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Role of Alpha/Beta Interferons in the Attenuation and Immunogenicity of Recombinant Bovine Respiratory Syncytial Viruses Lacking NS Proteins

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Received 27 February 2003/Accepted 6 May 2003

Alpha/beta interferons (IFN-α/β) are not only a powerful first line of defense against pathogens but also have potent immunomodulatory activities. Many viruses have developed mechanisms of subverting the IFN system to enhance their virulence. Previous studies have demonstrated that the nonstructural (NS) genes of bovine respiratory syncytial virus (BRSV) counteract the antiviral effects of IFN-α/β. Here we demonstrate that, in contrast to wild-type BRSVs, recombinant BRSVs (rBRSVs) lacking the NS proteins, and those lacking NS2 in particular, are strong inducers of IFN-α/β in bovine nasal fibroblasts and bronchoalveolar macrophages. Furthermore, whereas the NS deletion mutants replicated to wild-type rBRSV levels in cells lacking a functional IFN-α/β system, their replication was severely attenuated in IFN-competent cells and in young calves. These results suggest that the NS proteins block the induction of IFN-α/β gene expression and thereby increase the virulence of BRSV. Despite their poor replication in the respiratory tract of young calves, prior infection with virus lacking either the NS1 or the NS2 protein induced serum antibodies and protection against challenge with virulent BRSV. The greater level of protection induced by the NS2, than by the NS1, deletion mutant, was associated with higher BRSV-specific antibody titers and greater priming of BRSV-specific, IFN-γ-producing CD4+ T cells. Since there were no detectable differences in the ability of these mutants to replicate in the bovine respiratory tract, the greater immunogenicity of the NS2 deletion mutant may be associated with the greater ability of this virus to induce IFN-α/β.

Bovine respiratory syncytial virus (BRSV) is an enveloped, nonsegmented, negative-stranded RNA virus and is a major cause of respiratory disease in young calves (44). BRSV is closely related to human RSV (HRSV), which is a major cause of respiratory disease in young children (10), and the epidemiology and pathogenesis of infection with these viruses are similar (44). These features make BRSV infection in calves a good model for the study of HRSV. HRSV and BRSV belong to the Pneumovirus genus within the Paramyxoviridae family. One of the major differences between these genera is that the other Paramyxoviridae is the presence of two nonstructural (NS) genes called NS1 and NS2. These genes code for two proteins, which are abundantly transcribed in virus-infected cells. Comparison of the sequence of the NS proteins of BRSV with that of HRSV subgroup A and B reveals amino acid identities of 69 and 68% for the NS1 protein and 84 and 83% for the NS2 protein, respectively (8, 39).

The role(s) of the NS proteins is not fully defined. They are not essential for virus replication in vitro, although the growth of recombinant HRV and BRSV lacking these proteins is attenuated in cell culture (8, 25, 42, 51). There is evidence that the HRV NS1 protein coprecipitates with the M protein (19), and in experiments using HRV minigenomes, the NS1 protein appears to be a strong inhibitor of viral RNA transcription and replication (1). The NS2 protein also appears to be a transcriptional inhibitor but at a lower level than is the NS1 protein (1). NS2 protein colocalizes with the P and N proteins in infected cells (60) but does not coprecipitate with any viral protein (19). In addition, the NS1 and NS2 proteins of BRSV and HRV mediate resistance to the antiviral action of alpha/beta interferons (IFN-α/β) (3, 42).

Anti-IFN activity has been described for accessory proteins for a number of other negative-stranded RNA viruses. For example, the C protein of Sendai virus inhibits STAT1 activation by hampering phosphorylation and by increasing instability (21, 64) and the V protein of simian virus 5 inhibits the activation of IFN-responsive genes by targeting STAT1 for proteasome-mediated degradation (13). Other viral accessory proteins, such as influenza A virus NS1 protein and Bunyamwera virus NSs protein, inhibit the production of IFN-α/β (58, 61). Inhibition of the antiviral effect of IFN-α/β by HRV does not involve the inhibition of either IFN-α/β or IFN-γ signaling (64), and the exact molecular mechanisms employed by RSV are not known.

In addition to playing a key role in the innate control of virus replication, IFN-α/β have been shown to potently enhance immune responses (29, 30, 40). Consequently viruses that inhibit the production of IFN-α/β may also avoid control by the adaptive immune response. The fact that HRV is a poor
inducer of IFN-α/β in vivo compared with other human respiratory viruses (23, 33) may explain, at least in part, the lack of sustained protective immunity to RSV following natural infection.

Here we have analyzed the effects of deletion of the NS genes on the induction of IFN-α/β by BRSV and their role in establishing BRSV infection in young, gnotobiotic calves. The results obtained provide evidence for the importance of the NS genes in antagonizing the activation of IFN-α and IFN-β transcription as virulence determinants for BRSV and suggest a role for IFN-α/β in the immunogenicity of BRSV lacking the NS genes.

MATERIALS AND METHODS

Viruses and cells. Stocks of the Snook (53), 39J-2 (31) and 127 (45) strains of BRSV were prepared in fetal calf kidney (FCK) cells as described previously (45). Wild-type (WT) recombinant BRSV (rBRSV) and viruses lacking either the NS1 gene (NS1Δ), the NS2 gene (NS2Δ), or both (NS1ΔNS2Δ) were derived from full-length cDNA of BRSV strain AS5198 (36), variant Atuc5198 (GenBank accession no. AF692942), as reported previously (8, 42). Stocks of rBRSV were prepared in Vero cell monolayers that were infected at a multiplicity of infection (MOI) between 0.1 and 0.5 in Dulbecco’s modified Eagle’s medium (Gibco-BRL, Paisley, United Kingdom) containing 2% heated fetal calf serum (HFCS), as described previously. All recombinant virus stocks were checked by reverse transcriptase PCR (RT-PCR) for the presence of mRNA for the NS proteins (data not shown), and all virus stocks were free from contamination with bovine viral diarrhea virus.

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, which 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 bacterial media.

Virus titers were determined by plaque assay on FCK or Vero cell monolayers in 35-mm-diameter petri dishes (53) or by immunostaining of triplicate virus samples on Vero cell monolayers in 96-well plates with anti-F protein monoclonal antibody (MAB) 19 (48), 48 h postinfection (p.i.).

Bovine nasal fibroblasts (NT cells) were obtained from six healthy calves during necropsy. Nasal turbinate cells were washed extensively with phosphate-buffered saline (PBS) containing 40 μg of gentamicin (Sigma-Aldrich, Gillingham, United Kingdom)/ml and 25 μg of amphotericin B (Sigma-Aldrich)/ml. The epithelium was removed from the bone and was inoculated overnight at 4°C in modified minimal essential medium (MEM) with penicillin (10 IU/ml) (Gibco-BRL), streptomycin (10 μg/ml) (Gibco-BRL), gentamicin (40 μg/ml), 2.5 μg of amphotericin B/ml, 0.01 Kunitz units of DNase (DN-25) (Sigma-Aldrich)/ml, and 25 mM HEPES (Gibco-BRL), containing 10% of HFCS. The tissue were removed and the cells were trypsinized, seeded in vented flasks with fibroblast medium containing 5% HFCS, and incubated at 37°C in 5% CO2 in air. Ten million cells were plated into 35-mm-diameter six-well plates and were cultured in 2 ml of MEM (Gibco-BRL, Paisley, United Kingdom) containing 0.125 to 250 IU/ml of recombinant bovine IFN-α1 (Novartis, Basel, Switzerland). All samples were set up in duplicate and were cultured for an additional 24 h at 37°C. CAT expression in cell lysates was determined by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Mannheim, Germany), and the level of IFN-α/β in the test samples was determined by reference to the IFN-α standard.

RT-PCR in real time for analysis of IFN-α and IFN-β mRNA. Cytokine mRNA produced by NT cells or BAM following BRSV infection was quantified by using a method based on that of Kaiser et al. (26). Total RNA was extracted from cells lysed with RNeasy lysis buffer by using the RNeasy mini kit (Qiagen) with a DNase I digestion step (Qiagen) following the manufacturer’s instructions. Purified RNA was eluted in 40 μl of RNase-free water and was stored at −70°C. Total RNA was quantified by using the Ribogreen Kit (Molecular Probes Europe BV, Leiden, The Netherlands) according to the manufacturer’s instructions.

For both IFN mRNA and 28S rRNA-specific amplification, primers and probes were designed by using the Primer Express software program (PE Applied Biosystems). Details of the probes and primers are given in Table 1. Primers and probes were designed from the consensus obtained by multiple-sequence alignments of the bovine IFN-α and IFN-β sequences available in GenBank (for IFN-α, A00145, A00146, A00147, A00148, X93087, X90889, X90898, E00133, E00134, E00135, M11001, and Z46508; for IFN-β, M15477, M15478, and M15479) by using the Wisconsin CCG 9.1 package (55). The primers and probes were designed to detect all of the nine known bovine IFN-α gene products or all of the three known bovine IFN-β gene products. The primers and probes corresponded to areas containing at least seven mutations compared with the consensus sequences of the other subfamilies of IFN, and their specificity was checked by using plasmids containing different segments of bovine IFN-α, -β, -ω, or -τ (data not shown). IFN and 28S probes were labeled with fluorescent reporter dye 5-carboxyfluorescein at the 5’ end and with the quencher N,N,N,N’-tetramethyl-6-carboxyrhodamine at the 3’ end. Quantification was based on the increased fluorescence detected by ABI PRISM 7700 Sequence Detection system described previously. Detection of the target sequence by real-time PCR was possible only for the 5’ nuclear activity of the rT DNA polymerase during PCR amplification.

RT-PCR was performed by using Reverse Transcriptase qPCR Master Mix kit (Eurogenetic, Seraing, Belgium). The RT-PCR mixture consisted of 1× Master Mix Buffer (containing deoxyinosine triphosphates, deoxyguanosine triphosphate buffer, and Hot Goldstar DNA polymerase), 0.25 U of Moloney murine leukemia RT/24, 0.1 U of RNase inhibitor/μl, primers (concentrations, 300 nM for 28S and 100 nM for IFN-α, and 100 nM concentration of the specific probe, 15 ng of RNA, and RNSase-free water so that the volume added up to 25 μl. To quantify the RNA, the following cycle profile was used: 1
cycle of 48°C for 30 min and 96°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

To generate standard curves for IFN and 28S RNA, plasmids containing the specific amplified segment were used and were serially diluted in RNAse-free water to obtain between 10^3 and 10^10 copies in 5 μl. Each RT-PCR experiment contained Neo template controls, test samples, and serial dilutions of plasmids, and each was run in triplicate. Regression analyses of the mean values of replicate RT-PCRs for serial dilutions of the specific plasmids were used to generate standard curves. Results are expressed as the number of copies of IFN mRNA/10^6 copies of 28S rRNA.

Calves and experimental design. Gnotobiotic, BRSV-seronegative calves were derived, reared, and maintained individually in plastic isolators as described previously (12). To evaluate the virulence of different strains of WT BRSV and rBRSVs, groups of two to five gnotobiotic calves were infected at 2 to 3 weeks of age with approximately 10^6 PFU of virus in a volume of 20 μl, 10 μl administered intranasally (i.n.) and 10 μl administered intratracheally (i.t.). Nasopharyngeal swabs and 5 μl of blood were obtained daily to measure IFN-γ/H9251, IFN-β/H9253, and to monitor virus excretion from the nasopharynx. As controls, calves were inoculated i.n. and i.t. with 20 μl of control tissue culture fluid.

A clinical examination was 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., Harlow, United Kingdom). Calves were killed 6 days after challenge to determine the extent of gross pneumonia. From calves inoculated with rBRSV or different strains of BRSV were derived, reared, and maintained individually in plastic isolators as described above. Ten days after infection with rBRSV, calves were removed from the plastic isolators, mixed, and reared in a high-security, barrier-maintained facility. Six weeks after immunization, calves were challenged i.n. and i.t. with approximately 10^6 PFU of virus in a volume of 20 μl, 10 μl administered intranasally and 10 μl administered intratracheally.

Ten days after infection with rBRSV, calves were removed from the plastic isolators, mixed, and reared in a high-security, barrier-maintained facility. Six weeks after immunization, calves were challenged i.n. and i.t. with approximately 10^6 PFU of virus in a volume of 20 μl, 10 μl administered intranasally and 10 μl administered intratracheally.
We have extended these observations by comparing the growth of NS deletion mutants with that of WT virus in Vero cells, characterized by the lack of IFN-α/β genes (14, 59), and in low-passage bovine NT cells, which are competent for the production of IFN-α/β and which are used for the isolation of BRSV from clinical material (35). There were no significant differences in the growth of the NS deletion mutants and the parental full-length virus or the Snook strain of BRSV in Vero cells (Fig. 1). All viruses reached peak titers of between 8 × 10^5 and 5 × 10^6 PFU/ml after 3 or 4 days. For all of the viruses, giant cells were observed 2 days after infection and an increasing cytopathic effect was observed from day 3 to day 6. In contrast, the growth of all three deletion mutants was impeded in NT cells compared with that in Vero cells (Fig. 1). Furthermore, there were differences in the level of attenuation of the NS deletion mutants in NT cells. Thus, the growth of the ΔNS2 and ΔNS1/2 mutants was impeded to a greater extent than that of the NS1 deletion mutant. Whereas ΔNS1 mutant reached a peak titer of 4 × 10^5 PFU/ml 2 days after infection, the titers of ΔNS2 and ΔNS1/2 viruses decreased until day 4 after infection, when virus could no longer be recovered. The growth of BRSV Snook in NT and Vero cells was similar. However, WT rBRSV grew to lower titers in NT cells than in Vero cells. Cytopathic effects were observed in NT cells infected with WT BRSVs 3 to 6 days after infection but not in NT cells infected with the NS deletion mutants.

**Induction of IFN-α/β following infection of bovine NT cells with NS deletion mutants of BRSV.** Since the growth of the NS deletion mutants in IFN-competent NT cells was impeded compared with that in Vero cells, we investigated the ability of the mutant viruses to induce IFN-α/β in NT cells. NT cells, at a passage level of less than 14, were infected with WT or mutant viruses at an MOI of 0.01 with WT rBRSV, BRSV Snook (Snk), rBRSVΔNS1, rBRSVΔNS2, or rBRSVΔNS1/2, and levels of infectious virus produced each day were determined as described in Materials and Methods. Values for Vero cells were obtained from two independent experiments, each performed in duplicate, and for NT cells from one experiment in duplicate.

**FIG. 1.** Replication of NS deletion mutants of BRSV in simian Vero cells and in low-passage-number (5P) bovine NT cells. Vero or NT cells were infected at an MOI of 0.01 with WT rBRSV, BRSV Snook (Snk), rBRSVΔNS1, rBRSVΔNS2, or rBRSVΔNS1/2, and levels of infectious virus produced each day were determined as described in Materials and Methods. Levels of infectious virus produced each day were determined as described in Materials and Methods. Values for Vero cells were obtained from two independent experiments, each performed in duplicate, and for NT cells from one experiment in duplicate.

IFN-α/β was produced between 6 and 12 h p.i. and reached a plateau 12 h after infection with rBRSVΔNS2 or 24 h after infection with rBRSVΔNS1/2 (Fig. 2A). Although the ΔNS1 mutant did not induce detectable IFN-α/β in NT cells, the ΔNS1/2 mutant induced between 4 and 15 times more IFN-α/β than did rBRSVΔNS2. IFN-α/β induction by NT cells infected with rBRSVΔNS1/2 was influenced by the MOI, with increasing levels of IFN induced at higher MOIs (results not shown). However, even at an MOI of 1.0, IFN-α/β production was not detected in the supernatant of NT cells infected with ΔNS1 virus or WT rBRSV (results not shown).

Since infection of NT cells with WT rBRSV did not induce IFN-α/β, the ability of other WT BRSVs to induce IFN was investigated. The Snook, 391-2, and 127 strains of BRSV grew to similar titers in NT cells (Fig. 2F) and induced little or no IFN-α/β at an MOI of 0.1 (Fig. 2E). Thus, IFN-α/β production was not detected in NT cells infected with the Snook strain of BRSV and the highest levels of IFN-α/β induced by BRSV strains 391-2 and 127 were 3.75 and 2.15 IU/ml, respectively.

**Induction of IFN-α/β by BAM infected with NS deletion mutants.** Infection of BAM with NS deletion mutants resulted in induction of IFN-α/β in a pattern similar to that seen in NT cells (Fig. 3A). However, whereas infection of NT cells with
rBRSVΔNS1 induced little or no IFN-α/β, this virus induced low levels of IFN in BAM. The ΔNS2 mutant induced four to seven times more IFN than the ΔNS1 mutant and the ΔNS1/2 mutant induced 1.5-fold more IFN-α/β than did the ΔNS2 mutant. Although the NS deletion mutants appeared to be rapidly inactivated in BAM, as infectious virus could not be recovered 48 h after infection (Fig. 3B), infectious virus was essential for induction of IFN-α/β. Thus, UV-inactivated or antibody-neutralized viruses did not induce IFN-α/β in BAM (results not shown).

As seen in NT cells, WT rBRSV induced little or no IFN-α/β in BAM (Fig. 3A). However, this virus did not survive in BAM as well as other WT strains of BRSV did (Fig. 3B and C). Although two of the WT strains, 391-2 and 127, induced low levels (<10 IU/ml) of IFN-α/β in BAM (Fig. 3A), this did not appear to influence their survival in these cells. This is to be expected, since previous studies demonstrated that the NS proteins mediate resistance against IFN-α/β (3, 42).

Further studies on the interaction of rBRSV with BAM showed that there were large variations in the production of IFN-α/β by cells from different animals (Fig. 4). Nevertheless, the ΔNS2 mutant consistently induced significantly higher levels of IFN than did WT rBRSV. These variations may be related to the age and/or immune status of the cattle.

**Induction of IFN-α/β mRNAs in NT and BAM cells.** Production of IFN-α/β was confirmed by analysis of IFN-α and IFN-β mRNA in virus-infected NT cells with Taq-Man PCR. Following infection with the ΔNS2 or ΔNS1/2 mutant, NT cells produced both IFN-α and IFN-β mRNA (Fig. 5A), with the levels of IFN-β mRNA being greater than those for IFN-α. Although transcripts for IFN-α or -β were very low in NT cells infected with WT rBRSV (<1.2 copies/10^6 copies of 28S RNA) (Fig. 5A), IFN-β mRNA (6.16 copies/10^6 copies of 28S RNA) could be detected in NT cells infected with the ΔNS1 mutant. Low levels (0.78 to 3.7 copies/10^6 copies of 28S RNA) of IFN-β mRNA could also be detected in NT cells exposed to antibody-neutralized NS deletion mutants. These low levels of IFN-α or -β transcripts may be due to residual DNA contamination following treatment with DNase or incomplete neutralization of the mutant viruses. These results demonstrate that IFN-β mRNA is strongly synthesized and translated in NT cells infected with rBRSV lacking the NS2 gene but not in cells infected with WT virus, suggesting that the NS2 protein interferes with transcriptional activation of the IFN-β promoter.

Production of IFN-α/β by BRSV-infected BAM was confirmed by analysis of IFN-α and IFN-β mRNA. Whereas there was little or no IFN-α or -β mRNA in BAM infected with WT rBRSV or with the Snook strain of BRSV, transcripts for IFN-α were detected in BAM infected with the NS deletion mutants 24 h p.i. and decreased dramatically between 24 and 48 h p.i. (Fig. 5B). Levels of IFN-α mRNA in BAM infected with the ΔNS1, ΔNS2, and ΔNS1/2 mutants were 226-, 1,345-, and 1,831-fold greater, respectively, than that induced by WT rBRSV.

Differences in the types of IFNs produced by NT cells and BAM in this study were consistent with the known differences in the IFN response of fibroblasts and leukocytes. Thus, IFN-β is synthesized mainly by fibroblasts, which appear to produce
IFN-α only following IFN receptor activation by IFN-β (15). In contrast, IFN-α is synthesized predominantly by leukocytes and is independent of IFN-β synthesis in these cells (15).

WT rBRSV and ΔNS1 rBRSV inhibit production of IFN by rBRSVΔNS1/2. In order to determine if expression of the NS proteins by rBRSV inhibits the rBRSVΔNS1/2-mediated production of IFN-α/β, IFN levels were compared in rBRSVΔNS1/2-infected NT cells with or without superinfection by BRSV expressing the NS1 and/or the NS2 protein. Bovine NT cells were infected with the ΔNS1/2 mutant at an MOI of 0.05 for 2 h; the inoculum was removed and replaced by fresh medium containing 2% HFCS. Four hours p.i., cells were superinfected with WT virus or with the NS deletion mutants at an MOI of 0.5 or with fresh medium. Virus was adsorbed for 2 h at 37°C, the inoculum was removed, and the cells were incubated with fresh medium containing 2% HFCS. IFN-α/β production was determined at 24 and 48 h by using the MxA-CAT bioassay. Superinfection of rBRSVΔNS1/2-infected NT cells with either WT virus or the NS deletion mutant inhibited IFN-α/β production by 50 to 60% (Fig. 6). In contrast, superinfection with the NS2 deletion mutant increased IFN production by approximately 40%.

NS deletion mutants of BRSV are attenuated in calves. Since the NS deletion mutants appeared to have an attenuated phenotype in both NT cells and BAM, the replication of the mutants in the respiratory tract of 2-week-old, BRSV-seronegative, gnotobiotic calves was evaluated. Groups of three to six calves were inoculated simultaneously by the i.n. and i.t. routes with WT rBRSV or with the NS deletion mutants, and three calves in each group were killed 6 days after infection. The remaining calves were kept in isolation until challenged with virulent virus, 6 weeks after immunization. For comparison, the ability of other WT strains of BRSV to infect gnotobiotic calves was also investigated. Following infection, clinical signs of respiratory disease were not observed in any of the calves apart from those infected with the calf-passaged Snook strain of BRSV (Snook BAL), who developed increased respiratory rates 6 days p.i. These calves had extensive pneumonic consolidation at postmortem, 7 days after infection. WT rBRSV was not as virulent as either calf-passaged BRSV Snook or tissue culture-grown BRSV Snook but was not as attenuated as two other WT strains of BRSV studied (Table 2). Thus, the replication of WT rBRSV in the upper respiratory tract was similar to that in calves inoculated with a 10-fold-lower dose of tissue culture-grown BRSV Snook. Furthermore, virus was isolated in lower titers and less frequently from the lower respiratory tract of calves infected with WT rBRSV than that found in calves infected with a 10-fold-lower dose of tissue culture-grown BRSV Snook (Table 2). However, WT rBRSV appeared to replicate to higher titers in the bovine respiratory tract than the 391-2 or 127 strain of BRSV, although the numbers of calves infected with the latter viruses were small (Table 2).
The replication of the single-deletion NS mutants in the bovine respiratory tract was significantly reduced compared with that of WT rBRSV, and virus could not be isolated from either the nasopharynx or the lungs of calves infected with the double-deletion mutant (Table 2). Peak virus titers in the nasopharynx of calves infected with the single deletion mutants were 300- to 600-fold less than that in calves infected with WT rBRSV, and there were no significant differences in the replication of the ΔNS1 and ΔNS2 mutants. Virus was not detected in BAL cells from calves inoculated with either the NS1 or the NS2 deletion mutants but was isolated in low titers from a single sample of lung tissue from one calf in each group.

Whereas the lungs from all three calves inoculated with WT rBRSV showed some gross pulmonary consolidation, there was little or no gross pulmonary pathology in calves inoculated with any of the NS deletion mutants. Microscopic lesions in lungs from calves infected with WT rBRSV were similar to those reported previously in gnotobiotic calves experimentally infected with BRSV Snook (53) Thomas et al., 1984) and were characterized by a proliferative and exudative bronchiolitis with some accompanying alveolar collapse and peribronchial infiltration by mononuclear cells. Microscopic lesions were not observed in the lungs of any of the calves inoculated with the NS deletion mutants, and the pulmonary histology of these calves was indistinguishable from that in calves inoculated i.n. and i.t. with lysates from mock-infected Vero cells.

**Mucosal immunization with rBRSV lacking either NS1 or NS2 protects against challenge with virulent BRSV.** Groups of two or three gnotobiotic calves that had been inoculated i.n. and i.t. with WT, ΔNS1, ΔNS2 rBRSV, or noninfected Vero cell lysate 6 weeks previously were challenged with BRSV Snook in BAL. Despite the highly restricted replication of the ΔNS1 and ΔNS2 mutants in the respiratory tract of calves, infection with these viruses induced a serum antibody response (Table 3; Fig. 7A and B) and primed CD4+ T cells for production of IFN-γ (Fig. 7D). Although there were no detectable differences in the level of attenuation of the ΔNS1 and ΔNS2 mutants, the ΔNS2 mutant appeared to be more immunogenic than the ΔNS1 mutant. Thus, the number of BRSV-specific, IFN-γ-producing CD4+ T cells was greater in two of the calves immunized 6 weeks previously with the ΔNS2 mutant than in the three calves immunized with the ΔNS1 mutant (Fig. 7D). Furthermore, the levels of BRSV-specific serum antibodies detected by ELISA in calves immunized 6 weeks previously with the ΔNS2 mutant were similar to those induced by WT rBRSV and were eightfold greater than those induced by the
FIG. 6. Inhibition of rBRSVΔNS1/2-mediated IFN-α/β production by WT rBRSV or rBRSVΔNS1 in bovine NT cells. Triplet cultures of NT cells were infected with rBRSVΔNS1/2 at an MOI of 0.05 and 4 h later with rBRSV (WT), rBRSVΔNS1 (ΔNS1), or rBRSVΔNS2 (ΔNS2) at an MOI of 0.5. Levels of IFN-α/β were analyzed by using the MxA-CAT bioassay, as described in Materials and Methods. Results are expressed as the percent inhibition of IFN-α/β in rBRSVΔNS1/2-infected cells superinfected with the WT or ΔNS1 or ΔNS2 mutant 48 h after infection. The figure shows representative data from one of two independent experiments that gave similar results.

ΔNS1 mutant (Table 3). Serum-neutralizing antibodies in calves immunized with the ΔNS2 mutant were only sixfold less than those induced by WT rBRSV, whereas the titer of neutralizing antibody induced by the ΔNS1 mutant was 80-fold less than that induced by WT virus (Table 3). The IgG1 serum antibody response detected by ELISA appeared to be delayed in calves immunized with the NS deletion mutants compared with that in calves immunized with WT rBRSV (Fig. 7A). However, BRSV-specific IgG2 serum antibodies were detected earliest in calves immunized with the ΔNS2 mutant and titers were always highest in these animals, although the differences were not statistically significant (Fig. 7B).

Following challenge with virulent BRSV, there was a rapid increase in serum antibody titers in all immunized calves. Although serum antibody titers, as detected by ELISA, were similar in all immunized calves 6 days after challenge serum-neutralizing titers in calves immunized with WT rBRSV were 10- or 60-fold greater than those in calves immunized with the ΔNS2 or ΔNS1 mutant, respectively (results not shown). Differences in IgA titers in BAL from immunized calves were also detected 6 days after challenge. Thus, IgA titers in BAL from calves immunized with the ΔNS2 mutant were similar to those in calves immunized with WT rBRSV and were significantly higher than those in BAL from calves immunized with the ΔNS1 mutant or the control calves (P < 0.05) (Fig. 7C). BRSV-specific IgG2 antibodies were not detected in BAL from any of the calves.

Following challenge, clinical signs of respiratory disease were not observed in any calves. However, calves were killed on day 6 after challenge, which is at the time that clinical signs of disease are usually first detected. Nevertheless, pulmonary lesions were present in all of the control calves, whereas there was little or no pneumonia consolidation in calves immunized with either WT rBRSV or with either the ΔNS1 or the ΔNS2 mutant (Fig. 8). Animals previously immunized with WT rBRSV were highly resistant to subsequent challenge with BRSV Snook (Table 3). Following challenge, virus was not isolated from the nasopharynx or the lung tissue of calves immunized with WT rBRSV but was isolated in low titers from BAL cells of one calf. In contrast, control calves excreted high titers of virus from the nasopharynx starting from day 3 after challenge and high titers of virus were isolated from BAL cells and from all lung samples studied at postmortem (Table 3). Calves previously immunized with either of the NS deletion mutants were completely protected against BRSV infection in the lower respiratory tract. However, the level of protection against upper respiratory tract infection was not as great as that seen in calves immunized with WT rBRSV. Although the differences were not statistically significant, the level of protection against upper respiratory tract infection appeared to be greater in calves immunized with the ΔNS2 mutant than in those immunized with the ΔNS1 mutant. Thus, following challenge, all of the calves previously immunized with the ΔNS1

![Table 2: Evaluation of the virulence of rBRSV and rBRSV lacking the NS genes in calves](http://jvi.asm.org/Downloaded-from/February-27-2014-by-PENN-STATE-UNIV)
mutant excreted virus from the nasopharynx, whereas only one of the three calves immunized with the \( \text{NS2} \) mutant excreted virus.

**Induction of IFN-\( \alpha/\beta \) in vivo.** Following infection of gnotobiotic calves with the different rBRSV, levels of IFN-\( \alpha/\beta \) in serum and nasopharyngeal secretions obtained daily after infection and in BAL obtained at postmortem, 6 days after infection, were analyzed by using the MxA-CAT bioassay. Although viruses lacking the NS2 or NS1/2 gene induced high levels of IFN-\( \alpha/\beta \) in NT cells or BAM in vitro, IFN-\( \alpha/\beta \) could not be detected in sera, nasopharyngeal swabs (results not shown), or BAL (Table 2) from calves infected with these viruses or in calves infected with the \( \text{NS1} \) mutant. In contrast IFN-\( \alpha/\beta \) could be detected in BAL, obtained 6 days after infection, from calves infected with WT rBRSV or BRSV Snook, which did not induce IFN-\( \alpha/\beta \) in vitro (Table 2).

**TABLE 3.** Recombinant BRSV lacking NS1 or NS2 protects against challenge with virulent BRSV

<table>
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<tr>
<th>Immunizing virus</th>
<th>Inoculum dose (log(_{10}) PFU/ml)</th>
<th>No. of calves</th>
<th>Mean serum antibody titer on day 72</th>
<th>Nasopharyngeal excretion data</th>
<th>Virus titer in BAL cells</th>
<th>Presence of virus in lung tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT rBRSV ( \text{A}_{\text{True}} )</td>
<td>6.5</td>
<td>2</td>
<td>3.2 ± 0.01</td>
<td>2.4 ± 0.5</td>
<td>&lt;0.7</td>
<td>0</td>
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<tr>
<td>( \Delta\text{NS1} )</td>
<td>6.6</td>
<td>3</td>
<td>2.3 ± 0.3</td>
<td>0.5 ± 0.2</td>
<td>2.9 ± 0.8</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>( \Delta\text{NS2} )</td>
<td>6.6</td>
<td>3</td>
<td>3.1 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>1.1 ± 2.0</td>
<td>1.7 ± 2.1</td>
</tr>
<tr>
<td>Mock</td>
<td>None</td>
<td>3</td>
<td>&lt;1.5</td>
<td>&lt;0.5</td>
<td>4.4 ± 0.3</td>
<td>3.7 ± 0.6</td>
</tr>
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</table>

\( ^a \) Gnotobiotic calves were inoculated i.n. and i.t. with the indicated amounts of virus. After 72 days, calves were challenged i.n. and i.t. with 3.7 log\(_{10}\) PFU of BRSV Snook in BAL.

\( ^b \) Mean antibody titers (log\(_{10}\)) plus or minus standard deviation determined by ELISA with BRSV Snook-infected cell lysate as antigen.

\( ^c \) Mean neutralizing antibody titer (log\(_{10}\)) plus or minus standard deviation determined by a 50% plaque reduction assay using heat-inactivated sera and BRSV Snook.

\( ^d \) Mean peak titers (log\(_{10}\) PFU/ml) plus or minus standard deviation were calculated by using the peak virus titer achieved in the nasopharynx in each animal. The threshold of detection was 0.7 log\(_{10}\) PFU/ml, and samples lacking detectable virus were assigned a titer of 0.6 log\(_{10}\) PFU/ml.

\( ^e \) Mean log\(_{10}\) PFU plus or minus standard deviation of virus per ml in BAL cells sampled postmortem, 6 or 7 days after infection. The threshold of detection was 0.7 log\(_{10}\) PFU/ml, and samples lacking detectable virus were assigned a titer of 0.6 log\(_{10}\) PFU/ml (number of calves infected/total analyzed).

\( ^f \) Number of lung samples from which virus was isolated at postmortem/total analyzed. Three pieces of lung were sampled per animal.

FIG. 7. BRSV-specific antibody and CD4\(^+\)-T-cell responses induced by mucosal immunization with rBRSV in calves. Gnotobiotic calves were inoculated i.n. and i.t. with approximately 4 x 10\(^5\) PFU of rBRSV (WT) (n = 2), rBRSV\( \Delta\text{NS1} \) (\( \Delta\text{NS1} \)) (n = 3), rBRSV\( \Delta\text{NS2} \) (\( \Delta\text{NS2} \)) (n = 3), or noninfected Vero cell lysate (mock) (n = 3). Calves were challenged 6 weeks later with 5 x 10\(^3\) PFU of the Snook strain of BRSV in BAL. BRSV-specific IgG1 (A) and IgG2 (B) serum antibody responses were determined by ELISA. (C) BRSV-specific IgG1 and IgA antibody titers were determined by ELISA in BAL from immunized calves 6 days after challenge with BRSV Snook. (D) Number of BRSV-specific, IFN-\( \gamma \)-secreting CD4\(^+\) T cells, determined by ELISPOT assay, in peripheral blood 6 weeks after immunization. sfc, spot-forming cells.
large variations in the concentrations of IFN-α/β seen in BAL from calves infected with WT rBRSV may be the result of variations in the volume of PBS that was recovered during the BAL procedure. In general, IFN-α/β was detected in BAL only from animals in which the virus titers were greater than $10^5$ PFU of BAL cells/ml. These results suggest that, in vivo, induction of IFN-α/β in the lung correlates with the level of virus replication.

Induction of IFN-α/β appeared quite late after infection with WT BRSVs. Thus, in two of the mock-immunized calves that were infected with calf-passaged BRSV Snook, IFN-α/β could not be detected in BAL on day 1 or 3 of infection (results not shown), but 15.4 and 58.3 IU of IFN/ml of BAL were detected in these animals on day 6. The correlation between the induction of IFN-α/β and viral replication, in vivo, was supported by the observation that IFN-α/β could be detected in both nasal secretions and BAL from immunized calves that were not protected against challenge with virulent virus. Thus, low levels of IFN-α/β were detected in nasal secretions on days 3 and 6 after challenge of calves that had been previously immunized with either the ΔNS1 mutant or with noninfected Vero cell lysate (Fig. 9A). Similarly, IFN-α/β was detected only in BAL from control calves 6 days after challenge with virulent BRSV Snook and not in those calves previously immunized with either the ΔNS1 or ΔNS2 mutants (Fig. 9B) or WT rBRSV (<0.7 IU/ml).

**DISCUSSION**

Previous studies have demonstrated that the NS genes of BRSV are important in conferring resistance to the antiviral effects of IFN-α/β in vitro (42). We have extended these observations by demonstrating that the NS proteins are also involved in inhibiting the induction of IFN-α/β in vitro. In con-

<table>
<thead>
<tr>
<th>Immunising Virus</th>
<th>% pneumatic lesions after challenge with BRSV snook</th>
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<tbody>
<tr>
<td>wt</td>
<td><img src="https://example.com/image1.png" alt="Image" /></td>
</tr>
<tr>
<td>ΔNS1</td>
<td><img src="https://example.com/image2.png" alt="Image" /></td>
</tr>
<tr>
<td>ΔNS2</td>
<td><img src="https://example.com/image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Mock</td>
<td><img src="https://example.com/image4.png" alt="Image" /></td>
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**FIG. 8.** Immunization with rBRSV protects against the development of gross pneumatic lesions following challenge with the Snook strain of BRSV. Given is the percentage of lung showing macroscopic lung lesions 6 days after challenge with the Snook strain of BRSV in BAL in calves immunized by i.n. and i.t. inoculation with approximately $4 \times 10^6$ PFU of rBRSV (wt), rBRSVΔNS1 (ΔNS1), rBRSVΔNS2 (ΔNS2), or noninfected Vero cell lysate (Mock).

**FIG. 9.** Effect of immunization with NS deletion mutants on induction of IFNs-α/β in the respiratory tract following challenge with BRSV Snook. Gnotobiotic calves were inoculated i.n. and i.t. with approximately $4 \times 10^6$ PFU of rBRSVΔNS1 (ΔNS1) ($n = 3$), rBRSVΔNS2 (ΔNS2) ($n = 3$), or noninfected Vero cell lysate (Mock) ($n = 3$). Calves were challenged 6 weeks later with $5 \times 10^3$ PFU of the Snook strain of BRSV in BAL. Levels of IFN-α/β analyzed by using the MxA-CAT bioassay as described in Materials and Methods were determined in supernatants from nasopharyngeal swabs obtained after challenge with BRSV Snook (A) and in BAL (B) obtained 1 day prior to (D-1) or 6 days (D6) after challenge.
trast to WT BRSV, which induces little or no IFN-α/β in bovine NT cells or BAM, IFN-α/β was induced by NS deletion mutants of BRSV. The NS2 protein clearly had a greater role in inhibiting the production of IFN-α/β than the NS1 protein, but there was evidence that these proteins may cooperate in this function.

Initial studies demonstrated that, whereas replication of NS deletion mutants of BRSV was similar to that of WT BRSV in Vero cells, which are not able to induce IFN, they exhibited a restricted replication in bovine NT cells and in BAM. These findings are similar to those for NS deletion mutants of HRSV, which have a greater defect in replication in Hep-2 cells than in Vero cells (25). Differences in the level of attenuation of the NS deletion mutants of BRSV in bovine NT cells and BAM correlated with their ability to induce IFN-α/β. The more restricted replication of the ΔNS2 and ΔNS1/2 mutants was associated with the highest levels of IFN-α/β. The finding that IFN-β and IFN-α mRNAs are strongly synthesized and translated in NT cells and BAM, respectively, following infection with rBRSVs lacking the NS2 gene, compared with either WT viruses or the ΔNS1 mutant, together with the observation that the latter viruses inhibit IFN production by rBRSVΔNS1/2-infected NT cells, suggests that the NS2 protein strongly interferes with transcriptional activation of both the IFN-α and the IFN-β promoters.

The failure of the ΔNS2 mutant to inhibit induction of IFN-α/β by rBRSVΔNS1/2-infected NT cells and the absence of detectable IFN-α/β in supernatants from NT cells infected with the ΔNS1 mutant suggest that the NS1 protein alone is not able to inhibit induction of IFN-α/β in NT cells. However, the increased levels of IFN-β and IFN-α mRNA in rBRSVΔNS1-infected NT cells or BAM suggest that the NS1 protein does interfere with transcriptional activation of IFN promoters, although the effect is not as strong as that of the NS2 protein. It is possible that increased accumulation of the NS2 protein, which occurs in rHRSVΔNS1-infected cells (25), may limit the production of high levels of IFN-α/β by the ΔNS1 mutant. Differences in the levels of IFN mRNA and the bioactivity of IFN-α/β in supernatants from NT cells and BAM infected with the ΔNS1 mutant may reflect differences in the sensitivity of the MxA-CAT reporter gene assay to different subtypes of IFN-α/β produced by NT cells and BAM.

The production of IFN-α/β by rBRSVΔNS2-infected NT cells, which starts between 6 and 12 h after infection, reached a plateau 12 h after infection. This corresponds to the time when the NS1 protein can be clearly detected in RSV-infected cells (19). In the absence of both of the NS proteins, production of IFN-α/β does not reach a plateau until 24 h after infection of NT cells. These observations suggest that the NS1 protein may also inhibit a posttranscriptional stage of induction of IFN-α/β.

The accessory proteins of several negative-stranded RNA viruses are able to antagonize the IFN-α/β response. Some, like the C protein of Sendai virus and the V protein of simian virus 5, interfere with the activation of IFN-responsive genes (13, 64), while others, like the influenza A virus NS1 protein and the Bunyamwera virus NSs protein, directly inhibit the production of IFN-α/β (58, 61). The NS proteins of BRSV not only mediate resistance to the antiviral action of IFN but also antagonize IFN induction. The mechanisms by which BRSV suppresses induction of and escapes the antiviral effects of IFN-α/β are not known. Unlike other Paramyxoviridae, HRSV does not appear to block IFN-α/β or IFN-γ signaling (64). The NS1 protein of influenza virus and the NSs protein of Bunyamwera virus prevent activation of both NF-κB and IFN regulatory factor-3, possibly by sequestering double-stranded RNA (46, 58, 61). Similarly, IFN regulatory factor-3 is not activated in cells infected with WT BRSV; however, NF-κB is activated (B. Bossert and K.-K. Conzelman, submitted for publication). Studies to determine whether the NS proteins of BRSV are able to inhibit IFN synthesis on their own and to elucidate the precise mechanisms by which these proteins exert their effects are in progress.

Induction of IFN-α/β by HRSV in vitro is controversial, with some studies reporting IFN production by HRSV-infected human peripheral blood monocytes, macrophages, or pulmonary epithelial cells and others reporting a lack of IFN production in the same cells (2, 9, 22, 28, 32, 41). As seen in the present study of BRSV, these differences may be related to the strain of virus (32, 34) and/or the origin of the cells. Although there was some variation in the IFN response induced by different WT strains of BRSV and in the IFN response of BAM from different cattle, rBRSVs lacking the NS2 gene consistently induced significantly higher levels of IFN than any of the WT BRSVs. Rift Valley fever viruses containing mutations in the NSs gene are strong IFN-α/β inducers (4), and sequence variation in the NS genes of different strains of BRSV may be associated with differences in their ability to induce IFN. However, the increased synthesis of IFN-β by the ts1C mutant of HRsV compared with that of the parent virus or with two related temperature-sensitive mutants, is not associated with sequence variation in the NS genes, suggesting that other viral proteins may also play a role in the regulation of IFN induction by RSVs (32, 56).

The ability of NS deletion mutants and WT BRSV to induce IFN-α/β in NT cells and BAM correlated, at least in part, with their ability to replicate in the bovine respiratory tract. The 127 and 391-2 strains of BRSV induced higher levels of IFN-α/β, in vitro, and were more attenuated in vivo than the Snook strain of BRSV. Furthermore, the NS deletion mutants were highly attenuated in calves. However, we were unable to detect IFN-α/β in either respiratory secretions or sera from calves infected with any of the NS deletion mutants. Failure to detect IFN-α/β in BAL from calves infected with the NS deletion mutants may be related to the time of sampling. Since levels of IFN-α/β were maximal 24 h after in vitro infection of BAM with the NS deletion mutants, it is possible that IFN-α/β produced in vivo had declined to undetectable levels when BAL was obtained, 6 days after infection with the NS deletion mutants. Failure to detect IFN-α/β in nasopharyngeal swabs may be related to the limited area of the upper respiratory tract that is sampled by using nasopharyngeal swabs together with the presence of only a small number of virus-infected cells.

In contrast to findings from calves infected with the NS deletion mutants, IFN-α/β was detected in BAL and nasal secretions from calves that had been infected with either WT rBRSV or with BRSV Snook, and as seen in infants infected with HRSV (47), detection of IFN-α/β correlated with extensive virus infection and with a pulmonary inflammatory response. Although IFN-α/β can be detected in respiratory se-
creations from HRSV-infected individuals, HRSV is a poor inducer of IFN in vivo compared with other human respiratory viruses (23, 33). The major IFN-producing cell in the respiratory tract following BRSV infection is not known. Ciliated epithelial cells are the main sites of virus replication, although virus can also be detected in nonciliated cells and in both type I and type II pneumocytes (6, 7, 57). The ability of BRSV to induce IFN in plasmacytoid dendritic cells (DCs), which are major IFN-producing cells in vivo (11), is not known. However, neither peripheral blood monocyte-derived nor bone marrow-derived DCs produced IFN-α/β following infection in vitro with WT rBRSV or the Snook strain of BRSV. Furthermore, only low (< 6 IU/ml) levels of IFN-α/β were produced following infection of DCs with any of the ΔNS mutants (J.-F. Valarcher and G. Taylor, unpublished observations).

The replication of NS deletion mutants of BRSV in the bovine respiratory tract appeared to be more restricted than that of similar NS deletion mutants of HRSV in chimpanzees (52, 63). This may reflect differences in the virulence of the parental viruses, since rBRSV Atue51908 appears to be more attenuated in calves than the A2 strain of HRSV is in chimpanzees. The nasopharyngeal excretion of the WT rBRSV observed in this study was similar to that reported previously in 2-month-old conventional calves inoculated with the same virus (43). The low level of virus recovered from calves infected with the single-deletion mutants of BRSV may represent virus carried over from the initial inoculum. However, calves inoculated with these viruses developed a BRSV-specific immune response, suggesting that viral transcription had occurred in the respiratory tract.

Despite the highly restricted replication of rBRSVΔNS1 and rBRSVΔNS2 in the bovine respiratory tract, immunization with these viruses induced significant protection against subsequent challenge with a virulent BRSV. The immune response and resistance to BRSV challenge induced by the ΔNS2 mutant appeared to be greater than those induced by the ΔNS1 mutant. Thus, levels of BRSV-specific neutralizing serum antibodies, IgG2 serum antibodies, IgA antibodies in BAL, and priming of CD4+ T cells were greater in calves immunized with the ΔNS2 mutant than in those immunized with rBRSVΔNS1. Although a simple correlation between Ig isotype and cytokine response is not evident in cattle, IFN-γ has been shown to augment the production of IgG2, while interleukin 4 augments the production of IgG1 by bovine B cells in vitro (16, 17). Both IgG1 and IgG2 activate bovine complement, but IgG2 is the superior opsonizing subclass (37).

The greater immunogenicity of rBRSVΔNS2 in calves contrasts with that seen in chimpanzees immunized with NS deletion mutants of HRSV, where the serum antibody response and resistance to HRSV challenge induced by the ΔNS1 mutant were similar to those induced by the ΔNS2 mutant (52). The reasons for the greater immunogenicity of the ΔNS2 mutant than of the ΔNS1 mutant in calves are not clear. Whereas there were no detectable differences in the replication of the BRSV ΔNS1 and ΔNS2 mutants in the bovine respiratory tract, rHRSVΔNS2 grew to higher titers in the upper respiratory tract of chimpanzees than did rHRSVΔNS1 (52). It is therefore possible that undetectable differences in the levels of replication of the BRSV ΔNS mutants may have occurred in vivo. Alternatively, since IFN-α/β have profound immunomodulatory effects and stimulate the adaptive immune response, it is possible that the greater ability of rBRSVΔNS2 to induce IFN-α/β may contribute to the greater immunogenicity of this virus than that of the ΔNS1 mutant. IFN-α/β influence the differentiation, survival, and function of T cells and DCs, enhance primary antibody responses, promote Ig isotype switching, are important in the induction of IgA, and enhance priming of IFN-γ-secreting CD4+ T cells (5, 29, 40). The findings of higher levels of BRSV-specific serum-neutralizing antibody, IgG2 serum antibody, and IgA antibody in BAL than those found in calves immunized with rBRSVΔNS1 and increased numbers of IFN-γ-secreting CD4+ T cells in calves immunized with rBRSVΔNS2 compared with those found in calves immunized with rBRSVΔNS1 are consistent with the known adjuvant properties of IFN-α/β in vivo and with the known effects of recombinant human IFN-α on bovine B cells in vitro (18). Although we were unable to detect IFN-α/β either in the sera, nasal secretions, or BAL from calves infected with any of the NS deletion mutants, it is possible that induction of IFN-α/β early after infection was an important determinant in the enhanced priming of the immune response by the ΔNS2 mutant. Further studies are in progress to determine if a difference in the induction of IFN-α/β by ΔNS1 and ΔNS2 mutants in vivo is responsible for the greater immunogenicity of the ΔNS2 mutant.

The demonstration that NS deletion mutants of BRSV are highly attenuated in very young, seronegative, gnotobiotic calves, which are more permissive for BRSV replication than older, conventional calves, and that they induce protective immunity indicates that these viruses are promising candidates for vaccine development. Furthermore, these studies highlight the potential of IFN-α/β for improving the efficacy of BRSV vaccines.

ACKNOWLEDGMENTS

This work was supported by the European Commission (EC 5th FP-RSV Vacc QLK2-CT-1999-00443), the Department for Environment, Food and Rural Affairs of the United Kingdom, and the Deutsche Forschungsgemeinschaft (SPP1089, Co260/1-1).

We thank our Institute for Animal Health colleagues: John McCauley for helpful discussions, Keith Parsons for computing analysis, and Pete Kaiser for help in setting up RT-PCR in real time. In addition, we thank the animal technicians for the rearing and maintenance of experimental animals.

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