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Bovine respiratory syncytial virus lacking the virokinin or with a mutation in furin cleavage site RA(R/K)R^{109} induces less pulmonary inflammation without impeding the induction of protective immunity in calves

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The BRSV fusion (F) protein is cleaved at two furin consensus sequence sites, resulting in the generation of disulphide-linked F1 and F2 subunits and the release of an intervening peptide of 27 amino acids (pep27), which is converted into a biologically active tachykinin (virokinin). The role of the virokinin and the importance of one of the furin cleavage sites, FCS-2 [RA(R/K)R^{109}], in the pathogenesis of BRSV infection and in the subsequent development of immunity was studied in gnotobiotic calves infected with a recombinant BRSV (rBRSV) lacking pep27 (rBRSVΔp27) or with rBRSV108/109, which contains two amino acid substitutions in FCS-2 (RANN^{109}). Although replication of the mutant viruses and the parental wild-type (WT) rBRSV in the lungs was similar, the extent of gross and microscopic lesions induced by the mutant viruses was less than that induced by WT rBRSV. Furthermore, the numbers of eosinophils in the lungs of calves infected with the mutant viruses were significantly less than that in calves infected with WT virus. These observations suggest a role for the virokinin in the pathogenesis of BRSV infection. Following mucosal immunization with rBRSVΔp27, the levels of BRSV-specific serum antibodies were similar to those induced by WT virus. In contrast, the level of neutralizing antibodies induced by rBRSV108/109 was 10-fold lower than that induced by WT virus. Nevertheless, resistance to BRSV challenge induced by the mutant and WT viruses was similar, suggesting that neither pep27 nor FCS-2 plays a major role in the induction of protective immunity.

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INTRODUCTION

Bovine respiratory syncytial virus (BRSV) and human RSV (HRSV) are enveloped, non-segmented, negative-strand RNA viruses that belong to the genus *Pneumovirus* within the family *Paramyxoviridae*. BRSV and HRSV are major causes of respiratory disease in young calves and children, respectively (Stott & Taylor, 1985), and similarities in the epidemiology and pathogenesis of infection with these viruses make BRSV infection in calves a good model for the study of HRSV. The development of safe and effective vaccines against HRSV and BRSV faces similar problems, many of which remain unsolved. A live attenuated virus vaccine provides one of the most promising solutions, the development of which has been facilitated by the use of reverse genetics to manipulate the viral genome. Different strategies have been investigated such as incorporation of mutations, deletions, changing viral gene order, expression of additional genes and expression of genes of interest in a backbone from another virus (for review see Collins & Murphy, 2002). A major problem for the development of live attenuated RSV vaccines is obtaining the right balance between attenuation and induction of protective immunity (Wright *et al.*, 2000). Knowledge of the effects of each gene modification will increase our understanding of the pathogenesis of RSVs and will help to determine the most appropriate combination of genetic alterations to produce a safe and effective live vaccine.

The RSV genome encodes three glycoproteins: the small hydrophobic protein (SH), the large attachment protein (G) and the fusion protein (F). The G and F proteins mediate binding of virus to cells and F is also responsible for fusion of

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viral and cell membranes. Several studies indicate that both SH and G are dispensable for virus replication *in vitro* (Karger *et al.*, 2001). However, recombinant RSVs (rRSV) lacking these glycoproteins are attenuated *in vivo* (Collins & Murphy, 2002; Schmidt *et al.*, 2002), suggesting that they have accessory functions. The F protein, which is indispensable for virus replication, is synthesized as an inactive precursor, F0, which has to be proteolytically cleaved to become fusion-active. Cleavage, mediated by the endoprotease furin, occurs at two sites, FCS-1 ($RKRR^{136}$) and FCS-2 [$RA(R/K)R^{109}$], and results in the formation of F1 and F2 subunits linked by a disulphide bridge and in the release of an N-glycosylated peptide of 27 amino acids (pep27) (Gonzalez-Reyes *et al.*, 2001; Zimmer *et al.*, 2001). Cleavage at both sites is required for efficient syncytium formation and to allow changes in F protein structure from cone- to lollipop-shaped spikes (Begona Ruiz-Arguello *et al.*, 2002; Gonzalez-Reyes *et al.*, 2001; Zimmer *et al.*, 2001). In BRSV-infected cells *in vitro*, pep27 is further subjected to post-translational modifications and is converted into virokinin, a member of the tachykinin family (Zimmer *et al.*, 2003). Other known members of the tachykinin family are substance P, neurokinins A and B, haemokinin and endokinins A and B, all of which have proinflammatory and immunomodulatory properties (Patacchini *et al.*, 2004; Pennefather *et al.*, 2004). Tachykinins are produced by neurons and also by immune and inflammatory cells (Maggi, 1997). Recent results obtained *in vitro* suggest that the BRSV virokinin may cause bronchoconstriction, since it induces smooth muscle contraction (Zimmer *et al.*, 2003).

Previous studies demonstrated that neither the FCS-2 $RA(R/K)R^{109}$ nor pep27 were essential for replication of BRSV in cell culture (Zimmer *et al.*, 2002). However, mutant BRSV in which cleavage at the $RA(R/K)R^{109}$ motif was abolished did not grow as efficiently as the parental virus during early replication cycles and showed reduced syncytium formation. BRSV lacking pep27 also showed reduced syncytium formation, although replication was similar to that of the parental virus. In the present study, we have investigated the virulence, pathogenesis and immunogenicity of rBRSV lacking pep27 (rBRSVΔp27) and of rBRSV with two mutations, K108N and R109N, in FCS-2 $RA(R/K)R^{109}$ (rBRSV108/109) in young calves.

METHODS

Viruses and cells. Stocks of the Snook strain of BRSV (Thomas *et al.*, 1982) were prepared in fetal calf kidney (FCK) cells as described previously (Stott *et al.*, 1984). Wild-type (WT) rBRSV, virus mutated in FCS-2 of the F protein (rBRSV108/109) or virus containing a deletion of 25 amino acids in the F protein (Δ106–130), which comprises FCS-2 and most of pep27 (rBRSVΔp27), were derived from full-length cDNA of BRSV strain A51908 (Mohanty *et al.*, 1975), variant ATue51908 (GenBank accession no. AF092942), as reported previously (Zimmer *et al.*, 2002) (Fig. 1). Stocks of rBRSV were prepared in Vero cell monolayers, infected at an m.o.i. of between 0·1 and 0·5 in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) containing 2 % heated fetal calf serum, as described previously (Valarcher *et al.*, 2003). All recombinant viruses were checked by partial sequencing of the F gene containing the mutations, and all virus stocks were free from contamination with bovine viral diarrhoea virus and mycoplasmas.

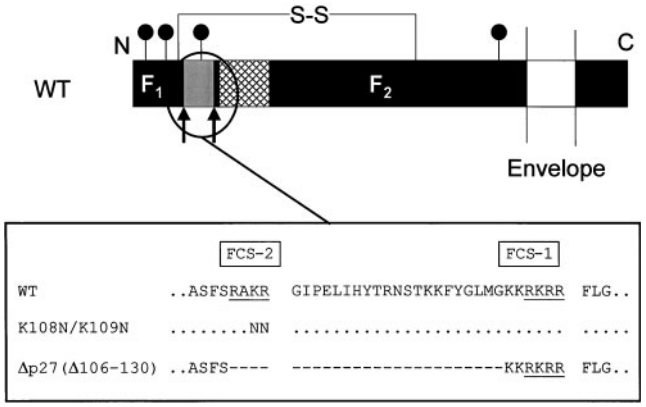

Fig. 1. Mutations introduced into the F protein of recombinant BRSVs. To be activated to its fusogenic form, the inactive precursor of the F protein (F_0) is cleaved by furin into F_1 and F_2 subunits linked by a disulfide bridge (S-S). The cleavage, which occurs at two sites (arrows), results in the exposure of a hydrophobic fusion peptide in the F_1 subunit (cross-hatched region) and in the release of a 27 amino acid peptide (p27) (shaded region). ●, Glycosylation sites. After further post-translational modifications, the BRSV p27 peptide is converted into a biologically active tachykinin (virokinin). A section of the BRSV F protein sequence comprising amino acids 102–139 is shown. The furin consensus sequences (FCS-2 and FCS-1) are underlined. Identical amino acids are indicated by dots and dashes indicate deletions. In this study, we have evaluated the virulence and immunogenicity of rBRSV with two mutations in the second furin cleavage site (rBRSV108/109) and rBRSV lacking amino acids 106–130 (rBRSVΔp27).

Virulent BRSV that produces clinical signs of respiratory disease in young gnotobiotic calves consisted of bronchoalveolar lavage (BAL) prepared from a gnotobiotic calf inoculated 6 days previously with the Snook strain of BRSV that had been passaged on two previous occasions in gnotobiotic calves. The BAL was free from other viruses, mycoplasmas and bacteria as assessed by inoculation of tissue culture or mycoplasma or bacteria media. Virus titres were determined 48 h post-infection (p.i.) by immunostaining of triplicate samples on Vero cell monolayers in 96-well plates with an anti-F protein mAb (mAb19) (Taylor *et al.*, 1992; Valarcher *et al.*, 2003). Virus titres in samples from nasopharyngeal swabs, BAL cells and lung homogenates were determined by plaque assay in Vero or FCK cells after 7 days incubation at 37 °C (Thomas *et al.*, 1984).

Calves and experimental design. Gnotobiotic, BRSV-seronegative calves were delivered and reared individually in plastic isolators as described previously (Dennis *et al.*, 1976). In order to evaluate the virulence of WT and F-mutant rBRSV, groups of three or four gnotobiotic calves were infected at 2–3 weeks of age with approximately 2×10^6 p.f.u. virus in a volume of 20 ml, 10 ml administered intranasally (i.n.) and 10 ml intratracheally (i.t.). Clinical examinations were performed twice a day following virus infection. Calves were killed 6 days after infection by intravenous injection of sodium pentobarbital (Euthatal; Merial Animal Health Ltd). At post-mortem examination, macroscopic lung lesions were recorded on a

standard lung diagram and the extent of pneumonic consolidation was expressed as percentage pneumonia. BAL was collected by irrigating the lungs from each calf with 400 ml PBS, as described previously (Taylor *et al.*, 1997). Cells, prepared from 100 ml of the BAL by centrifugation at 1200 ***g*** for 15 min at 4 °C, were resuspended in 5 ml lung buffer for virus isolation (Taylor *et al.*, 1995). Three pieces of pneumonic lung taken from three different lobes were homogenized in lung buffer to give a 20 % (w/v) suspension. Samples from nasopharyngeal swabs, BAL cells and lung homogenates were inoculated onto Vero or FCK cells to determine virus titres.

In order to evaluate the immunogenicity of the mutant rBRSV, four groups of three or four gnotobiotic calves were inoculated i.n. and i.t. with WT rBRSV, rBRSV108/109 or rBRSVΔp27 or with a suspension of non-infected Vero cell lysate as described above. Fourteen days p.i., calves were removed from the plastic isolators, mixed and reared in a high-security, barrier-maintained building. Six weeks p.i., calves were challenged i.n. and i.t. with approximately 10^3 p.f.u. virulent BRSV Snook. Serum samples and heparinized blood were obtained at 3-week intervals for analysis of serum antibodies and T-cell priming. Following challenge, nasopharyngeal swabs were obtained daily and calves were killed 6 days after challenge to determine the extent of gross pneumonic consolidation and the extent of virus infection in the lower respiratory tract.

All experiments were performed in compliance with the regulations of the UK Home Office Scientific Procedures Act (1986).

Virokinin ELISA. BAL supernatant from BRSV-infected calves was analysed for the presence of virokinin using a competitive ELISA as described previously (Zimmer *et al.*, 2003). Briefly, equal volumes of BAL supernatant and biotinylated virokinin were incubated with rabbit anti-virokinin serum bound to protein A-coated microtitre plates. The wells were washed and incubated with streptavidin–peroxidase complex. After washing, wells were incubated with 2,2′-azino-di-[3-ethylbenthiazoline sulfonate] diammonium salt peroxidase substrate (Roche Applied Science). The amount of biotinylated virokinin was determined by reference to a standard curve in the range 0·01–100 ng ml^{-1}.

Immunocytochemistry. Pieces of lung taken at post-mortem were snap-frozen in liquid nitrogen and cryostat sections were stained with mouse mAb19, specific for the RSV F protein (Taylor *et al.*, 1992), or with rabbit anti-virokinin serum (Zimmer *et al.*, 2003). The primary antibodies were detected with FITC-conjugated anti-mouse IgG (Sigma) or FITC-conjugated anti-rabbit IgG (Sigma).

Histology. Lung tissue for histology was fixed in 10 % neutral-buffered formalin and embedded in paraffin wax and sections were stained with carbol chromotrope. Eosinophils were counted in 100 high-power microscope fields on three lung sections from three or four calves infected with each rBRSV and from mock-infected control animals.

Serology. The presence of antibodies to BRSV in sera was determined by ELISA using a lysate prepared from FCK cells infected with the Snook strain of BRSV and a lysate of mock-infected cells as control antigen, as described previously (Taylor *et al.*, 1995). Neutralizing antibodies to BRSV were determined by a plaque-reduction assay on FCK cells using heat-inactivated serum as described previously (Kennedy *et al.*, 1988).

T-cell assays. BRSV-specific lymphocyte proliferative responses were analysed 6 weeks after immunization, as described previously (Taylor *et al.*, 1995). The proportions of BRSV-specific $CD8^+$ IFN-γ^+ T cells in peripheral blood were determined 6 weeks after immunization by flow cytometry as described previously (Gaddum *et al.*, 2003) and the proportion of BRSV-specific $CD4^+$ IFN-γ^+ T cells was determined in a similar way using mAb CC8 to detect $CD4^+$ T cells (Howard *et al.*, 1991).

RESULTS

Replication of rBRSV containing mutations in FCS-2 or lacking the virokinin was similar to that of WT rBRSV in the respiratory tract of calves

Previous studies have shown that, whereas replication of WT rBRSV and rBRSVΔp27 in cell culture was similar, replication of rBRSV108/109 was slightly retarded, and both mutant viruses showed reduced syncytium formation in Vero cells compared with that induced by the parental WT rBRSV (Zimmer *et al.*, 2002). In order to investigate the effects of these mutations on the virulence of BRSV *in vivo*, groups of six to eight gnotobiotic calves were inoculated simultaneously by the i.n. and i.t. routes with WT rBRSV or with the rBRSV F mutants.

The duration of virus excretion and the mean peak virus titres in the nasopharynx of calves infected with rBRSV108/109 or WT rBRSV were similar and greater than those observed in calves infected with Δp27 virus (Table 1). However, the differences between the three groups of calves were not statistically significant. In the lower respiratory tract, the mean virus titre in BAL cells from calves infected with Δp27 virus was similar to that obtained in animals infected with WT rBRSV (Table 1). Titres of rBRSV108/109 in BAL were approximately 20-fold greater than those in calves infected with either rBRSV WT or Δp27. Both of the F-mutant viruses were recovered more frequently from lung homogenates than was WT rBRSV at 6 days p.i. (Table 1). However, there were no significant differences in the mean titres of virus recovered from homogenates of three lung samples collected from each animal from the three groups of calves (Table 1).

F mutants induce less pulmonary eosinophil infiltration than WT rBRSV

Although the extent of gross pulmonary pathology was minimal in all calves at 6 days p.i., lesions were more extensive in calves infected with WT rBRSV than in those infected with the F mutants (Table 1). Microscopic lesions in lungs from calves infected with WT rBRSV were similar to, but less extensive than, those reported previously in gnotobiotic calves infected experimentally with BRSV Snook (Thomas *et al.*, 1984). Lesions were characterized by a proliferative and exudative bronchiolitis with accompanying alveolar collapse and a peribronchiolar infiltration by mononuclear cells. Microscopic lesions were also observed in lungs from calves inoculated with the F mutants, but were less extensive than those in calves infected with WT rBRSV. Whereas there were no significant differences in the numbers of neutrophils in BAL from calves infected with the F mutants or WT virus, there was a striking difference in the

Table 1. Evaluation of the virulence of rBRSV containing mutations in the FCS in calves

Two- to 3-week-old gnotobiotic calves were inoculated i.n. and i.t. with 6·3 $\log_{10}$ p.f.u. of the recombinant viruses shown. Titres are means ± SD, expressed as $\log_{10}$ p.f.u. ml^{-1}. The number of eosinophils in lung sections from calves infected with WT virus was significantly greater than that in calves infected with either rBRSV108/109 ($P<0·01$) or rBRSVΔp27 ($P<0·01$) or mock-infected calves (mean of 20·9 ± 26 eosinophils per 100 high-power fields; $P<0·01$).

Parameter	WT	108/109	Δp27
Number of calves	8	6	7
Nasopharyngeal excretion			
Mean peak virus titre*	2·8 ± 1·5	2·5 ± 0·8	1·6 ± 1·1
Mean duration (days)†	3·5 ± 2·2	3·0 ± 1·3	2·3 ± 2·0
Virus titre in BAL‡	2·0 ± 1·5 (2/4)	3·3 ± 0·4 (3/3)	1·9 ± 1·2 (2/3)
Virus in lung tissue§	4/12	6/9	5/9
Mean virus titre in lung tissue homogenate‖	1·1 ± 1·1 (2/4)	1·5 ± 0·9 (3/3)	1·3 ± 0·8 (2/3)
Pneumonic lesions (%)¶	4 ± 1·7	1 ± 0	1·3 ± 1·1
Mean no. eosinophils per 100 high-power fields	157 ± 123	7·2 ± 7·9	5·3 ± 4·1

*Calculated from peak virus titres in the nasopharynx of each animal. The threshold of detection was 0·7 $\log_{10}$ p.f.u. ml^{-1} and samples lacking detectable virus were assigned a titre of 0·6 $\log_{10}$ p.f.u. ml^{-1}.
†Length of time that virus was isolated from the nasopharynx between day 0 and day 6 p.i.
‡Determined at post-mortem, 6 or 7 days p.i. (number of calves infected/total analysed).
§Number of lung samples from which virus was isolated/total analysed. Three pieces of lung per animal were sampled from groups of three or four calves on day 6 or 7 p.i.
‖Determined in lung homogenate obtained from three pieces of pneumonic lung taken from three different lobes at post-mortem, 6 or 7 days p.i. (number of calves infected/total analysed).
¶Area of lung showing gross pneumonic consolidation.

extent of pulmonary eosinophil infiltration. Although only a minor component of the pulmonary cellular inflammatory response, eosinophils could be seen in the tissue surrounding the larger bronchioles migrating through the lamina propria and epithelium of calves infected with WT rBRSV (Fig. 2a). Although significantly less extensive than that which characterizes the BRSV vaccine-enhanced disease in calves (Schreiber *et al.*, 2000; Antonis *et al.*, 2003), the eosinophil influx was similar to that described previously in calves infected with the Snook strain of BRSV. In contrast, although there was an influx of cells in the lamina propria in calves infected with the F mutants, few of the cells were eosinophils (Fig. 2b, c). Analysis of 100 high-power microscope fields of lung sections showed that the numbers of eosinophils in calves infected with either of the two rBRSV F mutants were significantly smaller than those in calves infected with WT virus ($P<0·01$) (Table 1). In addition, damage to the bronchial epithelial cells appeared to be greater in calves infected with WT virus than in those infected with the F mutants (Fig. 2).

In order to determine whether there were differences in the levels of virokinin in BAL fluid from calves infected with WT rBRSV or the F mutants, BAL was performed on calves 3 and 6 days p.i. Using a competitive immunoassay, virokinin could not be detected in the BAL fluid from any of the calves.

Studies *in vitro* have demonstrated that pep27 of rBRSV108/109 remains attached to the F2 subunit and is transported to the cell surface, where it undergoes C-terminal maturation to produce a shorter peptide that terminates with the classical tachykinin motif (Zimmer *et al.*, 2003). Expression of this tachykinin epitope on the surface of cells infected with rBRSV108/109 can be detected using anti-substance P or anti-virokinin antibodies (Zimmer *et al.*, 2003). In order to determine whether expression of the tachykinin epitope could be detected in the lungs of calves experimentally infected with rBRSV108/109, sections of snap-frozen lung were stained with a mAb to the F protein and antiserum to substance P or antiserum to the virokinin. Whereas cells expressing the F protein were detected in lung sections from calves infected with rBRSV108/109, there was no reaction with either the substance P or virokinin antibody (results not shown).

Mucosal immunization of calves with rBRSV containing mutations in FCS-2 or lacking the virokinin protects against challenge with virulent BRSV

The BRSV-specific serum antibody response in groups of three or four gnotobiotic calves that had been inoculated i.n. and i.t. with WT, 108/109 or Δp27 rBRSVs or with non-infected Vero cell lysates was analysed by ELISA. There were no significant differences in the levels of antibodies as detected by ELISA in any of the infected calves (Table 2 and Fig. 3a). Similarly, there were no significant differences in the Ig isotypes of the BRSV-specific antibodies between

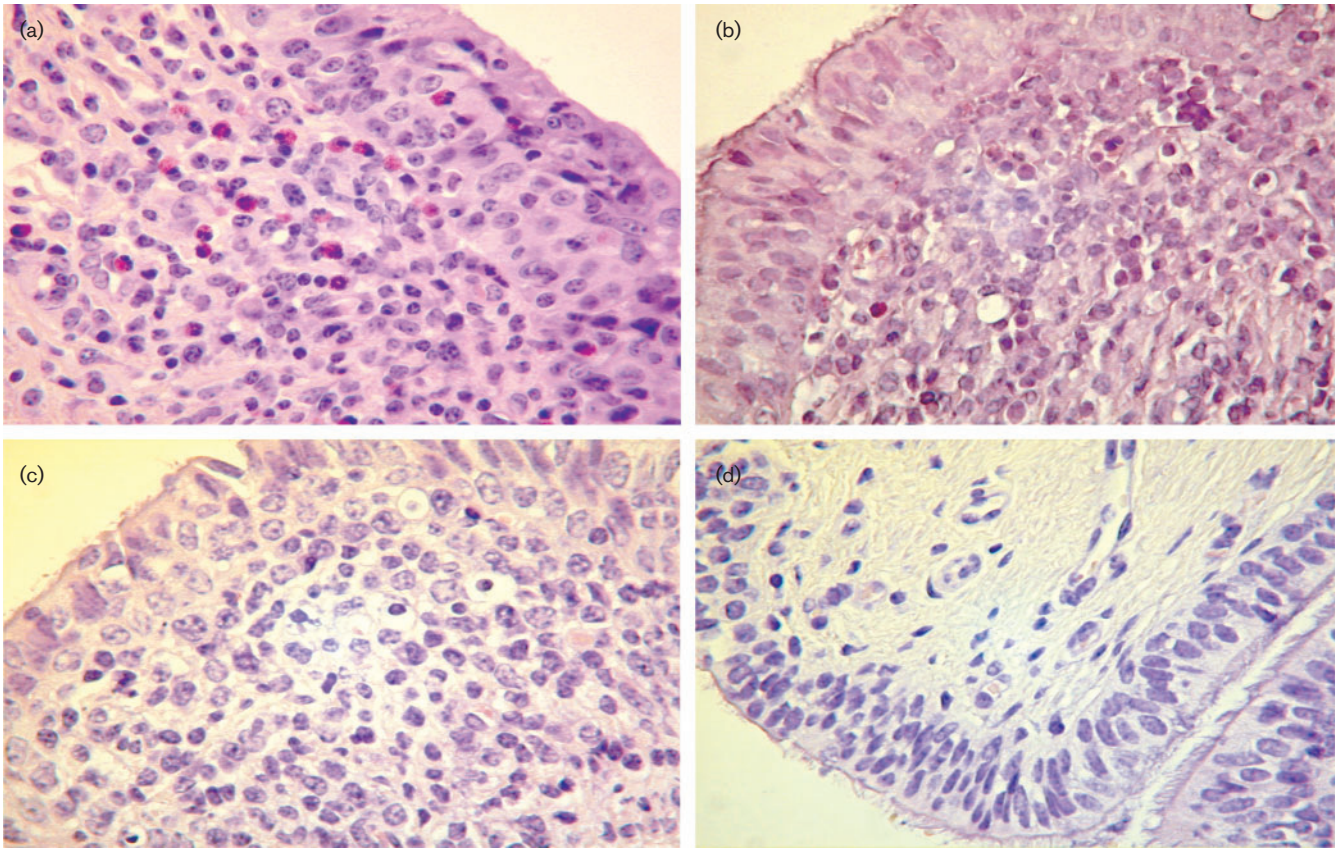

Fig. 2. F-mutant viruses induce less eosinophil infiltration in bronchial lamina propria than WT rBRSV. Groups of three or four gnotobiotic calves were infected i.n. and i.t. with approximately 2×10^6 p.f.u. WT rBRSV (a), rBRSV108/109 (b) or rBRSVΔp27 (c) or non-infected Vero cell lysate (d). Six days after infection, lung tissues were fixed in 10 % neutral-buffered formalin and embedded in paraffin wax and sections were stained with carbol chromotrope (eosinophils are stained red). Original magnification $\times 400$.

Table 2. Recombinant BRSVs containing mutations in the FCS protect against challenge with virulent BRSV

Gnotobiotic calves, inoculated with recombinant viruses or mock-infected as described in Table 1, were challenged i.n. and i.t. 6 weeks later with 3·7 $\log_{10}$ p.f.u. BRSV Snook in BAL. Titres are means ± SD, expressed as $\log_{10}$ p.f.u. ml^{-1} for virus or $\log_{10}$ for antibody.

Parameter	WT	108/109	Δp27	Mock
No. of calves	4	3	4	3
Mean serum antibody titre on day 42				
ELISA titre	3·5 ± 0·3	3·1 ± 0·5	3·4 ± 0·3	< 1·5
Neutralizing titre*	2·1 ± 0·7	1·0 ± 0·4	1·9 ± 0·5	< 0·5
Nasopharyngeal excretion				
Mean peak virus titre†	< 0·7	0·7 ± 0·2	0·7 ± 0·1	3·2 ± 1·1
Mean duration (days)†	0	0·5 ± 1·0	0·5 ± 0·6	3·3 ± 0·6
Virus titre in BAL†	0·9 ± 0·7 (1/4)	< 0·7 (0/3)	< 0·7 (0/4)	4·6 ± 1·0 (3/3)
Virus in lung tissue†	0/12	0/9	0/12	9/9

*Determined by a 50 % plaque-reduction assay using heat-inactivated sera and BRSV Snook.
†Calculated as described in Table 1.

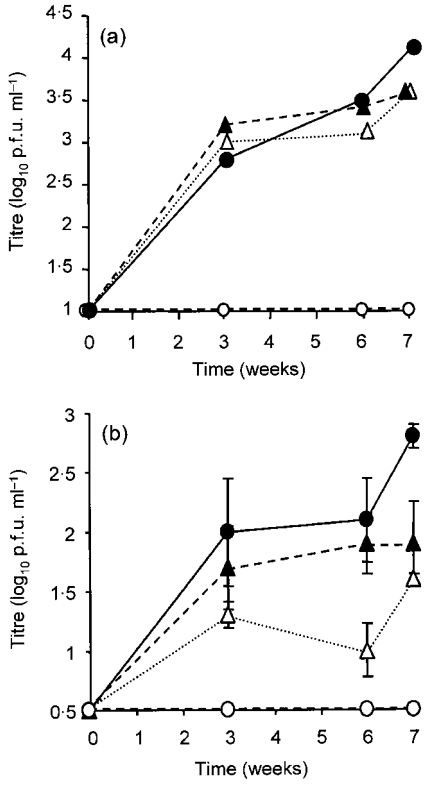

Fig. 3. BRSV-specific antibodies induced by mucosal immunization of calves with rBRSV. Gnotobiotic calves were inoculated i.n. and i.t. with approximately 2×10^6 p.f.u. WT rBRSV (●), rBRSV108/109 (△) or rBRSVΔp27 (▲) or non-infected Vero cell lysate (○). Calves were challenged 6 weeks later with 5×10^3 p.f.u. BRSV Snook. (a) BRSV-specific serum IgG antibody responses determined by ELISA. (b) BRSV-specific serum neutralizing antibody responses determined by a plaque-reduction assay in the absence of complement.

calves immunized with WT or F-mutant rBRSVs (data not shown). However, serum neutralizing antibody titres in calves infected with rBRSV108/109 were significantly lower 6 weeks after vaccination than those in calves infected with either Δp27 or WT rBRSVs (Table 2 and Fig. 3b) ($P < 0{\cdot}05$). Six days after challenge with the virulent Snook strain of BRSV, neutralizing antibody titres increased in calves that had been immunized with rBRSV WT or 108/109, but not in calves immunized with Δp27 (Fig. 3b).

At the time of challenge, there were no significant differences in the numbers of BRSV-specific $CD4^+$ IFN-γ^+ or $CD8^+$ IFN-γ^+ T cells between calves immunized with either of the rBRSV F mutants or WT virus and no significant differences in the BRSV-specific lymphocyte proliferative response (results not shown).

Following challenge, clinical signs of respiratory disease were not observed in any calves. Nevertheless, gross pneumonic lesions were present in all of the control calves and the extent was similar to that reported previously (Valarcher *et al.*, 2003). In contrast, there was little or no pneumonic consolidation in calves that had been immunized with either of the F-mutant or WT viruses. Furthermore, calves previously immunized with either of the F-mutant viruses were highly resistant to subsequent infection with BRSV Snook (Table 2). Following challenge, virus was isolated from the upper and lower respiratory tract of all control calves, previously inoculated with non-infected cell lysate. In contrast, little or no virus was isolated from the upper or lower respiratory tract of calves previously immunized with either of the F-mutant or WT viruses (Table 2).

DISCUSSION

Although the replication of rBRSV108/109 in cell culture was slightly retarded compared with that of WT rBRSV (Zimmer *et al.*, 2002), the replication of these viruses in the upper and lower respiratory tract of young calves was similar. The replication of rBRSVΔp27 in the bovine upper respiratory tract was reduced, but not significantly, compared with that of WT rBRSV. Nevertheless, the replication of these viruses in the bovine lower respiratory tract and *in vitro* was similar. Both mutant viruses showed reduced syncytium formation in cell culture compared with that of WT rBRSV and both induced a reduced pulmonary inflammatory response and less damage to bronchial epithelial cells than WT rBRSV *in vivo*. This reduction in pulmonary pathology did not appear to correlate with the level of virus replication in the lungs, which was similar for WT and mutant viruses. The differences in the inflammatory response may therefore be related to differences in the ability of the viruses either to cause a direct cytopathic effect or to induce an inflammatory response. However, neither HRSV infection of well-differentiated human airway epithelial cell (AEC) cultures *in vitro* (Zhang *et al.*, 2002) nor BRSV infection of well-differentiated bovine AEC (unpublished observations) results in any obvious cytopathic effects, as determined by light microscopy. These observations suggest that differences in pulmonary pathology induced by the F-mutant and WT rBRSVs may be related to differences in the ability of these viruses to induce an inflammatory response.

The role of the virokinin in the pulmonary inflammatory response and in eosinophil recruitment, in particular, is unclear. Both substance P and neurokinin A can contribute to eosinophil recruitment in the lungs of guinea pigs (Tiberio *et al.*, 2003), although a number of studies suggest that tachykinins do not act on their own to mediate neutrophil or eosinophil accumulation but play a role in granulocyte accumulation during an ongoing inflammatory process (Cao *et al.*, 2000; Numao & Agrawal, 1992). These observations correlate with preliminary studies in which we failed to demonstrate any chemotactic effects of a synthetic BRSV virokinin (Zimmer *et al.*, 2003) on freshly isolated bovine granulocytes *in vitro* (unpublished observations) or following i.n. inoculation of BALB/c mice with the synthetic virokinin (unpublished observations). If the virokinin plays

a role in eosinophil recruitment, its absence in rBRSVΔp27 could explain the small numbers of eosinophils in the lungs of calves infected with this virus. However, since cleavage at the mutated FCS-2 by exogenous trypsin has been demonstrated *in vitro* (Zimmer *et al.*, 2003), it is possible that the virokinin may have been produced by bronchial epithelial cells infected with rBRSV108/109 as a result of trypsin-like proteases produced by Clara cells *in vivo*. The failure to detect the uncleaved peptide on the surface of bronchial epithelial cells in the lungs of calves infected with rBRSV108/109 by immunostaining may indicate that cleavage had occurred *in vivo*. However, it was not possible to confirm this by detection of virokinin in BAL fluid. Thus, attempts to detect virokinin by ELISA in BAL fluid from calves infected with WT rBRSV or even the virulent Snook strain of BRSV were unsuccessful. This may be due to the sensitivity of the assay or because of the very short half life of the virokinin, which could be degraded by enzymes such as tryptase released by mast cells (Tam & Caughey, 1990; Zimmer *et al.*, 2001) or neutral endopeptidase located at the surface of AEC (Di Maria *et al.*, 1998). It is possible that there may be differences *in vivo* in the availability of active virokinin produced by rBRSV108/109 and WT rBRSV that are related to the site at which processing of the F protein takes place. Thus, cleavage at FCS-2 of the rBRSV108/109 F protein by extracellular trypsin-like proteases would take place at the apical surface of the bronchial epithelial cells, and the virokinin would be released into the bronchial lumen. In contrast, cleavage of the native F protein occurs within the trans-Golgi network, so that the virokinin would be formed intracellularly and its secretion might not be restricted to the apical surface of polarized cells.

The influx of inflammatory cells into RSV-infected respiratory tissues is associated with increased levels of a number of different chemokines, such as RANTES, MCP-1, IL-8 and MIP-1α (Domachowske *et al.*, 2001; Harrison *et al.*, 1999; McNamara *et al.*, 2005; Noah & Becker, 2000; Olszewska-Pazdrak *et al.*, 1998). Chemokines play an important role in the recruitment and activation of leukocytes, and MIP-1α and RANTES are potent chemoattractants for human eosinophils. A correlation between levels of MIP-1α and eosinophil cationic protein (ECP) in the lower airways of infants hospitalized with severe RSV disease has been demonstrated (Harrison *et al.*, 1999), and the presence of eosinophil degranulation products in the respiratory tract of such infants has led to the suggestion that eosinophil degranulation plays a role in the pathogenesis of RSV disease (Garofalo *et al.*, 1992; Harrison *et al.*, 1999). However, since pulmonary eosinophilia is not typically seen in infants hospitalized with HRSV infection, with the exception of a small proportion in the lungs of RSV-infected recipients of a formalin-inactivated HRSV vaccine (Prince *et al.*, 2001), it may be that the eosinophil response occurs early after RSV infection and is resolving by the time infants are hospitalized (Rosenberg & Domachowske, 2001). In our studies, differences in the eosinophil response between calves infected with the F mutants or WT rBRSV were not associated with significant differences in levels of expression of RANTES or MIP-1α mRNA in lung tissue, as determined by quantitative RT-PCR (unpublished observations). These observations support the suggestion that the BRSV virokinin is involved in the recruitment of eosinophils into the lung. However, this tachykinin is produced by BRSV and not by HRSV. Thus, the 27-mer peptide produced during the maturation of the HRSV F protein does not contain a tachykinin motif (Zimmer *et al.*, 2003), suggesting that the virokinin may be an additional factor to chemokines involved in eosinophil recruitment in BRSV infection.

Since the primary sequence of the virokinin is highly conserved in all BRSV strains, it has been suggested that it may be of benefit to the virus. The advantage to BRSV of recruiting eosinophils to the site of infection remains unclear, but this effect may be only one property of the virokinin. The biological role of activated eosinophils in RSV infection remains speculative, but it has been demonstrated that eosinophil products have an antiviral effect against HRSV (Rosenberg & Domachowske, 2001; Soukup & Becker, 2003). As a consequence and because virokinin is specific to BRSV, the production of the virokinin may explain the shorter duration of BRSV shedding in cattle compared with that of HRSV in man (Hall *et al.*, 1976; Valarcher *et al.*, 1999). This suggestion is supported by the more frequent isolation of the F mutants from lung tissues compared with that of the WT virus. On the other hand, recruitment of eosinophils to the site of virus infection may contribute to damage to the respiratory mucosa and ciliostasis (Hirata *et al.*, 1996; Hisamatsu *et al.*, 1990; Liu & Okuda, 1988), which could facilitate viral infection of epithelial cells. As mentioned previously, damage to the bronchial epithelium and loss of cilia were more apparent in lungs from calves infected with WT rBRSV than in those infected with either of the F mutants, and this correlated with the presence of eosinophils.

Mutations in FCS-2 or deletion of pep27 did not affect the induction of a protective immune response. Although tachykinins are potent immunomodulators (Goetzl & Sreedharan, 1992; Maggi, 1997; McGillis *et al.*, 1990), deletion of the 27-mer peptide did not appear to influence either the humoral or T-cell-mediated immune response to BRSV. However, mucosal immunization with rBRSV108/109, which is not cleaved at FCS-2, induced lower neutralizing antibody titres than immunization with either rBRSVΔp27 or WT rBRSV. The low neutralizing, but high ELISA, antibody titres induced by immunization with rBRSV108/109 could be due to loss of neutralizing epitopes in the F protein of this virus. However, rBRSV108/109 was recognized by F-specific neutralizing mAbs (unpublished observations). Despite the low levels of neutralizing antibodies, calves immunized with rBRSV108/109 were strongly protected against subsequent BRSV infection in the upper airways and completely protected in the lower airways.

Since the virokinin is capable of inducing smooth muscle contraction *in vitro*, it has been suggested that it may

contribute to the pathogenicity of BRSV. Several symptoms associated with severe BRSV infection, such as bronchoconstriction, excessive mucus production and oedema, are inducible by substance P and other tachykinins. However, the parental WT rBRSV was attenuated in calves, so it has not been possible to determine the contribution of the virokinin to the development of clinical signs of respiratory disease. Nevertheless, the findings from this study suggest a role for the BRSV F protein virokinin in the pulmonary inflammatory response and indicate that mutations in FCS-2 or deletion of pep27 do not significantly affect the induction of protective immunity.

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REFERENCES

Antonis, A. F., Schrijver, R. S., Daus, F., Steverink, P. J., Stockhofe, N., Hensen, E. J., Langedijk, J. P. & van der Most, R. G. (2003). Vaccine-induced immunopathology during bovine respiratory syncytial virus infection: exploring the parameters of pathogenesis. *J Virol* **77**, 12067–12073.

Begona Ruiz-Arguello, M., Gonzalez-Reyes, L., Calder, L. J., Palomo, C., Martin, D., Saiz, M. J., Garcia-Barreno, B., Skehel, J. J. & Melero, J. A. (2002). Effect of proteolytic processing at two distinct sites on shape and aggregation of an anchorless fusion protein of human respiratory syncytial virus and fate of the intervening segment. *Virology* **298**, 317–326.

Cao, T., Pinter, E., Al-Rashed, S., Gerard, N., Hoult, J. R. & Brain, S. D. (2000). Neurokinin-1 receptor agonists are involved in mediating neutrophil accumulation in the inflamed, but not normal, cutaneous microvasculature: an in vivo study using neurokinin-1 receptor knockout mice. *J Immunol* **164**, 5424–5429.

Collins, P. L. & Murphy, B. R. (2002). Respiratory syncytial virus: reverse genetics and vaccine strategies. *Virology* **296**, 204–211.

Dennis, M. J., Davis, D. C. & Hoare, M. N. (1976). A simplified apparatus for the microbiological isolation of calves. *Br Vet J* **132**, 642–646.

Di Maria, G. U., Bellofiore, S. & Geppetti, P. (1998). Regulation of airway neurogenic inflammation by neutral endopeptidase. *Eur Respir J* **12**, 1454–1462.

Domachowske, J. B., Bonville, C. A. & Rosenberg, E. S. (2001). Gene expression in epithelial cells in response to pneumovirus infection. *Respir Res* **2**, 225–233.

Gaddum, R. M., Cook, R. S., Furze, J. M., Ellis, S. A. & Taylor, G. (2003). Recognition of bovine respiratory syncytial virus proteins by bovine CD8+ T lymphocytes. *Immunology* **108**, 220–229.

Garofalo, R., Kimpen, J. L. L., Welliver, R. C. & Ogra, P. L. (1992). Eosinophil degranulation in the respiratory tract during naturally acquired respiratory syncytial virus infection. *J Pediatr* **120**, 28–32.

Goetzl, E. J. & Sreedharan, S. P. (1992). Mediators of communication and adaptation in the neuroendocrine and immune systems. *FASEB J* **6**, 2646–2652.

Gonzalez-Reyes, L., Ruiz-Arguello, M. B., Garcia-Barreno, B., Calder, L., Lopez, J. A., Albar, J. P., Skehel, J. J., Wiley, D. C. & Melero, J. A. (2001). Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. *Proc Natl Acad Sci U S A* **98**, 9859–9864.

Hall, C. B., Douglas, R. G., Jr & Geiman, J. M. (1976). Respiratory syncytial virus infections in infants: quantitation and duration of shedding. *J Pediatr* **89**, 11–15.

Harrison, A. M., Bonville, C. A., Rosenberg, H. F. & Domachowske, J. B. (1999). Respiratory syncytial virus-induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. *Am J Respir Crit Care Med* **159**, 1918–1924.

Hirata, A., Motojima, S., Fukuda, T. & Makino, S. (1996). Damage to respiratory epithelium by guinea-pig eosinophils stimulated with IgG-coated Sepharose beads. *Clin Exp Allergy* **26**, 848–858.

Hisamatsu, K., Ganbo, T., Nakazawa, T., Murakami, Y., Gleich, G. J., Makiyama, K. & Koyama, H. (1990). Cytotoxicity of human eosinophil granule major basic protein to human nasal sinus mucosa in vitro. *J Allergy Clin Immunol* **86**, 52–63.

Howard, C. J., Morrison, W. I., Mackay, C. R. & Splitter, G. A. (1991). Leukocyte antigens in cattle, sheep and goats. Proceedings of the First International Workshop on Leukocyte Antigens in Cattle, Sheep and Goats. *Vet Immunol Immunopathol* **27**, 1–94.

Karger, A., Schmidt, U. & Buchholz, U. J. (2001). Recombinant bovine respiratory syncytial virus with deletions of the G or SH genes: G and F proteins bind heparin. *J Gen Virol* **82**, 631–640.

Kennedy, H. E., Jones, B. V., Tucker, E. M., Ford, N. J., Clarke, S. W., Furze, J. M., Thomas, L. H. & Stott, E. J. (1988). Production and characterisation of bovine monoclonal antibodies to respiratory syncytial virus. *J Gen Virol* **69**, 3023–3032.

Liu, C. M. & Okuda, M. (1988). Injurious effect of eosinophil extract on the human nasal mucosa. *Rhinology* **26**, 121–132.

Maggi, C. A. (1997). The effects of tachykinins on inflammatory and immune cells. *Regul Pept* **70**, 75–90.

McGillis, J. P., Mitsuhashi, M. & Payan, D. G. (1990). Immunomodulation by tachykinin neuropeptides. *Ann N Y Acad Sci* **594**, 85–94.

McNamara, P. S., Flanagan, B. F., Hart, C. A. & Smyth, R. L. (2005). Production of chemokines in the lungs of infants with severe respiratory syncytial virus bronchiolitis. *J Infect Dis* **191**, 1225–1232.

Mohanty, S. B., Ingling, A. L. & Lillie, M. G. (1975). Experimentally induced respiratory syncytial viral infection in calves. *Am J Vet Res* **36**, 417–419.

Noah, T. & Becker, S. (2000). Chemokines in nasal secretions of normal adults experimentally infected with respiratory syncytial virus. *Clin Immunol* **97**, 43–49.

Numao, T. & Agrawal, D. K. (1992). Neuropeptides modulate human eosinophil chemotaxis. *J Immunol* **149**, 3309–3315.

Olszewska-Pazdrak, B., Casola, A., Saito, T., Alam, R., Crowe, S. E., Mei, F., Ogra, P. L. & Garofola, R. P. (1998). Cell-specific expression of RANTES, MCP-1 and MIP-1a by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. *J Virol* **72**, 4756–4764.

Patacchini, R., Lecci, A., Holzer, P. & Maggi, C. A. (2004). Newly discovered tachykinins raise new questions about their peripheral roles and the tachykinin nomenclature. *Trends Pharmacol Sci* **25**, 1–3.

Pennefather, J. N., Lecci, A., Candenas, M. L., Patak, E., Pinto, F. M. & Maggi, C. A. (2004). Tachykinins and tachykinin receptors: a growing family. *Life Sci* **74**, 1445–1463.

Prince, G. A., Curtis, S. J., Yim, K. C. & Porter, D. D. (2001). Vaccine-enhanced respiratory syncytial virus disease in cotton rats following

immunization with Lot 100 or a newly prepared reference vaccine. *J Gen Virol* **82**, 2881–2888.

Rosenberg, H. F. & Domachowske, J. B. (2001). Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens. *J Leukoc Biol* **70**, 691–698.

Schmidt, U., Breyer, J., Polster, U., Gershwin, L. J. & Buchholz, U. (2002). Mucosal immunization with live recombinant bovine respiratory syncytial virus (BRSV) and recombinant BRSV lacking the envelope glycoprotein G protects against challenge with wild-type BRSV. *J Virol* **76**, 12355–12359.

Schreiber, P., Matheise, J. P., Dessy, F., Heimann, M., Letesson, J. J., Coppe, P. & Collard, A. (2000). High mortality rate associated with bovine respiratory syncytial virus (BRSV) infection in Belgian white blue calves previously vaccinated with an inactivated BRSV vaccine. *J Vet Med B Infect Dis Vet Public Health* **47**, 535–550.

Soukup, J. M. & Becker, S. (2003). Role of monocytes and eosinophils in human respiratory syncytial virus infection in vitro. *Clin Immunol* **107**, 178–185.

Stott, E. J. & Taylor, G. (1985). Respiratory syncytial virus. Brief review. *Arch Virol* **84**, 1–52.

Stott, E. J., Thomas, L. H., Taylor, G., Collins, A. P., Jebbett, J. & Crouch, S. (1984). A comparison of three vaccines against respiratory syncytial virus in calves. *J Hyg* **93**, 251–261.

Tam, E. K. & Caughey, G. H. (1990). Degradation of airway neuropeptides by human lung tryptase. *Am J Respir Cell Mol Biol* **3**, 27–32.

Taylor, G., Stott, E. J., Furze, J., Ford, J. & Sopp, P. (1992). Protective epitopes on the fusion protein of respiratory syncytial virus recognized by murine and bovine monoclonal antibodies. *J Gen Virol* **73**, 2217–2223.

Taylor, G., Thomas, L. H., Wyld, S. G., Furze, J., Sopp, P. & Howard, C. J. (1995). Role of T-lymphocyte subsets in recovery from respiratory syncytial virus infection in calves. *J Virol* **69**, 6658–6664.

Taylor, G., Thomas, L. H., Furze, J. M., Cook, R. S., Wyld, S. G., Lerch, R., Hardy, R. & Wertz, G. W. (1997). 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. *J Gen Virol* **78**, 3195–3206.

Thomas, L. H., Gourlay, R. N., Stott, E. J., Howard, C. J. & Bridger, J. C. (1982). A search for new microorganisms in calf pneumonia by the inoculation of gnotobiotic calves. *Res Vet Sci* **33**, 170–182.

Thomas, L. H., Stott, E. J., Collins, A. P. & Jebbett, J. (1984). Experimental pneumonia in gnotobiotic calves produced by respiratory syncytial virus. *Br J Exp Pathol* **65**, 19–28.

Tiberio, I. F., Leick-Maldonado, E. A., Miyahara, L., Kasahara, D. I., Spilborghs, G. M., Martins, M. A. & Saldiva, P. H. (2003). Effects of neurokinins on airway and alveolar eosinophil recruitment. *Exp Lung Res* **29**, 165–177.

Valarcher, J. F., Bourhy, H., Gelfi, J. & Schelcher, F. (1999). Evaluation of a nested reverse transcription-PCR assay based on the nucleoprotein gene for diagnosis of spontaneous and experimental bovine respiratory syncytial virus infections. *J Clin Microbiol* **37**, 1858–1862.

Valarcher, J.-F., Furze, J., Wyld, S., Cook, R., Conzelmann, K.-K. & Taylor, G. (2003). Role of type I interferons in the attenuation and immunogenicity of recombinant bovine respiratory syncytial viruses (BRSV) lacking NS proteins. *J Virol* **77**, 8426–8439.

Wright, P. F., Karron, R. A., Belshe, R. B. & 15 other authors (2000). Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. *J Infect Dis* **182**, 1331–1342.

Zhang, L., Peeples, M. E., Boucher, R. C., Collins, P. L. & Pickles, R. J. (2002). Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J Virol* **76**, 5654–5666.

Zimmer, G., Budz, L. & Herrler, G. (2001). Proteolytic activation of respiratory syncytial virus fusion protein. *J Biol Chem* **276**, 31642–31650.

Zimmer, G., Conzelmann, K.-K. & Herrler, G. (2002). Cleavage at the furin consensus sequence RAK/KR^{109} and presence of the intervening peptide of the respiratory syncytial virus fusion protein are dispensable for virus replication in cell culture. *J Virol* **76**, 9218–9224.

Zimmer, G., Rohn, M., McGregor, G. P., Schemann, M., Conzelmann, K. K. & Herrler, G. (2003). Virokinin, a bioactive peptide of the tachykinin family, is released from the fusion protein of bovine respiratory syncytial virus. *J Biol Chem* **278**, 46854–46861.