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Impaired Antiviral Response and Alpha/Beta Interferon Induction in Mice Lacking Beta Interferon

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We have generated mice lacking the gene for beta interferon and report that they are highly susceptible to vaccinia virus infection. Furthermore, in cultured embryo fibroblasts, viral induction of alpha interferon and of 2-5A synthetase genes is impaired. We also show that beta interferon does not prime its own expression.

Interferons (IFNs) consist of a family of evolutionarily conserved proteins encoded by closely related and linked genes. Alpha/beta IFNs (IFN-α/β), represented by several IFN-α subtypes, IFN-β, and IFN-ω, bind to a common cell surface receptor, resulting in the activation of the Jak-STAT signal transduction system (7, 8) and leading to the transcriptional activation of IFN-stimulated genes. The products of these genes must account not only for the versatile antiviral effects of IFNs but also for their immunomodulatory and antiproliferative effects. The roles of some of these proteins, including 2-5A synthetase, in establishing an antiviral state have been described (25, 34, 38).

IFN plays an important role in the protection against infection by a large number of viruses, including vaccinia virus and other poxviruses (39). This is emphasized by the expression of a number of different anti-interferon strategies by viruses, including soluble IFN receptors and intracellular proteins that block the activities of key interferon-inducible genes (40).

IFN-α/β are expressed by cells within hours of infection by virus and can act in an autocrine or paracrine manner to limit the development and spread of viral infection. This primary role for IFN-α/β in vivo is confirmed in studies with mice lacking one of the two chains of the functional IFN-α/β receptor: these animals display an extreme sensitivity to infection by viral pathogens. However, these studies did not differentiate between the roles of IFN-α and IFN-β in the antiviral response (44).

Despite these advances in our knowledge, our understanding of the IFN-α/β system is still far from complete. First, it is not known why there are so many IFN-α/β genes. In mice, there are at least 12 IFN-α genes and a single IFN-β gene clustered on chromosome 4 (18), and the protein product of any one of these appears to be sufficient to generate an antiviral state in responsive cells (19). Second, it is not known how the role of IFN-β differs from that of the various IFN-α subtypes. Whereas the murine IFN-α genes have ~90% homology, murine IFN-β appears to be rather more divergent, with only ~55% homology to a murine IFN-α consensus sequence. Third, the exact nature of the inducers for IFN-α/β is uncertain. It is known that double-stranded RNA, produced during a variety of viral infections, can induce transcription of IFN-α/β genes, but nonviral inducers have also been described (4, 36, 41). Induction of the *IFN*β gene has been extensively studied in cell culture, leading to a detailed knowledge of *cis*-acting sequences and binding factors required for transcriptional induction (16); a similar but less complete analysis of IFN-α induction has been carried out (27). Nonetheless, the precise nature of the inducer for particular viral infections is unknown and details of the pathway(s) leading to transcriptional activation are still sketchy. Finally, the importance of the kinetics of IFN-α/β induction and the identity of the cellular source of IFN-α/β induction during an in vivo infection are unclear. Thus, although it is known that IFN-α/β can be induced in a wide variety of cell types, it is unclear whether induction in the initially infected cell type is sufficient for a proper defense or whether paracrine IFN activity on other cell types is important.

We reasoned that a mouse in which the endogenous IFN-β genes have been deleted would be useful in determining whether IFN-α can compensate for the loss of IFN-β. Furthermore, by replacing the IFN-β gene with a reporter, the same mice should yield information concerning the source, control, and timing of IFN-β gene expression. In a similar approach, another group (13) provided evidence for a role for IFN-β in IFN-α induction but did not establish whether the effect was of physiological significance.

Generation and preliminary analysis of IFN-β$^{-/-}$ mice. Targeting constructs were designed to delete the IFN-β gene and replace it with a reporter gene for green fluorescent protein (GFP) (30) or hCD2 (26) (Fig. 1a and b). The virus inducibility of the reporter genes was confirmed in human 293 cells stably transfected with these constructs (Fig. 1c and d). HM-1 embryonic stem cells (22) transfected with the constructs were screened by Southern blotting (46) and targeted clones were detected at a frequency of about 1 per 40 G418-resistant clones (Fig. 1e and f). Several targeted clones were separately injected into C57BL/6 blastocysts, and resulting chimeric mice were tested for germ line transmission of the transgene in crosses with C57BL/6 mice. One chimera carrying the MuβGFP/neo transgene showed efficient germ line transmission, and F1 heterozygotes from this chimera were identified by PCR analysis of tail biopsies. These IFN-β$^{+/-}$ offspring were crossed to generate IFN-β$^{+/+}$, IFN-β$^{+/-}$, and IFN-β$^{-/-}$ F2 pups as well as mouse embryo fibroblasts (MEFs) (17).

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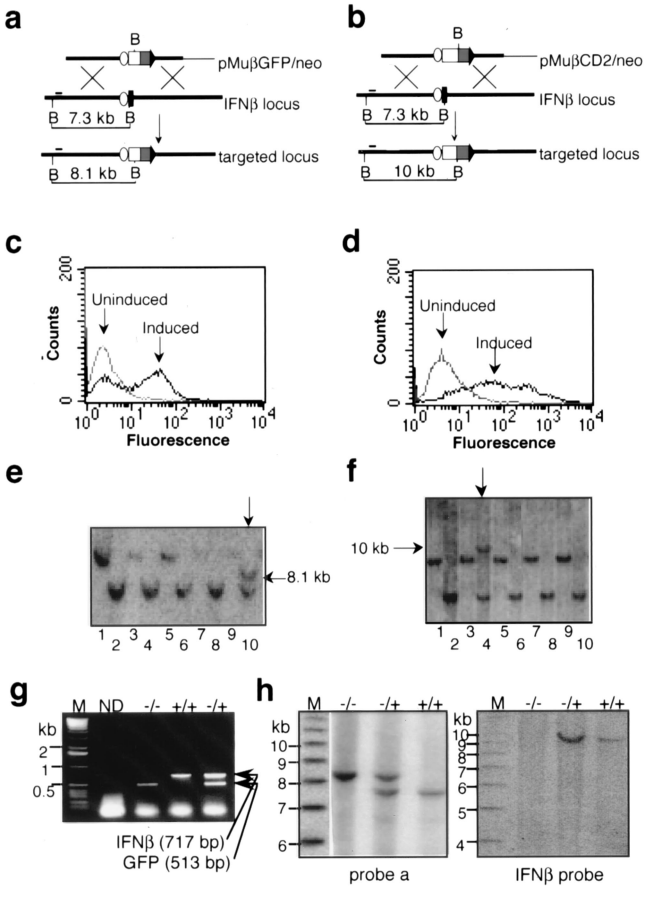

FIG. 1. Design and use of *IFN*β targeting constructs and genotyping of MEFs from an ES clone targeted with pMuβGFP/neo. Details of plasmid construction and conditions for cell growth and transfection are described elsewhere (9). (a) The targeting construct pMuβGFP/neo (*Not*I linearized; top), the *IFN*β locus (center), and the product of homologous recombination between them (bottom) are represented as follows: *IFN*β gene (black box), *IFN*β promoter region (ellipse), other DNA from the *IFN*β locus (thick black lines), GFP gene (white box; from pRSGFP [Clontech]), *neo* cassette (stippled box), *loxP* site (black triangle), and pBSKSII+ DNA (thin line). Key sites for *Bam*HI (B) and resulting fragments detectable by probe a (black bar) are shown. (b) As for panel a except for targeting construct pMuβCD2/neo; white box represents human CD2 gene. (c) Flow cytometric analysis of GFP expression in a G418-resistant 293 clone stably transfected with pMuβGFP/neo, either without induction or 48 h after induction by Sendai virus infection. (d) Flow cytometric analysis of CD2 expression in a pool of 293 G418-resistant 293 clones stably transfected with pMubCD2/neo, either without induction or 48 h after induction by Sendai virus infection. Conditions for electroporation, infection, and flow cytometry in panels c and d have been previously described (9) and are available on request. (e) Screening by Southern analysis for ES cell clones targeted with pMuβGFP/neo (18, 22). Analysis of *Bam*HI-digested DNA from 10 G418-resistant clones probed with probe a. The 7.3-kb band representing the unmodified *IFN*β locus migrates differently in odd- and even-numbered lanes because one comb was used for odd lanes and another for even lanes. A targeted clone and its diagnostic band are indicated by vertical and horizontal arrows, respectively. (f) As for panel e but with targeting construct pMuβCD2/neo. (g) Duplex PCR detection of *IFN*β and *GFP* genes in genomic DNA. ND, no template DNA. (h) Southern analysis of *Bam*HI-digested MEF DNA probed with probe a or IFN-β probe (a 460-bp *Bam*HI-*Kpn*I fragment of the *IFN*β gene). Size markers (M) are shown.

Genotypes of pups and MEFs were determined by PCR (Table 1) as illustrated for MEFs in Fig. 1g. In addition, Southern analyses of DNA from the MEFs showed the expected pattern of bands diagnostic for normal and targeted *IFN*β genes (Fig. 1h). The MEFs were also tested by reverse transcription-PCR (RT-PCR) for IFN-β and GFP mRNA induction following viral infection. As expected, IFN-β expression was not detected in IFN-$\beta^{-/-}$ MEFs, while a clear virus-inducible signal was detected in both IFN-$\beta^{+/-}$ and IFN-$\beta^{+/+}$ MEFs. Similarly, GFP expression was detectable following virus induction of both IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ MEFs but was not detected at all in IFN-$\beta^{+/+}$ MEFs (Fig. 2). The GFP reporter was therefore under the control of the virus inducible elements of the *IFN*β gene locus. A further observation from the induction of these MEF cells is that the inducibility of the GFP reporter is apparently the same in both IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ cells. This suggests that IFN-β does not have any marked effect on the inducibility of the *IFN*β promoter following virus induction. This contrasts with the transcription-enhancing activity reported by others (12, 20) for IFN-α/β on the *IFN*β regulatory elements.

Increased susceptibility of IFN-$\beta^{-/-}$ mice to viral infection. Because of the central role of IFN-α/β in host response to viral infection, we investigated the effect of deletion of the *IFN*β gene on the progression of vaccinia virus infection. Following intranasal inoculation at three doses of virus, the course of infection was monitored by measuring weight loss and other indicators of infection, as previously described (2, 42). The results (Fig. 3) show a dramatically increased susceptibility to infection in IFN-$\beta^{-/-}$ mice. At the lowest virus inoculum (10^3 PFU/animal), IFN-β-deficient mice showed signs of illness and weight loss and a single animal died. All other animals showed no signs of disease, cleared the infection, and recovered the initial weight loss. At both 10^4 and 10^5 PFU/animal, all the IFN-β-deficient mice succumbed to the infection after rapid weight loss and severe signs of illness. By contrast, the IFN-$\beta^{+/+}$ mice were more resistant to vaccinia virus, showing minor signs of illness and recovering from the infections, except for a single mortality at the intermediate virus dose. These data indicated that the IFN-$\beta^{-/-}$ animals are highly susceptible to vaccinia virus infection and succumb to doses that are sublethal to animals able to express IFN-β.

The replication of vaccinia virus in different organs of mice was also investigated (Fig. 4). The lower doses of viral inoculum (10^3 PFU/animal) resulted in significantly higher titers of virus in the IFN-$\beta^{-/-}$ mice than in IFN-$\beta^{+/+}$ animals, and this was particularly evident at the primary site of infection, the lungs. This difference in vaccinia virus replication was less evident at the higher inoculum tested (10^4 PFU/animal): at day 6 postinfection, vaccinia virus had replicated to similar levels in the absence or presence of IFN-β. It is interesting, however, that the IFN-$\beta^{+/+}$ mice recovered from the infection at 10^4 PFU/animal whereas the IFN-$\beta^{-/-}$ animals succumbed (Fig. 3). This suggests that IFN-β may play a role in the recovery from an established vaccinia virus infection.

Impaired IFN-α/β response in IFN-$\beta^{-/-}$ MEFs. As an indicator of the antiviral response, we used RT-PCR to measure induction of the gene encoding 2-5A synthetase in MEFs infected with virus. The results show a clear difference between IFN-$\beta^{-/-}$ MEFs, in which 2-5A gene expression was weak, and IFN-$\beta^{+/-}$ and IFN-$\beta^{+/+}$ MEFs, in which a robust induction was detected (Fig. 2). To explore the possible basis for the poor induction of 2-5A synthetase in IFN-$\beta^{-/-}$ MEFs, we measured IFN-α induction in the same RNA samples, again by RT-PCR. A recent study (23) has indicated that the IFN-α-4 subtype is induced earlier than other IFN-α subtypes in response to viral induction. An RT-PCR was therefore designed to detect all known IFN-α transcripts (Uα), another specific for the IFN-α-4 subtype (α4), and a third to detect all IFN-α transcripts except IFN-α-4 (Non-α4); all showed detectable induction in IFN-$\beta^{-/-}$ MEFs, although the response was clearly impaired compared to that in IFN-$\beta^{+/+}$ and IFN-$\beta^{+/-}$ MEFs (Fig. 2). The relatively low levels of the various IFN-α transcripts detected in RNA from IFN-$\beta^{-/-}$ MEFs was not caused by a low quality or

TABLE 1. PCR primers used in this study

Gene (assay)	Primers[a]	Cycles [no. (°C/s)]	$MgCl_2$ (mM)	Product size (bp)
IFN-β (genotyping)	5′-TGGGAAATTCCTCTGAGGCAG-3′ (S) 5′-CACTCATTCTGAGGCATCAACTGAC-3′ (A)	35 (94/30, 60/30, 72/60)	1.5–3	717
GFP (genotyping)	5′-GGTGAAGGTGATGCAACATACGG-3′ (S) 5′-TGTGGACAGGTAATGGTTGTCTGG-3′ (A)	35 (94/30, 60/30, 72/60)	1.5–3	513
IFN-β (RT-PCR)	5′-ACACAAGCTTAACCACCATGAACAACAGGTGGATCCTCCACGC-3′ (S) 5′-GTTAGGAATTCTCAGTTTTGGAAGTTTCTGGTAAGTCTTCG-3′ (A)	30 (94/30, 60/30, 72/60)	3	560
GFP (RT-PCR)	5′-GGTGAAGGTGATGCAACATACGG-3′ (S) 5′-TGTGGACAGGTAATGGTTGTCTGG-3′ (A)	30 (94/30, 60/30, 72/60)	3	513
Universal IFN-α	5′-ATGGCTAGGCYCTGTGCTTTC-3′ (S) 5′-TCTGAYCACCTCCCAGGCACA-3′ (A)	30 (94/60, 50/120, 72/180)	1.5	~500
IFN-non-α4	5′-ARSYTGTSTGATGCARCAGGT-3′ (S) 5′-GGWACACAGTGATCCTGTGG-3′ (A)	30 (94/30, 55/30, 72/30)	1.5	~104
IFN-α4	5′-CTGGTCAGCCTGTTCTCTAGGATG-3′ (S) 5′-TCAGAGGAGGTTCCTGCATCAC-3′ (A)	30 (94/30, 55/30, 72/30)	1.5	314
2-5A synthetase	5′-CCCCATCTGCATCAGGAGGTGGAG-3′ (S) 5′-AAGTCATAATACTTTGTCCAGTAG-3′ (A)	30 (94/30, 58/60, 72/60)	1.5	422
PGK	5′-CCTCCGCTTTCATGTAGAGGAAGA-3′ (S) 5′-GTAAAGGCCATTCCACCACCAA-3′ (A)	30 (94/60, 55/60, 72/60)	1.5	400

[a] S, sense; A, antisense.

quantity of IFN-$\beta^{-/-}$ RNA: control RT-PCR assays for transcripts encoding the housekeeping enzyme phosphoglycerate kinase (PGK) showed no difference between IFN-$\beta^{-/-}$, IFN-$\beta^{+/-}$, and IFN-$\beta^{-/-}$ MEFs (Fig. 2). While the signal for Non-α4 transcripts was particularly weak in IFN-$\beta^{-/-}$ cells, it was clearly higher at 12 h than at 0 h after infection. It therefore appears that all IFN-α genes are at least partially dependent on IFN-β for induction, although it remains possible that some species (e.g., Non-α4) are more dependent than others (e.g., α4).

The key finding in this report is that IFN-β is required to mount an antiviral response following infection of mice with vaccinia virus. Mice deficient in the IFN-α/β receptor have

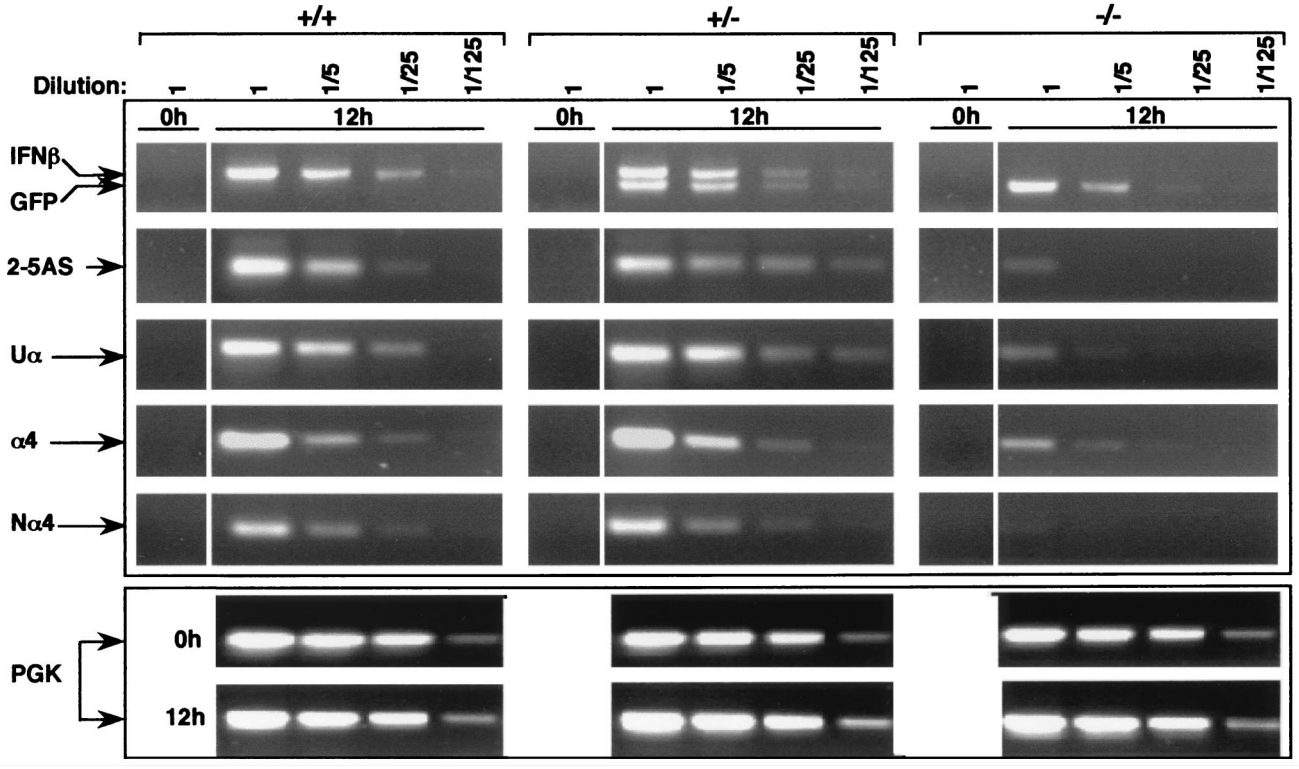

FIG. 2. RT-PCR assays for induction of IFN-α/β, GFP, and 2-5A synthetase transcripts in IFN-$\beta^{+/+}$, IFN-$\beta^{+/-}$, and IFN-$\beta^{-/-}$ MEFs. MEFs were infected with Sendai virus and harvested for RNA preparation either immediately (0 h) or 12 h later, as indicated. RNA (1 μg), prepared by lysis in guanidium isothiocyanate and density gradient centrifugation, was reverse transcribed (15-μl reaction mixtures containing avian myeloblastosis virus reverse transcriptase [Promega Biotech]) and, after the indicated dilutions, RT products (1 μl) were assayed by PCR for the following cDNAs: IFN-β, GFP, 2-5A synthetase (2-5AS), all known IFN-α subtypes (Uα), IFN-α-4 subtype (a4), all known IFN-α subtypes excluding IFN-α-4 (Nα4) and, as a positive control, phosphoglycerate kinase (PGK). Conditions for PCRs are summarized in Table 1.

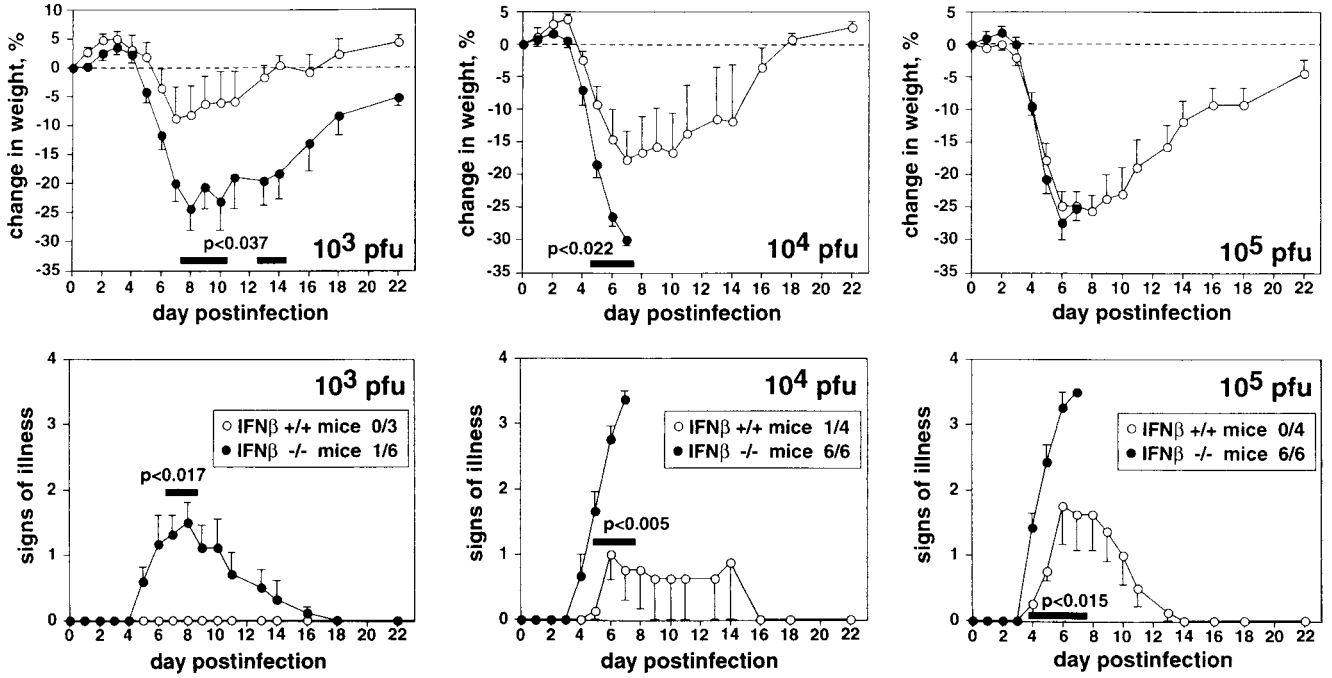

FIG. 3. Vaccinia virus infection in IFN-$\beta^{+/+}$ and IFN-$\beta^{-/-}$ mice. Groups of 7- to 9-week-old IFN-$\beta^{+/+}$ (open circles) or IFN-$\beta^{-/-}$ mice (closed circles) were intranasally infected with 10^3, 10^4, or 10^5 PFU of vaccinia virus strain Western Reserve. Every day, mice were individually weighed and monitored for signs of illness, scored from zero to four (ruffled fur, arched backs, and reduced mobility), or death. The mean percentage weight loss of each group ± the standard error of the mean, relative to the weight immediately preceding the infection, and the mean value of signs of illness ± the standard error of the mean in groups of mice infected with the indicated doses of virus, are shown. The horizontal bars indicate those days in which differences were statistically significant when analyzed by Student's *t* test, and the *P* values are shown. The number of mice per group that either died or were sacrificed due to severe infection is shown in the insets.

been reported to be more susceptible to viral infections, including vaccinia virus (43). However, because all IFN-α/β subspecies are induced under broadly similar conditions, act through a single receptor system, and have each been shown to have antiviral activities, the in vivo antiviral effect could be mediated by any or all IFN-α/β molecules, singly or in combination. The infection of IFN-$\beta^{-/-}$ mice with vaccinia virus provided an opportunity to evaluate the specific in vivo role of IFN-β in host defenses. The increased susceptibility of these animals to vaccinia virus was striking because the IFN-αs might be expected to compensate for loss of the single *IFN*β gene. That this is not the case indicates that IFN-β performs some unique role that is essential for a full antiviral response.

At least two mechanisms can be envisaged to explain such a unique role for IFN-β. In the first, *IFN*α and *IFN*β genes may be independently induced by viral infection, but IFN-β may specifically induce one or more genes that are required for full antiviral activity. Consistent with this possibility, there is evidence for IFN-β-specific signalling via the alpha/beta receptor (1, 6, 11, 28, 29, 33) and, in human fibrosarcoma cells at least, for a set of genes (including that encoding the double-stranded RNA-activated protein kinase, whose antiviral activity has been well studied [38]) that are preferentially or exclusively induced by IFN-β (10, 31). On its own, however, this model does not explain our observation, and that of others (13), that IFN-α induction is impaired in IFN-$\beta^{-/-}$ MEFs. A second mechanism that does not rely on differential signaling by IFN-β and IFN-α is suggested by this observation. Thus, it is possible that only *IFN*β is induced directly by viral infection and that IFN-α induction is a consequence of this initial IFN-β expression. Previous work showing that induction of *IFN*α, but not *IFN*β, requires protein synthesis (14) is consistent with this model, as are data (21, 35, 37, 45, 47) describing a direct pathway for *IFN*β induction by virus involving the transcription factor IRF-3. In a combination of these two mechanisms, viral infection might directly induce only IFN-β expression, and differential signaling by IFN-α and IFN-β could still be possible, with IFN-β specifically inducing IFN-α expression. The interesting observation that, in mice, IFN-α can be induced by IFN-β but IFN-β cannot be induced by IFNα (3), is probably most compatible with this model. Further experiments, including the injection of specific IFN-α/β subtypes into infected IFN-$\beta^{-/-}$ mice, are required to test these possibilities.

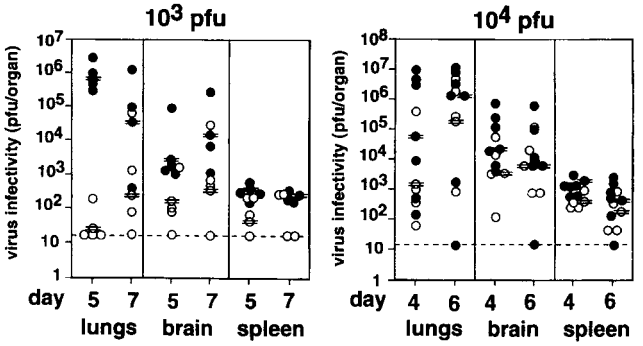

FIG. 4. Vaccinia virus replication in IFN-$\beta^{+/+}$ and IFN-$\beta^{-/-}$ mice (7 to 9 weeks old). Groups of IFN-$\beta^{+/+}$ (open circles) and IFN-$\beta^{-/-}$ mice (closed circles) were infected intranasally with 10^3 or 10^4 PFU of vaccinia virus strain Western Reserve per animal as previously described (2, 42). On the indicated days postinfection, animals were sacrificed and infectious virus in Dounce-homogenized lungs, spleen, and brain was determined by plaque titration on BS-C-1 cell monolayers. The geometric mean (=) and titers in independent mice, expressed as PFU per organ, are presented. The dashed line indicates the detection limit of the assay.

Vaccinia virus encodes a number of strategies to block IFN responses, including a soluble receptor for IFN-α/β (5, 42) that needs to be considered when interpreting results from infected mice. The vaccinia virus IFN-α/β receptor expressed from the strain Western Reserve binds to both mouse IFN-α and IFN-β with lower affinity than to the corresponding human IFNs (42). Furthermore, recent data indicate that this receptor does not block the antiviral effects of mouse IFN-β, suggesting a poor affinity for this species in vivo (V. P. Smith and A. Alcamí, unpublished data). Thus, in the mouse model we have used here, vaccinia virus does not modify the function of IFN-β.

A very recent study (23) has shown the *IFNα4* gene to differ from other *IFN*α genes and to be similar to the *IFN*β gene in its being induced particularly rapidly and without the need for de novo protein synthesis. It was also shown that *IFN*α genes other than *IFNα4* require Stat1 for induction. Other recent studies (21, 35, 37, 45, 47) have shown induction of *IFN*β to require viral modification of preexisting transcription factor IRF-3. It was therefore proposed (23) that *IFN*β and *IFNα4* are induced as a primary response to viral infection, via a pathway involving IRF-3 phosphorylation, and that secreted IFN-β and IFN-α-4 cause induction of the remaining *IFN*α genes via the type I receptor and the Jak-STAT pathway. Our data are only partly consistent with this model. Clearly some IFN-α inducibility remains in the absence of IFN-β and it is possible that this remaining induction represents the predicted IFN-α-4-dependent component. However, the fact that induction of IFN-α-4 itself is markedly compromised in the absence of IFN-β does not support a direct, and therefore presumably IFN-β-independent, mechanism for IFN-α-4 induction. Clearly, further experiments including deletion of the *IFNα4* gene are required to resolve these issues.

One of the aims of this study was to place a reporter gene at the *IFN*β locus so that the kinetics and cellular origin of IFN-β could be studied during an infection in vivo. Recent reports show that both GFP (15, 24) and CD2 (32) can be used successfully as reporters in targeted mice. We showed that the *GFP* or *CD2* reporter genes in the targeting constructs are induced by viral infection in human cells even when randomly inserted into the genome (Fig. 1c and d). RT-PCR analyses showed further that induction of GFP transcripts occurred in MEFs derived from targeted mice (Fig. 2). So far, however, we have been unable to detect GFP fluorescence by flow cytometry in virus-infected MEFs, although we have detected the induction of GFP in Western blots (R. Deonarain, unpublished results). It appears that a different form of GFP is required for the detection of GFP fluorescence in targeted MEFs, although targeted cell types that normally express IFN-β more abundantly than MEFs may yet allow induction to be followed by fluorescence.

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