PROPERTIES OF CORONAVIRUS IBV AFTER REMOVAL OF THE S1 SUBUNIT OF THE SPIKE GLYCOPROTEIN

Dave Cavanagh and Philip Davis

Houghton Poultry Research Station
Houghton
Huntingdon
Cambs. PE17 2DA, UK

INTRODUCTION

Our recent investigations of avian infectious bronchitis virus (IBV) have concentrated on the biological properties of the spike (S) glycoprotein. Previously we showed that the mature S is derived by cleavage of a precursor glycoprotein to yield two glycopolypeptides, S1 and S2[1] of approximately 514 and 625 amino acid residues respectively[1]. Each spike comprises two or three copies of each of S1 and S2[2,3], the two subunits not being held to each other by disulfide bonds[2,3]. The ease with which S1 but not the other virion proteins could be removed by urea or SDS led us to suggest that S was anchored in the membrane by S2 and that S1 was probably not in the membrane but formed most of the distal, bulbous part of S[3]. This view was strengthened when sequencing of the S gene showed that S2 had a highly hydrophobic sequence, typical of membrane-spanning domains, near its carboxy terminus[4].

Studies with purified IBV virion proteins indicated that only S induced virus neutralizing (VN) and hemagglutination-inhibiting (HI) antibody[5].

We report here observations on the properties of IBV after removal of S1 by urea. We conclude that S1 is not required for attachment to cells but that an intact S protein is required for some other function essential for virus infectivity[6]. Epitopes for the induction of neutralizing and hemagglutination-inhibiting (HI) antibodies are located on S1, and virus from which S1 has been removed cannot induce protection of the chicken trachea[7].

METHODS

The following methods have been described: preparation of radiolabelled IBV strains M41[8] and Beaudette[9] and b-propiolactone-inactivated non-labelled IBV-M41[2]; removal of S1 by urea[3,7]; assay of infectivity and virus neutralization (VN)[10]; assay of HI[11]; estimation of the mol.wt. of S1 released by urea[7]; Western blotting[7]; attachment of radiolabelled virus to chicken red blood

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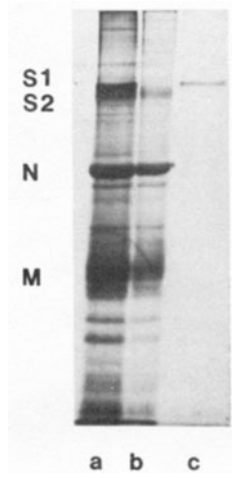

Fig. 1 SDS-PAGE of IBV-M41 (a) before and (b) after incubation with 6M urea and pelleting. The polypeptides that were released by the urea and which did not pellet are shown in (c). The gel contained 10% acrylamide and was silver stained.

cells (IBV-M41 and Beaudette) and chick kidney cells (IBV-Beaudette only)[6]. For immunization of chickens antigen was emulsified with an equal volume of Freund's complete adjuvant (Difco). 0.5ml 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-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 4 times over 5 months[7].

For the determination of tracheal protection chickens were challenged by intra-tracheal inoculation of 0.2ml of buffer containing 500 ciliostatic dose 50 of infectious virus. Four days later the chickens were killed and the tracheae removed. Virus was isolated either by swabs or from weighed pieces of trachea. For histology pieces of trachea were fixed, sectioned and stained with haematoxylin and eosin. Several sections from different parts of the trachea were examined. Tracheas 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 lower power microscopy. Ciliary activity associated with 50% or more of the epithelial cells was arbitrarily chosen as being indicative of protection[7].

RESULTS

Removal of S1 by Urea

IBV which had been incubated with 6M urea lacked S1 (Fig. 1a,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. 1c). With IBV Beaudette we observed that after 1h at 37°C in the absence of urea a small amount of S1 was released.

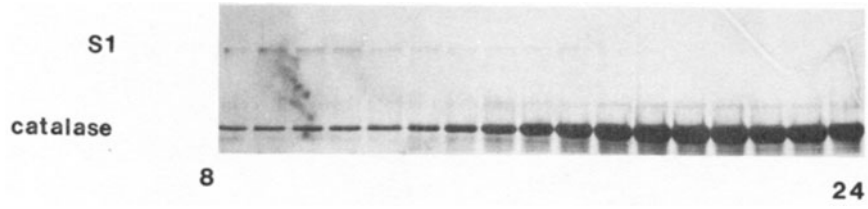

Fig. 2. Molecular weight estimation of S1 released from virions by urea. Virus was incubated with 6M urea and then pelleted. The unpelleted S1 was dialysed to remove urea and then co-sedimented with bovine catalase in a 5-20% sucrose gradient. After fractionation the polypeptides in each fraction were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

Intact S contains 2 or 3 copies of S1, deduced after co-sedimentation of purified S with catalase (mol.wt.260K). 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.

Effect of Urea on the Interaction of IBV With Cells

Treatment of IBV-M41 and Beaudette with urea reduced infectivity by more than 99% and abolished haemagglutination (HA). 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%. The same observation was made at high (260×10^7 cells/ml) and low (3×10^7 cells/ml) cell concentration, 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 resulted in a decrease in virus attachment but the extent was still approximately 30 to 60% of that of the intact virus.

Location of Neutralizing and HI Antibody Determinants on S

We have shown previously that VN and HI antibodies are induced by the S protein[5]. Preliminary experiments showed that intra-muscular inoculation of 3ug of inactivated IBV-M41 induced maximum titres of VN and HI antibody within four weeks[7]. We also showed that following such vaccination up to 40% of the chickens had some resistance to challenge with IBV since more than 50% of the ciliated epithelium of the trachea remained intact, whereas in chickens vaccinated with

Table 1.

Effect of removal of the S1 subunit on the induction of HI and VN antibody in groups of 10 chickens

S1[a] present	Serum titres (log_2)[b] HI Mean (range)	VN Mean (range)
+	10 (5-12)	6 (4-8)
-	3 (2-5)	3 (3)

[a] Chickens were inoculated once intra-muscularly with 10ug of intact or urea-treated inactivated IBV.

[b] Serum was collected 4 weeks after inoculation.

adjuvant only the ciliated epithelium was completely destroyed[7].

We then examined whether virus from which S1 had been removed could induce VN and HI antibodies and tracheal protection[7]. In the experiment of Table 1 all ten chickens inoculated with intact virus produced VN and HI antibodies and four were protected. In contrast none of the chickens inoculated with urea-treated virus produced VN antibody. The mean HI titre of serum from these chickens was 3 log_2 (negative); three chickens had very low positive titres (2 at 4 log_2, one at 5 log_2). None of the chickens which had received urea-treated virus resisted challenge. Similar results were obtained in a second experiment. In neither experiment, nor any of the others in which only intact virus was used, was there a correlation between serum VN or HI titres and tracheal protection.

To see if S1 alone could induce VN and HI antibody 9 chickens were inoculated with 3ug of S1 on each of four occasions over a 13 week period. AFter 3 inoculations all the sera were negative for VN and HI antibody but after 4 inoculations 4 chickens had positive HI titres and 2 of these contained VN antibody (Table 2). None of these birds were challenged with virus. A pool of the 4 sera with positive HI titres was used to probe Western blots[7]. The pool contained antibodies reactive with S1 and N (expected since the chickens had been inoculated with S1 contaminated by N) but not with S2 (Fig.3d). In contrast antibody raised against S2 excised from gels did react with S2 (Fig. 3a), and serum from previously infected chickens reacted well with S2 but poorly with S1 (Fig. 3d). 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.

Table 2.

Induction of VN and HI serum antibodies after four intramuscular inoculations of 3ug of S1

Chicken number	Serum titre (log_2) VN	HI
4	8	8
7	6	5
1	3	5
8	3	7
2	3	3
3	3	3
5	3	3
6	3	3
9	3	2

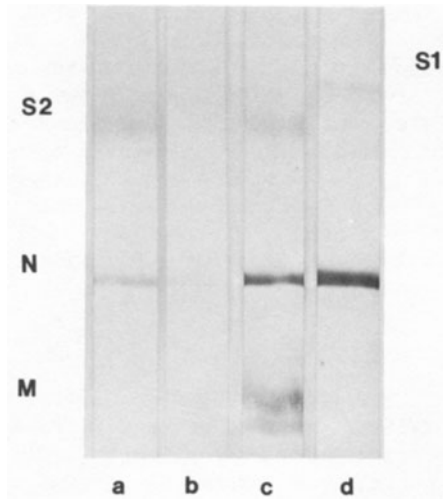

Fig. 3. Probing of Western blots containing IBV virion polypeptides with various chicken sera: (a) antiserum raised against S2 separated by electrophoresis; (b) normal chicken serum; (c) convalescent serum from IBV-infected chickens; (d) serum raised against a preparation of urea-released S1 containing 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.

DISCUSSION

Two monoclonal antibodies which neutralize IBV and inhibit HA have been shown to be specific for S1 of IBV-M41[12]. It was not surprising, therefore, to find that virus from which S1 had been removed was no longer infectious, could not cause HA or induce VN and HI antibody. Unexpectedly, however, the loss of HA activity 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.

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[13] 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.

Our studies with IBV, together with those of other coronaviruses[14,15,16,17], indicate that the S protein is required for the induction of protective immunity. The finding that IBV which lacked S1 failed to induce VN and HI antibody while S1 alone was able to do so indicates that the epitopes for these antibodies are part of S1. Moreover the induction of protective immunity would appear to require S1. However, the possiblity that S2 may be involved in these roles cannot be dismissed at this stage, for the reasons stated above. It is noteworthy that two monoclonal antibodies with VN and HI activity were able to bind to S1[12] which, after urea treatment, is monomeric (Fig. 2). Also the monomeric S1 had sufficient native structure to induce VN and HI antibody indicating that the conformation of at least one epitope is independent of the quaternary structure of the S protein.

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