (G_{110} isolate), yielded a total of 55 stable hybridomas, 44 of which were selected for this study (Tables 1 and 2). MAbs to HE immunoprecipitated a glycoprotein migrating with an M_r of 65K after reduction with 2-mercaptoethanol (2-ME) (Fig. 1, lanes 1 and 5). All anti-HE MAbs recognized the dimeric form of HE (125K), both from infected cell lysates and from octylglucoside-solubilized peplomers of purified BCV; none of these MAbs was able to bind to the SDS-denatured HE (data not shown). MAbs to HE identified epitopes which were common to all BCV isolates tested, as revealed by IIF.

MAbs to S glycoproteins, all of which immunoprecipitated the S precursor, pS or gp170, were classified as anti-S/gp105, anti-S/gp95 and anti-S/gp200, according to the polypeptide(s) they recognized. MAbs to S/gp105 precipitated a glycoprotein with an M_r of 105K together with pS from infected cell lysates or the 105K species alone when immunoprecipitations were done with radiolabelled detergent-disrupted virus (Fig. 1, lanes 2 and 4). Seven of 19 MAbs reacted with the SDS-denatured S/gp105 revealing a unique band at 105K on the Western blots (Table 2 and Fig. 2); the reduction of intrachain disulphide bridges, which did not alter the apparent mobility of S glycoproteins, resulted in a dramatic loss of antigenic reactivity as only MAb I16 bound to S/gp105 after reduction (Fig. 2, lanes 4 to 7). MAbs to S/gp105 were predominantly type-specific, two of them (MAbs A9 and E13) detecting a minor antigenic drift of the BCV Mebus strain (Table 2). Antigenic determinants conserved on OC43 and HEV were also recognized by anti-S/gp105 MAbs F7 and α8 (OC43), and I16 (HEV).

Five MAbs to S/gp95 immunoprecipitated a faint band at 95K, pS, a high M_r species (>200K) from infected cell lysates and S/gp95 together with a 200K moiety from purified virus preparations (Fig. 1, lanes 3 and 7). Four of these MAbs reacted strongly with a 95K protein and faintly with a high M_r species (≥200K) on Western blots (Fig. 2, lane 3). These four MAbs recognized a linear epitope conserved in two recombinant fusion proteins which were expressed in *Escherichia coli* transformed by pUEX plasmids containing two overlapping restriction DNA fragments corresponding to the N terminus of S2 (J. F. Vautherot, unpublished results).

From these results and by comparing the M_r deduced from the sequence with the M_r observed by PAGE, we infer that anti-S/gp105 MAbs recognize S1, the amino-terminal part of the S molecule. Five additional MAbs reacted only with pS and gp240 from infected cell lysates

Table 1. *Characterization of MAbs to HE*

MAb designation	Isotype/ species*	ELISA titre†	Neutralization titre†	HI titre
Y16	IgM/R	43700	10900	20
J18c	IgG2b	88000	17500	5120
J17b	IgG1	58000	3950	80
B4	IgG1	55000	400	2560
B7	IgG2a	275000	700	20480
F13	IgG3	37500	<300	5120
A12	IgG3	131000	<300	20480
G20	IgG2a	300000	750	640
J21	IgG	66000	<300	160
J10	IgG2a	71000	<300	640
J18b	IgG2a	64800	<300	1280
H17	IgG2a	101000	<300	2560
B13	IgG2a	8600	<300	0
B22	IgM	5450	<300	0

* Except for MAb Y16, a rat IgM MAb, all other anti-HE MAbs were produced in BALB/c mice.

† MAbs were purified from ascites fluids and concentrations were adjusted to 1 mg/ml for the first dilution. ELISA titres are expressed as the reciprocal of the dilution giving an absorbance of 0·8. Neutralization and HI titres are expressed as described in Methods.

Table 2. *Characterization of MAbs to S glycoproteins*

MAb designation	Isotype/ species*	ELISA titre†	Neutralization titre†	Polypeptide specificity‡		Antigenic cross-reactivity§			
				WB	RIPA	BCV G110-F15/ Mebus	MHV	HEV	OC43
C13	G1/M	100	6400	S/gp105	S/gp105	+/+	−	−	−
P11	G/R	130000	170	S/gp105	S/gp105	+/+	−	−	−
I11	G2a/M	8600	17000	S/gp105	S/gp105	+/+	−	−	−
I7	G2a/M	14700	97900	S/gp105	S/gp105	+/+	−	−	−
I9	G2a/M	15000	8800	−	S/gp105	+/+	−	−	−
J17a	G2a/M	11000	5400	−	S/gp105	+/+	−	−	−
G19	G2a/M	900	1600	−	S/gp105	+/+	−	−	−
Z17	G/R	470000	23200	−	S/gp105	+/+	−	−	−
B5	G2a/M	211000	46300	−	S/gp105	+/+	−	−	−
A20	G2a/M	100000	13000	−	S/gp105	+/+	−	−	−
J18a	G2a/M	72000	64300	−	S/gp105	+/+	−	−	−
E11	G2a/M	150	350	−	S/gp105	+/+	−	−	−
E19	G2a/M	27000	<100	−	S/gp105	+/+	−	−	−
H13	G2a/M	21000	<100	−	S/gp105	+/+	−	−	−
E13	G2a/M	300000	<100	−	S/gp105	+/−	−	−	−
A9	G2a/M	50250	10000	S/gp105	S/gp105	+/−	−	−	−
F7	G1/M	1300	<100	S/gp105	S/gp105	+/+	−	−	+
α8	G/R	60	<100	−	S/gp105	+/+	−	−	+
I16	G1/M	25	<100	S/gp105	S/gp105	+/+	−	+	+
I1	G2a/M	2600	<100	S/gp95	S/gp95	+/+	+	+	+
I22	G2a/M	640	<100	S/gp95	S/gp95	+/+	+	+	+
J22	G2a/M	1260	<100	S/gp95	S/gp95	+/+	+	+	+
G4	G2a/M	50	<100	S/gp95	S/gp95	+/+	+	+	+
H3	A/M	1400	<100	−	S/gp95	+/+	+	−	−
H14	G2a/M	ND	<100	−	S/gp95	+/+	−	+	−
B23	G2a/M	500	<100	−	gp200	+/+	+	+	+
E5	G2a/M	19000	<100	−	gp200	+/+	+	+	+
H7	G2a/M	4530	<100	−	gp200	+/+	+	+	+
H19	G2a/M	ND	<100	−	gp200	+/+	+	+	+
I12	G2a/M	1600	<100	−	gp200	+/+	+	+	+

* Immunoglobulin isotype/murine species immunized; M, mouse and R, rat MAbs.

† As in Table 1.

‡ Polypeptide specificities were assessed by reacting MAbs either in immunoprecipitation with radiolabelled viral proteins (RIPA) or on Western blots (WB).

§ Antigenic cross-reactivities of MAbs were tested in an IIF assay as described (Vautherot & Laporte, 1983).

or gp200 from virus preparations but did not bind to any other S product (Fig. 1, lanes 6 and 9). MAbs to S/gp95 and S/gp200 were mainly group-specific (Table 2).

Inhibition of viral functions by MAbs

MAbs were tested for their ability to neutralize the virus, and to inhibit haemagglutinating and esterase activities. MAbs to S1 (13 of 19) and HE (six of 14) neutralized BCV at titres ranging from 170 to 1×10^5, whereas MAbs to S2 or S/gp200 displayed no neutralizing activity (Tables 1 and 2), even in the presence of complement or sheep anti-mouse immunoglobulin. The highest neutralization titres were obtained with anti-S1 MAbs, and no correlation was seen between the binding efficiency to the antigen in ELISA and the capacity of MAbs to neutralize the virus. Neutralizing MAbs were also tested for the rate at which they inhibited the viral infectivity in a neutralization kinetics analysis. All neutralization curves were biphasic, with a maximum of neutralizing activity occurring during the first 10 min (Fig. 3). Kinetics of neutralization illustrate the lower efficiency of anti-HE MAbs compared to the anti-S1 MAbs in neutralization assays as anti-S1 MAbs neutralized 85 to 95% of the virus, whereas five of six anti-HE MAbs neutralized only 40 to 50% of the virus (Fig. 3). MAbs to HE (12 of 14) inhibited the agglutination of rat RBCs by BCV at dilutions up to 2×10^4 (Table 1). No correlation was found between the neutralizing and the HI activities (Table 1). MAbs to the S glycoproteins did not significantly inhibit the agglutination of mouse or rat RBCs by BCV, although some degree of HI was observed with anti-S1 MAbs A9 and E13 (data not shown).

All MAbs were tested for inhibition of AE and we found that MAb A12 alone inhibited the AE activity,

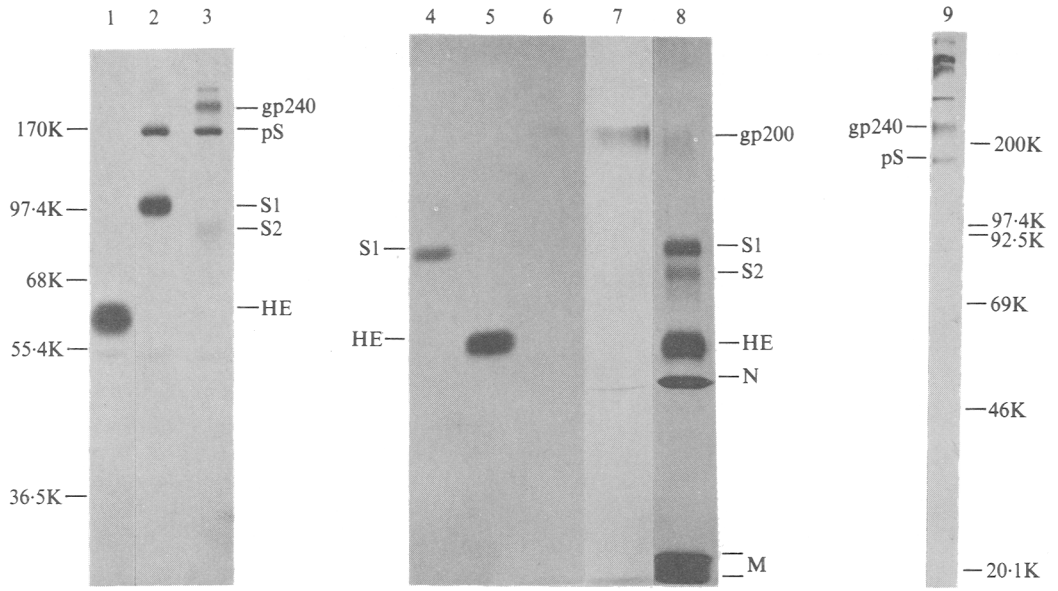

Fig. 1. Immunoprecipitation of viral proteins from BCV strain G_{110}. Viral polypeptides were precipitated from infected cell lysates labelled with ^{14}C- (lanes 1 to 3) or ^{3}H-labelled amino acids (lane 9), or from octylglucoside-disrupted ^{3}H-labelled virus (lanes 4 to 8) by a rabbit polyclonal antiserum (lane 8) or MAbs to HE (MAb A12, lanes 1 and 5), S1/gp105 (MAb I7, lanes 2 and 4), S2/gp95 (MAb I1, lanes 3 and 7) and gp200 (MAb E5, lanes 6 and 9). After dissociation, immunoprecipitates were separated on 10% polyacrylamide gels (lanes 1 to 3 and 9) or 5% to 15% polyacrylamide gradient gels (lanes 4 to 8). Positions of the M_r markers are shown on the right side for lanes 1 to 3 and left side for lane 9.

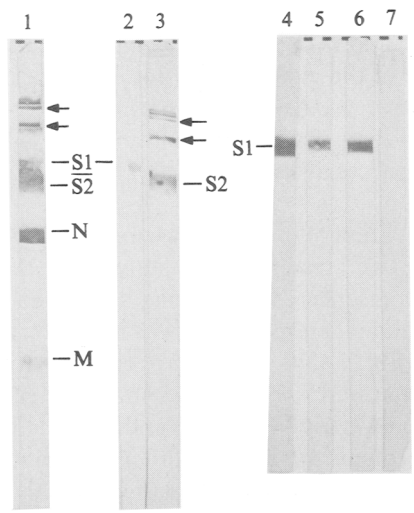

Fig. 2. Reactivity of representative MAbs to denatured BCV proteins. Purified BCV (G_{110} isolate) was dissolved in sample buffer with (lanes 5 and 7) or without (lanes 1 to 4 and 6) 2-ME, subjected to electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose sheets. Individual nitrocellulose strips were reacted with a rabbit polyclonal antiserum (lane 1), MAbs to S1/gp105 (MAb P11 in lanes 2, 6 and 7; MAb I16 in lanes 4 and 5) or MAb I22 to S2/gp95 (lane 3). The arrows point to the high M_r products identified by anti-S2 MAbs.

when pNPA (M_r 181·2) was used as the substrate. When 5-CFDA (M_r 460·4) was used instead of pNPA, anti-HE MAbs G20, B13 and A12 were able to inhibit the viral AE (Fig. 4). MAb G20 was the only one which displayed a significant neutralizing activity (Table 1).

Delineation of epitopes on S and HE by competitive antibody binding assays (ABAs)

Two of 44 MAbs (G4 and B23) showed a major loss in their binding ability after biotinylation, as measured in the direct binding assay. By comparing the neutralizing titres before and after conjugation we also found that the coupling reaction reduced the neutralizing activity of all anti-HE and six anti-S1 MAbs; however, anti-S1 MAbs I7, I11, J18a, Z17 and P11 displayed an enhanced neutralizing power after enzyme (four of five) or hapten conjugation (one of five) (data not shown). MAbs were first assayed for self-competition in a sequential test (two-step assay); those which were poor competitors were further tested using the simultaneous assay (one-step assay), as described by Delmas *et al.* (1986).

On S1, four independent sites were delineated using a panel of 19 MAbs in the sequential (sites B, C and D) and/or the simultaneous assay (sites A and B) (Fig. 5). The MAbs binding to unrelated epitopes did not compete for fixation as illustrated by the competition curves of MAbs C13, F7 and I16 for antigenic sites A, C and D respectively (Fig. 5). Twelve neutralizing and three non-neutralizing MAbs mapped in site B, which was further subdivided into subsites defined by MAbs showing a similar profile of reactivity. MAbs to S1-B1 enhanced the binding of labelled MAb E11, whereas MAbs to S1-B2 competed with MAb E11 (Table 3). MAbs to S1-B1 and -B2 did not compete with MAb A9,

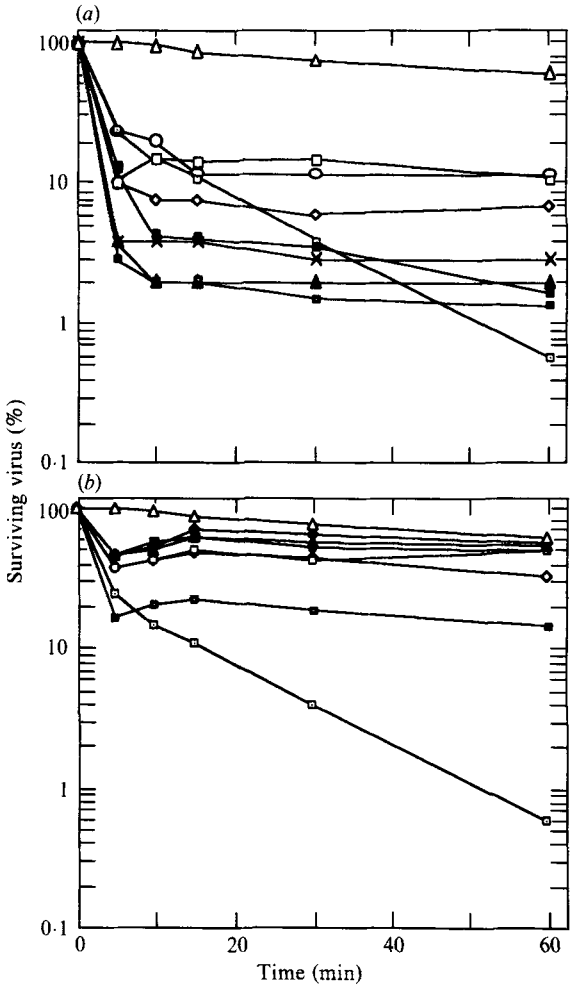

Fig. 3. Comparison of kinetics of BCV neutralization by hyperimmune mouse serum, MAbs to S1/gp105 and to HE. All MAbs used in these experiments were purified from ascites fluid by ammonium sulphate precipitation and their concentration was adjusted to 0·1 mg/ml. Kinetic experiments were performed as described, by incubating dilutions of antibodies with an equal volume of culture medium containing 10000 p.f.u./ml. Surviving virus was titrated at 10, 15, 30 and 60 min of incubation by plating on HRT-18 cells. The following symbols are used: polyclonal antisera from a hyperimmunized (⊡) or a control (△) mouse, MAbs A9 (○), J18a (◘), A20 (◇), C13 (■), 17 (□), Z17 (▲), I11 (×) to S1/gp105 in (*a*), and MAbs B7 (◆), Y16 (◘), B4 (◇), G20 (■), J18c (□) and A12 (▲) to HE in (*b*).

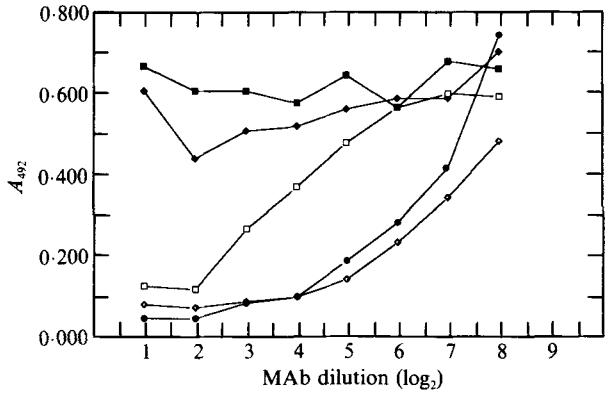

Fig. 4. Inhibition of AE activity by MAbs to HE. After contact with MAbs A12 (●), B7 (◆), B13 (□), G20 (◇) or H17 (■), residual AE activity of purified BCV was monitored by the conversion of the non-fluorescent 5-CFDA to fluorescent 5-CF by AE. Absorbances were read on a multichannel spectrophotometer at 492 nm.

defining S1-B4, unlike MAb J18a (S1-B3) which competed with all MAbs to site B except P11 (Fig. 5 and Table 3).

Competitive ABAs with weakly (J17a and G19) or non-neutralizing (E19 and H13) anti-S1 MAbs showed that they competed non-reciprocally with neutralizing MAbs. However analysis of the interactions between these MAbs and MAbs A9 and E13 strongly suggests that MAbs J17a and G19 bind to S1-B1/B2, and MAbs E19 and H13 to S1-B3 (Table 4). Competitive ABAs between MAbs H13 and E13 were strong and reciprocal (Table 4) whereas neutralizing MAbs mapping in S1-B1 and S1-B2 which strongly inhibited the fixation of H13 did not compete for fixation with E13.

These results illustrate the specificity of the competitive ABAs in delineating different sites and subdivisions, independent from the respective avidity of MAbs, and show that, within an epitope, differences in antibody avidity could be recognized, as all MAbs were tested in a reciprocal fashion (Table 3 and Fig. 5). We also verified that the size of the molecule did not influence the results of competitive ABAs by comparing the competitive binding curves of MAbs J18a, I11 and P11 which were conjugated successively to HRPO and biotin (data not shown).

The delineation of epitopes by competitive ABAs was confirmed by the results on the stability of the binding sites in the presence of denaturing agents, as MAb I16 defined a unique epitope which was not affected by SDS and 2-ME (Fig. 2) and MAb P11 reacted strongly with the SDS-denatured S1/gp105, when all other MAbs to site B reacted weakly (S1-B1, B3 and B4) or did not bind the viral protein (S1-B2). Another indication of the accuracy of our epitope map was indirectly obtained through the analysis of the reactivity of MAbs with BCV isolates, which revealed that subsite B4 (MAbs A9 and E13) was not conserved in the BCV Mebus strain (Vautherot & Laporte, 1983).

Two independent sites were defined on S2 (Table 5). Antigenic site S2-A, delineated by MAbs I22, J22 and I1, was resistant to SDS plus 2-ME and was highly conserved in the antigenic group, whereas S2-B (MAb H14) appeared to be SDS-labile and was present only on BCV and MHV (Table 2). MAb G4 was tentatively assigned to S2-A because of its pattern of reactivity in immunoblotting and IIF (Table 2). MAb H3 (S2-A′) competed non-reciprocally with MAbs in S2-A (Table 5), and also

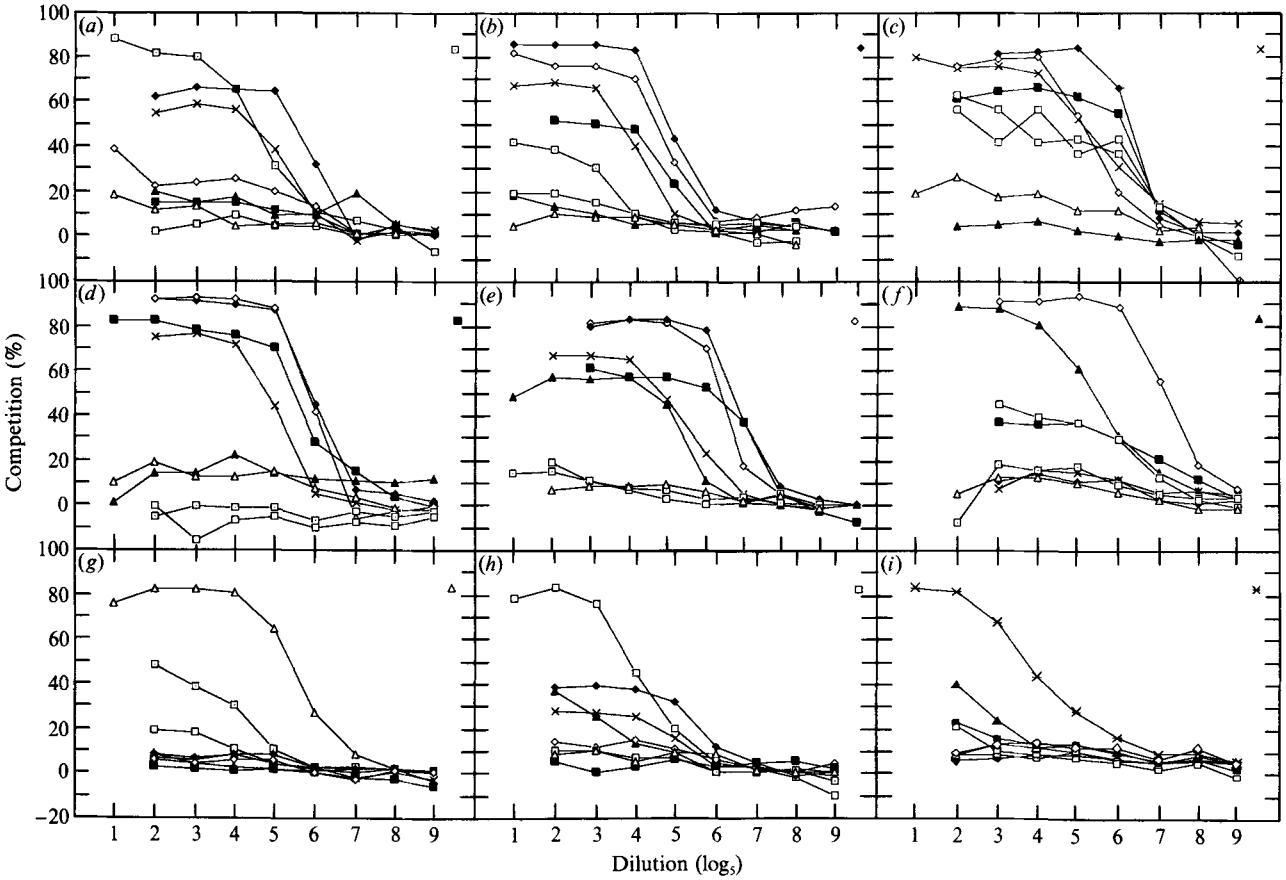

Fig. 5. Competitive ABAs using anti-S1 MAbs tested reciprocally as competitors and probes. The labelled MAbs used were (*a*) P11 (□), (*b*) I7 (◆), (*c*) I11 (×), (*d*) B5 (■), (*e*) J18a (◇), (*f*) A9 (▲), (*g*) I16 (△), (*h*) F7 (□) and (*i*) C13 (*). The ratio of probe/competitor antibody was adjusted to 1/100 or 1/500 for the first dilution and the unlabelled MAbs were subsequently diluted fivefold.

Table 3. *Delineation of antigenic domains on S1/gp105 by competitive ABA*

MAb competitor	Epitope site	Labelled MAb: C13*	P11	I11	I7	I9	Z17	B5	A20	E11	J18a	A9	E13*	F7	α8	I16
C13	A	+†														
P11			++	+	+/−		+/−			F°‡						+/−
I11			+	++	+	+	+	++	++	F°	+					
I7	B1		+	++	++	++	++	++	++	F°	++					
I9			+/−	+	+	+		+	++	F°	+					
Z17			+/−	++	+	++	+	++	++	++	+					
B5	B2			+	+	+	+	++	++	++	+					
A20				+	+/−	+		++	++	++	+					
E11	B3					F°				++						
J18a				++		++	+	++	++	++	++	++	++			
A9	B4									++	+	++	++			
E13										F°		+/−	++			
F7	C			+										++	++	
α8														+/−	++	
I16	D															++

* The MAbs were tested in the simultanous (one-step) assay.

† The competition reactions are: ++, 70 to 100% binding inhibition; +, 50 to 70% binding inhibition; +/−, 40 to 50% binding inhibition; blank, binding inhibition below 40%.

‡ The binding of the conjugated MAb was facilitated by the unlabelled MAb.

Table 4. *Binding characteristics of weakly or non-neutralizing MAbs mapping in antigenic site B (simultaneous assay)*

MAb competitor	Epitope site	Labelled MAb J17a	G19	E19	H13	E13	A9
J17a	B1/B2	+*	++	++	++		
G19		+	++	++	++		
E19	B2/B3	+	++	++	++	+/−	
H13		+	+	++	++	+	
E13	B4			+/−	++	++	+/−
A9				++	++	++	++

* The results of competition reactions are as presented in Table 3.

differed from the latter in its reactivity with the denatured S2 or with coronaviruses OC43 and HEV (Table 2). All anti-S/gp200 MAbs mapped in a unique site (Table 5) and reacted similarly with all coronaviruses tested (Table 2).

Identical experiments were performed using 14 MAbs against HE which mapped in two separate antigenic sites (Table 6). Epitope HE-A was delineated by MAb B22, and the remaining 13 MAbs apparently all interacted with one another, thus defining antigenic site HE-B. However, considering the results obtained using anti-HE-B MAbs in competitive ABAs, three subgroups of MAbs were tentatively identified. In each subgroup

Table 5. *Results of competitive ABAs between anti-S2 and anti-gp200 MAbs*

MAb competitor	Antigenic site	Labelled MAb I1	I22	J22	H3	H14	E5	H7	H19	I12
I1	S2-A	++*	+	+/−	+/−	F°†				
I22		++	+	+						
J22		+	+	+						
H3	S2-A′	+++	++	+++	+++					
H14	S2-B					+				
E5	gp200						+++	+++	+++	+++
H7							+++	+++	+++	+++
H19					+/−		+++	+++	+++	+++
I12							+++	+++	+++	+++

* The results of competition reactions are as presented in Table 3.

† The binding of the conjugated MAb was facilitated by the unlabelled MAb.

Table 6. *Epitope mapping of HE by competitive ABA*

MAb competitor	Epitope site	Labelled MAb B22	Y16	J18c	J17b	B4	B7	F13	G20*	A12*	J21*	J10	J18b	H17	B13
B22	A	+++†													
Y16			+	++	++	++	++	++	++	++	++	++	++	++	++
J18c				++	+	++	++	++	++	++	++	++	++	++	++
J17b	B1			++	+	+/−	++	++	++	++	++	++	++	++	++
B4				++	++	++	++	++	++	++	++	++	++	++	++
B7				+	+	+/−	++	++	++	++	++	++	++	++	++
F13								++	++	++	+	++	++	++	++
G20*	B2							+	++	++	++	+	++	F°‡	++
A12*								+	++	++	++	+	++	++	++
J21*									++	++	++	+	+	+	+
J10												++	+	+	
J18b	B3											++	++	++	++
H17												++	++	++	++
B13													+/−	+	+

* The MAbs were tested in the simultaneous (one-step) assay.

† The results of the competition reactions are as presented in Table 3.

‡ The binding of the conjugated MAb was facilitated by the unlabelled MAb.

Table 7. *Resistance to neutralization of escape mutants selected by anti-S1 MAbs*

Neutralizing MAb	Antigenic mutants										
	I7	I11	I9	J17a	G19	J18a	B5	A20	A9	Z17	C13
I7	++*	++	++		+						
I11	++	++	++	++	+						
I9	++	++	++	++	++	++					
J17a	++	++	++	++	++	++	++				
G19	++	++	++	++	++	++	++				
J18a					++	++	++	++			
B5							++	++			
A20							++	++			
A9									++		
Z17										++	
C13											++

* The resistance to neutralizing MAbs was rated as total, ++, when all mutants assayed were not neutralized by the selecting or related MAbs or as partial, +, when mutants showed a decreased sensitivity to the neutralizing MAb compared to the original BCV stock.

MAbs competed strongly and reciprocally. In addition, MAbs to HE-B1 prevented the binding of all the other MAbs to HE-B, whereas MAbs to HE-B2 could only prevent the binding of HE-B2 and -B3 MAbs. Strongly neutralizing MAbs were found directed against the HE-B1 (five of five) and -B2 (one of four) epitopes; inhibition of the AE activity was associated with MAbs at the same sites (HE-B1, one of five; -B2, two of four).

Selection and functional analysis of mutants resistant to neutralization by MAbs to S1 and HE

A library of mutants was selected; these were resistant to neutralization by 11 of 13 anti-S1 MAbs and two of six anti-HE MAbs. Mutant resistant to anti-S1 MAbs spontaneously arose from BCV stocks at frequencies ranging from 10^{-5} to $10^{-6.8}$ whereas the selection of escape mutants with anti-HE MAbs needed the use of a mutagenized virus stock (Delmas *et al.*, 1986) and yielded only two sets of mutants. Two to four mutants selected by each of the 11 anti-S1 MAbs were tested in a neutralization assay against all the selecting MAbs (Table 7). All MAbs mapping in subsite S1-B1 (I7, I11, I9 and J17a) selected mutants displaying a homogeneous pattern of reactivity to the four selecting MAbs and to MAb G19, with the exception of J17a mutants which were sensitive to neutralization by MAb I7 (Table 7). Resistance to neutralization by MAb J18a was a common feature of mutants selected by MAbs G19, J18a, B5 or A20; these mutants progressively lost their resistance to neutralization by MAbs I7 and I11 (G19 and J18a mutants), MAb I9 (B5 mutants), and MAbs J17a and G19 (A20 mutants); resistance to MAbs B5 and A20 appeared concomitantly in B5 and A20 mutants.

Mutants selected with MAbs Z17, A9 and C13 were resistant to their selecting MAb only. This functional analysis of mutants to anti-S1 MAbs confirmed the topographical studies, because (i) MAbs to site B selected mutants displaying cross-resistance, with the exception of Z17 and A9, (ii) between the pattern of resistance of mutants selected by MAbs to B1 (I7, I9, I11) and that of mutants selected by MAbs to B3 (A20) all intermediate profiles were found, suggesting a close interaction between the binding sites of these MAbs and (iii) MAbs delineating a unique epitope (S1-A, MAb C13) or a subsite which could be independent from the others affected by antigenic drift (S1-B4, MAb A9) selected mutants showing no cross-resistance.

The same set of experiments was performed by using the two escape mutants selected by anti-HE MAbs J17b and B7. The mutant selected by MAb JI7b was also resistant to four of six neutralizing anti-HE MAbs, the four neutralizing MAbs with equivalent or lower neutralization titres (Table 1), but was still neutralized by MAbs Y16 and J18c. Consequently MAb B7 also selected mutants which were resistant to neutralization by B7 and G20 but remained sensitive to neutralization by all the other neutralizing MAbs. Although partial, these results confirm the spatial organization of epitopes we described from competitive binding experiments.

Discussion

We have used a panel of 44 MAbs to extend our initial studies on the characterization of BCV peplomer glycoproteins, with a particular emphasis on the S subunits, S1 and S2. These MAbs were tested for their inhibitory activity toward viral replication, haemagglu-

tination and AE activity. The results of competitive ABAs were analysed and correlated, when possible, with results on the stability of binding sites to denaturing agents, the conservation of the antigenic determinants in coronavirus group 2 and the reactivity of MAbs with escape mutants.

The haemagglutinin (HE/gp65) elicited the production of MAbs binding to conformation-dependent epitopes on the mature dimeric protein. Considering the results of neutralization experiments, inhibition of haemagglutination and of AE activity, the anti-HE MAbs displayed some unique features.

The existence of neutralizing MAbs to HE has been reported (Deregt *et al.*, 1987), but we found a majority of non-neutralizing MAbs against HE in our panel and no correlation between their abilities to neutralize BCV and to inhibit haemagglutination. Furthermore the comparison with neutralizing anti-S1 MAbs revealed that the latter represented a higher percentage of the MAbs in the same class and displayed the highest neutralizing titres. The kinetics of neutralization confirmed these results, showing that anti-S1 MAbs neutralized BCV much more efficiently than did the anti-HE MAbs. Whether these differences are due to the existence of two mechanisms leading to viral inactivation remains to be studied.

As described for influenza C virus HE (Garcia-Sastre *et al.*, 1991), BCV HE cleaved a variety of synthetic substrates of defined M_r. We found that the inhibition of AE was strictly restricted to anti-HE MAbs, three of which inhibited the cleavage of 5-CFDA, whereas one of them (MAb A12) only inhibited AE as measured by the protection of pNPA. MAbs to influenza A virus neuraminidase and to NDV haemagglutinin–neuraminidase, the RDE of each virus, which inhibit the enzymatic degradation of neuraminlactose (M_r 633), are thought to interact with or near to the enzyme active centre; those which protect fetuin (M_r 48K) have been reported to act by steric hindrance (Jackson & Webster, 1982; Iorio & Bratt, 1984). Our results indicate that MAb A12, protecting the smaller substrate, pNPA, interacts with a critical site for AE activity without neutralizing the virus, unlike the chemical inhibitors which bind to the enzyme active centre and inhibit further viral replication (Vlasak *et al.*, 1988*a*). It should be noted that the three AE-inhibiting MAbs all displayed different characteristics of neutralization or HI, MAb G20 being the only one with a detectable neutralizing activity. Parker *et al.* (1990) observed a close association between the abilities of four MAbs to neutralize and to inhibit AE by using a different substrate to characterize AE [submaxillary mucin, M_r 400K (Downs & Pigman, 1969)]; these different assay conditions may well account for the discrepancies observed. MAbs to influenza C virus HE as well as polyclonal antisera to the virus did not protect synthetic substrates from AE, but did inhibit the receptor-destroying activity of the virus on chicken erythrocytes (Hachinoe *et al.*, 1989).

Competitive binding assays performed with 14 anti-HE MAbs allowed us to define two antigenic domains, HE-A defined by MAb B22 and HE-B defined by the 13 remaining MAbs. MAbs displaying the highest neutralizing titres competed for fixation with all the other MAbs to HE-B, indicating that they bound with a great avidity to a determinant consisting of several overlapping epitopes or induced allosteric changes at distant epitopes. The resistance to neutralization pattern of the two escape mutants was consistent with the results of competitive ABAs. Escape mutants resistant to anti-HE-B MAbs were selected with difficulty, as is reflected by their small number. Similar difficulties were reported by Delmas *et al.* (1986) for the selection of mutants resistant to neutralization by anti-S MAbs mapping in antigenic domain A, which could not be altered without a lethal effect on the virus. However, the existence of an important non-neutralized fraction might be the cause of the problems encountered. The data presented here strongly suggest that assembled epitopes within, or in close relationship with, antigenic domain HE-B are critical for rat RBC agglutination, AE activity and virus–cell interaction. Three overlapping antigenic domains representing four epitopes were defined on BCV E3(HE) by combining three different approaches, namely competitive ABAs, patterns of reactivity in RIPA and Western blotting (Deregt *et al.*, 1987). Interestingly, competitive ABAs with the four MAbs described by Deregt *et al.* (1987) showed that all MAbs bound to closely related epitopes, as described for HE-B MAbs.

The two S subunits of BCV were clearly identified by 25 MAbs, 19 binding to S1/gp105 and six to S2/gp95. A group of five MAbs was also isolated which recognized only the S precursor and a 200K glycoprotein. By using anti-S1 and anti-S2 MAbs to immunoprecipitate labelled virus proteins, we have shown that anti-S1 MAbs precipitate a unique band whereas anti-S2 MAbs react with S2 and S/gp200. The oligomerization of S has been well documented, both on cleaved (Vennema *et al.*, 1990) and uncleaved (Delmas & Laude, 1990) molecules and is proposed to depend on the S2 part of the protein (de Groot *et al.*, 1987). In this respect the presence of high M_r proteins precipitated by anti-S2 MAbs is not surprising and has been reported for anti-IBV S2 MAbs (Koch *et al.*, 1990). Whether gp200 is a S2 dimer or a full-length S molecule remains to be studied.

In contrast to HE, S1 elicited the production of a majority of MAbs displaying a strong neutralizing activity (Table 2 and Fig. 3). The S2 subunit was recognized by MAbs devoid of neutralizing activity, as detected in our test conditions, i.e. a plaque count

reduction assay performed on HRT-18 cells. These results are consistent with those previously published which reported that the major neutralization epitopes were located on the S1 of the IBV (Koch *et al.*, 1990), MHV (Gallagher *et al.*, 1990), TGEV (Delmas *et al.*, 1990; Correa *et al.*, 1990) and BCV (Yoo *et al.*, 1990) S molecule. However, neutralizing MAbs have been selected which reacted with a linear epitope on IBV (Kusters *et al.*, 1989) and MHV (Luytjes *et al.*, 1989) S2.

The 29 MAbs to the S glycoproteins were tested reciprocally as competitors and probes in competitive ABAs and defined four antigenic domains on S1, two on S2 and one on gp200. The 19 MAbs to S1 mapped in four independent domains (A to D), two of which (A and B) were delineated by neutralizing MAbs. Site A was defined by MAb C13 which interacted with no other MAbs in competitive ABAs, although showing impaired binding capacities after conjugation; MAb C13 recognized an SDS-resistant 2-ME-sensitive epitope and selected escape mutants which resisted neutralization only by MAb C13. A majority of MAbs, 12 neutralizing and three non-neutralizing, mapped in site B which was further subdivided on the basis of competitive ABAs. The greater stability of the B1 subsite to denaturing agents, and the resistance pattern of escape mutants selected by MAbs to subsites B2 and B3 confirmed the results from topographical studies. Furthermore the functional analysis showed that B4 could be modified independently of all other sites or subsites, thus confirming our previous observations (Vautherot *et al.*, 1983). The functional analysis also revealed that mutants selected by MAb Z17 had no linkage with any other mutant in site B. These discrepancies may be explained by the fact that mutants escape neutralization because of point mutations which alter binding sites in complex antigenic determinants made from several overlapping epitopes (Delmas *et al.*, 1990; Gallagher *et al.*, 1990).

Conjugation to a hapten yields a molecule which may have a spatial arrangement different from the original one, causing steric hindrance between MAbs binding to topographically separate epitopes (B. Delmas & H. Laude, personal communication). In this respect, we cannot exclude the possibility that B4 is an independent epitope interacting with B through an intermediate site delineated by MAbs J18a, E11 and non-neutralizing MAbs E19 and H13.

Site C was delineated by two MAbs, F7 and α8, one of which (F7) competed weakly but reciprocally with MAbs to B1 and B3 (competition levels up to 50%). We have cloned from a British BCV isolate two sets of mutants which differed only in their reactivity to MAbs F7 and α8; comparison of their sequences might help us to identify the amino acids critical for the conservation of antigenic site C. Site D was the only antigenic site on S1 resistant to denaturation and common to all haemagglutinating coronaviruses so far tested.

The spatial arrangement we present for S1 is more complex than that published previously (Deregt *et al.*, 1987), presumably because of the use of a larger panel of MAbs. The number of antigenic determinants delineated on S ranged from two on BCV (Deregt *et al.*, 1987), four on TGEV (Delmas *et al.*, 1986) to six on IBV (Koch *et al.*, 1990). Two neutralization epitopes have been delineated on BCV (Deregt *et al.*, 1987), MHV (Wege *et al.*, 1984) and TGEV (Delmas *et al.*, 1986), and four on IBV (Koch *et al.*, 1990). Yoo *et al.* (1991) recently located the binding site of neutralizing anti-BCV S1 MAbs to a domain between residues 324 and 720 in which the binding site could be made of two separate stretches of amino acids.

On S2 the six MAbs fell in two main groups, identifying two separate determinants, S2-A, a highly conserved linear epitope, and S2-B, a discontinuous epitope. MAb H3 appears to bind to a distinct epitope, S2-A′, close to, or interacting with, antigenic site S2-A. We have mapped the binding site of MAbs to S2-A and are verifying the immunogenicity of synthetic peptides analogous to this site.

It is noteworthy that on both S1 and HE we found non-neutralizing MAbs interacting in competitive ABAs with neutralizing MAbs. In the case of MAbs A9 and E13 all approaches confirmed the binding to a unique epitope of neutralizing MAb A9 and non-neutralizing MAb E13. By sequencing the neutralization-resistant mutants we aim to define more precisely the binding sites of our MAbs. It would be of great interest to elucidate whether the binding characteristics of individual MAbs might influence their neutralizing power.

We are indebted to René L'Haridon for his advice in fusion experiments. We gratefully acknowledge Dr H. Laude for critical comments. We thank Mrs A. M. Spite for typing this manuscript.

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(*Received 5 November 1991; Accepted 20 February 1992*)