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ORIGINAL ARTICLE

Human interferon-λ3 is a potent member of the type III interferon family

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Type III interferon (IFN) or IFN-λ is a recently discovered family of IFNs that signal through the same downstream transcription factors as type I IFN but use a separate receptor complex composed of the IL-10R2 and the unique IFN-λR1 receptor chains. We have established a simple and efficient expression system to produce highly pure and active IFN-λ of the three human IFN-λ isoforms (IFN-λ1, -λ2 and -λ3) and used this to compare the biological activity of the different IFN-λ subtypes. Surprisingly, we found IFN-λ3 to possess the highest specific activity of the human IFN-λ subtypes, exhibiting a twofold higher activity than IFN-λ1 and a 16-fold higher activity than IFN-λ2. Furthermore, in comparison with the commercially available preparations of IFN-λ1 and -λ2, we found our IFN-λ preparation to be superior in activity.
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Introduction

Interferons (IFNs) are class II[1] cytokines characterized by their cellular antiviral effects. IFNs are classified based on the type of receptors utilized (reviewed in Pestka *et al*.[2]). Type I IFN includes IFN-α, IFN-β and several other subtypes, all of which signal through the IFN-α/β receptor. Type II IFN includes only IFN-γ and signals through the IFN-γ receptor. Type I IFN induces antiviral, antiproliferative and immunomodulatory activities,[3] whereas type II IFN mainly induces immunomodulatory activities.[4] A novel class of IFNs, termed type III IFNs, has recently been discovered, which uses a receptor complex composed of the IL-10R2 and the IL-λR1 receptor chains.[5,6] Initially, named either IFN-λ (IFN-λ1, -λ2 and -λ3) or interleukin (IL)-29, -28A and -28B, we will refer to them as IFN-λ in this paper.

Both type I and III IFNs are expressed in response to viral infection;[7,8] however, in contrast to type I IFN, only a subset of cells express the IL-λR1 receptor chain and are responsive to type III IFN.[9,10] Although type I and type III IFNs utilize distinct receptor complexes, both type of IFNs have antiviral activity *in vitro*[6] as well as *in vivo*.[11] Similarly, type I IFN and type III IFN signal through the receptor-associated tyrosine kinases, JAK1/TYK2, which mediate the phosphorylation of transducers and activators of transcription (STAT).[12] This leads to an assembly of the IFN-stimulated gene factor-3 consisting of STAT1, STAT2 and IFN regulatory factor 9.[10] As both types of IFNs induce activation of IFN-stimulated gene factor-3, the profile of induced genes is highly similar.[10,13,14]

In addition to its antiviral activity, IFN-λ is also capable of modulating the Th1/Th2 response. Recently, IFN-λ1 has been shown to inhibit the production of IL-13 by T cells in a dose-dependent manner and, thus, down regulate the Th2 response.[15] Furthermore, IFN-λ acts on monocytes and stimulates the production of IL-6, IL-8 and IL-10.[16] Induction of IL-6 by IFN-λ might suggest a role in linking the innate immune response to the adaptive immune response. IL-10 induction can serve a complex role both by promoting B-cell survival and suppressing the production of pro-inflammatory cytokines. The inhibition of IL-13 secretion by monocytes can be of clinical relevance as both IL-13 and Th2 response is important in the development of asthma. This is further supported by a recent report that links deficient IFN-λ expression upon infection with Rhino virus infection to increased severity of asthma.[17]

Recent data showed promising *in vivo* antiviral activity of IFN-λ in a murine model,[11] suggesting that IFN-λ could be used therapeutically against viral infections. Treatment with IFN-α is often associated with substantial side effects.[18] As IFN-λ only affects a subset of cell types, treatment with IFN-λ could be associated with fewer complications.[19] Before clinical trials, however, one should determine the activity of the different subtypes of IFN-λ and establish an efficient expression system. IFN-λ1 has been expressed in several different bacterial and eukaryotic expression systems achieving half-maximal effective concentration (EC_{50}) values from 0.5 to 2 ng ml^{-1}.[5,6,13] IFN-λ2 has been expressed in insect and murine cells, resulting in rather poor specific activities with EC_{50} values of approximately 30 ng ml^{-1}.[6] On the

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basis of the high similarity in amino-acid sequence between IFN-λ2 and -λ3 (96% identity), it was inaccurately assumed that IFN-λ2 and -λ3 would have similar potency, and so the antiviral activity of IFN-λ3 has not been tested.

Here, we describe a simple and highly efficient protocol for the production of recombinant IFN-λ in *Escherichia coli*. We have produced all the three subtypes of IFN-λ in this system and tested their antiviral activity. Surprisingly, we found IFN-λ3 to be the most potent of the IFN-λ subtypes in an *in vitro* antiviral assay. Furthermore, we compare the activity of our IFN-λ preparations with that of commercial available preparations of IFN-λ1 and -λ2 and commercial IFN-α2. We found the activity of our *E. coli*-expressed IFN-λ to be superior to that of commercial preparations made in mammalian cells and that the activity of IFN-λ3 is within one order of magnitude of the activity of IFN-α2 in HepG2 cells.

Materials and methods

Cell lines

Human hepatocellular carcinoma cell lines, HepG2 and Huh7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Bovine kidney cell line, MDBK, was cultured in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. All incubations were performed at 37°C in a 5% CO_2 gassed incubator.

Protein expression and purification

Human IFN-λ1, -2 and -3 (residues 13–175, 13–175 and 13–178 of the mature polypeptides; accession codes NP 742151, NP 742150 and NP 742152) cDNA optimized for expression in *E. coli* was purchased from Geneart (Regensburg, Germany) and cloned into the pET-15b vector. A 6 × His-tag followed by a tobacco etch virus (TEV) protease cleavage site was added to the N termini. BL21 (DE3) *E. coli* cells transformed with the plasmids were grown at 37°C in Luria Bertani medium containing 100 μg ml^{-1} ampicillin and under continuous shaking until an OD_{600} of 0.4–0.6. The cells were then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside and incubated for another 12 h at 37 °C. Unless otherwise stated, the following steps were carried out at room temperature. After incubation, the cells were harvested by centrifugation, resuspended in lysis buffer (6 M guanidine hydrochloride, 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0)) and lysed with a high-pressure homogenizer. Cell debris was removed from the lysate by centrifugation (15 000 *g* for 30 min) and the supernatant was mixed with HIS-Select High Flow (HF) Nickel Affinity Gel (Sigma-Aldrich, Broendby, Denmark) and incubated at 4°C under continuous shaking for 12 h. Following incubation, nickel affinity gel beads were washed with wash buffer (8 M urea, 500 mM NaCl, 10 mM imidazole, 50 mM NaH_2PO_4 (pH 8.0)). The proteins were eluted from the nickel affinity gel with elution buffer (8 M urea, 100 mM NaCl, 250 mM imidazole, 20 mM Tris/HCl (pH 7.5)) and any disulfide bonds introduced by the *E. coli* cells (we observed intramolecular disulfide bonds in the proteins) were reduced by the addition of 30 mM dithiothreitol for several hours. In general, we achieved yields of approximately 15 mg protein per liter of *E. coli* culture after metal-ion affinity chromatography. From this point, all the following steps were carried out at 4 °C. Next, the proteins were refolded in the following way: first, they were diluted to a concentration of 0.5 mg ml^{-1} with dialysis buffer no. 1 (6 M urea, 100 mM NaCl, 50 mM Tris/HCl (pH 6.5)) and dialyzed against the same buffer for 12 h. Next, they were dialyzed against a refolding buffer (240 mM NaCl, 10 mM KCl, 0.5 M L-arginine, 0.75 M guanidine hydrochloride, 0.5% v/v triton X-100, 2 mM reduced glutathione, 0.5 mM oxidized glutathione, 50 mM 2[/V-morpholino] ethanesulfonic acid (pH 6.0)) for 24 h and finally, they were dialyzed against dialysis buffer no. 2 (200 mM NaCl, 5% (v/v) glycerol, 20 mM 2[/V-morpholino] ethanesulfonic acid (pH 6.5)) for another 24 h. During the last step, TEV protease was added for the last 12 h to cleave off the 6 × His-tag. Refolding and TEV protease cleavage generally resulted in yields ranging from 30 to 50%. After refolding, the precipitated protein was removed by centrifugation (15 000 *g* for 30 min) and the supernatant was mixed with an equal volume of ion exchange buffer (25 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 6.8)) and applied to a HiTrap SP FF column (GE Healthcare, Hilleroed, Denmark). The protein was eluted with a 15 ml linear gradient from 25 mM to 1 M NaCl at 0.5 ml min^{-1}. Fractions containing the protein were pooled and then concentrated to a volume of 1 ml using an Amicon Ultra centrifugal filter device before being applied to a HiLoad 16/60 Superdex 75 column (GE Healthcare). The protein was eluted with size exclusion buffer (140 mM NaCl, 3 mM KCl, 10 mM PO_4 (pH 6.5)) at 1 ml min^{-1}. Finally, the highly pure protein preparation was added to glycerol to a final concentration of 10% (v/v) and then stored at −80°C.

Reagents

Recombinant human IFN-λ1 and -2 were purchased from R&D Systems (Abingdon, UK) or PeproTech (London, UK). Recombinant human IFN-α2a was purchased from Chemicon International (Temecula, CA, USA) or PBL interferon source (Piscataway, NJ, USA).

Antiviral assays

Cytokines were diluted in 100 μl growth medium, in replicas of four for each concentration, in 96-well tissue culture plates. Two columns were used for cell control, and two for virus control, in which only growth medium was added. Cells were seeded at a density of 10^3 cells per well in 100 μl growth medium giving a total of 200 μl, and incubated for 24 h. After incubation, the growth medium was removed, and the virus was added in 100 μl growth medium. Encephalomyocarditis virus (EMCV) was added to a multiplicity of infection (MOI) of 0.04 against human hepatocellular liver carcinoma cell lines and against MDBK cells at an MOI of 4. Vesicular stomatitis virus (VSV) was added to an MOI of 0.01 against MDBK cells. The cell control wells were incubated in pure growth medium. After 20 h incubation with virus, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added, and the cells were incubated for an additional 4 h. Medium was then removed and the MTT resolved in 200 μl dimethyl sulfoxide before the

absorption was measured at 595 nm with background subtracted at 650 nm as a measure of cell viability.

Data treatment
Measured absorptions in the cytokine wells (Abs) were normalized as a percent of the cell control (CC) with the virus control (VC) subtracted, using the following equation: normalized cell survival (NCS) $=(\mathrm{Abs}-\mathrm{VC}/(\mathrm{CC}-\mathrm{VC}))\times 100$ to allow activity comparisons between plates. EC_{50}, efficacy and exponential slope values were calculated in GraphPad prism ver. 5.01 by fitting a sigmoidal equation to the plot. The equation used for fitting was:

$$\mathrm{NCS} = \mathrm{Baseline} + (\mathrm{Efficacy} - \mathrm{Baseline}) /(1 + 10\text{^}((\log \mathrm{EC}_{50} - X) * \mathrm{Slope}))$$

where efficacy is an estimate of maximal protection and slope describes the steepness of the curve. Values for baseline were restrained to zero.

Results

In vitro *refolding and purification of IFN-λ*
The cDNA encoding the mature form of human IFN-λ1, 2 or -3 were cloned into a pET-15b vector, and a 6 × His-tag followed by a TEV protease cleavage site was added to their N termini. This recombinant form of IFN-λ was expressed in *E. coli* and purified from inclusion bodies under denaturizing conditions by metal-ion affinity chromatography. The protein was then dialyzed against a refolding buffer allowing for the proper folding of the protein and the formation of disulfide bonds. After refolding, the precipitated protein was removed by centrifugation and the refolded protein was further purified by cation-exchange chromatography on a Hi-Trap SP FF column and size-exclusion chromatography on a HiLoad 16/60 Superdex 75 column. Representative chromatograms for the purification of IFN-λ3 are shown in Figures 1a and b. During size-exclusion chromatography, two peaks corresponding to a monomeric and dimeric form of the protein were observed at protein concentrations above 1 mg ml^{-1} (Figure 1b). The formation of the dimer was a dynamic process as the dimer appeared after increased protein concentrations above 1 mg ml^{-1}, and the protein reverted to a monomeric state after dilution (data not shown). This procedure resulted in a highly purified protein for all three subtypes of IFN-λ (Figure 1c). To ensure that the level of endotoxins did not interfere with our antiviral assays, we tested the first batches of IFNλ-3 with the E-TOXATE kit from Sigma. We could not detect any endotoxins (data not shown), although it should be noted that the E-TOXATE kit only gives a semiquantitative answer. Furthermore, we heat-inactivated IFN-λ3 at 95 °C for 20 min in the presence of 2-mercaptoethanol after which its antiviral activity was completely abolished (Supplementary Figure 1). This suggests that the level of endotoxins after our procedure of purification was too low to result in any interference in the antiviral assays.

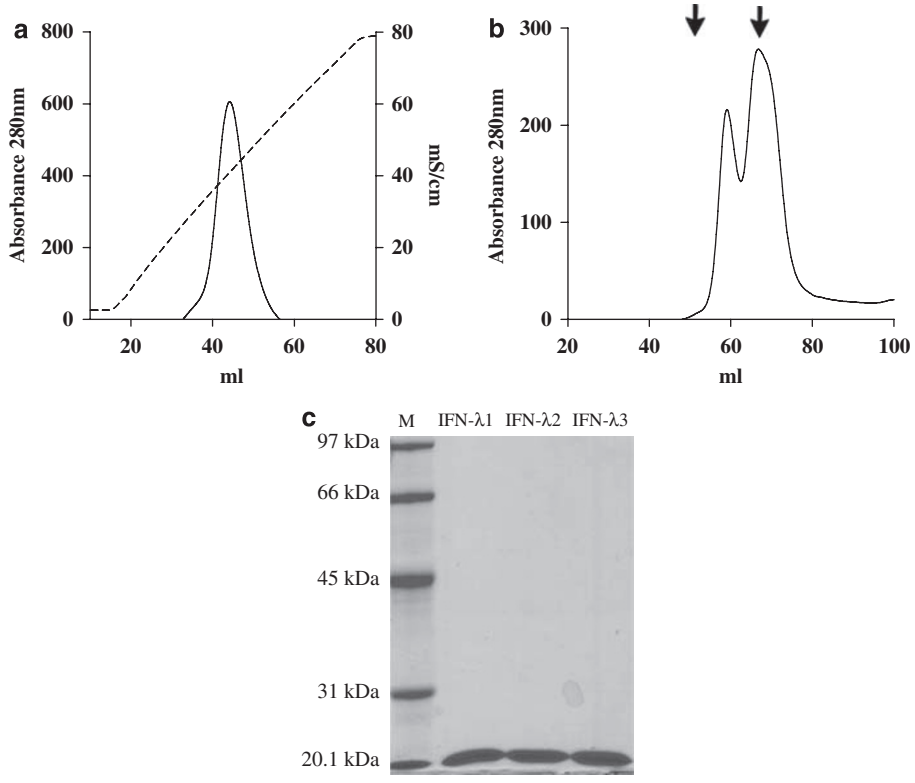

Figure 1 Expression and purification of IFN-λ. (**a**) Cation-exchange chromatography of IFN-λ3 on a HiTrap SP FF column. The salt gradient is shown as the conductivity in mS cm^{-1}. (**b**) Size-exclusion chromatography of IFN-λ3 on a HiLoad 16/60 Superdex 75 column. Markers indicated by arrows are chymotrypsinogen A 25.0 kDa and ovalbumin 43.0 kDa. (**c**) SDS-PAGE gel of final purified product of IFN-λ1, -λ2 and -λ3, respectively. Purified protein (2 μg) was loaded to a denaturing SDS-PAGE gel, and after electrophoresis, the gel was stained with coomassie blue. The expected sizes of IFN-λ1, -λ2 and -λ3 are 19.0, 18.6 and 18.4 kDa, respectively. IFN, interferon; PAGE, polyacrylamide gel electrophoresis.

Refolded IFN-λ3 is a highly active antiviral reagent in HepG2 cells

To measure the activity of the purified protein, an assay was established in which the ability of IFN-λ to protect cells from lysis by EMCV was measured. Cells were seeded in a 96-well microtiter plate and treated with the indicated amount of IFN for 24 h and then challenged with EMCV for 20 h. Each concentration of IFN was repeated four times in one plate and cell survival was measured by an MTT coloring assay.[20] One control group was left untreated (no IFN and no virus) and defined as 100% cell survival. A second control group was infected with the virus but not treated with IFN, and this group defined a zero % cell survival as well as a measure of background absorption. The data were fitted using the GraphPad Prism software, allowing for three variables: the EC_{50}, the efficacy (the maximal cell protection induced) and the exponential slope. The estimated values are given in Table 1 and Supplementary Table 1.

One experiment involves four replicates of each IFN concentration. However, this does not address variation originating from a number of random sources such as different protein preparations, small variation in cell growth conditions and so on. To address the general reproducibility of both the protein preparation and assay procedure, we performed four independent experiments with IFN-λ3, including two different batches of IFN-λ3 (Supplementary Figure 2). This confirmed a high degree of reproducibility for both assay procedure and protein purification. The data listed in Table 1 for IFN-λ3 are the mean of these four experiments, and the individual plots for the four IFN-λ3 assays can be seen in Supplementary Figure 2.

We obtained an EC_{50} value of 0.6 ng ml^{-1} for refolded IFN-λ3 that is comparable with the best preparations of IFN-λ1 reported in the literature and substantially lower than the reported values for IFN-λ2. Comparison of the activity of refolded IFN-λ1, -λ2 and -λ3, surprisingly, showed that IFN-λ3 is slightly more potent than IFN-λ1 and 16-fold more potent than IFN-λ2 (Figure 2a and Table 1).

Table 1 Antiviral activity

	HepG2 challenged with EMCV					
	EC_{50}	*95% CI*	*Slope*	*95% CI*	*Efficacy*	*95% CI*
IFN-λ3	0.61	0.56–0.68	0.90	0.84–0.97	88.93	87.38–90.49
IFN-λ2	9.84	8.10–11.95	0.74	0.67–0.81	108.50	103.50–113.50
IFN-λ1	1.27	1.06–1.53	0.77	0.68–0.86	97.02	93.66–100.40
IFN-λ1[a]	3.12	2.50–3.89	1.16	0.91–1.41	81.07	76.81–85.34
IFN-λ2[a]	29.18	20.95–40.64	0.97	0.76–1.17	57.30	51.24–63.36
IFN-λ1[b]	3.98	3.08–5.14	0.74	0.63–0.84	82.82	78.29–87.34
IFN-λ2[b]	8.01	6.22–10.32	0.80	0.69–0.91	71.15	66.82–75.48
IFN-α2b[c]	0.06	0.04–0.08	0.57	0.48–0.65	97.16	91.65–102.70
IFN-α2b[d]	0.05	0.04–0.06	0.68	0.60–0.76	86.39	82.92–89.86

Abbreviations: CI, confidence interval; EMCV, encephalomyocarditis virus; IFN, interferon.
[a]Purchased from R&D Systems.
[b]Purchased from PeproTech.
[c]Purchased from Chemicon.
[d]Purchased from PBL interferon source.

Refolded IFN-λ1 and -λ2 is superior to commercial preparations of IFN-λ1 and -λ2

We compared the activity of refolded IFN-λ with that of two different commercially available preparations of IFN-λ1 and -λ2, using the antiviral activity assay. According to the manufacturers, the preparations from R&D Systems are expressed in a mouse myeloma cell line and the preparations from PeproTech are expressed in *E. coli*. The EC_{50} values that we measured for the R&D Systems preparations of IFN-λ1 and -λ2 were well within the range of the specific activity given by the manufacturer. No specific activity was given for the PeproTech preparations of IFN-λ1 and -λ2. We observed a difference between refolded IFN-λ and the IFN-λ1 and -λ2 from commercial sources. Refolded IFN-λ1 was approximately threefold as potent as commercial IFN-λ1, whereas the two different commercial preparations of IFN-λ1 were equally potent (Figure 2b). Refolded IFN-λ2 was also approximately threefold more potent than the commercial preparation of IFN-λ2 from R&D Systems, whereas the commercial preparation of IFN-λ2 from PeproTech was equally potent to the refolded IFN-λ2 (Figure 2c). In summary, the specific activity of refolded IFN-λ3 is comparable or, possibly, superior to that of IFN-λ1. Although the specific activity varies between different modes of protein expression and between IFN-λ subtypes, the exponential slope is, however, comparable between all seven IFN-λ preparations (Table 1).

The activity of IFN-λ3 is approaching that of IFN-α2b

IFN-α2b is the standard IFN used in most research and clinical settings. We tested the antiviral activity of two different commercially available preparations of IFN-α2b in HepG2. The two commercial preparations have virtual identical activity (Figure 2d) and, compared with IFN-λ3, they were approximately 10-fold more potent. When comparing the curve slopes, a noticeable difference was observed between the two IFN-α2b preparations and IFN-λ3 (Figure 2d and Table 1).

As the activity of IFN-λ can vary significantly between different cell lines, we measured the antiviral activity in a different human liver cell line, HuH-7. Both IFNs showed weaker activities than they did in HepG2 cells with a lower slope, and only IFN-λ3 reached a maximum protection of 39% cell survival (Figure 3 and Supplementary Table 1). We used 400 ng ml^{-1} of IFN-λ3 as the highest dose in the antiviral activity assay; however, it is uncertain whether this dose is saturating in Huh-7 cells.

Antiviral activity in bovine kidney cells

The commercial suppliers of IFN-α2b utilize an assay that differs from the one used by ourselves to test the antiviral activity. To verify that our preparation of IFN-α2b had the activity specified by the manufacturer and to test for possible mishandling of IFN-α2b, we adapted their choice of virus and cell line.

MDBK (bovine kidney) cells were challenged with VSV (Figure 4a). We observed very similar activity profiles for the two different commercial IFN-α2b preparations. The observed potency was sevenfold weaker than the EC_{50} claimed by the manufacturer (Supplementary Table 1). It is worth noting that the exponential slope of the curve is much steeper than the exponential slope observed in human liver cells (Table 1, Supplementary Table 1). It was not possible to show any

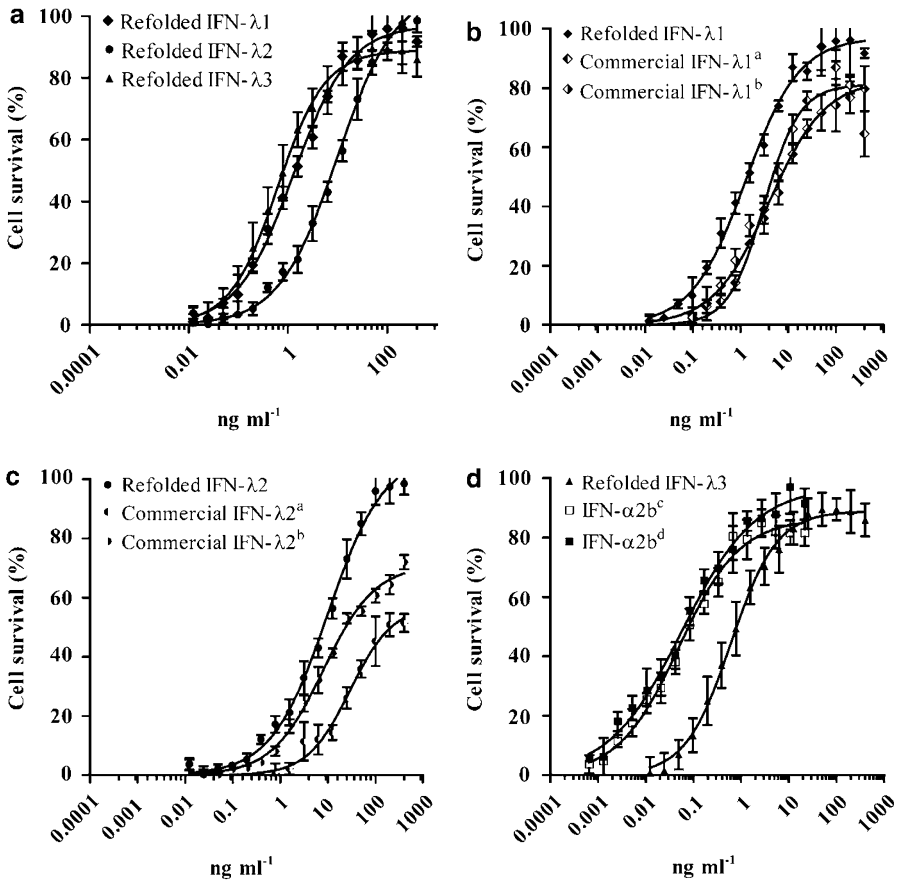

Figure 2 Antiviral effect of IFN concentrations in HepG2 cells challenged with EMCV. HepG2 cells were incubated with serial dilutions of IFN before being challenged with EMCV. The data shown for IFN-λ3 represent four separate plates, with each concentration performed in replicas of four. Data shown for the other IFNs represent one plate, with each concentration performed in replicas of four. [a]Purchased from R&D Systems. [b]Purchased from PeproTech. [c]Purchased from Chemicon. [d]Purchased from PBL interferon source. Mean ± s.d. from four replicates are shown. EMCV, encephalomyocarditis virus; IFN, interferon.

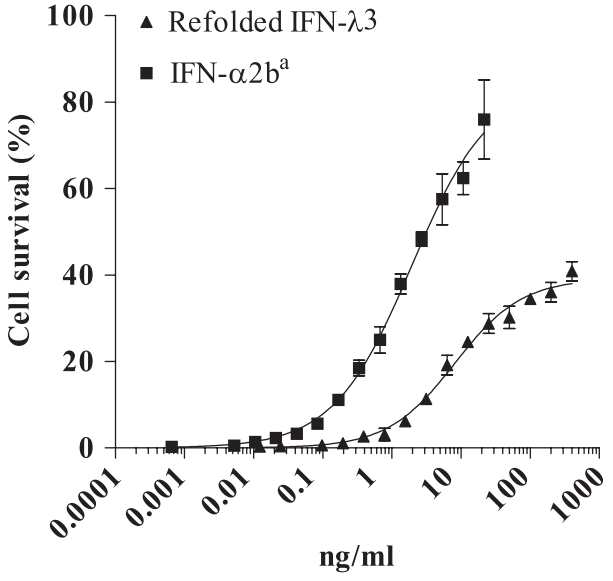

Figure 3 Antiviral effect of IFN in HuH-7 cells challenged with EMCV virus. HuH-7 cells were incubated with serial dilutions of IFN performed in replicas of four for each concentration before challenge with EMCV. [a]Purchased from Chemicon. Mean ± s.d. from four replicates are shown. EMCV, encephalomyocarditis virus; IFN, interferon.

activity for IFN-λ3 in MDBK cells against VSV even at concentrations of 400 ng ml^{-1} (data not shown).

Surprisingly, human IFN-λ3 showed antiviral activity in MDBK cells when challenged with EMCV (Figure 4b). However, compared with IFN-α2b, the potency is weak. IFN-α2b showed extreme potency against EMCV with EC_{50} values around 0.25 pg ml^{-1} compared with 3.4 ng ml^{-1} for IFN-λ3. Again the exponential slope was quite different from the exponential slopes observed in HepG2 and Huh-7 cells, but closer to the slopes of MDBK challenged with VSV (Table 1; Supplementary Table 1). Also, the amount of EMCV needed to lyse unprotected cells was 100-fold higher as compared with HepG2 cells, showing that these cells are highly resistant to EMCV infection.

Discussion

We have developed a protocol to express IFN-λ1, -λ2 and -λ3 in *E. coli* in inclusion bodies and subsequently refold the proteins *in vitro*. We further purified the protein through cation-exchange and size-exclusion chromatography and achieved a highly pure preparation. These preparations of IFN-λ are superior to the commercially available IFN-λ preparations produced in mammalian or bacterial cells, with regard to the antiviral activity. The production of recombinant protein in bacteria is very cost

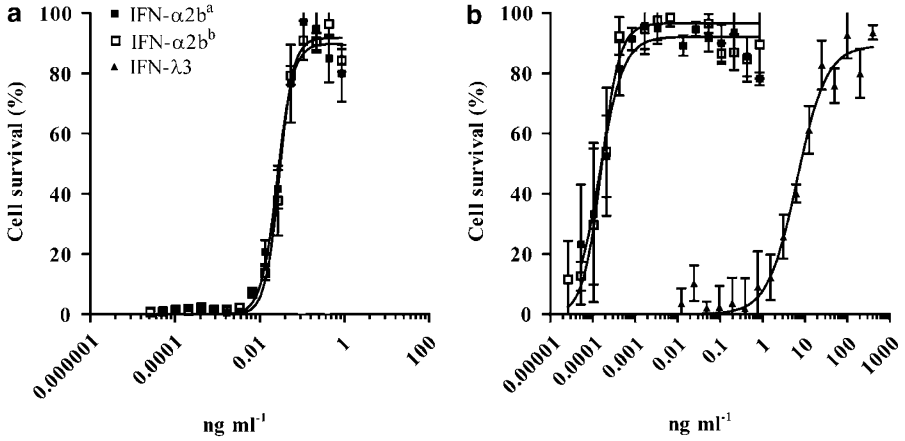

Figure 4 Antiviral effect of IFN concentration in MDBK cells challenged with virus. MDBK cells were incubated with serial dilutions of IFN performed in replicas of four for each concentration before the virus challenge. (**a**) MDBK cells challenged with VSV. (**b**) MDBK cells challenged with EMCV. [a]Purchased from Chemicon. [b]Purchased from PBL interferon source. Mean ± s.d. from four replicates are shown. EMCV, encephalomyocarditis virus; IFN, interferon; VSV, vesicular stomatitis virus.

effective and reduces the risk of contaminating the protein preparation with mammalian pathogens. Thus, the procedure we have described here can be used as a starting point for producing IFN-λ of a quality usable for clinical trials.

As human IFN-λ2 and -λ3 differ only at six amino-acid positions, it was expected that they would have comparable antiviral activities, and, therefore, the activity of IFN-λ3 has not been tested earlier. We produced IFN-λ1, -λ2 and -λ3 by the same protocol and compared their antiviral activities in HepG2 cells. We found that IFN-λ3 has a twofold lower EC_{50} value as compared with IFN-λ1 and a 16-fold lower EC_{50} value as compared with IFN-λ2. Thus, IFN-λ3 is the most active of the three types of IFN-λ in HepG2 cells. The activities of the commercially available preparations of IFN-λ1 were approximately threefold lower than those of the IFN-λ1 prepared by *in vitro* refolding, regardless of whether they were expressed in mammalian or bacterial cells. In the case of the commercial preparations of IFN-λ2, the activity of the mammalian-expressed IFN-λ2 from R&D Systems was threefold lower than that of the *in vitro* refolded IFN-λ2, whereas the bacterial-expressed IFN-λ2 from PeproTech was equal in activity to the *in vitro* refolded IFN-2. There can be several reasons for this including the increased purity of the IFN-λ prepared in our study.

The poor specific activities of IFN-λ2 observed by others and the absence of data for IFN-λ3 have led to questions as to the biological relevance of IFN-λ2 and IFN-λ3. Our data clearly demonstrate that IFN-λ3 is equal to or more potent than IFN-λ1 with regard to the antiviral activity. This would furthermore explain why mice do not have a functional IFN-λ1 gene[21] but do have functional IFN-λ2 and -λ3 genes. In contrast to the general expectations that IFN-λ2 and -λ3 are redundant proteins with similar antiviral activities, we demonstrate in our study a substantial difference in antiviral activity between the two (Figure 2a and Table 1). IFN-λ1 is glycosylated *in vivo*, but the assumed glycosylation site is lacking in IFN-λ2 or -λ3.[5] This suggests that IFN-λ2 and -λ3 are not glycosylated *in vivo* even though conclusive experiments have not been performed at this time. As we systematically obtained better or comparable EC_{50} values for IFN-λ1 prepared from *E. coli* than others have obtained from protein expressed in mammalian cells with normal glycosylation, there is no indication that glycosylation plays a role in receptor activation. As glycosylation serves to stabilize proteins found in the blood stream, it remains to be determined whether the lack of glycosylation of refolded IFN-λ3 affects its *in vivo* biological activities.

No concise mathematical model exists to describe the activation of IFN receptors and we therefore chose a simple model. We allowed for independent fitting of several parameters: the EC_{50} value, efficacy and the exponential slope (equivalent to the hill coefficient for allosteric activation). The fitted EC_{50} values translate into the IFN concentration at which half of the maximal cell survival is observed. As can be seen in Table 1 and Supplementary Table 1, this value depends upon both the cell system and the virus used for a given IFN.

The maximal cell survival expresses the ability of a given IFN to protect the cell from the virus at saturating concentrations of IFN. In reality, this parameter measures the cell density at the time the MTT assay is performed. This can be affected by several parameters; the anti-proliferative effect of IFN can affect this parameter in a negative way at high concentrations of IFN and the MOI used has a substantial effect on the cell survival. Obviously, the MOI used influences the EC_{50} determinations and, ideally, one should use a standard MOI in all IFN assays. It is, however, difficult to standardize virus administration across all cell lines and virus strains, and we systematically selected the lowest MOI that resulted in a complete lysis of cells not treated with IFN.

It is more difficult to give a direct biological interpretation of the exponential slope (or co-operativity). We, however, fitted four independent experiments for IFN-λ3 representing two independent protein preparations, and the fit of this value is highly reproducible (Supplementary Figure 1). It is worth noting that this value is relatively independent of the subtype of IFN-λ used, but highly dependent of the cell type used. The same is true for IFN-α2b. Thus, this parameter appears to be a characteristic of the receptor complex, signaling and gene activation rather than of the individual cytokine.

Our data highlight the importance of the cell types used when testing for IFN-induced antiviral activity. For

example, we observed a 13-fold difference between the EC_{50} values measured in HepG2 and HuH-7 cells. It is well documented that there is a direct connection between receptor expression and IFN-λ antiviral response.[5,10] Furthermore, the receptor for type III IFN is narrowly distributed compared with the type I IFN receptor.[19] We also observed that different cells respond rather differently to type I IFN, illustrated by an approximately 190-fold difference in EC_{50} values for IFN-α2b against EMCV between the HepG2 and MDBK cells. Thus, correct cell type selection is crucial when comparing the antiviral activity of type I and III IFNs. One might also question the relevance of defining the specific activity of human IFN-α in a bovine cell line that responds extremely well.

When comparing the antiviral activity of type I and type III IFNs against EMCV and VSV (both single-stranded RNA viruses) in bovine kidney cells, we see a striking difference. IFN-α2b differs by 100-fold in activity between EMCV and VSV, and we were unable to show any antiviral effects of IFN-λ3 against VSV even at a concentration of 400 ng ml^{-1}. This difference could be a measure of the different adaptations of VSV and EMCV as VSV is known to infect cattle and EMCV is more common in mice.

In conclusion, our data demonstrate that IFN-λ3 is equal, or superior, to IFN-λ1 in terms of antiviral activity. Furthermore, the glycosylation of IFN-λ1 is not needed for the interaction with the receptor. Despite a 96% sequence identity between IFN-λ2 and -λ3 there is a 16-fold difference in specific activity; this is surprising and it remains to be seen whether IFN-λ2 is to become redundant or it is acquiring novel functions. In the future, it would be interesting to see if the ability of IFN-λ to modulate the T-cell response described by others displays similar subtype specificity as does the antiviral effect.

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References

1 Langer JA, Cutrone EC, Kotenko S. The class II cytokine receptor (CRF2) family: overview and patterns of receptor–ligand interactions. *Cytokine Growth Factor Rev* 2004; **15**: 33–48.
2 Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* 2004; **202**: 8–32.
3 Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998; **67**: 227–264.
4 Young HA, Bream JH. IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications. *Curr Top Microbiol Immunol* 2007; **316**: 97–117.
5 Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK *et al*. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003; **4**: 69–77.
6 Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE *et al*. IL-28, IL-29 and their class II cytokine receptor IL-28R 1. *Nat Immunol* 2003; **4**: 63–68.
7 Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I *et al*. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol* 2004; **34**: 796–805.
8 Onoguchi K, Yoneyama M, Takemura A, Akira S, Taniguchi T, Namiki H *et al*. Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* 2007; **282**: 7576–7581.
9 Meager A, Visvalingam K, Dilger P, Bryan D, Wadhwa M. Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine* 2005; **31**: 109–118.
10 Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J Virol* 2007; **81**: 7749–7758.
11 Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections *in vivo*. *J Virol* 2006; **80**: 4501–4509.
12 Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV, Renauld JC. Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling. *J Biol Chem* 2004; **279**: 32269–32274.
13 Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H *et al*. Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* 2006; **44**: 896–906.
14 Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, Macdonald MR *et al*. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* 2006; **131**: 1887–1898.
15 Jordan WJ, Eskdale J, Srinivas S, Pekarek V, Kelner D, Rodia M *et al*. Human interferon lambda-1 (IFN-lambda1/IL-29) modulates the Th1/Th2 response. *Genes Immun* 2007; **8**: 254–261.
16 Jordan WJ, Eskdale J, Boniotto M, Rodia M, Kellner D, Gallagher G. Modulation of the human cytokine response by interferon lambda-1 (IFN-lambda1/IL-29). *Genes Immun* 2007; **8**: 13–20.
17 Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW *et al*. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006; **12**: 1023–1026.
18 Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 2006; **6**: 836–848.
19 Sommereyns C, Paul S, Staeheli P, Michiels T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells *in vivo*. *PLoS Pathog* 2008; **4**: e1000017.
20 Rubinstein S, Familletti PC, Pestka S. Convenient assay for interferons. *J Virol* 1981; **37**: 755–758.
21 Lasfar A, Lewis-Antes A, Smirnov SV, Anantha S, Abushahba W, Tian B *et al*. Characterization of the mouse IFN-lambda ligand-receptor system: IFN-lambdas exhibit antitumor activity against B16 melanoma. *Cancer Res* 2006; **66**: 4468–4477.

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