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## ENVELOPE PROTEINS OF AVIAN INFECTIOUS BRONCHITIS VIRUS: PURIFICATION AND BIOLOGICAL PROPERTIES

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Immunoabsorbents, made with monoclonal antibodies, were used to purify the spike and membrane proteins of infectious bronchitis virus (IBV). The purified proteins were inoculated into rabbits to produce antisera. The rabbit anti-spike sera neutralized the infectivity of the virus whereas the anti-membrane sera did not.

IBV-infected chickens produced antibodies to both the spike and membrane proteins. Both these antibodies were at their highest concentration about 9–11 days after inoculation, whereas neutralizing antibodies were present only at very low concentrations at that time. Neutralizing antibodies were at their highest concentration 21 days after inoculation. A second inoculation of virus at 42 days induced an anamnestic antibody response to the spike and membrane proteins and also for the neutralizing antibodies. The neutralizing, anti-spike and anti-membrane antibodies all reached highest concentrations 7–11 days after this inoculation. The advantages of purifying viral proteins using affinity chromatography with monoclonal antibodies are discussed.

envelope proteins    avian infectious bronchitis virus

### INTRODUCTION

Avian infectious bronchitis virus (IBV) is a coronavirus whose principal site of replication is the ciliated epithelial cells of the respiratory tract mucosa of chickens. Viral replication occurs in the cytoplasm of the cell and virions are formed by budding from the endoplasmic reticulum. There are three viral structural proteins: spike (S; peplomers), membrane (M) and nucleocapsid (N). S and M proteins are both glycosylated and parts of them are exposed at the surface of the virion. The spike protein consists of two glycopolypeptides, S1 and S2, which have molecular weights of 90 kDa and 84 kDa, respectively (Cavanagh, 1981). The membrane protein is present as a number of distinct species which have molecular weights ranging from 23 kDa to 34 kDa; the molecular weight differences are associated with the various degrees of glycosylation. N protein (54 kDa) is associated with the viral RNA.

The IBV spike protein, associated with the outer projections, plays an important part in the infection of cells. Chicken antisera to this protein (Cavanagh et al., 1984)

and spike-specific monoclonal antibodies (Mockett et al., 1984) can neutralize the infectivity of the virus. A similar function has been found for the spike protein of murine coronavirus MHV-4 (Collins et al., 1982; Fleming et al., 1983) and the porcine coronavirus TGEV (Garwes et al., 1978). The spike protein of IBV also contains strain-specific determinants (Mockett et al., 1984). The membrane protein appears to be a more highly conserved antigen and it is possible that only a small amount (approx. 1 kDa) is exposed at the viral surface (Bournnell et al., 1984). The nucleocapsid protein interacts with the viral RNA to form a helical nucleocapsid.

The objectives of this work were to produce immunoadsorbents using monoclonal antibodies which have been prepared previously (Mockett et al., 1984) and to purify the virus-coded proteins of the viral envelope in a single step in relatively large amounts. This allowed hyperimmune rabbit antisera to the proteins to be produced and tested for neutralizing antibodies. In addition the sequential humoral antibody response of chickens after IBV infection has been studied using the purified viral proteins and whole virus in ELISAs and compared to the results using the neutralization test.

## MATERIALS AND METHODS

### *Virus*

The Massachusetts M41 strain of IBV was grown in the allantoic cavities of 11-day-old embryonated chicken eggs and purified on isopycnic sucrose gradients as described by Cavanagh (1981).

### *Preparation of material for affinity chromatography*

Purified virus was pelleted in a  $6 \times 14$  ml rotor at  $70,000 \times g$  for 3 h at  $4^{\circ}\text{C}$  and resuspended in phosphate-buffered saline (PBS). An equal volume of PBS containing 4% (wt./vol.) NP40 was added, mixed using a Dounce homogeniser and incubated for 2 h at  $25^{\circ}\text{C}$ . The material was centrifuged for 5 min in an Eppendorf microcentrifuge and the resulting supernatant, containing soluble viral components, was used for the affinity chromatography purification.

### *Immunoadsorbent preparation*

Monoclonal antibodies (designated A38 and C124) to the spike and membrane proteins respectively of IBV strain M41 were prepared (Mockett et al., 1984). The gammaglobulin fraction of ascitic fluids containing either anti-spike or anti-membrane monoclonal antibodies was isolated by salt precipitation using a final concentration of 18% (wt./vol.)  $\text{Na}_2\text{SO}_4$ . For the spike immunoadsorbent 5.6 mg of gammaglobulin was coupled to 0.75 mg of CNBr-Sephrose 4B (Pharmacia) according to the manufacturers' instructions and for the membrane immunoadsorbent 5.5 mg was coupled to the same amount of gel. Unreactive groups on the gel were blocked using 1 M ethanolamine, pH 8.0, and any non-covalently bound proteins were

removed by repeated washings with 0.1 M NaHCO<sub>3</sub> buffer, pH 8.3, containing 0.5 M NaCl and 0.1 M acetic acid buffer, pH 4.4, containing 0.5 M NaCl. The immunoadsorbent was stored in PBS containing 0.2% NaN<sub>3</sub> at 4°C until used. It was washed twice with 3 M NH<sub>4</sub>SCN in PBS containing 0.1% octylglucoside, four times with PBS and twice with PBS containing 2% NP40 before use. All wash volumes were 10 ml.

#### *Affinity chromatography*

The solubilised virus preparation was mixed with the immunoadsorbent for 16 h at 4°C using a rotary stirrer. The gel was poured into a chromatography column and washed with PBS containing 0.1% NP40 (40 ml) and PBS containing 0.1% octylglucoside (10 ml). 3 M NH<sub>4</sub>SCN in PBS containing 0.1% octylglucoside was added and 10 fractions of 1 ml collected. The absorbance at 280 nm of each of the fractions was read using a SP1800 Pye Unicam spectrophotometer. The fractions in the absorbance peak were dialysed against PBS. A sample of each fraction was then subjected to electrophoresis in a polyacrylamide gel. Those fractions containing detectable viral protein were pooled and constituted the purified protein preparation.

#### *Polyacrylamide gel electrophoresis*

Ten per cent polyacrylamide slab gels containing SDS were used (Laemmli, 1970) and after electrophoresis samples were stained first with Coomassie Brilliant Blue R-250 and then silver (Morrisey, 1981).

#### *Antiserum production in rabbits*

New Zealand White rabbits were used. Samples of purified proteins were mixed with an equal volume of Freund's complete adjuvant and inoculated intramuscularly into the rabbits. A similar inoculation was given 1 wk later. After 5 wk the same antigen in incomplete Freund's adjuvant was inoculated subcutaneously. Five months later blood was collected from the ear vein and the resulting serum stored at -20°C until used.

#### *Antiserum production in chickens*

The Houghton Poultry Research Station line of Rhode Island Red chickens was used. IBV (M41) was inoculated intratracheally (500 ciliostatic dose fifty (CD 50); Darbyshire et al., 1979) into 8 chickens and sequential blood samples were taken from the wing vein (see Fig. 2 for times after inoculation). Sera from the blood samples were stored at -20°C until used. A serum pool for each time of sampling was made by mixing an equal volume of serum from each of the eight samples.

#### *Enzyme-linked immunosorbent assay (ELISA)*

Three different antigens were used for the ELISAs: spike protein, membrane protein and IB virus. The purified spike and membrane proteins were used at a dilution of 1 : 20 in carbonate buffer whilst the purified IB virus was used at a 1 : 100 dilution. Antigens

were adsorbed for 1 h at 37°C. In the second step chicken sera were serially diluted in 0.5 M NaCl containing 0.5% NP40 (saline/NP40) from an initial 6.64 log<sub>2</sub> dilution. Any specifically bound antibody was detected using a rabbit anti-chicken IgG serum (1 : 300) and a goat anti-rabbit IgG alkaline phosphatase conjugate (1 : 1,000) (Sigma), both diluted in saline/NP40. The substrate was *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer and the reaction stopped using 3 M NaOH. Each step was for 30 min at 37°C, the reaction volumes 50 µl and the plate was washed three times with PBS containing 0.1% Tween 20 between each step. Titres were calculated graphically (Mockett and Darbyshire, 1981) using an absorbance value of 0.1 as the cut off.

#### *Neutralization test*

The chicken antisera were tested for neutralizing antibody to IBV as described by Darbyshire et al. (1979). Rabbit sera were precipitated with 18% (wt./vol.) Na<sub>2</sub>SO<sub>4</sub> and the resulting gammaglobulin tested because rabbit sera have high concentrations of non-specific inhibitors of IB virus replication.

## RESULTS

The viral proteins purified by affinity chromatography are shown in Fig. 1. The spike protein, which is composed of two polypeptides, was the only protein detected in the two fractions shown using the sensitive silver staining procedure. Similarly the membrane protein was not contaminated with other proteins, although this protein did not stain as well as the spike. There were other stained bands present, but these are artifacts sometimes observed, even in the absence of protein, with this staining procedure. The purification was highly reproducible. The purified viral proteins were inoculated into rabbits and the gammaglobulin fraction of the antisera tested for neutralizing activity. Only the anti-spike gammaglobulin neutralized the virus. However the membrane protein was a good immunogen because the rabbit anti-membrane sera tested in the ELISA had high activity against the whole virus; in fact the titres were higher than those of the rabbit anti-spike sera (see Table 1).

The results of testing sera from IBV-infected chickens for antibody to spike and membrane proteins showed that both anti-spike and anti-membrane antibodies were produced early after infection (see Fig. 2). Peak titres were between 9 and 11 days after infection. The antibody response to the whole virus had a similar profile. However, the

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Fig. 1. Affinity chromatography used to purify the spike and membrane proteins of IBV. Two affinity columns were made by linking an anti-spike monoclonal antibody to one lot of CNBr-Sepharose and an anti-membrane monoclonal antibody to another. Detergent-solubilised virus was mixed with the columns and any specifically bound material was eluted using 3 M NH<sub>4</sub>SCN and dialysed against PBS buffer. The eluted material was subjected to PAGE and silver stained. Material eluted from the anti-spike column, lanes B1, B2, and the anti-membrane column, lanes C1, C2, is shown. Purified IBV is shown in lanes labeled A, stained using Coomassie Brilliant Blue R-250.

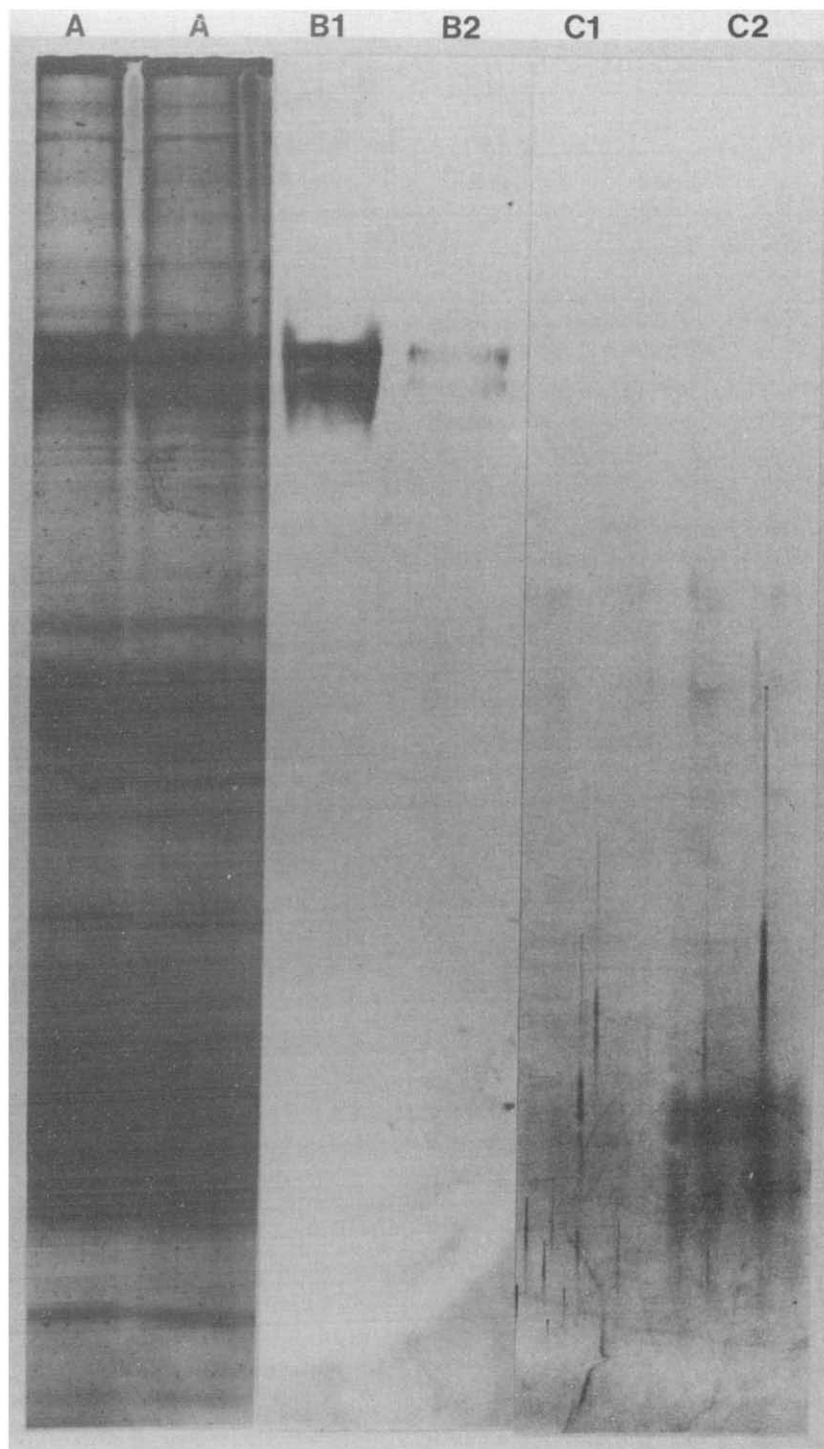


TABLE 1

Reactions of the rabbit anti-spike and anti-membrane sera in the serum neutralization (SN) test and ELISA

Serological test	Rabbit sera				
	Control	Anti-spike	Anti-spike	Anti-membrane	Anti-membrane
SN	- <sup>a</sup>	6.61	5.18	-	-
ELISA (whole virus)	-	12.62	8.73	15.28	15.61

<sup>a</sup> Negative (SN ≤ 4.00, ELISA ≤ 5.64) SN and ELISA values are log<sub>2</sub>.

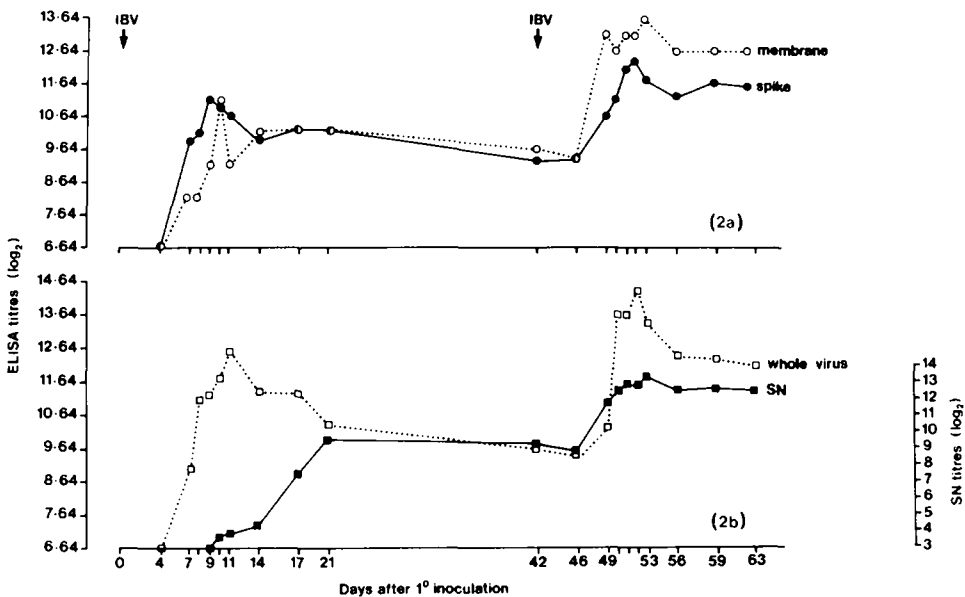


Fig. 2. IBV-specific antibody titres, determined by ELISA using whole virus, spike or membrane protein and SN, of sera from chickens inoculated with IBV. The profiles of specific antibodies to spike protein (—) and membrane protein (.....) detected by ELISA are shown in 2a and antibodies to the whole virus (□) (detected by ELISA) and neutralizing antibodies (■) are shown in 2b. The pooled serum was tested for each time of sampling.

pattern of neutralizing antibody was different. Neutralizing antibody was first detected at 10 days and reached a peak at about 21 days after infection.

The second inoculation of virus induced an anamnestic antibody response. The ELISA detected a similar increase in antibody titres using spike, membrane and whole virus – the peak was at 10–11 days after infection. The neutralizing antibody response was also similar and the peak titres were at 11 days after infection which contrasted to the slow rise to the peak titres after the primary inoculation.

## DISCUSSION

This paper describes the application of affinity chromatography using monoclonal antibodies for the purification of the two viral structural proteins present at the surface of the IB virion – spike and membrane. A previous report has described procedures for the purification of these viral proteins and also nucleocapsid protein, the only other major structural protein (Cavanagh, 1983). IBV was solubilised in NP40 detergent and centrifuged in a sucrose gradient containing this detergent in order to purify the nucleocapsid protein. The addition of 1 M NaCl to the sucrose solutions was required for the purification of the spike and membrane proteins, as they co-migrated in gradients containing low salt concentrations. However, the nucleocapsid protein could not be purified in gradients containing high salt concentrations. The yield of material from these gradients was relatively low, due to the limited number of fractions which contained purified viral components. In other studies (Cavanagh, 1984) purified spike material contained some nucleocapsid protein and the membrane preparation contained other proteins which were thought to be of cellular origin.

There are a number of advantages in using affinity chromatography. By making use of the specificity of the antibody pure material can be isolated, even from a crude mixture of proteins. The method is very quick and easy and the immunoabsorbent can be used several times. Thus, relatively large amounts of purified material can be obtained. The availability of spike and membrane proteins in a highly purified form will allow more biochemical, structural and immunological studies to be done.

The conditions used to solubilise the virus did not dissociate the two spike polypeptides, therefore, both S1 and S2 were detected in the material eluted from the anti-spike immunoabsorbent.

The results of experiments using the rabbit antisera to the viral proteins confirmed the biological importance of the spike protein as only antibodies to this protein neutralized the infectivity of the virus.

The chicken, about 10 days after an IBV infection, has antibodies to both the spike and membrane proteins in its serum but only very low concentrations of neutralizing antibodies. The profile of neutralizing antibodies shown in this paper agrees with previous published findings (Holmes, 1977; Mockett and Darbyshire, 1981; Hawkes et al., 1983). The results show that anti-spike antibodies produced early after infection are non-neutralizing, as assessed by our *in vitro* technique. This raises the question as to the function of these antibodies in the chicken. Previous evidence has shown only the spike protein to be capable of eliciting neutralizing antibodies. There is a possibility that the anti-spike antibodies could be neutralizing *in vivo* and the function of the anti-membrane antibodies could be similar. The possible role of these antibodies in protection remains to be resolved.

Purified viral proteins can be used to determine which is responsible for protecting the chicken from infection. Protection tests such as the ciliostasis (Darbyshire, 1980) and the mixed infection (*Escherichia coli* and IBV) (Smith et al., 1985) tests are



available. It is only by using methods such as affinity chromatography that sufficiently large amounts of pure viral proteins can be made available to enable such tests to be done.

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