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Monoclonal antibodies differentiate between the haemagglutinating and the receptor-destroying activities of bovine coronavirus

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A relatively simple and sensitive method is described which enables the effect of monoclonal antibodies (MAbs) on the receptor-destroying enzyme (RDE) and the haemagglutination (HA) activity of bovine coronavirus (BCV) to be analysed in one assay. A lysate of HRT-18 cells infected with the L9 strain of BCV was found to have a higher RDE:HA ratio than purified virus. At 4 °C the lysate induced an HA pattern which completely disappeared upon raising of the temperature to 37 °C. This L9-infected cell lysate was used to determine the HA inhibition (HAI) titres of MAbs directed against the surface glycoproteins S and HE of BCV. Thereafter, the test plates were incubated at 37 °C to enable the ability of the MAbs to prevent elution of virus from BCV–erythrocyte complexes to be assessed. No inhibition of RDE was detectable with MAbs against glycoprotein S, which had HAI titres ranging from 1:16 to 1:128. On the other hand, MAbs directed against glycoprotein HE had similar HAI titres, but they inhibited elution of 8 HA units of BCV at titres of up to 1:65000.

Bovine coronavirus (BCV) is an enteropathogen which causes severe diarrhoea in neonatal calves (Mebus *et al.*, 1973) and which is implicated aetiologically in winter dysentery of adult cattle (Saif *et al.*, 1988). BCV represents one of the better characterized haemagglutinating coronaviruses. Four major structural proteins are associated with infectious BCV (King & Brian, 1982; Deregt *et al.*, 1987; St Cyr-Coats *et al.*, 1988), two of which are a phosphorylated nucleocapsid protein of M_r 50K to 54K and the integral membrane protein M, consisting of a family of glycoproteins of 23K to 26K.

King *et al.* (1985) identified a haemagglutinin with an approximate M_r of 62K in the reduced, and of 124K in the non-reduced form. This structural protein forms the short spikes of the viral envelope (Doughri *et al.*, 1976). Acetylesterase (AE) activity is associated with this glycoprotein, which is referred to as haemagglutinin–esterase (HE) (Vlasak *et al.*, 1988*a*; Cavanagh *et al.*, 1990). The enzyme is able to inactivate cellular receptors for BCV by hydrolysing an ester bond to release acetate from C-9 of sialic acid. The gene encoding HE is located upstream of the S gene, and encodes a protein of 424 amino acids (Parker *et al.*, 1989; Kienzle *et al.*, 1990).

The S glycoprotein is the third envelope-associated protein and forms the longer surface projections characteristic of BCV (Doughri *et al.*, 1976). The nucleotide sequence of the S gene of BCV encodes 1363 amino acids, with an N-terminal signal sequence and a transmembrane sequence near the C-terminal end. Cleavage of the S glycoprotein into proteins S1 and S2 is predicted to occur at an RRSRR or RRSVR sequence at positions 764 to 768 (Zhang *et al.*, 1991; Parker *et al.*, 1990; Boireau *et al.*, 1990; Abraham *et al.*, 1990). The N-terminal moiety is the S1 glycoprotein and that at the C terminus represents the S2 subunit (Spaan *et al.*, 1988). Cleavage of the S protein precursor is required for cell fusion activity (Storz *et al.*, 1981; Sturman *et al.*, 1985). The functions of the S protein include attachment to susceptible cells, fusion and induction of neutralizing antibodies, as shown by other coronaviruses.

BCV has been found to use *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) as a receptor for attachment to cells (Vlasak *et al.*, 1988*b*; Schultze *et al.*, 1990); HE is capable of binding to Neu5,9Ac$_2$-containing receptors (Schultze *et al.*, 1991*a*). Recently, evidence has shown that the S protein also recognizes Neu5,9Ac$_2$ as a receptor determinant and that it is a more potent haemagglutinin than HE (B. Schultze *et al.*, unpublished results).

The aim of our investigation was to determine the interactions of the receptor-binding and the receptor-destroying enzyme (RDE) activities of BCV with

chicken erythrocytes by using monoclonal antibodies (MAbs) against the S and HE glycoproteins, and to explore their capacity to prevent the viral AE from inactivating receptors on BCV–erythrocyte complexes.

The cell culture-adapted BCV prototype L9 was used (Mebus *et al.*, 1973; St Cyr-Coats *et al.*, 1988). HRT-18 cells were grown in Dulbecco's modified Earle's medium with 5% bovine foetal serum. Serum-free medium was used to propagate the virus in these cells and to determine the infectivity by plaque assay (Storz *et al.*, 1981). BCV was purified from the culture fluid of three 250 cm^2 culture flasks containing L9-infected cells as described (Wege *et al.*, 1979; Hussain *et al.*, 1991). The sediment of infected HRT-18 cells was suspended in 7·5 ml PBS, pH 7·4, sonicated three times for 15 s at a power setting of 4 and centrifuged at 3000 *g* for 30 min. The supernatant fluid is referred to as the L9-infected cell lysate. Uninfected HRT-18 cells were treated similarly and used as a control.

The haemagglutination (HA) test was done according to Herrler *et al.* (1985) employing a 0·5% suspension of adult chicken erythrocytes. The HA titre was determined after incubation for 1 h at 4 °C. Thereafter, the plates were incubated at 37 °C to determine the titre of the RDE activity. Viral AE is able to inactivate the receptors for BCV at this temperature. The BCV–erythrocyte aggregate disappeared in those dilutions of L9-infected cell lysate which contain a sufficient amount of RDE, and the red blood cells settle at the bottom of the well, as they do in controls incubated in the absence of haemagglutinating antigen. The RDE titre was determined as the reciprocal value of the highest dilution causing complete disappearance of the HA pattern. The activities of purified virus and the L9-infected cell lysate are compared in Table 1. Purified virus caused HA at a titre of 1024 and receptor inactivation was detectable at dilutions up to 1:32. The L9-infected cell lysate had an HA titre of 128, which disappeared completely after the temperature was raised to 37 °C, indicating that the RDE titre was at least 128. Lysates of uninfected HRT-18 cells did not react visibly with chicken erythrocytes. The infectivity of the purified virus and infected cell lysate was 7×10^7 and $1{\cdot}5 \times 10^6$ p.f.u./50 µl. One HA unit corresponds to $6{\cdot}8 \times 10^4$ and $1{\cdot}2 \times 10^4$ p.f.u., whereas 1 RDE unit is $2{\cdot}2 \times 10^6$ and $1{\cdot}2 \times 10^4$ p.f.u. of the respective preparations.

The L9-infected cell lysate was used to assess the effect of four MAbs directed against the S protein of BCV. The production and properties of these antibodies, as well as the preparation of ascites fluid, have been reported (Hussain *et al.*, 1991). MAbs 43, 44, 16 and 31 bound to the S protein in mildly denatured Western blots, but not after standard denaturation. They had ELISA titres as high as 10000. MAbs 43 and 44 neutralized strain L9 of BCV at titres exceeding 640. MAb S2/1, described by El-Ghorr *et al.* (1989), and MAbs S2/4 and S2/7 had identical properties. These MAbs, which are directed against HE of the S2 strain of BCV, were reactive in indirect immunofluorescence, HA inhibition (HAI) and neutralization tests. All three anti-HE MAbs competed fully in competitive ELISA.

Table 1. *Infectivity, AE, HA and RDE activities of purified BCV and L9-infected cell lysate*

	BCV L9		
Viral activity*	Purified virus	Infected cell lysate	Uninfected cell lysate
P.f.u.	7×10^7	$1{\cdot}5 \times 10^6$	NT†
AE‡	0·225	0·366	0·210
HA§	1024	128	<8
RDE‖	32	128	<8
HA:RDE	32:1	1:1	–
P.f.u./HA unit	$6{\cdot}8 \times 10^4$	$1{\cdot}2 \times 10^4$	–
P.f.u./RDE unit	$2{\cdot}2 \times 10^6$	$1{\cdot}2 \times 10^4$	–

* All BCV activities relate to 50 µl volumes, with the exception of the AE, which was determined with 5 µl.

† NT, Not tested.

‡ Absorbance at 405 nm after a 5 min reaction with 1 mM-*p*-nitrophenyl acetate.

§ Reciprocal value of highest dilution producing complete agglutination of chicken erythrocytes after 1 h.

‖ Reciprocal value of highest dilution producing complete disaggregaton of BCV–erythrocyte complexes after 1 h at 37 °C. The RDE titre may be higher for the L9-infected cell lysate.

The effect of these MAbs on the HA and RDE activity of the lysate, and on the HA activity of purified BCV is shown in Table 2. MAbs directed against the S protein (43, 44, 16 and 31) inhibited the HA activity exclusively. MAbs directed against HE (S2/1, S2/4 and S2/7) inhibited the HA activity of both purified virus and cell lysate. Additionally, the latter antibodies prevented the RDE of the lysate from dissolving the agglutination pattern. The RDE activity was inhibited at titres of 32768 and 65536, 256- to 512-fold higher than the HAI titre. Polyclonal serum 1745 and 10 sera from experimentally or naturally infected calves which neutralized BCV infectivity had HAI activity, whereas the potential for inhibiting RDE activity was below the limit of detection.

Several MAbs against HE have been tested previously and found to bind one overlapping and three distinct antigenic sites. The analysis of these MAbs did not include their potential for inhibiting the AE or RDE activity (Deregt & Babiuk, 1987; El-Ghorr *et al.*, 1989; Deregt *et al.*, 1989). The finding that MAbs against both S protein and HE are able to inhibit the HA activity might be interpreted to show that the combined action of both glycoproteins is required for agglutination of chicken erythrocytes or that they have a similar binding

Table 2. *Inhibition of the HA and RDE activities of purified BCV and L9-infected cell lysate by MAbs against the surface glycoproteins S and HE*

MAb	Western blot reaction with	L9-infected cell lysate		Purified BCV
		HAI*	RDEI†	HAI
43	S	128	<128	256
44	S	128	<128	512
16	S	128	<128	512
31	S	16	<16	64
S2/1	HE	128	65536	128
S2/4	HE	128	32768	128
S2/7	HE	128	32768	128
1745	S, HE, N and M	128	<128	128
Normal serum	–	<8	<8	<8

* Reciprocal value of highest MAb dilution inhibiting agglutination of chicken erythrocytes by BCV (8 HA units) after 1 h at 4 °C.

† Reciprocal value of highest MAb dilution inhibiting RDE of BCV. The effect of RDE was judged by the disaggregaton of BCV–erythrocyte complexes after incubaiton for 1 h at 37 °C, followed by incubation for 6 h at room temperature. RDEI titres below those of HAI were not detectable in this assay.

function. However, recent evidence has shown that isolated S protein is a very efficient haemagglutinin, whereas HE on virus particles is able to agglutinate rat and mouse erythrocytes, but not those of chickens (Schultze *et al.*, 1991 *a*, *b*). The HAI activity of the MAb against HE is probably produced by steric hindrance rather than specific inhibition of a receptor-binding protein. The S protein of non-haemagglutinating coronaviruses has a function in the attachment of virus to receptors of susceptible cells in the infectious process (Boyle *et al.*, 1987). The binding of the S protein of BCV to erythrocytes implies that S protein also attaches to cellular receptors during initiation of infection. The strong reactivity of MAbs with HE in the RDE test shows that activity is its predominant function. The role of AE in BCV infections has yet to be defined. RDE was inhibited only by anti-HE MAbs. The assay described here is highly sensitive, as indicated by the titres, and it should be useful for future analyses of the role of AE or RDE in the infectious process of haemagglutinating coronaviruses.

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