Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 534–539

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Measurement of neutralising antibodies to type I interferons by gene expression assays specific for type 1 interferon-inducible 6-16 mRNA

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ARTICLE INFO

Article history:
Received 8 August 2008
Received in revised form 7 November 2008
Accepted 10 November 2008
Available online 19 November 2008

Keywords:
Interferons
Molecular bioassays
Gene induction
Neutralising antibodies

ABSTRACT

Human type I interferon products have been approved for the treatment of several diseases, though neutralising antibodies against them may develop and reduce therapeutic efficacy. Traditionally, potencies of human interferons (IFNs) and of neutralising antibodies (NAbs) against them are quantified by antiviral assays. These are being increasingly replaced by less cumbersome and faster bioassay methods. Since IFNs exert their biological effects by binding to receptors on target cells and stimulating the expression of IFN-inducible genes, measurement of transcribed mRNAs can form the basis of functional bioassays. In this study we have used two approaches, quantitative reverse transcription-polymerase chain reaction (qPCR) and branched DNA (bDNA), to develop efficient, sensitive and robust non-viral assays to quantify type I IFNs *per se* and NAbs in sera from patients treated with either IFNβ or IFNα2a. We show the rapid (4 h) induction of the type I IFN-inducible 6-16 mRNA in A549 lung carcinoma cells is sensitively and reproducibly concentration-dependent for both IFNβ and IFNα2a stimulation, is quantifiable by either approach, and is readily adaptable for the detection and measurement of NAbs against type I IFNs. Quantitative neutralisation of IFN-stimulated 6-16 mRNA expression was achieved in both assays when sera from patients receiving IFNβ or IFNα2a therapy known to contain NAbs against these IFNs were tested. Their rapid and potentially automatable performance strongly suggests these assays could be used in a clinical setting to monitor the development of neutralising antibodies in patients receiving IFN therapy.

1. Introduction

Interferons (IFNs), secreted cytokines with antiviral, antiproliferative and immunomodulatory activities, have been extensively tested as therapeutics in infectious, malignant and chronic autoimmune diseases. To-date, clinical-grade IFNα2a products have been approved for the treatment of chronic hepatitis virus B and C infections and a restricted number of rare cancers, e.g., chronic myeloid leukemia and hairy-cell leukemia. In contrast, IFNβ is widely used in the treatment of relapsing-remitting multiple sclerosis (RRMS) [1]. However, development of neutralising antibodies (NAbs) against such IFN products is associated with reduced therapeutic efficacy in a proportion of patients who develop them while on prolonged treatment regimes [2]. Thus, it is important to monitor patients regularly for the presence of NAbs. Several different assay systems have been developed to measure IFN potency, chief among which are, partly for historical reasons, antiviral assays (AVA) [3]. In AVA, the inhibitory action of IFNs is often determined by measurement of the reduction in cytopathic effect (CPE) of a cytolytic virus in IFN-treated cells [3,4]. These assays, commonly known as cytopathic effect-reduction (CPER) assays, can also be used to measure antagonists of IFNs, such as NAbs, where the protective CPER induced by IFN activity is reduced in proportion to the concentration of NAbs in the test sample [5]. These assays are laborious and time-consuming and frequently display poor reproducibility, mostly due to the inherent variability of challenge virus infection and replication. Thus, to improve reproducibility, alternative bioassays have been developed that do not require virus challenge. One recently favoured for quantifying NAbs is a 2-step protocol involving the induction of the protein MxA by IFN in an A549 cell bioassay followed by the quantitative measurement of MxA by ELISA [6–9]. However, this assay, termed the MxA protein assay (MPA) is as time-consuming as a CPER assay and the ELISA requires specific anti-human MxA antibodies that are not commercially available. Further developments include bioassays based on the IFN-induced expression of a reporter gene. These assays use cells stably transfected with a plasmid containing, for example, the MxA promoter linked to an enzyme such as firefly luciferase. While such assays have been used for IFN potency determinations [3,10], and only more recently for measuring NAbs to IFNs [11,12], they require a genetically modified cell line which is not commercially available. More recently, a new approach to the detection of NAbs to IFNs has made use of sensitive quantitative reverse transcription-polymerase chain reaction (qPCR) technology to determine the

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doi:10.1016/j.jpba.2008.11.010

bioactivity of IFNβ, by measuring the induction of IFN-responsive genes [11,13]. In this study, we have further investigated the utility of assays based on the measurement of gene expression for the detection of NAbs to IFNs. We describe the development of two assays for the measurement of mRNA transcribed from the IFN-inducible gene 6-16, which has recently been shown to encode a 34 kDa glycosylated protein G1P3 localised in mitochondria [14] and is transcriptionally up-regulated in type I IFN treated human cell lines [15,16]. The first assay, using qPCR, requires a shortened period of incubation of the cells with IFN (allowing the qPCR assay to be completed in a single day after cell seeding) and will sensitively measure the potency of IFNα and IFNβ preparations. The second assay uses branched DNA technology to measure 6-16 mRNA levels. Branched DNA quantifies gene expression by directly measuring mRNA without the requirement for RNA extraction and cDNA synthesis and is easily adapted to high throughput applications. Furthermore, we demonstrate that both assays can be used to quantify NAbs present in patient sera.

2. Materials and methods

2.1. Cell culture, IFN and human sera

The A549 human lung carcinoma cell line (CCL 185), originally obtained from ATCC (Manassas, USA), was cultured in DMEM (Sigma–Aldrich Co Ltd., Dorset, UK) supplemented with 10% heat-inactivated foetal bovine serum (HIFBS), 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37 °C, 5% CO_2. Cells were maintained by trypsinisation of the confluent cell monolayer every 5–7 days. The IFNα2a and IFNβ used were the WHO international standards 95/650 [17] and 00/572 [18], respectively.

Serum samples from multiple sclerosis (MS) patients were generously provided by Dr. F. Bagnato (NINDs, formerly at University of Rome, Italy) for the assay of IFNβ NAbs. All samples were taken with informed consent and local Ethical Committee approval, coded and stored at −20 °C. A pre-treatment sample (T0) and a sample (T21) taken at 21 months from the beginning of therapy were evaluated by antiviral assay and the IFN gene expression assays. For the assay of IFNα2a NAbs, serum from chronic myeloid leukemia patients was kindly donated by Dr. D. Russo [19]. Serum from four patients was obtained, with informed consent and local Ethical Committee approval, between 9 and 64 months post-treatment and combined before evaluation by antiviral assay and the IFN gene expression assay.

2.2. IFN antiviral assays

Antiviral cytopathic effect assays were carried out as previously described [4,10]. Briefly, A549 cells were trypsinized and resuspended as a single cell suspension at 5×10^5 cells/ml of cell growth medium (DMEM + 10% HIFBS). Cells were seeded into 96-well microtitre plates at 5×10^4 cells/well (0.1 ml/well) and incubated at 37 °C, 5% CO_2 for 16 h. Serial dilutions of human IFNα2a or IFNβ were prepared from reconstituted WHO International Standards for IFNα2a (95/650: 250 ng/ml) and IFNβ (00/572: 200 ng/ml), respectively, in cell growth medium and then transferred to the assay plates in duplicate rows. Following a 24 h incubation at 37 °C, the culture medium was replaced by 0.1 ml maintenance medium (DMEM + 2% HIFCS) containing encephalomyocarditis virus (EMCV) in all wells, except "no virus" cell controls, to give multiplicity of infection (m.o.i.) of approximately 0.1 plaque forming units (pfu) per cell, based on EMCV infectivity in the A549 cell line. The assay plates were incubated again for 24 h at 37 °C before processing using amido-blue black stain, where cells were washed with PBS and then stained with 0.05% amido blue black in 0.1 M sodium acetate buffer for 30 min at room temperature (RT). The stained monolayers were then fixed with 4% formalin-acetate, washed, dried, stain eluted with 0.1 M sodium hydroxide and the absorbance read at 620 nm (Spectramax 340PC, Molecular Devices). Dose–responses were plotted graphically as absorbance versus IFN concentration. The relative protection of IFN at each concentration tested was calculated as a percentage using the maximum absorbance value and plotted versus IFN concentration.

For evaluation of serum samples for the presence of NAbs to IFNβ and IFNα2a, serial dilutions of test sera were pre-incubated with diluted IFNβ or IFNα2a (10 laboratory units {LU: the LU is defined as the smallest amount of IFN that produces the endpoint of the assay} per milliliter) for 2 h before addition to cells. Assays were processed as described above. The NAb titre was calculated as the dilution of serum that reduced 10 LU/ml of IFN to 1 LU/ml (the normal endpoint of antiviral assays) and was expressed as 10-fold reduction units (TRU) per milliliter according to the Kawade formula [5,20].

2.3. Real-time PCR assays

2.3.1. IFN treatment

A549 cells were cultured in 96-well plates (5×10^4 cells; 0.1 ml/well) in cell growth medium (DMEM + 10% HIFBS) for 16 h. Cells were then exposed to IFNα2a or IFNβ (0–10,000 pg/ml) in assay media (DMEM + 2% HIFBS) for 4 h. In subsequent experiments, IFN was pre-incubated with human serum positive for IFNβ or IFNα2a NAbs for 2 h prior to incubation with cells. For these neutralisation assays, the fixed IFN concentration in LU/ml was calculated from dose–responses of IFN-stimulated increases in 6-16 gene expression.

2.3.2. Isolation of total RNA

Total RNA was extracted from control (unstimulated) and IFN-stimulated A549 cells employing RNeasy spin-column technology with a DNase incubation step to remove residual genomic DNA (Qiagen, Crawley, UK). cDNA was synthesized simultaneously from all RNA samples using MMLV-RT Superscript II (Promega, Southampton, UK). Briefly, oligo$(dT)_{18}$ (1 μg, Promega) and random primers (1 μg, Promega) were added to the RNA (9.5 μl), and the mixture was heated (70 °C, 5 min) to remove secondary RNA structure and then cooled on ice. Dithiothreitol (10 mM), dATP, dCTP, dTTP, and dGTP (all 0.5 mM, Promega), recombinant ribonuclease inhibitor (80 units, RNAsin, Promega), MMLV-RT (200 units), and diethyl pyrocarbonate-treated water were added to make the final volume 20 μl, and the mixture was incubated at 42 °C for 50 min. MMLV-RT was inactivated by heating at 70 °C for 15 min. The cDNA was diluted 1:3 with water and used immediately in PCR reactions or stored at −20 °C for future use. An aliquot of RNA was not reverse-transcribed and was diluted with tRNA and stored at −80 °C to be used to check for genomic contamination (minus RT control).

2.3.3. qPCR

PCR reactions were set up using a CAS automated liquid handling system (Corbett Research UK, Cambridge) with intron-spanning primers specific for the 6-16 gene (GenBank reference BT006850) and a housekeeping gene (GAPDH, GenBank reference NG_007073). Primers were designed (Primer 3 software) so that both 6-16 and GAPDH could be amplified in the same PCR reaction. Primer sequences for human 6-16 were forward; 5′-TGG TCT GCG ATC CTG AAT G-3′, reverse; 5′-CAG GGC ACC AAT ATT ACC TAT GA-3′ and the final PCR product was 111 bp. Human GAPDH primer sequences were forward; 5′-GTC AGT GGT GGA CCT GAC CT-3′, reverse; 5′-CCC TGT TGC TGT AGC CAA AT-3′ and the final PCR product was 251 bp. PCR reactions contained 2× Sensimix (Quantace, London, UK), 50x SYBR green (Quantace), forward and reverse primers (500 nM final concentration), and either sample cDNA (2 μl, 1:3 dilution) or stan-

dard DNA (2 μl, generated from a purified PCR product of the gene of interest or housekeeping gene). PCR products were purified according to the manufacturer's instructions using the Geneclean Turbo kit (Q-Biogene, Cambridge). PCRs were performed on a Rotorgene 6000 (Corbett Research UK, Cambridge) and included 40 cycles consisting of a denaturation step at 95 °C for 5 s, annealing for 10 s at 55 °C, and a 72 °C extension phase for 10 s. Fluorescence measurements were taken at the end of the 72 °C extension phase. The amplification product of each primer pair was subjected to melting point analysis and subsequent gel electrophoresis to ensure specificity of amplification. Data was analysed using two-standard curve and delta–delta ct software. Gene expression was quantified relative to the expression of a housekeeping gene (whose expression had previously been shown not to change with IFNα2a or IFNβ treatment in A549 cells, data not shown) to normalise for differences in tissue loading. Data were then expressed as percentage of maximal 6-16 gene expression relative to an untreated control.

2.4. Branched DNA assays

2.4.1. IFNβ treatment

A549 cells were cultured in 96-well plates (5×10^4 cells; 0.1 ml/well) in cell culture medium (DMEM + 10% HIFBS) for 16 h. Cells were then exposed to IFNβ (0–2500 pg/ml) in assay medium (DMEM + 2% HIFBS) for 4 h. In subsequent experiments, IFNβ was pre-incubated with human serum positive for IFNβ NAbs for 2 h prior to incubation with cells.

2.4.2. Branched DNA plate preparation and signal amplification

Expression of the IFN-stimulated gene, 6-16, and housekeeping genes GAPDH or hypoxanthine phosphoribosyltransferase 1 (HPRT1) in IFNβ treated A549 cells were analysed using branched DNA technology (Quantigene 2.0, Panomics, Italy). Branched DNA plates were prepared according to manufacturer's instructions. Briefly, IFNβ treated A549 cells were lysed in 350 μl lysis buffer (Quantigene 2.0, Panomics) plus proteinase K (0.5 μg/μl) for 30 min at 37 °C. From the cell lysate, 7 μl (equivalent to 1000 cells) was transferred in duplicate or triplicate to the capture plate (Quantigene 2.0, Panomics). Remaining lysed cells were stored at −80 °C. The relevant probe sets for 6-16, GAPDH or HPRT1 (20 μl, Quantigene 2.0, Panomics) were added to the cells or to blank wells as a background control, and lysis buffer added to a total volume of 100 μl. The capture plate was then incubated in a hybridisation oven (Hybridiser HB-1D, Techne, Staffordshire, UK) at 54 °C for 16 h. The plate was then processed according to manufacturer's instructions, with pre-amplifier, amplifier and alkaline phosphate probe

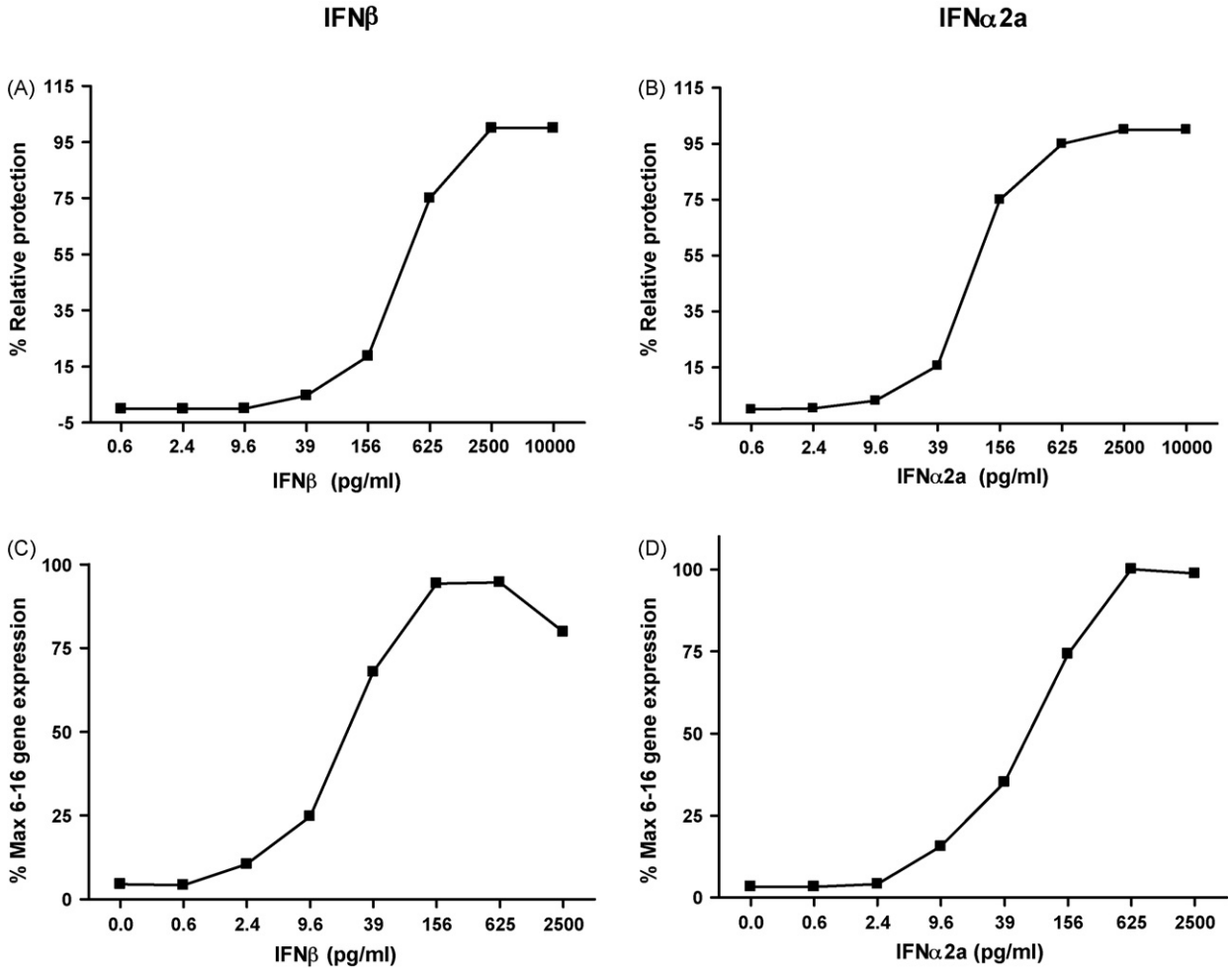

Fig. 1. Type 1 interferon bioactivity assessed by CPER and qPCR assays. IFN bioactivity in A549 cells was assessed by CPER assay (A and B) and by qPCR assay (C and D). For CPER assay, starting concentrations of 10,000 pg/ml IFNβ and IFNα2a were serially diluted four-fold to generate dose–response curves. Graphs are representative dose–responses of IFNβ (A) or IFNα2a (B) concentration vs percentage relative protection, where 10,000 pg/ml IFN was taken as providing 100% protection and 0.6 pg/ml IFN as 0% protection. For qPCR assay, starting concentrations of 2500 pg/ml IFNβ and IFNα2a were serially diluted four-fold to generate dose–response curves. Graphs are representative dose–responses (from six separate assays) of IFNβ (C) or IFNα2a (D) concentration vs percentage of maximal 6-16 gene expression in A549 cells (relative to an untreated control—corresponding to a 20-fold induction with IFNβ and 40-fold induction with IFNα2a).

hybridisation steps. As the final step, a chemiluminescent substrate (100 μl, Quantigene 2.0, Panomics) was added to the capture plate wells and incubated for 5 min before reading in a microplate luminometer (The Reporter, Turner Biosystems, CA, USA) with a 0.2 s integration time. Data were obtained as relative light units (RLU) and the coefficient of variation (CV) was calculated for each sample on the bDNA plate to ensure variation was kept at a minimum. All CVs were below 15%. To normalise for differences in tissue loading, expression of 6-16 was quantified relative to the expression of the housekeeping genes, GAPDH or HPRT1, after background RLU from control wells was subtracted. Data were then expressed as percentage of maximal 6-16 gene expression relative to an untreated control.

3. Results and discussion

3.1. Measurement of IFN bioactivity by antiviral CPER and qPCR assays

Most reported data on the potency of IFN preparations and the activity of NAbs against IFNs has been generated using the CPER assay [5]. In this study, concentration-dependent dose–responses were demonstrated for both IFNβ and IFNα2a in A549 cells using antiviral CPER assays. Fig. 1 shows representative dose–responses for IFNβ (Fig. 1A) and IFNα2a (Fig. 1B) expressed as percentage relative protection against viral CPE provided by the IFN. These data showed increasing protection was afforded to A549 cells from 39 pg IFNβ/ml and from 9.6 pg IFNα2a/ml, indicating a higher antiviral effect of the IFNα2a preparation under the conditions used. However, maximum percentage relative protection was seen at an IFN concentration of 2500 pg/ml for both IFNβ and IFNα2a.

In parallel experiments to the CPER assays, using A549 cells plated under the same conditions, qPCR assays were used to determine IFN activity by quantifying the expression of IFN-inducible gene mRNA levels. Although previous studies reported concentration-dependent increases in MxA mRNA in response to 6.5 h IFN stimulation [11,13], we found IFN preparations did not reproducibly stimulate its expression in a dose-responsive manner after 4 h (the maximal time that would allow the subsequent processing steps of the qPCR assay to be completed in a single day) (data not shown). We therefore turned to the more rapidly induced 6-16 gene as the basis of qPCR assays. Dose–response curves demonstrate reproducible, concentration-dependent, up-regulation of 6-16 gene expression after 4 h stimulation of A549 cells with IFNβ (Fig. 1C) and IFNα2a (Fig. 1D), with maximal 6-16 mRNA levels for both IFNs at a concentration of 625 pg IFN/ml. Data from all assays (for both IFNβ and IFNα2a) are shown in Table 1 as mean values ±SEM. From these data, the limit of detection for both assays was 2.4 pg IFN/ml and the dynamic range was from 2.4 to 156 pg IFN/ml.

Table 1
Mean 6-16 gene expression in IFNβ- and IFNα2a-treated A549 cells measured by qPCR and bDNA assays. Data is expressed as mean ± SEM from 5 or 6 assays.

IFN concentration (pg/ml)	qPCR		bDNA
	IFNβ	IFNα	IFNβ
0.0	4.8 ± 0.10	2.61 ± 0.36	2.60 ± 0.53
0.6	4.86 ± 0.30	2.82 ± 0.39	nd
2.4	8.59 ± 1.51	3.74 ± 0.34	3.54 ± 0.28
9.6	27.07 ± 0.98	14.27 ± 2.08	4.46 ± 0.61
39.0	55.67 ± 10.24	38.01 ± 2.41	12.05 ± 3.13
156.0	86.44 ± 7.96	81.84 ± 5.83	42.10 ± 10.62
625.0	89.65 ± 4.08	89.81 ± 6.46	78.53 ± 10.56
2500.0	87.04 ± 2.79	95.04 ± 3.78	87.00 ± 12.74

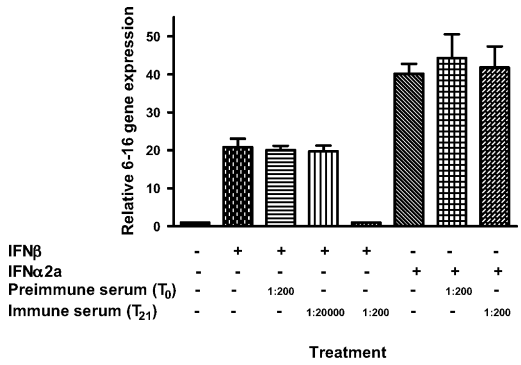

Fig. 2. Measurement of NAbs to IFNβ by assessment of 6-16 gene expression. The expression of the 6-16 gene, relative to an untreated control, was measured by qPCR in A549 cells treated with 156 pg/ml IFNβ or 156 pg/ml IFNα2a with or without dilutions (1:200 or 1:20,000) of serum from a patient prior to receiving IFNβ treatment (T0) or 21 months after commencement of treatment (T21). Data shown is mean ± SEM of duplicate assays. The expression of the 6-16 gene was significantly reduced by the addition of immune serum (T21, 1:200) to IFNβ-treated cells ($P < 0.001$ vs IFNβ alone), with no significant effect of the pre-immune serum (T0) at the same dilution ($P > 0.05$ vs IFN alone). The data also demonstrate that 6-16 gene expression was unaffected by the addition of both pre-immune serum and immune serum to IFNα2a treated cells ($P > 0.05$ vs IFNα2a alone) demonstrating specificity of the NAbs for IFNβ.

3.2. Measurement of neutralising antibodies to IFNβ by antiviral CPER and qPCR assays

To assess whether the measurement of 6-16 gene expression could be used to detect IFNβ NAbs, A549 cells were treated with 156 pg/ml IFNβ or IFNα2a with or without dilutions of serum from a RRMS patient prior to receiving IFNβ therapy (T0; 1:200 dilution) or 21 months after commencement of treatment (T21; 1:200 and 1:20,000 dilutions) (Fig. 2). Expression of 6-16 gene was significantly ($P < 0.001$) reduced by addition of immune T21 serum at 1:200 to IFN-treated cells, with no significant effect of the pre-immune serum (T0) at the same dilution ($P > 0.05$). Larger dilutions, e.g., 1:20,000, of immune T21 serum had no significant inhibitory effect ($P > 0.05$). Furthermore, 6-16 gene expression was unaffected by the addition of both pre-immune T0 serum and immune T21 serum to IFNα2a treated cells ($P > 0.05$) demonstrating specificity of the NAbs for IFNβ. To obtain NAb titres from this assay for comparison with those from antiviral CPER assays, and to investigate whether the assay could be extended to measure NAbs to IFNα2a, further assays were performed using serial dilutions of immune sera (1:200–1:20,000 for IFNβ; 1:200–1:10,000 for IFNα2a) pre-incubated with a fixed concentration of IFNβ or IFNα2a (approximately 10 LU/ml). Fig. 3 shows dilution-dependent neutralisation of IFNβ- or IFNα2a-stimulated 6-16 gene expression (Fig. 3A and B) obtained with dilutions of NAb containing serum from patients undergoing IFNβ or IFNα2a therapy, respectively. Using the Kawade approach [20], a neutralising titre of 4151 ten-fold reducing units (TRU)/ml was calculated for immune T21 serum containing NAbs to IFNβ, in close agreement with a titre of 4800 TRU/ml obtained from analysis of data generated by antiviral CPER assays (Fig. 3C). Reduction of 6-16 mRNA levels was also used to calculate the titre of NAbs to IFNα2a in a sample of combined sera from patients undergoing IFNα2a therapy. This immune serum effectively neutralised IFNα2a-stimulated 6-16 gene expression (Fig. 3B) with a titre of 648 TRU/ml. A 'right shift' in the neutralisation curve was observed in comparison to that obtained by antiviral CPER assay (Fig. 3D), from which a neutralising titre of 1531 TRU/ml was calculated. Such differences in NAb titres between different assay methods are not entirely unexpected and have been reported in other comparative studies using these and other assays platforms [11,21].

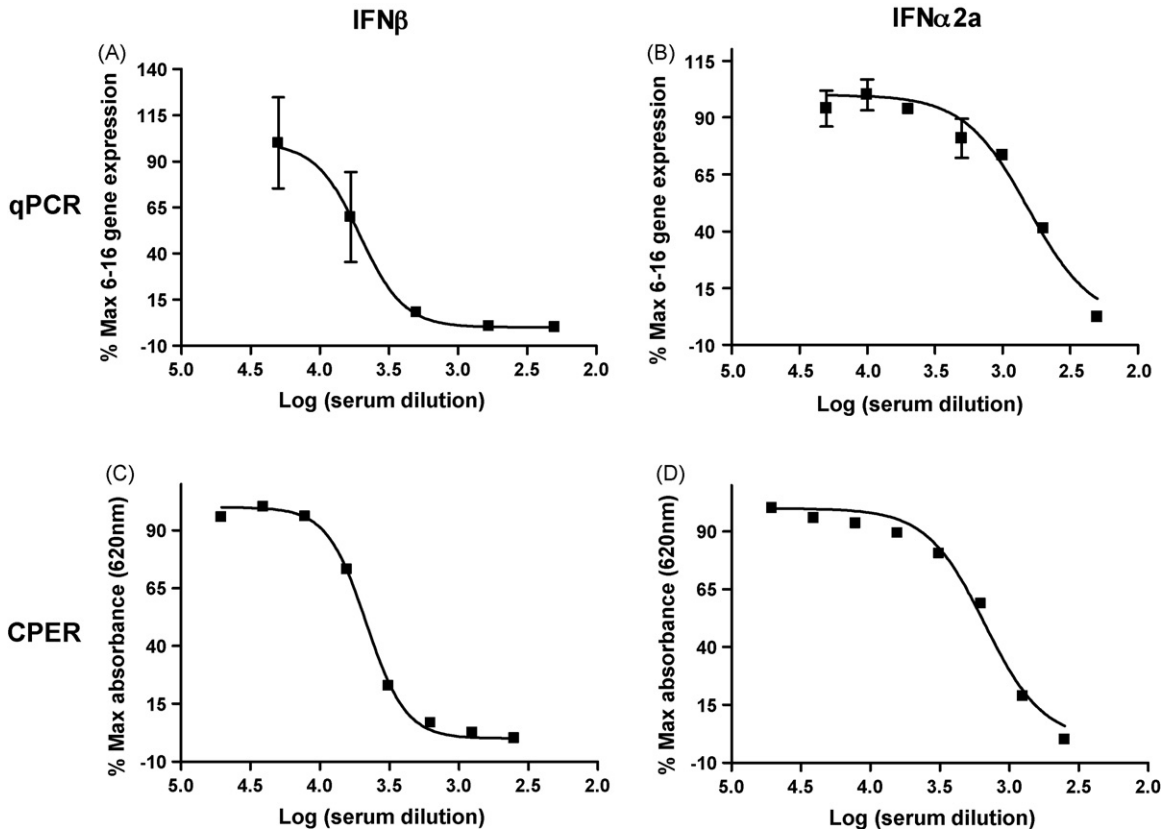

Fig. 3. Quantification of neutralising activity of NAbs to IFNs. The activities of IFNβ (A) and IFNα2a (B) NAbs were measured by qPCR in A549 cells treated with IFNβ or IFNα2a plus a dilution series of patient immune serum. Mean data are expressed as percentage of maximal 6-16 gene expression ± SEM (n = 3 assays). Calculated titres for NAbs were 4151 TRU/ml (IFNβ) and 648 TRU/ml (IFNα2a). The activities of IFNβ (C) and IFNα2a (D) NAbs were also assessed by CPER assay. Mean data are expressed as percentage of maximal absorbance ± SEM from duplicate (IFNβ) or triplicate (IFNα2a) assays. Calculated titres for NAbs were 4800 TRU/ml (IFNβ) and 1531 TRU/ml (IFNα2a) when assessed by CPER assay.

3.3. Branched DNA assays

The measurement of IFN NAbs using qPCR is undoubtedly faster than measurements using the CPER assay but is more expensive. The requirement for a real-time thermal cycler for qPCR, and in many cases a semi-automated RNA extraction platform, does involve an initial financial outlay, though these instruments are becoming more commonplace in laboratories. In addition, the combined requirement to extract and purify RNA, generate cDNA and amplify by PCR is a potential source of variation and error. To further investigate the utility of measurements of gene expression for the detection of NAbs to IFN, we used bDNA technology as an alternative method for the assay of IFN-induced changes in 6-16 mRNA levels. This technique has previously been shown to have utility in the measurement of NAb activity to protein therapeutics [22]. Briefly, RNA molecules are quantified directly in cell lysate through a process of sequential nucleic acid hybridisation steps—the RNA binds to a set of capture extenders, non-specific background is reduced by binding of a set of blocking probes and, finally, a set of label extenders bind to facilitate signal amplification [23]. Signal amplification is mediated by a bDNA amplifier coupled to multiple luciferases, which amplify the reporter signal. As a result, the technique is highly sensitive and eliminates the need to purify RNA, reverse transcribe the RNA into cDNA and then perform PCR. This simplification removes the potential bias introduced during the reverse transcription and amplification steps of RT-PCR and facilitates automation, making bDNA amenable to high-throughput applications. Fig. 4 demonstrates comparative dose responses of IFN-stimulation, in parallel experiments, assessed by qPCR (Fig. 4A) and bDNA (Fig. 4B). The data obtained from bDNA assays show reproducible, concentration-dependent, up-regulation of 6-16 gene expression upon treatment of A549 cells with IFNβ for 4 h with maximal 6-16 mRNA expression at 625 pg IFNβ/ml (Fig. 4B), which is in good agreement with parallel qPCR assays (Fig. 4A). Data shown are expressed as percentage of the maximal increases in 6-16 mRNA relative to an untreated control (corresponding to a 25-fold induction for bDNA assays). Combined data from all assays are shown in Table 1 as mean values ±SEM. From these data, the limit of detection for the bDNA assay was 2.4 pg IFNβ/ml with a dynamic range from 2.4 to 625 pg IFNβ/ml. Next, we investigated whether bDNA assays could detect and quantify NAbs to IFNβ. Thus, a dilution series of immune T21 serum (1:200–1:20,000) was pre-incubated with a fixed concentration of IFNβ to determine neutralising activity (Fig. 4C). In these proof-of-concept experiments, restrictions on sample capacity did not permit the inclusion of IFNβ dose–responses in every bDNA assay plate. As such it was not possible to accurately estimate NAb titres in terms of TRU/ml. Instead, to enable comparison with qPCR and CPER assay systems, we determined the dilution of serum which inhibited the IFNβ-stimulated responses, as shown in Fig. 4B, by 50% to give a value for an IC_{50} (expressed as a reciprocal of dilution). From these data, the IC_{50}, calculated from bDNA experiments, was 5697, which correlated well with IC_{50} values calculated by qPCR and CPER assays which were 5146 and 4657 respectively, demonstrating excellent agreement among the different assays for the measurement of the neutralising activity of NAbs.

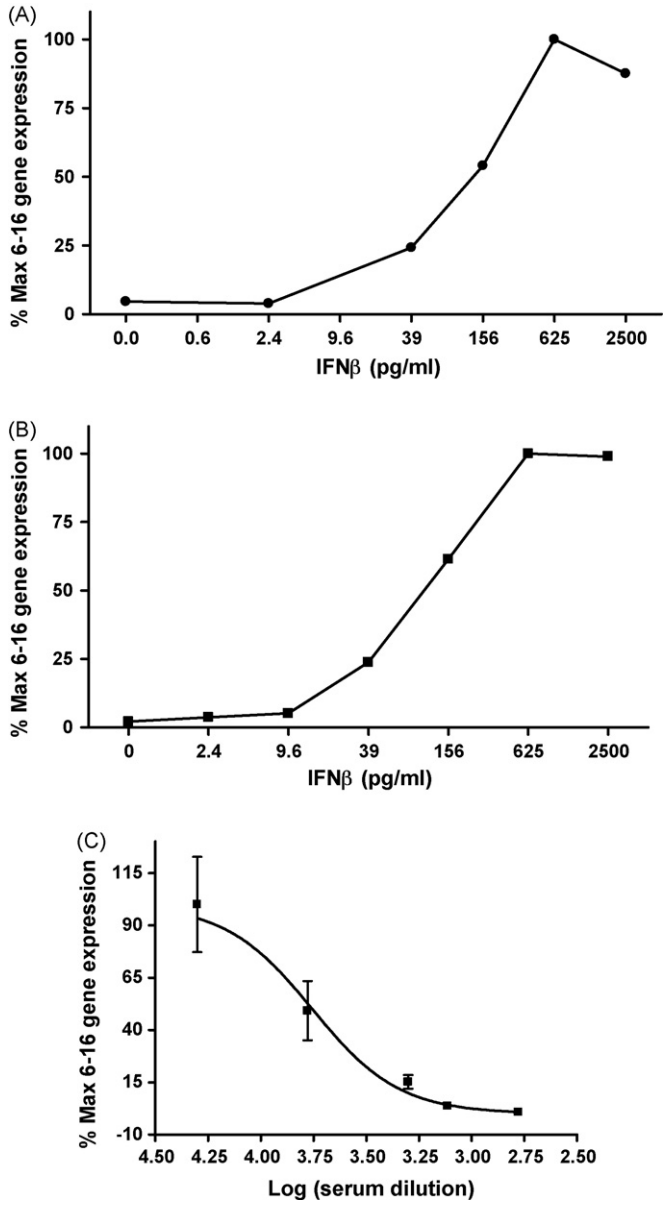

Fig. 4. IFNβ bioactivity and NAb activity assessed by bDNA assay of 6-16 gene expression. The expression of the 6-16 gene was also quantified by bDNA assay in A549 cells treated with serial four-fold dilutions of 2500 pg/ml IFNβ to generate dose–response curves. Panel B shows a representative dose–response from five separate bDNA assays and data are shown as IFNβ concentration vs percentage of maximal 6-16 gene expression. Panel A shows a representative dose–response from parallel qPCR assays for comparative purposes. The neutralising activity of IFNβ NAbs in patient serum was also determined by bDNA assay (Panel C) in which A549 cells were treated with IFNβ plus a dilution series of patient immune serum. Mean data are expressed as percentage of maximal 6-16 gene expression ± SEM ($n = 3$ assays). The calculated IC_{50} value (the dilution of serum which inhibited the IFNβ-stimulated response by 50%) was 5697 (expressed as a reciprocal of dilution).

4. Conclusion

The data presented further highlight the utility of measurements of IFN-inducible gene expression for developing assays to quantify the potency of IFN preparations. Not only would such assays be suitable for IFN product release purposes, but also, since they are readily adapted to quantifying neutralising activity of NAbs to IFN, for monitoring for the appearance of NAbs in patients undergoing IFN therapies. The expression of the IFN-inducible gene 6-16 can be quantified by qPCR assay in a single day and by bDNA assay in two days. This is considerably faster than existing assays based on the reduction of CPE of a viral challenge and eliminates the need to handle viruses. In addition, both qPCR and bDNA assays are amenable to almost complete automation, greatly reducing the 'hands on' time required by the operator. Both assays have the potential for use as a clinical screen for the development of IFN NAbs or for monitoring the efficacy of IFN treatment and although bDNA technology may be more expensive than qPCR in terms of reagent costs, the requirement for equipment is limited to a luminometer, which is likely to be available in many clinical laboratories. In addition, the simplified sample preparation of the bDNA assay lends itself to multi-plate high-throughput analysis of patient samples. qPCR is a more rapid assay platform but requires a relatively expensive initial outlay for a real-time thermal cycler, and although the development of 384-well machines means the current restrictions on the capacity of real-time thermal cyclers is less of a constraint, the RNA extraction step is often the limiting factor for sample throughput. The choice of technology for the measurement of neutralising antibodies to IFN is therefore dependent on both sample capacity and available resources.

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