

Type I interferon subtypes produced by human peripheral mononuclear cells from one normal donor stimulated by viral and non-viral inducing factors

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ABSTRACT. Through the activation of Toll-like receptors (TLRs) or cytosolic RNA helicases, a large number of pathogenic or synthetic components can induce the transcription of genes coding for type I interferons (IFNs). This family of related cytokines includes notably, a single IFN- β protein and 13 different IFN- α subtypes, whose biological activities are probably not the same. The aim of this study was to characterize the type I IFN subtypes produced *in vitro* by human peripheral blood mononuclear cells (PBMCs) in response to specific inducers. Thus, PBMCs obtained from a single donor, were exposed to various agents including Sendai virus, *Herpes simplex virus-1* (HSV-1), poliovirus-IgG complexes and serum from a patient with systemic lupus erythematosus (SLE). Six hours later, mRNA was extracted and amplified by RT-PCR using primers which recognize IFN-B mRNA and the different IFN-A mRNA subtypes. IFN-A subtypes were identified by cloning and sequencing the amplification product. Antiviral activity was assayed in supernatant at 18 hours. Human PBMCs were found to express constitutively type I IFNs mRNA. Antiviral activity and expression of IFN-A and IFN-B mRNA increased with each inducing agent. Although almost all the IFN-A subtypes were detected, their relative abundance appeared to be dependent upon the inducing agent. Incubation of PBMCs with a neutralizing monoclonal antibody directed against the type I IFN receptor (IFNAR) did not affect the level of antiviral activity in the supernatant of induced PBMCs. Our results suggest that the level of IFN- α expressed by PBMCs cells is independent of IFNAR feedback signalling and that the nature of the inducing agent modifies the pattern of IFN-A subtypes preferentially expressed by these cells.

Keywords: induction, type I interferon subtypes, virus, human PBMC, systemic lupus erythematosus

Type I interferons (IFNs) are produced during the initial stages of the innate immune response, following the interaction of conserved molecular components of pathogens with Toll-like receptors (TLRs) or cytosolic RNA helicases. IFNs play a determining role in the subsequent development of the innate and later antigen-specific adaptive immune responses and constitute an important component of host defence against virus infection prior to the production of specific antiviral antibody. In man, type I IFNs constitute a family of at least 20, closely related IFN- α genes and a single IFN- β gene clustered on the short arm of chromosome 9 [1]. These intronless genes encode 13 functional, structurally related, IFN- α subtypes and a single IFN- β molecule. Although the biological signifi-

cance of the existence of several closely related IFN- α proteins is unclear, several reports suggest that IFN- α subtypes exhibit quantitatively distinct patterns of antiviral, growth inhibitory, and natural killer (NK) cell-stimulatory activities [2, 3]. The biological activity of all the IFN- α subtypes and of IFN- β is mediated by binding to a common, ubiquitously expressed heterodimeric receptor composed of the type I IFN receptor (IFNAR)-1 and IFNAR-2 subunits.

Virus infection of fibroblasts has been reported to induce predominantly IFN- β , while virus infection of human peripheral blood mononuclear cells (PBMCs) leads to the production of numerous IFN- α subtypes. Feldman *et al.* [4] showed that, *in vitro*, enveloped viruses but not non-enveloped viruses elicit an IFN- α response from PBMC and that non-monocytic, natural IFN- α -producing cells were responsible for the majority of IFN- α production. Although IFN- α subtypes are produced by the appropriate

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stimulation of virtually all cells, plasmacytoid dendritic cells (pDCs), derived from both myeloid- and lymphoid-committed progenitors, produce up to a thousand-fold more type I IFN- α than other cell types [5, 6].

Numerous enveloped viruses induce production of IFN- α in PBMCs *in vitro* including Sendai virus and other paramyxoviridae, *Herpes simplex* virus (HSV), influenza virus, and vesicular stomatitis virus (VSV) [4]. The IFN- α inducing capacity of enteroviruses, poor IFN inducers by themselves, is dramatically enhanced in the presence of virus-specific IgG [7]. Viral nucleic acids (RNA or DNA) produced during virus replication or contained in the viral genome are thought to be the principal signal for inducing the transcription of type I IFN genes via TLRs [8]. Other virus components are also capable of inducing the production of type I IFNs however, including certain viral glycoproteins [9, 10], although the relevant signalling pathways have not been identified except TLR4 for the VSV glycoprotein [11]. IFN- α is also produced, during the course of certain pathological conditions, in which virus does not appear to be involved, including familial encephalopathy-Aicardi-Goutieres syndrome [12] or the active phase of systemic lupus erythematosus (SLE) [13, 14]. Recent evidence suggests that type I IFN system is critically involved in the pathogenesis of SLE [15]. A variety of non-viral IFN inducers have also been described, including prokaryotic, unmethylated CpG containing DNA [16], synthetic double-stranded RNA (poly[I:C]) [17], lipopolysaccharide [18], imiquimod derivatives [19], single-stranded RNA [20], and some short interfering RNA [21].

It is important to determine the pattern of IFN subtypes produced in relation to the type of inducer, given that type I IFNs present distinct biological activities which could influence outcome under certain pathological conditions. Thus, we have analyzed IFN subtypes in response to different inducers; an enveloped DNA virus, an enveloped RNA virus, an inactivated RNA poliovirus-antibody immune-complex, and a sample of serum from a patient with SLE. Variation in IFN- α subtype production was examined as a function of time following induction with HSV-1, and neutralizing antibody against the IFNAR1 chain of the human type I IFN receptor was used with each inducer to determine the effect of IFN- α feedback on IFN- α production.

METHODS

Cells and virus

A suspension of peripheral blood cells enriched in mononuclear cells was collected from a single healthy donor during a blood platelet donation using a cell separator (Etablissement Français du Sang, Paris). PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, France), washed twice in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and stored in aliquots with 10% dimethylsulfoxide under liquid nitrogen.

HSV-1 (Shealey strain) was propagated and titered on Vero cells. Stocks of Sendai virus (E72) were obtained after infection of embryonated chicken eggs and had a titer of 10^8 infectious doses/ml. Formalin-inactivated poliovirus

vaccine was obtained from Pasteur Merieux MSD (Lyon, France). It contained a mixture of three poliovirus serotypes.

IFN- α induction in PBMCs

Human PBMCs (1.5×10^6 cells) were incubated at 37°C under 5% CO₂ in 0.5 ml RPMI supplemented with 10% FBS with or without the inducing agent. After a period of incubation of 6 or 18 hours, PBMCs were pelleted and lysed in a guanidinium isothiocyanate-phenol solution (TRIzol®, Invitrogen, France). The samples were stored at -80°C until extraction of total RNA. Simultaneously, supernatants were harvested and used to assay IFN- α .

For induction, Sendai virus and HSV-1 were added to PBMC cultures at a final concentration of one infectious unit per cell. Poliovirus vaccine was added to the cells after pre-incubation for 45 min with 500 µg/ml of polyvalent human IgG (Tegeline®, prepared by the Laboratoire Français du Fractionnement et des Biotechnologies, France). This IgG preparation contained a high titer of neutralizing antibodies against poliovirus. Fifty microlitres of a serum sample, collected from a patient with active SLE and known to induce IFN- α from donor's PBMCs, were also used as an inducing agent.

In some experiments, PBMCs were incubated, prior stimulation, with different concentrations (10 to 200 µg/ml) of a neutralizing monoclonal antibody (MAb) directed to type I IFN receptor (IFNAR1, clone 64G12) [22]. A MAb to Mx protein (a generous gift of Michel Horisberger), was used to check, by immunofluorescent staining, the ability of the IFNAR-MAb to inhibit the activity of recombinant IFN- α 2 Introna (Schering-Plough, Levallois-Perret, France) on PBMCs.

IFN- α assay

Supernatants from control and stimulated PBMC cultures were collected 18 hours after induction, subjected to serial two-fold dilutions in a 96 micro-well plate, and IFN- α content was determined by cytopathic reduction in vesicular stomatitis virus (VSV)-infected Madin-Darby bovine kidney (MDBK) cells [23]. A laboratory reference of human IFN- α , which had been standardized against the NIH Ga 023-902-530 reference preparation, was included with each titration. Interferon titers are expressed as international units per milliliter.

Polyclonal antibodies to IFN- α 2 were obtained from the serum of a patient monitored for the presence of antibodies during treatment with recombinant IFN- α 2. The serum titered 1/12,500 against 10 IU /ml of IFN- α 2 and was used at a dilution of 1/10 in the neutralisation test.

Amplification of type I interferon mRNA

Total RNA was isolated from 1.5×10^6 PBMC by homogenization in TRIzol®, followed by addition of chloroform and precipitation of the samples with isopropanol following by washing with 70% ethanol. Before reverse-transcription, the pellet was treated with 40 U of RNase-free DNase I (Roche Molecular Biochemicals, France) for 30 min at 37°C to eliminate possible DNA contamination. DNase was then removed using a second extraction step. The absence of genomic DNA was verified for each

sample by the lack of amplification in the absence of reverse transcription. The RNA extract was then transformed into cDNA by reverse-transcription using random hexamers as primers. IFNA, IFNB and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA were then amplified from this mixture by PCR. A consensus oligonucleotide primer pair was designed to amplify the coding sequence of all known IFNA genes. The predicted size of amplification product was 375-bp, corresponding to the coding sequence from amino acids 27 to 151 of the mature IFN protein. The sequences of oligonucleotides used were as follows: sense primer (5'-TTCCTCCTGYYTGAWGGACAGA-3'), antisense primer (5'-GATCTCATGATTCTGCTCTGACA-3'). A second primer pair was chosen to create a 470-bp PCR product from the IFNB cDNA [24]. A portion of 165-bp of the G3PDH cDNA, which is expressed constitutively in all tissues, was amplified to verify adequate nucleic acid extraction, reverse transcription and amplification. An aliquot of the cDNA corresponding to the extraction of approximately 50,000 cells was amplified. The specific DNA fragments were detected by ethidium bromide staining after electrophoresis through a 1.5% agarose gel, followed by DNA blotting and hybridization with digoxigenin-labeled oligonucleotide probes as described previously [7].

Cloning and sequencing of IFN-A PCR products

Purified IFN-A PCR products were cloned into the pGEM[®]-T Easy Vector (Promega, France) and used to transform *E. coli* JM109 competent cells according to the manufacturer's instructions. After overnight incubation at 37°C, the white colonies were selected and the presence of the insert was checked by PCR with T3/T7 promoter primer pairs. For each of the inducing conditions, the DNA from randomly selected clones was sequenced using the T7 promoter primer on a ABI 377 sequencer with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA). The nucleotide sequences were then analyzed and compared to IFNA sequences available from the GenBank database in order to identify the subtype represented by each clone. A PCR product obtained from genomic DNA extracted from PBMCs was analyzed to verify the ability of the cloning and sequencing procedures employed to detect all IFN- α subtypes.

Statistical analysis

The distributions of IFN- α subtypes were compared by Fisher's exact test. When pairwise group comparisons were performed after a global test, Hochberg's corrections for multiple testing were used. All tests were two-sided at a 0.05 significance level.

RESULTS

Expression of type I interferons after exposure of PBMC to inducing agents

The antiviral activity present in the supernatant of PBMC cultures 18 hours after exposure to a variety of inducing agents was determined using the MDBK/VSV bioassay. Mean IFN titers of 37, 600, 2,000, and 3,000 IU/ml were

observed following induction with serum from a patient with SLE, poliovirus-IgG complexes, HSV-1 and Sendai virus respectively. The antiviral activity detected was characterized as principally IFN- α by immunoneutralization with specific antisera [7]. IFN activity was not detected in un-induced PBMC or in control cultures, exposed to IgG alone, or to serum samples from healthy subjects, using a bioassay with a limit of detection of 2 IU/ml. The expression of type I IFN was also studied at the transcriptional level. As the type I IFN genes are intronless, RNA extracts were treated with DNase I before reverse-transcription (RT) in order to remove traces of genomic DNA. A control PCR reaction without RT, confirmed the absence of genomic DNA in each RNA preparation. Constitutive expression of both IFN-A and IFN-B was detected in RNA extracted six hours after culture of human PBMCs in the absence of inducer. Expression of both IFN-A and IFN-B mRNA was markedly enhanced however, six hours after incubation of human PBMCs with each of the four inducers studied: Sendai virus, HSV-1, poliovirus-IgG complexes and serum from a patient with SLE (data not shown).

In order to determine whether IFN genes are subject to autocrine regulation in this system, PBMCs were incubated, before induction, in the presence of a neutralizing MAb, clone 64G12, directed against the IFNAR1 chain of the human type I IFN receptor. Incubation of PBMCs with this MAb at concentrations ranging from 10 to 200 μ g/ml prior to induction with the different agents did not cause any significant variation in antiviral activity detected in the supernatant. In contrast, incubation of either frozen or fresh PBMCs with the anti-IFNAR1 MAb, at a concentration of 100 μ g/ml (but not 10 μ g/ml), caused a marked decrease in the expression of the IFN-induced protein MxA, determined by immunofluorescence staining, following treatment of PBMCs with 10 or 1,000 IU/ml of recombinant human IFN- α 2.

Identification of IFNA subtypes produced constitutively and after stimulation by inducing agents

The subtype composition of IFNA mRNA present in PBMCs six h after induction was determined by cloning and sequencing of the PCR products. The IFNA1 and IFNA13 genes, which differ only in a few nucleotides in the 5' and 3' non-coding regions, have identical coding sequences and give rise to a single protein species [25]. The RNA transcripts of these genes cannot be distinguished by the procedure employed in this study. The ability of the procedures employed to detect all IFN- α subtypes was verified by analysis of 32 clones obtained from amplification of genomic DNA extracted from un-induced PBMC collected from the single donor. All the IFN-A subtypes were detected except A6, A8, and A14 in proportions ranging from 6.3 to 18.8%.

Five IFN subtypes were expressed constitutively (A4, A5, A8, A17, A21), of which IFNA5 comprised 65.5% of the total (table 1). Six hours post-induction, all the IFNA subtypes were detected except IFN-A6, which did not give rise to detectable transcripts in any of the experiments. The IFN-A6 gene contains a deletion of 12 nucleotides, from position -61 to -73, within its putative promoter region that may prevent expression [26].

Table 1
IFN- α subtypes (%) identified by sequencing of randomly selected clones derived by RT-PCR from RNA isolated from human PBMCs

Number of clones tested	Uninduced PBMCs n = 29	Induced PBMCs n = 136
IFNA1/A13	0	17.6
IFNA2	0	8.1
IFNA4	10.3	6.6
IFNA5	65.5	17.6
IFNA6	0	0
IFNA7	0	4.4
IFNA8	3.5	5.9
IFNA10	0	1.5
IFNA14	0	5.9
IFNA16	0	2.9
IFNA17	3.5	14.0
IFNA21	17.2	15.4

PBMCs : peripheral blood mononuclear cells. Inducing agents were Sendai virus, HSV-1, poliovirus-IgG complexes and SLE (systemic lupus erythematosus) serum. The most predominant subtypes are indicated in boldtype. Comparison between these two distributions : $p = 0.00013$ (Fisher's exact test).

For all of the inducers studied, the most abundant subtypes detected were IFNA1, A5, A17 and A21, comprising 17.6, 17.6, 14 and 15.4% respectively of the clones analyzed, while A2, A4, A7, A8 and A14 were detected at a frequency of between 6 to 8% each. IFN subtypes A10 and A16 were more rarely detected. Comparison of IFN subtype distribution using Fisher's exact test revealed a significant difference ($p = 0.00013$) between constitutive and induced expression in PBMC. Eight to 10 different subtypes were detected for each of the four inducers studied (table 2). IFNA21 was the most frequent IFN subtype induced with serum from a patient with SLE, while IFNA8 occurred more frequently following induction with Sendai virus than with the other inducers tested. HSV-1 induced a different profile of expression from the three other inducers tested, and was characterized by the absence of IFNA2. Furthermore, polyclonal antibodies directed against IFN- α 2 did not neutralise, even partially, the IFN activity induced by HSV-1. Although a statistically significant difference was observed in the distributions of the IFNA subtypes obtained with the four inducers ($p=0.0035$) when compared globally, no statistically significant differences were observed, when one distribution was compared individually with another (Fisher's exact test with Hochberg's correction). Nevertheless, the variation in the level of expression of the IFNA2, A8, A14, A21 subtypes appeared to be greater than that of subtypes A5, A7, A10, and A16 at least with the inducers studied.

Analysis of IFN- α expression six and 18 hours after induction of PBMCs with HSV-1 revealed a statistically significant difference in IFNA subtype expression with respect to time ($p=0.006$). Thus, the relative expression of IFNA2, A4, A10, and A16 mRNA increased between six and 18 hours post-induction (figure 1). The proportions of IFNA5, A7, A21 mRNA remained unchanged while the expression of IFNA1, A8, A14 and A17 decreased markedly between six and 18 hours post-induction.

DISCUSSION

In order to determine whether the pattern of human type I IFN subtype expression is specific to a particular inducer, mRNA was extracted from human PBMCs stimulated with various inducers, amplified by RT-PCR using universal primers designed to recognise IFN-B mRNA and all the human IFN- α mRNA, cloned, and multiple clones sequenced. Subcloning and sequencing is considered to be the most reliable procedure for identifying individual IFN- α subtypes since the nucleotide differences between the genes encoding IFN- α subtypes are too small to discriminate between them by hybridisation with specific probes. To evaluate the intrinsic variability of the techniques employed, IFNA PCR products were first cloned from genomic DNA using the same primers that were to be used for mRNA, and variations in cloning frequency were shown to be within acceptable limits. Furthermore, in order to avoid genetic variation among individuals, IFN subtype expression was analysed using mRNA extracted from PBMCs from a single donor.

Constitutive expression of certain IFN- α subtypes was detected in human PBMCs in agreement with previous reports [27]. Although IFNA5 was the most abundant subtype expressed constitutively, as has been reported previously [24], constitutive expression of IFNA21 and A4, more rarely IFNA8 and A17 was also detected. Each of the inducing agents tested induced a mixture of IFN- α subtypes in a pattern that appeared to be dependent upon the nature of the inducing agent. Although previous studies have shown that different IFN- α subtypes are expressed following induction, few data are available on their respective quantitative abundance. Induction with Sendai virus resulted in marked increases in the expression of IFNA1, A2, A5, A8 and A17, which together represent more than 85% of total IFN mRNA. Previous studies have also reported IFNA1 to be a major component of virus-induced IFN production in human cells, even though the specific anti-viral activity of IFN- α 1 is markedly less than that of other IFN subspecies such as IFN- α 2 [3]. Thus,

Table 2
IFN- α subtypes (%) identified by sequencing of randomly selected clones derived by RT-PCR from RNA isolated from human PBMCs six hours after stimulation

Inducing agents Number of clones tested	HSV-1 n = 34	Sendai virus n = 32	Poliovirus-IgG n = 34	SLE serum n = 36
IFNA1/A13	23.5	18.8	11.8	16.6
IFNA2	0	15.6	11.8	5.6
IFNA4	2.9	3.1	11.8	8.3
IFNA5	14.7	18.8	20.6	16.6
IFNA7	5.9	3.1	2.9	5.6
IFNA8	5.9	15.6	0	2.8
IFNA10	2.9	0	0	2.8
IFNA14	14.7	0	8.8	0
IFNA16	2.9	0	2.9	5.6
IFNA17	8.9	18.8	20.6	8.3
IFNA21	17.7	6.2	8.8	27.8

PBMCs : peripheral blood mononuclear cells. SLE : systemic lupus erythematosus. The most predominant subtypes are indicated in boldtype. Global comparison of the distributions obtained with the 4 inducing agents : $p=0.0035$ (Fisher's exact test). Each distribution was also compared to the others but no one combination lead to a statistically significant result.

Nyman *et al.* [28] showed that IFN- α 1 and IFN- α 2 constituted the major IFN components (approximately 50%) of a highly purified IFN- α preparation obtained from Sendai virus-induced human PBMCs. Seven other IFN- α subtypes were also identified as minor components of the IFN preparation. Other studies have reported IFN- α 1, IFN- α 2 and IFN- α 4 to be major components, and IFN- α 5, IFN- α 7, IFN- α 8 and IFN- α 14 to be minor components of virus-induced PBMCs [24, 26].

Even though we did not study IFN subtype production in individual cellular components of human PBMCs, the pattern of IFN- α subtype expression observed following treatment of human PBMCs from a single donor with HSV-1 was similar to that previously reported for HSV-1 infected pDCs [29]. Our results differ from those obtained

using an HSV-1-infected cell lysate instead of the infectious virus employed in the present study [30]. It is of interest that IFN- α 8 and IFN- α 2, which exhibit a high specific antiviral activity [31], are minor components of HSV-1-induced IFN production and major components of Sendai virus-induced IFN production in human PBMCs. The differences in the pattern of IFN subtype expression in Sendai virus and HSV-1-induced PBMCs may reflect differences in the phenotype of the IFN-producing cell in response to a particular inducing agent. Thus, both monocytes and pDCs may be recruited by Sendai virus in contrast to the recruitment of pDCs only with HSV-1. Indeed, monocytes have been identified as the predominant IFN- α -producing cells in response to Sendai virus infection of human PBMCs, but not in response to HSV-1 infection [5, 32].

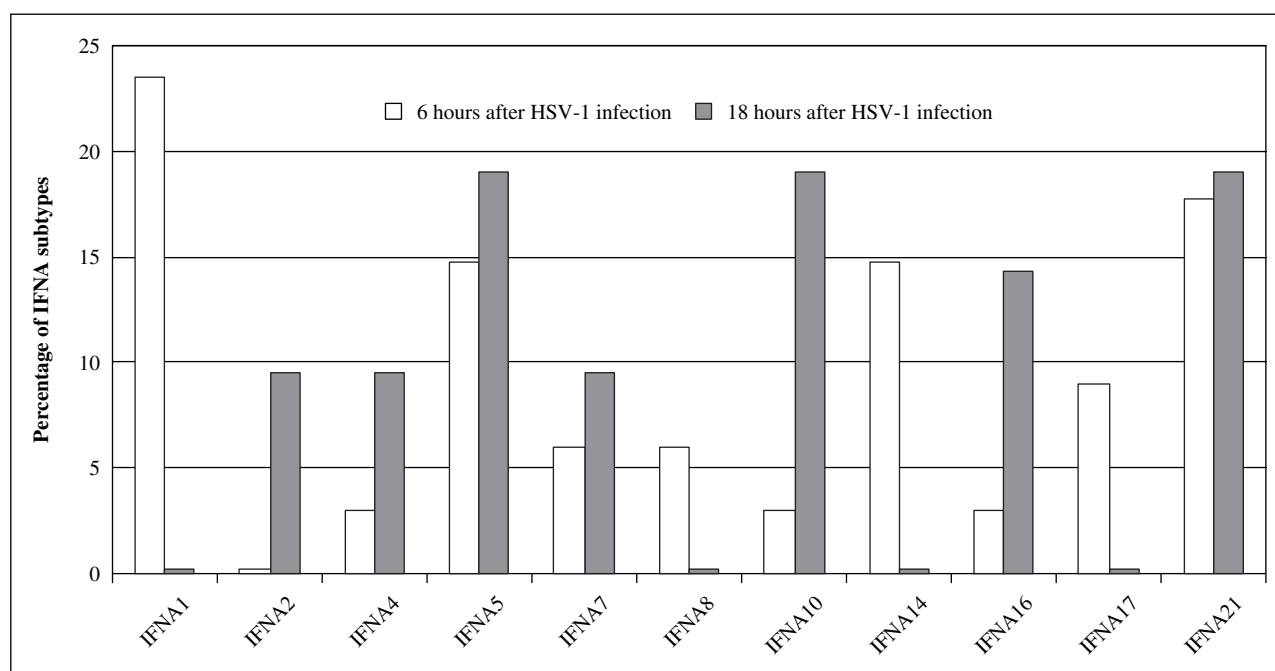


Figure 1

Distribution of the IFN- α subtypes expressed 6 and 18 hours after infection of human PBMCs with HSV-1. Data obtained by sequencing of 34 clones (6 h) and 21 clones (18 h), see materials and methods. Comparison between these two distributions : $p = 0.006$ (Fisher's exact test).

To our knowledge, no data are available concerning the pattern of IFN subtype expression produced in response to the other inducing agents analysed in this study, namely serum from a patient with SLE and a preparation of poliovirus-antibody complexes. The results obtained in this study show that IFNA21, which has a relatively low specific antiviral activity, was the predominant IFN subtype produced in response to induction with serum from a patient with SLE. Although the cells which produce IFN in response to serum from patients with SLE, or inactivated poliovirus-antibody complexes are supposed to be pDCs as in the case of HSV-1 virus induction [6, 7, 33], some differences were observed in the pattern of IFN subtype expression observed in response to these inducing agents. Such differences may be due to the diversity of activation of Toll-like receptors involved in type I IFN production. Although various viral components are able to activate several different TLRs [34], the available data do not allow a particular pattern of IFN subtype expression to be linked specifically to activation of either TLR-7, 8, or 9 signalling through the MyD88 pathway [35] or via TLR3 and the MyD88 independent pathway [36]. Indeed, single stranded RNA from inactivated polioviruses or Sendai virus may activate TLR-7 and -8, while double-stranded RNA from the replicative form of Sendai virus may also activate TLR3 [37]. Furthermore, unmethylated CpG-rich DNA present in immune complexes contained in serum from patients with SLE [38] and HSV-1 DNA may activate TLR9 [39].

It has been reported previously that the levels of IFN- α and IFN- β mRNA are maximal at 6h post infection and decline some 20 fold at 21 hours after induction [29]. Thus, most of the reported studies analysing IFN subtype expression in PMBCs have examined IFN production at early rather than late time points. We have shown however, that the pattern of IFN subtype expressed after HSV-1 stimulation varies as a function of time. This observation needs to be confirmed by other experiments. In our study with human PBMC, a neutralizing MAbs directed against the IFNAR1 chain of the human IFN receptor did not change the level of antiviral activity assayed at 18 hours. The initial production of type I IFN seems independent of IFN receptor feedback regulation. In agreement with studies in mice showing that unlike fibroblasts, pDCs can produce IFN- α early after induction and that this process is IFN receptor-independent [40, 41]. This capacity of pDCs to produce rapidly type I interferons is probably due to constitutive expression of IRF7 in these cells [41, 42].

The results of this study emphasise the importance of analysing IFNA subtypes expression in order to improve our understanding of the biological significance of the differential regulation of specific IFN- α genes. The differences in IFN- α subtype expression observed with different inducing agents may be related to differences in TLR signaling resulting in differences in the regulation of individual IFN genes at the transcriptional level or to differences in the type of stimulated cells. Further studies will be required in order to determine the relative influence of such factors on IFN- α subtype expression. Detailed characterisation of subtypes of type I interferon produce in the course of SLE disease would be very helpful at a moment when several anti-interferon approaches are being developed as candidate treatment for this disease.

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