The Journal of Immunology

Antiviral Activities of Individual Murine IFN-α Subtypes In Vivo: Intramuscular Injection of IFN Expression Constructs Reduces Cytomegalovirus Replication

Wen-Shuz Yeow, Cassandra M. Lawson and Manfred W. Beilharz

J Immunol 1998; 160:2932-2939; ;
http://www.jimmunol.org/content/160/6/2932

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Antiviral Activities of Individual Murine IFN-α Subtypes In Vivo: Intramuscular Injection of IFN Expression Constructs Reduces Cytomegalovirus Replication[1]

Wen-Shuz Yeow, Cassandra M. Lawson,[2] and Manfred W. Beilharz

The IFN-α cytokines belong to a multigene family. However, the in vivo biological functions of each of the IFN-α subtypes is unknown. Recently, we developed an experimental model in which the tibialis anterior muscles of mice were transfected in situ with naked DNA plasmids encoding an IFN transgene. Here we use this model to investigate the in vivo effect of the expression of three murine IFN-α subtypes (*A1*, *A4*, and *A9*) on murine CMV replication in C57BL/6, BALB/c, and A/J mice. CMV was shown to replicate in the tibialis anterior muscles of mice for at least 6 days and induced an inflammatory infiltrate. However, mice expressing the IFN-α transgenes showed a marked reduction in the peak titers of virus replication, with less severe inflammation in the muscles compared with control mice that were inoculated with blank vectors. Moreover, mice expressing the IFN-α1 transgene had significantly lower CMV titers in the inoculated muscle than mice expressing either the IFN-α4 or the IFN-α9 transgenes. Furthermore, IFN-α/β receptor knockout mice had markedly higher levels of CMV replication in the tibialis anterior muscles than the wild-type parental strain (129/Sv/Ev) following IFN-α1 transgene inoculation, suggesting that the protection observed is due to host cell-mediated IFN signaling. These data provide the first evidence indicating that there are in vivo differences in the antiviral efficacy of the IFN-α subtypes. ***The Journal of Immunology*****, 1998, 160: 2932–2939.**

The type 1 IFNs belong to a multigene family that includes multiple IFN-α subtypes, a single IFN-β, IFN-τ, and IFN-ω (1). Humans express >15 IFN-α (*IFNA*) subtypes, a single β (*IFNB*), and one ω (*IFNW*) located together on chromosome 9 (2). Similarly in the mouse, there are >10 IFN-α subtypes and one IFN-β subtype (3, 4), all of which are located on chromosome 4 (5). Collectively, these IFNs have diverse biologic functions, including antiviral (6, 7), antiproliferative (8), and immunomodulatory activities (9–11) mediated via signal transduction pathways (12). Although evidence from in vitro studies suggests differences in the biologic effects of the subtypes (13–16), the in vivo significance of these findings remains obscure. Some researchers have hypothesized that the multiple IFN-α subtypes are a series of evolutionary duplications of an original gene. It may therefore be the case that these subspecies have an equivalent function in vivo. Alternatively, it has been hypothesized that each subtype may have evolved specific functions (17). Increased knowledge of the activities of the individual subtypes in vivo will enhance utilization of such subtypes for improved clinical efficacy over the current usage of either a mixture of IFN subspecies or a single rIFN subtype.

One approach to study the functions of individual IFN subtypes in vivo was made using transgenic mice (18–21). Transgenic mice were created with either murine *IFNA1* or *IFNB* under the control of the metallothionein I promoter and unexpectedly expressed the gene only in the testes (IFN-α and -β) and liver (IFN-β) upon induction with cadmium. IFN was only detected in the sera of the IFN-β transgenic animals. However, both transgenic lines showed inhibition of spermatogenesis, resulting in sterility of male mice. No further experimental work with these animals has been reported. We have also unsuccessfully attempted to produce transgenic mice expressing the murine IFN-α1 and IFN-α4 genes under the control of a mutated (non-leaky) metallothionein IIA promoter (W.-S. Yeow and M. W. Beilharz, Department of Microbiology, University of Western Australia, Perth, Australia, unpublished observations). It is likely that even low levels of expression of the IFN transgene in the developing embryo are conditionally lethal.

The advent of naked DNA transfer technology provided an alternative approach to the manipulation of gene expression in vivo (22, 23). The persistence of the plasmid allows ongoing expression of the transgene, and this finding has been pursued for gene therapy (24). Immunization with plasmids expressing viral genes has induced protective immune responses, and these plasmids are being developed for improved vaccines (25, 26). Recently, we have developed a mouse experimental model in which the tibialis anterior (TA)[3] muscles were transfected in situ with naked DNA plasmids encoding an IFN transgene (27). In this model, a mammalian expression vector carrying *MuIFNA9* was injected into the mouse skeletal TA muscle. Dot-blot analysis showed that the *IFNA9* transcript was present in the DNA-inoculated muscle. Biologic IFN activities were found in both muscle homogenates and sera of inoculated animals for up to 2 mo post-DNA injection. This model allows for the first time investigations into the comparative

Department of Microbiology, University of Western Australia, Queen Elizabeth II Medical Center, Nedlands, Perth, Western Australia, Australia

Received for publication August 25, 1997. Accepted for publication November 25, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Australian National Health and Medical Research Council (Grant 961302).

[2] Address correspondence and reprint requests to Dr. Cassandra Lawson, Department of Microbiology, University of Western Australia, Queen Elizabeth II Medical Center, Nedlands, Perth, Western Australia 6009, Australia. E-mail address: cassiel@cyllene.uwa.edu.au

[3] Abbreviations used in this paper: TA, tibialis anterior; MCMV, murine cytomegalovirus; IFNAR1, the alpha chain of the interferon-α/β receptor; B6, C57BL/6; pfu, plaque-forming unit; MOBS, mouse osmolarity-buffered saline; IE, immediate early; p.i., postinfection; HCMV, human cytomegalovirus.

0022-1767/98/$02.00

biologic efficacies of individual IFN-α subtypes. With this experimental model, we have chosen in the present study to compare the protective levels of a panel of three mouse IFN-α subtypes (namely, MuIFN-α1, -α4, and -α9) expressed in mice challenged with murine CMV (MCMV).

Type I IFNs play an important role in early defense mechanisms against MCMV infection. The administration of antiserum specific for type I IFN increased the susceptibility of adult mice to MCMV (28). The mouse genotype (*H-2* and non-*H-2* loci) determines the level of resistance to MCMV infection, with C57BL/6 (*H-2*^{b}) mice being two- to fourfold more resistant to lethal challenge of virus, and A/J (*H-2*^{a}) mice being twofold more susceptible to virus infection compared with BALB/c (*H-2*^{d}) mice (29, 30).

In this paper, we report the in vivo effects of three individual subtypes of IFN-α on MCMV replication in skeletal muscle. Three murine IFN-α genes (*A1*, *A4*, and *A9*) were individually subcloned into a mammalian expression vector and were used to inoculate the TA muscles of C57BL/6, BALB/c, and A/J mice, which were subsequently challenged with MCMV i.m. (TA). Expression of *MuIFNA1*, *-A4*, and *-A9* reduced MCMV replication in vivo to different extents in each of the mouse strains. The greatest antiviral effects were observed for *MuIFNA1*. The effects of IFN were presumably mediated by host cell signaling events, since IFN-α/β receptor (IFNAR1) knockout mice could not use the plasmid-expressed IFNs for protection against virus infection, in contrast to the parental wild-type mouse strain. Together, these findings provide supporting evidence for the hypothesis that individual IFN subtypes may have differential biologic functions in vivo.

Materials and Methods

Mice

Specific pathogen-free female C57BL/6 (B6), A/J, and BALB/c female mice were purchased from Animal Resources Center (Murdoch, Western Australia) and housed under minimal disease conditions. All mice used were between 6 and 8 wk of age. Specific pathogen-free male IFN-α/β receptor (IFNAR1) knockout mice (derived from M. Aguet, University of Zurich, Zurich, Switzerland) and the parental wild-type strain (129/Sv/Ev) were obtained from Drs. Alistair Ramsay and Malcolm Lawson (University of Western Australia).

Virus

The K181 strain of MCMV was prepared as a salivary gland homogenate from infected 3-wk-old female BALB/c mice and stored in the gas phase of liquid nitrogen. Virus titers in infected mice were quantitated by plaque assay of 20% tissue homogenates (30) and expressed as mean pfu/ml of 100% tissue homogenates.

Anesthetized mice were inoculated with a 25-μl injection volume of MCMV diluted in mouse osmolarity-buffered saline (MOBS) i.m. into each TA muscle. Both muscles were injected with half the specified dose of MCMV per mouse.

Plasmid constructs

The mammalian expression vector, pkCMVint.blank (Fig. 1*A*), was donated by VICAL (San Diego, CA). This vector contains the human CMV immediate-early (IE-1) gene enhancer/promoter and intron A for transcription initiation coupled with the SV40 polyadenylation signal. The construction of pkCMVint.*IFNA9* (Fig. 1*B*), encoding the full-length *MuIFNA9* gene, has previously been described in detail (27). The cloning of the full-length *MuIFNA4* gene cassette into pkCMVint involved an initial subcloning of the *Eco*RI/*Hin*dIII *IFNA4* cassette present in pBR322 (3) into the shuttle vector pBluescript II KS$^+$ (Stratagene, La Jolla, CA) and then from the shuttle vector to the *Pst*I/*Sal*I site of pkCMVint. This construct was designated pkCMVint.*IFNA4* (Fig. 1*C*). The mammalian expression vector construct containing the full-length *MuIFNA1* gene also driven by CMV IE-1 enhancer/promoter (pCMVint.*IFNA1*; Fig. 1*D*) was provided by Dr. Alistair Ramsay (John Curtin School, Canberra, Australia). This expression vector was obtained from Drs. James Arthos and James Mullins (31) (Stanford University School of Medicine, Stanford, CA).

Large-scale plasmid preparations were obtained from terrific broth cultures of transformed *Escherichia coli* (XL1-Blue) using standard DNA extraction procedures with LiCl precipitation. DNA integrity was checked by agarose gel electrophoresis, and concentrations were determined by spectrophotometric analysis. Yields of 10 to 15 mg of plasmid/L of culture were routinely obtained. All DNA construct preparations were performed under identical conditions.

Muscle regeneration

To induce muscle regeneration by crush injury (27), mice were anesthetized with 2.5% avertin, fur was removed with depilatory cream from the hind legs, and TA muscles were exposed by surgical incision of the skin. The TA muscles were partially sheared away from the underlying bones with forceps and crushed in three equally spaced locations along the muscles for 3 s using artery forceps. The incisions were sutured, and the mice allowed to recover for 5 days before inoculation with the DNA constructs.

In separate experiments, muscle regeneration was induced by bupivacaine. Anesthetized mice were injected bilaterally in the TA muscles with 25 μl of 0.5% bupivacaine 5 days before inoculation of the DNA constructs.

Mouse injection of DNA constructs

Anesthetized (2.5% avertin) mice were inoculated with 200 μg of DNA plasmids in a 25-μl volume of saline bilaterally in the TA muscles by inserting the needle longitudinally into the muscle. Similarly, control groups of mice were given the blank expression vectors. The expression ability of these plasmids in vivo was confirmed using a similar vector that contained the *lacZ* gene cassette driven by the CMV IE-1 enhancer/promoter and intron A (pVR-1412, VICAL), and positive staining for β-galactosidase was found in the DNA-injected TA muscle (9 days post-DNA; data not shown).

Dot-blot analysis

Dot-blot analysis was performed to determine the presence of plasmid in the transfected muscles. The human CMV IE-1 enhancer/promoter probe (654 bp) was obtained as a *Spe*I- and *Sac*II-cut fragment from pkCMVint-.blank (nucleotides 272–925), purified from agarose with Geneclean (BIO 101, Inc., La Jolla, CA), and labeled with [α-^{32}P]dCTP using random primers (Megaprime DNA labeling kit, Amersham International, Aylesbury, U.K.). The sequence of the probe has no homology with viral sequences of MCMV (GenBank U68299). DNA extracts were prepared from TA muscles of mice on day 7 post-DNA injection and dot-blotted onto Hybond-N$^+$ membranes (Amersham International). Hybridization to the labeled probe was performed at 60°C for 5 h (6 $\times$ SSC/7% SDS and 100 μg/ml salmon sperm DNA).

IFN bioassay

The TA muscles were collected from mice at 7 days post-DNA inoculation and treated at pH 2 to remove acid-labile IFNs, and IFN content was titrated in an in vitro bioassay using encephalomyocarditis virus-infected L929 cells as previously described (27). The standard murine IFN-α/β (Lee Biomolecular, San Diego, CA; 1000 IU/ml) was also titrated in the bioassay as a control.

Histology

Muscles were removed, fixed in 10% formalin-buffered saline, and paraffin embedded. Sections (5 μm) were stained by hematoxylin and eosin and observed microscopically for evidence of pathology. Sections were also stained by the immunoperoxidase method using hyperimmune sera from MCMV-infected mice to reveal virus-infected cells (32). Whole TA muscles from mice injected with the plasmid containing the *lacZ* gene cassette were fixed with 4% paraformaldehyde and stained for the presence of β-galactosidase using X-gal, frozen in liquid nitrogen, and then sectioned and counterstained with eosin as previously described (33).

Results

MCMV replication in the TA muscles

To establish the kinetics of MCMV replication in a skeletal muscle compartment, we inoculated mice with MCMV bilaterally in the TA muscles. B6 mice were given a sublethal dose of MCMV (4×10^4 pfu/mouse), and the amount of virus present in the muscles at 4 h and 1, 2, 3, 4, and 6 days postinfection (p.i.) was titrated by

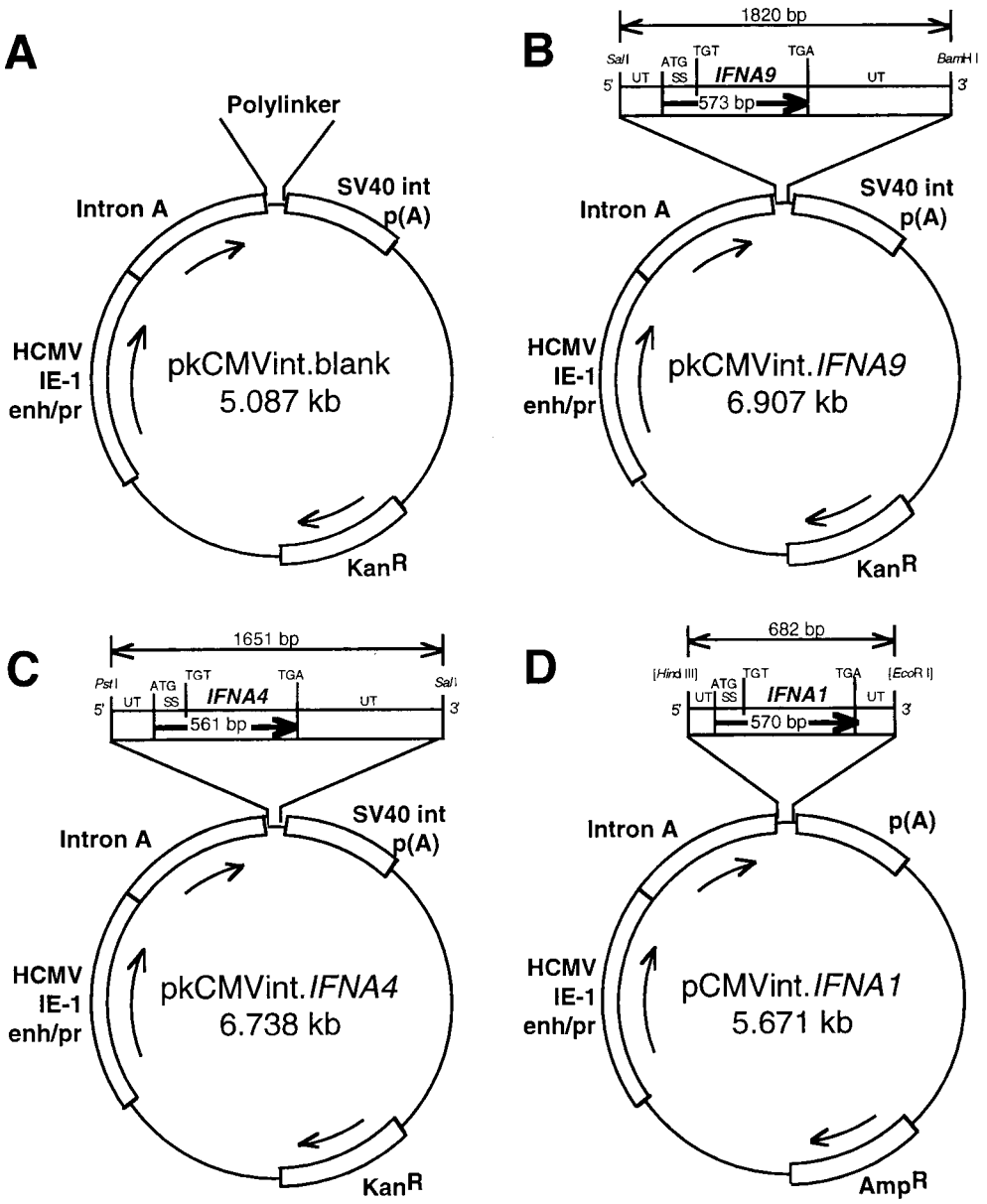

FIGURE 1. Plasmid maps of the CMV promoter-based expression vector constructs. *A*, pkCMVint.blank is the mammalian expression vector without any IFN transgene. *B*, pkCMVint.*IFNA9* encoding the full-length mouse *IFNA9* gene (573 bp), including the signal sequence (SS) located 69 nucleotides upstream of the first cysteine (TGT) of the mature protein. *C*, pkCMVint.*IFNA4* encoding the full-length mouse *IFNA4* gene (561 bp), including the signal sequence (SS) located 69 nucleotides upstream of the first cysteine (TGT) of the mature protein. *D*, pkCMVint.*IFNA1* encoding the full-length mouse *IFNA1* gene (570 bp), including the signal sequence (SS) located 69 nucleotides upstream of the first cysteine (TGT) of the mature protein. UT, untranslated regions. See *Materials and Methods* for details of cloning.

plaque assay (three mice per time point; Fig. 2). Within 24 h of MCMV inoculation, the virus titers in the muscles had decreased significantly ($p < 0.05$) before increasing steadily with time. Virus replication peaked on day 3 and was almost cleared by day 6 p.i. The kinetics of MCMV replication in the muscles of a more susceptible mouse strain (A/J) were also investigated following inoculation of the same dose of MCMV as that used to infect B6 mice. There was also a significant decrease ($p < 0.005$) in the MCMV titer in A/J muscles within 24 h of infection followed by an increase in the MCMV titer (Fig. 2), such that by day 3 the titer was approximately fourfold higher than that obtained from B6 muscles. Thus, MCMV replicated to markedly higher titers in the susceptible A/J mice compared with those in the resistant B6 mice, and no evidence of clearance was seen by day 4 p.i. Immunoperoxidase staining of TA muscle sections from the above MCMV-infected mice revealed further evidence of virus-infected cells (data not shown), providing confirmation of virus replication in the skeletal muscle tissue.

MuIFNA9 *expression reduces MCMV replication in vivo*

Previously, we have described the construction, delivery, and expression of IFN-α9 in our mouse model (27). Preliminary experiments were conducted to assess the antiviral activity of *MuIFNA9* in B6 mice following MCMV infection in vivo. B6 mice (five mice per group) were injected with 200 μg of plasmid constructs carrying the full-length gene of *MuIFNA9* driven by the human cytomegalovirus (HCMV) IE enhancer/promoter (pkCMVint.*IFNA9*; Fig. 1*B*). Control groups of five mice received either no DNA or the blank expression

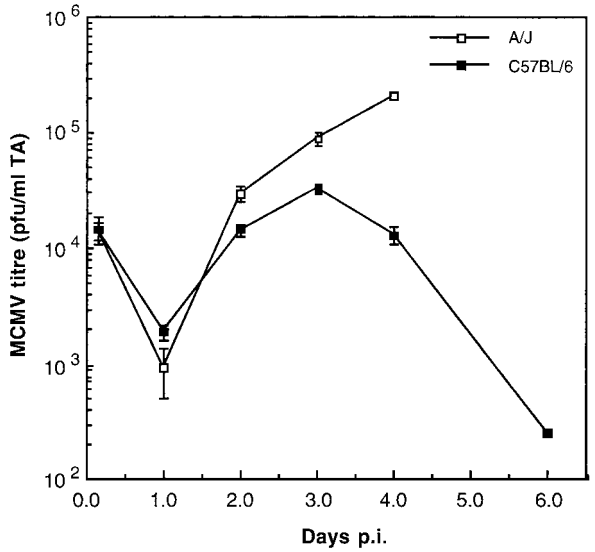

FIGURE 2. Kinetics of MCMV replication in the TA muscles of C57BL/6 and A/J mice. MCMV (4×10^4 pfu/mouse) was injected bilaterally into the TA muscles of anesthetized mice. At specific times p.i., the muscles were collected and homogenized, and the amount of MCMV present in the individual mouse muscle homogenates (three mice per group) was titrated by plaque assay. The mean virus titer (plaque-forming units per milliliter of TA muscle ± SEM) is shown.

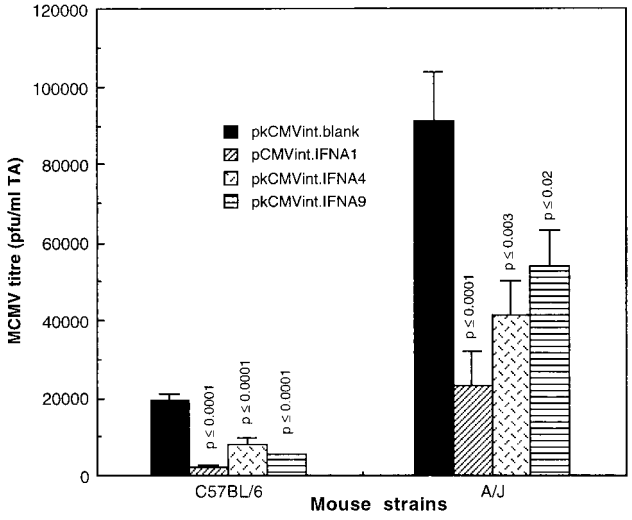

FIGURE 3. The antiviral activity of the *MuIFNA1* subtype is greater than those of the *MuIFNA4* and *MuIFNA9* subtypes at reducing MCMV replication in the TA muscles of C57BL/6 and A/J mice. Plasmid DNA constructs (200 μg in MOBS) were injected bilaterally into regenerating TA muscles of adult mice 5 days after bupivacaine treatment. Seven days following DNA injections, the mice were challenged with MCMV. The challenge doses (4×10^4 pfu/mouse in C57BL/6 mice and 2×10^4 pfu/mouse in A/J mice) were injected bilaterally into the TA muscles (five five mice per group). MCMV titers were determined on day 3 p.i. by plaque assay of the individual mouse muscle homogenates and are expressed as mean plaque-forming units per milliliter of TA muscle ± SEM. The mean reduction in virus titer in mice injected with plasmid constructs encoding IFN transgenes was compared with that in mice injected with the blank vector. The *p* values describe the significant reduction of MCMV titers by the IFN transgene.

vector, pkCMVint.blank (Fig. 1*A*). Regenerating TA muscles were induced by crush injury 5 days before DNA injection. Seven days post-gene transfer, MCMV (4×10^4 pfu/mouse) was injected bilaterally into the TA muscles. These muscles were sampled on both day 3 and day 4 p.i., around the time of peak virus replication in B6 mice (see Fig. 2), and the levels of MCMV present in the muscle homogenates were determined by plaque assay.

In control mice, in which no DNA was injected into the crush-injured muscles, MCMV titers obtained on both day 3 and day 4 p.i. were similar to the MCMV titers determined earlier for the kinetics experiment (Fig. 2). Since there was a small degree of protection observed in mice immunized with the blank vector compared with control mice that were not injected with plasmid DNA, all comparisons of plasmid constructs were performed with mice receiving the blank expression vector as the baseline. On day 3 p.i., the time when peak MCMV titers were found, the mean level of MCMV titer determined in muscles expressing the *MUIFNA9* transgene (3.58×10^3 pfu/ml of TA ± 8.78×10^2) was significantly lower ($p \leq 0.04$) than that determined in muscles injected with the blank vector (6.43×10^3 pfu/ml of TA ± 1.00×10^3). Furthermore, on day 4 p.i., virus titers were significantly lower in mice inoculated with pkCMVint.*IFNA9* (2.14×10^3 pfu/ml of TA ± 6.24×10^2; $p \leq 0.02$) than in mice inoculated with blank vector (7.95×10^3 pfu/ml of TA ± 1.95×10^3).

Antiviral activities of MuIFNA1, MuIFNA4, *and* MuIFNA9 *in vivo*

We next wished to compare individual murine IFN-α subtypes for their relative antiviral activities in vivo using our mouse model of MCMV i.m. infection. Mammalian expression plasmids encoding the genes for murine IFN-α4 and IFN-α1 were constructed (see *Materials and Methods*; Fig. 1, *C* and *D*). Three mouse strains (B6, A/J, and BALB/c, eight mice per group) were injected with 200 μg of plasmid constructs carrying the full-length gene of *MuIFNA1*, *MuIFNA4*, or *MuIFNA9* driven by the HCMV IE enhancer/promoter (pCMVint.*IFNA1*, pkCMVint.*IFNA4*, and pkCMVint.*IFNA9*, respectively). Control groups of mice received the blank expression vector, pkCMVint.blank (Fig. 1*A*). The regenerating TA muscles were induced by bupivacaine inoculation 5 days before DNA injection. This alternative method to crush injury for the induction of muscle regeneration was used in all additional experiments. Seven days after DNA injection, half of the TA pools from three mice (of the eight) were collected and used for the determination of plasmid in the transfected muscle by dot-blot analysis and for the estimation of IFN protein titers by bioassay. All the plasmids (pkCMVint.blank, pCMVint.*IFNA1*, pkCMVint.*IFNA4*, and pkCMVint.*IFNA9*) from muscle extracts of DNA-injected A/J mice showed similar hybridization signal intensities (the sensitivity limit was 50 pg detected in the 1/10 dilution of DNA samples; data not shown), suggesting that the plasmids were maintained at comparable levels in the muscles. DNA extracts were hybridized to the human CMV IE promoter/enhancer-labeled probe a fragment (657 bp) of the pkCMVint. vector. A further DNA sample of murine salivary gland-derived MCMV was included in the dot blot and resulted in no specific hybridization with the human CMV IE probe. Production of IFN proteins in the TA muscle from B6 mice injected with the different IFN constructs was evidenced by bioassay of muscle homogenates; results ranged from 24 to 150 IU/TA (24, *IFNA9*; 75, *IFNA4*; 150, *IFNA1*). Whereas B6 mice injected with the blank vector did not have detectable levels of IFN titer (≤1.7 IU/TA).

The remaining five mice (of the eight) were challenged with virus 7 days after DNA injection. MCMV was injected bilaterally into the TA muscles of the mice. B6 mice received 4×10^4 pfu/mouse, while the more susceptible strains (A/J and BALB/c) received a lower virus dose (2×10^4 pfu/mouse). The levels of

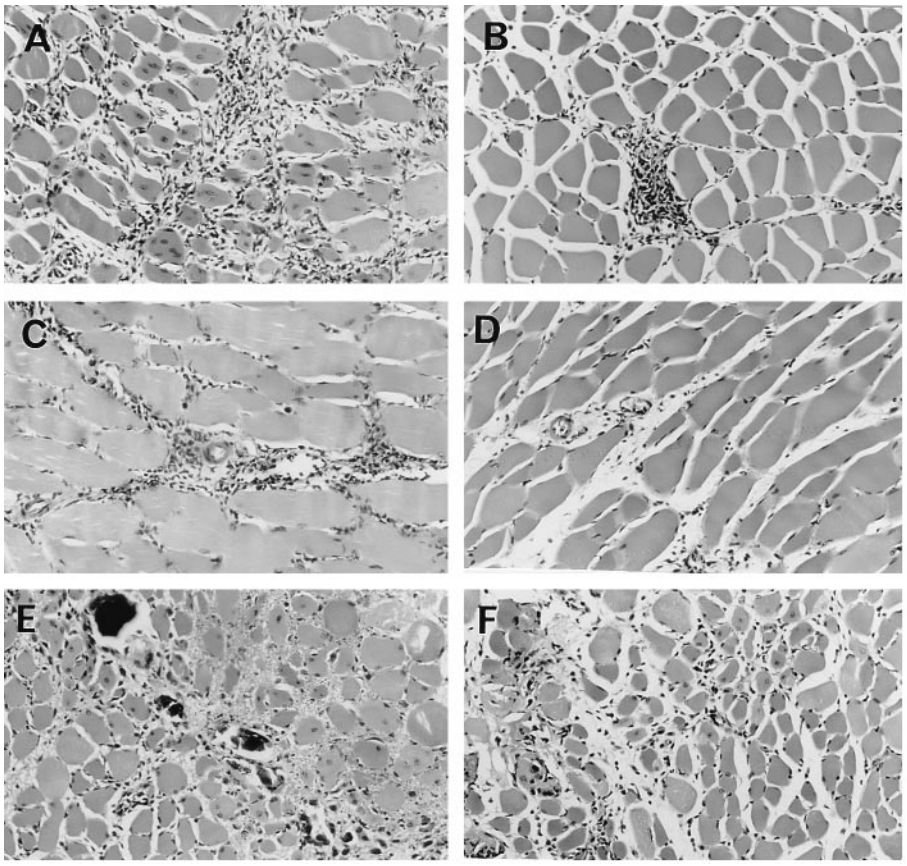

FIGURE 4. Histopathology of IFN transgene-expressing TA muscle of mice challenged with MCMV. *A*, Severe and diffuse cellular infiltration associated with necrotic myofibers is present in muscle from MCMV-infected B6 mice injected with pkCMVint.blank plasmid. *B*, A mild focal infiltrate is present in muscle from MCMV-infected B6 mice injected with pCMVint.*IFNA1*. *C*, Perivascular infiltration is present in muscle from MCMV-infected B6 mice injected with pkCMVint.*IFNA4*. *D*, A mild perivascular infiltration is present in muscle from MCMV-infected B6 mice injected with pkCMVint.*IFNA9*. *E*, Muscle from MCMV-infected BALB/c mice injected with pkCMVint.blank plasmid, showing marked necrosis and evidence of calcified lesions associated with cellular infiltration. *F*, Muscle from MCMV-infected BALB/c mice injected with pCMVint.*IFNA1* showing diffuse cellular infiltration. All muscle sections shown are from mice treated with bupivacaine to induce muscle regeneration, inoculated with DNA 5 days post-bupivacaine treatment, challenged i.m. (TA) with MCMV on day 7 after DNA delivery, and infected with MCMV for 3 days Magnification, ×160; hematoxylin-eosin stain.

MCMV present on day 3 p.i. in the muscle homogenates of all the mouse strains investigated were determined by plaque assay.

The bupivacaine-injured muscles of B6 mice that were transfected with an IFN transgene (*A1*, *A4*, or *A9*) had significantly reduced ($p \leq 0.0001$) MCMV titers compared with the muscles of control B6 mice given the blank vector (Fig. 3). Histologically, this is supported by reduced inflammation of the muscle from *MuIFNA1* (Fig. 4*B*), *MuIFNA4* (Fig. 4*C*), and *MuIFNA9* (Fig. 4*D*) transfected B6 mice compared with muscle from mice given the blank vector (Fig. 4*A*). Furthermore, B6 mice transfected with the *MuIFNA1* transgene showed a greater reduction (8.0-fold) in virus replication than the mice transfected with *MuIFNA4* or *MuIFNA9* (2.4- and 3.5-fold, respectively).

Similarly, when a different mouse genotype was used (A/J mice), muscles that were transfected with IFN transgenes showed lower MCMV titers than muscles transfected with the blank vector, confirming the observation with B6 mice. A/J mice transfected with the *MuIFNA1* transgene also showed markedly reduced MCMV titers (4.0-fold) than those in mice transfected with either *MuIFNA4* or *MuIFNA9* (2.2- or 1.7-fold, respectively). The efficacy of *MuIFNA1* transgene was further seen in BALB/c mice. Significantly lower MCMV titers ($p \leq 0.006$) were determined in mice given the *MuIFNA1* transgene than in mice given blank vector (data not shown). The hematoxylin- and eosin-stained transverse muscle sections from BALB/c mice showed a more severe inflammatory response in mice given the blank vector (Fig. 4*E*) than in mice given pCMVint.*IFNA1* (Fig. 4*F*). Moreover, the MCMV titers from the liver and spleen of BALB/c mice given pkCMVint.*IFNA1* were markedly lower (13- and 20-fold, respectively) than those in mice transfected with the blank vector (data not shown).

We were also interested to test whether our model of IFN transgene delivery was effective when virus infection was further delayed to 2 wk post-DNA inoculation. MCMV was injected (2×10^4 pfu/mouse) bilaterally into the TA muscles of A/J mice 14 days post-DNA injection (five mice per group). The levels of MCMV present in the muscle homogenates, as determined by plaque assay, are shown in Figure 5. Again, each of the *MuIFN* subtypes significantly reduced MCMV replication, and *MuIFNA1*-transfected mice showed the lowest MCMV titer compared with that in mice transfected with the other DNA constructs.

Antiviral activities of MuIFNA1 *in IFNAR1 knockout mice in vivo*

We next compared the effect of *MuIFNA1* expression on MCMV replication in mice depleted of functional IFN-α/β receptors (IFNAR1 knockouts) and in the wild-type parental strain (129/Sv/Ev). MCMV was injected (2×10^4 pfu/mouse) bilaterally into the TA muscles of the IFNAR1 knockout and wild-type mice 7 days post-*MuIFNA1* injection (six mice per group). The mean titers of MCMV present in the muscle homogenates are shown in Table I. IFNAR1 knockout mice had 19.4-fold higher levels of MCMV replication in the muscles than wild-type mice following *MuIFNA1* inoculation. Moreover, the MCMV titers from liver and spleen of wild-type mice were markedly lower (8.2- and 265.3-fold, respectively) than those in the IFNAR1 knockout mice.

Discussion

Our experimental mouse model of IFN transgene expression in vivo has allowed us to compare the antiviral activities of individual IFN-α subtypes (*A1*, *A4*, and *A9*). Each of the three IFN-α subtypes investigated could independently reduce MCMV replication in the TA muscle compartment, where both the transgene and virus were administered. The primary observation from this study suggests, for the first time, that there are differences in the biologic

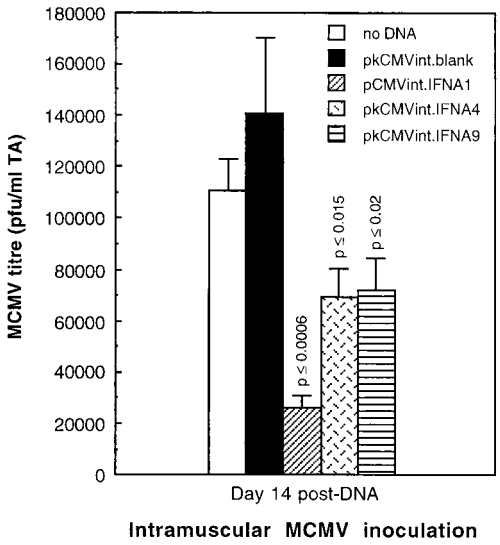

FIGURE 5. The protection by the *MuIFNA1* subtype is still greater than that by the *MuIFNA4* and *MuIFNA9* subtypes at reducing MCMV replication in the TA muscles of A/J mice when challenged with the virus 14 days after DNA injections. Plasmid DNA constructs (200 μg in MOBS) were injected bilaterally into regenerating TA muscles of adult mice 5 days after bupivacaine treatment. Fourteen days following DNA injections, the mice were challenged with MCMV (2×10^4 pfu/mouse) bilaterally into the TA muscles (five five mice per group). MCMV titers were determined on day 3 p.i. by plaque assay of the individual mouse muscle homogenates and are expressed as mean plaque-forming units per ml TA muscle $\pm$ SEM. The mean reduction in virus titer in mice injected with plasmid constructs encoding IFN transgenes was compared with that in mice injected with the blank vector. The *p* values show the significant reduction of MCMV titers by the IFN transgenes.

efficacies of the IFN-α subtypes in vivo. Indeed, the MCMV titers in TA muscles of B6, A/J, and BALB/c mice transfected with the *MuIFNA1* transgene were consistently lower than the MCMV titers in TA muscles transfected with the *MuIFNA4* or *MuIFNA9* transgene. Furthermore, *MuIFNA1*-transfected mice had lower MCMV titers in liver and spleen, indicating partial protection from virus dissemination to these organs following i.m. virus challenge. Thus, mice allowed to express the *MuIFNA1* transgene for 1 wk before virus infection appeared better protected against MCMV challenge than mice given either *MuIFNA4* or *MuIFNA9* transgene.

In some cases partial protection was afforded by the blank vector via a non-IFN-specific mechanism. This has also been noted in other models of DNA vaccines including studies of influenza virus (C. M. Lawson and S. L. Epstein, National Institute of Allergy and Infectious Diseases and Food and Drug Administration, National Institutes of Health, unpublished observations). DNA sequences in the backbone of the plasmid containing cytosine guanine (CG) dinucleotides in a particular base context may have immunostimulatory effects on the immune system (34). Since we used the data obtained from the control group of mice injected with the blank vector as the baseline for comparison with other groups of mice injected with the vector constructs containing the IFN insert genes, we may be underestimating the degree of protection.

In considering the potential in vivo interactions of several genes and their effects on IFN transcription, we observed no hybridization of MCMV genes with the human CMV IE promoter/enhancer. Therefore, we believe that *trans*-activation of IFN expression through the virus would be unlikely and would be expected to occur equally with each of the individual plasmid constructs, since they have identical promoters. It is known that a rIFN (human/mouse hybrid) can turn off transcription of mouse CMV IE genes due to the negative regulation of IE enhancer sequences (35). However, since IFN does not appear to alter the mRNA levels of human CMV IE genes (36), putative IFN-α-responsive elements might be missing in human CMV IE genes. Our studies suggest that there is unlikely to be a strong negative regulation of murine IFN on the HCMV IE genes resulting in the termination of IFN transgene expression, since newly synthesized IFN from the plasmid does not turn off detectable plasmid IFN production in the TA, as assessed kinetically over a 2-mo period (27). Furthermore, the regulatory effect of murine IFN subtypes (endogenous and plasmid coded) on the human CMV IE promoter (if any) would be expected to occur equally with each of the individual plasmid constructs.

Many in vitro studies have shown differences in the biologic activities with either human IFN-α subtypes (37–41) or mouse IFN-α subtypes (15, 16). However, it has not been possible until now to conduct comparative studies of the different IFN-α subtypes in vivo. Early attempts to develop an in vivo model for such studies of the biologic activity of type 1 IFN subtypes have involved making transgenic mice. The failure of these mice to reproduce and/or to survive embryologic life makes this an ineffective approach (18–20) (W.-S. Yeow and M. W. Beilharz, unpublished observations; B. Williams, unpublished observation). With the advent of naked DNA technology, we were able to successfully develop an in vivo model in which the expression of a specific IFN subtype predominates (27).

The finding of this study, that the MuIFN-α1 subtype appeared to be more protective than the MuIFN-α4 or the MuIFN-α9 subtype in vivo, was unexpected. Bioassay of the muscle homogenates taken on day 7 after plasmid inoculation for type I IFN titers varied from 24 to 150 IU in C57BL/6 mice. However, it must be emphasized that this read-out of titers reflects a wide array of in vivo interactions of the IFN subtype with its environment. The sp. act. of these subtypes in vivo are not known. However, the specific antiviral activities have been determined from in vitro experiments using MuIFN-α1, -α4, and -α9 (15, 16) (S. J. Boyer and M. W.

Table I. *Antiviral activities of the IFN-α transgene expressed intramuscularly are receptor-mediated*

Organ[a]	MCMV Titer[b]		Reduction[c]
	IFNAR1 knockout	Wild-type (129/Sv/Ev)	
TA muscle	$1.04 \times 10^5 \pm 1.07 \times 10^4$	$5.38 \times 10^3 \pm 8.35 \times 10^2$	19.4 ($p \leq 0.0001$)
Liver	$2.04 \times 10^3 \pm 6.69 \times 10^2$	$2.50 \times 10^2 \pm 0.00$	8.2 ($p \leq 0.04$)
Spleen	$7.74 \times 10^4 \pm 1.01 \times 10^4$	$2.92 \times 10^2 \pm 4.17 \times 10^1$	265.3 ($p \leq 0.0007$)

[a] pCMVint.*IFNA1* (200 μg in MOBS) was injected bilaterally into regenerating TA muscles of adult mice 5 days after bupivacaine treatment.
[b] Seven days following DNA injections, mice were challenged with 2×10^4 pfu of MCMV injected bilaterally into TA muscles (six mice per group). MCMV titers were determined at day 3 p.i. by plaque assay of the individual mouse muscle homogenates. Data are expressed as mean pfu/ml organ $\pm$ SEM.
[c] Mean reduction (*n*-fold) in virus titer obtained from wild-type mice compared to that obtained from IFNAR1 (IFN-α/β receptor) knock-out mice. The *p* values describe the significant reduction of MCMV titers by the IFN transgene.

Beilharz, unpublished observations). The in vitro findings indicated that the antiviral activity of MuIFN-α4 was 2-fold higher than that of MuIFN-α1 or MuIFN-α9, that the antiproliferative activity of MuIFN-α4 was approximately 10-fold higher than that of MuIFN-α1 or MuIFN-α9, and that the NK cell activity of MuIFN-α9 was higher than MuIFN-α4 and MuIFN-α1. Thus, one would not have predicted that the MuIFN-α1 subtype would be more protective in vivo against a viral infection than the other two subtypes, especially the MuIFN-α4 subtype. We emphasize that the in vitro data were obtained with the use of a different challenge virus (encephalomyocarditis virus) and mouse L cells, and is thus limited and cannot be used for comparisons in the present in vivo study. The importance of determining functional differences in vivo is highlighted by the above contrasting data. Indeed, further detailed molecular analysis beyond our general assumptions, including the idea that the rate of production of each IFN subtype in situ at a molar level is equivalent, need to be undertaken, which would allow direct comparisons and strengthen our claim that there are differential antiviral differences in vivo among the IFN subtypes.

Our present in vivo study probably reflects the pleiotropic nature of IFNs in their capacities at inducing multiple biologic effects and affecting the host immune response (9, 10). Firstly, the principal antiviral effect of IFN against MCMV is at the level of inhibiting the transcription of the MCMV IE gene (42). Secondly, IFN was known to be a negative growth factor for skeletal muscle by inhibiting myogenesis in vivo (43–45). Thirdly, type 1 IFNs provide an early and rapid nonspecific immune response (for example, by augmenting the proliferation and activation of NK cells) to limit the extent of viral spread before Ag-specific responses can more fully control the infection. It is not possible from the present data to establish whether the protective effect of IFN was mediated by each of the mechanisms listed above or by a combination of the mechanisms. However, the MuIFN-α1 subtype was more efficient at triggering such a protective response to MCMV challenge than were the other two subtypes in vivo. The relationship between the different subtypes and the immune responses that they trigger is presently under further study in our laboratory. For example, the importance of augmenting the NK cell responses is being investigated in NK cell-deficient (beige) mice and in congenic strains of mice depleted of NK cells.

While the mechanism(s) by which one IFN-α subtype can trigger a better protective response than another subtype against the same viral challenge is not defined at present, a potential mechanism may involve priming (46, 47). This phenomenon, defined as an enhanced response to an IFN-inducing agent following pretreatment with IFN, has been described both in vitro (46–50) and in vivo (51). This effect was shown to be IFN dose dependent (48, 51) and was determined to be a nonantiviral function of IFN (49). Priming of human leukocytes with a mixture of human IFN-α subtypes was shown to selectively increase the levels of some IFN-α subtypes but not others (50). Although the priming ability of human IFN-α1 was shown to be equivalent to that of IFN-α2 using human peripheral blood leukocytes (52), the priming abilities of the other individual IFN-α subtypes are not known. In the present study the MuIFN-α1 transgene could be a superior primer over the other IFN-α transgenes at enhancing the expression of a panel of IFN subtypes that act synergistically to induce a protective response to the subsequent MCMV infection. We are currently in the process of determining the panel of IFN subtypes that were expressed in the TA muscles in response to MCMV challenge to determine whether its composition and/or magnitude has been changed.

In our in vivo study, mice depleted of functional IFN-α/β receptors (knockout mice) were shown to be poor responders to the protective effects of *MuIFNA1* compared with mice with such receptors. We expected the parental wild-type strain (129/Sv/Ev), which has a similar susceptibility level to MCMV infection as A/J mice, to display virus titers in the same order of magnitude as A/J mice ($9 \times 10^4 \pm 1 \times 10^4$ pfu/ml of TA). Thus, the titers obtained from 129/Sv/Ev mice injected with the *IFNA1* plasmid demonstrate a strong protective effect. On the other hand, the level of MCMV replication in the knockout mice was significantly higher than that in all other mouse strains tested with IFN-α/β receptors. These data clearly establish that the protective effect of IFN is receptor mediated.

Overall, our study demonstrates for the first time that there are in vivo differences in the relative biologic efficacies of the different MuIFN-α subtypes during a virus infection. Although many more questions have now been raised by these data, the mouse model we described should be capable of allowing experimental resolution of these issues. We are currently investigating whether the same MuIFN subtype (α1) can induce a better protective immune response in animals challenged with different viruses. Collectively, these studies will ultimately provide an improved rationale for effective clinical use of the IFNs.

Acknowledgments

We are grateful to VICAL for providing the pkCMVint expression vector, and to Dr. Alistair Ramsay for providing the pCMVint.*IFNA1* construct and the IFNAR1 knockout mice. We also thank the staff at Pathcentre for histology services.

References

1. Johnson, H. M., F. W. Bazer, B. E. Szente, and M. A. Jarpe. 1994. How interferons fight disease. *Sci. Am. 270:40.*
2. Samuel, C. E. 1991. Antiviral actions of interferon: interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology 183:1.*
3. Kelley, K. A., and P. M. Pitha. 1985. Characterization of a mouse interferon gene locus. I. Isolation of a cluster of four α interferon genes. *Nucleic Acids Res. 13:805.*
4. Dandoy, F., E. De Maeyer, F. Bonhomme, J. L. Guenet, and J. De Maeyer-Guignard. 1985. Segregation of restriction fragment length polymorphism in an interspecies cross of laboratory and wild mice indicates tight linkage of the murine interferon-β gene to the murine interferon-α gene. *J. Virol. 56:216.*
5. Kelley, K. A., C. A. Kozak, F. Dandoy, F. Sor, D. Skup, J. D. Windass, J. DeMaeyer-Guignard, P. M. Pitha, and E. DeMaeyer. 1983. Mapping of murine interferon-α genes to chromosome 4. *Gene 26:181.*
6. Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proc. R. Soc. London B 147:258.*
7. Gresser, I. 1990. Biological effect of interferons. *Soc. Invest. Dermatol. 95:S66.*
8. Fleischmann, C. M., and W. R. Fleischmann, Jr. 1988. Differential antiproliferative activities of IFNs α, β and γ: kinetics of establishment of their antiproliferative effects and the rapid development of resistance to IFNs α and β. *J. Biol. Regul. Homeostatic Agents 2:173.*
9. Moore, M. 1983. Interferon and the immune system. II. Effect of interferon on the immune system. In *Interferon: From Molecular Biology to Clinical Application. Thirty-fifth Symposium of the Society for General Microbiology*. D. C. Burke and A. G. Morris, eds. Cambridge University Press, Cambridge, p. 181.
10. Belardelli, F. 1995. Role of interferons and other cytokines in the regulation of the immune response. *Acta Pathol Microbiol Immunol Scand 103:161.*
11. Belardelli, F., and I. Gresser. 1996. The neglected role of type I interferon in the T-cell response: implications for its clinical use. *Immunol. Today 17:369.*
12. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science 264:1415.*
13. Goren, T., D. G. Fischer, and M. Rubinstein. 1986. a. Human monocytes and lymphocytes produce different mixtures of α-interferon subtypes. *J. Interferon Res. 6:323.*
14. Borecky, L. 1989. Interferon after 30 years. *Acta Virol. 33:378.*
15. Beilharz, M. W., N. Swaminathan, C. M. Lai, P. M. Pitha, and S. J. Boyer. 1991. Relative antiviral activity of in vitro-synthesized murine interferon-α4 and -α1. *J. Interferon Res. 11:9.*
16. Swaminathan, N., C. M. Lai, S. J. Boyer, M. W. Beilharz, and S. P. Klinken. 1992. Biological activities of recombinant murine interferons α1 and α4: large difference in antiproliferative effect. *Antiviral Res. 19:149.*

17. Finter, N. B. 1991. Why are there so many subtypes of α-interferons? *J. Interferon Res. (Special Issue) 11:185.*
18. Iwakura, Y., M. Asano, Y. Nishimune, and Y. Kawade. 1988. Male sterility of transgenic mice carrying exogenous mouse interferon-β gene under the control of the metallothionein enhancer-promoter. *EMBO J. 7:3757.*
19. Hekman, A. C., J. Trapman, A. H. Mudler, J. L. M. van Gaalen, and E. C. Zwarthoff. 1988. Interferon expression in the testes of transgenic mice leads to sterility. *J. Biol. Chem. 263:12151.*
20. Iwakura, Y., M. Hayashi, and M. Asano. 1993. Transgenic mice carrying interferon genes. *Mol. Reprod. Dev. 36:245.*
21. Cases, O., I. Seif, C. Babinet, and E. De-Maeyer. 1994. Transgenic mice carrying the interferon-beta gene placed under the control of a constitutive promoter. *J. Interferon Res. 14:S206.*
22. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Ascadi, A. Jani, and P. L. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. *Science 247:1465.*
23. Blechynden, L. M., M. A. Lawson, H. Tabaria, M. J. Garlepp, J. Sherman, N. Raben, and C. M. Lawson. 1997. Myositis induced by naked DNA immunization with the gene for histidyl-tRNA synthetase. *Hum. Gene Ther. 8:1469.*
24. Ascadi, G., G. Dickson, D. R. Love, A. Jani, F. S. Walsh, A. Gurusinghe, J. A. Wolff, and K. E. Davies. 1991. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. *Nature 352:815.*
25. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, Y. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science 259:1745.*
26. Davies, H. L., M. L. Michel, and R. G. Whalen. 1993. DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody. *Hum. Mol. Genet. 2:1847.*
27. Lawson, C. M., W.-S. Yeow, C. M. Lee, and M. W. Beilharz. 1997. In vivo expression of an interferon alpha gene by intramuscular injection of naked DNA. *J. Interferon Cytokine Res. 17:255.*
28. Grundy (Chalmer), J. E., J. Trapman, J. E. Allan, G. R. Shellam, and C. J. M. Melief. 1982. Evidence for a protective role of interferon in resistance to murine cytomegalovirus and its control by non-*H-2*-linked genes. *Infect. Immun. 37:143.*
29. Grundy (Chalmer), J. E., J. S. Mackenzie, and N. F. Stanley. 1981. Influence of *H-2* and non-*H-2* genes on resistance to murine cytomegalovirus infection. *Infect. Immun. 32:277.*
30. Allan, J. E., and G. R. Shellam. 1984. Genetic control of murine cytomegalovirus infection: Virus titres in resistant and susceptible strains of mice. *Arch. Virol. 81:139.*
31. Lu, S., J. Arthos, D. Montefiore, Y. Yasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. Letvin, M. Wyand, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. *J. Virol. 70:3978.*
32. Bartholomaeus, W. N., H. O'Donoghue, D. Foti, C. M. Lawson, G. R. Shellam, and W. D. Reed. 1988. Multiple autoantibodies following cytomegalovirus infection: virus distribution and specificity of autoantibodies. *Immunology 64:397.*
33. Vitadello, M., M. V. Schiaffino, A. Picard, M. Scarpa, and S. Schiaffino. 1994. Gene transfer in regenerating muscle. *Hum. Gene Ther. 5:11.*
34. Sato, Y., M. Roman, H. Tighe, D. Lee, M. Corr, M.-D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science 273:352.*
35. Gribaudo, G., S. Ravaglia, A. Caliendo, R. Cavallo, M. Garigalo, M. G. Martinotti, and S. Landolfo. 1993. Interferons inhibit onset of murine immediate-early gene transcription. *Virology 197:303.*
36. Stinski, M. F., D. R. Thomsen, and J. E. Rodriguez. 1982. Synthesis of human cytomegalovirus-specified RNA and protein in interferon-treated cells at early times after infection. *J. Gen. Virol. 60:261.*
37. Weck, P. K., S. Apperson, L. May, and N. Stebbing. 1981. Comparison of the antiviral activities of various cloned human interferon-α subtypes in mammalian cell cultures. *J. Gen. Virol. 57:233.*
38. Bell, D. M., N. J. Roberts, Jr., and C. B. Hall. 1983. Different antiviral spectra of human macrophage interferon activities. *Nature 305:319.*
39. Fish, E. N., K. Banerjee, and N. Stebbing. 1983. Human leukocyte interferon subtypes have different antiproliferative and antiviral activities on human cells. *Biochem. Biophys. Res. Commun. 112:537.*
40. Langer, J. A., J. R. Ortaldo, and S. Pestka. 1986. Binding of human alpha-interferons to natural killer cells. *J. Interferon Res. 6:97.*
41. Weber, H., D. Valenzuela, G. Lujber, M. Gubler, and C. Weissmann. 1987. Single amino acid change that render human IFN-α2 biologically active on mouse cells. *EMBO J. 6:591.*
42. Landolfo, S., G. Gribaudo, A. Angeretti, and M. Gariglio. 1995. Mechanisms of viral inhibition by interferons. *Pharmacol. Ther. 65:415.*
43. Lough, J., S. Keay, J. L. Sabran, and S. E. Grossberg. 1982. Inhibition of chicken myogenesis in vitro by partially purified interferon. *Biochem. Biophys. Res. Commun. 109:92.*
44. Multhauf, C., and J. Lough. 1986. Interferon-mediated inhibition of differentiation in a murine myoblast cell line. *J. Cell. Physiol. 126:211.*
45. Garrett, K. L., M. D. Grounds, M. A. L. Maley, and M. W. Beilharz. 1992. Interferon inhibits myogenesis in vitro and in vivo. *Basic Appl Myol 2:291.*
46. Isaacs, A., and D. C. Burke. 1958. Mode of action of interferon. *Nature 182: 1073.*
47. Lockart, R. Z., Jr. 1963. Production of an interferon by L cells infected with Western equine encephalomyelitis virus. *J. Bacteriol. 85:556.*
48. Friedman, R. M. 1966. Effect of interferon treatment on interferon production. *J. Immunol. 96:872.*
49. Stewart, W. E., II, L. B. Gosser, and R. Z. Lockart, Jr. 1971. Priming: a nonantiviral function of interferon. *J. Virol. 7:792.*
50. Goren, T., D. G. Fischer, and M. Rubinstein. 1986. b. Priming of leukocytes selectively increases the level of some interferon-α subtypes and not others. *Biochim. Biophys. Acta 887:80.*
51. Rosztoczy, I. 1986. Study of the in vivo priming effect of interferon in mice. *J. Gen. Virol. 67:2731.*
52. Fuchsberger, N., G. Karayianni-Vasconcelos, H.-L. Kauppinen, V. Hajnicka, and P. Kontsek. 1995. Priming with interferon-α1 or interferon-α2 enhances the production of both subtypes simultaneously. *J. Interferon Cytokine Res. 15:637.*