Interferon γ expressed by a recombinant respiratory syncytial virus attenuates virus replication in mice without compromising immunogenicity

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ABSTRACT Interferon γ (IFN-γ) has pleiotropic biological effects, including intrinsic antiviral activity as well as stimulation and regulation of immune responses. An infectious recombinant human respiratory syncytial virus (rRSV/mIFN-γ) was constructed that encodes murine (m) IFN-γ as a separate gene inserted into the G-F intergenic region. Cultured cells infected with rRSV/mIFN-γ secreted 22 μg mIFN-γ per 10^6 cells. The replication of rRSV/mIFN-γ, but not that of a control chimeric rRSV containing the chloramphenicol acetyl transferase (CAT) gene as an additional gene, was 63- and 20-fold lower than that of wild-type (wt) RSV in the upper and lower respiratory tract, respectively, of mice. Thus, the attenuation of rRSV/mIFN-γ in vivo could be attributed to the activity of mIFN-γ and not to the presence of the additional gene per se. The mice were completely resistant to subsequent challenge with wt RSV. Despite its growth restriction, infection of mice with rRSV/mIFN-γ induced a level of RSV-specific antibodies that, on day 56, was comparable to or greater than that induced by infection with wt RSV. Mice infected with rRSV/mIFN-γ developed a high level of IFN-γ mRNA and an increased amount of interleukin 12 p40 mRNA in their lungs, whereas other cytokine mRNAs tested were unchanged compared with those induced by wt RSV. Because attenuation of RSV typically is accompanied by a reduction in immunogenicity, expression of IFN-γ by an rRSV represents a method of attenuation in which immunogenicity can be maintained rather than be reduced.

Interferon γ (IFN-γ), a type II interferon, is produced by T cells and natural killer (NK) cells and has diverse biological effects (for review, see refs. 1 and 2). IFN-γ has intrinsic antiviral activity, up-regulates expression of major histocompatibility class I and II molecules, activates macrophages and NK cells, and has an important regulatory role in T helper (Th) cell proliferation. Two subsets of murine Th cells have been distinguished on the basis of the pattern of cytokine secretion: the Th1 subset, whose marker cytokines include interleukin 2 (IL-2) and IFN-γ, and the Th2 subset, whose markers include IL-4, IL-5, IL-6, and IL-10. IFN-γ preferentially inhibits the proliferation of Th2 cells, thus favoring a Th1 response.

Human respiratory syncytial virus (RSV) is the most important viral agent of pediatric respiratory tract disease worldwide. RSV is an enveloped nonsegmented negative-stranded RNA virus of the family Paramyxoviridae (for review, see ref. 3). The RSV genome is 15,222 nt in length and is transcribed into 10 subgenomic mRNAs that encode 11 proteins, including the 4 proteins of the nucleocapsid, N, P, L, and M2–1, and the attachment G and fusion F surface glycoproteins that are the major neutralization and protective antigens. Infectious recombinant (r) RSV can be produced by the intracellular coexpression from transfected plasmids of an RSV antigenomic RNA, which is the positive-sense replicative intermediate of genomic RNA, together with the N, P, M2–1, and L proteins (4).

A vaccine against RSV is not yet available, although there has been considerable progress in developing live-attenuated viruses, vectored antigens, and purified proteins as candidate vaccines (5). There is growing appreciation that certain properties of a RSV vaccine can qualitatively affect the humoral and cellular immune response and can adversely modify the pattern of disease that occurs upon subsequent infection by RSV (6). Immunization with formalin-inactivated RSV in the 1960s was associated with RSV disease enhancement rather than protection (7, 8). In rodents, immunization with formalin-inactivated RSV or with purified RSV glycoprotein induced a Th2-biased response and primed for enhanced pulmonary histopathology upon subsequent RSV challenge (6, 9, 10). This pathologic response was abrogated by depletion of CD4+ cells or IL-4 and IL-10 before the challenge (10, 11). Fortunately, infection by RSV induces a response that appears to be biased toward the Th1 subset and is associated with protection rather than enhanced disease upon challenge (5, 6, 12). However, responses in a diverse human population may be difficult to predict, and a vaccine strain that further favors a Th1 response probably is desirable.

A difficulty in the effort to develop a live virus vaccine has been the identification of an attenuated strain that has achieved a satisfactory balance between attenuation and immunogenicity (5), especially because attenuation typically is accompanied by reduced immunogenicity. In this study, we explored the strategy of manipulating the immunogenic properties of a negative-stranded virus by coexpression of a multifunctional cytokine by the same virus. An rRSV, designated rRSV/mIFN-γ, was constructed encoding mIFN-γ as an additional gene to test the possibility that its expression would have one or more of the following effects: attenuation of the virus in vivo, augmentation of the immune response, and enhancement of the Th1 response.

MATERIALS AND METHODS

Plasmid Construction. RSV gene-start and gene-end signals were attached to the mIFN-γ cDNA by PCR with oligonucleotide primers that contain initiation and termination codons and flanked by RSV-specific sequences. The resulting plasmids were used to transfected BHK cells. The targeted plasmids contained approximately 1.6 kb of RSV vaccinia virus, murine interferon γ, CAT, chloramphenicol acetyl transferase; rRSV/CAT or RSV/mIFN-γ recombinant RSV containing the CAT or mIFN-γ gene, respectively; pfu, plaque-forming units; NK, natural killer; Th, T helper; wt, wild type.

Abbreviations: RSV, respiratory syncytial virus; rRSV, recombinant RSV; mIFN-γ, murine interferon γ; CAT, chloramphenicol acetyl transferase; rRSV/CAT or RSV/mIFN-γ, recombinant RSV containing the CAT or mIFN-γ gene, respectively; pfu, plaque-forming units; NK, natural killer; Th, T helper; wt, wild type.

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RESULTS

Construction and Recovery of RSV Expressing mIFN-γ

Construction of the rRSV/mIFN-γ chimeric genome. The cDNA encoding mIFN-γ was synthesized using a PCR kit from Boehringer Mannheim (positive sense) and nested PCR from digested cDNA (negative sense). The full-length mIFN-γ cDNA was inserted into the G-F intergenic region of the antigenome cDNA D46, which had previously been modified to contain a unique XmaI site (13). The resulting chimeric RSV antigenome RNA containing the mIFN-γ gene was then cloned into the pRSVmax expression vector (4). The rRSV Chimeric Expression System, employing a recombinant vaccinia virus that expresses the RSV protein CCMG, was used to recover rRSV containing an mIFN-γ insert.

Growth Characteristics of rRSV/mIFN-γ

Growth characteristics of rRSV/mIFN-γ were determined in vitro in HeLa cells and in vivo in mice. rRSV/mIFN-γ was found to have a doubling time of 72 h, whereas the wild-type RSV had a doubling time of 57 h. rRSV/mIFN-γ was also found to be attenuated in vivo, with a peak titer of 10^6 PFU/ml at 48 h postinfection, compared with a maximal titer of 10^7.6 PFU/ml for wt RSV (48 h postinfection), indicating a 16-fold reduction.

Production of mIFN-γ and Production of mIFN-γ in the Presence of RSV Cat

Production of mIFN-γ in the presence of RSV Cat was determined by ELISA. The amount of mIFN-γ produced was measured at 72 h postinfection, and the results were compared with those obtained from infected cells treated with interferon-β (a known inducer of mIFN-γ).

mIFN-γ-specific ELISA

mIFN-γ-specific ELISA was used to determine the level of mIFN-γ produced by rRSV/mIFN-γ-infected cells. The results showed a significant increase in mIFN-γ production compared to control groups, indicating that rRSV/mIFN-γ was able to express mIFN-γ in vivo.

Growth of rRSV/mIFN-γ in the Presence of mIFN-γ

Growth of rRSV/mIFN-γ in the presence of mIFN-γ was determined by measuring the plaque size and morphology of rRSV/mIFN-γ-infected cells. The results showed a significant reduction in plaque size and an increase in the number of plaques compared to control groups, indicating that mIFN-γ had a inhibitory effect on the growth of rRSV/mIFN-γ.

Discussion

The results presented here demonstrate that rRSV/mIFN-γ can be used as a potential vaccine candidate against RSV infection. The attenuated phenotype of rRSV/mIFN-γ, coupled with the ability to express mIFN-γ in vivo, makes it a promising candidate for further study.

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References


times postinfection (Fig. 3). The concentration of mIFN-γ was 0.1 ng/ml 8 h postinfection, the earliest time tested. 1.8 μg/ml at 40 h, and reached a maximum 4.4 μg/ml at 120 h, which corresponds to 22 μg per 10⁸ cells.

**Replication, Immunogenicity, and Protective Efficacy of rRSV/mIFN-γ in BALB/c Mice.** To evaluate replication of rRSV/mIFN-γ *in vivo*, mice were infected intranasally with 10⁶ pfu of rRSV/mIFN-γ, rRSV/CAT, or wt RSV. Animals were sacrificed on day 3, 4, or 5 postinfection, and the concentration of the virus in the upper (nasal turbinates) and lower (lungs) respiratory tract was determined by plaque assay. Replication of rRSV/mIFN-γ was reduced relative to wt RSV by up to 63- and 20-fold in the upper and lower respiratory tracts, respectively (Fig. 4). In contrast, replication of rRSV/CAT was not significantly different from that of wt RSV, showing that the presence of an additional foreign gene of comparable size per se did not attenuate RSV replication in mice.

Serum samples were collected on days 0, 28, and 56 from mice infected with rRSV/mIFN-γ, rRSV/CAT, or wt RSV and were analyzed by an RSV-specific and antibody isotype-specific ELISA and by an RSV-neutralization assay (Table 1). The levels of IgA antibodies induced by the viruses were not significantly different. There was a significant increase (4-fold) of the total IgG specific to RSV F protein in mice vaccinated with rRSV/mIFN-γ compared with animals vaccinated with wt RSV or rRSV/CAT on day 56, but not on day 28. The titer of IgG1 antibodies was not significantly different between viruses on day 28, but on day 56 the mean titer of IgG1 from mice immunized with rRSV/mIFN-γ was higher than that of mice immunized with wt RSV (reciprocal 12.1 log₂ versus 9.3 log₂; \( P < 0.05 \)) or rRSV/CAT. In contrast, the mean titer of IgG2a on day 56 was decreased for mice immunized with rRSV/mIFN-γ compared with wt RSV (9.6 log₂ versus 11.6 log₂; \( P < 0.001 \)). Neutralizing antibody titers of mice infected with RSV/mIFN-γ compared with wt RSV and rRSV/CAT were marginally lower on day 28 but were modestly higher on day 56 (12.3 versus 11.2, log₂; \( P < 0.2 \)).

To evaluate protective efficacy, five mice from the groups described above were challenged on day 56 by the intranasal instillation of 10⁶ pfu per animal of wt RSV. Four days later, the mice were sacrificed and nasal turbinates and lungs were harvested for virus quantitation. Challenge virus was not detectable in animals that had been infected previously with RSV/mIFN-γ, and only a very low level of replication was observed in the upper respiratory tract in animals previously infected with wt RSV (data not shown).

**Pulmonary Cytokine mRNAs.** The levels of mRNAs encoding selected cytokines were determined in the lungs of mice infected with RSV/mIFN-γ or wt RSV to determine whether the level of mIFN-γ mRNA synthesis was increased and whether its synthesis affected the level of other Th1 or Th2 cytokine mRNAs. Five mice each from groups infected with RSV/mIFN-γ, wt RSV, or placebo were sacrificed on days 1 and 4 after infection or days 1 and 4 after challenge with wt RSV on day 28 (days 29 and 32). Total lung RNA was isolated and analyzed for selected cytokine mRNAs by a commercial ribonuclease protection assay (Fig. 5). This direct assay reflects the concentration of an mRNA at the site of interest at a given time and precludes possible artifacts because of *in vitro* manipulation of harvested cells. The mRNA levels were determined for the Th1 marker cytokines IL-2 and IFN-γ, the Th2 marker cytokines IL-4, IL-6, and IL-10, and the IL-12 p40 protein, which is the inducible component of the IL-12 heterodimer.

Fig. 5 shows an autoradiograph of an assay of IFN-γ and IL-12 p40 mRNAs in lungs of five individual animals harvested 4 days after immunization with the indicated virus. Increased accumulation of mIFN-γ was seen in the rRSV/mIFN-γ-infected animals, and a slight, but statistically significant, increase in IL-12 p40 mRNA was seen in the rRSV/mIFN-γ-infected animals compared with those infected with wt RSV. The results from this and other gels were quantitated with a PhosphorImager (Molecular Dynamics), and the mean value for each set of five mice was expressed as a percentage of the mouse L-32 housekeeping gene mRNA in the same gel lane (Fig. 6).
DISCUSSION

A chimeric virus, rRSV/mIFN-γ, was constructed that expresses the mIFN-γ gene as a separate mRNA from an additional transcriptional unit placed eighth in the gene order, between the G and F genes. This virus directed the synthesis of high levels of mIFN-γ in cell culture. Growth of rRSV/mIFN-γ in cell culture was reduced 16-fold compared with wt RSV. However, the magnitude of this effect was comparable to that observed for rRSV/CAT, which contains the CAT gene in the same genome location. Thus, we attribute the growth restriction in vitro to the presence of the foreign gene rather than to its encoded product. That the expression of mIFN-γ did not inhibit viral growth in human HEp-2 cells is not surprising because human IFN-γ and mIFN-γ share only 40% amino acid sequence identity.

Replication of rRSV/mIFN-γ in BALB/c mice was reduced 63- and 20-fold in the upper and lower respiratory tract, respectively, compared with wt RSV. In contrast, rRSV/CAT assayed in parallel was not restricted compared with wt RSV, indicating that the attenuation of rRSV/mIFN-γ in vivo was not a result of the presence of the additional gene per se, but rather was a consequence of expression of mIFN-γ. Because the growth restriction in vivo operated early in infection, it seems likely that it was due to effects of the expressed mIFN-γ on innate immunity, such as induction of oligoadenylate synthetase and the resulting antiviral cascade or possibly the activation of NK cells and macrophages, rather than to effects on adaptive immunity. That the growth of rRSV/mIFN-γ was restricted only 63-fold or less suggests that IFN-γ is not the major effector of resistance to RSV. For another respiratory virus, influenza A virus, expression of IFN-γ by the host was not needed for an efficient immune response, although its absence resulted in a Th2-biased antibody and cytokine response (15).

The question of whether the coexpression of IFN-γ during RSV infection could further bias T cell proliferation in favor of a Th1 response was addressed by analyzing the pattern of cytokine mRNA and RSV-specific antibody isotypes. Infection with wt RSV was associated with increases in mRNA for the Th1 marker IFN-γ but not IL-2, for the Th2 markers IL-6 and IL-10, and for IL-12 p40, which is produced primarily by monocytes and macrophages. Infection with rRSV/mIFN-γ resulted in an increased level of IFN-γ mRNA and a slightly increased (less than 2-fold) level of IL-12 p40 mRNA over that
observed with wt RSV. The increase in mIFN-γ mRNA presumably was due, at least in part, to that expressed by the recombinant virus. The increase in the IL-12 p40 mRNA probably was a result of IFN-γ-mediated activation of its monocyte/macrophage source, although this was not observed previously in vitro (15). Animals infected with rRSV/mIFN-γ did not exhibit differences from wt RSV in the level of mRNAs for the other Th1 marker, IL-2, or for the Th2 markers IL-6 or IL-10. There were modest increases in total IgG and IgG1 RSV-specific antibodies in mice immunized with rRSV/mIFN-γ compared with wt RSV, with the latter antibodies being a marker for a Th2 response (17). There also was a modest decrease in IgG2a, a marker for a Th1 response (17). Thus, neither the cytokine nor the antibody response was consistent with an increased bias toward Th1 markers either upon the initial infection with rRSV/mIFN-γ or after challenge with wt RSV.

Mice immunized with wt RSV or rRSV/mIFN-γ were highly resistant to RSV challenge. Despite its growth restriction in vivo, rRSV/mIFN-γ induced titers of total IgG against RSV F protein and RSV-neutralizing serum antibodies that were higher than that induced by wt RSV. Our previous results (18) showed that chimpanzees vaccinated with RSV cpts248/404, a candidate live-attenuated virus vaccine, developed lower titers of RSV-neutralizing antibodies compared with wt RSV-immunized animals (7.9 log2 versus 11.1 log2, a 9.2-fold difference), suggesting a correlation between the level of RSV replication and its immunogenicity. That the antibody response to rRSV/mIFN-γ was increased moderately overall despite its reduced level of virus replication is encouraging for the development of a live-attenuated RSV vaccine.

We have shown previously (19) that rodents such as mice or cotton rats mount extremely efficient immune responses to RSV antigens, whereas immunogenicity is usually much less when evaluated in nonhuman primates or human volunteers. This might be especially important for young infants, whose antibody response to RSV has been shown to be reduced (20). Thus, differences in antigenicity that could be very significant
in humans often are not detected in rodents because of their greater immunoresponsiveness to RSV antigen. Furthermore, replication of RSV in rodents is very restricted, such that only a small percentage of pulmonary cells are infected and disease typically does not occur. It is very likely that the effect of IFN-γ on attenuation, immunogenicity, or reactivity will be greater in a fully permissive host. To evaluate this, we presently are constructing an rRSV that contains human rather than murine IFN-γ. Evaluation of this virus in chimpanzees, which is the animal that most resembles humans with regard to RSV replication, disease, and immunogenicity, should yield a more realistic picture of the effects of the coexpressed IFN-γ.

One possible complication, namely, that expression of the human cytokine in infected cultured primate cells will hamper the preparation of vaccine lots, presumably could be obviated by using cells from a different species.

A number of cytokine genes have been inserted into recombinant DNA viruses, mostly vaccinia virus, revealing effects on attenuation, pathogenicity, and immunogenicity (refs. 21 and 22; for review, see ref. 23). In a poxvirus, expression of IFN-γ or type 1 IFN attenuated the virus for the host, but this attenuation was accompanied by a decreased humoral immune response (24–26). Expression of IFN-γ by a simian immunodeficiency virus (SIV) lacking the nef gene resulted in further attenuation of the SIV mutant for monkeys, but the cytokine insert was very unstable after several weeks of replication, and the attenuation was accompanied by a decrease in the humoral immune response to SIV glycoprotein (27). In the present work, we have extended this strategy to a vaccine development program for a negative-stranded virus. The results demonstrate that it is possible to attenuate the virus while maintaining immunogenicity, a result previously attained only in the case of expression of IL-2 by a vaccinia virus vector (28). The present results are encouraging and indicate that the coexpression of IFN-γ represents a new type of attenuation of nonsegmented negative-stranded RNA viruses, one that reduces virus growth without compromising immunogenicity.

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