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Coronavirus IBV: Virus Retaining Spike Glycopolypeptide S2 but Not S1 Is Unable to Induce Virus-neutralizing or Haemagglutination-inhibiting Antibody, or Induce Chicken Tracheal Protection

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SUMMARY

Avian infectious bronchitis coronavirus (IBV) inactivated by β-propiolactone induced partial protection of the trachea in up to 40% of chickens following one intramuscular inoculation 4 to 6 weeks prior to challenge. Retention of an intact tracheal ciliated epithelium 4 days after challenge was the criterion of protection. There was no correlation between protection and serum titres of virus-neutralizing (VN) and haemagglutination-inhibiting (HI) antibody, which were maximal at about 4 weeks after inoculation. Virus from which the S1 but not the S2 (spike-anchoring) spike glycopolypeptide had been removed by urea did not induce protection or VN or HI antibody. Four intramuscular inoculations of monomeric S1 induced VN and HI antibody in two and four chickens respectively. These results indicate that VN and HI antibodies are induced primarily by S1, that intact spikes are a major requirement for the induction of protective immunity and that this property is probably associated with S1.

INTRODUCTION

Apart from the internally situated nucleocapsid (N) protein, avian infectious bronchitis coronavirus (IBV) has two other proteins. One of these, the membrane or matrix (M) glycoprotein appears to have only a small part protruding at the outer surface of the virus membrane (Cavanagh *et al.*, 1986*a*). In contrast, the peplomer or spike (S) glycoprotein has most of its mass externally exposed. S is an oligomeric protein comprising two or three copies of each of two glycopolypeptides, S1 [mol. wt. about 90000 (90K)] and S2 (about 84K; Cavanagh, 1983*a*, *b*), derived by cleavage of a precursor glycopolypeptide, So (Stern & Sefton, 1982; Cavanagh *et al.*, 1986*b*). Purified S but not M or N induced both virus-neutralizing (VN) and haemagglutination-inhibiting (HI) antibodies in chickens (Cavanagh *et al.*, 1984). The finding that S1 but not S2 could be removed from virions by urea led to the proposal that S2 served to anchor S in the membrane while S1 might form the major part of the bulbous distal end of S (Cavanagh, 1983*b*). This conclusion was strengthened by the discovery, following nucleotide sequencing, that the order of S1 and S2 within So is amino (N)-terminus–S1–S2–carboxy (C)-terminus, that S1 has a signal sequence and that the C-terminus of S2 has a 44 residue hydrophobic sequence and a 26 residue hydrophilic tail similar to the anchor sequences of other integral membrane proteins (Binns *et al.*, 1985). The purpose of the work described herein was twofold: firstly, to determine which of S1 and S2 induces serum VN and HI antibody, and secondly, to ascertain if intact S is required for the induction of protective immunity in the chicken trachea.

METHODS

Virus preparation. The M41 strain of IBV was used throughout. Virus was grown in embryonated eggs and purified in sucrose gradients as described by Cavanagh (1983*b*). Prior to inoculation of chickens the virus was inactivated by two additions of 0·05% β-propiolactone (Sigma), with incubation at 37 °C for 5 h after each addition.

Removal of S1 by urea. Virus from sucrose gradients in NET buffer (100 mM-NaCl, 1 mM-EDTA, 10 mM-Tris–HCl pH 7·2) was incubated for 1 h at 37 °C with 6 M-urea. After a twofold dilution the virus was loaded into a centrifuge tube containing 25% and 55% (w/w) sucrose in NET buffer and centrifuged at 90000 g_{max} for 3 h at

20 °C in a 6 × 38 ml swing-out rotor (MSE). The virus, without S1, was at the 25/55% sucrose interface. The supernatant fluid containing the S1 was dialysed against 5 mM-Tris–HCl pH 7·2 containing urea at 1 M (8 h), 0·5 M (16 h), 0·25 M (8 h) and no urea (20 h). The material was then lyophilized and dissolved in water to give a 20-fold concentration. The S1 was then centrifuged as described above, at 4 °C; this depleted the amount of N protein which contaminated the S1. The S1 in the supernatant was used for inoculation into chickens and for estimation of the mol. wt. of S1.

Immunization of chickens. Antigen in NET buffer was emulsified with an equal volume of Freund's complete adjuvant (Difco). A 0·5 ml amount was inoculated into each leg of specified pathogen-free Rhode Island Red chickens at 7 weeks of age. Convalescent serum was obtained from chickens which had been infected with IBV strain M41 and bled 4 weeks later. An antiserum specific for S2 was produced by grinding polyacrylamide gel which contained S2, emulsifying with Freund's complete adjuvant and inoculating chickens intramuscularly four times over 5 months.

Determination of tracheal protection. Chickens were challenged by intratracheal inoculation of 0·2 ml NET buffer containing 500 median ciliostatic doses (CD_{50}) of infectious virus. Four days later the chickens were killed and the tracheae removed. Virus was isolated either by swabs (Darbyshire & Peters, 1984) or from weighed pieces of trachea (Darbyshire, 1985). For histology, pieces of trachea were fixed in formal sublimate, sectioned and stained with haematoxylin and eosin. Several sections from different parts of the trachea were examined. Tracheae were scored as exhibiting protection when 50% or more of the epithelium was intact. Other pieces of trachea were cut into rings and 10 rings observed by low power microscopy (Darbyshire, 1980). Ciliary activity associated with 50% or more of the epithelial cells was arbitrarily chosen as being indicative of protection.

Assays. Virus infectivity and VN were assayed in tracheal organ cultures (Darbyshire *et al.*, 1979). Titres were expressed as $\log_{10} CD_{50}$. The HI test was performed in round-bottomed microtitre plates (Sterilin) as described by Mockett & Darbyshire (1981). For the ELISA, virus, suitably diluted in 50 mM-carbonate buffer pH 9·6, was used to coat flat-bottomed Dynatech disposable microelisa plates at 4 °C overnight. After washing in 0·5 M-NaCl containing 0·1% Tween 20 the plates were blocked by incubation at 37 °C for 30 min with a 5% solution of skimmed milk powder (Marvel) in carbonate buffer. After washing, twofold dilutions of serum, starting at 1/100, in 0·5 M-NaCl, 5% Marvel and 0·1% Tween 20 were added for 60 min at 37 °C. After washing, a 1/1000 dilution of rabbit anti-chicken IgG serum (Nordic) conjugated to horseradish peroxidase (RaChIgG–PO) was added for 1 h at 37 °C. A solution containing 100 mM-sodium acetate, 50 mM-sodium dihydrogen phosphate, 2·5 mM-hydrogen peroxide and 2 mM-2,2′-azino-bis(3-ethylbenzthiazolinesulphonic acid) was added after washing. After development of the green colour at 37 °C the reaction was stopped by addition of an equal volume of 10% SDS and the absorbance at 405 nm was measured. Protein was assayed according to the procedure of Schaffner & Weissman (1973).

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Polypeptides were analysed in 10% or 5 to 10% acrylamide slab gels and silver-stained (Cavanagh, 1983*b*).

Mol. wt. estimation of S1 released by urea. S1 was co-sedimented with bovine catalase through 5 to 20% (w/v) sucrose gradients, the position of the polypeptides was determined by SDS–PAGE after fractionation of the gradients and the mol. wt. of S1 was calculated (Cavanagh, 1983*b*).

Western immunoblotting. Viral polypeptides were separated in 5 to 10% acrylamide gels and then soaked for 30 min in a solution containing 20 mM-Tris and 192 mM-glycine pH 8·3. The polypeptides were then transferred to 0·45 μM-nitrocellulose (NC) (Schleicher & Schüll) using a Bio-Rad Trans-Blot cell system and Bio-Rad power supply, model 160/1.6. The buffer was 20 mM-Tris, 192 mM-glycine, 20% methanol, 0·1% SDS, pH 8·3 and electrophoresis was for 16 h at 30 V and 0·1 A. The buffer was then removed, water added and discarded and fresh buffer without SDS added and electrophoresis continued for 2 h. The NC was submerged in Ponceau S (Helena Laboratories, Texas, U.S.A.) for 5 min to stain the polypeptides, rinsed in water and cut into strips. The NC was blocked with 0·5% Brij 58 (polyoxyethylene 20 cetyl ether; Sigma) for 3 h at 37 °C with agitation. The blocking solution was then removed and replaced with a 1/100 dilution of serum in NET buffer plus 0·1% Brij 58. After overnight incubation at 4 °C with agitation the strips were washed with several changes of NET buffer for 1 h. RaChIgG–PO, diluted 1/1000 in NET, was then added for 3 h at 37 °C. After washing thoroughly the enzyme substrate was added; this was made by adding 1 ml of a 3 mg/ml solution of 4-chloro-1-naphthol (Sigma) in methanol to 5 ml NET plus hydrogen peroxide at 10 mM. After development of the blue colour the NC was washed with water and then stored dry in the dark.

RESULTS

Removal of S1 by urea

IBV which had been incubated with 6 M-urea lacked S1 (Fig. 1*a*, *b*). The supernatant recovered after sedimentation of the urea-treated virus contained predominantly S1, there being a small amount of N detected in some preparations (Fig. 1*c*).

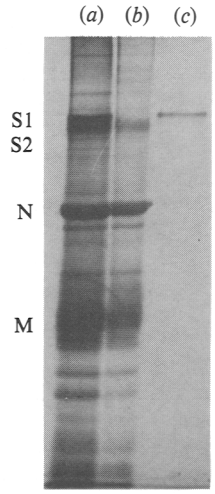

Fig. 1

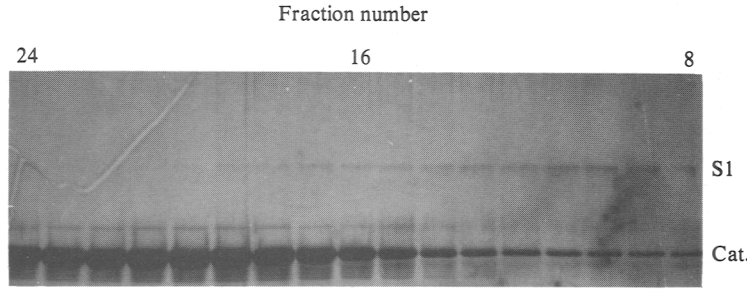

Fig. 2

Fig. 1. SDS-PAGE of IBV strain M41 (*a*) before and (*b*) after incubation with 6 M-urea and pelleting. The polypeptides that were released by the urea and which did not pellet are shown in (*c*). The gel contained 10% acrylamide and was silver-stained.

Fig. 2. Molecular weight estimation of S1 released from virions by urea. Virus was incubated with 6 M-urea and then pelleted. The unpelleted S1 was dialysed to remove urea and then co-sedimented with bovine catalase (Cat.) in a 5 to 20% sucrose gradient. After fractionation the polypeptides in each fraction were identified by SDS-PAGE in a gel containing 10% acrylamide. The polypeptides were visualized by silver staining. Sedimentation was from right to left.

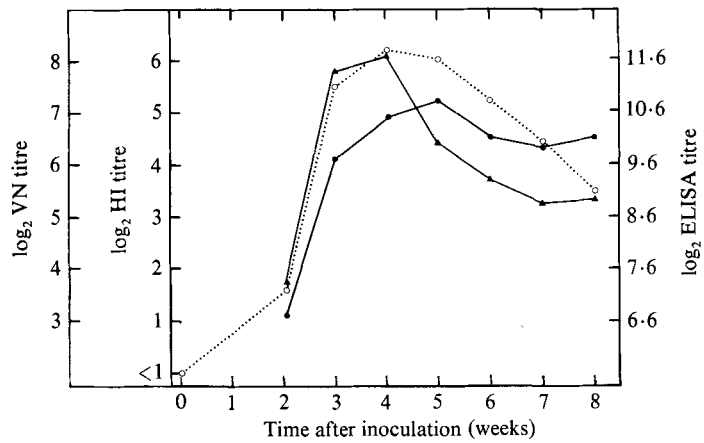

Fig. 3. Titres of anti-IBV serum antibody following the intramuscular inoculation of chickens with 3 μg of inactivated IBV with Freund's complete adjuvant. ●, VN assay; ○, HI assay; ▲, ELISA.

Molecular weight of S1 released by urea

Intact S contains two or three copies of S1, deduced after co-sedimentation of purified S with catalase (mol. wt. 260K) (Cavanagh, 1983*b*). After co-sedimentation of urea-released S1 with catalase (Fig. 2) it was subsequently calculated, in two experiments, that the S1 had a mol. wt. of 82K and 85K. Given that the mol. wt. of fully denatured S1 in SDS–polyacrylamide gels is about 90K this indicates that the S1 released by urea is monomeric.

Humoral antibody response to inoculation with inactivated IBV

VN, HI and ELISA titres of serum antibody to IBV were maximal at about 4 weeks after intramuscular inoculation with 3 μg of inactivated IBV (Fig. 3). By 8 weeks the HI and ELISA titres had decreased by about 3 $\log_2$ whereas the VN titres had decreased only about 1 $\log_2$.

Table 1. *Induction of VN and HI antibody and tracheal protection by one intramuscular inoculation of inactivated IBV*

Expt. no.	Virus inoculum (μg)	Serum antibody (log_2)* VN	HI	Virus not isolated†‡	Tracheal cilia beating†	Tracheal epithelium intact†	Tracheal protection indicated†
1	5	4	4	+	–	ND§	–
	5	5	7	+	+	ND	+
	5	9	9	–	–	ND	–
	5	4	5	–	–	ND	–
	25	6	5	+	+	ND	+
	25	5	6	+	+	ND	+
	25	8	8	–	–	ND	–
	100	9	7	–	–	ND	–
	100	8	7	+	+	ND	+
	100	6	5	–	–	ND	–
	100	5	6	–	–	ND	–
2	10	5	3	–	–	–	–
	10	5	6	+	–	–	–
	10	8	9	+	–	+	+
	10	7	7	+	–	–	–
	10	8	8	+	+	+	+
	10	7	8	–	–	–	–
	10	8	9	+	+	+	+
	10	8	8	–	–	–	–
	10	6	9	+	–	–	–
	10	7	7	–	–	–	–
	10	8	9	+	+	–	–
	10	9	10	+	+	+	+
	10	9	7	+	–	–	–
	10	8	10	+	–	–	–
	10	5	6	+	–	–	–
	10	7	8	+	–	–	–
	10	8	8	+	+	+	+
	None	3	3	–	–	–	–
	None	3	3	–	–	–	–
	None	3	3	–	–	–	–
	None	3	3	–	–	–	–
	None	3	2	–	–	–	–
	None	3	2	–	–	–	–
	None	3	0	–	–	–	–
	None	3	0	–	–	–	–

* Serum was collected 4 weeks after inoculation. Each horizontal line contains data from an individual chicken.
† Chickens were challenged 7 weeks after inoculation and tracheae removed 4 days later.
‡ Virus isolation was attempted by swabbing the removed trachaea.
§ ND, Not done.

Induction of a protective immune response by inactivated IBV

To assess protection of the trachea following live vaccination, Darbyshire (1980) challenged chickens at 3 weeks after vaccination, removed the tracheae 3 or 6 days later and observed ciliary activity of tracheal rings. Retention of ciliary activity was taken as evidence that the trachea was protected against IBV infection. Conclusions with regard to protection based on the histological observation of an intact ciliated epithelium correlated well with deductions made by observation of ciliary motility (Darbyshire & Peters, 1984). In this communication, we have used both criteria by which to judge whether inactivated virus had induced protection of the trachea. Only tracheal rings and sections which showed 50% or more ciliary activity or intact ciliated epithelium respectively were regarded as being indicative of protection.

In one experiment, groups of chickens were inoculated once intramuscularly with different

Table 2. *Effect of the removal of S1 from IBV on the induction of VN and HI antibody and tracheal protection*

Inoculum (10 μg)	Serum titre (log_2)* VN	HI	Infectious virus recovered (log_{10} CD_{50}/0·1 g trachea)†‡	Tracheal cilia beating†	Tracheal epithelium intact†	Tracheal protection indicated†
Control virus	8	12	1	+	+	+
	6	12	2·0	+	+	+
	4	6	2·5	+	+	+
	7	9	1	+	+	+
	7	10	1	–	–	–
	7	10	1	–	–	–
	8	12	1·9	–	–	–
	4	10	4·8	–	–	–
	7	10	2·8	–	–	–
	4	5	5·0	–	–	–
Urea-treated virus	3	4	1·5	–	–	–
	3	2	1·8	–	–	–
	3	3	2·7	–	–	–
	3	3	3·9	–	–	–
	3	5	3·9	–	–	–
	3	3	4·1	–	–	–
	3	2	4·1	–	–	–
	3	4	4·6	–	–	–
	3	3	5·3	–	–	–
	3	2	5·9	–	–	–
No virus	3	2	3·9	–	–	–
	3	3	4·1	–	–	–
	3	2	4·6	–	–	–
	3	2	4·8	–	–	–
	3	2	5·1	–	–	–

* Serum was collected 4 weeks after inoculation. Each horizontal line contains data from an individual chicken.
† Chickens were challenged 5 weeks after inoculation and tracheae removed 4 days later.
‡ Virus isolation was attempted by homogenizing weighed amounts of trachea.

amounts of inactivated virus. There were no significant differences between the mean serum VN or HI titres of the groups inoculated with 5, 25 or 100 μg of virus (Table 1, expt. 1). Of four chickens inoculated with 1 μg of virus, three and one had negative VN and HI titres respectively, while all chickens which received 0·2 μg of virus were negative in both tests (data not shown). Four of 11 (36%) chickens were judged to have resisted challenge on the basis of ciliary activity. Virus could not be recovered from the tracheae of these four chickens whereas it was found in all except one of the unprotected chickens (Table 1). In this and all other experiments both protected and unprotected chickens that had been challenged had subepithelial oedema in the lamina propria and a generalized lymphocytic infiltration.

In a second experiment, five of 17 (29%) chickens were protected following inoculation with 10 μg of virus (Table 1, expt. 2). Virus was not isolated from these five birds or from eight of the remaining 12 chickens which had been inoculated with inactivated virus. In contrast, virus was recovered from all of eight chickens inoculated with Freund's adjuvant only.

Neither of these experiments, or others, provided evidence of any correlation between serum VN or HI titres and tracheal protection. When chickens received a second inoculation of inactivated virus 4 weeks after the first the HI titres 4 weeks later were increased on average by only 1 log_2 and there was no increase in the number of chickens showing tracheal protection.

Inoculation of chickens with virus lacking S1

The above experiments established that one intramuscular inoculation of inactivated IBV could induce tracheal protection in some chickens. We therefore investigated the capacity of

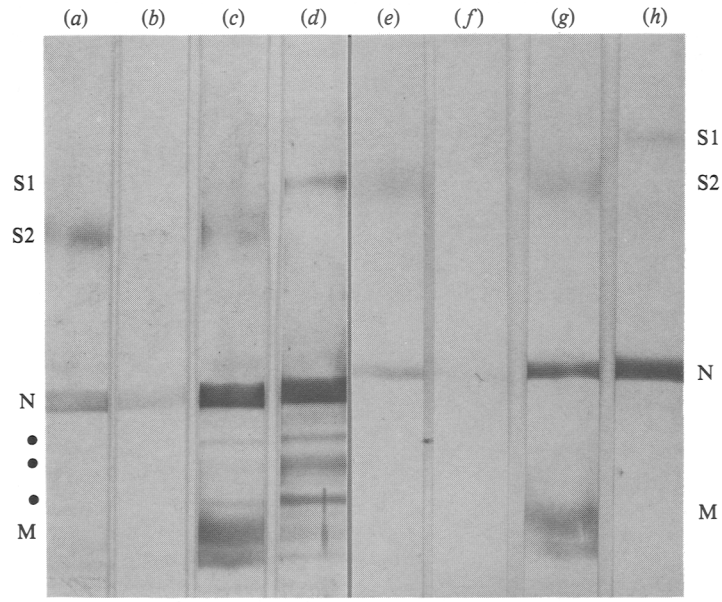

Fig. 4. Probing of Western blots containing IBV polypeptides with various chicken sera: (*a*, *e*) antiserum raised against S2 separated by SDS–PAGE; (*b*, *f*) normal chicken serum; (*c*, *g*) convalescent serum from IBV-infected chickens; (*d*, *h*) serum raised against a preparation of urea-released S1 containing some N protein. Sera were diluted 1/100 and bound antibody was detected by the addition of rabbit anti-chicken IgG conjugated to horseradish peroxidase followed by the enzyme substrate 4-chloro-1-naphthol. (*a*) to (*d*) show the side of the nitrocellulose which was adjacent to the polyacrylamide gel during Western blotting while (*e*) to (*h*) show the reverse side. The filled circles indicate the positions of three polypeptides which are structurally related to N.

urea-treated virus, which lacked S1, to induce not only VN and HI antibody but also tracheal protection. In the experiment shown in Table 2, all ten chickens inoculated with intact virus developed VN and HI titres, and four were protected. In contrast, of those chickens inoculated with urea-treated virus none had VN antibody, none was protected and only three had positive HI titres and these were very low.

Seven of the control virus group, including all the protected chickens, had 2·5 $\log_{10}$ CD_{50} or less of infectious virus per 0·1 g of trachea at 4 days after challenge, compared with only two of the group which had received urea-treated virus and none of five which had received adjuvant only (Table 2).

In a second experiment, the results with regard to VN and HI titres were essentially the same as those in the experiment of Table 2. Two of ten birds inoculated with intact virus were scored as protected. None of ten chickens inoculated with urea-treated virus was protected. These results show that intact spikes are required for the induction of VN and HI antibody and for tracheal protection following parenteral vaccination and suggest that S1 has a direct role in these properties.

Inoculation of chickens with S1 alone

To see if S1 alone could induce VN and HI antibody, nine chickens were inoculated with 3 μg S1 on each of four occasions over a 13 week period. After three inoculations all the sera were negative for VN and HI antibody but after four inoculations four chickens had positive HI titres and two of these contained VN antibody (Table 3). None of these birds was challenged with virus. A pool of the four sera with positive HI titres was used to probe Western blots. This immunoblotting showed that the serum pool reacted with S1 and N and to a low, possibly non-specific degree, with M but did not react with S2 (Fig. 4*d*, *h*). A convalescent IBV serum reacted weakly with S1, moderately with S2 and strongly with N and M (Fig. 4*c*, *g*) while normal

Table 3. *Induction of VN and HI antibodies by intramuscular inoculation of S1 glycopolypeptide*

Serum titre (log_2)* after			
Three inoculations		Four inoculations	
VN	HI	VN	HI
3	2	8	8
3	1	6	5
3	1	3	5
3	1	3	7
3	2	3	3
3	1	3	3
3	2	3	3
3	2	3	3
3	2	3	2

* Each horizontal line contains data from an individual chicken.

chicken serum gave a weak reaction with N only (Fig. 4*b*, *f*). Chickens which had been inoculated with S2 excised from polyacrylamide gels had antibodies which reacted with S2 (Fig. 4*a*, *e*). These results confirm the conclusion that the absence of detection of S2 by the anti-S1 serum pool is a consequence of the absence of anti-S2 antibody and that the VN and HI antibodies in this serum pool had been induced by S1. Some antibody reacted non-specifically with N and, to a lesser extent, with M (Fig. 4*a*, *b*).

DISCUSSION

The populations of serum antibodies measured by the VN, HI and ELISA assays increased at a similar rate, peak titres being reached at about 4 weeks after inoculation of inactivated virus. This is in contrast to the situation following vaccination with infectious IBV. Although HI and ELISA titres were maximal about 2 weeks after infection VN antibody was only just detectable at this time, becoming maximal approximately 2 weeks later (Mockett & Darbyshire, 1981; Hawkes *et al.*, 1983). The greater rate of decline in HI and ELISA than VN titres following inactivated vaccine was, however, similar to the observations made following infection.

IBV which lacked S1 failed to induce VN and HI antibody while S1 alone was able to do so. This indicates that the epitopes for these antibodies are part of S1. The immunogenicity of the purified S1 was inferior to that of purified but intact spikes (Cavanagh *et al.*, 1984). While this may in part be because smaller amounts of S1 (3 μg) than whole spikes (20 μg) were used for each inoculation at least three other factors probably account for this. Firstly, intact IBV spikes aggregate at their narrow ends to form rosettes; such aggregates are likely to be more immunogenic than individual spikes (Morein & Simons, 1985). Secondly, not only was the urea-released S1 not aggregated but the S1 subunit had been dissociated into S1 monomers. Thirdly, some regions of S1 had probably been irreversibly denatured following dissociation from S2 and other S1 molecules, possibly resulting in the loss of some epitopes associated with the induction of VN and HI antibodies. This may be why some chickens did not produce any detectable VN and HI antibody following inoculation with S1 alone. Mockett *et al.* (1984) produced two VN and HI monoclonal antibodies to IBV which immunoprecipitated urea-dissociated S1 but did not react with S2. Since a polypeptide which mediates haemagglutination is most likely to be situated at the distal end of a spike protein these results collectively lend support to the view that S1 forms the major part of the distal, bulbous part of the spike protein (Cavanagh, 1983*b*).

Some mice immunized with spike protein from murine hepatitis coronavirus (MHV) resisted viral challenge whereas all mice which had been immunized with the M or N protein died after challenge (Hasony & Macnaughton, 1981). Passively administered anti-spike monoclonal antibodies protected mice against lethal disease caused by the neurotropic JHM strain of MHV (Wege *et al.*, 1984; Buchmeier *et al.*, 1984). Inoculation of pregnant sows with the spikes of transmissible gastroenteritis coronavirus (TGEV) induced the formation of VN antibody which

could be detected in colostrum as well as in serum (Garwes *et al.*, 1978/79). The colostrum of one sow contained VN antibody of the IgA class; the piglets of this sow survived viral challenge whereas the litters of other sows which had been inoculated with spikes died after challenge. These results and ours with IBV indicate that the spike protein is the major inducer of protective immune responses against these coronaviruses. Our results go further in that they suggest that the S1 subunit is the dominant part of the spike with respect to the induction of protection. However, the possibility that S2 may be involved cannot be dismissed at this stage since the S2 of urea-treated virus probably has a changed configuration, in part because of the removal of S1 and partly because of possible direct denaturing effects of urea on S2.

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