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## Monoclonal Antibodies to the S1 Spike and Membrane Proteins of Avian Infectious Bronchitis Coronavirus Strain Massachusetts M41

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### SUMMARY

We have established four murine hybridoma cell lines which secrete specific antibody to avian infectious bronchitis virus (IBV) strain Massachusetts M41. Two monoclonal antibodies reacted with the spike protein and two reacted with the membrane protein. The specificity of the monoclonal antibodies for the external structural proteins was detected by immunoprecipitations using radiolabelled virus. The reactions of the monoclonal antibodies showed that one (S1) of the two glycopoly-peptides associated with the spike protein has a strain-specific region involved in neutralization and haemagglutination, and the membrane protein has antigenic determinants which are present on the three strains of IBV tested (M41, Beaudette and D41).

Avian infectious bronchitis virus (IBV), in common with other members of the *Coronaviridae*, contains three structural proteins: spike (S; peplomer), membrane (M) and nucleocapsid (N) (Siddell *et al.*, 1983). The spike protein of IBV comprises two glycopolypeptides, S1 and S2, of 90K (90 000 mol. wt.) and 84K respectively (Cavanagh, 1983*a, b*). Parts of both S and the glycosylated M protein (30K) are present at the virion surface. The N protein (54K) is associated with the viral RNA. Several monoclonal antibodies have been established which react with the three structural proteins of another coronavirus, murine hepatitis virus (Collins *et al.*, 1982; Fleming *et al.*, 1983). The majority of monoclonal antibodies to the spike glycoprotein neutralized the virus whilst those to the other structural proteins did not, although some anti-membrane protein monoclonal antibodies can neutralize the virus if complement is present. Our aims were (i) to establish monoclonal antibodies to the spike glycoprotein of IBV and determine whether they would neutralize the virus, (ii) to ascertain whether the antigenic determinants involved could be localized to either of the two glycopolypeptides which comprise the spike protein and (iii) to investigate the properties of any other virus-specific monoclonal antibodies generated by the inoculation procedure.

The Massachusetts M41 strain of IBV was grown in embryonated eggs and purified using sucrose gradients (Cavanagh, 1981). The purified virus was solubilized in NP40 and centrifuged in a sucrose gradient containing the detergent (Cavanagh, 1983*a*). Spike-containing fractions were identified using PAGE, pooled and treated with Amberlite XAD-2 monobed resin (BDH) to remove the detergent. The spike-containing preparation was inoculated (in 50% Freund's complete adjuvant) intraperitoneally into BALB/c mice on day 1 and reinoculated on day 30. The same antigen in Freund's incomplete adjuvant was inoculated intraperitoneally on day 53 and intravenously (in PBS) on day 57. The volume of each inoculum was 0.2 ml. Three days after the final inoculation the spleen was removed and a cell suspension prepared. The spleen cells were fused with the cells of the myeloma cell line P3-NSI-Ag4-1, essentially as described by Köhler & Milstein (1975). After 10 to 14 days, the Linbro wells were examined and those which contained hybridomas were tested for IBV-specific antibody in an ELISA as described by Mockett *et al.* (1984) except that the coating antigen was the spike-enriched preparation, used at

a 1:15 dilution. The cells from any well which gave a positive reaction were cloned twice at limiting dilution using mouse peritoneal macrophages as a feeder layer. The established cell lines were stored in liquid nitrogen vapour and inoculated intraperitoneally into Pristane (Aldrich Chemical Co.)-primed mice, to produce ascitic fluid.

The specificity of the monoclonal antibodies was shown using radiolabelled virus, Protein A-Sepharose 4B (Sigma) and hybridoma culture supernatant in radioimmunoprecipitation assays. [<sup>35</sup>S]Methionine-labelled virus was purified from de-embryonated eggs (Cavanagh, 1981). Treatment of the purified virus with 4 M-urea and subsequent centrifugation resulted in a supernatant containing S1 glycopolyptide and virions devoid of S1 in the pellet (Cavanagh, 1983*b*). Protein A-Sepharose 4B was swollen in phosphate-buffered saline (PBS) pH 7.4 containing 0.01% (w/v) sodium azide. To 1 ml of this gel 9 ml of Sepharose 4B was added, mixed, made as a 50% (v/v) suspension in PBS/0.01% sodium azide and stored at 4 °C until required. Radiolabelled virus (90000 d.p.m.) was dissociated at 25 °C for 30 min in PBS containing 0.9% (v/v) Triton X-100, 0.1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. Radiolabelled S1 glycopolyptide (30000 d.p.m.) and virus lacking S1 (90000 d.p.m.) were prepared for immunoprecipitations as described above. Hybridoma culture fluid (400 µl) was added and incubated for 1 h at room temperature prior to the addition of 500 µl of 1:10 Protein A-Sepharose gel (50%, v/v). The mixture was left overnight at 4 °C. The gel was washed in 150 mM-NaCl, 5 mM-EDTA, 50 mM-Tris-HCl (NET) buffer containing 0.05% NP40 three times before adding 400 µl of solubilizer (Laemmli, 1970). After heating at 90 °C for 5 min the fluid was removed and subjected to SDS-PAGE in a 10% acrylamide tube gel. The gel was processed as described by Cavanagh (1981). The monoclonal antibody subclass was determined in an ELISA using the appropriate IgG subclass-specific reagents from Nordic Immunological Laboratories (Maidenhead, U.K.). The monoclonal antibodies were also tested for reactions in ELISA tests using three purified viruses as antigens: M41, Beaudette and D41. Ascitic fluid from each of the hybridomas was tested for neutralization of the M41 strain using tracheal organ cultures. End-points were based on the presence or absence of ciliostasis (Darbyshire *et al.*, 1979). Any fluids that neutralized the M41 strain were tested against 13 other IBV strains: H52, H120 (Intervet Laboratories, Milton, Cambs., U.K.), MM (Salisbury Laboratories, Reading, Berks., U.K.), VF69-149, VF70-873, HVI-140, VF70-861, Gray, 591, D41 (Darbyshire *et al.*, 1979), D207, D3128, D3896 (Kouwenhoven *et al.*, 1983). Haemagglutination inhibition (HI) tests were done using ascitic fluids essentially as described by Mockett & Darbyshire (1981).

The results of the experiments showed that the treatment of purified IBV with NP40 and the subsequent centrifugation on an isopycnic sucrose gradient containing the detergent proved a useful method for obtaining a preparation enriched in S; there was some contamination with N and M proteins. S was largely located in fractions near the top of the gradient while fractions containing principally N were located lower in the gradient (Fig. 1). Virus-specific antibodies were detected in mice that had been inoculated with spike-enriched material. A 1:1000 dilution of immune mouse serum gave an absorbance value of 0.5 or greater in the ELISA. Sera from non-inoculated mice, at a 1:50 dilution, did not react. The spleen cells from an immunized mouse were used in a fusion and hybridomas were observed after 2 weeks growth in HAT-containing medium in about 25% of the wells. Fluids from 19 wells reacted with spike-enriched material in an ELISA. However, only four hybridomas, designated A13, A38, C5 and C124 were cloned by limiting dilution on mouse macrophage cells and grown in mice to produce ascitic fluid. The specificity of the monoclonal antibody for the virus was shown by immunoprecipitations using radiolabelled virus. The radiolabelled immunoprecipitates were analysed by SDS-PAGE in tube gels and the results are given in Fig. 2. The hybridoma clones A13 and A38 reacted with the spike protein; both S1 and S2 glycopolyptides were immunoprecipitated (Fig. 2*b*). The conditions used to solubilize the virus did not dissociate S1 from S2 and this accounts for their co-precipitation. The hybridoma clones C5 and C124 reacted with the membrane protein (Fig. 2*d*). In all these experiments some immunoprecipitation of nucleocapsid protein was observed. The non-specific nature of the reaction was shown by the precipitation of nucleocapsid protein with a monoclonal antibody to a fowl adenovirus [egg drop syndrome 1976 virus (Mockett *et al.*, 1984)]. Further immunoprecipitations (Fig. 3) using radiolabelled

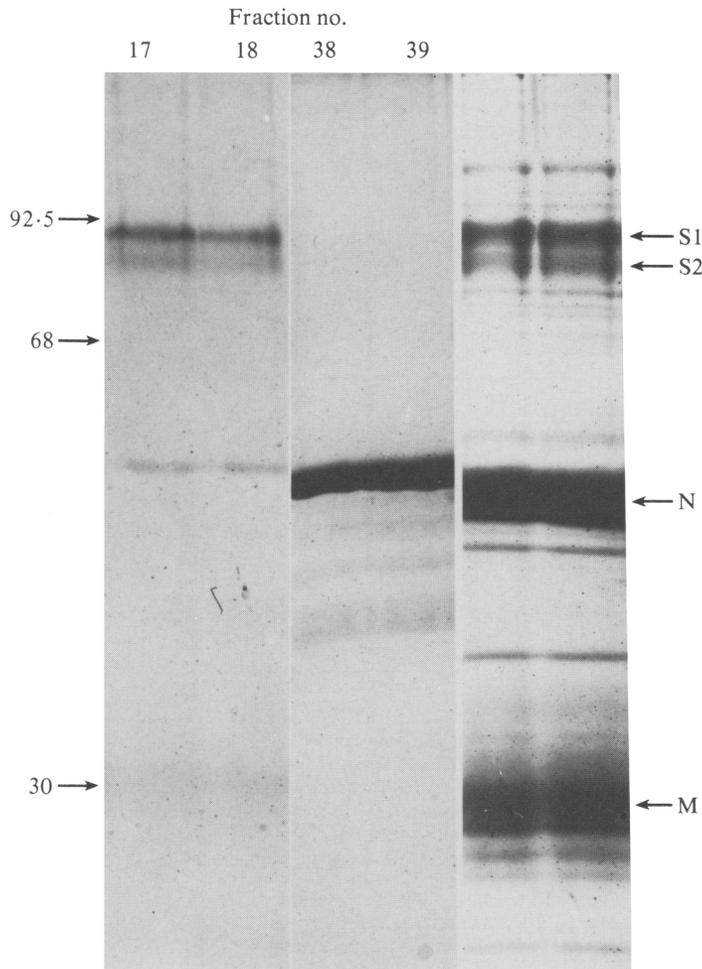


Fig. 1. Production of spike-enriched material. Purified IBV (M41) was solubilized in 2% (w/v) NP40 and centrifuged in a 10 to 55% (w/w) sucrose gradient in NET containing 0.1% (w/v) NP40. After fractionation the proteins were detected by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. Results from fractions 17, 18, 38 and 39 are shown, with purified virus for comparison. Figures indicate the molecular weight ( $\times 10^{-3}$ ) of the marker proteins used. S1 and S2 are the spike polypeptides, M the membrane protein and N the nucleocapsid protein.

S1 and virus lacking S1 showed that the A13 monoclonal antibody reacted with the S1 glycopolyptide.

The anti-spike monoclonal antibodies, A13 and A38, neutralized the M41 strain of IBV, with titres of 1:40000 and 1:9500 respectively. However, they did not neutralize any of the other 13 strains of IBV tested, including H52, H120, MM, VF69-149, VF70-873 and HVI-140 which are classified on the basis of neutralization tests as Massachusetts-like (Darbyshire *et al.*, 1979). The two anti-membrane monoclonal antibodies did not neutralize the M41 virus. Both A13 and A38 ascitic fluids inhibited the haemagglutination of chicken red blood cells by the virus. The titres for A13 and A38 were 1:512 and 1:4096 respectively. Ascitic fluids C5 and C124 did not inhibit haemagglutination. The subclass of the IgG immunoglobulin was determined by ELISA as IgG2b for the A13 monoclonal antibody and IgG2a for A38, C5 and C124 monoclonal antibodies. The monoclonal antibodies A13 and A38 both reacted with the spike protein and neu-

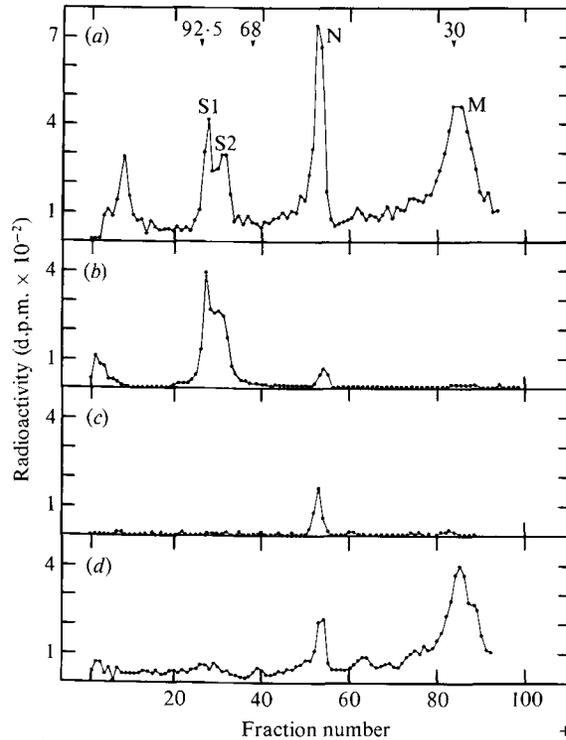


Fig. 2. Immunoprecipitation of [ $^{35}\text{S}$ ]methionine-labelled IBV (M41) viral protein using monoclonal antibody. The radioactivity in each fraction from an SDS-polyacrylamide tube gel (10% acrylamide) is shown for (a) purified virus, and material immunoprecipitated with (b) an anti-spike monoclonal antibody (A13), (c) an anti-egg drop syndrome 1976 virus monoclonal antibody and (d) an anti-membrane monoclonal antibody (C124). Figures indicate the molecular weight ( $\times 10^{-3}$ ) of the marker proteins used. S1 and S2 are the spike polypeptides, M the membrane protein and N the nucleocapsid protein.

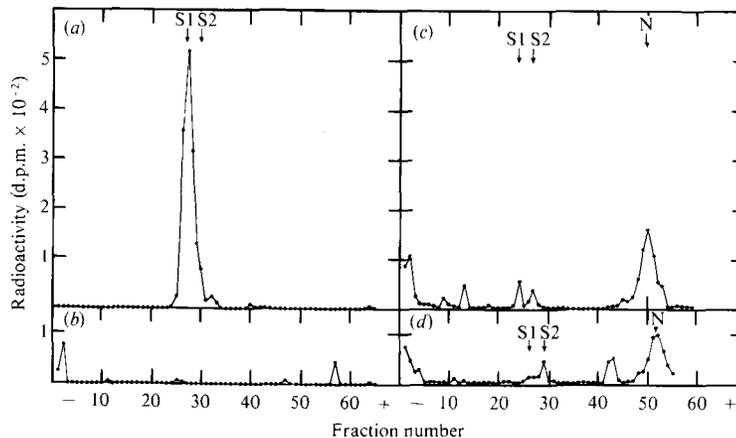


Fig. 3. Immunoprecipitation of [ $^{35}\text{S}$ ]methionine-labelled S1 polypeptide of IBV using monoclonal antibody. Radiolabelled S1 polypeptide and virions lacking this polypeptide were prepared and used in the immunoprecipitation tests. The radioactivity in each fraction from an SDS-polyacrylamide tube gel (10% acrylamide) of material immunoprecipitated after mixing S1 and (a) anti-spike monoclonal antibody (A13) or (b) anti-membrane monoclonal antibody (C124) is shown. Also material immunoprecipitated after mixing virion lacking S1 and (c) anti-spike monoclonal antibody (A13) or (d) anti-membrane monoclonal antibody is shown. Only that part of the gel containing polypeptides greater than 50K is shown. S1 and S2 show the positions of the spike polypeptides and N the nucleocapsid protein.

tralized the virus but were of a different IgG subclass. It was possible they reacted with the same epitope. The observation that ascitic fluid of either hybridoma successfully blocked the reaction of the tissue culture fluid of the other in an ELISA using the appropriate IgG subclass-specific reagents provided evidence that this was the case. The inhibition was reduced as the ascitic fluid was diluted. The A13 and A38 monoclonal antibodies reacted only with the M41 strain whereas the C5 and C124 reacted with the three IBV strains tested (M41, Beaudette and D41).

Cavanagh (1983*b*) suggested that each spike protein molecule of IBV is composed of two or three of each of S1 and S2 glycopolypeptides with the latter anchored in the membrane of the virus. Both the anti-spike monoclonal antibodies produced in this study reacted specifically with the S1 glycopolypeptide and were able to neutralize the infectivity of the virus *in vitro*. This finding, and that of Cavanagh *et al.* (1984) who demonstrated the neutralization of the virus by polyvalent chicken antisera produced after the inoculation of spike protein, clearly indicates the importance of the spike protein in viral replication. The spike protein of murine coronavirus is involved in neutralization and cell fusion (Collins *et al.*, 1982; Fleming *et al.*, 1983).

The two anti-spike monoclonal antibodies were both M41 strain-specific whereas polyclonal chicken antiserum to the M41 strain also neutralized some other strains (Darbyshire *et al.*, 1979). This indicates that the M41 strain possesses more than one antigenic determinant involved in neutralization although antibody binding to a single determinant can achieve neutralization. The spike protein of murine coronaviruses shows considerable antigenic variation between strains (Fleming *et al.*, 1983) and this is probably the case with the spike protein of IBV strains. With the aid of monoclonal antibodies the variation in antigenic structure between strains of IBV may be resolved. Both anti-spike monoclonal antibodies also inhibited haemagglutination. This suggests that the haemagglutination and neutralization sites on the viral spike may be identical or are so close to one another that antibody attaching to one site affects binding to the other, or that antibody attaching to one site may sterically alter the structure of the other site. However, there is evidence to suggest that the sites involved in haemagglutination and neutralization are different. Mockett & Darbyshire (1981) showed that the HI and serum neutralization (SN) patterns of antibody response in chickens after infection with IBV were different. HI antibodies were at peak levels 10 days after infection, whereas SN antibodies were negligible at 10 days and reached a peak at about 23 to 30 days. Also, strains which do not cross-react in the SN test do so in the HI test. A comparison of the neutralization and HI titres of the two anti-spike monoclonal antibodies showed that one (A13) was relatively more efficient at neutralization than at inhibiting haemagglutination whereas the converse was true for the other (A38). A possible explanation for these observations is that there is an overlap area between the HI and SN sites on the spike. Monoclonal antibodies to a number of other viruses have been produced which react with both the HI and neutralization sites (Carter *et al.*, 1982; Heinz *et al.*, 1983; Nishikawa *et al.*, 1983; Sonza *et al.*, 1984). Thus, monoclonal antibodies to the overlap area of the spike protein of IBV would both neutralize and inhibit haemagglutination and, depending on exactly where they attached, could have more neutralization than HI activity or vice versa. The haemagglutination determinants outside the overlap area could be major immunogens for the chicken and this could explain the different HI and SN antibody profiles after infection. Previous attempts to produce IBV-specific monoclonal antibodies using IBV grown in eggs resulted in large numbers of monoclonal antibodies to egg proteins (A. P. A. Mockett, unpublished observations). To overcome this problem, a spike-enriched fraction was prepared and used to produce IBV spike-specific monoclonal antibodies in this study. However the material was still contaminated with enough membrane protein to produce monoclonal antibodies to this protein. Experiments with these monoclonal antibodies showed that the three strains of IBV tested all contained at least one highly conserved antigenic determinant. The reactions of monoclonal antibodies to the membrane protein of murine coronavirus also showed that this was a highly conserved protein (Fleming *et al.*, 1983). The suggested reason for this antigenic conservation is a stringent structural requirement for maintenance of the membrane protein function.

In conclusion, the monoclonal antibodies we have produced have provided information on the antigenic structure of IBV. These data confirm the major role of the coronavirus spike protein in infection. The monoclonal antibodies may be used to further our knowledge of the coron-

avirus by, for example, making possible the purification of the viral polypeptides and facilitating studies on the antibody response of chickens to these isolated components.

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