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SHORT COMMUNICATION

Novel Nonviral Bioassays for Mouse Type I and Type III Interferon

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We used embryo fibroblasts from Mx2-Luc transgenic mice that express *Firefly* luciferase under control of the interferon (IFN)-regulated mouse Mx2 promoter to develop simple nonviral bioassays for type I and type III IFN. Since type III IFN is acid-labile, Mx2-Luc fibroblasts detected the presence of type I IFN in acid-treated biological samples with high sensitivity and selectivity. For selective detection of type III IFN, we employed embryo fibroblasts from Mx2-Luc mutant mice that lack functional receptors for type I IFN. The sensitivity of this latter assay remained comparatively low, presumably because type III IFN receptors are not abundantly present on fibroblasts. The main advantages of our novel IFN assays are that they are easy to perform, yield fast results, and can be used in laboratories that are not licensed for work with infectious agents. Further, the type I IFN assay has superior sensitivity than commercially available enzyme-linked immunosorbent assay systems.

Type I and type III interferons (IFN) constitute a first line of defense against viral infections in vertebrates (Dumoutier and others 2004; Haller and others 2006, 2009). Type I IFN of mice comprises 14 different IFN-α subtypes, IFN-β, IFN-κ, IFN-ε, and IFN-ζ (limitin). Signaling of all these type I IFN molecules occurs via a heterodimeric IFN-α/β receptor complex (IFNAR1/2) (Haller and others 2006, 2007). By contrast, the type III IFN family members, IFN-λ2 and IFN-λ3 in the mouse, use the heterodimeric interleukin-28 receptor α/interleukin-10 receptor β complex for signaling (Dumoutier and others 2004). In spite of using different receptors, both type I and type III IFN activate transcription factors STAT-1 and STAT-2 (Dumoutier and others 2004; Zhou and others 2007) induce a similar set of IFN-stimulated genes and, as a consequence, exhibit similar biological activities (Zhou and others 2007; Ank and others 2008; Mordstein and others 2008; Ank and Paludan 2009; Gad and others 2009).

A wide variety of assays is available for measuring IFN in biological samples (Canosi and others 1996; Meager 2002; Schwarz and others 2004; Francois and others 2005). However, quantification of mouse type I and type III IFN by enzyme-linked immunosorbent assay (ELISA) is cost intensive, and conventional bioassays are cumbersome, as they require infectious virus. We now developed nonviral bioassays for type I and type III IFN that are based on cells from Mx2-Luc transgenic mice that express *Firefly* luciferase under control of the IFN-regulated mouse Mx2 promoter. In these mice, the luciferase reporter gene is activated strongly and selectively by type I and type III IFN (Pulverer and others 2010).

Initial studies with fibroblast cultures from 14-day-old embryos or ear tissue of adult Mx2-Luc mice did not yield satisfactory results. Background expression of the reporter gene in the absence of exogenous IFN was variable and usually unacceptably high (data not shown). We reasoned that the problem might be due to spontaneous expression of the IFN-β gene in a few cells of the culture as previously described (Koerner and others 2007; Lienenklaus and others 2009). To evaluate this possibility, we crossed Mx2-Luc mice to IFN-β-deficient (BKO) mice and selected for offspring that carry the luciferase transgene but lack functional IFN-β genes. Embryo fibroblasts from Mx2-Luc-BKO mice showed greatly reduced background expression of the transgene as hypothesized. To analyze the kinetics of the IFN response, Mx2-Luc-BKO indicator cells were seeded at $\sim 2\times10^5$ cells per well into 24-well dishes. The cells were subsequently treated with *Escherichia coli*–produced mouse IFN-αA (Sigma), mouse IFN-β (supernatant of human 293T cells transfected with a mouse IFN-β cDNA expression construct) or mouse IFN-λ3 (supernatant of human 293T cells transfected with mouse IFN-λ3 cDNA expression construct) for 8, 16, or 24 h. Luciferase activity in the cell lysates was then measured using a commercial kit (Promega, Cat. no. E1500). We used the various IFN preparations at 10 and 100 U/mL, as determined by a standard antiviral assay using L929 cells and VSV for IFN-αA and IFN-β. The activity of the IFN-λ preparation was determined with a bioassay that measures effects on proliferation of BWLICR2 cells that overexpress the ligand-binding α-chain of the human IL28 receptor complex (Chi and others 2006).

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The Mx2-Luc-BKO indicator cells responded fast and vigorously to IFN-α and IFN-β, and maximal expression of the reporter gene was observed within 8 h (Fig. 1A and 1B). By contrast, the reporter cells responded only weakly to treatment with IFN-λ (Fig. 1A) or IFN-γ (Fig. 1C). The response to type I IFN was very fast. We observed substantial reporter gene activation at 4 h and almost maximal activation at 6 h poststimulation (Fig. 1B). At concentrations of IFN-α (Fig. 1A)

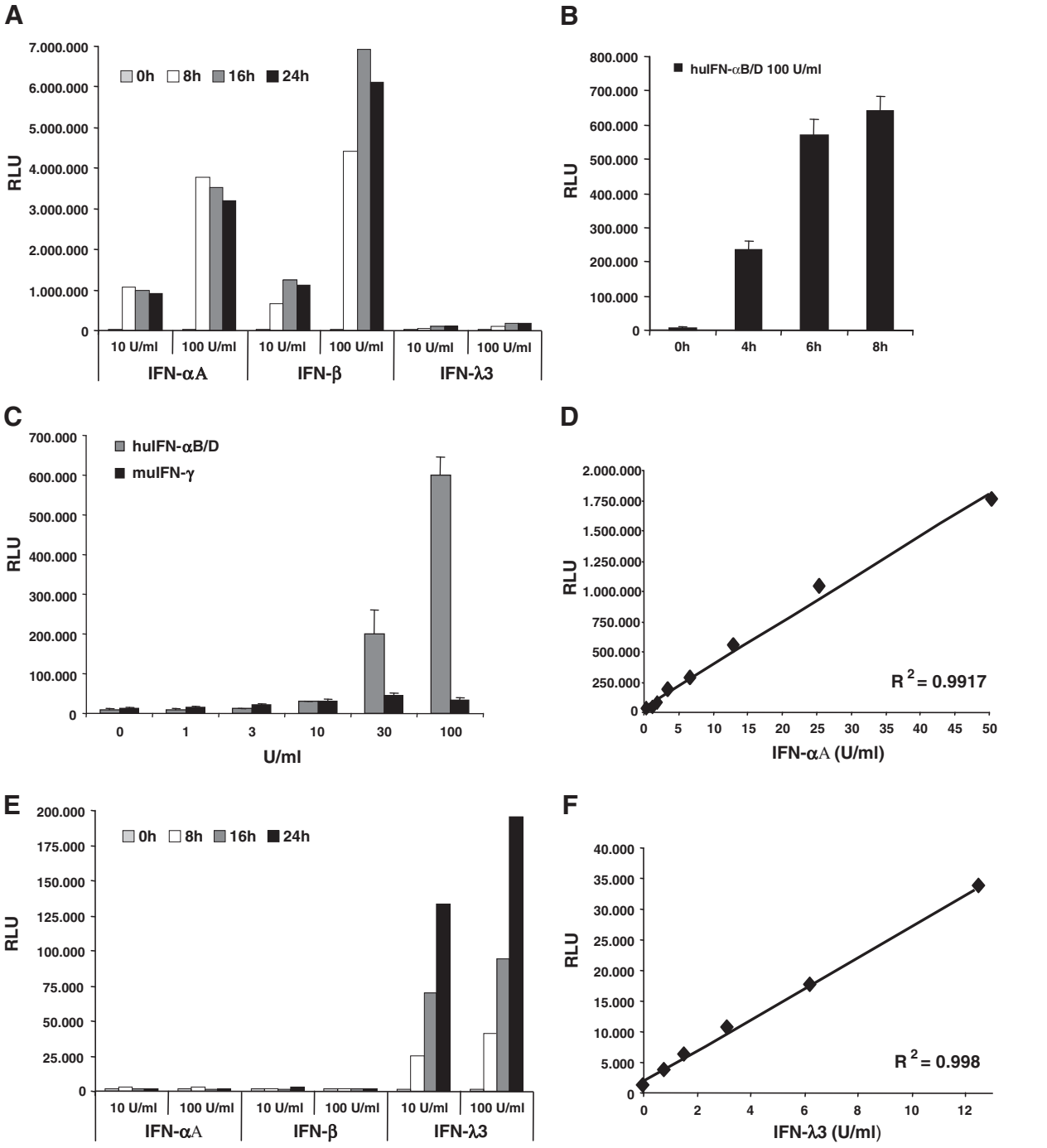

FIG. 1. IFN responses of reporter cells carrying an Mx2 promoter-controlled luciferase construct. Cells in 24-well plates were treated for the indicated times with 10 or 100 U/mL of *Escherichia coli*–produced mouse IFN-αA (Sigma), mouse IFN-β (supernatant of transfected human 293T cells), mouse IFN-λ3 (supernatant of transfected 293T cells), hybrid IFN-αB/D, or mouse IFN-γ (R&D Systems). Cells were then lysed in 50 μL of lysis buffer per well (Promega), and luciferase activity was measured using a kit (Promega). IFN responses of Mx2-Luc-BKO **(A–D)** and Mx2-Luc-IFNAR **(E–F)** reporter cells were analyzed. Representative results from 3 independent experiments are shown. To generate standard curves **(D, F)**, the indicator cells were treated with the indicated doses of IFN-αA or IFN-λ3, respectively. Data points within the linear range of the assays (1 to 50 U/mL for Mx2-Luc-BKO, and 1 to 12.5 U/mL for Mx2-Luc-IFNAR) were plotted. Linear regression analysis was performed. Representative standard curves of at least 5 independent experiments are shown. RLU, relative light units; R^2, coefficient of regression; IFN, interferon.

Table 1. Detection of Type I and III Interferon in Biological Samples by Enzyme-Linked Immunosorbent Assay and Bioassay

	ELISA (pg/mL)[a]			*Bioassay (U/mL)*[b]	
	IFN-αA	*IFN-β*	*IFN-λ3*	*Type I IFN*	*Type III IFN*
Supernatants of L929 cells infected with					
No virus	<1	<1	<1	<1	<1
RVFV clone 13	4×10^3	4×10^3	<10	4×10^4	<10
SC35M-delNS1	1.2×10^3	9×10^2	<10	1×10^4	<10
in vivo					
Control serum	<10	<10	15.5	<10	<10
Serum of RVFV clone 13-infected IFNAR mice	2×10^5	8.6×10^5	1.5×10^3	3.7×10^6	<10
Control liver lysate	–	–	<10	–	<10
Liver lysate of RVFV clone 13-infected IFNAR mice	–	–	2.7×10^3	–	<10
Control lung lysate	<10	<10	2.6×10^2	<10	<10
Lung lysate of SC35M-delNS1-infected IFNAR-IL28R mice	1.3×10^3	8×10^2	1.4×10^3	3.5×10^3	<10

[a]ELISA kits specific for mouse IFN-αA (PBL), IFN-β (PBL), or IFN-λ3 (IL-28 ELISA; RayBio) were used as described by the manufacturers. The indicated values represent the average of at least 3 independent experiments.

[b]Type I and type III IFN activities in acid-treated samples were measured using Mx2-Luc-BKO and Mx2-Luc-IFNAR cells, respectively. The indicated values represent the average of at least 3 independent experiments.

ELISA, enzyme-linked immunosorbent assay; IFN, interferon; RVFV, Rift valley fever virus.

or IFN-β (data not shown) between 1 and 50 U/mL, the dose–response curve of the Mx2-Luc-BKO cells showed almost perfect linearity. The assay performed similarly well if confluent cell monolayers in 96-well format culture dishes were stimulated with IFN (data not shown).

To determine whether our Mx2-Luc transgenic mice can also be used to develop an assay for the specific detection of IFN-λ, we crossed Mx2-Luc mice with IFNAR1-deficient mice to generate offspring that contained the luciferase transgene but lacked functional receptors for type I IFN (Mx2-Luc-IFNAR mice). As expected, embryo cells from such mice failed to respond to either IFN-α or IFN-β, but continued to respond to IFN-λ (Fig. 1E). The detection limit of this bioassay was ~1 U/mL, which equals the sensitivity of a nonrelated bioassay (Chi and others 2006). Careful evaluation revealed a linear dose response if IFN-λ was used at concentrations between 1 and 15 U/mL in our bioassay (Fig. 1F). The response of the indicator cells to IFN-λ was substantially slower than the response to type I IFN. Further, the IFN-λ response reached a maximum only after some 24 h (Fig. 1E). Maximal luciferase values of IFN-λ-treated Mx2-Luc-IFNAR cells remained some 50 times lower than maximal values of IFN-α- and IFN-β-treated Mx2p-Luc-BKO cells, presumably due to rather poor expression of functional IFN-λ receptors on mouse fibroblasts (Sommereyns and others 2008).

We next set out to determine whether our novel bioassays could reliably measure type I and III IFN in biological samples. The samples analyzed included supernatants of mouse L929 cells infected with either influenza A virus mutant SC35M-delNS1 or Rift valley fever virus (RVFV) mutant clone 13 that both lack IFN-antagonistic factors and therefore induce high levels of type I and type III IFN (Bouloy and others 2001; Kochs and others 2007). Our collection of biological samples further included serum and organ extracts of mice that were infected with either

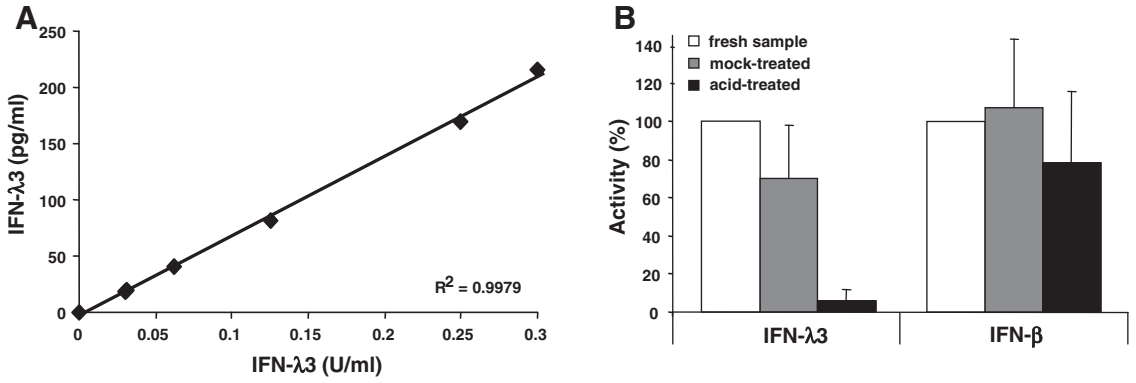

FIG. 2. Specific activity and acid sensitivity of mouse IFN-λ3. **(A)** Correlation of activity and physical presence of mouse IFN-λ3 in supernatants of transfected human 293T cells by bioassay and enzyme-linked immunosorbent assay (RayBio). Results from one of two independent experiments are shown. R^2: coefficient of regression. **(B)** Comparison of acid stability of IFN-λ3 and IFN-β. Samples of 1,000 U/mL of mouse IFN-λ3 or mouse IFN-β were treated with neutral buffer or with acid (0.1 M glycine, pH 2) for 24 h at 4°C before dialysis against phosphate-buffered saline (pH 7.3). The biological activities of the fresh reference preparations were set to 100%. The indicated values represent the average of at least 3 independent experiments. The standard deviation is indicated.

SC35M-delNS1 or RVFV clone 13. Analysis by commercial ELISA kits revealed that supernatant of L929 cells infected with RVFV clone 13 contained ~4,000 pg/mL of IFN-α and 4,000 pg/mL of IFN-β, but virtually no IFN-λ (Table 1). Supernatant of L929 cells infected with influenza virus SC35M-delNS1 contained slightly less IFN-α and IFN-β but, again, no IFN-λ. Serum of RVFV-infected mice contained high levels of IFN-α and IFN-β, and moderate levels of IFN-λ (Table 1). Moderate levels of IFN-λ were also detected in liver extracts of RVFV-infected mice. Lungs of mice infected with SC35M-delNS1 contained substantial levels of IFN-α, IFN-β, and IFN-λ as determined by ELISA (Table 1).

The various samples were next analyzed with our novel bioassays. To avoid potential interference by infectious viral particles in the samples that might induce endogenous IFN, virus was inactivated by dialysis against glycine buffer (pH 2) for 24 h followed by dialysis against neutral pH buffer for another 24 h as previously described (Schwarz and others 2004). Acid-treated supernatants of L929 cells infected with RVFV clone 13 or SC35M-delNS1 contained 4×10^4 and 1×10^4 U/mL of type I IFN, respectively (Table 1). In agreement with the ELISA data, no type III IFN was detected in supernatants of virus-infected L929 cells by our bioassay. Acid-treated sera of RVFV-infected mice contained 3.6×10^6 U of type I IFN, but seemingly no type III IFN (Table 1). Substantial levels of type I IFN, but no type III IFN, were detected by our bioassays in acid-treated extracts of lungs from SC35M-delNS1-infected mice (Table 1). Since one international unit of type I IFN corresponds to ~10 pg of protein [PBL manufacture's manual (Pestka 1986)], these results indicated that our novel type I IFN bioassay performs very accurately. Detection of type I IFN in the organs of infected mice by our bioassay was thus at least 10-fold more sensitive than by ELISA, presumably because our assay integrates signals for all different type I IFN species. By contrast, our type III IFN bioassay failed to detect IFN-λ activity in samples that scored positive by ELISA, suggesting either that our type III IFN bioassay is not sensitive enough or that IFN-λ loses activity during acid treatment.

To evaluate these 2 different possibilities, we first correlated specific biological activity and physical presence of mouse IFN-λ3 (as determined by ELISA) in supernatants of cDNA-transfected human 293T cells. Results (Fig. 2A) showed that ~700 pg/mL of IFN-λ3 was required for 1 U of activity in our bioassay, which corresponds to a specific activity of IFN-λ3 in our bioassay of $\sim1.4\times10^6$ U/mg. This result is consistent with findings by Dellgren and coworkers (2009), who also noted a much weaker antiviral activity of human IFN-λ3 against vesicular stomatitis virus and encephalomyocarditis virus compared to human IFN-α2b in bovine kidney cells. In addition, several groups showed that there is a direct connection between receptor expression and IFN-λ antiviral response (Kotenko and others 2003; Zhou and others 2007; Dellgren and others 2009).

To determine if acid instability contributed to the fact that our bioassay failed to detect type III IFN in biological samples, we compared the acid stability of recombinant mouse IFN-λ3 and mouse IFN-β that were both produced by transfection of human 293T cells with suitable expression constructs. The activity of IFN-β in crude supernatants of transfected 293T cells decreased by only about 20% upon acid treatment (Fig. 2B), demonstrating a high degree of acid resistance of IFN-β as previously described (Levy-Koenig and others 1970; Balkwill and others 1983). By contrast, our IFN-λ preparation lost >90% of its activity during acid-treatment (Fig. 2B). A control sample also lost some activity during mock treatment, suggesting that IFN-λ is not only less resistant to acid treatment but may further be intrinsically less stable than type I IFN. Thus, the rather poor performance of the type III IFN bioassay on biological samples appeared, at least in part, to be due to low acid stability of mouse IFN-λ.

In summary, we established rapid nonviral bioassays that can specifically detect either type I or type III IFN of mouse origin. We found that the type III IFN bioassay is of limited use for the analysis of virus-containing samples as acid treatment, which removes virus infectivity, also destroys the activity of IFN-λ. On the other hand, this particular property of IFN-λ is fortunate, because a simple acid treatment of biological samples eliminates unwanted crosstalk and renders our novel type I IFN bioassay highly selective. Our assays are based on nontransformed embryo cell cultures, a fact that will limit their widespread use. At present, we try to find cell immortalization protocols that have no negative effect on background activity of the assays. The main advantages of our novel IFN assays are that they are easy to perform, yield fast results, and can be used in laboratories that are not licensed for work with infectious agents. Further, the type I IFN assay has superior sensitivity than commercially available ELISA systems.

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Author Disclosure Statement

No competing financial interests exist.

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