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Research paper

Improved spectrophotometric human interferon-gamma bioassay

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A B S T R A C T

Interferon gamma (IFNγ) is a cytokine involved in many anti-viral and immunoregulatory processes. One of the major mechanisms through which IFNγ exerts these effects is by inducing expression of indoleamine 2,3 dioxygenase-1 (IDO1), an enzyme that catalyses the first, rate-limiting step of the kynurenine pathway. In this pathway, tryptophan can be catabolised to many products, including picolinic acid and nicotinamide adenine dinucleotide. However, in endothelial cells, the pathway ends at the production of kynurenine. This is due to little or no expression of enzymes that metabolise kynurenine. Production of kynurenine has been used as an indicator of human IDO1 activity, and hence as an hIDO1 bioassay. Due to IFNγ's ability to induce IDO1 expression, kynurenine production can also be a measure of human IFNγ (hIFNγ) bioactivity. Previously, the levels of hIFNγ have been commonly determined by anti-viral assays, high performance liquid chromatography and ELISA. Apart from their technical complexity, these assays are costly and only the anti-viral assay measures bioactive IFNγ. Here, we report the development of an improved IFNγ spectrophotometric bioassay using a human brain endothelial cell line (HBEC 5i). The method is sensitive, easy to perform and cost efficient.

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1. Introduction

Interferons are a family of proteins known for their antiviral and antiproliferative effects. In humans, three distinct forms of interferon have been identified: interferon alpha, beta and gamma, respectively abbreviated IFNα, IFNβ and IFNγ (Lengyel, 1982). All have antiviral and immunoregulatory properties, but Type 1 interferons (IFNα, IFNβ) are more involved in the former whereas Type 2 interferon (IFNy) has a predominantly immunoregulatory role (Bach et al., 1997; Schroder et al., 2004).

Bioactive IFNy has been measured by an interferon anti-viral assay known as the cytopathic protection effect (CPE) assay (Green et al., 1981; Sato et al., 1984; Svedersky et al., 1984). Enzyme-linked immunosorbent assay (ELISA) for IFNy is also commercially available and often used for its measurement (Gibson and Kramer, 1989; Mazurek et al., 2001). However, a limitation of ELISA is that it detects only the protein itself without reflecting its activity.

One of the major mechanisms through which IFNγ exerts its antiviral, antiproliferative and immunomodulatory effects is via the induction of tryptophan catabolism along the kynurenine pathway (Tashiro et al., 1961; Pfefferkorn, 1984; Yoshida et al., 1988; Munn et al., 1999). This is a pivotal pathway involved in numerous physiological and pathophysiological processes, which at the first step is catalysed by three enzymes (Tryptophan 2,3 dioxygenase, TDO; Indoleamine 2,3-dioxygenase 1, IDO1; Indoleamine 2,3-dioxygenase 2, IDO2) that differ in their tissue distribution and expression (Ball et al., 2009). Of these, only IDO1 is reportedly inducible by IFNγ, with its activity being highly increased in pathophysiological or pathological conditions such as cancer, malaria, microbial infection, Alzheimer disease, schizophrenia and organ transplantation (Munn et al., 1998; Sanni et al., 1998; Hansen et al., 2000, 2004; Okamoto et al., 2005; Ino et al., 2006; Takao et al., 2007; Lin et al., 2008; Wang et al., 2010; Weng et al., 2011). The induction of IDO1 is through

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72 an IFNγ-stimulated response element (ISRE) flanking the
73 gene (Caplen and Gupta, 1988; Sen and Lengyel, 1992). Upon
74 the induction of IDO1 activity, tryptophan is metabolised to
75 N-formyl-kynurenine, which can then be converted to kyn-
76 urenine and other metabolites by enzymes present in the tissue
77 (Owe-Young et al., 2008; Ball et al., 2009). Based on this, IFNγ
78 bioactivity has been assessed indirectly by high performance
79 liquid chromatography (HPLC) through measurement of the
80 substrate, tryptophan, and indirect product, kynurenine (Byrne
81 et al., 1986; Mailankot and Nagaraj, 2010). Despite being
82 commonly used, HPLC is a laborious method. Colorimetric
83 measurement of kynurenine with Ehrlich's reagent also has
84 been described (Daubener et al., 1994).
85 It was recently reported that, kynurenine in endothelial
86 cells cannot be converted to subsequent products of the
87 pathway due to the absence or low expression of the necessary
88 enzymes (Hunt et al., 2006; Owe-Young et al., 2008). This
89 fundamental characteristic of endothelial cells suggested that
90 they would be ideal candidates for a human IFNγ bioassay,
91 since they are known to upregulate IDO1 expression in
92 response to the cytokine both in vivo (Hansen et al., 2000)
93 and in vitro (Weiser, 2007; Wang et al., 2010).
94 In this study, we report an optimised and improved
95 spectrophotometric human IFNγ bioassay using an endothelial
96 cell line, human brain endothelial cell (HBEC) 5i. The assay is
97 more sensitive than IDO1 RT-qPCR and simpler to perform than
98 HPLC, ELISA or viral proliferation assays.

99 **2. Materials and methods**

100 *2.1. Cell culture and treatments*

101 The HBEC 5i cell line was cultured in DMEM-F12 without
102 phenol red, supplemented with 10% foetal bovine serum
103 (FBS), 100 μg/mL streptomycin and 100 U/mL of penicillin
104 (15070-063, GIBCO, Life Technologies). Primary human brain
105 microvascular endothelial cells (HBMEC) (Angio Proteomie,
106 cAP-0002) were grown in 100 μL endothelial basal medium
107 EBM-2 (Clonetics CC3156, LONZA) supplemented with 5%
108 (v/v) FBS, ascorbic acid (5 μg/mL), hydrocortisone (1.4 μmol/L),
Q2 109 chemically defined lipid concentrate (CDLC) (1:100 dilution),
110 HEPES (10 mmol/L) and β-FGF (1 ng/mL). Cells were plated at a
111 density of 2×10^4 per well in 96-well flat bottom plates
112 (Corning® Costar® 3599). The following day, the medium was
113 replaced with 100 μL of fresh medium supplemented with
114 400 μmol/L L-Tryptophan with or without recombinant human
115 IFNγ (AbD Serotoc PHP050l) or peripheral blood mononuclear
116 cells (PBMC, Australian Red Cross Blood Service, Sydney)
117 supernatant. For the neutralising experiments, 1 μg/mL of
118 human IFNγ antibody (monoclonal mouse IgG2A Clone 25718,
119 R&D Systems) was mixed with the supplemented medium
120 containing recombinant IFNγ or PBMC supernates, before
121 addition to the plated cells. The cells were further incubated for
122 48 h before supernates were harvested for the kynurenine assay
123 and cells lysed for RNA extraction.

124 *2.2. Stimulating IFNγ expression in peripheral blood*
125 *mononuclear cells (PBMC)*

126 Each well of a 24-well plate (Corning® Costar® 3524) was
127 coated with 220 μL of 1 μg/mL of anti-human CD-3 functional
128 grade purified monoclonal antibody (Clone HIT3a, Catalog no.
129 16-0039-85, eBioscience) overnight prior to the addition of
130 2×10^6 PBMC in DMEM. The cells were incubated at 37 °C for
131 72 h, medium collected thereafter and frozen at −80 °C
132 until further use. To examine whether products of the
133 kynurenine pathway, potentially produced by activated PBMC,
134 might interfere with measurements of kynurenine produced
135 by endothelial cells, a known concentration of kynurenine
136 (200 μmol/L) was added to anti-CD3 activated PBMC superna-
137 tant and phenol red-free 10% FBS supplemented DMEM. The
138 two samples were further diluted in cell medium and assayed
139 using the spectrophotometric method.

140 *2.3. Kynurenine assay*

141 Protein was precipitated from the supernates (80 μL) by
142 the addition of trichloroacetic acid (final concentration 4%
143 w/v) and centrifugation at 16,100 rcf for 10 min at 4 °C. After
144 centrifugation, 90 μL of the supernate was added to an equal
145 volume of 2% w/v Ehrlich's reagent, namely 4-(Dimethylamino)
146 benzaldehyde (SIGMA-156477) in glacial acetic acid (AnalaR,
147 BDH 100015 N). The absorbance was read on a spectropho-
148 tometer (SPECTRAmax 190, Molecular Devices) at a wave-
149 length of 485 nm.

150 *2.4. Reverse transcription quantitative polymerase chain*
151 *reaction (RT-qPCR)*

152 RNA extraction was performed using the RNeasy QIAGEN
153 kit, according to the manufacturer's protocol, and one-quarter
154 of the eluted RNA was used to synthesise cDNA. Briefly, RNA
155 was heat-denatured (70 °C, 5 min) and primed with random
156 hexamers (0.5 μg; 1 μL). Then, 2 μL of 20 mmol/L dNTPs,
157 50 U/μL U Bioscript and its supplied 1× FS reaction buffer
158 (Bioline) were added and the 20 μL reaction incubated for
159 45 min at 37 °C. Following heat denaturation (95 °C for 2 min)
160 the cDNA was diluted 10-fold with water and used for
161 RT-qPCR. The RT-qPCR reaction (20 μL) contained 9 μL cDNA,
162 0.1 μmol/L of each primer in 1× KAPA SYBR Fast Universal
163 qPCR Master Mix (KP-KK4602). Amplification was performed
164 in a Rotorgene 3000 (Corbett Research) with 40 cycles of 95 °C
165 for 15 s followed by 60 °C for 45 s. Expression of *IDO1* was
166 calculated with the ΔΔCt method, with normalisation to the
167 *HPRT1* reference gene. These genes have similar amplification
168 efficiencies, as assessed using serial dilutions of cDNA, and the
169 purity of the PCR products was assessed by melting curve
170 analysis. The primer sequences were: 5′ GGA GCT ACC ATC TGC
171 AAA TCGT 3′ and 5′ TGG CTT GCA GGA ATC AGG AT 3′ for
172 human IDO1 and 5′ CGT CTT GCT CGA GAT GTG ATG 3′ and 5′
173 GGG CTA CAA TGT GAT GGC CT 3′ for human HPRT.

174 *2.5. Cytometric bead array (CBA)*

175 Human IFNy levels in the PBMC supernatants were ana-
176 lysed using an optimised CBA using the CBA human soluble
177 protein master buffer kit (Becton Dickinson Biosciences,
178 Catalog no: 558264) And A Human Soluble Ifnγ Flex Set (Bd
179 Biosciences, Cat. no: 560111). Using the principles of CBA, the
180 fluorescence intensity of hIFNγ was measured on the flow
181 cytometer (Cytomics FC500; Beckman Coulter, Fullerton,

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