Human Reproduction Vol.23, No.9 pp. 2095–2103, 2008 doi:10.1093/humrep/den207
Advance Access publication on June 20, 2008

Antiviral responses of human Leydig cells to mumps virus infection or poly I:C stimulation

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BACKGROUND: The immuno-privileged status of the testis is essential to the maintenance of its functions, and innate immunity is likely to play a key role in limiting harmful viral infections, as demonstrated in the rat. In men mumps virus infects Leydig cells and has deleterious effects on testosterone production and spermatogenesis. The aim of this study was to test whether mumps virus infection of isolated human Leydig cells was associated with an inhibition of their innate antiviral defences. METHODS: Leydig cell production of mRNA and protein for interferons (IFNs) and of three antiviral proteins—2′5′ oligoadenylate synthetase (2′5′OAS), double-stranded RNA-activated protein kinase (PKR) and MxA—was investigated, in the absence or presence of mumps virus or viral stimuli including poly I:C, a mimetic of RNA viruses replication product. RESULTS: Stimulated or not, human Leydig cells appeared unable to produce routinely detectable IFNs α, β and γ. Although the level of PKR remained unchanged after stimulation, the expression of 2′5′OAS and MxA was enhanced following either mumps virus or poly I:C exposure ($P < 0.05$ versus control). CONCLUSIONS: Overall, our results demonstrate that mumps virus replication in human Leydig cells is not associated with a specific inhibition of IFNs or 2′5′OAS, MxA and PKR production and that these cells display relatively weak endogenous antiviral abilities, as opposed to their rat counterparts.

Keywords: humans; Leydig cells; mumps virus; interferons; antiviral proteins

Introduction

Viral infections of the testis represent a threat to the species as they may lead to the sexual dissemination of pathogens. Furthermore, virus replication can result in decreased production of spermatozoa ultimately causing sterility, and/or testosterone deficiency (reviewed in Dejucq and Jegou, 2001). The immuno-privileged status of the testis is essential to the maintenance of its endocrine and exocrine function (Maddocks and Setchell, 1990) and its disruption through the infiltration of blood-borne leukocytes associated with the pathogen-induced inflammation of the testis, named orchitis, is known to contribute to the impairment of sperm production (reviewed in Hedger and Meinhardt, 2003). In this context, it is important to understand whether innate immunity can limit replication of viruses in the human testis and hence the harmful intrusion of acquired immune cells, and how some viruses may escape this defence system.

Interferons α/β (type I IFNs) are crucial components of the innate immune system involved in the cellular antiviral defence system. Following the binding of IFNs α/β to their specific receptor, JAK/Stat signalling cascade induces the activation of transcription of several antiviral proteins, among which the best characterized are the 2′5′ oligoadenylate synthetase (2′5′OAS), double-stranded RNA-activated protein kinase (PKR) and MxA (reviewed in Stark *et al*., 1998; Goodbourn *et al*., 2000; Sen, 2001; Katze *et al*., 2002). These proteins can also be directly activated by viral gene products through an IFN-independent pathway (reviewed in Sen, 2001). IFN-induced proteins create an antiviral state in infected cells by inhibiting various stages of viral replication (Stark *et al*., 1998; Goodbourn *et al*., 2000). However, some viruses have evolved mechanisms to counteract the antiviral host responses (Stark *et al*., 1998; Goodbourn *et al*., 2000; Sen, 2001; Katze *et al*., 2002).

We showed previously that in the rat testis exposed to a virus the testosterone-producing Leydig cells synthesized large amounts of IFNs α/β (Dejucq *et al*., 1998) as well as of the antiviral proteins 2′5′OAS, PKR and MxA (Melaine *et al*., 2003). Other testicular somatic cells (i.e. macrophages, Sertoli cells and peritubular cells) were also found to display strong antiviral responses, whereas germ cells responded very weakly, if at all, to virus exposure (Dejucq *et al*., 1995, 1997, 1998; Melaine *et al*., 2003). Interestingly, the strong antiviral innate immunity of the rat testis correlates to the absence of naturally occurring viral orchitis in this animal species. In contrast, several viruses may cause orchitis in men. Among them, the mumps virus is well known for its testicular tropism and for inducing inflammation, decreased androgen production and degeneration of the seminiferous epithelium that can lead to sterility (reviewed in Dejucq and Jegou, 2001). As shown in the testicular interstitial tissue *in vivo* (Bigazzi *et al*., 1968; Bjorvatn, 1973), the mumps virus has been found to replicate in Leydig cells and be associated with a decrease in testosterone production *in vitro* (Le Goffic *et al*., 2003). Interestingly, the latter study reported that mumps virus infection of human Leydig cells did not seem to induce IFN production by these cells (Le Goffic *et al*., 2003), in contrast to their rat counterpart exposed to another paramyxoviridae virus, the Sendai virus (Dejucq *et al*., 1998). To determine whether the deleterious effects of the mumps virus on the human testis functions resulted from its ability to circumvent antiviral responses, the present study aimed at comparing the antiviral defence capabilities of human Leydig cells following either mumps virus infection or exposure to other viral stimuli including poly I:C, a mimetic of RNA viruses replication product that, unlike live viruses, is devoid of inhibitory effects on the IFN system.

Materials and Methods

Antibodies, cells and virus

Mouse monoclonal antibodies against mumps virus proteins (clone 11–046) and herpes simplex virus type 2 (HSV-2) glycoprotein (clone CHA437) were purchased from Argene SA (Varilhes, France) while rabbit polyclonal anti-human MxA (Ronni *et al*., 1993) and Annexin V (Casado *et al*., 1999) were generously provided by Dr Ilkka Julkunen (Department of Microbiology, National Public Health Institute, Helsinki, Finland) and Dr Françoise Russo-Marie (Institut Cochin, Paris, France), respectively. Mouse monoclonal anti-human PKR (Francois *et al*., 2000) and LH receptor (clone LHR29) (Meduri *et al*., 1997) were, respectively, gifts of Dr Eliane Meurs (Pasteur Institute, Paris, France) and Pr Edwin Milgrom (INSERM U135, Le Bicêtre hospital, Saint Cloud, France). MRC-5, 293T and Vero cell lines were obtained from the European Collection of Cell Cultures (http://www.ecacc.org.uk). Martin Darby bovine kidney (MDBK) cell line and peripheral blood mononuclear cells (PBMCs) were obtained from the University Hospital (Pontchaillou, Rennes, France) and maintained as previously described (Ruffault *et al*., 1992, 1995). The Vit-MA strain of mumps virus, isolated from a patient at the Reims hospital (France), was amplified in Vero cells and titrated by plaque assay. Mumps virus titre was expressed as plaque forming units (PFU) as previously described (Le Goffic *et al*., 2003). Titrated stock of Sendai virus, vesicular stomatitis virus (VSV) and HSV-2 were obtained from the University Hospital (Pontchaillou, Rennes, France). Human immunodeficiency virus (HIV)-2_{ROD} gp105 (EVA621) was produced using a baculovirus expression system (EU program EVA, MRC, UK).

Isolation and culture of human Leydig cells

The protocol was approved by the local ethics committee of University of Rennes and informed consent was obtained from the donors. Normal testes, obtained from prostate cancer patients not subjected to any hormone therapy (age range 60–80 years), were transported on ice immediately after orchidectomy in fresh medium. The clinical status of the patients revealed no reproductive abnormalities or testicular infections. Leydig cell isolation was performed as previously described (Willey *et al*., 2003). The occurrence of fully developed spermatogenesis was verified by transillumination examination of the freshly isolated seminiferous tubules (Willey *et al*., 2003; Roulet *et al*., 2006). In order to eliminate the remaining monocytes/macrophages, a depletion step was performed using magnetic CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of Leydig cells preparations was >98%, as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase (HSD) and flow cytometry analysis for LH receptor (Meduri *et al*., 1997; Willey *et al*., 2003). The contaminants were a few CD45 positive leukocytes, alkaline phosphatase positive peritubular cells (Anthony and Skinner, 1989) and round non-adherent haploid to tetraploid germ cells identified on their morphological appearance and ploidy profile in flow cytometry (Pfitzer *et al*., 1982) (<1% each).

Cell stimulation and Leydig cell infection

Isolated Leydig cells and cellular positive controls (PBMC or MRC-5) were incubated for various periods of time (as specified in the text and figure legends) with either 25 μg/ml of synthetic double-stranded RNA (poly I:C; Amersham Biosciences, Piscataway, NJ, USA) (optimum concentration determined by preliminary dose–response experiments using 1–100 μg/ml of poly I:C) or 1000 U/ml of recombinant IFN α2a (PBL Biomedical laboratories, NJ, USA) (Der *et al*., 1998). In addition, primary Leydig cell cultures were exposed or not (control) to mumps virus [multiplicity of infection (m.o.i) of 0.1 PFU/cell] (Le Goffic *et al*., 2003) for 6–96 h as specified in the text, HSV-2 [m.o.i of 10 $TCID_{50}$ (tissue culture infectious dose affecting 50% of the cultures)/cell] for 20 h (Malmgaard *et al*., 2004), or to the envelope glycoprotein (gp105) from HIV-2_{ROD} (1 μg/ml for 20 h) (Gessani *et al*., 1994), a virus previously shown to infect Leydig cells (Willey *et al*., 2003). Productive infection of Leydig cells by mumps virus was consistently observed between 72 and 96 h post-viral exposure. Infected cells formed syncitia, in the cytoplasm of which mumps proteins were detected by immunocytochemical staining as previously described (Le Goffic *et al*., 2003). Leydig cells were also permissive to HSV-2 replication as assessed by their rounded morphological appearance and positive immunocytochemical staining with anti-HSV-2 protein antibodies.

IFN detection: enzyme-linked immunosorbent assay and bioassay

Cultured cell supernatants from at least three different donors were assayed using enzyme-linked immunosorbent assay (ELISA) kits specific for IFNs α (subtypes αA, α2, αA/D, αD, αK and α4b), IFN β (both from Biosource Europe SA, Nivelles, Belgium) and IFN γ (CYTELISA, Cytimmune Science Inc.®, MD, USA), with a sensitivity range of 10–500 pg/ml, 8.25–660 UI/ml and 7.81–500 pg/ml, respectively, and an inter-assay accuracy of less than 10%, 15% and 11% and an intra-assay accuracy of 5%, 9% and 7% for IFNs α, IFN β and IFN γ, respectively. The bioactivity of IFNs

α/β was measured in samples from at least three different donors in three independent experiments using a standard microcytopathic inhibition assay derived from the method of Rubinstein *et al*. (1981), using MDBK cells (a bovine kidney cell of epithelial origin expressing the IFN α/β receptor), VSV as the challenge virus and recombinant IFN α2a (PBL Biomedical Laboratories) to standardize inter-assay variations, as previously described (De Maeyer *et al*., 1982). For both techniques, a supernatant of PBMC cells infected for 48 h by Sendai virus was used as a positive control.

RNA extraction and cDNA synthesis

Total RNA was extracted from cultured Leydig cells using the Rneasy mini kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's instructions. RNA was then submitted to DNase digestion to remove contaminating genomic DNA and reverse-transcribed into complementary DNA (cDNA) as previously described (Willey *et al*., 2003).

RT–PCR analysis

Forty nanogram of equivalent RNA was used in each RT–PCR. Amplification was carried out using 35 cycles [except 30 cycles for human IFN α/β receptor, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] in a Trio-Thermoblock thermocycler (Biometra, Goettingen, Germany) as previously described (Dejucq *et al*., 1998). Primers used for RT–PCR were either described previously [human IFN α/β receptor (Lutfalla *et al*., 1992), IFN γ (Taya *et al*., 1982), IFN α1 (Nagata *et al*., 1980), interleukin (IL)-6 (Cudicini *et al*., 1997), β-actin (Ponte *et al*., 1984)] or designed according to the Primer 3 software (Rozen and Skaletsky, 2000), all presented as forward (5′) then reverse (3′), and 5′ to 3′: human consensus IFN α (designed to detect 12 IFN α sub-types): 5′ primer, CTGTCCTCCATGAGVTG (V is a degenerate base coding for either A, C or G. Oligonucleotides generated from this sequence are a heterogenous population of these three distinct species); 3′ primer, CATGATTTCTGCTCTGACAACC; human IFN β: 5′ primer, TCA GAGCTCCTGTGGCAAT; 3′ primer, CTGACTATGGTCCAGGC ACA; human CD45: 5′ primer, TCAGGGAAAGAAAGTGGTGC, 3′ primer, AAGAAGGAGCCCTGATTTCC; human 3β-HSD: 5′ primer, GCCTGTTGGTGGAAGAGAAG; 3′ primer, TGTGGGT CTTAACG CACAAG; human TLR3: 5′ primer, CTTAGCACGGCTCTGGAAAC, 3′ primer, AAGAGTTCAAAGGGGGCACT; human TLR7: 5′ primer, TGCTCTGCTCTCTTCAACCA, 3′ primer, TGGTCCAGTCTGTGAA AGGA; human TLR8: 5′ primer, TGCTGCAAGTTACGGAATGA, 3′ primer, ATTTTGCAGCCCTTGAAATG; human GAPDH: 5′ primer, TGGATATTGTTGCCATCAATGACC, 3′ primer, GATGGCATGG ACTGTGGTCATG. The amplification products were validated by direct sequencing using an automated DNA sequencer (373 DNA sequencer, Applied Biosystems, Foster city, CA, USA). cDNA integrity, as well as absence of genomic DNA contamination, was checked by GAPDH amplification with primers designed in two subsequent exons, thus generating different sizes for genomic DNA versus cDNA amplification products.

Quantitative real-time PCR

Relative quantification of mRNAs encoding PKR, MxA and the 40 kD isoform of 2′5′OAS (2′5′OAS p40) was performed using the TaqMan technology. PCR was carried out on 4 and 40 ng of equivalent RNA run in duplicate, with the ABI7500 (Applied Biosystems), using commercially available target probes (with the exception of the 2′5′OAS p40) and master mix (Applied Biosystems): Hs00169345_m1 (PKR), Hs00182073_m1 (MxA), Hs99999901_s1 (ribosomal 18S). PCR primers and the TaqMan probe for the p40 2′5′OAS were designed using the primer Express software (Applied Biosystems) and purchased from Eurogentec (Angers, France) and Applied Biosystems, respectively. The relative gene expression in four independent cultures was normalized to the level of ribosomal 18S by the use of the comparative CT method, as described in the Applied Biosystems User bulletin no. 2 (P/N 4303859). The non-parametric unpaired Kruskal–Wallis test was used to assess differences between control and stimulated cultures. $P < 0.05$ was considered statistically significant.

Western blotting

Leydig cell cultures were lysed using radioimmunoprecipitation assay (RIPA) buffer supplemented with 5 μl/ml protease inhibitor cocktail and 1 mM 4-(2-Aminoethyl) benzenesulphonyl fluoride hydrochloride (AEBSF) (both Sigma). Protein quantification, electrophoresis on polyacrylamide gel and electro-transfer were performed as previously described (Guillaume *et al*., 2001). The membrane was then blocked overnight at 4°C with tris-buffered saline (TBS) 0.01% Tween 20 (TBST) supplemented with 5% non-fat milk. Following washes in TBST, the membrane was incubated for 1–2 h at room temperature in 1% non-fat milk-TBST containing the primary antibody at the following dilutions: MxA 1:25 000; PKR 1:200; Annexin V 1:15 000. Bands were visualized using the appropriate horseradish peroxidase secondary antibody (Jackson Immunoresearch Laboratories Europe, Suffolk, UK and Amersham Biosciences) and the enhanced chemiluminescence (ECL)+ system, according to the manufacturer's instructions (ECL plus, Amersham Biosciences).

Lentiviral vector production

The transfer vector plasmid pHRsin-cppt-SEW containing the enhanced green fluorescent protein (eGFP) reporter gene under the control of the ubiquitous spleen focus forming virus (SFFV) promoter (Demaison *et al*., 2002), the pMD.G plasmid encoding the VSV envelope (Naldini *et al*., 1996) and the multideleted packaging plasmid pCMV8.91 (Gray *et al*., 1998) were generously provided by Dr Stuart Neil (UCL, London). The IFNβ-pGL3 plasmid containing the luciferase reporter gene under the control of the human IFN β promoter (Lin *et al*., 2000) was a gift from Dr Eliane Meurs. The construction of the pHRsin-cppt-lark-IFN β vector plasmid was realized in the viral vector production plate-forme (INSERM U649, Nantes, France). Briefly, the SFFV strain P long terminal repeat sequence was removed from pHRsin-cppt-SEW using BamH1 and EcoR1 enzymes (fragment 7748–8256; filled with klenow enzyme) and subsequently replaced by the IFN β promoter fragment, previously removed from the IFN β-pGL3 plasmid using the EcoR1 enzyme (fragment 24–327; filled with klenow enzyme).

Pseudotyped vectors were produced by transient lipofectamine transfection (Invitrogen SARL, Cergy Pontoise, France) of three plasmids into 293T cells: pHRsin-cppt-lark-IFNβ or pHRsin-cppt-SEW transfer vector plasmid, the packaging construct plasmid pCMV8.91 and the VSV-G envelope plasmid pMD.G, and stocks of virus titrated as previously described (Demaison *et al*., 2002). For the pseudotyped lentiviral vector particles containing the pHRsin-cppt-lark-IFNβ plasmid, the eGFP expression was measured following stimulation with poly I:C (25 μg/ml).

Transduction of Leydig cells by lentiviral vectors

Purified Leydig cells plated for 48 h in a 12-well plate were infected with pseudotyped lentiviral vector particles containing either the pHRsin-cppt-lark-IFNβ plasmid (called IFN β promoter below) or the pHRsin-cppt-SEW plasmid (called SFFV promoter below) at an m.o.i of 40, in Dulbecco's modified Eagle's medium F12 medium

with 10% fetal calf serum. Three to four days post-transduction with the pseudotyped lentiviral vector containing the SFFV promoter (positive control), *c.*100% of the cells were positive for eGFP expression as assessed by fluorescent microscopy observation. This time point was chosen to start stimulating the IFN β promoter-transduced Leydig cells with 25 μg/ml of poly I:C or with 20 μg/ml of lipopolysaccharide (LPS) (Sigma). Following stimulation, eGFP expression was checked regularly (every 6–12 h) for several days under a fluorescent microscope. Leydig cells were identified by light microscopy and by fluorescent immunostaining for LH receptor expression using a previously described anti LH receptor antibody (Le Goffic *et al.*, 2003) and a secondary rhodamine conjugated donkey anti-mouse IgG (dilution 1/50; Jackson Immunoresearch Laboratories Europe).

Results

Absence of IFNs in human Leydig cells

Using specific ELISA assays, neither unstimulated Leydig cells nor Leydig cells from three different donors exposed to mumps virus for various durations (6, 12, 24, 48 or 72 h) were found to produce IFNs α, β or γ. To determine whether this lack of detection was specific to mumps infection, Leydig cells were stimulated with poly I:C, a well-known potent inducer of IFNs α/β. Very low concentrations of IFNs α and β (respectively, 14 and 13 pg/ml), at the limit of detection of the assay, were occasionally detected in cultures exposed to poly I:C for 6–24 h. Using a bioassay based on the detection of the antiviral properties of IFNs α/β (Rubinstein *et al.*, 1981), Leydig cell supernatants from at least three independent cultures incubated in the absence or presence of mumps virus or poly I:C stimulation for 20 h were accordingly devoid of any significant activity. Similarly, Leydig cell exposure to either HSV-2 or HIV-2 gp105 did not lead to any detectable antiviral activity in the cell culture supernatants, further indicating that the lack of IFN production by Leydig cells was independent of the nature of the viral stimuli. The results of our assays were in line with the data obtained by RT–PCR which showed absence or very low IFN α, β and γ transcript levels in our Leydig cell cultures stimulated by poly I:C for 5 or 10 h (Fig. 1). In contrast, transcripts for the specific Leydig cell marker 3βHSD and for IL6 that are constitutively expressed by Leydig cells (Cudicini *et al.*, 1997) were consistently detected (Fig. 1). Thus, the very weak production of IFNs α/β detected in some Leydig cell cultures stimulated by poly I:C was very likely due to the few contaminating leukocytes (<1%) present in our Leydig cell preparations. Viral RNA sensors are known to be essential to the induction of transcription of IFN and IFN-induced genes in virus-infected or poly I:C-stimulated cells (reviewed in Sen, 2001). We thus looked for their expression in Leydig cells. Using RT–PCR, we failed to detect transcripts encoding the viral single-stranded RNA sensors TLR7/8 in three independent Leydig cells preparations (Fig. 2). However, a strong expression of the poly I:C agonist TLR3 mRNA was demonstrated in all Leydig cell cultures tested (Fig. 2). TLR3 has been demonstrated to be necessary for the recognition of poly I:C by different cell types (Alexopoulou *et al.*, 2001). These results suggest that although Leydig cells may not be equipped to recognize single-stranded RNA viruses such as the mumps virus, their lack/poor IFN expression in response to poly I:C is unlikely to result from absence of recognition.

To further test the ability of Leydig cells to activate the transcription of IFN genes, Leydig cell cultures were transduced with either a lentivector containing the eGFP reporter gene under the control of either an exogenous IFN β promoter or

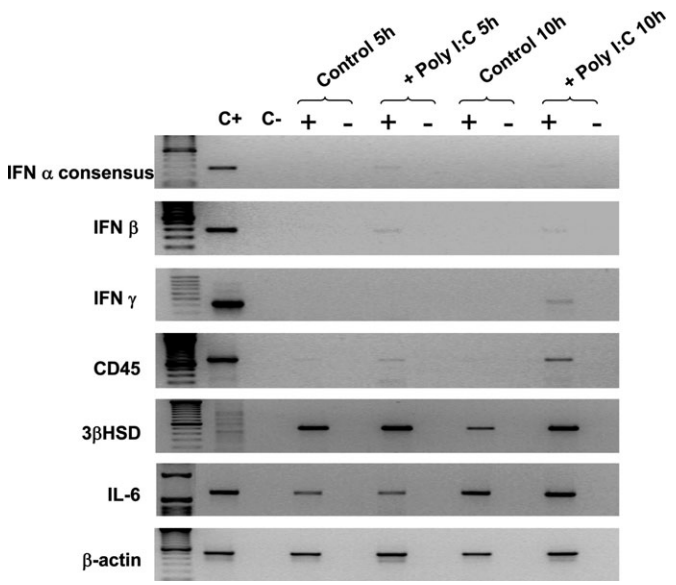

Figure 1: IFNs α, β, γ and cell marker transcripts detection in Leydig cells.
RT–PCR was performed to detect mRNAs encoding: a consensus region for the 12 human IFNs α, IFN β, IFN γ, CD45 (haematopoietic cell marker), 3β-HSD (Leydig cell marker), IL-6 (produced by Leydig cells) (all 35 cycles of PCR) and β-actin (30 cycles) in human Leydig cell cultures stimulated or not (control) for 5–10 h by poly I:C. For each mRNA sample, both a reverse transcriptase (RT) reaction (+) and a negative control reaction without RT (−) were performed (C+: positive control represented by PBMC stimulated 24 h by poly I:C; C−: negative control).

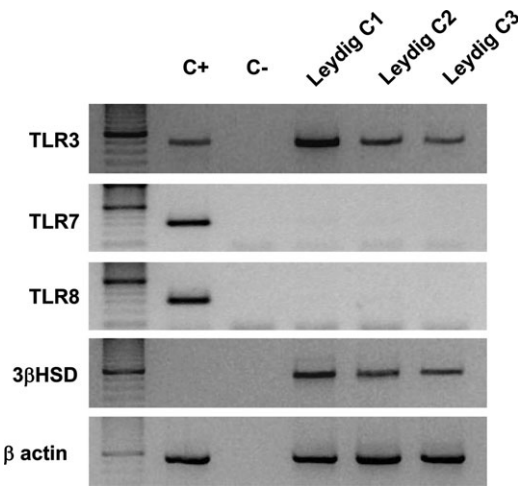

Figure 2: TLRs expression by human Leydig cells.
RT–PCR was performed to detect mRNAs encoding TLR3, TLR7 and TLR8 in independent Leydig cell cultures from three donors (Leydig C1–3) (C+: positive control represented by PBMC stimulated for 24 h by poly I:C; C−: negative control).

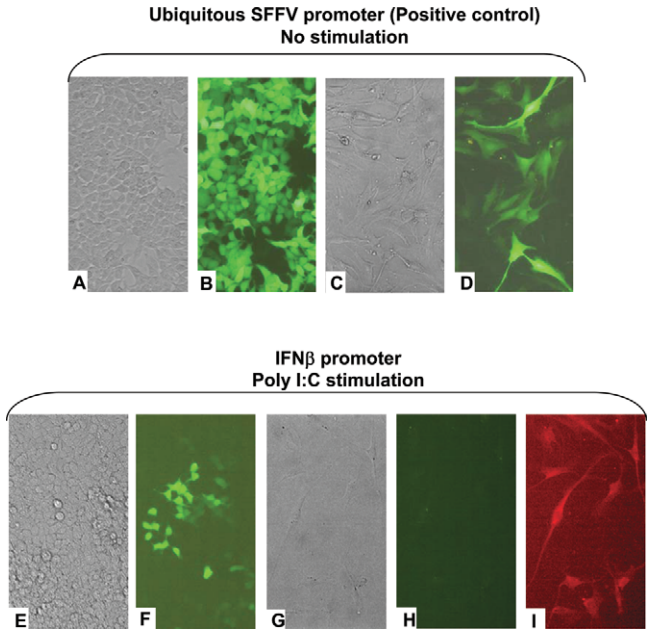

Figure 3: Transduction of human Leydig cells with an exogenous IFN β promoter and poly I:C stimulation.
293T cells (**A** and **B**) and human Leydig cells (**C** and **D**) were transduced for 48 h with a VSV pseudotyped vector construct containing the enhancer region of the ubiquitous spleen focus forming virus (SFFV) promoter inserted upstream of the eGFP open reading frame (positive control). The efficacy of transduction was assessed by checking eGFP fluorescence which appeared at 3–4 days post-transduction, in both 293T cells (A and B) and Leydig cells (C and D). At this time point, 293T cells (**E** and **F**) and Leydig cells (**G**–**I**) transduced with the IFNβ promoter were stimulated with poly I:C. eGFP expression was then checked regularly (every 6–12 h) for up to 5 days. Leydig cells were identified on morphological criteria (C and G) and by immunostaining for LH receptor (I). Although fluorescence was consistently detected in IFN β promoter-transduced 293T cells stimulated with poly I:C, this stimulus had no effect on the transduced Leydig cells. The results presented are representative of three independent Leydig cell cultures. ×200 magnification.

an SFFV ubiquitous promoter—the latter representing a positive control to assess the efficacy of transduction in Leydig cells. In addition, the functionality of these two constructs was verified by checking eGFP fluorescence in transduced 293T cells: 293T cells transduced with the ubiquitous SFFV promoter were consistently fluorescent in the absence of stimulation (Fig. 3A and B) whereas 293T transduced with the IFN β promoter displayed fluorescence when exposed to poly I:C (Fig. 3E and F), demonstrating the validity of the two constructs. Similarly, a large majority of Leydig cells transduced with the SFFV promoter were positive for eGFP expression (Fig. 3C and D), thus showing the efficacy of transduction in this cell type. In contrast, fluorescence was never detected in human Leydig cell cultures transduced with the IFN β promoter and exposed to poly I:C for various durations (Fig. 3G–I). We additionally tested the effect of stimulation by LPS (a potent bacterial IFN α/β inducer) on IFN β promoter's transduced Leydig cells, but no fluorescence was ever observed (data not shown). These results show a lack of activation of the exogenous IFN β promoter in human Leydig cells in response to different stimuli, which explains the absence of IFN expression in stimulated Leydig cells.

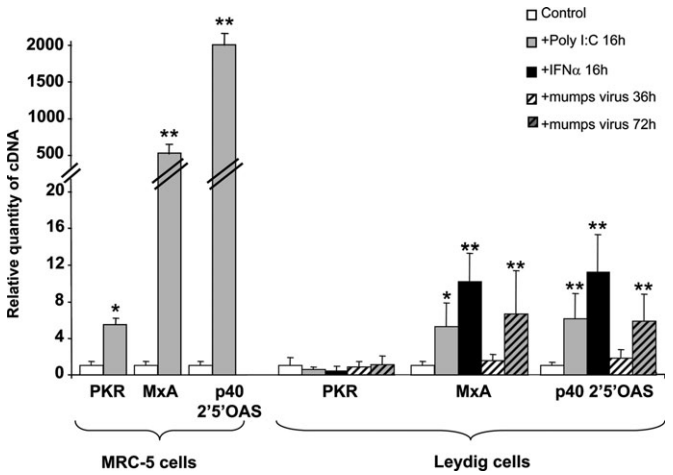

Figure 4: Quantification of transcripts encoding antiviral proteins in human Leydig cells exposed to mumps virus, poly I:C or IFNa.
Real-time quantitative RT-PCR analysis of double-stranded RNA-activated protein kinase (PKR), MxA and the 40 kD isoform of 2′5′ oligoadenylate synthetase (p40 2′5′OAS) transcript expression in human Leydig cells stimulated by poly I:C (25 μg/ml), IFN α (1000 U/ml) or infected by the mumps virus (multiplicity of infection 0.1 PFU/cell) for the indicated durations. The results of independent Leydig cell cultures from four donors were pooled (± SEM). MRC-5 cells stimulated by poly I:C were used as a positive control. Stars indicate statistical difference between controls and stimulated cultures (Kruskal–Wallis test, $^{**}P < 0.05$, $^{*}P < 0.15$).

Presence of PKR, MxA and p40 2′5′oas in human Leydig cells

Whether poly I:C or exogenous IFN α stimulation of Leydig cells would lead to the production of antiviral proteins and how it would compare with mumps virus infection was then investigated. Quantitative RT–PCR showed that the transcripts levels of PKR, MxA and p40 2′5′OAS were consistently enhanced in MRC-5 cells exposed to poly I:C, used as a positive control (Fig. 4). The constitutive low Leydig cell PKR transcriptional levels remained unchanged following exposure to either poly I:C or IFN α for 16 h or to mumps virus for 36 or 72 h (Fig. 4). In contrast, a significantly increased expression of p40 2′5′OAS transcripts was observed in Leydig cells exposed to either poly I:C or IFN α for 16 h. MxA mRNA expression was also significantly enhanced in response to IFN α and was marginally stimulated by poly I:C (Fig. 4). Of note, however, is that their maximum induction levels were always much lower than those in stimulated MRC-5. Following 72 h of mumps virus exposure, MxA and 2′5′OAS transcripts were significantly stimulated, in the same order of magnitude as with poly I:C. In contrast, no induction was observed at 36 h post-infection, suggesting that viral replication triggered the effect (Fig. 4). These results were substantiated at the protein level as Leydig cell MxA levels appeared stimulated after exposure to either poly I:C or IFN α for 24 h. This stimulation was maintained for up to 72 h (Fig. 5). Mumps virus also enhanced MxA protein expression from 72 h up to 96 h post-exposure (Fig. 5). In contrast, the constitutive expression of PKR by Leydig cells was not modified by any of those stimulations (Fig. 5).

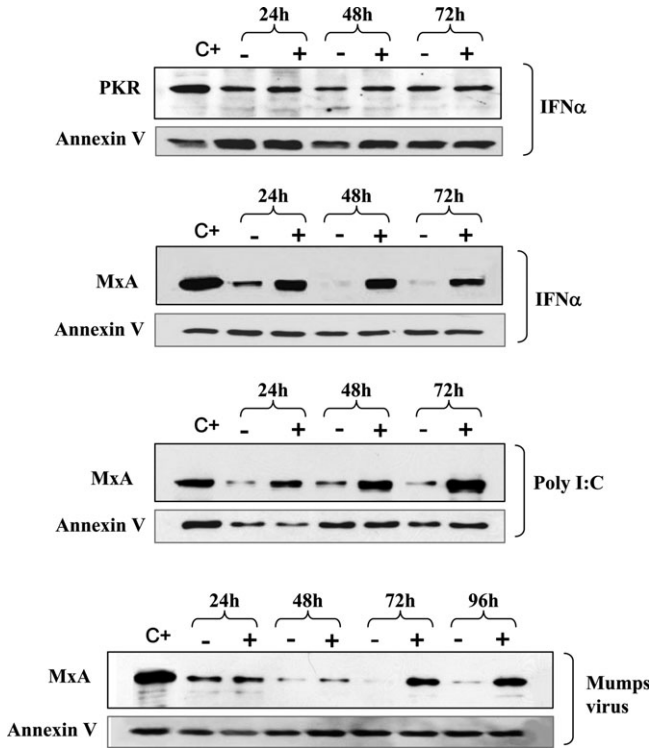

Figure 5: Antiviral proteins expression in human Leydig cells exposed to mumps virus, poly I:C or IFNa.
Western-blot analysis of PKR and MxA protein expression in Leydig cells in the absence (−) or presence (+) of IFN α (MxA and PKR), poly I:C (MxA) (24–72 h) or mumps virus (MxA) (24–96 h).
C+: positive control represented by PBMC stimulated for 24 h with poly I:C. Annexin V represents the loading control. The results presented are representative of independent Leydig cell cultures from three donors.

Discussion

Within the testis, the testosterone-producing Leydig cells play crucial paracrine and endocrine roles in the maintenance of spermatogenesis. Being strategically located in the vicinity of interstitial tissue blood vessels, these cells represent the first testicular line of defence against blood-borne viruses. Indeed, in the rat, Leydig cells exhibit high antiviral activities (Dejucq *et al*., 1998; Melaine *et al*., 2003) and are not known to be infected by any viruses. In men, mumps virus causes disruption of spermatogenesis (Dejucq and Jegou, 2001). Isolated human Leydig cells have been shown to support mumps virus replication, leading to a fall in testosterone production (Le Goffic *et al*., 2003).

In our attempt to address the differences in infectivity between human and rat testis, we demonstrate here that, in contrast to the rat Leydig cells, human Leydig cells do not represent a significant source of IFNs α, β and γ *in vitro*, whether exposed to different viral stimuli, including mumps virus or to poly I:C, or not. This reinforces previous observations from our laboratory about the lack of IFN α/β production in response to mumps infection (Le Goffic *et al*., 2003) and demonstrates that this effect is not specific to mumps virus infection. This unusual response of Leydig cells is very unlikely to be due to the age of the donors as their testes displayed full spermatogenesis as assessed by transillumination, a cardinal criteria to establish the functioning of the testis and therefore of Leydig cells. Furthermore, our Leydig cell preparations expressed IL-6 mRNA, similar to Leydig cells obtained from younger donors (Cudicini *et al*., 1997). Although the donors had prostate cancer, this cancer has never been reported to impact on testis function. The testis is a rare site for prostate cancer metastasis and all the testes samples used in this study displayed normal morphology. A whole transcriptomic analysis on testes obtained from prostate cancer patients and from vasectomized men as well as normal versus cancerous prostate did not show any major alterations in testicular gene expression in prostate cancer patients (F. Chalmel, personal communication). In fact, testes are removed in hormone-dependent prostate cancer patients as they represent the main source of testosterone favouring the growth of the tumour, further attesting proper Leydig cell function. Since IFN γ is usually the product of specialized immune cells, its absence in human Leydig cells was not unexpected. In contrast, the very weak/lack of production of IFNs α/β by Leydig cells following poly I:C stimulation was surprising as poly I:C represents a potent inducer of IFNs α/β in most eukaryotic cells studied (reviewed in Goodbourn *et al*., 2000; Katze *et al*., 2002; Hengel *et al*., 2005). Other viral stimuli (HSV-2, HIV-2 gp105) also failed to induce IFN α/β production by Leydig cells, further indicating that human Leydig cells are not IFN producers. We also demonstrated that the lack of IFN β is not due to a defective endogenous IFN β gene or to high IFN β mRNA instability when we showed that poly I:C or LPS stimulation of Leydig cells transduced with an exogenous IFN β promoter failed to trigger transcriptional activation, while both poly I:C and LPS respective recognition receptors and signalling pathways (reviewed in Uematsu and Akira, 2007) are nevertheless functional. Thus, LPS is able to enhance human Leydig cell IL-1 and IL-6 (Cudicini *et al*., 1997) and these cells express the poly I:C sensor TLR3 transcript and produce antiviral proteins following poly I:C exposure. In most body cells, including the fibroblastoid cell types to which Leydig cells belong, the expression of both IFN β and a subset of IFN-stimulated genes (ISGs) has been shown to be induced early, whereas the expression of the full spectrum of IFNs and ISGs occurs as a second wave dependent on IFN β secretion (Goodbourn *et al*., 2000; Hiscott, 2007). Therefore, it is possible that the lack of Leydig cell IFN β prevents the activation of IFN α genes and ISGs that are not directly activated by viral infection. The repression of the IFN β gene in Leydig cells could be due to either a lack in one or several transcription factors (reviewed in Hiscott, 2007), or to specific negative factor(s), as the human IFN β promoter is constitutively under negative control and a large number of repressors have been described in various cell types (Lopez and Navarro, 1998; Nourbakhsh and Hauser, 1999; Ren *et al*., 1999), some of them preventing IFN induction following virus or poly I:C stimulation (Barlow *et al*., 1984).

In humans, other type I IFNs (ω, ε, κ) have been described in particular cell types (Samarajiwa *et al*., 2006). The negative results obtained with the bioassay used in this study, which is

able to detect all functional type I IFNs as they all bind to the same receptor, indicated that indeed human Leydig cells are very unlikely type I IFNs producers with antiviral activities. A newly discovered third type of IFNs (IFNs λ) shares with IFNs α/β the same Jak/Stat signalling pathway, driving expression of a common set of genes, and exhibits similar antiviral activity (Uze and Monneron, 2007). Since IFNs λ bind to a distinct membrane receptor, they would not be detected in the bioassay used here. However, to date IFNs λ have never been reported to be selectively induced compared with IFNs α/β. These different IFNs appear to be co-produced in response to all inducers studied, strongly suggesting IFNs α/β and λ are regulated by a common mechanism (Uze and Monneron, 2007). Therefore, although IFNs λ production by Leydig cells cannot be excluded, it seems unlikely in light of the current knowledge.

In order to control infection, Leydig cells may display antiviral responses in an IFN-independent fashion. Alternatively, the antiviral state of Leydig cells could rely on IFN secretion by neighbouring cells. To test these hypotheses, we investigated the ability of Leydig cells, stimulated or not, to produce the three well-characterized human proteins playing a major role in regulating virus infection, namely PKR, 2′5′OAS and MxA (Stark *et al*., 1998; Goodbourn *et al*., 2000; Sen, 2001; Katze *et al*., 2002) in response to poly I:C, mumps virus or exogenous IFNα. All stimuli increased expression of MxA and 2′5′OAS, whereas PKR remained unchanged. Although the same antiviral protein expression profiles were observed in the three conditions tested, the kinetics of induction differed between poly I:C /IFN α and mumps virus. The delayed response observed following virus exposure suggests that viral replication is needed for the stimulated 2′5′OAS and MxA expression to occur, probably via products of mumps virus replication (most likely dsRNA). Since Leydig cell IFN production was never detected following mumps virus infection, the virus probably directly stimulates MxA and 2′5′OAS transcription. The fact that mumps virus is known in other systems to impair IFNs α/β and γ signalling (Kubota *et al*., 2001, 2002, 2005), leading to poor induction of 2′5′OAS, PKR and MxA after IFN treatment (Fujii *et al*., 1999), further sustains the hypothesis of an IFN-independent pathway in human Leydig cells. Direct induction by viruses or poly I:C of a subset of ISGs, including 2′5′OAS (Grandvaux *et al*., 2002) and MxA (Nicholl *et al*., 2000; Preston *et al*., 2001), has been previously shown in the absence of IFN (reviewed in Sen, 2001; Hengel *et al*., 2005). When compared with poly I:C or IFN α stimulation, we observed no inhibition of the expression of the antiviral proteins studied following mumps virus infection of Leydig cells. The failure of these two proteins to prevent mumps replication could be due to their delayed induction, although we cannot rule out a block of their functions by mumps virus proteins. However, this feature, described for several viruses, has never been reported for mumps virus (reviewed in Goodbourn *et al*., 2000; Sen, 2001).

PKR is generally present at low levels in a number of cell types, its expression being increased by IFN (Le Page *et al*., 2000). However, IFN stimulation of human Leydig cells did not lead to an enhanced PKR mRNA and protein expression. Similarly, neither poly I:C nor mumps virus modified PKR constitutive expression. Thus, rather than virus-induced specific inhibitions of the antiviral responses of Leydig cells, the combined lack of IFN expression, basal low levels of PKR refractory to stimulation, together with the delayed stimulation of p40 2′5′OAS and MxA expression could create the environment favourable to mumps virus replication.

Systemic treatment with IFN α has been described as beneficial in three studies on patients with mumps orchitis: the occurrence of testicular atrophy and subsequent infertility was prevented in all patients tested (Erpenbach, 1991; Ruther *et al*., 1995; Ku *et al*., 1999). This benefit may arise from the development of an antiviral state prior to mumps virus infection in Leydig cells that are, as demonstrated by our results, responsive to exogenous IFN α, thus compensating for their delayed expression of antiviral proteins that, in the absence of IFN signalling, only occurs secondary to mumps replication. IFN α treatment may limit Leydig cell infection and hence the spread of the virus in the testis. This in turn would reduce tissue inflammation and consecutive testicular atrophy leading to sterility.

In summary, mumps virus replication in Leydig cells was not associated with a specific inhibition of IFNs or 2′5′OAS, MxA and PKR production. Compared with their rat counterparts (Dejucq *et al*., 1995, 1997, 1998; Melaine *et al*., 2003), our study reveals that human Leydig cells have relatively weak antiviral capabilities (absence of IFN production) and delayed responses to infection. Thus in the rat, several somatic testicular cell types, among which the most potent was Leydig cells, were able to produce high levels of IFNs and antiviral proteins in response to Sendai virus—a virus that belongs to the same viral family as the mumps virus (paramyxoviridae). Whether other human testicular cell types (e.g. resident macrophages, Sertoli cells, as identified in the rat) display stronger innate defences or whether the human testis as a whole possesses weaker antiviral capacities than the rat testis, remains to be investigated.

Acknowledgement

We thank Cécile Chevrier for statistical analysis and Simon Rainsford for English proofreading.

Funding

This work was supported by Inserm, Organon and French research ministry.

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Submitted on November 14, 2007; resubmitted on April 30, 2008; accepted on May 7, 2008