TOPOLOGICAL AND FUNCTIONAL ANALYSIS OF EPITOPES ON THE S(E2) AND HE(E3) GLYCOPROTEINS OF BOVINE ENTERIC CORONAVIRUS

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SUMMARY

Monoclonal antibodies (Mabs) were selected which reacted with bovine enteric coronavirus S and HE. Mabs to S were used to identify 2 cleavage products of S, S/gp105 and S/gp90. Monoclonals to S/gp105 and HE neutralised the virus; only Mabs to the latter inhibited haemagglutination and acetyl-esterase activity. Topological distribution of epitopes was studied on these 3 glycoproteins by means of competition binding experiments. Two independent epitopes were characterised on HE, 4 on S/gp105, and 2 on S/gp90. Neutralising Mabs defined one major site on both S/gp105 and HE; however a minor neutralisation epitope was also delineated on S/gp105. Functional mapping using neutralisation-resistant mutants confirmed the topological distribution of epitopes on S/gp105.

INTRODUCTION

Bovine enteric coronavirus (BCV) is a well characterised enteric virus which causes enteritis in newborn calves (1), as well as chronic shedding (1) and/or acute diarrhoea in adult cows (2). BCV has 4 major structural proteins, a nucleoprotein {N}, a trans-membrane protein {M } and 2 N-glycosylated proteins, S and HE, which constitute the peplomers (3,4,5). S is a large complex protein, processed from an intracellular precursor (gp170) which is further glycosylated to yield a transient gp190 (5), which, in turn, is proteolytically cleaved into glycoproteins with Mr of 90 to 105 kD (5). One of the cleavage products, gp105 (6) or gp100/E2 (5) was reported to elicit neutralising antibodies (6-7). HE, the haemagglutinin (8-9), is a disulphide-linked dimer (3,4,5), also recognised by neutralising Mabs(7), and recently shown to be responsible for the receptor-destroying activity of BCV(10).

The aims of the present investigation were i)to assess the specificity of Mabs to the different glycoproteins, with a particular concern for the cleavage products of S, ii) to characterise the viral functions inhibited by these Mabs, iii) to present an epitope map of the different glycoproteins and compare these maps with the results of functional and antigenic analyses.

MATERIAL AND METHODS

Viruses and cell-lines

Isolation and characterisation of the different BCV isolates (11), as well as their cultivation (3,6,11), titration (12), and purification from infected HRT 18 cells (3) have been described.

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Production, purification (13), initial characterisation (6) of Mabs and their titration by neutralisation, ELISA, or haemagglutination-inhibition (HAI) (4,6,9,11), have also been reported.

Immune precipitation and Western Blotting

Isotopic labelling and immune precipitation of viral proteins were performed as previously described (6-14). For Western Blotting, virus proteins were first separated by PAGE (6), and transferred to a nitrocellulose sheet by transverse electrophoresis (15) in a Milliblot SDE electroblotting apparatus {Millipore} at 2,5 mA /cm^2 for 30 min. Mabs reacting with denatured virus proteins were detected using a sheep anti-mouse IgG conjugated to alkaline phosphatase {Biosys} and BCIP/NBT {Gibco-BRL} as substrate.

Selection of BCV mutants resistant to neutralisation by Mabs

The procedure followed was essentially similar to the one described for the selection of TGEV neutralisation escape mutants (14). Mutants resistant to neutralisation by their selecting Mab after 3 subcloning experiments were tested in a virus neutralisation test (V.N.T) using a panel of neutralising Mabs.

Labelling of monoclonal antibodies

Purified Mabs were either conjugated to horseradish peroxidase (HRPO), using the glutaraldehyde two-step method (16), or biotinylated (17).

ELISA for binding and competition binding of monoclonal antibodies

Binding assays for HRPO or biotin-labelled Mabs were performed in conditions that were similar to those of ELISA for unlabelled Mabs (11). Detection of biotinylated Mabs necessitated an additionnal step i.e. incubation with HRPO-labelled streptavidin {Amersham}, and 3 washs before incubation with the HRPO substrate { T.M.B Microwell Peroxidase System- Kirkegaard and Perry Lab.}.

Competition binding assays between Mabs were performed as described (14), except that detection of bound labelled Mabs was based on an enzymatic assay.

RESULTS

Specificity and biological functions of monoclonal antibodies

Biological functions relevant to each specificity were determined by testing Mabs in neutralisation, HAI and inhibition of acetyl-esterase assays.

Monoclonal antibodies to HE.

All Mabs to HE precipitated a 125 kD glycoprotein which was resolved in two monomers of 65 kD in the presence of reducing agents (fig. 1, lanes C and L). None of these Mabs recognised the denatured protein in Western Blotting. Six of the 14 anti-HE Mabs displayed a complement independent neutralising activity (Table 1). All neutralising and 6 non-neutralising Mabs inhibited haemagglutination of rat red blood cells (RRBC) by BCV (Table 1). Mabs were also tested in a rapid acetyl-esterase inhibition assay for their ability to protect a synthetic substrate of acetyl-esterase {para-nitrophenyl-acetate-PNPA; Aldrich} from cleavage by HE. Mab A12, a non-neutralising Mab, had a significant inhibitory effect on the enzymatic activity (data not shown).

Monoclonal antibodies to S.

Mabs to S were separated in two classes according to their immune precipitation pattern and reactivity in Western Blotting.

Mabs to S/gp105 precipitated gp105 together with its precursor gp170 from infected cell lysates (Fig.1, lanes A, B, and D), and gp105 alone from labelled virus disrupted with non-ionic detergents. Some Mabs of this specificity were also able to react with gp105 after denaturation with SDS alone (Mabs I16, P11, I7, I9, I11), or SDS and 2-ME (Mab I16).

Mabs to S/gp90 precipitated a 90 kD species together with the precursor gp170 and high Mr products from infected cell-lysates (Fig.1, lanes E,I,M). Four Mabs reacted with the denatured reduced gp90 in Western Blotting (data not shown). Expression in pUEx vector of a stretch of 109 amino-acids (aa) covering the N terminus part of S2 {carboxy-end cleavage fragment} and 28 aa at the C-terminus of S1 {Amino-end cleavage fragment}, yielded a ß-galactosidase fusion protein (P.Boireau and N. Woloszin, unpublished results) which was recognised by Mabs to S/gp90 (Table 1, subsite B2). A majority of Mabs to S/gp105 could neutralise BCV, but none of the anti S/gp90 had any effect on the viral replication in HRT 18 cells (Table 1).

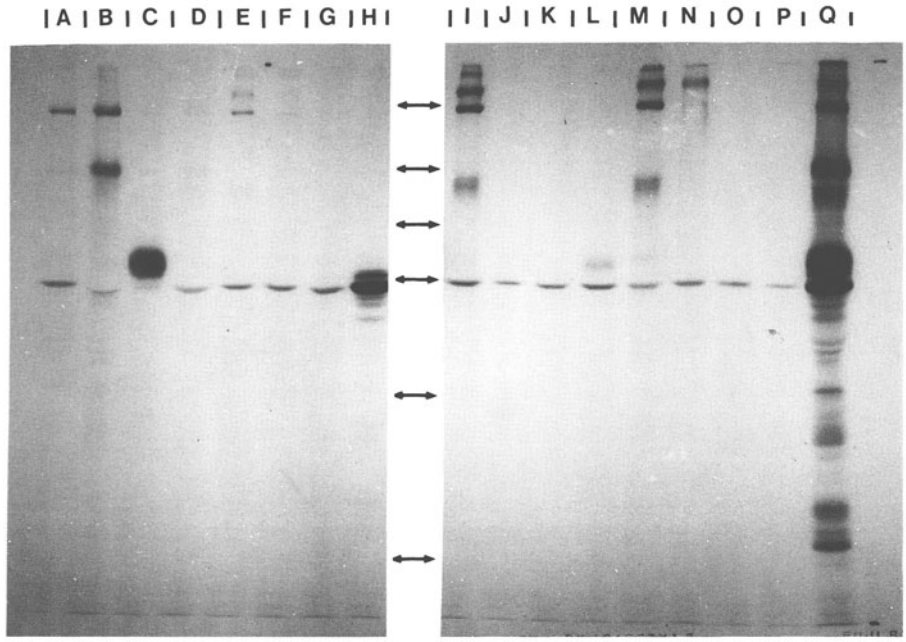


Fig. 1. PAGE analysis of ^{14}C labelled polypeptides immunoprecipitated from HRT 18 cells infected with BCV G110 isolate. In lanes A, B, D, Mabs C13, B5, and F15 precipitated gp170 and S/gp105. Lanes C and L, Mabs A12 and J10 reacted with gp65, the HE monomer. Mabs H3, J22, and G4, in lanes E, I, M respectively, precipitated S/gp90, gp170, and high Mr products.. Lane Q, precipitation of viral polypeptides by a rabbit hyperimmune antiserum. Molecular mass markers migrated to positions identified by double arrows, and were, from top to bottom, 170 kD, 97,4 kD, 68 kD, 55 kD, 36,5 kD, and 21,5 kD.

Delineation of epitopes

Competitive binding ELISA.

Labelling of Mabs had no major adverse effect on the antibody binding characteristics (except for 2 anti-S/gp90 Mabs), when binding curves of labelled and unlabelled Mabs were compared. A sequential competition binding assay against saturating amounts of homologous unlabelled Mab showed that the binding of a majority of Mabs was inhibited from 65 to 98%. Mabs which competed poorly in the sequential assay were retested in a simultanous assay and gave satisfactory results (self competition at 70 to 99%).

Table 1. Characterisation of monoclonal antibodies to S and HE

Specificity	Site or subsite	Number	Neutralisation	HAI	Western Blotting SDS	Western Blotting SDS/2ME	Antigenic analysis
HE(E3)	A1	6	+	+	–	–	BCV
	A2	7	–	+	–	–	BCV
	B	1	–	–	–	–	BCV
S/gp105	A	1	+	–	–	–	BCV
	B1	1	+	–	+	–	BCV
	B2	4	+	–	+	–	BCV
	B3	6	+	–	–	–	BCV
	B4	2	+[a]	–	–	–	BCV[b]
	C	2	–	–	–	–	BCV & OC43
	D	1	–	–	+	+	BCV, OC43, & HEV
S/gp90	A	2	–	–	–	–	BCV & HEV
	B1	1	–	–	–	–	BCV & MHV
	B2	4	–	–	+	+	BCV,OC43,HEV, &MHV

[a] Mab A9 only neutralised the virus
[b] NCDCV (Mebus isolate) was not recognised by B4 Mabs

On HE, 2 independent epitopes (A, B) were delineated using 14 biotin-labelled Mabs. Non reciprocal competitions between Mabs of site A (Table 1) were observed. These may reflect differences in Mab avidity as well as conformational modifications of HE upon binding with neutralising Mabs. Epitope B was unequivocally delineated by Mab B22 which was only self-competing and had no effect on the binding of the other Mabs.

On S/gp90, two epitopes were identified using 6 biotin-labelled Mabs. Epitope B was further subdivided according to the reactivity of Mabs to denatured S/gp90 (subgroup B1 and B2 in Table 1).

On S/gp105, 4 independent sites were defined by using 18 Mabs (Fig.2). Site A was defined by Mab C13, a neutralising monoclonal antibody. All other neutralising Mabs mapped in site B, which could also be further subdivided on the basis of competition binding results (Fig.2).

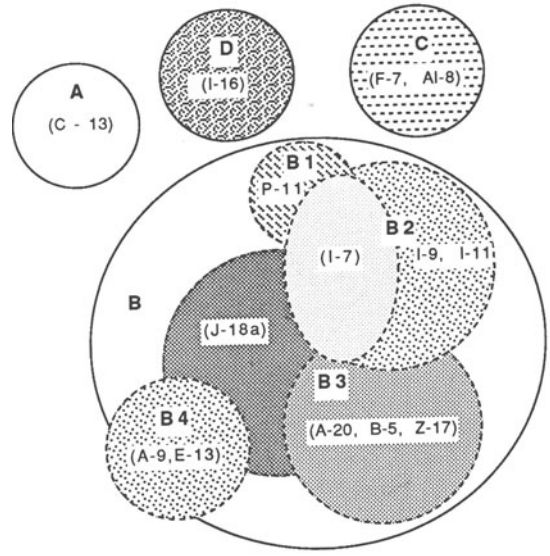


Fig. 2. Epitope map showing the 4 independent sites on S/gp105. Mabs delineating the different sites and subsites are indicated in brackets.

Subsite B1 was defined by Mab P11 which reciprocally competed by 40 to 50% with Mabs in subsite B2 (I7, I9, I11), but not with the other Mabs in site B.

Subsite B2 and B3 were differentiated on the basis of their reactivity to Mabs P11 and E11. Mabs of subsites B1 and B2 enhanced the fixation of labelled Mab E11, whereas Mabs of B3, together with E11 and J18a competed strongly with E11.

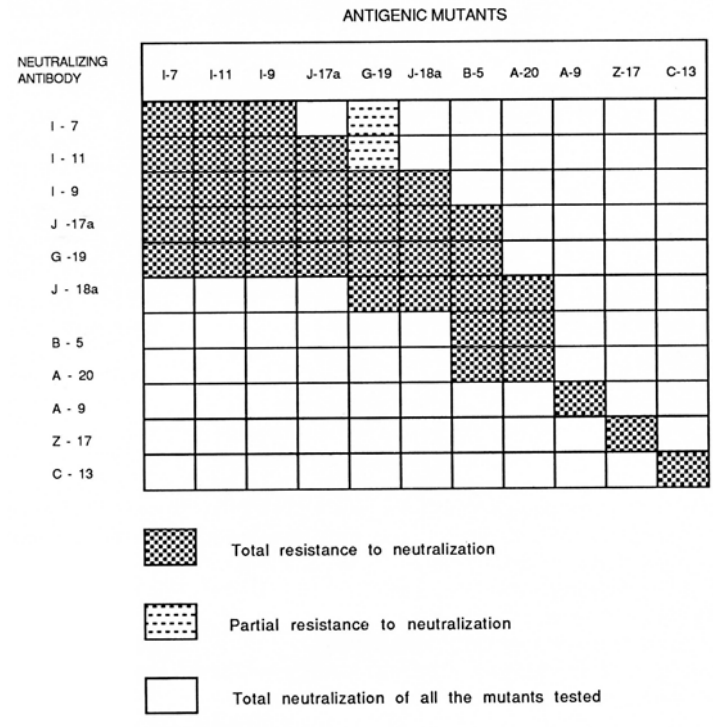
Subsite B4 was delineated by Mab A9 which competed reciprocally and strongly with Mab J18a only. Mab E13, a non-neutralising Mab, also mapped in this subsite, but did not reciprocally compete with Mab J18a.

Sites C and D were characterised as independent epitopes defined by non-neutralising Mabs F7 and Alfa8 (site C), and I16 (site D) (Fig.2).

Selection of neutralisation resistant mutants and functional mapping of S/gp105.

Neutralisation epitopes were further characterised by selecting a library of mutants that were resistant to neutralisation by anti S/gp105 Mab. These mutants were tested in a virus neutralisation test against anti-S/gp105 neutralising Mabs. Mutants which were selected by Mab I7, I11, I9, and J17a (subsite B2 as defined by competition binding experiments) were resistent to neutralisation by these Mabs. However some discrepancies were observed in the reactivity of J17a mutants which were neutralised by Mab I7 (Table 2). G19 mutants were only partially sensitive to neutralisation by Mabs I7 and I11 (Table 2), as both Mabs neutralised the 3 G19 mutants tested at a lower titre than the original virus (data not shown). Resistance to neutralisation by Mab J18a was the only common feature of mutants G19, J18a, B5, and A20, which progressively lost their resistance to Mab I9 (B5 mutants), G19 and J17a (A20 mutants), while acquiring resistance to Mabs B5 and A20. Mutants selected by Mabs A9, Z17, and C13 were found to resist only to their selecting Mab.

Table 2. Resistance to neutralisation of BCV mutants



Antigenic analysis

Monoclonal antibodies were tested in an indirest immunofluorescence assay (11) for their reactivity with different BCV isolates and OC43, HEV and MHV (Table 1).

Mabs to S/gp105 delineating sites A and B reacted with BCV specific determinants. Futhermore Mabs mapping in site B4 did not recognise the NCDCV isolate of BCV (11), thus confirming the results of competition binding experiments. Anti-site C and D Mabs reacted also with OC43 (Mab F7 and Alfa8, site C) or with OC43 and HEV (Mabl16, site D).

Mabs to S/gp90 reacted with antigenic determinants which were found on BCV and HEV (site A Mabs), BCV and MHV (subsite B1 Mabs), and all the coronaviruses tested in antigenic group 2(subsite B2) (Table 1).

Anti-HE Mabs reacted with all the BCV isolates so far tested, and Mabs Y16 and H17 also recognised HEV and OC43.

DISCUSSION

Bovine enteric coronavirus has two large envelope glycoproteins, S and HE, which form the viral spikes and interact with the cellular receptor(s), thus mediating the entry of the virion in the sensitive cells (10) and modulating the immune response (18).

Monoclonal antibodies reacting with S identified the 2 cleavage products of this glycoprotein, S/gp105 and S/gp90. By comparison between the Mr of the immuneprecipitation products and the molecular masses predicted from sequence data (P.Boireau et al, in the press),we suggest that S/gp105 corresponds to S1 (amino terminus of S)and S/gp90 to S2 (carboxyl terminus of S). Expression in a procaryotic vector of a set of overlapping fragments covering the entire S2 showed that the antibody binding region of Mab to S/gp90 (subsite B2) mapped in a stretch of 109 aa extending from position 741 to 850. As this fragment contains 28 aa of S1, additionnal work is needed to remove the 28 N terminal aa in order to ascertain that binding of these Mabs is restricted to the S2 subunit. The linear epitope defined by these antibodies appears to be different from the one delineated on the S2 of MHV (19); the latter extends from aa 848 to aa 856, corresponding to aa 899 to 907 on BCV S2 (P.Boireau , personnal communication). Work is in progress to map more precisely the antibody binding sites of Mabs defining linear epitopes on S1 and S2.

Virus neutralisation was mediated by anti-S1/gp105 and anti-HE Mabs, but HAI and inhibition of acetyl-esterase activity were displayed by anti-HE Mabs only. The finding that all neutralising epitopes so far delineated on the S glycoprotein of BCV presumably map in the S1 subunit is in good correlation with results obtained for the characterisation of neutralising Mabs against IBV (20), and antibody binding sites on TGEV S glycoprotein (Delmas et al-this volume).

Competition binding experiments enabled us to define 2 non-overlapping epitopes on HE, 4 on S1/gp105, and 2 on S2/gp90. Epitopes defined on HE were SDS sensitive and functional analysis did not allow further differentiation of site A Mabs. All neutralising Mabs to S1/gp105 mapped in two sites, A and B, whereas sites C and D were defined by non-neutralising Mabs. Site B could be subdivided according to the results of competition binding experiments. These results were in good correlation with the analysis of SDS resistance of epitopes, and antigenic relationships. Functional mapping of S1/gp105 confirmed the importance of site B and the differences between subsites B2 and B3. For S2/gp90 also, the combination of the results of competition binding experiments with the antigenic analysis and SDS resistance of epitopes allowed us to confirm the topological map and to subdivide site B in two subsites, B1 and B2.

Previous publications reported the existence of 2 independent neutralising epitopes on gp100/E2 (S1/gp105) and 4 overlapping epitopes on E3 (HE) (7). In this report we describe a more complex epitope distribution for S1/gp105. By using a large panel of Mabs in competition binding experiments and functional analysis, it was possible to define a major neutralisation site which could be subdivided in 4 , a minor neutralisation epitope, and 2 additional independent sites defined by non-neutralising Mabs.

Mapping of antibody binding sites will be indispensable in unambiguously identifying the amino-acid sequences recognised by neutralising Mabs on S and HE.

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