Expression of the Infectious Bronchitis Virus Spike Protein by Recombinant Vaccinia Virus and Induction of Neutralizing Antibodies in Vaccinated Mice

By FIONA M. TOMLEY,* A. P. ADRIAN MOCKETT, MICHAEL E. G. BOURSNELL, MATTHEW M. BINNS, JANE K. A. COOK, T. DAVID K. BROWN† AND GEOFFREY L. SMITH†

Houghton Poultry Research Station, Houghton, Huntingdon, Cambridgeshire PE17 2DA and 1Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP U.K.

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SUMMARY

A cDNA clone of the infectious bronchitis virus (IBV) spike protein gene has been recombined into vaccinia virus. Cells infected with the recombinant virus synthesized IBV spike antigen which was recognized by antibody raised against purified spike protein. Immunofluorescence showed that the IBV spike antigen was transported to the infected cell surface membrane and immunoprecipitation showed the presence of the glycosylated 180K mol. wt. polypeptide precursor of the two spike subunits S1 and S2 that comigrated with this antigen from IBV-infected cells. Vaccinated mice produced antibody that recognized the IBV spike antigen by ELISA and which neutralized IBV infectivity as shown by ciliostasis tests on tracheal organ cultures.

INTRODUCTION

Infectious bronchitis virus (IBV) is a respiratory pathogen of the fowl which is of economic importance to the poultry industry. IBV-associated disease is controlled by the use of 'live' attenuated and inactivated virus vaccines. Problems are encountered in the use of each type of vaccine and there is scope for improvements in both efficacy and the minimization of side effects. The emergence of variant strains of IBV may also necessitate changes in the vaccination regimes currently employed by the poultry industry.

IBV is the type species of the Coronaviridae, a family of large, positively stranded RNA viruses, and possesses three major structural proteins (for review, see Siddell et al., 1983). The nucleocapsid protein is found in close association with the viral RNA. The membrane protein is a heterogeneous glycosylated polypeptide that protrudes slightly from the virion surface. The spike protein is the major surface projection and gives the virus its characteristic 'corona'. Each projection comprises two or three copies of two glycopolypeptides, S1 (90K) and S2 (84K) which are derived by proteolytic cleavage of the precursor spike protein (Cavanagh, 1983 a, b; Stern & Sefton, 1984). Nucleotide sequencing of the spike gene has predicted an N-terminal hydrophobic signal sequence which is not present in the mature protein and a near C-terminal hydrophobic region which may act to anchor the spike in the viral envelope (Binns et al., 1985).

Virus-neutralizing antibody is directed against the spike protein. Strain-specific monoclonal antibodies that neutralize virus infectivity and inhibit haemagglutination bind to S1 protein (Mockett et al., 1984). Chickens inoculated with gradient-purified spike protein and rabbits inoculated with immunoaffinity-purified spike protein both produce virus-neutralizing
antibodies (Cavanagh et al., 1984; Mockett, 1985). No evidence implicates other virus proteins as targets for antibody-mediated virus neutralization. Despite this, protective immune responses have not yet been obtained in birds inoculated with purified spike protein. This may be attributable to the lack of sufficient quantity of purified spike protein for immunization experiments.

An alternative approach to the production of spike protein free of other IBV antigens is to express its gene in a eukaryotic vector system. We chose to use vaccinia virus since the recombinant virus retains its infectivity, and has a broad host range. Additionally, other eukaryotic viral glycoproteins have been correctly glycosylated and transported within recombinant vaccinia virus-infected cells (for review, see Mackett & Smith, 1986). The infectivity of recombinant vaccinia viruses has enabled animals to be vaccinated and protected against subsequent challenge with influenza virus (Smith et al., 1983b), herpes simplex virus (Paolelli et al., 1984; Cremer et al., 1985), hepatitis B virus (Moss et al., 1984), rabies virus (Kiény et al., 1984; Wiktor et al., 1984), vesicular stomatitis virus (Mackett et al., 1985) and human respiratory syncytial virus (Ball et al., 1986; Elango et al., 1986). This protection may be attributable to the ability of the recombinant viruses to induce both antibody and cytotoxic T cell responses specific for the foreign gene product (Bennink et al., 1984, 1986; Wiktor et al., 1984; Yewdell et al., 1985).

In this paper we show that a cDNA clone of IBV spike gene directs synthesis of spike antigen after insertion into vaccinia virus. The spike antigen is glycosylated and transported to the cell surface where it is recognized by specific antisera. Mice vaccinated with the recombinant virus produced antibodies against spike that can neutralize virus infectivity as shown by a ciliostasis test in tracheal organ culture.

METHODS

**Virus, cell cultures and plasmids.** Vaccinia virus, WR strain, was used throughout. African green monkey kidney cells (CV-1) and human 143 thymidine kinase-negative (TK-) cells were grown in Eagle's MEM, supplemented with 10% foetal calf serum. Plaque assays and virus purifications were as described by Mackett et al. (1985). Plasmid pMB179 containing the entire spike gene of IBV Beaudette within a 5.3 kb insert, and plasmid pGS20 containing the vaccinia virus 7.5K promoter flanked by TK sequences have been described (Binns et al., 1985; Mackett et al., 1984).

**Molecular cloning.** Restriction endonucleases and DNA-modifying enzymes were used according to manufacturers' instructions. Recombinant DNA manipulations were carried out essentially as described by Maniatis et al. (1982). The recombinant plasmid pSB1 was constructed by excising the spike-coding sequence from pMB179 with TthIII and XbaI, purifying the fragment from agarose (Dretzen et al., 1981) and ligating it into SmaI-cut, alkaline phosphatase-digested pGS20. The orientation of the inserted spike fragment was checked by restriction and the precise sequence surrounding the junction between the 7.5K vaccinia virus promoter and the spike-coding sequence checked by chemical sequencing (Maxam & Gilbert, 1980).

**Construction of recombinant virus.** A TK- recombinant vaccinia virus containing the IBV spike gene was generated by transfection of CV-1 cells infected with wild-type (wt) vaccinia virus with plasmid pSB1 using previously described methods (Mackett et al., 1984). Lysates from cells infected with TK- recombinant plaques were screened with nick-translated 32P-labelled pMB179 or with anti-spike antibody followed by 125I-labelled staphylococcal Protein A (Mackett et al., 1985). Virus was plaque-purified twice, amplified and finally purified on sucrose density gradients. The recombinant virus was called vSP19-1.

**Immunofluorescence.** Indirect immunofluorescence tests were carried out on monolayers of CV-1 cells grown on microscope slides and infected at 30 p.f.u./cell with either wt vaccinia virus or recombinant vSP19-1 virus. Cells were fixed at 14 h post-infection with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed monolayers were incubated with 0.5% bovine serum albumin in PBS at 20º C for 1 h and then with rabbit anti-IBV Beaudette serum for 1 h at 20º C. After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti-rabbit antibody for 1 h at 20º C, washed with PBS and photographed under u.v. illumination.

**Immunoprecipitation.** CV-1 cells (25 cm² bottles) were infected with wt vaccinia virus or recombinant vSP19-1 virus at 30 p.f.u./cell, with IBV at 5 p.f.u./cell or mock-infected. For radiolabelling, the cells were washed and incubated for 30 min with methionine-free medium, then incubated with 100 µCi (per bottle) of 35S]methionine in methionine-free medium from 3 to 6 h post-infection. Lysates and immunoprecipitations were prepared as detailed by Smith et al. (1983a).

**Animal inoculations.** Six-week-old BALB/c mice were used. Wt vaccinia virus or recombinant vSP19-1 was inoculated intraperitoneally (i.p.) into groups of six mice (5 x 10⁷ p.f.u./mouse). Serum samples were collected at 3
weeks post-vaccination when the mice were revaccinated with the appropriate vaccinia virus (2 x 10^8 p.f.u./mouse). Further serum samples were collected 2 and 4 weeks later.

**Examination of mouse sera by ELISA.** Purified IBV Beaudette was prepared from infected allantoic fluid as described previously (Mockett & Cook, 1986). A 1:50 dilution of virus in 50 mM-carbonate buffer pH 9.6 was used to coat the wells of a PVC microelisa plate (Dynatech Laboratories, Alexandria, Va., U.S.A.). After 18 h at 4°C, the plate was washed in PBS containing 0.1% Tween 20 (PBST) using an automatic microelisa plate washer (Skatron). Serial dilutions of mouse sera, diluted in PBST from an initial 5-64 log_2 dilution, were added. Specifically bound mouse antibodies were detected using a goat anti-mouse IgG conjugate labelled with alkaline phosphatase (Sigma) at 1:1000 in PBST and p-nitrophenyl phosphate substrate (1 mg/ml in diethanolamine buffer). The plate was washed with PBST between each step. The volume of each reagent was 50 µl and each incubation was for 30 min at 37°C. The final reaction was stopped with 3 M-NaOH and A_05 measured in an automatic microelisa reader (Dynatech).

**Neutralization test in tracheal organ cultures.** A standard ciliostasis test was performed using chick embryo tracheal organ cultures (Darbyshire et al., 1979). Doubling dilutions of mouse anti-vaccinia virus or anti-recombinant vSP19-1 sera were reacted with an equal volume of a single concentration of IBV strain M41 (100 ciliostatic doses) for 30 min at room temperature. Residual infectivity was assayed in tracheal organ cultures (five tubes per dilution) and neutralizing antibody titres were calculated by the method of Reed & Muench (1938).

**RESULTS**

**Construction and genome analysis of recombinant vSP19-1**

The IBV spike gene (3672 bases) was inserted downstream of a vaccinia virus promoter in plasmid insertion vector pGS20 (Mackett et al., 1984). This placed the initiating methionine codon of the IBV gene approximately 80 nucleotides away from the early RNA start site of the 7.5K vaccinia virus promoter. Forty-two of these nucleotides were derived from upstream of the spike gene open reading frame. Nucleotide sequencing (Maxam & Gilbert, 1980) confirmed the orientation of the IBV gene and that there were no additional ATG codons introduced into this 80 nucleotide region during cloning. Since the 7.5K gene promoter is transcribed both early and late during infection, but from different initiation sites, we expected the IBV spike antigen to be expressed throughout infection. Plasmid pSB1 was used to generate recombinant virus vSP19-1 using previously described methods (Mackett et al., 1984).

The position of the IBV gene within recombinant vSP19-1 was analysed by extraction of genomic DNA from purified vSP19-1, restriction endonuclease digestion, agarose gel electrophoresis and Southern blot hybridizations (Fig. 1). Using a 32P-labelled probe for the vaccinia virus HindIII J fragment a band of 5 kb was detected in HindIII-cut wt virus DNA. In HindIII-cut vSP19-1 DNA this 5 kb was absent and replaced by bands of 5-6 and 3-4 kb corresponding to the predicted structure of this region of the genome. Hybridization of a duplicate filter with 32P-labelled pMB179 DNA showed that only the 5-6 and 3-4 kb bands contained IBV cDNA. These data confirmed that the IBV spike gene was inserted into the TK gene within the HindIII J fragment.

**Expression of the spike antigen**

To test for expression of the IBV spike gene we first used indirect immunofluorescence on recombinant vSP19-1-infected cells (Fig. 2). Cells were fixed with freshly prepared paraformaldehyde, to prevent membrane permeabilization, and then reacted with rabbit serum raised against IBV Beaudette followed by fluorescein-conjugated goat anti-rabbit serum. As shown in Fig. 2, cells infected with recombinant virus vSP19-1 showed strong surface fluorescence whereas wt virus-infected cells showed only background fluorescence. These data demonstrate expression of the IBV spike antigen and indicate that transport of the spike protein to the cell surface is attributable to intrinsic properties of this protein itself and not to a cooperative effect of other IBV proteins. Similar data were obtained using rabbit antiserum raised against purified spike antigen.

To analyse the polypeptide composition of the spike antigen in vSP19-1-infected cells, [35S]methionine-labelled cell lysates were immunoprecipitated with rabbit anti-Beaudette IBV serum. Fig. 3 shows that a polypeptide of approximately M_r 180000 (180K) was immunoprecipitated from CV-1 cells infected with recombinant virus vSP19-1 (lane 10) but not
Fig. 1. Genomic DNA was extracted from purified recombinant vaccinia virus (lanes 1 and 3) or wt virus (lanes 2 and 4) and digested with HindIII. DNA fragments were resolved by electrophoresis through a 0.8% agarose gel, then blotted bidirectionally onto duplicate nitrocellulose sheets. After denaturation and fixation of the DNA to the sheets they were probed with either 32P-labelled DNA from vaccinia virus HindIII J fragment (lanes 1 and 2) or 32P-labelled DNA from pMB179 corresponding to the IBV spike gene. An autoradiograph is shown. The sizes of the DNA fragments are indicated in kb and were calculated using the vaccinia HindIII J fragments as markers.

from wt vaccinia virus-infected (lane 9) or mock-infected (lane 8) cells. This band comigrates with spike antigen precursor synthesized in IBV-infected CV-1 cells (lane 12). The similar size of these two bands indicates that the primary translation product had been glycosylated to the same extent in IBV- and recombinant vaccinia virus-infected CV-1 cells. More direct evidence for glycosylation came from the results of labelling and immunoprecipitation in the presence of tunicamycin (lanes 11 and 13). In recombinant vaccinia virus- and IBV-infected cells tunicamycin greatly reduced the amount of the 180K protein compared with controls while having little effect on overall level of protein synthesis (lanes 1 to 6). From these experiments it is clear that the 180K spike precursor was not significantly cleaved into S1 and S2 subunits in CV-1 cells infected with either recombinant vaccinia virus or IBV. Pulse-chase experiments with recombinant vaccinia virus-infected cells confirmed that no significant cleavage to S1 and S2 subunits occurred even after a 6 h chase. Similar data were obtained in human 143 TK- cells. Despite lack of cleavage, the spike protein evidently was transported to the cell surface indicating that transport is not dependent upon cleavage. Similar observations have been made for the influenza virus haemagglutinin (HA) expressed by transfection (White et al., 1982) or in
recombinant vaccinia virus-infected cells (Smith et al., 1983b). Despite lack of cleavage the spike polypeptide still reacted strongly with anti-Beaudette virus antibodies.

Inoculation of mice

Mice were vaccinated with either wt vaccinia virus or recombinant vSP19-1 at 0 and 3 weeks and sera were collected at 3, 5 and 7 weeks after the first vaccination. These sera were tested for anti-IBV antibodies by ELISA measurement, and for anti-IBV neutralizing antibodies using a ciliostasis test. The results are shown in Table 1. In the ELISA the target antigen was immobilized IBV Beaudette, the homologous virus strain. Three weeks after the first vaccination with recombinant vSP19-1, the mice had produced low but measurable titres of anti-IBV antibodies. These increased considerably after the second inoculation and were around eightfold higher at 7 weeks. Sera from mice inoculated with wt vaccinia virus remained at background level. In the neutralization test, it was not possible to use IBV Beaudette as the target virus since this non-pathogenic laboratory strain is only very poorly ciliostatic. However, IBV Beaudette is antigenically related to the pathogenic strain M41 which causes ciliostasis and so can be used in the neutralization test. At 3 weeks after the first inoculation, mouse anti-vSP19-1 serum had a neutralization titre of 1:25 against the M41 strain compared with a titre of 1:10 for sera from mice inoculated with wt vaccinia virus. By 7 weeks after the first, and 4 weeks after the second inoculation, the neutralization titre of the mouse anti-vSP19-1 serum had risen to over 1:40, whereas that of the anti-wt serum remained at 1:10. The neutralization titres of sera from mice inoculated with recombinant virus were therefore low, but substantially higher than those of the controls.

Discussion

The spike protein of IBV is the target for virus-neutralizing antibodies (Cavanagh et al., 1984; Mockett, 1985). Although other virus polypeptides may play a role in protection and recovery from infection in the chicken, the spike is an excellent candidate for use in novel IBV vaccines.
Fig. 3. Immunoprecipitation of IBV spike antigen from infected cells. CV-I cells were mock-infected (lanes 1 and 8), or infected at 30 p.f.u./cell with wt vaccinia virus (lanes 2 and 9) or recombinant virus vSP19-1 (lanes 3, 4, 10 and 11), or at 5 p.f.u./cell with IBV strain Beaudette (lanes 5, 6, 12 and 13). At 3 h post-infection with vaccinia virus or 6 h post-infection with IBV, cells were washed and incubated in methionine-free medium for 30 min. [3S]Methionine (100 μCi/ml) was added for 3 h in the absence (lanes 1 to 3, 5, 8 to 10 and 12) or presence (lanes 4, 6, 11 and 13) of tunicamycin at 10 μg/ml. After washing three times in PBS, cells were lysed in NP40 buffer and lysates mixed with rabbit anti-IBV Beaudette serum. Immunoprecipitated (lanes 8 to 13) or total polypeptides (lanes 1 to 6) were separated by electrophoresis through a 12.5% polyacrylamide gel and an autoradiograph was prepared from the dried gel. Sizes ($\times 10^{-3}$) of protein mol. wt. markers (lane 7) and position of the spike antigen (S) are indicated.

Table 1. Induction of anti-spike antibodies and virus-neutralizing antibodies in mice inoculated with recombinant vSP19-1

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>ELISA titres on day</th>
<th>Neutralization titres* on day</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 21 35 49</td>
<td>0 21 35</td>
</tr>
<tr>
<td>Vaccinia (wt)</td>
<td>0† 0 0 0</td>
<td>&lt;10 &lt;10 &lt;10</td>
</tr>
<tr>
<td>vSP19-1</td>
<td>0 180 820 1350</td>
<td>&lt;10 &gt;25 &gt;40</td>
</tr>
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* Virus neutralization titres are the dilutions of sera at which 100 median ciliostatic doses of IBV strain M41 are neutralized in chicken tracheal organ cultures.
† All titres are mean values from two individuals. Mice were inoculated with $5 \times 10^7$ p.f.u. of purified wt vaccinia virus or recombinant vSP19-1 at day 0 and a further $2 \times 10^8$ p.f.u. at day 21.
In this paper we describe the expression of a cDNA clone of the IBV Beaudette spike gene in recombinant vaccinia virus. Cells infected with the recombinant vaccinia virus express the IBV spike gene as a 180K glycosylated polypeptide which is transported to the cell surface membrane. During productive IBV infection the 180K polypeptide is cleaved into S1 and S2 subunits. This did not occur in recombinant vaccinia virus-infected or IBV-infected CV-1 cells but the spike protein was nonetheless transported to the cell surface membrane. Clearly this demonstrates that spike cleavage is not required for transport. Similar observations have been made with the influenza virus HA expressed from a recombinant vaccinia virus expressing HA. The lack of cleavage did not prevent recognition by spike-specific antibodies or induction of virus-neutralizing antibodies. Attempts to cleave the spike protein on the infected cell membrane by addition of exogenous trypsin were not successful.

The immunogenicity of the spike protein expressed by recombinant vaccinia virus was demonstrated by vaccination of mice. Sera taken from groups of mice inoculated with recombinant vSP19-1 produced antibody which bound to purified IBV Beaudette in ELISA, whereas sera from wt vaccinia virus-vaccinated animals did not. That this antibody was neutralizing was demonstrated by its ability to neutralize the infectivity of the virulent M41 strain of IBV in a neutralization test in tracheal organ culture. Cross-neutralization between Beaudette and M41 is poor and Chomiak et al. (1963) reported that Beaudette antiserum did not neutralize M41 infectivity in chicken embryos. Despite this, sera taken from vaccinia virus recombinant vSP19-1-vaccinated mice contained antibodies which neutralized M41 IBV infectivity as shown by the ciliostasis test. These data indicate that the IBV spike protein expressed by a recombinant poxvirus vector could potentially be used as a live IBV vaccine. To pursue this approach further it will be necessary to express spike gene cDNAs of pathogenic IBV strains in poxvirus vectors whose natural hosts include birds.

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REFERENCES


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