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Coronavirus IBV: Removal of Spike Glycopolypeptide S1 by Urea Abolishes Infectivity and Haemagglutination but Not Attachment to Cells

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SUMMARY

Urea has been used to remove the S1 spike glycopolypeptide from avian infectious bronchitis virus (IBV) strains M41 and Beaudette, without removing the S2 spike-anchoring glycopolypeptide. Reduction of the pH to 2·9 did not cause release of S1 although some S1 was released spontaneously from IBV Beaudette at pH 7·4. Virus that lacked S1 was no longer infectious or able to cause haemagglutination (HA). However, radiolabelled IBV that lacked S1 attached to erythrocytes and chick kidney cells to the same or similar extent as did intact virus. Treatment of IBV with a phospholipase C preparation, required to make IBV cause HA, did not increase binding of IBV to erythrocytes. The results indicate that while the attachment to cells of virus that lacks S1 is qualitatively different from that of intact virus, the decline in infectivity is the consequence of the loss of some other spike function.

Avian infectious bronchitis virus (IBV) causes economically important disease in the domestic fowl. Of the three structural proteins, one, the nucleocapsid (N) protein, is within the lumen of the virus formed by the virus membrane while the other two proteins, the membrane (M) and spike (S) glycoproteins, are partially exposed at the outer virus surface. Only a small, glycosylated portion of M protein appears to be exposed at the virion surface (Boursnell *et al.*, 1984; Cavanagh *et al.*, 1986*a*). In contrast, most of the S glycoprotein is exposed. S comprises two or three copies of each of two glycopolypeptides, S1 [mol. wt. about 90000 (90K)] and S2 (about 84K) derived by cleavage of a precursor glycopolypeptide (Stern & Sefton, 1982; Cavanagh *et al.*, 1986*b*). S1, but not S2, M or N proteins, can be removed by urea (Cavanagh, 1983*b*), which led to the proposal that S was anchored in the membrane by S2 while S1 might form the major part of the distal, bulbous end of S. This view was supported by the finding that of two monoclonal antibodies which neutralized IBV and inhibited haemagglutination (HA) both reacted with S1 (Mockett *et al.*, 1984). Also, nucleotide sequence analysis has shown that S2, which forms the carboxy (C)-terminal half of the precursor, has a C-terminal membrane-anchoring structure (Binns *et al.*, 1985). We predicted, therefore, that removal of S1 by urea would result in the loss of infectivity and HA activity, as a consequence of the failure of the virus to attach to cells. This paper describes observations and experiments to test these ideas.

IBV was radiolabelled with [^{35}S]methionine in de-embryonated chicken eggs (IBV strain M41, Cavanagh, 1981) or chick kidney (CK) cells (IBV strain Beaudette; Stern *et al.*, 1982) and unlabelled virus was grown in embryonated eggs (Cavanagh, 1983*a*). Virus lacking S1 was produced by incubating IBV with various concentrations of urea for 1 h at 37 °C (Cavanagh, 1983*b*). The virus particles were then sedimented through 25% (w/w) sucrose onto a 55% sucrose pad using a 6 × 14 ml swing-out rotor (MSE) at 90000 g_{max} for 3 h at 20 °C. No S1 was detected in these preparations after analysis of samples by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Virus infectivity was titrated in chicken tracheal organ cultures (IBV M41; Darbyshire *et al.*, 1976) or by plaque assay in CK cells (IBV Beaudette; Stern *et al.*, 1982) and HA was assayed in microtitre plates (Mockett & Darbyshire, 1981). SDS–PAGE was performed in 10% slab gels and polypeptides were located by fluorography (Cavanagh, 1983*b*). For attachment experiments bovine serum albumin (BSA) was added (1 mg/ml) to [^{35}S]methionine-

Fig. 1. SDS–PAGE of polypeptides, mostly S1, released after incubation of [^{35}S]methionine-labelled IBV Beaudette with (*a*) no urea, (*b*) 4 M-urea and (*c*) 6 M-urea followed by sedimentation of the virus particles.

labelled virus which was then dialysed against phosphate-buffered saline (PBS) containing 0·5 mM-calcium chloride and 2·0 mM-magnesium chloride. CK cell monolayers in both 60 mm diam. plastic dishes and in Linbro plates were used. The cells were washed three times with PBS and were then incubated with 150 µl of virus at 37 °C for various times. The cells were then washed, scraped off the plastic, pelleted, and digested overnight at room temperature with 0·5 ml of a 9:1 solution of NCS tissue solubilizer (Amersham) in water prior to addition of scintillant. For attachment to erythrocytes freshly collected cells were washed in PBS and 0·2 ml vol. of cells incubated at 37 °C with an equal volume of virus in microcentrifuge tubes. The cells were then washed twice prior to digestion. For erythrocyte concentrations of less than 10^8 per ml the cells were resuspended in 20 µl water and digested with 250 µl NCS overnight at room temperature. For cells at $2{\cdot}6 \times 10^9$ per ml digestion was with 12 ml of a 1:1 solution of Soluene-350 (Packard) and isopropanol. After 1 h at 40 °C and overnight at room temperature the digest was divided into 1·5 ml aliquots and incubated at 40 °C for 10 min with 500 µl hydrogen peroxide (100 vol.). To this was added 15 ml of a scintillation cocktail containing 9 parts Instagel (Packard) and 0·5 M-HCl for 2 h at room temperature prior to the estimation of radioactivity. Lower concentrations of erythrocytes required less Soluene-350 for digestion.

Stern & Sefton (1982) reported that when newly synthesized IBV Beaudette was incubated in cell culture medium at 37 °C for 18 h most of the S1 was absent from the virus after pelleting. While we were unable to reproduce this finding we did observe that, following a 1 h incubation, a small amount of S1 was released from IBV Beaudette which was acting as a control during a urea-treatment experiment (Fig. 1). In another experiment IBV M41 was incubated at 37 °C for 10 min in NET buffer (100 mM-NaCl, 1 mM-EDTA, 10 mM-Tris–HCl pH 7·2) adjusted to several pHs down to pH 2·9 using acetic acid. After pelleting through 25% sucrose in NET buffer at 20 °C the pellets were resuspended, the amount of radiolabel determined by scintillation counting and the polypeptides analysed by SDS–PAGE. There was no significant difference in the amounts of virus that pelleted and there were no differences in polypeptide composition.

Treatment with urea abolished infectivity (Table 1) and HA (Table 2). It was considered likely that the loss of these two properties was a consequence of the inability of the virus without S1 to attach to cells. However, this proved not to be the case. Preliminary experiments showed that intact IBV did attach to erythrocytes. The percentage of virus that attached varied among experiments but the amount that attached was generally low, even when cell concentrations as high as 10^9 per ml or greater were used (Fig. 2; Table 3). In the experiment of Fig. 2, the unattached IBV M41 from the five sets of samples were re-exposed to five further sets of erythrocytes at 1×10^9 per ml for 30 min at 37 °C. Between 27 and 40% of the virus attached. This emphasized that the attachment of IBV to chicken erythrocytes was inefficient.

Virus which had had S1 removed attached to erythrocytes almost to the same extent as did intact virus; the decrease in attachment was never more than 50% (Table 3). The same observation was made at high (260×10^7 cells/ml) and low (3×10^7 cells/ml), the latter being the approximate concentration of cells used in the HA assay. IBV Beaudette was used to examine the attachment of virus to monolayers of CK cells, since this strain of virus replicates well in these cells. The extent of attachment of intact virus was never more than 20%. Removal of S1

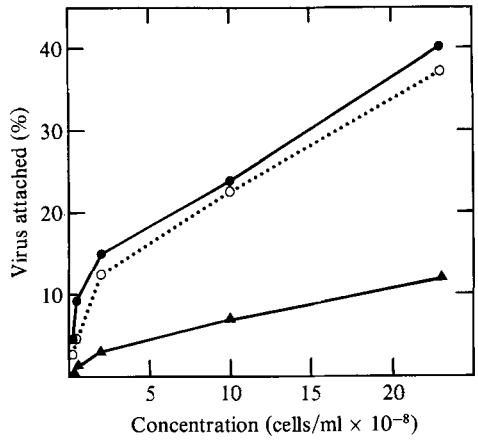

Fig. 2. Attachment of [^{35}S]methionine-labelled IBV strains M41, Connecticut and Beaudette to different concentrations of erythrocytes. After incubation of the virus and cells for 30 min at 37°C the cells were washed, solubilized and the associated radioactivity determined. ●, M41; ○, M41, a second preparation; ▲, Beaudette.

Table 1. *Effect of urea on the infectivity of IBV*

Expt. no.	IBV strain	Urea concn. (M)	(4) Infectivity titre (log_{10}/ml)*	(5) Amount of virus d.p.m./ml (× 10^{-3})	(5) Amount of virus A_{260}	Specific infectivity (4) ÷ (5)	Specific infectivity % decrease
1	M41	0	6·2	113	–	14·0	–
		2	3·5	122	–	<0·03	99·8
		6	<2·8	125	–	<0·01	>99·9
2	M41	0	7·2	–	2·7	5·8 × 10^6	–
		3	<2·9	–	4·6	<1·7 × 10^2	>99·9
3	Beaudette	0	4·8	203	–	0·3	–
		6	<3·0	155	–	<0·01	>98
4	Beaudette	0	4·9	375	–	0·2	–
		6	<3·0	265	–	<0·01	>98

* IBV M41 was titrated in organ cultures (CD_{50}/ml) whereas IBV Beaudette was titrated in CK cells (p.f.u./ml).

Table 2. *HA titres of intact and urea-treated IBV M41 with and without phospholipase C treatment*

Expt. no.	Phospholipase C treatment	log_2 HA titre Intact virus	log_2 HA titre Urea-treated virus
1	–	<1	2
	+	5	3
2	–	<1	<1
	+	8	<1
3*	–	3	2
	+	8	3

* In this experiment two additional controls were included; buffer alone gave an HA titre of 1 log_2 whereas buffer plus phospholipase C (5 units/ml, as used to treat virus) without any virus gave a titre of 2 log_2. HA titres of 3 log_2 or less are considered negative.

Table 3. *Effect of the removal of S1 by urea on the capacity of IBV to attach to red blood cells (RBC) and CK cells*

Expt. no.	IBV strain	Urea treatment	RBC concn. ($\times 10^{-7}$)	CK monolayers	Virus attachment (%) at 37 °C (after min) 5	15	30	60	120
1	M41	–	260	–	28	–	30	–	–
		+	260	–	26	–	28	–	–
	Beaudette	–	260	–	37	–	39	–	–
		+	260	–	20	–	23	–	–
	M41	–	260	–	44	–	81	–	–
		+	260	–	42	–	54	–	–
2	M41	–	3	–	–	20	22	25	
		+	3	–	–	20	19	20	–
	Beaudette	–	3	–	–	22	22	23	–
		+	3	–	–	12	13	14	–
3	Beaudette	–	–	+	–	–	10	12	14
		+	–	+	–	–	4	7	10
4	Beaudette	–	–	+	–	–	3	4	7
		+	–	+	–	–	1	2	3

resulted in a decrease in virus attachment but the extent was still approximately 30 to 60% of that of the intact virus (Table 3).

Most strains of IBV agglutinate erythrocytes undetectably unless the virus has been concentrated and then incubated with semi-purified preparations of phospholipase C (Bingham *et al.*, 1975; Alexander & Chettle, 1977). This is illustrated in Table 2 for IBV M41. On another occasion, two semi-purified preparations of IBV M41 each had HA titres of 1 $\log_2$ and 10 $\log_2$ before and after phospholipase C treatment respectively. To see if phospholipase C treatment increased the attachment of IBV M41 to erythrocytes, radiolabelled virus with and without phospholipase C treatment was incubated for 90 min with 1×10^7, 3×10^7 and 9×10^7 cells/ml. There was no difference between the extent of attachment of the control virus and the phospholipase C-treated virus (data not shown).

Our observation that some S1 was lost spontaneously from IBV Beaudette, although not to the same extent as described by Stern & Sefton (1982), shows that the forces that keep S1 and S2 together are not strong. Since S1 and S2 are not held together by disulphide bonds (Cavanagh, 1983*b*), and as the spike proteins of several viruses do not induce membrane fusion unless the pH is reduced to about 5 or 6 to cause a conformational change in the spike protein (White *et al.*, 1983) we wondered if S1 might be released from the virus by decreasing the pH. However, there was no change in the polypeptide composition of the virus even when the pH had been reduced to 2·9.

Two monoclonal antibodies which neutralize IBV and inhibit HA have been shown to be specific for S1 of IBV M41 (Mockett *et al.*, 1984). As anticipated, virus from which S1 had been removed by urea was no longer infectious or able to agglutinate erythrocytes. Unexpectedly, however, this was not a consequence of a reduction in the extent to which the virus attached to cells since this was affected little or not at all. Thus, our results show that failure of a virus to cause HA does not necessarily mean that the virus has not attached to the erythrocytes. Similarly, attachment alone is not sufficient to cause HA. Also, in the case of IBV, since virus lacking S1 was able to attach to cells this indicates that attachment can be mediated by other molecules at the virion surface, e.g. the S2 and/or the M glycopolypeptides. Observations made with other viruses relate to this. Addition of sialic acid to the haemagglutinin protein of influenza virus destroyed HA activity but not the capacity to attach to cells or infectivity (Lakshmi & Schulze, 1978). Proteolytic removal of the glycoproteins of lymphocytic choriomeningitis virus did not decrease infectivity (Bruns & Lehmann-Grube, 1984). Rous sarcoma virus (RSV) from which the spikes had been removed enzymically had a 40-fold

decreased infectivity but only a twofold reduction in cell attachment (Notter *et al.*, 1982). An RSV mutant which lacked spikes was still able to attach to cells to an extent 20% of that of the parent vitus (Notter *et al.*, 1982). A mutant of Sendai virus which lacked the haemagglutinin–neuraminidase protein was unable to infect conventional host cells which contained gangliosides (sialoglycolipids) known to act as receptors for the parent virus (Markwell *et al.*, 1985). However, the fusion protein, not normally considered to be a cell attachment protein, was able to mediate attachment of the mutant to Hep G2 cells which contain a lectin able to bind asialoglycoproteins containing glycans with terminal galactose or *N*-acetylgalactosamine residues. Lastly, although the attachment of influenza virus is normally mediated by the haemagglutinin protein the neuraminidase spike of an N9 subtype avian influenza virus has HA activity (Laver *et al.*, 1984). In view of these reports it is perhaps not surprising that IBV can attach to cells in the absence of S1, although in intact virus this may be the primary cell attachment protein.

Despite the attachment of IBV without S1 to cells the virus was not infectious. One possible explanation is that the virus was no longer able to fuse with cell membranes in order to release the RNA genome into the cytoplasm, a prerequisite of replication. That the spike protein is necessary for membrane fusion has been shown by Sturman & Holmes (1983) for murine hepatitis coronavirus. Whether fusion induction is primarily a property of S1 or of S2 is unknown. Although it is tempting to speculate that fusion is induced by S1, on the basis that virus which lacks S1 is not infectious, it has to be borne in mind that the configuration of S2 will undoubtedly have been changed to some degree as a consequence of the absence of S1 in addition to possible changes induced irreversibly by urea.

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