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JC virus induces altered patterns of cellular gene expression: Interferon-inducible genes as major transcriptional targets

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Abstract

Human polyomavirus JC (JCV) infects 80% of the population worldwide. Primary infection, typically occurring during childhood, is asymptomatic in immunocompetent individuals and results in lifelong latency and persistent infection. However, among the severely immunocompromised, JCV may cause a fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML). Virus–host interactions influencing persistence and pathogenicity are not well understood, although significant regulation of JCV activity is thought to occur at the level of transcription. Regulation of the JCV early and late promoters during the lytic cycle is a complex event that requires participation of both viral and cellular factors. We have used cDNA microarray technology to analyze global alterations in gene expression in JCV-permissive primary human fetal glial cells (PHFG). Expression of more than 400 cellular genes was altered, including many that influence cell proliferation, cell communication and interferon (IFN)-mediated host defense responses. Genes in the latter category included signal transducer and activator of transcription 1 (STAT1), interferon stimulating gene 56 (ISG56), myxovirus resistance 1 (MxA), 2′5′-oligoadenylate synthetase (OAS), and *cig5*. The expression of these genes was further confirmed in JCV-infected PHFG cells and the human glioblastoma cell line U87MG to ensure the specificity of JCV in inducing this strong antiviral response. Results obtained by real-time RT-PCR and Western blot analyses supported the microarray data and provide temporal information related to virus-induced changes in the IFN response pathway. Our data indicate that the induction of an antiviral response may be one of the cellular factors regulating/controlling JCV replication in immunocompetent hosts and therefore constraining the development of PML.

Keywords: Polyomavirus; JCV; PML; Interferon stimulating genes; *cig5*; MxA; U87MG; Primary human fetal glial cells; Transfection; Infection; Microarray; Real-time PCR; Copy numbers; Chimeras; JCV-SV40 chimera; Mad1/SVEDelta

Introduction

JC virus (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating neurodegenerative disease, occurring in individuals severely immunocompromised as the result of AIDS, advanced malignancy or organ transplantation (Berger, 2003a; Berger and Major, 1999). Prior to 1980, PML was a rare disease, but, in the AIDS era, 3–5% of AIDS patients are diagnosed with PML (Antinori et al., 2001; Berger, 2003b; Snider et al., 1983). In immunocompetent subjects, primary JCV infection is typically asymptomatic, and viruria is common. After primary infection, JCV may persist in the kidney and possibly at other sites in the human body (Dorries, 2001). Reactivation occurs under conditions of impaired immunocompetence and results in the destruction of oligodendrocytes, the myelin-forming cells, for which JCV has a strong tropism (Padgett and Walker, 1973; Padgett and Walker, 1983).

The JCV genome is comprised of a double-stranded circular DNA divided into early and late gene-coding regions between which lies a regulatory region containing the viral promoter–enhancer sequences and the origin of replication (Frisque, 1983). Large T antigen, the main viral regulatory protein, specifies several activities through which the virus commandeers the cellular metabolic machinery for production of virions. Glial-specific expression of T antigen, via the early viral promoter, contributes to JCV's neurotropic

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behavior. During the late stage of the virus replication cycle, capsid proteins are synthesized from alternatively spliced mRNAs, transported to the nucleus and assembled into progeny virions. In addition, the late transcripts also encode agnoprotein, which influences various stages of the viral lytic cycle, including expression and translation of viral late proteins and viral replication via physical interactions with T antigen (Endo et al., 2003; Safak et al., 2002).

In vitro, JCV has a highly restricted host range, making pathogenesis studies challenging. Primary human fetal glial (PHFG), a heterogeneous population of glial cells, remains the most permissive system in vitro to propagate JCV, although transformed derivatives of these cells also support moderate to efficient production of infectious virions (Major et al., 1985; Mandl et al., 1987). Our laboratory has recently shown that the human glial cell line (U87MG) derived from human glioblastoma can efficiently support JCV replication and virion production (P. Ananthula et al., unpublished observations).

Infection of cells by viruses typically elicits a potent and dramatic shift in the transcriptional activity of host cellular genes. These changes have been shaped by co-evolution of the host and pathogen and mainly reflect the strategies developed by the virus to facilitate its own survival. The hallmark of the host response is the expression of interferons (IFN), best known for their antiviral properties (Samuel, 2001). As a countermeasure to this response, many viruses encode proteins that interfere with the products of IFN-stimulated genes (ISG). A better understanding of viral–host interactions will be central to our ability to control the pathogenic potential of JCV in its human host. Since modulation of host cell gene expression by JCV is likely to be important to altering the cellular environment to facilitate viral replication, the primary objective of this study was to identify cellular responses that are specifically regulated by JCV.

We employed cDNA microarray technology as a means to simultaneously compare expression profiles of a large number of host genes in PHFG cells following transfection with a full-length infectious clone of JCV(Mad1). The host response to JCV was analyzed by hybridizing mRNA extracted from JCV- and mock-transfected populations of cells to Affymetrix gene chips. Our results indicate significant induction of several ISG, including signal transducer and activator of transcription1 (STAT1), myxovirus (influenza virus) resistance 1 (MxA), 2′5′-oligoadenylate synthetase 2 (OAS2), ISG56 and *cig5*. Immunoblot analyses were performed to correlate increased mRNA expression with elevated protein levels. The association between ISG expression and JCV infection has not been reported previously. Thus, to further validate that the antiviral response was specific to JCV infection, altered expression of key ISG was analyzed in JCV-infected PHFG and U87MG glioblastoma cells at different time points by real-time RT-PCR.

Results

JCV DNA replication and transcription following transfection of PHFG cells

Three PHFG cell cultures derived from independent biological specimens were transfected with infectious JCV(Mad1) DNA. Thirty seven percent of PHFG cells transfected with pcDNA3.1/CT-GFP expressed GFP at 48 h post-transfection as measured by flow cytometry (data not shown). The efficiency and success of transfections were further confirmed using DNA replication and RT-PCR-based assays. The profiles of JCV DNA replication following transfection, as determined by Southern blotting, showed low levels at day 5 and significantly higher levels at days 10 and 15 (Fig. 1A). All three biological specimens produced similar results.

The mRNAs coding for T antigen and agnoprotein or VP1 were detected by RT-PCR at 3 and 5 days post-transfection, respectively, and the amounts of viral transcripts increased steadily over 15 days (Fig. 1B). JCV DNA replication is initiated upon expression of T antigen; replication is detectable as a faint band on Southern blots by day 5 post-transfection (Fig. 1A). The temporal difference in the detection of T antigen mRNA and JCV DNA replication is due to differences in the sensitivity of the two assays. Therefore, gene expression profiles of mock- and JCV-transfected PHFG cells were compared on day 10 following transfection using microarray chip analysis.

Microarray analysis of PHFG cells after JCV transfection

Total RNA samples were prepared from the JCV- and mock-transfected PHFG cells on day 10 at a time when early and late viral transcription are active (Fig. 1) and detectable secondary

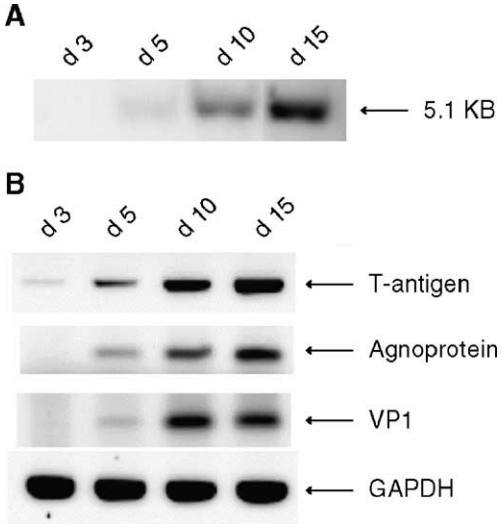

Fig. 1. Replication of JCV DNA in transfected PHFG cells. (A) DNA was extracted on days 3, 5, 10, and 15 following transfection, and JCV DNA replication was measured using a modified *Dpn*I replication assay (Ziegler et al., 2004). After digestion of the DNA with *Dpn*I and *Eco*RI, DNA fragments were separated by gel electrophoresis, transferred to a nylon membrane and hybridized with linear full-length ^{32}P-labeled JCV DNA. The membrane was exposed to a phosphor screen, and the signal was detected by scanning the screen with the Molecular Imager FX system (Bio-Rad). The 5.1-kb band represents the replicated JCV DNA. (B) RNA was extracted on days 3, 5, 10 and 15 following transfection, and cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). One microliter of cDNA was used in all PCR reactions, except for GAPDH, in which 0.5 μL of cDNA was used. PCR was conducted using primers specific for genes encoding T antigen, agnoprotein and VP1.

infection has not yet occurred (Walker and Frisque, 1986). Gene expression profiles were determined using Affymetrix U133A chips that include oligonucleotides corresponding to 14,500 human genes. For analysis, target RNA preparations of JCV-transfected cells were compared with mock-transfected cells used as baseline. Differential expression of 410 genes was observed, of which 265 genes were up-regulated, and 145 genes were down-regulated. Genes, whose expression levels changed 2-fold or more, either up or down, were identified for further analysis.

Our data demonstrate an increase in the levels of expression (>2-fold) of 51 cellular genes that encode proteins involved in cell cycle regulation, cell growth, transcriptional activation, cell communication and signal transduction (Table 1). Importantly, we observed a profound up-regulation of several ISG. At least 15 of the 51 genes that exhibited a 2-fold or greater increase in expression were IFN-responsive genes, including MxA, OAS2, STAT1, *cig5*, and ISG56 (Table 1). Interferons bind to their cognate receptors and initiate a signaling cascade that involves the STAT family of transcription factors. STAT1, a component of IFN-stimulated gene factor 3 (ISGF3) (Fu et al., 1990), that binds and activates interferon-stimulated response elements (ISRE) was induced 3-fold during JCV replication. Upon activation by phosphorylation, STAT1 forms hetero- or homo-dimers, translocates to the nucleus and induces production of many proteins including MxA, OAS2/3, ISG56 (also known as IFIT1) and IFI6–16, via binding to ISRE (Darnell et al., 1994).

Transcripts of a few genes associated with cell cycle regulation/communication, such as the cyclin D2 (2-fold), cyclin E (1.8-fold), transforming growth factor-β (TGF-β) (1.8-fold) and TGF-β receptor1 (TGF-βR1) (2-fold), which have been predicted to influence JCV–host interactions (Radhakrishnan et al., 2003), were up-regulated. Finally, no obvious biological patterns of genes suppressed by JCV could be identified.

Real-time RT-PCR analysis of ISG and TGF-β after JCV transfection

To extend findings obtained in the microarray experiments, expression of nine genes whose fold change ranged from very high, low, and no change (NC) was examined by real-time RT-PCR on three independent preparations of PHFG cells transfected with JCV DNA (Table 2). GAPDH, which was not significantly regulated in JCV-transfected cells as noted in the microarray experiments, was used as a reference gene for normalization in parallel with the gene of interest. The induction of each gene was expressed as a relative fold induction in comparison to the control. The amplification plot of the PCR data was used to determine the threshold cycle (CT) (Pfaffl, 2001). The results for all genes tested concurred with the microarray data and revealed a high consistency between the three RNA preparations from the independent biological specimens. However, we observed some differences in the level of induction measured by the RT-PCR and microarray assays. For example, the fold induction for *cig5* and MxA gene expression was 3 to 7 times higher when measured by real-time RT-PCR vs. microarray assay. Since our microarray results demonstrated increase in the expression of two members of the P56 family, ISG56 and ISG60, which are highly induced by IFN, dsRNA and many viruses, we conducted real-time analysis of ISG56. Our data indicated substantial induction of the mRNA transcript of ISG56 gene in all three JCV-transfected PHFG cell preparations. Interferon regulatory factors (IRF) such as IRF3 and IRF7 are critical components of the transcription complexes that recognize the IFN-stimulated response element (ISRE). JCV replication did not alter the level of IRF3 transcript in either microarray or real-time experiments, while IRF7 was increased by two-fold. However, IFI44, which is one of the prominent genes induced by activated IRF3, was increased using microarray (37.8-fold), and real time RT-PCR (5.3-fold). In addition, we observed a slight up-regulation (1.5 to 1.8-fold) of TGF-β using both techniques at day 10 after JCV transfection.

Evaluation of cellular MxA and cig5 protein expression in transfected cells

To corroborate that the induced transcription of cellular genes is manifested at the protein level, we conducted Western immunoblot analysis of the cellular MxA and cig5 proteins as examples of up-regulated IFN-inducible genes. PHFG cells transfected with pcDNA3.1-GFP were used as an additional control to rule out the possibility that induction of these genes was due to the introduction of a foreign DNA. MxA and cig5 protein profiles were examined at four time points following transfection (Fig. 2). At day 3 post-transfection, MxA protein levels were low and close to the basal level in mock-transfected cells. The amount of MxA protein increased significantly by day 5 post-transfection and then decreased slightly on days 10 and 15 post-transfection; the levels were higher at each of these time points than in the mock- and GFP-transfected PHFG cells. The highest level of cig5 protein was observed early after transfection and was considerably higher as compared to the two controls (Fig. 2). The amounts of β-actin protein remained constant at all time points.

Expression of ISG is induced in JCV-infected PHFG and U87MG cells

The products of ISG are induced in response to virus infection and act on infected as well as uninfected cells to activate a global antiviral state. Since our microarray data identified interferon-inducible genes as the subset of genes most influenced following JCV transfection, we asked whether similar cellular gene activation occurred during a viral infection. Studies performed in our laboratory demonstrate that the U87MG glioblastoma cell line supports JCV replication and transcription leading to infectious virion production (P. Ananthula et. al., unpublished observation). It has been proposed that U87MG cells are a convenient and alternative cell culture system for PHFG cells for studying JCV pathogenesis. Real-time RT-PCR analysis of four ISG (STAT1, ISG56, *cig5* and MxA) was performed at different time points on RNA extracted from JCV-infected U87MG and PHFG cells

Table 1
Profile of differentially expressed genes

Gene name or product	Gene ID	Probe set ID	GenBank accession no.	Fold change
Interferon-inducible genes				
Interferon-induced protein 44	IFI44	214059_at	BE049439	37.8
Interferon, alpha-inducible protein 27	IFI27	202411_at	NM_005532	16.5
2′,5′-oligoadenylate synthetase 1, 40/46 kDa	OAS1	202869_at	NM_016816	10.8
Myxovirus (influenza virus) resistance 1	MX1	202086_at	NM_002462	8.3
Viperin	cig5	213797_at	AI337069	7.3
Interferon, alpha-inducible protein (clone IFI-15K)	ISG15	205483_s_at	NM_005101	6.5
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1/ISG56	203153_at	NM_001548	6.3
Interferon-induced transmembrane protein 1	IFITM1	214022_s_at	AA749101	4.4
Interferon-induced protein with tetratricopeptide repeats 4	IFIT4/ISG60	204747_at	NM_001549	3.2
Interferon, alpha-inducible protein (clone IFI6–16)	G1P3	204415_at	NM_022873	3.0
Signal transducer and activator of transcription 1, 91 kDa	STAT1	209969_s_at	BC002704	3.0
2′,5′-oligoadenylate synthetase 2, 69/71 kDa	OAS2	204972_at	NM_016817	2.6
2′,5′-oligoadenylate synthetase 3, 100 kDa	OAS3	218400_at	NM_006187	2.6
Interferon regulatory factor 7	IRF7	208436_s_at	NM_004030	2.0
Interferon-induced with helicase C domain 1	IFIH1	219209_at	NM_022168	2.0
Cell growth, cell cycle and cell proliferation genes				
Bone marrow stromal cell antigen 2	BST2	201641_at	NM_004335	5.7
Alpha1 adrenergic receptor	ADRA1A	211489_at	D32201	4.3
Nuclear cap bindin protein subunit 1, 80 kDa	NCBP1	209519_at	BG108193	3.6
Cyclin D2	CCND2	200952_s_at	AI635187	2.0
Solute carrier family 4, sodium bicarbonate cotransporter	SLC4A7	210286_s_at	AF053755	2.0
Epithelial membrane protein 3	EMP3	203729_at	NM_001425	2.0
Microtubule-associated protein, RP/EB family, member 3	MAPRE3	214270_s_at	AI885178	2.0
Tumor protein p53 (Li-Fraumeni syndrome)	TP53	211300_s_at	K03199	2.0
Transcription factors				
ZFX mRNA for transcription activator, isoform 3	ZFX	217176_s_at	X59740	2.2
Zinc finger protein 426	ZNF426	205964_at	NM_024106	2.1
SP110 nuclear body protein	SP110	209761_s_at	AA969194	2.0
Zinc finger protein, transcription factor 8	TCF8	210875_s_at	U12170	2.0
Zinc finger protein ZNF286 (ZNF286)	ZNF286	220250_at	NM_020652	2.0
Cell communication/signal transduction				
Similar to islet cell autoantigen	ICA1	211740_at	BC005922	4.1
Integrin, beta 8	ITGB8	205816_at	NM_002214	2.8
Alpha1 adrenergic receptor	ADRA1A	211489_at	D32201	2.8
Lectin, galactoside binding	LGALS8	210731_s_at	AL136105	2.4
Protein kinase C, iota	PRKCI	209677_at	L18964	2.0
Transforming growth factor, beta receptor I	TGFBR1	206943_at	NM_004612	2.0
Miscellaneous				
N-myristoyltransferase 1	NMT1	201158_at	AI570834	6.2
Hypothetical protein FLJ20035	FLJ20035	218986_s_at	NM_017631	6.1
Macrophage stimulating 1 hepatic growth factor-like	MST1	213382_at	AL137798	3.5
KIAA0117 gene	KIAA0117	214942_at	D38491	3.1
Hect domain and RLD 6	HERC6	219352_at	NM_017912	3.0
Programmed cell death 4	PDCD4	202730_s_at	NM_014456	2.8
Cytochrome P450, subfamily 1	CYP1A2	207608_x_at	NM_000761	2.8
Cytochrome P450, subfamily XIB	CYP11B1	214610_at	AV702430	2.7
DC31 mRNA	_	209483_s_at	AF255793	2.7
Lysosome associated membrane glycoprotein	LAMP1	201551_s_at	J03263	2.5
Family with sequence similarity 46, member A	FAM46A	221766_s_at	AW24667	2.4
Early endosome antigen 1, 162 kDa (EEA1)	EEA1	204841_s_at	NM_003566	2.1
Hypothetical protein FLJ21168	FLJ21168	204666_s_at	NM_0250	2.1
Mn containing superoxide dismutase	SOD2	216841_s_at	X15132	2.0
Phospholipid scramblase 1	PLSCR1	202430_s_at	NM_021105	2.0
Mammalian LIN-7 protein 3	LIN7C	219399_at	NM_018362	2.0
HLA class I heavy chain	HLA-A	211799_x_at	U62824	2.0
Down-regulated genes				
Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	204285_s_at	AI857639	−4.3
CGI-146 protein	PNAS-4	222158_s_at	AF229834	−3.2
Yippee-like 1	YPEL1	213996_at	NM_013313	−3.0
Williams Beuren syndrome chromosome region 20C	WBSCR20C	213670_x_at	AI768378	−3.0
Ribosomal protein S11	RPS11	213350_at	BF680255	−2.7
Immunoglobulin superfamily, member 4C	IGSF4C	215259_s_at	AC005525	−2.6

Table 1 (*continued*)

Gene name or product	Gene ID	Probe set ID	GenBank accession no.	Fold change
Down-regulated genes				
Protein phosphatase 1, regulatory (inhibitor) subunit 12A	PPP1R12A	201602_s_at	BE737620	−2.6
Secreted frizzled-related protein 1	SFRP1	202036_s_at	AF017987	−2.5
Twist homolog	TWIST	213943_at	X99268	−2.4
Potassium channel tetramerization domain containing 12	KCTD12	212188_at	AA551075	−2.2
H3 histone, family 3A	H3F3A	213826_s_at	AA292281	−2.0
Nuclear pore complex interacting protein	NPIP	204538_x_at	NM_006985	−2.0
Cytoskeleton-associated protein 4	CKAP4	200998_s_at	AW029619	−2.0

(Table 3). Infection of both PHFG and U87MG cells with 10 and 50 HAU of JCV led to a 2- to 12-fold increase in the levels of STAT1, ISG56, *cig5* and MxA transcripts at days 8 and 14 post-infection. Increasing the multiplicity of infection 10-fold (500 HAU) enhanced the up-regulation of these same genes in U87MG cells by approximately 1 to 2 orders of magnitude. A sharp increase in the STAT1, ISG56, *cig5* and MxA transcripts between days 5 and 8 post-infection, correlating with the rapid increase in copies of JCV genomes determined by real-time PCR (Table 3), was noted. As determined by real-time RT-PCR, the expression of IFN-α and IFN-β in JCV-transfected PHFG cells (Table 2), and in JCV-infected PHFG and U87MG cells (data not shown), did not change significantly.

Discussion

JCV, an opportunistic pathogen, lytically infects oligodendrocytes and causes the progressive neurodegenerative disease, PML. In PML patients, JCV primarily targets oligodendrocytes, although a few reports suggest that JCV infects astrocytes and granule cell neurons (Du Pasquier et al., 2003; Enam et al., 2004; Richardson-Burns et al., 2002; von Einsiedel et al., 2004). In vitro, PHFG cells mimic the in vivo tissue milieu, in which both oligodendrocytes and astrocytes support efficient JCV replication. We therefore chose to transfect this oligodendrocyte-rich heterogeneous population of glial cells with an infectious molecular clone of JCV(Mad1) to study the impact of viral replication on host gene expression. Differential expression of 410 genes was observed on day 10 post-transfection, of which 265 genes were up-regulated and 145 genes were down-regulated. Surprisingly, there were no genes among the down-regulated genes that are repressed more than 4.3-fold, and no easily discernible families could be identified (Table 1). However, among the up-regulated genes, 51 genes were induced 2-fold or more, most notable being antiviral defense response genes, including several interferon responsive genes. These latter findings have not been reported previously.

Efficient production of IFN is an essential component of the host's defense against virus invasion, and the binding of IFN to cell surface receptors results in the induction of a set of ISG that mediate antiviral action (Darnell et al., 1994). Recent evidence suggests that many ISG are also induced by dsRNA or by other viral products in the absence of IFN. Sarkar and Sen have recently used the term "viral stress-inducible genes" (VSIG) to describe a set of genes induced by three independent mechanisms: virus infection, IFN and dsRNA (Sarkar and Sen, 2004). Initially, it was thought that induction of these genes by viruses or dsRNA was a consequence of IFN production. However, it has been unequivocally established that these genes can be induced independent of IFN (Bandyopadhyay et al., 1995; Daly and Reich, 1993; Sarkar and Sen, 2004). In naive cells, the basal level of VSIG is very low, but they are strongly induced transiently upon exposure to viral stress. In our experimental system, the up-regulated ISG, such as STAT1, OAS, MxA and ISG56, belong to the VSIG family. Among these, STAT1, which was up-regulated three-fold in our experimental system, is the

Table 2
Microarray and real-time RT-PCR analysis of IFN-inducible genes and TGF-β in JCV-transfected cells

Gene	GenBank no.	Relative change in the level of expression[a]		
		Microarray	Real-time RT-PCR	
			Mean	Range
cig5	AI337069	7.3	50.9	33–68
STAT1	BC002704	3	4.13	3.1–6
MxA	NM_002462	8.3	27.8	17–38
IFIT1 (ISG56)	NM_001548	6.3	7.9	1.8–27
IFI44	BE049439	37.8	5.3	2.2–10.6
IRF3	NM_001571	NC	NC	0.3–0.6
TGF-β	BF061658	1.8	1.51	1.4–2.2
IFN-α	NM_002176	NC	NC	0.1–0.8
IFN-β	BC093757	NC	NC	0

NC, no change.
[a] Compared to mock-transfected cells.

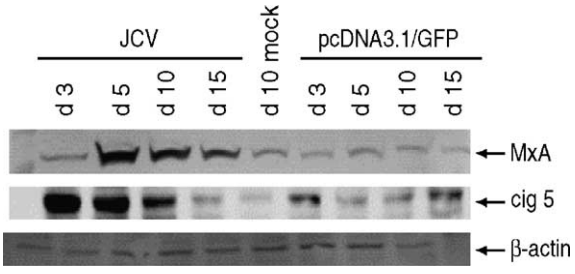

Fig. 2. Western blot analysis of MxA, cig5 and β-actin in JCV-transfected PHFG cells. Western blot analysis was performed on protein extracts from mock, pcDNA3.1/GFP and JCV-transfected cells harvested on days 3, 5, 10 and 15. Forty micrograms of protein from whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated with mouse monoclonal anti-MxA (1:1000 dilution, gift from Dr. Otto Haller), rabbit polyclonal anti-cig5 (1:1000 dilution, gift from Dr. Keh-Chuang Chin) and mouse anti human-β-actin (1:1000 dilution, Sigma). Bands were visualized following incubation with alkaline-phosphatase-conjugated secondary antibodies (Bio-Rad) for MxA and β-actin and horseradish-peroxidase-conjugated secondary antibody (Amersham Biosciences) for cig5 following the manufacturers' instructions. β-actin was used as an internal control.

Table 3
JCV copy numbers and fold induction of IFN-inducible genes at different time points in JCV-infected cells

Cells/HAU	Gene-Fold induction[a]	JCV copies/mL[b]	Days after infection with JCV			
			Day 3	Day 5	Day 8	Day 14
		Copy numbers	4.7×10^6	2.6×10^7	1.0×10^8	5.9×10^8
U87MG/10HAU	MxA		NC	1.1	1.4	3
	STAT1		1.5	2.1	5.5	5.4
	*cig*5		1.8	4.6	9.15	12.2
	ISG56		2.9	1.3	4	4.6
		Copy numbers	2.7×10^8	3.3×10^8	2.7×10^9	3.4×10^9
U87MG/500HAU	MxA		1.1	1.7	233	378
	STAT1		7.1	12.5	541	552
	*cig*5		46	51.1	3326	3962
	ISG56		3.2	3.1	26.3	33.2
		Copy numbers	9.4×10^6	1.0×10^7	1.2×10^8	1.0×10^9
PHFG/10HAU	MxA		NC	NC	1.2	2.1
	STAT1		NC	NC	NC	3
	*cig*5		0.5	0.9	4	4.2
	ISG56		NC	NC	NC	2.3
		Copy numbers	5.5×10^6	8.8×10^7	2.8×10^8	4.4×10^9
PHFG/50HAU	MxA		0.4	0.6	2.1	11.5
	STAT1		1.3	1	2	2.6
	*cig*5		1.4	1	3	10.5
	ISG56		1.5	1.8	2.1	3.6

NC, no change; HAU, hemagglutination unit.
[a] Real-time RT-PCR was performed.
[b] Real-time PCR was performed.

principal regulator of the IFN response pathway. Genes, such as MxA, OAS1, OAS2 and ISG56K, have STAT1-binding elements in their promoters and are regulated by STAT activation. MxA protein, which was significantly increased in JCV-transfected PHFG cells, is a dynamin superfamily GTPase that interferes with viral replication (Frese et al., 1996; Kochs and Haller, 1999a, 1999b). The 2′5′ OAS proteins activate RNase L and lead to the degradation of viral mRNAs (Floyd-Smith et al., 1981). ISG56, which belongs to the P56 family of proteins, is one of the most highly induced genes in response to IFN, dsRNA and many viruses. P56 family member contains multiple tetratricopeptides motifs, which bind to eIF-3. This interaction causes impairment of eIF-3 and results in inhibition of viral protein synthesis.

Our data also demonstrate for the first time the induction of a unique IFN-inducible gene, *cig5* or viperin, following JCV replication in human cells. First reported by Chin and Cresswell, this antiviral protein is expressed in response to human cytomegalovirus (HCMV) infection, and its envelope protein glycoprotein B is shown to inhibit viral replication (Chin and Cresswell, 2001). HCMV, a member of herpesvirus group, and JCV are structurally unrelated but share some epidemiological and pathogenetic features, such as high prevalence in the human population, latency in kidney and asymptomatic primary infection in childhood. Impaired immunocompetence results in the reactivation of these viruses leading to severe life-threatening nervous system complications.

Cellular IFN regulatory factors, IRF3 and IRF7, are a family of transcriptional regulators and are important in the context of viral induction of IFN genes and ISG. IRF3, a key transcriptional activator, is a subunit of dsRNA-activated transcription factor complex (DRAF) (Lin et al., 1999). It is directly activated by dsRNA or by virus infection, translocates to the nucleus, binds to the transcriptional co-activator p300/CAB and contributes to the activation of IFN α/β responsive genes (Bandyopadhyay et al., 1995; Daly and Reich, 1993; Hummer et al., 2001). Though our data do not show direct induction of IRF3, many of the genes induced by IRF3 such as ISG56, ISG60, OAS and *cig5* (Grandvaux et al., 2002) are up-regulated in the presence of JCV. Similar to IRF3, IRF7, which is up-regulated by 2-fold in our experimental system, is a critical determinant for induction of the IFN genes and functions in part by a positive feedback mechanism (Marie et al., 1998).

We infected PHFG and U87MG cells with JCV virions to ensure that alterations to the expression of ISG were not non-specific events caused by transfection of cells with JCV DNA. Induction of STAT1, ISG56, *cig5* and MxA genes by JCV in both cell types following infection confirms that this response is specifically mediated by JCV. Moreover, induction of antiviral genes was dose-dependent, with the expression levels of four genes being elevated 10- to 100-fold when the viral inoculum was increased from 10 to 500 HAU per plate of cells. It may only reflect the cumulative response of a larger number of infected cells by 500 HAU. The higher dose of infection did not alter the time at which this slowly replicating virus underwent a rapid increase in DNA replication in either primary or immortalized cells. The approximate 5- to 10-fold increase in JCV copy number at day 8 post-infection corresponded to the increase in antiviral response (Table 3). This dramatic increase in ISG expression may only reflect the cumulative response of a larger number of infected cells by 500 HAU of JCV.

As there is no significant change in the transcripts of IFN-α and IFN-β in both transfected and infected cells, the induction of ISG as observed in our data is likely to be an IFN-

independent event. One explanation for an IFN-independent signaling cascade could be the activation of ISG by virus-specific dsRNA. There are several stages during the replication cycle of JCV when dsRNA could occur as an intermediate product of transcription. The genome of JCV is divided into early and late transcription units, with the early genes and late genes being transcribed from opposite DNA strands in opposite directions (Frisque et al., 1984). This arrangement has been shown in mouse polyomavirus and SV40 to lead to greater than genome-length transcripts, having the potential to form dsRNA molecules (Hersh et al., 1984; Kumar and Carmichael, 1997). Currently, it is unknown whether such dsRNAs form in cells infected with JCV.

Several viruses target the IFN signaling system and elicit immune responses during infection. The induction is more pronounced in RNA viruses, like Hantaan virus, human parainfluenza virus and Rous sarcoma virus (Garcia-Sastre et al., 1998; Kong et al., 2003; Nam et al., 2003). Most DNA viruses, such as adenoviruses and human papillomavirus, use multiple mechanisms to debilitate STAT-mediated antiviral responses that are presumed to help evade host immune surveillance. Nevertheless, the JCV-induced response described in this report is very similar to that induced by several herpesviruses, such as Epstein–Barr virus, herpes simplex virus, Kaposi's sarcoma-associated herpesvirus and HCMV. All of these viruses activate IFN-responsive genes, such as MxA, OAS and IFI 6–16 (Browne et al., 2001; Mossman et al., 2001; Poole et al., 2002; Ruvolo et al., 2003; Zhu et al., 1998). The effect of the accumulation of these antiviral products on JCV replication is not clear at this point. However, it may be possible that these gene products collectively contribute to restricting JCV growth initially and slowing down spread of the virus to neighboring cells in healthy immunocompetent hosts. JCV might employ this strategy to develop a long-term association with the host.

A recent study examined JCV's effects on cellular gene expression using microarray analysis of RNAs extracted from human fetal astrocyte cultures 15 days following infection with the JCV-SV40 chimera, Mad1/SVEdelta (Radhakrishnan et al., 2003). This chimeric virus consists of JCV coding region sequences and a hybrid JCV-SV40 promoter–enhancer. Expression of several cellular genes was reported to be up-regulated, including those involved in cell cycle regulation (Radhakrishnan et al., 2003). We also found enhanced transcription of cell cycle regulatory genes, supporting the hypothesis that JCV promotes cell cycle progression to facilitate viral DNA replication (Radhakrishnan et al., 2003). Strikingly, Radhakrishnan et al. (2003) did not report the up-regulation of any IFN-inducible genes identified in the current study, suggesting that differences in the cell culture system and virus employed greatly influence the outcome of such experiments.

In conclusion, our studies represent a starting point for the analysis of links between JCV and cellular defense responses. Our microarray results identify novel genes altered by the JCV–host interaction. Enhanced production of proteins encoded by ISG in response to JCV infection would be expected to induce an antiviral state. Although this response is not sufficient to block virus replication, it may modulate the infection and limit spread of JCV from cell to cell, thereby explaining the slow replication and subtle cytopathogenic effects of JCV-infected PHFG cells. Deciphering the molecular mechanisms, by which JCV induces changes in cellular gene expression, will enhance our understanding of the basic biology of the virus–host interaction and ultimately aid in elucidating the pathogenesis of PML.

Materials and methods

Cell culture

PHFG cells were obtained from therapeutically aborted 10- to 14-week-old fetuses from the Kapiolani Medical Center for Women and Children (KMCWC) in Honolulu, Hawai'i, and from the University of Washington, after receiving approval from the KMCWC institutional review board. Fetal tissues were processed, and cells were maintained as described previously (Swenson et al., 1996). The human glioblastoma cell line (U87MG) was obtained from the American Type Culture Collection (Rockville, MD) and propagated in Eagle's Minimum Essential Medium (EMEM) containing 10% FBS and 1% penicillin and streptomycin.

JCV transfection and infection

Oligodendrocyte-rich PHFG cell cultures, prepared from three different biological specimens, were transfected using Lipofectine, a lipid-based transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, PHFG cells were grown to 60–70% confluency in 35-mm or 100-mm plates. The cells were washed twice with Opti-MEM medium (Gibco, Grand Island, NY) and transfected with circular full-length JCV(Mad1) DNA devoid of vector sequences. Viral DNA, 0.1 or 1 μg, was added to 35-mm or 100-mm plates, respectively, and incubated for 6 h at 37 °C. For control experiments, transfections were conducted with Lipofectine alone, without DNA. To determine the transfection efficiency at 48 h, PHFG cells were transfected with the GFP plasmid pcDNA3.1/CT-GFP (Invitrogen), and the percentage of fluorescent cells was measured by flow cytometry.

For infection experiments, PHFG and U87MG glioblastoma (5×10^5) cells were seeded on 35-mm plates grown to 60–70% confluency and infected with 10, 50 or 500 hemagglutinating units (HAU) of prototype JCV(Mad1) for 3 h and washed three times with their respective serum-free culture medium. Cells were maintained for the duration of the experiment in their respective culture medium supplemented with serum.

Southern blot analysis

Cells were harvested on days 3, 5, 10 and 15 following transfection, and total genomic DNA was extracted with the DNAeasy™ tissue kit (Qiagen, Valencia, CA) (Ziegler et al.,

2004). DNA was digested with *Eco*RI and *Dpn*I for 16–20 h, precipitated and suspended in 10 μL 1× TE. *Dpn*I selectively digests transfected plasmid DNA, which is methylated during prokaryotic replication; DNA replicated in PHFG cells is resistant to *Dpn*I cleavage. Digested DNA was electrophoresed on a 0.8% agarose gel and blotted onto nylon membranes (Schleicher and Schuell, Keene, NH). Membranes were UV cross-linked and hybridized with ^{32}P-labeled linear full-length JCV DNA.

RNA extraction

Cells were washed twice with PBS and lysed directly on the plates with lysis buffer (RNeasy Mini Total RNA Isolation Kit, Qiagen Inc.). Total RNA was extracted from lysates of transfected and infected cells, in duplicate, on days 3, 5, 10 and 15 following transfection using the RNAeasy kit according to the manufacturer's protocol (Qiagen Inc.). Genomic DNA contamination was eliminated by digesting the RNA with RNase-free DNase (Ambion, Austin, TX). The yield and purity of each RNA sample were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). The 28S/18S RNA ratios of all RNA specimens as determined by the Bioanalyzer were between 1.6 and 2.

Microarray analysis

Biotinylated cRNA targets were prepared from 10 μg aliquots of the total RNA extracted from JCV-transfected PHFG cells at day 10 following the Affymetrix protocol. Briefly, total RNA was reverse transcribed to first-strand cDNA using Superscript II reverse transcriptase (Invitrogen) promoted by T7-(dT)24 oligomer, and second-strand synthesis was performed using T4 DNA polymerase. cDNA was transcribed in vitro and labeled using biotin-UTP and biotin-CTP to produce biotin-labeled cRNA (Bioarray High Yield RNA transcript labeling kit, ENZO, Farmingdale, NY). cRNA target integrity was determined with an Affymetrix Test 3 array, which contains probe sets representing a subset of characterized genes from various organisms, including housekeeping genes of mammals, plants and eubacteria. Prehybridization to GeneChip Test 3 array was performed to determine the quality of the labeled target prior to its analysis using the Affymetrix U133A chip. These arrays contain cDNA oligomers that are complementary to 18,400 transcripts and variants, including 14,500 well-characterized human genes (Affymetrix, Santa Clara, CA). Hybridization was conducted for 16 h at 45 °C with rotation at 60 rpm. Four prokaryotic genes (bioB, bioC, bioD and cre) were added to the hybridization cocktail as internal controls. Gene chip arrays were scanned at 570 nm with a gene array scanner and analyzed with Microarray Analysis Suite 5.0 software and Data Mining Tool (Affymetrix). For comparison of fluorescence intensity (average difference) values among JCV- and mock-transfected chips, the average difference values for each JCV-transfected gene chip were normalized to that of the mock-transfected chip and the change in expression levels was calculated (Fig. 3). A gene was considered up- or down-regulated if its expression increased or decreased by 2-fold or more compared to the mock-transfected cells.

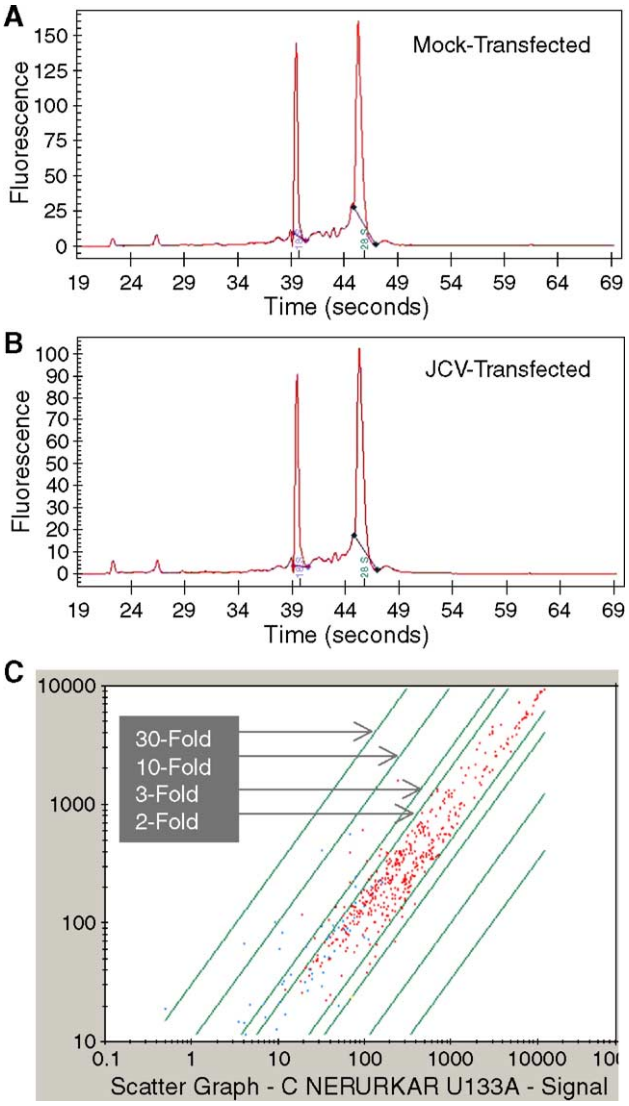

Fig. 3. Purity and integrity of RNA extracted on day 10 from mock (A)- and JCV (B)-transfected PHFG cells. The ratios of 28S/18S RNA were 1.75 and 1.8 for mock- and JCV-transfected cells, respectively. (C) Scatter plot demonstrating the variation in fluorescent signal intensities between mock- and JCV-transfected PHFG cells. Each dot represents the signal for one gene. Changes in 2-, 3-, 10- and 30-fold are highlighted by parallel lines flanking the center diagonal line.

Real-time RT-PCR for cellular genes

RNA samples from all three batches of transfected PHFG cells, including those analyzed by microarray, and from infected PHFG and U87MG cells, were assayed by real-time RT-PCR using SYBR Green (Bio-Rad iCycler, Hercules, CA) to confirm changes in the expression of selected genes. Total RNA from each sample (1 μg) was denatured in the presence of random primers at 65 °C for 10 min and thereafter chilled on ice. A master mix of first-strand buffer, 0.1 M DTT, 40 U of recombinant ribonuclease inhibitor and 25 U of Superscript II reverse transcriptase (Invitrogen), was added to the tube and incubated

for 10 min at 25 °C and then for 50 min at 42 °C followed by incubation at 70 °C for 15 min. Real-time RT-PCR was performed on 2 μL of diluted cDNA in duplicate with both internal and no template controls. Primer sequences used for amplification of *cig5*, STAT1, MxA, interferon-inducible protein 56 (ISG56), interferon regulatory factor (IRF3), IFI44, IFN-α and IFN-β genes were designed using the Beacon Designer 2.0 (PREMIER Biosoft International, Palo Alto, CA) primer design software (Table 4). The housekeeping gene GAPDH was used as an internal control for normalization in parallel with each gene of interest. PCR cycling conditions were 95 °C for 5 min, 36–38 cycles of 95 °C for 10 s, 57 °C for 10 s and 72 °C for 15 s, and the melt curve starting from 60 °C to 90 °C. Each reaction was performed at least three times, with duplicate samples, to verify reproducibility. PCR product intensity data were normalized relative to GAPDH, and the relative fold change was calculated using the Pfaffl method (Pfaffl, 2001).

Real-time PCR for JCV copy number

JCV DNA amplification and quantitation were performed in the Bio-Rad iCycler iQ Multicolor Real-time PCR Detection System using 1 μL of template DNA, Bio-Rad 2× iQ™ SYBER® Green supermix and 12.5 pmol each of forward and reverse primers specific for JCV T antigen gene (Table 4) in a final reaction volume of 20 μL. Thermal cycling was initiated with a first denaturation step of 10 min at 95 °C followed by 35 cycles of 95 °C for 10 s and 62 °C for 10 s, and the amplification fluorescence was read at 60 °C. A standard curve for the quantification of JCV DNA was constructed using serial dilutions of a plasmid containing the entire linear genome of JCV(Mad1). Dynamic range of detection was determined by preparing 10-fold serial dilutions of JCV DNA in the range of 100 pg to 10 fg that represented 1.8×10^9 to 1.8×10^5 copies of JCV DNA, respectively. All experiments were performed at least twice, and samples were run in triplicate each time. The amounts of JCV DNA in experimental samples were calculated from the standard curve and were expressed as copies of viral DNA per mL of total DNA.

Table 4
Primers employed for amplification of IFN-inducible, JCV and GAPDH genes

Gene		Sequence	Amplicon size
T-antigen	F	CTC AGG TAG GCC TTT GGT CTA AA	350 bp[a]
	R	GCT ATT CAA GGG GCC AAT AGA C	
	F	AGA GTG TTG GGA TCC TGT GTT TT	90 bp[b]
	R	GAG AAG TGG GAT GAA GAC CTG TTT	
Agno	F	GGA TTT TTG CAC AGG TGA AGA C	90 bp[a]
	R	TGG TTC AGG CAA AGC ACT GTA	
VP1	F	CAT GAC AAT GGT GCA GGA AAG CC	185 bp[a]
	R	CGC CTT GTG CTC TGT GTT CAT TAC	
GAPDH	F	AGT TAG CCG CAT CTT CTT TTG C	100 bp[a]
	R	CAA TAC GAC CAA ATC CGT TGA CT	
*cig*5	F	TGG TGA GGT TCT GCA AAG TAG A	126 bp[c]
	R	TCA CAG GAG ATA GCG AGA ATG TC	
STAT1	F	TGC TCC TTT GGT TGA ATC CCC	92 bp[c]
	R	GGA ATT TTG AGT CAA GCT GCT GA	
MxA	F	AGT ATG GTG TCG ACA TAC CGG A	145 bp[c]
	R	GAG TCT GGT AAA CAG CCG AAT G	
ISG56	F	CTTGAGCCTCCTTGGGTTCG	137 bp[c]
	R	GCTGATATCTGGGTGCCTAAGG	
IFI44	F	AAG GGC ATG TAA CGC ATC AGG	151 bp[c]
	R	CCG CCT TCT TTC TCA CTC AGC	
IRF3	F	GAC GCT CAC CAC GCT ATG	171 bp[c]
	R	GCA GGT CCA CAG TAT TCT CC	
IFN-α	F	CTC CTT TCT CCT GCC TGA AG	170 bp[c]
	R	AAG TGT CTC ATC CCA AGT AGC	
IFN-β	F	CTC TCC TGT TGT GCT TCT CC	152 bp[c]
	R	GTC AAA GTT CAT CCT GTC CTT G	

[a] RT-PCR primers for amplification of JCV and GAPDH genes.
[b] Real-time PCR primers to determine JCV copy numbers.
[c] Real-time RT-PCR primers to amplify IFN-inducible genes.

RT-PCR for JC viral genes

cDNA was synthesized from RNA extracted from JCV-transfected and JCV-infected cultures at various time points using the aforementioned protocol. One microgram of RNA was used for cDNA synthesis, and 1 μL of cDNA was used for amplification of JCV T antigen, agnoprotein and VP1 genes (primer sequences in Table 4) in a GeneAmp Thermal Cycler 9700 (Perkin-Elmer, Wellesley, MA). The GAPDH gene was amplified as a control with each RT-PCR. The cycling conditions were 95 °C for 5 min, 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s (28 cycles) and a final extension step at 72 °C for 5 min. The amplicons were electrophoresed on a 2% agarose gel, and the ethidium bromide fluorescence was visualized after scanning with a Bio-Rad Molecular Phosphorimager.

Western immunoblots for detection of cellular proteins

Total cellular protein extracts were prepared from mock- and JCV-transfected PHFG cells. Cells were washed with cold PBS and lysed with 250 μL NP40 lysis buffer, consisting of 20 mM Tris pH 7.5, 120 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM DTT, 10 mM NaF, 2 mM sodium vanadate, 1 mM PMSF and 1% protease inhibitor cocktail (Sigma catalog number P8340). Total protein was separated by centrifuging the lysates for 10 min at 14,000 rpm. Protein concentration was assayed using the Bradford technique (Bio-Rad), and 40 μg of protein extracts was boiled in Laemmli buffer for 10 min and fractionated on 10% SDS-polyacrylamide gels. Proteins were transferred by electro-blotting onto nitrocellulose filters (Bio-Rad) and blocked for 2 h in TBS plus 0.3% Tween and 5% BSA.

Filters were incubated with primary antibody for 2 h at room temperature in the blocking solution. The immunoblots were washed in TBS with 0.5% Tween (TTBS) three times for 15 min each and incubated with the appropriate secondary antibody in the blocking solution (5% BSA) for 1 h followed by three washes with TTBS. The bound goat alkaline-phosphatase-conjugated anti-mouse secondary antibody was detected using a color development kit (Bio-Rad), and donkey HRP-conjugated anti-rabbit secondary antibody was developed, using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Primary antibodies included mouse anti-human β-actin (Sigma), mouse monoclonal anti-MxA (gift from Dr. O. Haller) and rabbit polyclonal anti-cig5 (gift from Dr. K.-C. Chin).

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