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# Role of Type I IFNs in the *in Vitro* Attenuation of Live, Temperature-Sensitive Vaccine Strains of Human Respiratory Syncytial Virus

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The contributions of type I interferons (IFNs) to the *in vitro* attenuation of three temperature-sensitive (Ts) subgroup A and one subgroup B deletion mutant RSV strains were evaluated. The ability of these vaccine viruses to induce IFNs at their permissive and restrictive temperatures and their sensitivity to the antiviral effects of exogenous I IFNs were tested in human lung epithelial A549 cells. Our results show that the highly attenuated and immunogenic subgroup A vaccine strain Ts1C produced higher levels of IFN-$\beta$ than its parent RSS-2 or two related strains, Ts1A and Ts1B, at their permissive temperature. Growth of RSV-infected A549 cultures at restrictive temperatures or prior UV inactivation of the virus abolished the observed induction of IFN-$\beta$, suggesting a strict requirement of viral replication for cellular IFN induction. The enhanced induction of IFN-$\beta$ by the highly immunogenic Ts1C at permissive temperature may be an advantageous characteristic of a live intranasal vaccine candidate. The subgroup B strain RSV B1 and its mutant *cp-52* (with SH and G gene deletions) both induced similar but low levels of IFN-$\beta$. Hence the observed overattenuation of *cp-52* in human infants is probably not due to enhanced IFN induction during its replication in the host. The ability of *cp-52*, which does not express the SH and G proteins, to induce IFN-$\beta$ levels similar to those of its parent strain suggests that these viral proteins may not have a role in the induction of IFN-$\beta$ in the host. In addition, both subgroup A and B mutants and their respective parent strains were similarly resistant to the antiviral effects of exogenous IFN-$\alpha$ or -$\beta$. Therefore, increased sensitivity of the mutants to IFNs does not seem to contribute to their attenuation. 

## INTRODUCTION

Respiratory syncytial virus (RSV)-induced lower respiratory tract disease remains a major threat to the health of infants and young children, resulting in an estimated 90,000 hospitalizations and 4500 deaths a year in the United States (MMWR update 1998). RSV belongs to the genus *Pneumovirus* of the family of Paramyxoviridae and contains single-stranded negative-sense RNA as its genome (Collins *et al.,* 1996). The genome of RSV strain A2 consists of 15,222 nucleotides (nt) and encodes two surface glycoproteins (F, G) that are involved in viral adsorption and fusion and are the primary components that elicit neutralizing antibodies during RSV infection (Johnson *et al.,* 1987a,b). Two RSV subgroups, A and B, can be distinguished based on sequence variations within the F and G genes (Johnson and Collins, 1988; Johnson *et al.,* 1987b). A number of prototypic subgroup A and B strains are being used in the development of RSV vaccines (Crowe *et al.,* 1996; Firestone *et al.,* 1996; Karron *et al.,* 1997a,b; Whitehead *et al.,* 1999).

Vaccination of infants with Formalin-inactivated RSV (FI-RSV) during the early 1960s resulted in exacerbated disease in response to natural infection due to Th2-biased immune responses (Kim *et al.,* 1969; Connors *et al.,* 1994). Live, attenuated, temperature-sensitive (Ts) RSV mutants hold a great deal of promise as potential vaccines due to their restricted replication in the host and their ability to produce balanced humoral and cellular immune responses (Crowe, 1999). However, one of the difficulties in developing live RSV vaccines has been the identification of attenuated strains that have achieved a satisfactory balance between attenuation and immunogenicity because attenuation was often associated with reduced immunogenicity (Crowe *et al.,* 1996).

The induction of the IFN class of cytokines following viral infection is known to play a significant role in the host-mediated viral attenuation. The efficiency of this so-called "innate immunity" is often dependent on the interplay between the ability of host cells to produce IFN in response to infection and the sensitivity of virus replication to the antiviral effects of these host-induced IFNs.

A number of viruses have, however, developed complex and elaborate mechanisms to evade this first line of host defense (Ploegh, 1998; Smith, 1996). Analyses of measles and Sendai (SeV) virus strains have led to the identification of distinct viral subpopulations that differ in their induction of and sensitivity to IFNs (Carrigan and

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Kabacoff, 1987; Carrigan and Knox, 1990; Mattana and Viscomi, 1998). Several reports also suggest a correlation between a viral Ts phenotype and sensitivity to IFNs, as is the case for vaccinia and influenza viruses (Atkinson and Lancashire, 1976; Diaz-Guerra *et al.,* 1993; Hatada *et al.,* 1999). It is possible that the enhanced induction of IFNs in the host or increased sensitivity of virus replication to IFNs contributes to the overall attenuation of some of the Ts or host range mutants strains of RSV. Moreover, the types and levels of immune responses produced *in vivo* in response to viral infection are often dependent on balanced induction and coordinated expression of a variety of cytokines including IFNs (Whitton and Oldstone, 1996). Thus, any imbalance in the well-orchestrated cytokine expression during infection by live, mutant vaccine viruses can lead to over- or underattenuation of the virus, thus affecting its immunogenicity, and more important, to undesirable inflammatory or immunopathological consequences in the host (Pullan and Hey, 1982; Crowe, 1999).

Little information is available regarding the IFNs induced during the replication of the live, attenuated RSV vaccine candidates that are currently being used in clinical trials. Although experimental RSV infection has been shown to induce Th1-biased protective immune responses in animal models (Graham *et al.,* 1993; Connors *et al.,* 1994; Openshaw, 1995), the immune responses elicited by a diverse human population may be difficult to predict. Therefore, a vaccine strain that further favors protective cellular and humoral immune responses through the induction of appropriate cytokines would be desirable. A potential strategy would be to identify mutant virus strains that naturally induce cytokines such as type I or II IFNs or IL-2 in the host during their replication and to study their effects on their attenuation and possibly immunogenicity.

In this study, three RSV subgroup A Ts mutant strains, Ts1A, Ts1B, Ts1C (with restriction temperatures of replication at 39, 38, and 37°C, respectively), and one subgroup B deletion mutant, *cp-52,* were tested in human lung epithelial A549 cells for (a) their ability to induce type I IFNs at their permissive and restrictive temperatures and (b) their sensitivity to the antiviral effects of exogenous type I IFNs. These RSV subgroup A and B mutants possess defined genetic lesions that contribute to their Ts and/or attenuated phenotypes (Tolley *et al.,* 1996; Karron *et al.,* 1997a; Crowe *et al.,* 1996). Although mutations at nt positions 731 and 736 of the viral polymerase are considered responsible for the Ts and attenuation phenotypes of these subgroup A strains (Tolley *et al.,* 1996), very little is known about other factors that may have contributed to their attenuation phenotype. In addition, immunization of human adult volunteers with these RSV strains produced less severe disease and, in the case of Ts1C, also resulted in sero-conversion levels that were indistinguishable from the RSS-2 parent (Tolley *et al.,* 1996), thus making it a favorable vaccine candidate. However, the reason(s) for its enhanced immunogenicity compared to Ts1A or Ts1B is not known.

RSV B1 *cp-52*/2B5, hereafter referred to as *cp-52*, is a subgroup B mutant strain derived from the RSV B1 wild-type virus through 52 passages at low temperature (Crowe *et al.,* 1996). This mutant contains a substantial genomic deletion that eliminates the synthesis of the SH and G glycoproteins in RSV-infected cells, possibly contributing to its attenuated phenotype (Karron *et al.,* 1997a). Clinical trials demonstrated that *cp-52* was overattenuated in seronegative human infants despite its ability to replicate in Vero (simian kidney origin) cells to an extent similar to that of its wild-type parent (Karron *et al.,* 1997a). The reason(s) for the overattenuation of this mutant in infant subjects is not known. Further study of the replication of this strain in human lung cells and the cytokine factors such as IFNs that are induced during infection may lead to a better understanding of their role in the infectivity and attenuation of this virus. In addition, the role, if any, of the missing RSV G and SH glycoproteins in the induction IFNs leading to the attenuation of viral replication may be evaluated. Glycoproteins of some of the related paramyxoviruses such as Parainfluenza types 1 (SeV) and 4 (PIV4) or Newcastle Disease Virus (NDV) have been shown to induce IFN in the absence of viral replication (Ito and Hosaka, 1983; Ito *et al.,* 1994; Jestin and Cherbonnel, 1991), and it is not known if RSV glycoproteins have a similar capability. If RSV glycoproteins alone are capable of inducing IFNs in the host, they may influence the attenuation and immune responses of the recombinant chimeric vaccine candidates (Jin *et al.,* 1998; Whitehead *et al.,* 1999).

In this study, we hypothesized that the attenuation of some of these RSV mutant strains may be in part due to variations either in their ability to induce IFNs in the host or in their sensitivity to IFNs. We tested this hypothesis by infection of human pulmonary epithelial A549 cell line with attenuated or their parent RSV strains and evaluated their ability to induce IFNs or to replicate in the presence of exogenously added IFNs. Finally we attempted to find a correlation, if any, between our cell culture data and the available information from previous *in vivo* studies on the attenuation and immunogenicity of these vaccine candidates (Tolley *et al.,* 1996).

## RESULTS

### Induction of antiviral activity in A549 cells by RSV mutants

To determine whether the observed attenuation of RSV subgroup A and B strains is related to an increase in the production of type I IFNs, A549 cells were infected with the mutants and their parent strains and analyzed for their ability to induce IFN at permissive temperature (32°C). Supernatants and cellular RNA

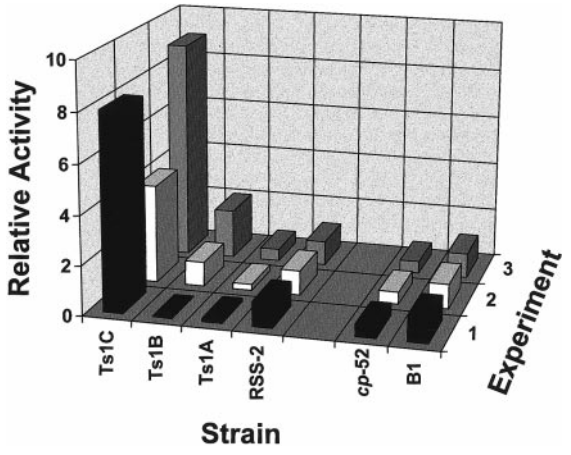


FIG. 1. Production of antiviral activity by A549 cells infected by RSV subgroup A and B strains. Approximately $1 \times 10^6$ aged A549 cells were infected with indicated viruses at a m.o.i. of 0.5 PFU/cell, were adsorbed for 12 h at 32°C, and were harvested at 48 h p.i. Infectious virus from infected culture supernatants was UV inactivated and assayed for antiviral activity by plaque-reduction assay in MDCK cells with vesicular stomatitis virus (VSV) as the challenge virus. Assay results represent endpoint dilutions required to reduce VSV induced CPE by 50%. Results from three independent experiments were shown. The activities of the RSV subgroup A and B strains were normalized relative to their respective parent virus.

were harvested 48 h post-infection (p.i.) and evaluated for the presence of antiviral activity and IFN mRNA, respectively. As shown in Fig. 1, the subgroup A RSV mutant strains Ts1A and Ts1B induced antiviral activity that was comparable to or lower than that of their parental strain RSS-2, while Ts1C, the most-attenuated mutant (restrictive temperature, 37°C), induced antiviral activity that ranged from four- to ninefold greater than that of its parent. This was consistently observed in three independent experiments. In contrast, both subgroup B strains induced low antiviral activity and the level of antiviral activity induced by *cp-52* mutant was about half of that induced by its parent (Fig. 1). These data indicate that during *in vitro* infection of A549 cells, RSV and its attenuated mutant viruses are capable of stimulating low but detectable antiviral activity that is functional against vesicular stomatitis virus (VSV)-induced CPE in MDCK cells. The most-attenuated RSV strain induced the most antiviral activity at permissive temperature. Similar results were observed even with increasing virus loads of 1 or 3 PFU/cell, tested at 32°C (data not shown). We also evaluated the samples shown in Fig. 1 for antiviral activity on WISH cells and found that, although this cell line was less sensitive to IFN-$\beta$, the relative antiviral activities were identical to those determined on MDCK cells (data not shown). Neutralization by antisera raised against specific IFNs further indicated that the antiviral activity in the virus-infected cell supernatants was due to IFN-$\beta$ (data not shown).

## Induction of IFN-$\beta$ mRNA by RSV vaccine candidates

We next used IFN-specific RT–PCR to determine whether the antiviral activity observed in A549 cell supernatants infected with RSV parent and vaccine strains was associated with an increase in intracellular type I IFN ($\alpha$ or $\beta$) mRNA. The conditions for detection of IFN mRNA were optimized by incubating the A549 cell cultures for 7 days before viral infection (aging) and also by incubating the infected cultures at 32°C. Total cellular RNA from each sample was analyzed for the induction of IFN-$\alpha$, IFN-$\beta$, RSV nucleoprotein (RSV N), and ribosomal S14 (RS14) mRNA. As shown in Fig. 2A, A549 cells were permissive to infection by RSS-2 and its attenuated Ts mutants as the level of RSV N protein mRNA present in each mutant sample is similar to that in its respective parent. IFN-$\alpha$ was not detected in any of the samples tested. In contrast, while very low or no IFN-$\beta$ mRNA was detected in cells infected with RSS-2, Ts1A, or Ts1B strains, infection with the Ts1C strain induced significantly higher levels of IFN-$\beta$ mRNA than any of its relatives. Although the levels of IFN-$\beta$ mRNA induced could not be reliably quantitated, these results were consistent with the relative antiviral activity induced by these viruses (Fig.1). The observed differences in IFN-$\beta$ mRNA levels do not appear to be due to differences in viral replication or RNA sample amounts as indicated by the similar quantities of RSV N and the housekeeping RS14 mRNA specific products. Similarly we found that *cp-52* induced levels of IFN-$\beta$ mRNA similar or slightly higher than those of its parent virus, RSV B1, under similar replication conditions (Fig. 2A).

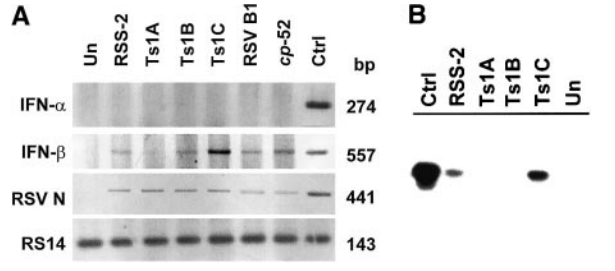


FIG. 2. Induction of IFN mRNA by RSV subgroup A and B viral strains. Approximately $1 \times 10^6$ aged A549 cells were infected with indicated viruses at an m.o.i. of 0.5 PFU/cell, were adsorbed for 12 at at 32°C, and were harvested at 48 h p.i. (A) RT–PCR analysis of IFN mRNA. RT–PCR performed on total RNA from RSV-infected samples using oligonucleotide primers specific to amplify 247 and 557 bp IFN-$\alpha$ and IFN-$\beta$ mRNA products, respectively. RT–PCR of RSV N mRNA and Ribosomal S14 (RS 14) transcripts produced expected-size products of 441 and 143 bp, respectively, and indicate the level of viral replication and RNA sample loading. (B) Southern blot analysis of RSV subgroup A strains. RT–PCR products were separated on 2.0% agarose-TBA gel, transferred to NYTRAN membranes and hybridized with a $^{32}$P-labeled cDNA probe derived from cloned human IFN-$\beta$. The samples for Southern blot analysis were from a similar but separate experiment. Un, uninfected sample; Ctrl, control IFN-$\alpha$ or -$\beta$ PCR product from A549 chromosomal DNA or control RSV N PCR product from a RSV N containing plasmid.

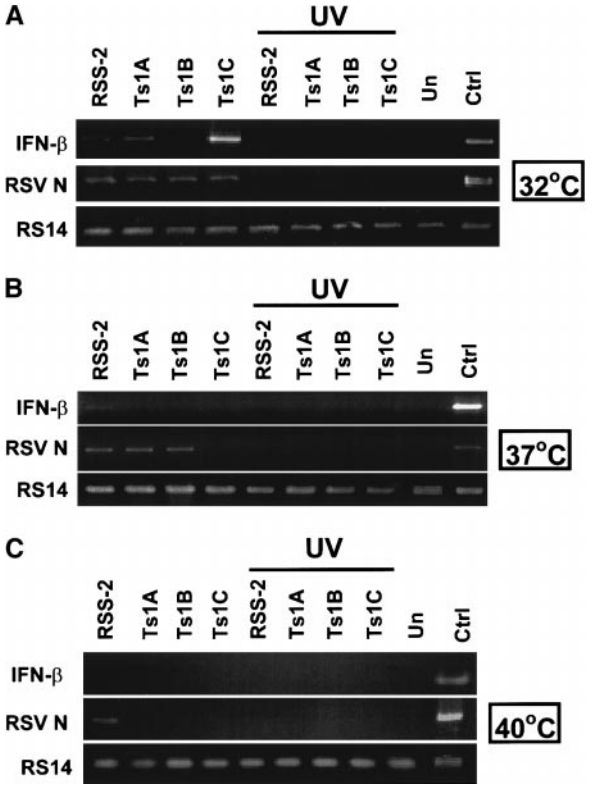


FIG. 3. Induction of IFN-β mRNA by RSV subgroup A strains at permissive and restrictive temperatures. Approximately $1 \times 10^6$ aged A549 cells were infected with indicated infectious or UV-inactivated viruses at (A) permissive (32°C), (B) physiological (37°C), and (C) restrictive (40°C) temperatures and harvested at 48 h p.i. RT–PCR analysis of the total RNA was performed with specific primers for IFN-β, RSV N or ribosomal S14 RNA as described. Un, uninfected sample; Ctrl, control IFN-β PCR product from A549 chromosomal DNA or control RSV N PCR product from a RSV N containing plasmid.

The specificity of the RT–PCR products obtained by using IFN-β-specific primers was confirmed by Southern blot analysis. As shown in Fig. 2B, the [$^{32}$]P-dCTP-labeled IFN-β probe selectively hybridized to the 557-bp IFN-β PCR product generated from the RNA of RSS-2- and Ts1C-infected samples but not from the RNA of the uninfected control sample. Low hybridization signals were detected in the Ts1A and Ts1B sample lanes after long exposure (data not shown). PCR amplification of chromosomal DNA isolated from untreated A549 cells served as the positive control in these experiments.

### Induction of IFN-β mRNA at permissive or restrictive temperatures

We next evaluated whether the induction of IFN-β was dependent on viral replication by infecting A549 cells with infectious or UV-inactivated viral strains at permissive (32°C), physiological (37°C), or restrictive temperatures (40°C). RSV B1 and *cp-52* were evaluated at 32 and 37°C. Results of these analyses are shown in Figs. 3 and 4.

At the permissive temperature of 32°C, the subgroup A strains RSS-2, Ts1A, and Ts1B produced only low levels of IFN-β mRNA when compared to Ts1C (Fig. 3A). These differences did not appear to be due to the differences in viral replication or unequal RNA amounts since RSV N protein and RS14 amplification products are similar in these samples. In each case, UV inactivation of viral replication (as evidenced by the absence of viral N mRNA) effectively ablated the observed IFN-β mRNA induction in these samples.

Next, the ability of these vaccine strains to replicate and induce IFN-β mRNA at physiological (37°C) temperatures was evaluated. As shown in Fig. 3B, RSS-2, Ts1A, and Ts1B replicated equally well at 37°C, while Ts1C did not, confirming its restriction at this temperature. With regard to the IFN induction, RSS-2 induced a very low level of IFN-β mRNA, while none was detected in any of the vaccine strains. UV treatment abolished even the small induction of IFN-β mRNA seen by RSS-2, further supporting the requirement of viral replication for IFN induction. Similarly, at 40°C (the restriction temperature for all three mutants), Ts1A, Ts1B, and Ts1C were unable to replicate at this temperature and therefore did not induce IFN-β mRNA. Although RSS-2 was capable of replication, IFN-β mRNA was not detected at this temperature.

In the case of RSV subgroup B strains, infection with B1 and *cp-52* at 32°C induced similar levels of IFN-β mRNA (Fig. 4A). Each of these viral strains replicated

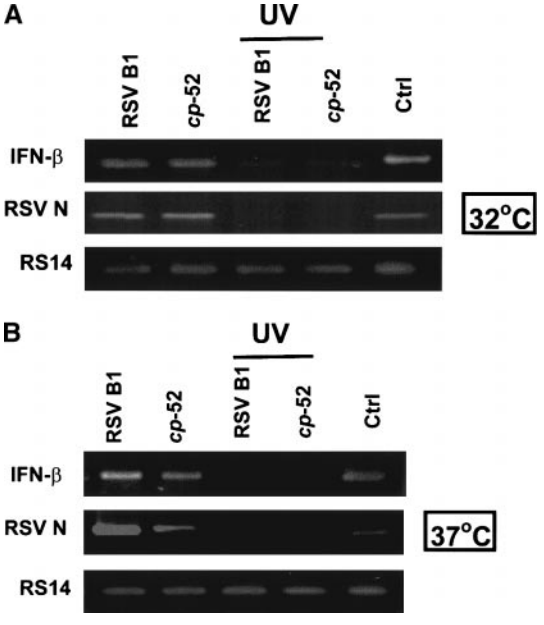


FIG. 4. Induction of IFN-β mRNA by RSV subgroup B strains at 32°C and 37°C. Approximately $1 \times 10^6$ A549 cells were infected with infectious or UV-inactivated viruses at 32°C (A) or 37°C (B) and harvested at 48 h p.i. RT–PCR analysis of the total RNA was performed with specific primers for IFN-β, RSV N or S14 ribosomal RNA as described. Un, uninfected sample; Ctrl, control IFN-β PCR product from A549 chromosomal DNA or control RSV N PCR product from a RSV N containing plasmid.

TABLE 1

Induction of IFN-$\beta$ Protein by RSV Strains

| Strain | IFN-$\beta$ (IU/ml[a]) | | |
|---|---|---|---|
| | 32°C | 37°C | 40°C |
| RSS-2 | <20 | <20 | <20 |
| Ts1A | <20 | <20 | <20 |
| Ts1B | <20 | <20 | <20 |
| Ts1C | 487 | <20 | <20 |
| RSVB1 | 20 | 113 | ND |
| RSVB*cp*52 | 64 | <20 | ND |
| RSVA2 | ND | 156 | ND |
| PIV3 | ND | 628 | ND |

*Note.* Values derived from a representative experiment. ND, not determined.

[a] Minimum detection limit of 20 IU/ml.

equally well in A549 cells at 32°C as suggested by the similar levels of cDNAs for RSV N mRNA. The production of IFN-$\beta$ mRNA by B1- and *cp-52*-infected cultures was also dependent on viral replication since UV inactivation abolished the observed IFN induction. Analyses performed at 37°C indicate that both B1 and *cp-52* induce IFN-$\beta$ mRNA at this temperature, with *cp-52* inducing slightly lower levels than B1 (Fig. 4B). The parental B1 strain replicated to levels noticeably higher than *cp-52* at this temperature as indicated by the levels of RSV N products although samples contained equal amounts of RNA (similar levels of RS14 products). UV inactivation once again ablated the viral replication and IFN-$\beta$ mRNA induction.

### Detection of IFN-$\alpha$ or IFN-$\beta$ proteins by ELISA

Since we have detected antiviral activity in the supernatants and IFN-$\beta$ mRNA in the cells of cultures infected with RSV subgroup A and B vaccine strains at 32°C, we used IFN-specific ELISA analyses to determine which IFN species were present in these samples. IFN-$\alpha$ was not detected in any of the samples tested by ELISA (data not shown), which correlated with the lack of IFN-$\alpha$ specific PCR products observed in these samples (Fig. 2). Variable levels of IFN-$\beta$ were detected in these samples as shown in Table 1.

Among the RSV subgroup A strains, only the Ts1C mutant induced detectable levels of IFN-$\beta$ protein at 32°C (Table 1). IFN-$\beta$ protein was not detected in any of the subgroup A infected samples at 37°C or 40°C, consistent with the very low or lack of IFN-$\beta$ mRNA seen by RT–PCR at these temperatures (Table 1 and Figs. 3B and 3C).

At 32°C, infection of A549 cells by RSV subgroup B mutant *cp-52* induced a low but detectable level of IFN-$\beta$ protein while a lower level of IFN-$\beta$ protein was induced by the RSV B1 parent. In contrast, at 37°C, while the B1 strain produced a moderate amount of IFN-$\beta$ protein, *cp-52* failed to induce any IFN-$\beta$ protein.

To gain some perspective on the level of IFN production by these RSV vaccine strains, we also evaluated the levels of IFN-$\beta$ protein induced by another RSV wild-type strain A2 or PIV3 at 37°C, their usual temperature of growth and replication. Data in Table 1 indicate that at 37°C, RSV A2 induced a significant amount of IFN-$\beta$ compared to RSS-2 wild-type virus or its derived mutants. Only wild-type RSV B1 induced comparable or slightly lower amounts. As expected, PIV3, a known inducer of IFN-$\beta$ (Gao *et al.,* 1999), induced the most IFN-$\beta$, thus validating the intrinsic ability of the A549 cell line to produce IFN-$\beta$ in response to viral infection.

### Effect of type I IFNs on virus yields of subgroup A and B RSV vaccine strains

The attenuated phenotype exhibited by the RSV subgroup A and B vaccine strains may be, in part, due to an increased sensitivity to the antiviral affects of the type I IFNs. To test this possibility, A549 cells were pretreated with varying amounts of IFN-$\alpha$ or IFN-$\beta$ for 16 h to stimulate host cell antiviral pathways. After IFN priming, cell cultures were infected with each of RSV strains. PIV3, a known IFN-sensitive virus, served as the positive control in these experiments. Viral yields were determined as described under Materials and Methods.

Addition of increasing amounts of IFN-$\alpha$ significantly inhibited viral yield of PIV3 as expected and at the highest dose of 10,000 IU/ml, PIV3 viral yield was reduced to 0.21% ( 500-fold reduction) of the untreated control (Table 2). In contrast, each of the RSV subgroup A strains was relatively unaffected by similar doses of IFN-$\alpha$ with the maximum dose inhibiting viral replication to ~10–20% of the control sample. The Ts1C mutant appeared to be slightly more sensitive at low dose (10 IU/ml) of IFN-$\alpha$ than RSS-2, Ts1A, or Ts1B strains ($P < 0.05$) although at the highest dose, its yield was decreased to the levels similar to its relatives.

Analysis of the affect of exogenous IFN-$\beta$ on the viral yields of subgroup A strains is also presented in Table 2. In contrast to the effects of priming with IFN-$\alpha$, PIV3 was also less sensitive to IFN-$\beta$ at similar doses, although increasing doses of IFN-$\beta$ resulted in a progressive inhibition of PIV3 replication. Subgroup A strains RSS-2, Ts1A, Ts1B, and Ts1C were all only minimally sensitive to IFN-$\beta$ priming with about one- to fourfold reduction in their yields, although Ts1C seemed slightly more sensitive to IFN treatment ($P < 0.05$) than its relatives.

Similarly, results in Table 3 indicate that both the viruses of RSV subgroup B are also resistant to the antiviral effects of both IFN-$\alpha$ and IFN-$\beta$ with a decrease in virus yield to ~8–20% (12- to 5-fold decrease) of the untreated controls.

TABLE 2

Effect of IFN Priming[a] on Replication of RSV Subgroup A Strains

| Dose (IU/ml) | Virus yield[b,c] (%) | | | | |
|---|---|---|---|---|---|
| | PIV3 | RSS-2 | Ts1A | Ts1B | Ts1C |
| IFN-α | | | | | |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 10 | 27 ± 1.5 | 76 ± 0.5 | 70 ± 2.7 | 90 ± 2.2 | 51 ± 2.2 |
| 100 | 7.2 ± 0.2 | 39 ± 1.1 | 56 ± 2.5 | 47 ± 2.5 | 38 ± 2.5 |
| 1,000 | 0.89 ± 0.1 | 25 ± 1.6 | 45 ± 1.2 | 28 ± 1.7 | 15 ± 1.7 |
| 5,000 | 0.23 ± 0.03 | 19 ± 1.5 | 31 ± 3.1 | 18 ± 1.4 | 12 ± 1.4 |
| 10,000 | 0.21 ± 0.02 | 15 ± 2.3 | 19 ± 3.7 | 12 ± 2.7 | 11 ± 2.7 |
| IFN-β | | | | | |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 10 | 96 ± 4.5 | 90 ± 4.6 | 93 ± 6.6 | 95 ± 3.1 | 54 ± 3.5 |
| 100 | 54 ± 13 | 85 ± 2.7 | 78 ± 9.2 | 91 ± 0.8 | 52 ± 1.0 |
| 1,000 | 5.1 ± 1.0 | 79 ± 7.4 | 75 ± 2.3 | 81 ± 1.9 | 45 ± 1.5 |
| 5,000 | 1.6 ± 0.2 | 80 ± 8.3 | 69 ± 5.5 | 81 ± 3.7 | 39 ± 2.0 |
| 10,000 | 1.4 ± 0.4 | 66 ± 2.8 | 67 ± 7.9 | 75 ± 1.2 | 30 ± 2.7 |

[a] $1 \times 10^6$ A549 cells were treated with the indicated dose of human IFN prior to viral infection.

[b] Viral yield was expressed as percentage of control and calculated as: $\frac{\text{Virus yield (+IFN)}}{\text{Virus yield (−IFN)}} \times 100$.

[c] Data represent averages or means ± SD of duplicate (PIV3) or triplicate experiments (RSS-2, Ts1A, Ts1B, and Ts1C).

## DISCUSSION

In this study we investigated whether the attenuation of either subgroup A Ts strains Ts1A, Ts1B, and Ts1C or the subgroup B attenuated strain, *cp-52,* is related to variations in their ability induce or their sensitivity to type I IFNs (α or β). Our data demonstrated that among RSV subgroup A strains, Ts1C, the most attenuated Ts mutant produced significantly higher levels of antiviral activity, IFN-β mRNA, and protein compared to its related strains RSS-2, Ts1A or Ts1B, at the lowest temperature permissive to the growth of all of them. Therefore, the specific mutations accumulated in Ts1C may contribute not only to its attenuation and immunogenicity but also to its ability to induce IFN-β at its permissive temperature. However, infection by Ts1A, -1B, -1C strains at their restrictive temperatures (40 or 37°C) failed to induce antiviral activity, IFN-β mRNA or IFN-β protein. This clearly demonstrates that although RSV Ts1C is inherently capable of inducing IFN-β, the induction of IFN-β by this virus is critically dependent upon its ability to replicate at a given temperature. The inability of nonreplicative virus to induce IFN-β was independently confirmed in experiments where UV-inactivation abolished both virus replication and IFN-β induction. Therefore, we conclude that although the additional mutations present in Ts1C may be responsible for its increased ability to induce IFN-β and its attenuation at permissive temperature, IFN-β may not contribute to its attenuation at ≥37°C since IFN-β was not induced at this temperature.

In the upper respiratory tract, i.e., nose, where the temperatures are slightly lower (32–34°C) than the core body temperature (37°C), Ts1C may be able to induce higher levels of IFN-β than its relatives. This may be relevant in clinical trials using these RSV strains. In an adult clinical trial where volunteers were immunized by intranasal inoculation (Tolley *et al.,* 1996), 9% of the Ts1C vaccinees developed upper respiratory tract illness

TABLE 3

Effect of IFN Priming[a] on Replication of RSV Subgroup B Strains

| Dose (IU/ml) | Virus yield[b,c] (%) | | |
|---|---|---|---|
| | PIV3 | RSV B1 | *cp-52* |
| IFN-α | | | |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 10 | 0.80 ± 0.2 | 50 ± 1 | 59 ± 22 |
| 100 | 0.52 ± 0.2 | 35 ± 12 | 38 ± 16 |
| 1,000 | 0.64 ± 0.1 | 28 ± 3 | 21 ± 15 |
| 5,000 | 0.48 ± 0.03 | 17 ± 9 | 23 ± 19 |
| 10,000 | 0.58 ± 0.3 | 18 ± 7 | 21 ± 13 |
| IFN-β | | | |
| 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 10 | 75 ± 25 | 83 ± 4 | 66 ± 24 |
| 100 | 12 ± 3.7 | 51 ± 33 | 41 ± 13 |
| 1,000 | 0.20 ± 0.14 | 29 ± 27 | 16 ± 7 |
| 5,000 | 0.19 ± 0.11 | 17 ± 13 | 10 ± 4 |
| 10,000 | 0.22 ± 0.17 | 17 ± 14 | 7.9 ± 3 |

[a] $1 \times 10^6$ A549 cells were treated with the indicated dose of human IFN prior to viral infection.

[b] Viral yield was expressed as percentage of control and calculated as: $\frac{\text{Virus yield (+IFN)}}{\text{Virus yield (−IFN)}} \times 100$.

[c] Data represent averages or means ± SD of duplicate (PIV3) or triplicate experiments (RSV B1, *cp-52*).

(URTI) and had a mean clinical severity score of 3.1. In contrast, 42% of subjects inoculated with RSS-2 developed URTI that was noticeably more severe than those of the Ts1C vaccinees (mean severity score of 13.7). In addition, Ts1C was as immunogenic (68% seroconverted) as its parent RSS-2 (70% seroconverted), while Ts1A and Ts1B seroconverted only 35 and 50% of the volunteers, respectively. The enhanced immunogenicity and vaccine response rates produced by Ts1C may relate to its ability to induce higher levels of IFN-$\beta$ than Ts1A and Ts1B at 32°C. However, it is not known if the nasal washes from the Ts1C vaccinees contain levels of IFN-$\beta$ higher than those obtained from other vaccinees. It is possible that the immunomodulatory effects of an increased induction of IFN-$\beta$ contribute to the increased immunogenicity of Ts1C, making it a better vaccine candidate with a practical advantage. Even if one argues that its attenuation is only due to the increased temperature sensitivity of its polymerase, the enhanced immunogenicity and vaccine response rates of Ts1C, possibly due to increased IFN-$\beta$ induction, would be desired characteristics of a favorable vaccine candidate.

One of the recent strategies that utilizes the ability of immunomodulatory cytokines to enhance the immunogenicity of the live vaccines is the simultaneous expression of cloned cytokine genes by recombinant vaccine viruses. Toward achieving this goal, types I and II IFN as well as IL-2 genes have been cloned and expressed by recombinant DNA and RNA viruses with varying success (Flexner *et al.,* 1990; Leong *et al.,* 1994; Rolf and Ramshaw, 1997; Giavedoni *et al.,* 1997; Bembridge *et al.,* 1998; Karaca *et al.,* 1998; Bukreyev *et al.,* 1999). In one case, the expression of the IFN-$\gamma$ gene by a recombinant RSV led to viral attenuation and favorable immunogenicity (Bukreyev *et al.,* 1999). Our present data further support that selection of natural RSV Ts mutants capable of inducing higher levels of type I IFNs may be a viable and rational strategy for achieving desired levels of attenuation coupled with enhanced immunogenicity.

We also tested an alternative possibility in which the attenuation of Ts1C, compared to its relatives, is due to its increased sensitivity to type I IFNs in A549 cells. Our results (Table 2) indicate that while Ts1A and Ts1B are almost identical to their parent RSS-2 in their resistance to IFN-$\alpha$ and IFN-$\beta$, Ts1C is slightly more sensitive to both IFNs as evidenced by the reduction in its virus yields. We are evaluating if this increased sensitivity is due to the enhanced induction of endogenous IFN-$\beta$ during infection. The observed differences between Ts1C and its relatives were statistically significant ($P < 0.05$) especially at the lower doses of IFN-$\alpha$ and at all doses of IFN-$\beta$, although the overall reduction in virus yields was 10-fold at any dose of IFN for any given virus. This level of reduction in virus yields is not generally considered very significant because the yields of many IFN-sensitive viruses are decreased by ≥100-fold even at lower doses of IFNs. From our data it appears that increased sensitivity to IFN-$\beta$ *per se* does not have a major effect in reducing viral replication and does not seem to contribute to their attenuation. We previously showed that another related wild-type strain, RSV A2, is similarly resistant to both type I IFNs and MxA, an IFN-inducible antiviral protein (Atreya and Kulkarni, 1999). Therefore it would appear that the mutations accumulated in the vaccine strains do not increase their sensitivity to the antiviral effects of type I IFNs.

The overattenuation (lack of viral replication) of *cp-52* observed in seronegative children (Karron *et al.,* 1997a) may be due to the induction of IFNs in human respiratory tract in response to infection by *cp*-52. We tested this possibility in human lung epithelial A549 cells that are capable of producing IFNs in response to viral infection. At 32°C, *cp-52* produced antiviral activity and IFN-$\beta$ mRNA levels that are very similar to or within twofold difference of those of its parent virus. The overall levels of antiviral activity induced by both subgroup B viruses were very low, and hence the observed differences in the antiviral activity induced by these two viruses may not be significant. From our *in vitro* studies using A549 cells, there does not seem to be a significant induction of IFN-$\beta$ in response to *cp-52* infection that would account for its overattenuation when compared to its parent virus. We also tested the relative sensitivity of *cp-52* and RSV B1 to both IFN-$\alpha$ and -$\beta$ and found that both viruses were similarly resistant to these IFNs (Table 3), and hence increased sensitivity of *cp-52* to type I IFNs does not seem to contribute to its overattenuation.

It is also interesting to note that both the RSV B1 and *cp-52* strains induce similar but low levels of IFN-$\beta$ despite the absence of the SH and G proteins in the mutant virus. This indicates that these RSV glycoproteins may not have any obvious role in either suppressing or inducing IFN-$\beta$ in the host, upon viral infection. This finding is in contrast to the related paramyxoviruses (e.g., SeV, PIV4, and NDV) whose surface glycoproteins alone were shown to induce IFNs in the absence of any viral replication (Ito and Hosaka, 1983; Ito *et al.,* 1994; Jestin and Cherbonnel, 1991). In addition, the upregulation of MHC Class I gene expression by UV-inactivated PIV3 was also suggested to be due to the induction of IFN-$\beta$ by viral glycoprotein (Gao *et al.,* 1999). However, recent studies with other negative-strand RNA viruses such as VSV indicate that mutations in viral polymerase may also be responsible for regulation of IFN induction in permissive cell cultures (Awaya *et al.,* 1999).

In addition, our results (Table 1) demonstrate for the first time an apparent difference between RSS-2 and RSV A2 wild-type strains in their abilities to induce IFN-$\beta$ at 37°C in A549 cells. Despite their high degree of homology (96.6%), RSS-2 differs from RSV A2 in having at least 34 deletions and two insertions in its genome (Tolley *et al.,* 1996). One of the major deletions occurs in the 3′

noncoding region of its P gene. It remains to be determined with recombinant A2 virus if this deletion, in fact, affects IFN-β induction.

One apparent contradiction that was noticed in our studies is that at 37 or 40°C, although RSS-2 was able to replicate, no IFN-β mRNA, protein, or antiviral activity was detected. The difficulty in detecting IFN-β mRNA at higher temperatures (37 and 40°C) may be due to the extremely low level of IFN-β induction by this strain or it may relate to a decreased IFN-β mRNA stability (Cavalieri *et al.,* 1977). In fact, enhanced production of IFN has been reported in NDV-infected Namalwa and rabbit cells in response to lowering the culture from 37 to 32 or 25°C. This was suggested to be due to prolonged translation of polysome-stabilized IFN mRNA (Kognovitskaya *et al.,* 1987; Kojima and Yoshida, 1974; Maehara *et al.,* 1980).

The data presented here are, to the best of our knowledge, the first report demonstrating the induction of IFN-β mRNA, protein, and antiviral activity in the human epithelial A549 cell line in response to infection with these RSV vaccine candidates. Our data also indicate that the *in vitro* induction of IFN-β by Ts1C is coincident with enhanced immunogenicity observed *in vivo*. The data also indicate that the RSV G and SH proteins have no obvious role in the induction of or sensitivity to these type I IFNs during viral infection. Studies with several different RSV subgroup A and B virus mutant strains are currently in progress to further determine the role of type I and type II IFNs in their attenuation and immune responses.

## MATERIALS AND METHODS

### Cell culture, viruses, and reagents

Human IFN-α was purchased from Sigma Chemical Co. (St. Louis, MO) and ELISA reagent kit for detection of IFN-α was obtained from Endogen (Woburn, MA). Human IFN-β and the ELISA kit for detection of IFN-β were both purchased from Bio-Source International (Camarillo, CA). Polyinosinic-polycytidylic acid (poly IC) and DEAE-Dextran were obtained from Pharmacia (Piscataway, NJ). Human pulmonary epithelial carcinoma (A549), African green monkey kidney (BS-C-1) and human laryngeal epithelial carcinoma (HEp-2) cell lines and human *Parainfluenza* type 3 strain C243 were all obtained from American Type Culture Collection (ATCC, Manassas, VA). RSV A2 strain has been described (Atreya and Kulkarni, 1999). RSV B1 and *cp-52* strains (Karron *et al.,* 1997a) were a gift from Dr. Brian Murphy (NAID, NIH). The RSV strains RSS-2, Ts1A, Ts1B, and Ts1C were kindly provided by Dr. Craig R. Pringle (University of Warwick, Coventry, UK) and have been previously described (Tolley *et al.,* 1996). Human IFN-β cDNA (Raj and Pitha, 1981) was kindly provided by Dr. Paula Pitha-Rowe (Johns Hopkins Oncology Center, Baltimore, MD).

### Preparation of cell and viral stocks

A549, BS-C-1, and HEp-2 cell lines were maintained in Earle's minimal essential medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine (L-Gln), 1% GASP, and 2.5 μg/ml Amphotericin B (Quality Biological, Gaithersburg, MD). RSS-2 and derived mutants were propagated in BS-C-1 cells. Briefly, BS-C-1 monolayers were adsorbed with viruses at 37 (RSS-2) or 32°C (Ts1A, Ts1B, Ts1C) at a multiplicity of infection (m.o.i.) of 0.1 PFU/cell for 4 h. RSV B1 and *cp-52* strains were propagated in Vero cells at 37 and 32°C, respectively. Subsequently, viral inoculum was removed and replaced with EMEM containing 2% FBS and antibiotics. Infection was allowed to proceed for an additional 48–96 h or until the observed cytopathic effects (CPE) were approximately 80%. Cell monolayers and supernatants were collected and centrifuged at 1400 rpm for 10 min. Supernatants were removed and supplemented with 1/20 volume of 2 M $MgSO_4$ and 2 M HEPES buffer (pH 7.4). RSV A2 and PIV3 were propagated in HEp-2 cells at 37°C after infection at an m.o.i. of 0.1 PFU/cell for 48–72 h as described previously (Atreya and Kulkarni, 1999). Viral titers were determined on HEp-2, Vero, or BS-C-1 monolayers by plaque assay (Kirsch and Johnson, 1963).

### Viral infections of A549 cells

A549 cells were plated onto six-well tissue culture plates, were grown to confluence, and were aged, i.e., incubated for 7 days before infection (Friedman, 1979). Approximately $1 \times 10^6$ cells were infected with viruses at an m.o.i. of 0.1 or 0.5 PFU/cell (or as specified) and allowed to adsorb for 2 or 12 h, respectively. Cell monolayers were washed and were further incubated in 1 ml of EMEM containing 2% FBS for 48 h or as specified.

### Interferon bioassay

Supernatants from RSV-infected cell cultures were harvested and stored at −70°C until further analysis. Virus in the supernatant was inactivated by UV treatment and the antiviral activity due to IFNs was determined in a plaque-reduction assay performed in MDCK and/or WISH cells with VSV as the challenge virus (Gresser *et al.,* 1974). Assays results represent the endpoint dilutions required to produce 50% reduction in VSV-induced CPE (Gresser *et al.,*1974). The antiviral activities induced by the RSV subgroup A and B mutant strains were normalized relative to those induced by their respective parent viruses.

### ELISA

The concentrations of IFN-α and IFN-β protein in virus-infected cell supernatants were determined by quantitative enzyme-linked immunosorbent assay (ELISA). Twofold dilutions of experimental samples were pre-

TABLE 4

Oligonucleotide Primers Used For RT–PCR Analysis[a]

| Oligonucleotide | Orientation | Sequence (5′-3′) | Product (bp) |
|---|---|---|---|
| IFN-$\alpha$ | 5′-3′ | TCCATGAGATGATCCAGCAG | 274 |
| | 3′-5′ | ATTTCTCGCTCTGACAACCTCCC | |
| IFN-$\beta$ | 5′-3′ | GCTCTCCTGTTGTGCTTCTCCAC | 557 |
| | 3′-5′ | GAGGCACAGGATCCGAGATCTTCAGTTTCG | |
| RSV N protein | 5′-3′ | TAACTCAAAGCTCTACATCAT | 441 |
| | 3′-5′ | ACGCGTCGACCACAGCTTCTATGAAGTG | |
| RS14 | 5′-3′ | GGCAGACCGAGATGAATCCTCA | 143 |
| | 3′-5′ | CAGGTCCAGGGGTCTTGGTCC | |

[a] IFN-$\beta$ forward and RSV N protein primer design was based on reported mRNA sequences for human IFN-$\beta$ and RSV A2 N proteins, respectively. All others were as previously reported (Noah and Becker, 1993; Ronni *et al.*, 1997).

pared in duplicate and assayed against serial dilutions of respective standards as suggested by the manufacturer. Sample concentrations were determined from the standard curves. Minimal levels of detection for IFN-$\alpha$ and IFN-$\beta$ were 25.6 pg/ml and 20 IU/ml, respectively.

## RT–PCR

Total RNA was extracted from control or RSV-infected A549 cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Five micrograms of total RNA was incubated with 7 Units (U) of DNase I (Life Technologies) for 15 min at 37°C, followed by inactivation of the enzyme by adding 2.5 mM EDTA at 70°C for 15 min. Equal amounts (2.5 $\mu$g) of each sample were reverse transcribed in the presence or absence of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Life Technologies) at 37°C for 1 h in a final volume of 20 $\mu$l RT mixture containing 2.5 $\mu$g/ml oligo(dT)$_{12-18}$, 3 mM $MgCl_2$, 50 mM Tris–HCl, 75 mM KCl, 250 $\mu$M each dNTPs, 10 mM dithiothreitol (DTT). Enzyme activity of M-MLV RT was inactivated by heating at 70°C for 15 min, and then one-tenth-volume (2 $\mu$l) aliquots were subjected to amplification by polymerase chain reaction (PCR). PCR amplifications were carried out in 25 $\mu$l reaction volume containing 10 mM Tris–HCl (pH 9.0 at 25°C), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 25 mM $MgCl_2$, 250 $\mu$M each of dNTPs, 1.5 U *Taq* DNA polymerase (Promega, WI), and 15 pmol of each primer. PCR reactions were heat denatured at 94°C for 3 min immediately before amplification. Amplifications were carried out for 30 cycles using a denaturation temperature of 94°C for 30 s, annealing temperatures of 51°C (RSV N) or 54°C (IFN-$\beta$, RS14) for 30 s, and a primer extension temperature of 70°C for 30 s. Forward and reverse primers for the gene targets, namely, IFN-$\alpha$, IFN-$\beta$, RSV N, and Ribosomal S14 (RS14), were designed from published sequences as shown in Table 4 and were synthesized by the Peptide and Nucleic Acid Laboratory, FDA.

## Southern blot analysis

PCR reactions to detect IFN-$\beta$ cDNA were performed as described in the previous section. PCR products were separated in 2.0% agarose–TBA gel and transferred to 0.2-$\mu$m NYTRAN (Schleicher and Schuell, Keene, NH) membranes using TURBOBLOTTER (Schleicher and Schuell, Keene, NH), a rapid downward transfer system, per manufacturer's protocols. Southern blot hybridization was performed at 65°C essentially as described (Sambrook *et al.,* 1989) except for the hybridization buffer (500 mM $NaPO_4$, 10% dextran sulfate, 1% SDS, 10 mM EDTA, 1 M NaCl with 100 $\mu$g/ml freshly denatured, sheared salmon-sperm DNA). The $^{32}$P-labeled probe generated by random-primer labeling (Life Technologies), contained human IFN-$\beta$ coding sequence (Raj and Pitha, 1981). Blot was placed under cellophane and exposed to BioMax X-ray film (Kodak, Rochester, NY) at −70°C in the presence of intensifying screens for 1, 4, or 24 h.

## Determination of virus yields

Priming of A549 cells with IFN-$\alpha$ or -$\beta$ before viral infections was carried out essentially as described (Atreya and Kulkarni, 1999). Briefly, confluent A549 monolayers in six-well plates were primed with 10, 100, 500, 1000, 5000, or 10,000 IU/ml doses of IFN-$\alpha$ or IFN-$\beta$ for 16 h followed by virus infection at an m.o.i. of 0.1 PFU/cell. Previous studies indicated that both IFN-$\alpha$ and -$\beta$ at the amounts used were not cytotoxic to A549 cells (Atreya and Kulkarni, 1999). Unprimed virus-infected cultures were included as an internal control for each plate of cells infected with a given virus. PIV3, a known interferon-sensitive virus (Zhao *et al.,* 1996), was included in each set of experiments. Cells were adsorbed with virus for 4 h, were washed, and then were incubated for 48 h in EMEM containing 2% FBS and antibiotics. Infectious virus yields using 48-h cell culture supernatants were determined by plaque assay in HEp-2 cells (Kirsch and Johnson, 1963). The infectious virus yields of the IFN-treated samples were determined as follows (Atreya and

Kulkarni, 1999): (number of plaques per ml of culture supernatant obtained from IFN-treated samples) divided by (number of plaques per ml of culture supernatant obtained from untreated samples) × 100. Statistical significance of the affect of IFN priming on viral yields was evaluated by two-way analysis of variance (ANOVA) of ranked data using SIGMASTAT software.

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## REFERENCES

Atkinson, J. P., and Lancashire, C. L. (1976). The induction of interferon by temperature-sensitive mutants of Sindbis Virus: Its relationship to double-stranded RNA synthesis and cytopathic effect. *J. Gen. Virol.* **30**, 157–165.

Atreya, P. L., and Kulkarni, S. (1999). Respiratory syncytial virus strain A2 is resistant to the antiviral effects of type I interferons and human MxA. *Virology* **261,** 227–241.

Awaya, M., Marcus, P. I., and Sekellick, M. J. (1999). Regulation of interferon induction by vesicular stomatitis virus L-protein. Abstract W 32–6. 18th Annual Meeting (July 10–14) American Society for Virology, University of Massachusetts, Amherst.

Bembridge, G. P., Lopez, J. A., Cook, R., Melero, J. A., and Taylor, G. (1998). Recombinant vaccinia virus co-expressing the F protein of respiratory syncytial virus (RSV) and interleukin-4 (IL-4) does not inhibit the development of RSV specific memory cytotoxic T lymphocytes, whereas priming is diminished in the presence of high levels of IL-2 or gamma interferon. *J. Virol.* **72,** 4080–4087.

Bukreyev, A., Whitehead, S. S., Bulreyeva, N., Murphy, B. R., and Collins, P. L. (1999). Interferon γ expressed by a recombinant respiratory syncytial virus attenuates virus replication in mice without compromising immunogenicity. *Proc. Natl. Acad. Sci. USA* **96,** 2367–2372.

Carrigan, D. R., and Kabacoff, C. M. (1987). Identification of a nonproductive, cell-associated form of measles virus by its resistance to inhibition by recombinant human interferon. *J. Virol.* **61,** 1919–1926.

Carrigan, D. R., and Knox, K. K. (1990). Identification of interferon-resistant sub-populations in several strains of measles virus: Positive selection by growth of the virus in brain tissue. *J. Virol.* **64,** 1606–1615.

Cavalieri, R. L., Havell, E. A., Vilcek, J., and Pestka, S. (1977). Induction and decay of human fibroblast interferon mRNA. *Proc. Natl. Acad. Sci. USA* **74,** 4415–4419.

Collins, P. L., McIntosh, K., and Chanock, R. M. (1996). Respiratory syncytial virus. *In* "Fields Virology" (Fields, B.N., Ed.), pp. 1313–1351. Raven Press, New York.

Connors, M., Giese, N. A., Kulkarni, A. B., Firestone, C. Y., Morse, H. C., and Murphy, B. R. (1994). Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/C mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. *J. Virol.* **68,** 5321–5325.

Crowe, J. E., Jr. (1999). Host responses to respiratory virus infection and immunization. *Curr. Top. Microbiol. Immunol.* **236,** 191–214.

Crowe, J. E., Jr., Bui, P. T., Firestone, C. Y., Connors, M., Elkins, W. R., Chanock, R. M., and Murphy, B. R. (1996). Live subgroup B respiratory syncytial virus vaccines that are attenuated, genetically stable, and immunogenic in rodents and non-human primates. *J. Infect. Dis.* **173,** 829–839.

Diaz-Guerra, M., Kahn, J. S., and Esteban, M. (1993). A mutation of the nucleoside triphosphate phosphohydrolase I (NPH-I) gene confers sensitivity of vaccinia virus to interferon. *Virology* **197,** 485–491.

Firestone, C. Y., Whitehead, S. S., Collins, P. L., Murphy, B. R., and Crowe, J. E.Jr. (1996). Nucleotide sequence analysis of the respiratory syncytial virus subgroup A cold-passaged (cp) temperature sensitive (ts) cpts-248/404 live attenuated virus vaccine candidate. *Virology* **225,** 419–422.

Flexner, C., Moss, B., London, W. T., and Murphy, B. R. (1990). Attenuation and immunogenicity in primates of vaccinia virus recombinants expressing human interleukin-2. *Vaccine* **8,** 17–21.

Freidman, R. M. (1979). Induction and production of interferon. *Methods Enzymol.* **58,** 292–296.

Gao, J., De, B. P., and Banerjee, A. K. (1999). Human parainfluenza virus type 3 up-regulates major histocompatibility complex class I and II expression on respiratory epithelial cells: Involvement of a STAT1- and CIITA-independent pathway. *J. Virol.* **73,** 1411–1418.

Giavedoni, L., Ahmad, S., Jones, L., and Yilma, T. (1997). Expression of gamma interferon by simian immunodeficiency virus increases attenuation and reduces post-challenge virus load in vaccinated rhesus macaques. *J. Virol.* **71,** 866–872.

Graham, B. S., Henderson, G. S., Tang, Y. W., Lu, X., Neuzil, K. M., and Colley, D. G. (1993). Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. *J. Immunol.* **151,** 2032–2040.

Gresser, I., Bandu, M. T., Brouty-Boye, D., and Tovey, M. (1974). Pronounced antiviral activity of human interferon on bovine and porcine cells. *Nature* **251,** 543–545.

Hatada, E., Saito, S., and Fukuda, R. (1999). Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells. *J. Virol.* **73,** 2425–2433.

Ito, Y., and Hosaka, Y. (1983). Component(s) of Sendai virus that can induce interferon in mouse spleen cells. *Infect. Immun.* **39,** 1019–1023.

Ito, Y., Bando, H., Komada, H., Tsurudome, M., Nishio, M., Kawano, M., Matsumura, H., Kusagawa, S., Yuasa, T., and Ohta, H. (1994). HN proteins of human parainfluenza type 4A virus expressed in cell lines transfected with a cloned cDNA have an ability to induce interferon in mouse spleen cells. *J. Gen. Virol.* **75,** 567–572.

Jestin, V., and Cherbonnel, M. (1991). Interferon-induction in mouse spleen cells by the Newcastle disease virus (NDV) HN protein. *Ann. Rech. Vet.* **22,** 365–372.

Jin, H., Clarke, D., Zhou, H. Z.-Y., Cheng, X., Coelingh, K., Bryant, M., and Li, S. (1998). Recombinant human respiratory syncytial virus (RSV) from cDNA and construction of subgroup A and B chimeric RSV. *Virology* **251,** 206–214.

Johnson, P. R., and Collins, P. L. (1988). The fusion glycoprotein of human respiratory syncytial virus of subgroups A and B: Sequence conservation provides a structural basis for antigenic relatedness. *J. Gen. Virol.* **69,** 2623–2628.

Johnson, P. R., Olmsted, R. A., Prince, G. A., Murphy, B. R., Alling, D. W., Walsh, E. E., and Collins, P. L. (1987a). Antigenic relatedness between glycoproteins of human respiratory syncytial virus subgroups A and B: Evaluation of the contributions of F and G glycoproteins to immunity. *J. Virol.* **61,** 3163–3166.

Johnson, P. R., Spriggs, M. K., Olmsted, R. A., and Collins, P. L. (1987b). The G glycoprotein of human respiratory syncytial viruses of sub-

groups A and B: Extensive sequence divergence between antigenically related proteins. *Proc. Natl. Acad. Sci. USA* **84,** 5625–5629.

Karaca, K., Sharma, J. M., Winslow, B. J., Junker, D. E., Reddy, S., Cochran, M., and McMillen, J. (1998). Recombinant fowlpox viruses co-expressing chicken type I IFN and Newcastle disease virus HN and F genes: Influence of IFN on protective efficacy and humoral responses of chickens following in ovo or post-hatch administration of recombinant viruses. *Vaccine* **16,** 1496–1503.

Karron, R. A., Buonagurio, D. A., Gergely, L., Whitehead, S. S., Aguzzi, A., Clements-Mann, M. L., Hart, C. A., Randolph, V. B., Udem, S. A., Murphy, B. R., and Sidhu, M. S. (1997a). Respiratory Syncytial Virus (RSV) SH and G proteins are not essential for viral replication in vitro: Clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. *Proc. Natl. Acad. Sci. USA* **94,** 13961–13966.

Karron, R. A., Wright, P. F., Crowe, J. E. J., Clements-Mann, M. L., Thompson, J., Makhene, M., Casey, R., and Murphy, B. R. (1997b). Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. *J. Infect. Dis.* **176,** 1428–1436.

Kim, H. W., Canchola, J. G., Brandt, C. D., Pyles, G., Chanock, R. M., Jensen, K., and Parrott, R. H. (1969). Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am. J. Epidemiol.* **89,** 422–434.

Kirch, A. L., and Johnson, K. M. (1963). A plaque assay for respiratory syncytial virus. *Proc. Soc. Exp. Biol. Med.* **112,** 583.

Kognovitskya, A. I., Bokhonko, A. I., Mamontova, T. V., and Orlova, T. G. (1987). The influence of high temperature on human cells in culture, their sensitivity to viruses and their interferon-producing capacity. *Acta Virol.* **31,** 7–12.

Kojima, Y., and Yoshida, F. (1974). Enhanced production of interferon by temperature shift-down from 37°C to 25°C in rabbit cell cultures stimulated with Newcastle disease virus. *Jpn. J. Microbiol.* **18,** 217–222.

Leong, K. H., Ramsay, A. J., Boyle, D. B., and Ramshaw, I. A. (1994). Selective induction of immune responses by cytokines co-expressed in recombinant fowl pox virus. *J. Virol.* **68,** 8125–8130.

Maehara, N., Komatsu, K., Shimoda, K., Makino, S., Nagano, Y., and Matumoto. M. (1980). Enhanced production of virus-inhibiting factor (interferon) in human diploid cells by ultraviolet irradiation and temperature shift-down after stimulation with Newcastle disease virus. *Microbiol. Immunol.* **24,** 907–914.

Mattana, P., and Viscomi, G. C. (1998). Variations in the interferon-inducing capacity of Sendai virus sub-populations. *J. Interferon. Cytokine. Res.* **18,** 399–405.

MMWR Update: Respiratory syncytial virus activity—United States, (1997)–1998 Season. *Morbidity Mortality Weekly Rep.* **46**, 1163–1165.

Noah, T. L., and Becker, S. (1993). Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. *Am. J. Physiol.* **265,** L472–L478.

Openshaw, P. J. (1995). Immunity and immunopathology to respiratory syncytial virus. The mouse model. *Am. J. Respir. Crit. Care Med.* **152,** S59–S62.

Ploegh, H. L. (1998). Viral strategies of immune evasion. *Science* **280,** 248–253.

Pullan, C. R., and Hey, E. N. (1982). Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. *Br. Med. J.* (Clin.Res. Ed.) **284,** 1665–1669.

Raj, N. B., and Pitha, P. M. (1981). Analysis of interferon mRNA in human fibroblast cells induced to produce interferon. *Proc. Natl. Acad. Sci. USA* **78,** 7426–7430.

Rolf, M. S., and Ramshaw, I. A. (1997). Recombinant viruses as vaccines and immunological tools. *Curr. Opin. Immunol.* **9,** 517–524.

Ronni, T., Matikainen, S., Sareneva, T., Melen, K., Pirhonen, J., Keskinen, P., and Julkunen, I. (1997). Regulation of IFN-alpha/beta, MxA, 2′,5′-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells. *J. Immunol.* **158,** 2363–2374.

Sambrook, J., Fritch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," p. 9.31–9.57. Cold Spring Harbor Press, Plainview, NY.

Smith, G. L. (1996). Virus proteins that bind cytokines, chemokines or interferons. *Curr. Opin. Immunol.* **8,** 467–471.

Tolley, K. P., Marriott, A. C., Simpson, A., Plows, D. J., Matthews, D. A., Longhurst, S. J., Evans, J. E., Johnson, J. L., Cane, P. A., Randolph, V. B., Easton, A. J., and Pringle, C. R. (1996). Identification of mutations contributing to the reduced virulence of a modified strain of respiratory syncytial virus. *Vaccine* **14,** 1637–1646.

Whitehead, S. S., Hill, M. G., Firestone, C. Y., St. Claire, M., Elkins, W. R., Murphy, B. R., and Collins, P. L. (1999). Replacement of the F and G proteins of respiratory syncytial virus (RSV) subgroup A with those of subgroup B generates chimeric live attenuated RSV subgroup B vaccine candidates. *J. Virol.* **73,** 9773–9780.

Whitton, J. L., and Oldstone, M. B. A. (1996). Immune response to viruses. *In* "Fields Virology" (B.N. Fields *et al.*, Eds.), pp. 345–374. Raven Press, New York.

Zhao, H., De, B. P., Das, T., and Banerjee, A. K. (1996). Inhibition of human parainfluenza virus—3 replication by interferon and human MxA. *Virology* **220,** 330–338.