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Immunology

Vaccinia virus recombinants expressing chimeric proteins of human immunodeficiency virus and γ interferon are attenuated for nude mice

(chimeric proteins/lymphokines/vaccine development)

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ABSTRACT **We have developed a method for attenuating vaccinia virus recombinants by expressing a fusion protein of a lymphokine and an immunogen. Chimeric genes were constructed that coded for γ interferon (IFN-γ) and structural proteins of the human immunodeficiency virus type 1 (HIV-1). In this study, we describe the biological and immunological properties of vaccinia virus recombinants expressing chimeric genes of murine or human IFN-γ with glycoprotein gp120, gag, and a fragment of gp41. All fusion proteins retained the antigenic characteristics of both IFN-γ and HIV as shown by immunoblot analysis. However, the antiviral activity of IFN-γ could be demonstrated only for the IFN-γ–gag fusion protein. In contrast, the attenuating activity of IFN-γ for nude mice was retained by all of the recombinants, albeit at various rates. Unlike the antiviral activity, the attenuating activity of IFN-γ was not species specific. Implications for the development of attenuated live recombinant vaccines for AIDS are discussed.**

Vaccinia virus (VV) recombinant (rVV) vaccines have been developed for a number of important diseases of humans and animals. Two rVV vaccines currently undergoing field testing have great promise for the eradication of rabies (1, 2) and rinderpest (3, 4), diseases that have seriously impacted human and animal populations for centuries. Currently, rVVs are also being evaluated as candidate vaccines against the human immunodeficiency virus (HIV) (5). The HIV antigens expressed by rVV are correctly myristylated, glycosylated, proteolytically processed, and transported to the appropriate cellular compartment (ref. 6 and references therein). Moreover, experimental animals and human volunteers who were vaccinated with rVV expressing HIV antigens developed detectable levels of humoral and cell-mediated immunity to the viral proteins (6, 7).

A major safety concern regarding the use of rVV has been emphasized by reports of clinical complications in HIV-infected individuals after immunization against smallpox (8) or the administration of an experimental rVV vaccine (9). Therefore, such safety considerations must be addressed before introducing live recombinant viruses into the environment. The rapidly expanding AIDS epidemic, particularly in Africa and Asia, demands that live vaccines pose no health threat to populations, especially those who may already be immunosuppressed by other infectious diseases.

In this study, we report an approach for the attenuation of live recombinant vaccines by the construction of chimeric rVV expressing fusion proteins of human or murine γ interferon (HuIFN-γ or MuIFN-γ) with various immunogens derived from HIV-1. In nude mice, we have demonstrated reduced pathogenicity of several rVVs expressing these fusion proteins. Furthermore, we provide evidence that the antiviral and attenuating activities of IFN-γ have different mechanisms of action. Unlike the antiviral activity, the attenuating activity of IFN-γ is not species specific. Moreover, we show attenuating activity by IFN-γ in the absence of any detectable antiviral activity.

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MATERIALS AND METHODS

Viruses and Cells. VV, strain WR (obtained from B. Moss, National Institute of Allergy and Infectious Diseases, Bethesda, MD), was propagated in human 143 thymidine kinase (TK)-deficient (TK^-) osteosarcoma cells. Encephalomyocarditis virus (EMCV; obtained from K. Anderson, Genentech) was propagated in human A549 cells and in murine L cells. Human 143 TK^-, human A549, CV-1 (monkey kidney cell line), and murine L cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum and antibiotics.

Antibodies. Rat anti-MuIFN-γ and rabbit anti-HuIFN-γ were provided by N. Burnette, Amgen Biologicals. Chiron was the source for murine monoclonal antibodies against HIV glycoprotein gp120 (anti-HIVgp120) and HIV protein p25 (anti-HIVp25) and for goat polyclonal antisera against HIV envelope protein env5B (anti-HIVenv5B).

Chimeric Gene Construction and Expression. Oligonucleotides were synthesized by the phosphoramidite method by using Applied Biosystems 380A and 380B DNA synthesizers. The synthetic gene for HuIFN-γ has been described (10, 11). The gene encoding mature MuIFN-γ was chemically synthesized in a similar manner by using 2 22-mer and 18 43-mer oligonucleotides. For each gene, additional DNA was synthesized that encoded each natural signal peptide sequence.

Genes encoding the pre-IFN-γ sequences were cloned into the plasmid pUC18. For insertion of HIV-1 coding sequences into these plasmids, we excised, as *Nco* I/*Sal* I fragments, the gp120, the gag polyprotein precursor, and the gp41 env5B fragment coding sequences from previously described yeast expression plasmids (12, 13). The gp41 env5B fragment includes the bases coding for amino acids 556–677 of gp160, which corresponds to a hydrophilic domain in the amino-terminal half of gp41.

Abbreviations: VV, vaccinia virus; rVV, recombinant VV; HIV-1, human immunodeficiency virus type 1; IFN-γ, γ interferon; TK, thymidine kinase; EMCV, encephalomyocarditis; pfu, plaque-forming units; NK, natural killer; MuIFN-γ and HuIFN-γ, mouse and human IFN-γ; vMuIFN-γ, vHuIFN-γ, and similarly named rVV, rVV expressing MuIFN-γ, HuIFN-γ, and related fusion proteins.

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The strategy for the construction of rVVs has been described (14). A rVV expressing glycoprotein gp160 of the SF2 strain of HIV-1 was developed in the same manner as the one described by Chakrabarti *et al.* (15). The appropriate expression of proteins was demonstrated by immunoblot (Western) analysis as described (4). Antibodies were diluted in Tris-buffered saline solution containing 5% nonfat dried milk throughout the experiments. The protein bands were visualized by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates for the alkaline phosphatase-labeled secondary antibodies.

Antiviral Activity. The antiviral activities of IFN-γ and fusion proteins were determined by the prevention of cytopathic effects of EMCV on murine L and human A549 cell lines (16). Briefly, human TK-143 cells were infected with rVVs at a multiplicity of infection of 10 and incubated for 24 hr. Supernatants were then harvested, filtered through 0.2-μm filters, and serially diluted in DMEM for the determination of IFN-γ titers. Aliquots of 50 μl of these dilutions were placed in 96-well plates, and 10^4 cells of the appropriate type in 100 μl of DMEM with 10% fetal calf serum were added to each well. After 24 hr of incubation, the cells were challenged with the minimum dose of EMCV [10^4 plaque-forming units (pfu) per well] that gave 100% cytopathic effects in IFN-γ-untreated cells. The units of IFN-γ are expressed as the reciprocal of the dilution of sample that gave 50% protection against the challenge virus.

Animal Studies. The attenuating effect of IFN-γ on rVV virulence was determined in nude mice. Athymic *nu/nu* BALB/c mice (5–6 weeks old) were purchased from Simonsen Laboratories (Gilroy, CA). The animals were housed in isolated sterile cages and were challenged i.p. with either 10^6 or 10^7 pfu of rVV in a final volume of 200 μl of sterile phosphate-buffered saline (PBS). Mice were examined daily for 60 days, and the mortality rate for each group was determined.

Macrophage and Natural Killer (NK) Cell Inhibition. Carrageenan and silica (0.012 μm) were purchased from Sigma. Sterile mixtures of Carrageenan at 1.2 mg/ml and at 0.4 mg/ml of silica in PBS were prepared. Groups of 10 nude mice were injected i.p. with 0.5 ml of either stock mixture, and a control group received PBS alone. Carrageenan was administered on days −2, −1, 0, and +1; silica and PBS were given on days −1, 0, and +1. On day 0, the mice were injected i.p. with 5×10^6 pfu of rVV expressing MuIFN-γ (vMuIFN-γ), and their survival rate was monitored for a period of 1 month.

RESULTS

rVV Construction. Chimeric IFN-γ–HIV-1 genes were constructed in the plasmid pUC18 that was modified by the inclusion of a synthetic polylinker. A schematic representation of these fusion genes and their flanking restriction sites is shown in Fig. 1. This vector allowed for the excision of each chimeric gene as a *Sma* I fragment and subsequent insertion into the VV vector pSC11.

The correct expression of IFN-γ, HIV-1, and IFN-γ–HIV chimeric genes by rVV was demonstrated by Western blot analysis (Fig. 2). The IFN-γ and HIV fusion proteins were recognized both by specific anti-IFN-γ and anti-HIV-1 antibodies. Bands of the same molecular weight reacting with two different antibodies indicated that the two proteins formed part of a single molecule. Furthermore, the observed

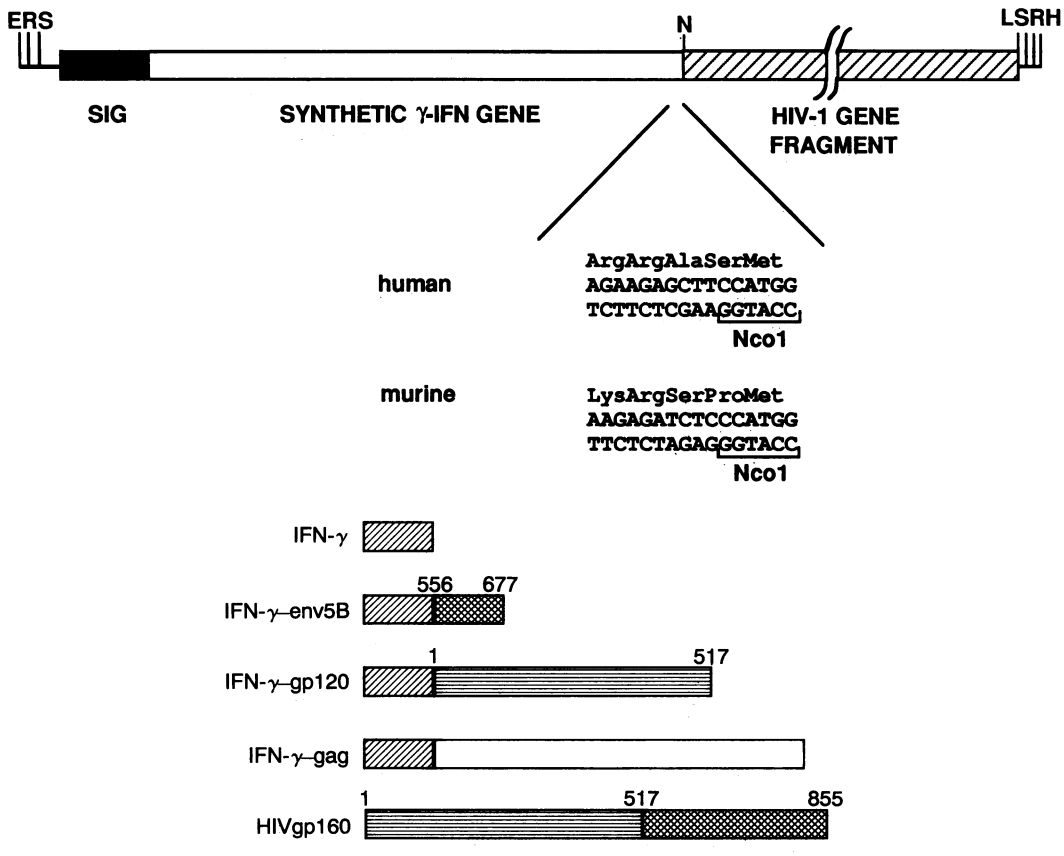

FIG. 1. Schematic representation of chimeric gene constructions. (*Upper*) Diagram depicting the strategy used for the construction of genes encoding IFN-γ–HIV-1 fusion proteins (IFN-γ–env5B, IFN-γ–gp120, and IFN-γ–gag). Highlighted are the polylinker cloning sites (E, *Eco*RI; R, *Eco*RV; S, *Sma* I; N, *Nco* I; L, *Sal* I; H, *Hin*dIII), the encoded secretory signal sequence (SIG), and the DNA and protein sequences around the junction of the chimeric gene. (*Lower*) Proteins expressed by rVV, including IFN-γ (hatched boxes), IFN-γ–HIV fusion proteins, and HIVgp160 (striped and crosshatched boxes for gp120 and gp41 regions, respectively). Numbers indicate the gp160 amino acids. The open box represents the HIV gag protein.

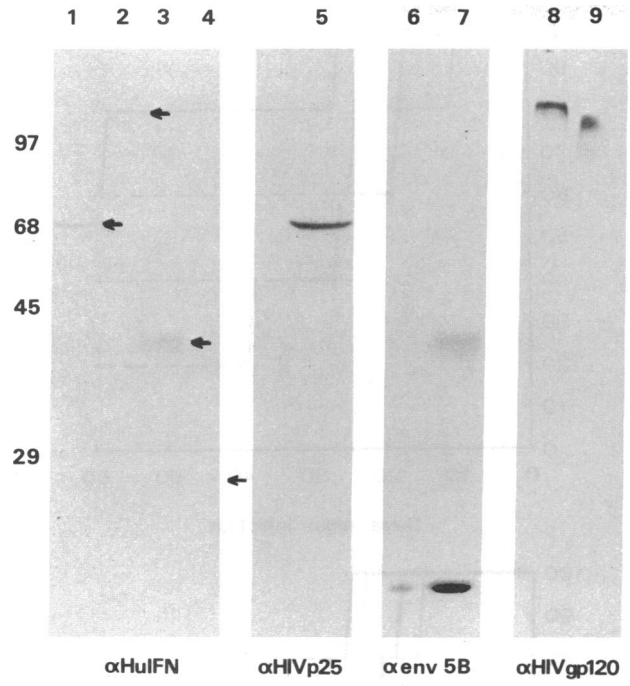

FIG. 2. Western blot analysis of fusion proteins expressed by rVV. TK-143 cells were infected with rVVs expressing HuIFN-γ–gag (vHuIFN-γ–gag) (lanes 1 and 5), HuIFN-γ–gp120 (vHuIFN-γ–gp120) (lanes 2 and 9), HuIFN-γ–env5B (vHuIFN-γ–env5B) (lanes 3 and 7), HuIFN-γ (vHuIFN-γ) (lane 4), and HIVgp160 (vHIVgp160) (lanes 6 and 8). After 24 hr of infection, cells were processed, and proteins were separated by SDS/10% PAGE. Proteins were then transferred to an Immobilon P membrane for immunodetection. Note that the proteins have the predicted antigenic determinants and molecular mass (in kDa) when probed with antibodies to either HuIFN-γ (αHuIFN) or HIV-1 structural proteins (αHIVp25, αenv5B, and αHIVgp120).

and predicted molecular weights of the fusion proteins were equivalent.

IFN-γ-Induced Antiviral Activity. The antiviral activities induced by IFN-γ and IFN-γ–HIV-1 fusion proteins were determined by the prevention of the cytopathic effects of EMCV in homologous and heterologous cells; the results are summarized in Table 1. In homologous cell lines, HuIFN-γ or MuIFN-γ expressed by VV had antiviral titers of >30,000 units/ml. In contrast, fusion proteins of IFN-γ (murine or human) with HIVgp120 and HIVenv5B lacked any detectable antiviral activity. The only fusion protein with detectable antiviral activity was IFN-γ–gag; however, this protein had approximately 10% (3000 units/ml) of the antiviral activity of the recombinant virus expressing IFN-γ alone. As anticipated, IFN-γ-induced antiviral activity was species specific. MuIFN-γ or MuIFN-γ–HIV-1 fusion protein had no detectable antiviral activity on human A549 cells. Conversely, HuIFN-γ or HuIFN-γ–HIV-1 fusion protein lacked detectable antiviral activity in murine L cells.

Attenuation of VV for Nude Mice. Nude mice were challenged with 10^6 or 10^7 pfu of rVVs (Fig. 3). Those rVVs expressing MuIFN-γ or MuIFN-γ–HIV-1 fusion protein with any detectable level of antiviral activity—namely, vMuIFN-γ and vMuIFN-γ–gag—were completely attenuated (100% survival) for nude mice (Fig. 3 *A* and *C*). On the other hand, vHuIFN-γ or rVVs expressing HuIFN-γ–HIV-1 fusion proteins were only partially attenuated for nude mice (Fig. 3 *B* and *D*). Similarly, partial attenuation was observed for rVVs expressing MuIFN-γ fusion proteins lacking detectable antiviral activity. No attenuation (0% survival) was observed in a group of nude mice challenged with a mixture of vHuIFN-γ and vHIVgp160 or vMuIFN-γ and vHIVgp160. The injection of 10^7 pfu of vHIVgp160 was highly lethal to nude mice; no survivals were recorded 10 days after injection.

Macrophage Inhibition. Carrageenan and silica, two particulate substances known to inhibit macrophage activity (17), were used to determine the role of these cells in the IFN-γ-induced clearance of VV in nude mice. No detectable difference in response to challenge with vMuIFN-γ was observed in groups of nude mice treated with carrageenan and silica or in an untreated control group (Table 2).

DISCUSSION

We have described the construction and biological characterization of several rVVs that express single IFN-γ molecules or fusion proteins of IFN-γ (murine and human) and HIV-1 structural proteins (SF2 isolate). We have preferred to express these as chimeric proteins rather than separate molecules in rVVs; this guarantees the presentation of a molecule that generates both immunoregulatory and antigenic functions in responding cells. These chimeric molecules consist of IFN-γ linked to gp120, a hydrophilic portion of gp41 encompassed by amino acids 556–677 (env5B), or the gag precursor p53. The TK locus of VV was used exclusively for the generation of all rVVs. The fusion proteins expressed by rVVs were authentic and had the predicted molecular mass. Only single bands that reacted specifically with both anti-IFN-γ and anti-HIV-1 antibodies were detected, indicating the presence of a single fusion protein and lack of degradation (Fig. 2).

Antiviral Activities of Fusion Proteins. Although we demonstrated the antigenic authenticity of the IFN-γ portion of the fusion proteins, no detectable antiviral activity could be demonstrated for any of the fusion proteins except IFN-γ–gag, which had only 10% of the antiviral activity of IFN-γ alone (Table 1). While the human and murine IFN-γ genes are 60% conserved at the amino acid sequence level, their antiviral activity is species specific (18). The antiviral activity and species specificity of IFN-γ or the IFN-γ–gag fusion protein expressed by rVV were identical to the corresponding natural IFN-γ.

It has been shown that antibodies directed against the carboxyl terminus of MuIFN-γ neutralize the antiviral activity, and this activity was also lost after deletion of the first nine amino acids. This indicates that both the amino- and carboxyl-terminal portions of MuIFN-γ are functionally important and that they may be sterically distant from one another (ref. 19 and references therein). It has been demonstrated, however, that the amino terminus of HuIFN-γ is more critical for the stability of the tertiary structure of the

Table 1. IFN-γ-induced antiviral activity of fusion proteins expressed by rVVs in different cell lines

	Antiviral activity, % of homologous IFN-γ							
Cell line	HuIFN-γ	HuIFN-γ–env5B	HuIFN-γ–gp120	HuIFN-γ–gag	MuIFN-γ	MuIFN-γ–env5B	MuIFN-γ–gp120	MuIFN-γ–gag
Human A549	100	<0.1	<0.1	10	<0.1	<0.1	<0.1	<0.1
Murine L	<0.1	<0.1	<0.1	<0.1	100	<0.1	<0.1	10

Human B-143 TK⁻ cells were infected with rVVs expressing different fusion proteins and the supernatants were processed as described in *Materials and Methods*.

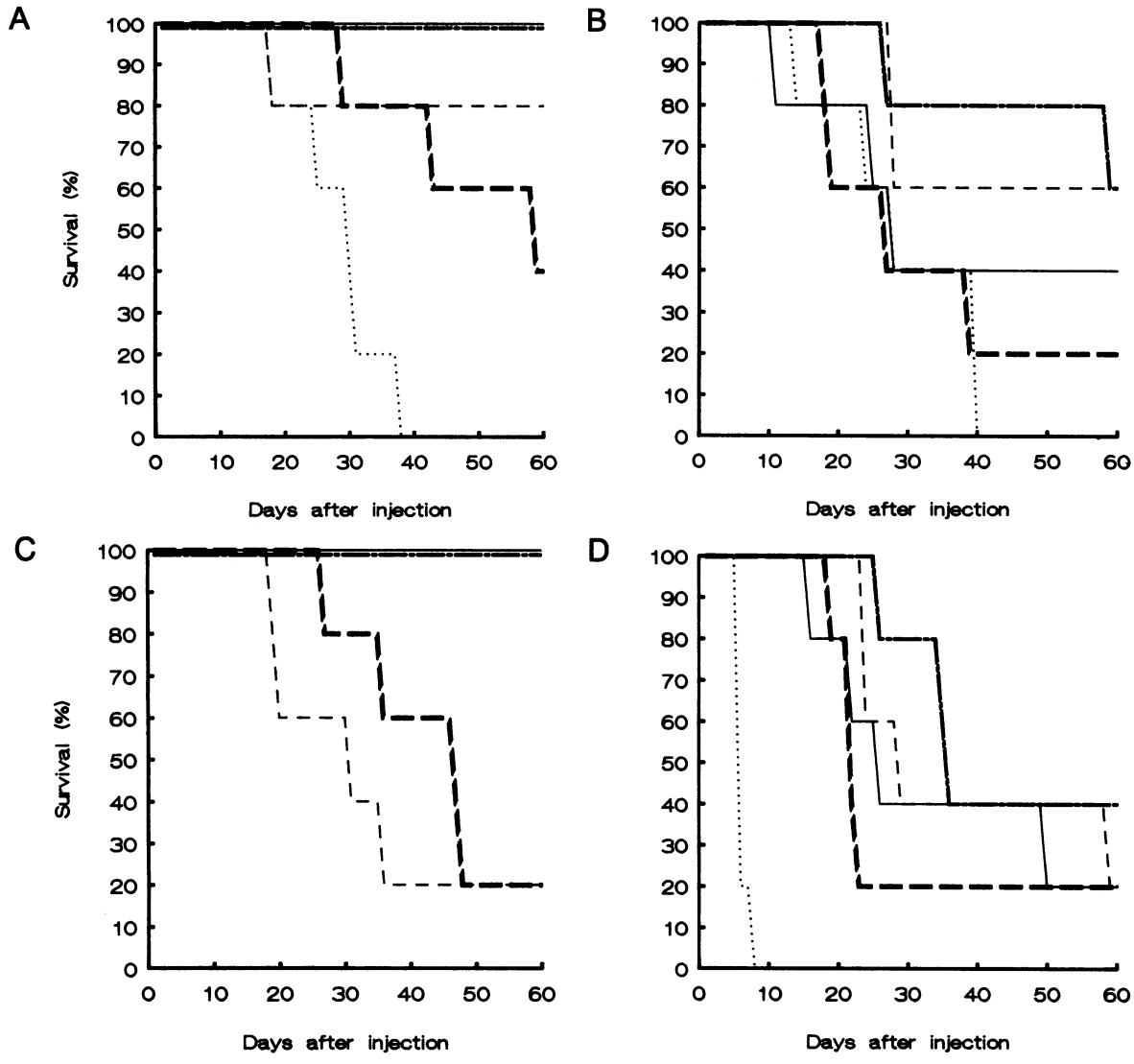

FIG. 3. Attenuation of rVVs in nude mice. Nude mice (five per group) were inoculated with 10^6 (*A* and *B*) or 10^7 (*C* and *D*) pfu of rVV, and survival rates were recorded daily for a period of 60 days. (*A* and *C*) Nude mice injected with vMuIFN-γ (—), with rVVs expressing MuIFN-γ–HIV-1 fusion proteins [vMuIFN-γ–env5b (---), vMuIFN-γ–gp120 (•••), and vMuIFN-γ–gag (·-·)], or with a mixture of vMuIFN-γ and vHIVgp160 [vMuIFNγ/gp160 (···)]. (*B* and *D*) Nude mice injected with vHuIFNγ (—), vHuIFN-γ–env5b (---), vHuIFN-γ–gp120 (•••), vHuIFN-γ–gag (·-·), vHIVgp160 (···) (*D* only), or a mixture of vHuIFN-γ and vHIVgp160 [vHuIFNγ/gp160 (····) (*B* only)].

molecule, which governs both receptor binding and biological activity of the lymphokine (20). As a result, we decided to make chimeric gene constructs that have the HIV proteins linked to the carboxyl-terminal end of the IFN-γ molecule. However, it appears that even this modification has altered the structure of IFN-γ and that this steric interaction is strictly related to the conformation of the fusion protein and not to the size of the fused fragment. This is best illustrated by HIV gag, which is 3 times as large as the env5B fragment; nevertheless, it was IFN-γ–gag and not IFN-γ–env5B that retained antiviral activity. Whether this absence of antiviral activity is due to the inability of certain fusion proteins to form dimers of IFN-γ (including the amino terminus of one subunit and the carboxyl terminus of the other) that are essential for receptor binding has been investigated (20).

Attenuating Activity of Fusion Proteins. Safety considerations must be addressed before introducing live recombinant viruses into the environment. Three strategies have been used to develop an attenuated rVV vaccine: (*i*) construction of the rVV with the attenuated strain (Wyeth) used worldwide in

Table 2. Survival of nude mice to challenge with 5×10^6 pfu of vMuIFN-γ after depletion of macrophages and NK cells with carrageenan and silica

Treatment/challenge	Day of treatment				Survival*
	−2	−1	0	+1	
Carr/vMuIFN-γ	Carr	Carr	Carr/vMuIFN-γ	Carr	10/10
Sil/vMuIFN-γ	—	Sil	Sil/vMuIFN-γ	Sil	10/10
PBS/vMuIFN-γ	—	PBS	PBS/vMuIFN-γ	PBS	10/10
Carr	Carr	Carr	Carr	Carr	4/4
Sil	—	Sil	Sil	Sil	4/4

Carr, carrageenan and 1.2 mg/ml i.p.; Sil, silica at 0.4 mg/ml i.p.
*Mice inoculated/mice alive 45 days after inoculation.

successful smallpox eradication; (*ii*) expression of heterologous genes in the TK locus of the virus, since it has been shown that insertional inactivation of the TK gene further attenuates the virus (21); and (*iii*) coexpression of lymphokine genes. We and others have shown that coexpression of interleukin 2 and MuIFN-γ in VV completely abolishes the lethal effects of the virus in nude mice (22–24). We have now extended these studies by assessing the attenuating effects of IFN expressed as fusion proteins with HIV-I structural proteins.

The mechanism by which VV expressing lymphokine genes is cleared from these immunocompromised animals has not yet been determined. Our studies were conducted in nude mice that were 5–6 weeks old, and no functional T-cell immunity is detected in these *nu/nu* mice until the age of 6 months (25). Nude mice that were injected with 10^7 pfu of vMuIFN-γ and later sacrificed never showed VV titers of $>2.4 \times 10^2$ pfu/100 mg of tissue (spleen and liver), and no virus could be recovered after 7 days postinjection (data not shown). Macrophages, NK, and B cells are active in athymic nude mice (26). However, the inhibition of NK cell and macrophage activity by the administration of silica and carrageenan particles did not affect the ability of nude mice to eliminate vMuIFN-γ, suggesting that these cells do not play a major role in the attenuation observed. It has also been shown that, in normal mice, vMuIFN-γ do not enhance NK cell activity above that induced by VV alone (24). In addition, anti-VV antibodies did not play a role in the observed attenuation, since there were no detectable antibodies present in the surviving nude mice (data not shown) (23, 27).

Relationship Between Attenuating and Antiviral Activity of IFN-γ. We have investigated the relationship between the attenuating and the antiviral activities of IFN-γ and have made the following four observations. (*i*) No significant difference is observed in the kinetics of *in vitro* replication of rVVs producing IFN-γ as compared with rVV producing other antigens, indicating the lack of interference in rVV replication by the lymphokine. For example, no difference was observed in the kinetics of replication of vMuIFN-γ, vHuIFN-γ, and vHIVgp160 in murine or human cells (data not shown). (*ii*) vMuIFN-γ or rVVs expressing MuIFN-γ–HIV-1 fusion proteins with any detectable antiviral activity *in vitro* also exhibit 100% attenuation for nude mice (Fig. 3 *A* and *C*). (*iii*) IFN-γ exhibits attenuating activity in the absence of detectable antiviral activity. Nevertheless, all rVVs expressing either human or murine IFN-γ or IFN-γ–HIV-1 fusion proteins with no detectable antiviral activity *in vitro* are partially attenuated for nude mice. In these groups survival rates of 40–60% and delayed onset of illness have been observed (Figs. 1 and 2). This may indicate that the attenuating activity of IFN-γ is not species specific. (*iv*) The addition of a second rVV that does not express IFN-γ completely abrogates the attenuating activity of a rVV expressing IFN-γ. For example, the vMuIFN-γ/vHIVgp160 mixture was 100% lethal to nude mice. This is probably due to the infection of some cells with vHIVgp160 only, resulting in a productive disseminated infection and death of the mice.

Recently, in a phase 1 human trial, individuals vaccinated with rVV expressing HIVgp160 showed a strong T-cell response against both homologous and heterologous strains of whole virus (5). Similarly, rhesus macaques vaccinated with rVV expressing simian immunodeficiency virus SIV_{mac}-gag developed $CD8^+$ cytotoxic T lymphocytes (CTL) directed against epitopes of the gag protein (7). $CD8^+$ CTLs may play a major role in the containment of the spread of HIV infection, and a number of linear CTL epitopes are located on the HIV gag protein (28, 29). Consequently, we believe that the fusion of HIV gag with HuIFN-γ or MuIFN-γ would have little or no effect on the configuration of linear CTL determinants and probably would not alter their immunogenicity. We have also demonstrated that rVVs expressing the IFN-γ–gag fusion proteins retain antiviral activity and are 100% attenuated for nude mice. Therefore, we predict that vHuIFN-γ–gag recombinant vaccines should be safe and able to elicit $CD8^+$ CTL responses in vaccinates.

In summary, we have shown that rVVs expressing fusion proteins of MuIFN-γ or HuIFN-γ and HIV-I antigens are attenuated for immunocompromised mice. These studies with different fusion proteins may assist in the design of a candidate vaccine against HIV-1 based on safety and improved immunogenicity.

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