Recombinant vaccinia viruses expressing the F, G or N, but not the M2, protein of bovine respiratory syncytial virus (BRSV) induce resistance to BRSV challenge in the calf and protect against the development of pneumonic lesions

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The immunogenicity and protective efficacy of recombinant vaccinia viruses (rVV) encoding the F, G, N or M2 (22K) proteins of bovine respiratory syncytial virus (BRSV) were evaluated in calves, the natural host for BRSV. Calves were vaccinated either by scarification or intratracheally with rVV and challenged 6 to 7 weeks later with BRSV. Although replication of rVV expressing the F protein in the respiratory tract was limited after intratracheal vaccination, the levels of serum and pulmonary antibody were similar to those induced following scarification. The serum antibody response induced by the F protein was biased in favour of IgG1 antibody, whereas the G and the N proteins induced similar levels of IgG1:IgG2, and antibody was undetectable in calves primed with the M2 protein. The F protein induced neutralizing antibodies, but only low levels of complement-dependent neutralizing antibodies were induced by the G protein, and antibody induced by the N protein was not neutralizing. The F and N proteins primed calves for BRSV-specific lymphocyte proliferative responses, whereas proliferative responses were detected in calves primed with the G protein only after BRSV challenge. The M2 protein primed lymphocytes in only one out of five calves. Although there were differences in the immune responses induced by the rVVs, the F, G and N, but not the M2, proteins induced significant protection against BRSV infection and, in contrast with the enhanced lung pathology seen in mice vaccinated with rVV expressing individual proteins of human (H)RSV, there was a reduction in lung pathology in calves.

Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory disease in both cattle and young children (Stott & Taylor, 1985) and the pathology of the disease in both species is remarkably similar (Aherne et al., 1970; Thomas et al., 1984). However vaccination, particularly in children, has not been effective and on occasions certain vaccines, such as formalin-inactivated human (H)RSV, have resulted in exacerbation of disease (Kim et al., 1969). There is a need, therefore, to fully understand the contribution of each RSV protein to immunity to enable a rational approach to vaccine design to be made.

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Vaccinia virus recombinants (rVV) expressing the fusion (F) or attachment (G) proteins of HRSV induce resistance to pulmonary replication of HRSV in mice (Wertz et al., 1987; Stott et al., 1987), cotton rats (Stott et al., 1987; Olmsted et al., 1986) and monkeys (Olmsted et al., 1988). However the same recombinants in chimpanzees, a species in which HRSV disease closely resembles that of man, were much less effective (Collins et al., 1990; Crowe et al., 1993). Furthermore, there are conflicting findings on the effects of these rVV on the development of pulmonary pathology after HRSV challenge. Thus, microscopic lesions in vaccinated cotton rats are similar to those observed in control animals undergoing a primary HRSV infection, whereas increased pulmonary histopathology is observed in vaccinated mice (Connors et al., 1992a; Stott et al., 1987; Openshaw et al., 1992). The exacerbated histopathology observed in vaccinated mice may reflect the greater tendency of the mouse to develop pulmonary lesions upon
reinfection. In contrast, infection in children or calves does not predispose to more severe respiratory disease upon subsequent RSV infection, but leads to less severe disease. These observations highlight the need to investigate the effects of sensitization to individual RSV proteins on RSV infection in a natural host.

Initial studies in calves with the mouse-adapted WR strain of VV expressing the F protein of the A2 strain of HRSV failed to demonstrate evidence of protection against challenge with BRSV. However, the serum antibody response at the time of challenge, 3 weeks after vaccination, was low when compared with that observed in mice vaccinated with the same recombinant (G. Taylor, E. J. Stott & L. H. Thomas, unpublished observations). The poor immune response in calves may have been related to the use of the mouse-adapted WR strain of VV. Therefore rVV were constructed using the Copenhagen (Cop) strain of VV. Recombinant VV-Cop expressing the F protein of BRSV completely protected mice against infection with HRSV (G. Taylor and others, unpublished observations).

In the present study, rVV expressing either the F protein of HRSV or the F, G, N or M2 protein of bovine (b)RSV were compared for their ability to restrict BRSV replication in the respiratory tract of calves and for their effect on the development of lung pathology after challenge with BRSV. We also analysed the effect of route of vaccination on induction of immunity, the class of antibody produced in serum and lungs and the ability of the rVV to prime bovine lymphocytes for a BRSV-specific lymphocyte proliferative response.

Methods

- **Viruses and cells.** Recombinant VV expressing the F protein of the A2 strain of HRSV (vF317) (Wertz et al., 1987) or the F, G or N proteins of the 391-2 strain of BRSV (Lerch et al., 1990, 1991; Amann et al., 1992) were constructed as described previously but using the Copenhagen strain of VV instead of the mouse-adapted WR strain. A cDNA containing the M2 gene was generated by reverse transcription on mRNA extracted from BRSV 391-2-infected bovine nasal turbinate cells followed by PCR amplification. The cDNA was then cloned using standard techniques into the BamHI site of transfer vector pAB 191 (Bail et al., 1986) in the positive orientation with respect to the VV7.5K promoter. Recombinant VVM2bov was generated as described previously using VV-Cop (Wertz et al., 1987; Lerch et al., 1990, 1991; Amann et al., 1992). Expression of the M2 protein from the vector vM2 was confirmed by immunoprecipitation with a monoclonal antibody (MAb 6) specific for the M2 protein (Taylor et al., 1984) of radiolabelled proteins from vM2-infected cells followed by analysis on SDS-PAGE (data not shown). Recombinant VV were grown and assayed on HTK cells. For inoculation of calves, rVV were purified over a 35% sucrose gradient.

- **Animals and experimental design.** Seronegative, Friesian cross calves were produced by hysterotomy and reared without colostrum in a high security, barrier-maintained building. Seven separate experiments were carried out using calves whose ages ranged between 20 and 86 days at the time of vaccination. Calves were randomly allocated to groups and vaccinated by either intradermal scarification (i.d.) or intratracheal (i.t.) inoculation with 1 to 2 × 10⁶ p.f.u. rVV. In experiments 1 and 2, swabs were taken from skin vaccination sites, rectum, nasopharynx and conjunctivae of vaccinated calves daily for 10 days and from one sentinel unvaccinated calf, housed in direct contact with vaccinated calves (experiment 1), to monitor VV shedding. Skin reactions to vaccination were measured. Six to seven weeks after vaccination, calves were challenged with 1 × 10⁵ p.f.u. of BRSV in a volume of 20 ml of 10 ml administered by the i.t. and 10 ml by the intranasal (i.n.) route. Nasopharyngeal swabs were taken at 1–2 day intervals following challenge to monitor BRSV excretion. Serum samples were obtained at weekly intervals and lung wash (LW) samples, obtained by instilling approximately 100 ml phosphate-buffered saline (PBSa) into the lungs via 3 mm portex tubing, were taken immediately prior to vaccination and at the time of challenge. Calves were killed 7 days after challenge by intravenous pentobarbitone Na. Following slaughter, lungs were excised, macroscopic lesions recorded on a standard lung diagram and expressed as % pneumatic consolidation. Three lobes were clamped off (usually right apical, right cardiac and left cardiac lobes) and a tracheobronchial lung wash (TBLW) made with 200 to 300 ml PBSa. The TBLW was centrifuged at 300 g for 15 min and the cells resuspended in 5 ml lung buffer (Taylor et al., 1984) for virus isolation, and a sample of the supernatant was retained for antibody analysis. The clamped lobes were excised and representative portions taken for virology, histology and immunofluorescence. Three pieces of pneumatic lung, taken from different lobes, were homogenized in lung buffer to give a 20% (w/v) suspension. Homogenates were centrifuged at 9000 g for 1 min and the supernatant assayed for virus.

- **Serology.** Antibody responses to BRSV and VV were determined by ELISA (Taylor et al., 1992). The BRSV antigen was composed of detergent-treated CK cells infected with the Snook strain of BRSV. Sham-infected cells treated in the same way were used as control antigen. The VV antigen was composed of detergent-treated HTK cells infected with rVV expressing the F protein of BRSV (vFbov); sham-infected cells treated in the same way were used as control antigen. Isotype-specific antibody responses to BRSV were examined by ELISA as described previously (Taylor et al., 1995). Neutralizing antibodies to BRSV were detected by a plaque reduction assay on secondary CK cells as described previously (Kennedy et al., 1992).

- **Lymphocyte proliferation assay.** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood as described previously (Taylor et al., 1995) and restimulated with BRSV antigen prepared from frozen and thawed CK cells infected with the Snook strain of BRSV (Taylor et al., 1987). Lysate from uninfected CK cells was used as control antigen. After 5 days incubation, cultures were labelled with 1 μCi [methyl-³H]thymidine per well, incubated for a further 18 h and harvested. All assays were performed in triplicate. Because c.p.m. in cultures incubated with control antigen ranged from 84 to 16000, the lymphocyte proliferative responses were expressed as the ratio of c.p.m. in cultures stimulated with BRSV antigen to that in cultures stimulated with control antigen and expressed as the stimulation index (S.I.). An S.I. greater than 3 was considered to be a significant response.

- **Immunolabelling of BRSV in lung.** Lung tissue for immunocyto-LOGY was snap-frozen in liquid nitrogen and stored at −20 °C prior to cryostat sectioning. BRSV antigen was detected using MAbs 8 and 16, specific for the M2 (22 kDa) and F proteins respectively (Taylor et al., 1984, 1992), followed by FITC-conjugated goat anti-mouse IgG (Sigma).
Results

Replication of rVV in calves

Following scarification with vF317 or vFbov, VV was isolated only from swabs taken from the site of scarification up to 9 days post-vaccination and from vaccination scabs up to 16 days post-vaccination. Local skin reactions to both recombinants following scarification were similar and comprised, at peak size 7 days post-vaccination, a raised scab, 10 to 20 cm², which resolved progressively until no lesion was recognizable after 1 month. There was no evidence of spread of VV to a sentinel, unvaccinated animal.

Following i.t. inoculation of calves with vFbov, LW samples were obtained at days 6, 9 and 15 after vaccination in experiment 2, and at days 4, 7 and 14 in experiment 3. VV was isolated in low titres (5 p.f.u./ml) on one day only from the nasopharynx of two of three calves vaccinated by the i.t. route 3 days previously in experiment 2, but not from any of the calves in experiment 3. Virus was not isolated from LW or from any other site examined. There was no increase in the number of cells in the LW after i.t. vaccination (results not shown) and a febrile response was not recorded in any of the calves.

Antibody responses induced by vaccination

The titre of serum antibody to VV was maximal 6 weeks after vaccination and remained fairly constant for the duration of the experiment (Fig. 1a). Serum antibody to BRSV was first detected by ELISA 2 weeks after vaccination with vF recombinants, and continued to increase to a plateau at about 5–6 weeks after vaccination (Fig. 1b). Antibody titres at the time of BRSV challenge (day 42) were slightly lower in calves vaccinated with vF317 compared with those given vFbov. There were no differences in the titres of ELISA antibody to BRSV in sera or LW, from calves vaccinated i.d. with vFbov compared with those vaccinated i.t., 6 weeks previously (Table 1). The titres of neutralizing antibodies to BRSV in sera, 6 weeks after vaccination, ranged from 1:25 to 1:126 (Table 1). Seven days after BRSV challenge there was a 10-fold increase in serum antibody titres as detected by ELISA, and neutralizing titres increased by 10- to 300-fold. Similarly, ELISA antibody in LW increased 10- to 100-fold, 7 days after BRSV challenge.

Seven weeks after vaccination with vFbov, the level of serum antibody to BRSV was about 25-fold less than that induced by vFbov (Table 1) and antibody did not neutralize BRSV in the absence of complement. However, in the presence of rabbit complement, a neutralizing titre of 1:6 was detected at the time of challenge and increased about 5-fold to a titre of 1:40, 7 days after BRSV challenge (Table 1). There was a similar increase in serum ELISA antibody after challenge.

Following vaccination with vN, the titre of ELISA serum antibody was similar to that induced by vG and increased between 2.5- to 10-fold after BRSV challenge (Table 1). Antibody induced after vaccination with vN did not neutralize BRSV. ELISA antibody to BRSV was not detected in sera from calves vaccinated with vM2.

Class-specific antibody responses

Six weeks after vaccination with vFbov, by either the i.d. or i.t. route, IgG1, IgG2 and IgM antibodies to BRSV were detected in sera, although one calf vaccinated i.d. failed to develop IgG2 antibodies to RSV (Fig. 2a). The predominant isotype was IgG1 and the ratio of IgG1:IgG2 in calves vaccinated either i.d. or i.t. was approximately 25:1, excluding the calf which failed to develop IgG2 antibodies. The increase in serum antibody after challenge was mainly due to increases in IgG1, IgG2 and IgA antibodies (Fig. 2a). The increase in serum IgA antibody to BRSV was most pronounced, with undetectable serum IgA at the time of challenge, increasing to a titre of 1:20000, 7 days after challenge (Fig. 2a). However, IgG1 antibody remained the predominant antibody isotype after challenge. In contrast, serum antibody to BRSV was detectable only in low titres, 7 days after infection of control calves, with IgM being the predominant antibody (Fig. 2).
Six weeks after vaccination, low titres of antibody were detected in LW from vF-vaccinated calves (Fig. 2a). Although there were no significant differences in the mean titres of antibody in LW from calves vaccinated by the i.d. or i.t. routes, if experiments 2 and 3 are taken together, IgG1 antibody was detected in LW from five out of six calves vaccinated i.t. compared with only one out of three calves vaccinated i.d. IgA antibody to BRSV was detected in LW from only one calf vaccinated i.t. (Fig. 2a). Seven days after BRSV challenge, LW antibody titres were increased by 10- to 100-fold when compared with those at the time of challenge. This increase was due to increases in titres of all antibody isotypes, with IgG1 and IgA as the predominant isotypes (Fig. 2a). In contrast, antibody titres to BRSV were low or undetectable in LW from control calves 7 days after infection.

Seven weeks after vaccination with vGbov, IgG1, IgG2 and IgM antibodies were detected in sera, with the levels of IgG1 and IgG2 antibodies being similar (IgG1:IgG2 ratio of 1:6:1) (Fig. 2a). Seven days after challenge with BRSV, there was an increase in all antibody isotypes. BRSV-specific serum IgA was undetectable at the time of vaccination and increased to a titre of 1:500, 7 days after BRSV challenge. Antibody was detected only in low titres in LW from vaccinated calves 7 days after BRSV challenge, and IgG2 was the predominant antibody (Fig. 2b).

Vaccination with vN induced IgG1, IgG2 and IgM antibodies to BRSV and the titres of IgG1 and IgG2 antibodies were similar (Fig. 2c). There was only a small increase in serum antibody after BRSV challenge, whereas IgG1 and IgG2 antibodies were increased 10- to 100-fold in the LW after challenge.

**Lymphocyte proliferative responses**

Following vaccination with rVV expressing the F protein, transient BRSV-specific lymphocyte proliferative responses were detected in two out of three calves scarified with vF317, in all three calves scarified with vFbov and in two out of six calves vaccinated i.t. Seven days after challenge, BRSV-specific proliferative responses were detected in five of six calves.

### Table 1. Antibody responses to RSV in calves immunized with rVV expressing individual BRSV proteins and challenged with BRSV

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Vaccine* Route</th>
<th>Mean antibody titres at challenge</th>
<th>Mean antibody titres post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum</td>
<td>Lungwash</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISA†</td>
<td>Neut.†</td>
</tr>
<tr>
<td>1.</td>
<td>vF317 i.d.</td>
<td>3.1 ± 4</td>
<td>2.1 ± 5</td>
</tr>
<tr>
<td></td>
<td>None i.d.</td>
<td>&lt; 1.5</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>2.</td>
<td>vFbov i.d.</td>
<td>3.6 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>vFbov i.t.</td>
<td>3.8 ± 0.01</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>None i.t.</td>
<td>&lt; 1.5</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>3.</td>
<td>vFbov i.t.</td>
<td>3.9 ± 0.2</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>None i.t.</td>
<td>1.8 ± 0.3</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>4.</td>
<td>vGbov i.d.</td>
<td>3.0 ± 0.4</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>None i.d.</td>
<td>&lt; 1.5</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>5.</td>
<td>vNbov i.d.</td>
<td>3.3 ± 0.3</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td></td>
<td>None i.d.</td>
<td>&lt; 1.5</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>6.</td>
<td>vNbov i.d.</td>
<td>2.9 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>None i.d.</td>
<td>&lt; 1.5</td>
<td>ND</td>
</tr>
<tr>
<td>7.</td>
<td>vM2bov i.d.</td>
<td>&lt; 1.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Calves were vaccinated with 1–2 × 10⁶ p.f.u. rVV and challenged 6–7 weeks later with BRSV. Calves were killed 7 days after challenge.
† Mean antibody titre (log₁₀ ± SD) detected by ELISA using BRSV strain 391-2 or Snook as antigen, 6 or 7 weeks after vaccination.
‡ Mean neutralizing antibody titre (log₁₀ ± SD) detected by 50% plaque reduction assay using BRSV, strain Snook, and with rabbit complement (vG-vaccinated calves) or without rabbit complement (vF and vN-vaccinated calves).
ND, Not determined.
vaccinated by the i.d. route with either vF317 or vFbov, compared with only one out of six calves vaccinated by the i.t. route and only one out of seven control animals. The lymphocyte proliferative responses of the vF-primed calves from experiments 1, 2 and 3 have been amalgamated and are shown in Fig. 3(a).
BRSV-specific lymphocyte proliferative responses were not detected in any of the calves following vaccination with vGbov. However, 7 days after BRSV challenge, a significant response was detected in three out of five vaccinated calves compared with none of the five control animals (Fig. 3b). Following vaccination with vNbov, proliferative responses were detected in three out of five calves and, after BRSV challenge, a strong proliferative response was detected in all five vaccinated calves compared with none of the control animals (Fig. 3c). Only one calf vaccinated with vM2 developed a proliferative response and the response in this animal increased almost 10-fold following BRSV challenge (Fig. 3d).

**Effect of vaccination on BRSV infection**

Following challenge of vF-primed calves, BRSV was shed in low titres from the nasopharynx of ten out of twelve vaccinated calves and six out of eight control animals. In experiments 1 and 3, the nasopharynx of some calves was colonized with an antibiotic-resistant strain of *Pseudomonas aeruginosa*, which compromised isolation of BRSV. The low titres recorded may therefore, in some measure, have been due to contamination of cells in the plaque assay. Nevertheless, in experiment 2, BRSV was excreted for fewer days in vaccinated calves when compared with control animals (P < 0.05) (Table 2). Seven days after challenge, BRSV was isolated from the TBLW of four out of nine vaccinated calves compared with four out of five control animals (experiments 2 and 3). If experiments 2 and 3 are combined, there was a significant reduction in virus titres in TBLW from calves vaccinated i.t. with vF compared with control animals (P < 0.02). Titres of virus in lung homogenates taken at slaughter, 7 days after challenge, were low; therefore, lung tissue was examined for the presence of BRSV antigen by immunofluorescence using MAbs specific for RSV. Three representative sections from each of three lung lobes were examined from each calf. Virus antigen was detected in significantly fewer lung samples of vF-vaccinated animals when compared with controls in all three experiments (Table 2). There was widespread distribution of BRSV antigen in the majority of the lung samples taken from the control animals (Fig. 4a), whereas viral antigen in the lungs of vaccinated calves was limited to two or three areas containing less than a dozen infected cells (Fig. 4b).

Following challenge of vG-primed calves, BRSV was isolated from the nasopharynx of all calves and there was no difference in either the levels of virus isolated or in the duration of virus excretion from vaccinated calves when compared with controls. Although virus was not isolated from the TBLW of either vG-primed or control calves, virus antigen was detected in significantly fewer lung sections from vG-primed calves compared with controls (Table 2).

Calves were vaccinated with either 1 or 2 × 10^8 p.f.u. of
Effect of vaccination on lung lesions

The mean % pneumonia consolidation, 7 days after challenge with BRSV, in experiments 1 to 6 was greater in control than in vaccinated calves, whereas in experiment 7, the extent of pneumonia consolidation in calves vaccinated with vM2 was similar to that seen in control animals (Table 2). If the results from experiments 1 to 3 are combined, there is a significant reduction in the extent of pneumonia lesions in the vF-primed calves when compared with controls ($P < 0.001$). Similarly, pneumonia lesions in vG-primed calves were significantly less than those observed in control animals ($P < 0.05$), and, if experiments 5 and 6 are combined, pneumonia lesions in vN-primed calves were significantly less than those in controls ($P < 0.05$).

Pulmonary histopathology of control animals in all seven experiments revealed the typical proliferative, non-suppurative exudative alveolitis and bronchiolitis associated with BRSV infection (Fig. 5, Fig. 6b). Bronchiolitis and alveolitis was also seen in all five calves vaccinated with vM2. However, in animals vaccinated with vF, vG or vN the histopathology was essentially restricted to peribronchial and alveolar lymphoid cell infiltration, which in the majority of calves, particularly those primed with vG, appeared as peribronchial lymphoreticular hyperplasia (Fig. 6a). This enhanced activity of

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**Table 2. Effect of immunization with rVV expressing RSV proteins on resistance of calves to BRSV**

Significant protection at $P < 0.05$ (*); $P < 0.025$ (**); $P < 0.001$ (***) $; P < 0.001$ (****). Data were analysed using linear logit regression analysis for RSV in lungs, Student's $t$-test for virus titres and the Mann-Whitney U-test for pneumonia lesions. No, Not determined.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Vaccine of calves (p.f.u.)</th>
<th>Mean duration (days)</th>
<th>Virus peak titre†</th>
<th>LW virus titre†</th>
<th>RSV in lungs§</th>
<th>% pneumonia lesions¶</th>
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<tbody>
<tr>
<td>1.</td>
<td>vF317 i.d. 5</td>
<td>1.7 ± 0.6</td>
<td>ND</td>
<td>ND</td>
<td>9/27*</td>
<td>1 ± 1.6</td>
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<tr>
<td></td>
<td>None</td>
<td>0.3 ± 0.6</td>
<td>ND</td>
<td>ND</td>
<td>26/27</td>
<td>5 ± 2.5</td>
</tr>
<tr>
<td>2.</td>
<td>vFbov i.d. 5</td>
<td>1.7 ± 0.6*</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.7</td>
<td>8/27***</td>
<td>&lt; 1</td>
</tr>
<tr>
<td></td>
<td>vFbov i.t. 5</td>
<td>1.3 ± 0.6*</td>
<td>0.7 ± 0.2</td>
<td>&lt; 0.7</td>
<td>5/27***</td>
<td>&lt; 1</td>
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<tr>
<td></td>
<td>None</td>
<td>3.0 ± 0.0</td>
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<td>14/18</td>
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<td>3.</td>
<td>vFbov i.t. 5</td>
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<td>&lt; 0.7</td>
<td>0/27***</td>
<td>0</td>
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<td>1.7 ± 0.6</td>
<td>1.8 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>10/27*</td>
<td>2 ± 1.3</td>
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<td>4.</td>
<td>vGbov i.d. 5</td>
<td>2.4 ± 1.1</td>
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<td>&lt; 0.7</td>
<td>1/13**</td>
<td>2 ± 2.0*</td>
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<td></td>
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<td>2.6 ± 0.9</td>
<td>1.5 ± 0.4</td>
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<td>10/15</td>
<td>7 ± 3.0</td>
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<td>5.</td>
<td>vNbov i.d. 5</td>
<td>3.8 ± 1.3</td>
<td>1.1 ± 0.5</td>
<td>&lt; 0.7</td>
<td>4/15*</td>
<td>1 ± 1.0</td>
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<td>None</td>
<td>4.2 ± 0.8</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 1.4</td>
<td>12/15</td>
<td>6 ± 5.0</td>
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<td>1.4 ± 0.9***</td>
<td>1.8 ± 0.7</td>
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<td>7.</td>
<td>vM2 i.d. 5</td>
<td>3.8 ± 0.8</td>
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<td>&lt; 0.7</td>
<td>9/15</td>
<td>5 ± 3.5</td>
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<td>2.7 ± 1.2</td>
<td>1.1 ± 0.4</td>
<td>1.1 ± 0.8</td>
<td>9/9</td>
<td>6 ± 3.1</td>
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</table>

† Mean log$_{10}$ p.f.u./ml ± SD. The lowest level of virus detectable was 10$^{6.7}$ p.f.u./ml and samples lacking detectable virus were assigned a titre of 10$^{6.9}$ p.f.u./ml.
‡ LW, tracheobronchial lung wash taken at slaughter 7 days after challenge with BRSV.
§ RSV antigen in lungs was detected by immunofluorescence staining of three or nine lung sections per calf. No. specimens positive/no. examined.
¶ Area of the lungs showing pneumonia consolidation expressed as the mean ± SD, 7 days after inoculation with RSV.
lymphoid tissue extended to bronchial lymph nodes in which follicles were more numerous and larger, and germinal centres were prominent. Mild exudative bronchiolitis was recognized in three of twelve vF-primed calves, one of five vG-primed calves and five of ten vN-primed animals, but in these cases the lesion was confined to fewer than three small bronchioles of
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Fig. 6. Comparison of lymphoid cell responses 7 days after BRSV challenge. H & E; bar marker 100 µm. (a) Peribronchiolar lymphoreticular hyperplasia and absence of bronchiolitis in a calf vaccinated with vF. (b) Typical non-suppurative proliferative bronchiolitis with peribronchiolar infiltration, but no lymphoreticular hyperplasia in a control, non-vaccinated calf.

Discussion

Cumulative evidence from a number of animal models has already established the importance of the F and G proteins in conferring protection against infection with HRSV, but this is the first report in which the contribution of individual RSV proteins to protective immunity has been studied in a natural host. The present study confirms the importance of the F and G proteins in protection against RSV infection and also demonstrates that vaccination with vN can restrict BRSV replication in both the upper and lower respiratory tract. Furthermore, in contrast with the enhanced lung pathology seen after HRSV challenge in mice vaccinated with rVV expressing the F, G or N proteins, there was a reduction in lung pathology in calves immunized with these proteins. The lack of protection in calves vaccinated with vM2 when compared with unvaccinated animals indicated that protection induced by the rVV was antigen-specific. Although the titres of infectious virus isolated from control calves were low, there was widespread distribution of BRSV antigen in the lungs, and this was significantly reduced in the lungs of calves vaccinated with either vF, vG or vN. The low titres of BRSV isolated from calves may reflect the poor ability of low tissue-culture passaged BRSV to replicate in tissue culture.

The response of cattle to rVV resembles that reported in chimpanzees (Collins et al., 1990) in that the dermal reaction was localized and non-systemic, and the serological response to HRSV was poor when compared with that observed in mice and cotton rats. Levels of BRSV-specific antibody in calves, 3 weeks after vaccination, were similar to those observed previously in calves given the WR strain of rVV (unpublished observations). Therefore, VV-Cop appeared to be no better as a vector in calves than the WR strain. Further, VV shedding patterns and skin reactions post-vaccination with both recombinants reflected this similarity. The reasons for the poor immunogenicity of rVV in chimpanzees and calves when compared with mice may be related to the species specificity of the immunomodulatory proteins that are expressed by VV. For example, the B8R open reading frame of VV encodes an IFN-
γ-binding protein that inhibits the biological activity of human and bovine, but not mouse IFN-γ (Alcamí & Smith, 1995). The expression of such a protein may reduce the immunogenicity of the virus in cattle when compared with mice.

A comparative evaluation of the efficacy of rVV expressing influenza or Sendai virus proteins administered i.d. or i.n. in mice showed that whereas i.d. vaccination protects the lungs from virus challenge, only i.n. vaccination Protects both the upper and lower respiratory tract (Small et al., 1985; Takao et al., 1997). These observations suggest that in order to protect the entire respiratory tract from infection, rVV vaccines should be inoculated via the respiratory route. We therefore investigated the immune response induced in calves by i.t. inoculation of vFbov. Although replication of vF in the respiratory tract appeared to be limited after i.t. inoculation, the level of BRSV-specific antibodies and of protection against BRSV challenge induced were similar to those in calves vaccinated by the i.d. route. Both routes primed calves for a rapid IgA response in the serum and lungs after BRSV challenge. This contrasts with studies in mice where replication of rVV in the lungs and nasal passages was necessary in order to induce IgA antibody in these sites (Takao et al., 1997). The origin of the TBLW IgA antibody in calves scarified with vF and challenged with BRSV is not clear. However, the observation that the ratios of IgG1:IgA in sera and TBLW after challenge are different suggests that the IgA is either produced locally or selectively transported into the lungs. The G protein, but not the N protein, also appeared to prime for a serum IgA response after BRSV challenge. Since IgA was undetectable in the lungs of vG-primed calves even after BRSV challenge, further work is needed in order to determine if protection against infection induced by vG can be improved by vaccination via the respiratory route.

There was a marked difference in the ability to detect BRSV-specific antibodies in F- and G-primed calves. This did not appear to be related to differences in the amounts of F and G proteins in the ELISA antigens, since, MAbs specific for these proteins gave similar titres against the ELISA antigen. Although antigenic differences have been identified in the G protein of BRSV and antibody induced in calves vaccinated with vg appears to be subgroup-specific (Furze et al., 1997), the Snook strain, used to challenge the calves and in serological assays, is antigenically similar to the 391-2 strain, which was used to produce the rVV (Furze et al., 1994). The failure to detect BRSV-specific antibodies in calves vaccinated with vM2 may be related to the amount of M2 protein in the ELISA antigen, although the end-point titre of a MAb specific for the M2 protein was 1:100,000.

The mechanisms of protection induced in calves by the F, G and N proteins are not known. Long-term protection against HRSV infection induced in mice by the F and G proteins appears to be mediated by antibodies (Connors et al., 1992b; Gaddum et al., 1996). Despite differences in the ability of antibodies induced by the F and G proteins to neutralize BRSV, the G protein appeared to be as effective as the F protein in protecting the lung against BRSV infection. The ability of the N protein to induce resistance to BRSV infection in the calf, 6 weeks after vaccination, even in the upper respiratory tract, contrasts with studies in BALB/c mice in which the low level of resistance to HRSV challenge observed 9 days after vaccination with vN administered both i.n. and i.p. had largely waned by day 28 (Connors et al., 1991). The transient resistance induced by the N protein in mice may have been mediated by primary cytotoxic T lymphocytes (CTL) induced by i.p. and i.n. vaccination with vN, although scarification of mice with vN does not appear to prime HRSV-specific CTLs (Alwan et al., 1993). The F and M2 proteins prime HRSV-specific CTLs in BALB/c mice (Alwan et al., 1993). The F protein also appears to prime BRSV-specific CTLs in sheep immunized with baculovirus-expressed F protein administered with Quil-A (Sharma et al., 1996). Analysis of the ability of rVV expressing the F, G, N or M2 proteins to prime bovine class I-restricted CTL responses may provide information on the role of these cells in resistance to BRSV infection in the calf.

The different patterns of pulmonary histopathology observed in BALB/c mice sensitized to different HRSV proteins and challenged with HRSV appear to be related to priming of different T cell subsets by HRSV proteins (Openshaw et al., 1988, 1992; Alwan et al., 1994). Thus, the G protein primes CD4+ cells with production of Th2 cytokines, including IL-5, and induces pulmonary eosinophilia after HRSV challenge. In contrast, the F protein primes cytolytic CD8+ cells and CD4+ cells with a Th1 cytokine profile and induces lung haemorrhage and a neutrophil influx after HRSV challenge. Although differences in the ability of the F and G proteins to prime bovine T cells for proliferative responses were observed in this study, the patterns of cytokines produced by bovine T cells after restimulation in vitro by BRSV were not analysed. Since, the G protein of BRSV did not appear to induce pulmonary eosinophilia in calves after BRSV challenge, it will be important to characterize the cytokines produced by bovine lymphocytes primed by different BRSV proteins.

In conclusion, the F, G and N, but not the M2, proteins of BRSV induce resistance to BRSV challenge in the calf and protect against the development of pneumonic lesions. The mechanisms of resistance induced in calves by these proteins are not clear. Thus, the F protein induced neutralizing antibodies, whereas the levels of neutralizing antibodies induced by the G protein were low and the N protein did not induce neutralizing antibody. Furthermore, the F protein primed calves for an IgG1 and IgA response in the lungs after BRSV challenge, whereas there was little or no antibody in the lungs of calves that had been vaccinated with vG. Since there was evidence of T cell priming by the F, G and N proteins, analysis of the cytokine responses of the primed T cells and the ability of these proteins to prime bovine CTL will facilitate studies on the role of T cells in resistance to BRSV infection in the calf.
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