Suppression of the Induction of Alpha, Beta, and Gamma Interferons by the NS1 and NS2 Proteins of Human Respiratory Syncytial Virus in Human Epithelial Cells and Macrophages

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Wild-type human respiratory syncytial virus (HRSV) is a poor inducer of alpha/beta interferons (IFN-α/β). However, recombinant HRSV lacking the NS1 and NS2 genes (ΔNS1/2) induced high levels of IFN-α and -β in human pulmonary epithelial cells (A549) as well as in macrophages derived from primary human peripheral blood monocytes. Results with NS1 and NS2 single- and double-gene-deletion viruses indicated that the two proteins function independently as well as coordinately to achieve the full inhibitory effect, with NS1 having a greater independent role. The relative contributions of the individual NS proteins were the converse of that recently described for bovine RSV (J. F. Valarcher, J. Furze, S. Wyld, R. Cook, K. K. Conzelmann, and G. Taylor, J. Virol. 77:8426–8439, 2003). This pattern of inhibition by HRSV NS1 and NS2 also extended to the newly described antiviral cytokines IFN-α1, -2, and -3.

Human respiratory syncytial virus (HRSV) is the most common cause of viral bronchiolitis and pneumonia in infants and children worldwide, and a vaccine is needed (9, 10). HRSV belongs to the genus Pneumovirus of the family Paramyxoviridae and has single-stranded, negative-sense RNA as its genome (10). One of the differences between the members of the genus Pneumovirus and other members of Paramyxoviridae is that Pneumovirus species express two putative nonstructural proteins, NS1 and NS2, from separate mRNAs encoded by the first two genes in the viral gene order. Recombinant HRSVs in which the NS1 and/or NS2 genes have been deleted singly or in combination (ΔNS1, ΔNS2, and ΔNS1/2 viruses) exhibit reduced replication in cultured cells that are competent to produce alpha interferon (IFN-α) and IFN-β, as well as in mice, monkeys, and chimpanzees, but replicate more like wild-type (wt) HRSV in Vero cells that lack the IFN-α/β genes (15, 16, 21–23, 25, 28). Clinical trials of recombinant HRSV (rHRSV) vaccine candidates lacking NS1 or NS2 are under way or in preparation. Bovine RSV (BRSV) is an animal counterpart of HRSV. It appears to be broadly expressed, are inducible by double-stranded RNA (dsRNA) or virus infection, bind to a heterodimeric receptor (albeit one distinct from that of IFN-α/β), activate the same JAK/STAT signal transduction pathway, up-regulate genes that render cells resistant to infection, and potentially augment the subsequent adaptive immune response. Thus, IFN-λ appears to be an IFN-α/β-like cytokine with pleiotropic effects that include potent antiviral activities.

The rHRSV ΔNS1, ΔNS2, and ΔNS1/2 viruses were constructed in previous work and are derivatives of the recombinant version of the wt A2 strain (rA2) (21–23, 25). All of the viruses used in this study were grown in Vero cells and purified by centrifugation through a sucrose gradient to ensure the absence of IFN in the viral inocula. The identities and purity of the specific virus stocks used in these experiments were confirmed by reverse transcription (RT)-PCR. The expression levels of IFN-α/βs were evaluated in A549 cells, an established line of human type II alveolar epithelial cells, and in human macrophages. The macrophages were prepared by incubating human peripheral blood monocytes in Iscove’s medium containing 20 ng of recombinant human granulocyte-monocyte colony-stimulating factor/ml, 10% human serum, and 1% sodium pyruvate for 1 week, after which residual nonadherent cells were removed by washing.

We compared the growth of the mutant viruses to that of wt rA2 in A549 cells (Fig. 1a), which are competent for the expression of IFN-α/β, unlike Vero cells (Fig. 1b), which lack the structural genes for these cytokines. Cells were infected at an input multiplicity of infection (MOI) of 0.01 PFU per cell for addition, we investigated the effect of HRSV infection and of deleting NS1 and/or NS2 on the expression of the newly described antiviral cytokines IFN-α1, -2 and -3 (alternatively designated interleukin 29 (IL-29), -28A, and -28B, respectively) (17, 20). These cytokines differ genetically and structurally from INF-α/β but share the following characteristics. They appear to be broadly expressed, are inducible by double-stranded RNA (dsRNA) or virus infection, bind to a heterodimeric receptor (albeit one distinct from that of IFN-α/β), activate the same JAK/STAT signal transduction pathway, up-regulate genes that render cells resistant to infection, and potentially augment the subsequent adaptive immune response. Thus, IFN-λ appears to be an IFN-α/β-like cytokine with pleiotropic effects that include potent antiviral activities.

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staining with anti-F monoclonal antibodies (21), and the mean titers
were derived. Plaques were visualized by immuno-

\[ \text{MOI of 0.01 PFU/cell with wt recombinant HRSV (rA2),} \]

Triplicate cultures of A549 (a) or Vero (b) cells were infected at an

\[ \text{in Vero cells, although the difference was less dramatic; for} \]

Virus titers were determined by plaque assay. The cell line from which

\[ \text{and the housekeeping mRNA \( \beta \)-actin was labeled with} \]

Total RNA was extracted from cell pellets by using TRIzol reagent (Invitrogen) and the RNeasy total RNA isolation kit (QIAGEN). Prior to qRT-PCR, RNA was treated with DNase 1 (QIAGEN) to remove any contaminating DNA, the absence of which was confirmed in control experiments in which the reverse transcriptase enzyme was omitted (data not shown). qRT-PCR was performed by using reagents from the Brilliant two-step qRT-PCR kit (Stratagene). RT was performed with random primers and total intracellular RNA. PCR primers and TaqMan probes were designed for the single species of IFN-\( \beta \), the multiple species of IFN-\( \alpha \), the several species of IFN-\( \lambda \), and the housekeeping mRNA \( \beta \)-actin were designed using Primer 3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) software (Table 1). Human IFN-\( \alpha \) exists as at least 13 species; based on their high degree of se-

\[ \text{The primers are identified as forward or positive-sense (F), reverse (R), or probe (P).} \]

\[ \text{IFN-\( \alpha \) set 1 includes IFN-\( \alpha \)-1, -6, and -13.} \]

\[ \text{IFN-\( \alpha \) set 2 includes IFN-\( \alpha \)-4, -5, -8, -10, -14, -17, and -21.} \]

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the reporter dye 5-carboxyfluorescein (FAM) at the 5' end and black-hole quencher (BHQ1) at the 3' end. All IFN probes were labeled with the reporter dye 5'-HEX at the 5' end and BHQ1 at the 3' end. Reactions contained a passive reference dye, which is not involved in amplification and which served to normalize the probe reporter dye signals. The use of differential reporter dyes (FAM and HEX) allowed qRT-PCRs to be done for each IFN mRNA of interest and the β-actin housekeeping mRNA to be duplexed (performed simultaneously in the same tube), and thus each result was normalized to the internal housekeeping-mRNA control. In addition, each set of PCRs was run in parallel with a single-standard cDNA preparation made from RNA from A549 cells infected with ΔNS1/2 and known to contain significant levels of IFN mRNA; this cDNA was run as a 10-fold dilution series that was used to make a curve for the purposes of quantitation and comparison between runs and for confirming the linearity of each PCR.

The response of IFN-α to infection with wt rA2 and the NS deletion mutants and controls is shown in Fig. 2a for time points 14-, 18-, and 26-h p.i.; at the other time points there was only minimal expression, and there is no data shown. Infection with the ΔNS1/2 virus resulted in a 300-fold increase in the expression of the combined species at 18-h p.i. compared to that for mock-treated cells, which diminished thereafter. Infection with ΔNS1 resulted in an 18-fold increase that peaked at 26 h, whereas the remaining viruses were associated with levels that were threefold or less that of the mock-infected control. In particular, infection with UV-inactivated wt rA2 as a control did not induce a detectable IFN-α response, implying a need for viral RNA synthesis. Infection with wt HPIV3 as another control was also inefficient in inducing IFN-α, implying that HPIV3 also strongly inhibits the induction of IFN-α. Similar results with UV-HRSV and HPIV3 were observed with IFN-β (Fig. 2b) and -λ (Fig. 2c and d).

In the case of IFN-β (Fig. 2b), infection of A549 cells with the ΔNS1/2 virus resulted in a 40,000-fold increase in the expression level of IFN-β mRNA compared to that for mock-infected cells. The accumulation of IFN-β mRNA peaked at 18-h p.i. and diminished thereafter. This pattern of a sharp peak followed by rapid decay is consistent with the induction of IFN-β mRNA in response to dsRNA, where the half-life of the mRNA was calculated to be 18 min (6, 12, 19). The peak induction of IFN-β in response to dsRNA occurs within 1.5 h (6), and the considerably longer time frame in response to HRSV infection presumably reflects a requirement for extensive HRSV RNA replication and transcription. In comparison, the peak values of wt rA2, HPIV3, ΔNS1, and ΔNS2 were 2010, 3220, 22,600, and 6,635 times those of the mock-infected control, and thus each of these viruses induced a substantial response, even if it was much less than that of ΔNS1/2. This finding is consistent with the reported induction of IFN-β in epithelial cells by wt HRSV (14). The kinetics of accumulation of intracellular IFN-β mRNA was generally the same for each of the HRSVs, although the peak for the ΔNS1 virus was at 26 h rather than 18 h in this particular experiment (Fig. 2b), reflecting experimental variability. Thus, the highest induction of IFN-β mRNA was associated with deletion of the NS1 and NS2 genes together, followed, respectively, by deletion of NS1 and NS2.

We also examined the expression of IFN-λ by using primers that differentiated between IFN-λ1 (Fig. 2c) and -λ2/3 (Fig. 2d). In each case, ΔNS1/2 induced an increase in the accumulation of IFN-λ1 and -2/3 that peaked at 18-h p.i. and declined thereafter. Cells infected with ΔNS1 also displayed modestly elevated levels of IFN-λ, peaking at 26 h. Thus, the time frame of the increase and decrease of mRNA was similar for all of the IFN-α/βs.

The secretion of IFN-α and -β from infected A549 cells was quantified by antigen-capture enzyme-linked immunosorbent assay (ELISA) of the medium supernatants (Fig. 3). The secretion of IFN-α faithfully reflected the results of the qRT-PCR and was the greatest for ΔNS1/2, reaching a level of

![FIG. 2. Expression levels of mRNA encoding human IFN-α (a), -β (b), -λ1 (c), or -λ2/3 (d) in A549 cells infected with wt rA2, ΔNS1, ΔNS2, ΔNS1/2, or HPIV3 or in mock-infected A549 cells. mRNA was measured by qRT-PCR using specific primers and Taqman probes (Table 1). The IFN-α primers were designed as two sets, each of which detected multiple species due to the high degree of sequence relatedness. Set 1 detected IFN-α1, -6, and -13, and set 2 detected IFN-α4, -5, -8, -10, -14, -17 and -21 (Table 1). For each sample, the expression level of each IFN mRNA was calculated in relation to the expression level of β-actin and expressed as a fold increase compared to that for the mock-infected sample. Representative data from one of two independent experiments that gave similar results are shown. There was minimal expression at the 2-, 6-, 10-, 38-, and 50-h time points, and no data is shown for these time points.](http://jvi.asm.org/11032/4365)
nearly 5,000 IU of IFN-α/H9251/ml, followed by that for H9004/NS1 (Fig. 3a). In the case of IFN-α/H9252/Fig. 3b), the pattern of secretion did not completely match the results of qRT-PCR. In particular, the peak level of secretion in response to H9004/NS1 was somewhat higher than that of H9004/NS1/2, and the amount of secretion in response to wt rA2 was unexpectedly high. Similar results were obtained in independent experiments. The reason for the inconsistent correspondence between the IFN-α/H9252/qRT-PCR and the ELISA is not known. One possible factor is that the level of secretion of IFN-α/H9252 was very high, up to 6,000 to 12,000 IU/ml, and it might be that its production or secretion by A549 cells was not completely responsive to intracellular mRNA levels. Another possibility is that some of the mRNA measured by qRT-PCR was not translatable, reflecting the rapid post-transcriptional repression of IFN-α synthesis in which translatability of the mRNA is lost before the body of the mRNA is degraded (11, 18). A previous study with Sendai virus indicated that viral factors can influence IFN-β mRNA translatability (11), which might cause a further difference between the various viruses studied here. Nonetheless, the IFN-β ELISA did confirm the most important point, that the ΔNS1 and ΔNS1/2 mutants were associated with the highest levels of IFN-β secretion. The secretion of IFN-α-1, -2 and -3 was not monitored due to the lack of an established, specific, functional or antibody-based assay.

We also investigated the expression of IFN-α/β/β in response to wt and mutant HRSVs in macrophages, immune cells that are exposed to HRSV during pulmonary infection. Monocyte-derived macrophages were infected with the NS mutants or UV-rA2 in the same manner as the A549 cells, and total cellular RNA was extracted 2-, 14-, 26-, and 38-h p.i. in three independent experiments by using cells from two donors. The expression of IFN-α was strongly induced in cells infected with the ΔNS1/2 virus and, to a lesser extent, with ΔNS1 (Fig. 4a). A similar pattern was observed for IFN-β; in the experiment shown, the expression level in response to the ΔNS1 virus was nearly equal to that of ΔNS1/2 (Fig. 4b), whereas in other experiments the response to ΔNS1 was intermediate between that of ΔNS1/2 and the control viruses. A similar pattern was observed in the case of IFN-λ1 (Fig. 4c) and -λ2/3 (Fig. 4d), with the response being the greatest to the ΔNS1/2 virus followed by that for the ΔNS1 virus. For each of the IFNs, the peak of mRNA accumulation in the macrophages was 26-h p.i., with some experiment-to-experiment variability. Specifically, in the particular experiment shown in Fig. 4c, the peak level of IFN-λ1 was at 38-h rather than 26-h p.i., but in other experiments it was at 26-h p.i. (data not shown). There was also
experiment-to-experiment variability in the background levels of mRNA, which were high in this experiment (Fig. 4c and d) but not in other experiments. However, the general pattern was consistent. At the protein level (Fig. 5), the pattern of secretion of IFN-α and -β was consistent. At the protein level (Fig. 5), the pattern of secretion of IFN-α and -β was consistent with the qRT-PCR data, although the maximum levels, 410 IU of IFN-α/ml, were much lower than that observed with A549 cells. Also, the balance between IFN-α set 1 and set 2 in the qRT-PCR was the converse of that observed for A549 cells. In each case, expression levels were the greatest for ∆NS1/2, followed by those for ∆NS1, with the other viruses inducing little IFN.

Since it is likely that viral dsRNA is important in inducing the IFN responses observed here, we compared the magnitude of intracellular viral RNA synthesis by wt rA2 and the NS gene deletion viruses. Analysis of the intracellular accumulation of viral genomic RNA and the viral mRNAs and proteins during single-cycle growth in both IFN-competent HEp-2 and IFN-lacking Vero cells revealed at most only modest reductions for the NS deletion viruses compared to those for wt rA2, differences that did not appear to be cell type-specific (15; M. N. Teng, K. M. Spann, and P. L. Collins, unpublished data). In addition, we measured the intracellular accumulation of viral genomic RNA in A549 cells and macrophages 14-h p.i. by using TaqMan probes and primers specific to the N and F regions of the RSV genome (data not shown). The reaction was made specific to genomic RNA by using a positive-sense primer for RT. Genomic RNA was chosen for measurement, since it presumably would be the duplex partner that would be limiting in the formation of intracellular viral dsRNA. For both cell types, the accumulations of genomic RNA by wt rA2, ∆NS1, ∆NS2, and ∆NS1/2 were within a twofold range and thus were very similar and should not be a factor in these virus-to-virus comparisons.

We then tested the ability of wt rA2 and the ∆NS1 and ∆NS2 single-gene-deletion mutants to inhibit the induction of IFN by the ∆NS1/2 double-gene-deletion mutant. A549 cells were infected with wt rA2, ∆NS1, ∆NS2, or ∆NS1/2 or were mock infected and were incubated for 8 h. One culture from each set was processed to purify intracellular RNA, and the remaining cultures were mock superinfected or superinfected with ∆NS1/2. The cultures were incubated for an additional 12 h and processed to purify intracellular RNA. The expression levels of IFN mRNA were measured by qRT-PCR.

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fected or infected with ΔNS1/2 and not in cells that had originally been infected with wt rA2, ΔNS1, or ΔNS2. Thus, superinfection by ΔNS1/2 failed to induce a further induction of IFN in cells expressing NS1 and/or NS2, supporting the idea that each of these proteins, together or alone, inhibits the induction of IFN.

The production of IFN-α/βs is an early response to virus infection. IFN-α/βs mediate the autocrine and paracrine induction of a wide array of genes. Some of these gene products, including 2-5A synthetase, RNA-dependent protein kinase PKR, adenosine deaminase, and Mx, are potent antiviral effectors that establish an intracellular antiviral state (14). Other IFN-stimulated gene products, such as major histocompatibility complex proteins and antigen-processing proteins, have the potential to enhance the adaptive immune response (13, 14).

IFN-α/βs are also potent cytokines that enhance cell-mediated responses of both innate and adaptive immunity, including Th1 helper cells, dendritic cells, NK cells, and cytotoxic T cells (1, 2, 12). The BRSV NS1 and NS2 proteins had previously been shown to counteract the IFN-induced intracellular antiviral state (3, 19). Also, while this manuscript was in preparation, Vallarcher et al. and Bossert et al. (4, 24) showed that the BRSV NS1 and NS2 proteins together inhibit the induction of IFN-α/β and that BRSV NS2 had the greater independent inhibitory activity. In the case of HRSV, the two NS proteins also cooperate to inhibit the induction of IFN-α/βs. Each also appeared to have a significant independent role, but NS1 rather than NS2 had the greater independent role, reflecting an apparent species-specific difference. Previous studies of the rHRSV mutants in chimpanzees showed that the ΔNS1 virus was significantly more attenuated than the ΔNS2 virus (23, 25), and similar results were observed with BALB/c mice (M. N. Teng and P. L. Collins, unpublished data). This finding is consistent with the interpretation that IFN is responsible for the greater attenuation of ΔNS1 in vivo.

In the present paper, deletion of the HRSV NS proteins also resulted in the induction of the newly described cytokines IFN-λ1 and -λ2. While the full range of their biological properties remains to be described, IFN-λ has been shown to have an antiviral potency comparable to that of IFN-α (17, 20). IFN-λ had been shown to be inducible by dsRNA or by infection with several different viruses, and the present paper shows that IFN-λ is comparable to IFN-α/β with regard to the kinetics of induction, the transient nature of induction, the apparent short half-life of the mRNA, induction in both epithelial cells and macrophages, and inhibition by the HRSV NS proteins.

A vaccine for HRSV is presently not available, but live attenuated viruses, including some involving deletion of the NS1 or NS2 gene (16, 23, 25), appear to be promising candidates and are under development and in clinical trials (26). Ideally, an HRSV vaccine should be given to infants of 1 month of age or less, given the very young age at which infants become susceptible to HRSV disease (9, 10). Immune responses in this age group are reduced due to immunologic immaturity and the immunosuppressive effects of maternally derived serum antibodies, posing a major obstacle to HRSV immunoprophylaxis. The immunologic immaturity of the young infant extends to IFN-α/β responses (7), and a pediatric HRSV vaccine that does not further inhibit these responses might be particularly advantageous. It has also been hypothesized that wt HRSV infection suppresses the adaptive immune response, as suggested by the recent observation that virus-specific CD8+ T cells induced during HRSV infection of BALB/c mice exhibited reduced cytolytic activity, cytokine secretion, and development of memory cells (8). It would be interesting to determine whether these defects are mitigated by an increased IFN-α/β response, such as during infection by ΔNS1/2 or ΔNS1. In addition, although wt HRSV is a poor inducer of IFN-α/β, the IFN that is produced appears to play a regulatory role that minimizes pathogenic, Th2-associated components of the immune response in the BALB/c mouse model (12). Thus, an HRSV vaccine virus that has a reduced ability to block the synthesis and signaling of IFN-α/β has the potential for both increased immunogenicity and decreased reactogenicity. Indeed, the BRSV ΔNS2 virus appeared to be more immunogenic than the BRSV ΔNS1 virus when administered to the respiratory tracts of calves, suggesting that increased synthesis of IFN-α/β indeed correlated with improved immunogenicity (24). Comparison of the HRSV ΔNS1 and ΔNS2 viruses in studies with chimpanzees did not reveal a difference in immunogenicity (23, 25), but this comparison was complicated by the difference in replication efficiency between the two viruses and by the small number and outbred nature of the animals. The double-deletion ΔNS1/2 virus would be the best choice for a robust IFN-α/β response, but a BRSV ΔNS1/2 virus was overattenuated in calves (24), and an HRSV ΔNS1/2 virus was overattenuated in African Green monkeys (16). However, the poor replication observed in these studies might have been exaggerated due to the use of a laboratory strain in the case of BRSV and a semipermissive animal model in the case of HRSV. Thus, these studies might not accurately predict the level of attenuation of the HRSV ΔNS1/2 virus in infants, particularly since the attenuation might be reduced due to the reduced IFN-α/β response in that age group. Thus, the HRSV ΔNS1 and ΔNS1/2 viruses are attractive vaccine candidates.

The views in this article are those of the authors and do not reflect the official policy or position of the Food and Drug Administration or the United States Government.

While this work was in progress, the NIAID laboratory received support from Wyeth for the development of live-attenuated vaccines against HRSV.

REFERENCES
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