EARLY ANTIVIRAL DEFENSE PATHWAYS

Ph.D. Thesis

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CONTENTS

LIST OF PUBLICATIONS

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ABBREVIATIONS

AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease-activating factor-1
ATF	activating transcription factor
BrdU	bromodeoxyuridine
CBP	CREB binding protein
cdk	cyclin dependent protein kinase
CREB	cAMP responsive element binding protein
crmA	cytokine response modifier A
CRS	congenital rubella syndrome
CTL	cytotoxic T lymphocyte
DFF-45	DNA fragmentation factor-45
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
ds	double-stranded
EBV	Epstein-Barr virus
eIF-2	eukaryotic translation initiation factor-2
EMSA	electrophoretic mobility shift assay
FACS	fluorescence-activated cell sorter
FADD	Fas-associated death domain protein
FLIP	FLICE-inhibitory protein
Grg	Groucho-related gene
HBV	hepatitis B virus
HCV	hepatitis C virus
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HMG	high mobility group family of proteins
HPV	human papillomavirus
HVS	herpesvirus saimiri
ICAD	inhibitor of caspase-activated DNase
IE	inducible element

IFN	interferon
IFNR	interferon receptor
IκB	inhibitory subunit of NF-κB
IL	interleukin
ISG	interferon-stimulated gene
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulated response element
IU/ml	international unit/ml
JAK	Janus kinase
LAK	lymphokine-activated killer cell
M-CSF	macrophage-specific colony stimulating factor
MHC	major histocompatibility complex
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NDV	Newcastle disease virus
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NRE	negative regulatory element
NRF	NF-κB-repressing factor
NSP	non-structural protein
P/CAF	p300/CBP-associated factor
PARP	poly(ADP-ribose) polymerase
PBMC	human peripheral blood mononuclear cells
PFU/ml	plaque forming unit/ml
PIG	p53-induced gene
PKA	protein kinase A
PKC	protein kinase C
PKR	interferon-induced double-stranded RNA-activated protein kinase
pRb	protein product of the retinoblastoma tumor suppressor gene
PRD	positive regulatory element
PTPC	permeability transition pore complex
RID	receptor internalization and degradation
RNA	ribonucleic acid

RNA Pol II	RNA polymerase II
ROS	reactive oxygen species
RT-PCR	reverse transcription coupled polymerase chain reaction
RV	rubella virus
ss	single-stranded
SSI	STAT-induced STAT inhibitor
STAT	signal transducer and activator of transcription
SV	Sendai virus
TBP	TATA-binding protein
TFII	transcription factor II
Th1	$CD4^+$ T helper type lymphocyte
TK	tyrosine kinase
TLE	transducin-like enhancer of split
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR-associated death domain protein
TUNEL	terminal deoxynucleotidyltransferase (TdT)-mediated digoxigenin-dUTP end-labeling assay
vIAP	viral inhibitor of apoptosis
VRE	virus-responsive enhancer element
VSV	vesicular stomatitis virus

INTRODUCTION

Viruses are mobile genetic elements that replicate intracellularly and spread by moving from one cell to another. Diseases caused by viruses occur in several different forms, including acute, latent, persistent, abortive and slow infections, depending on both the virulence of viruses and the physiological state of infected cells (reviewed in 1).

To control infections, and to protect genetic-, as well as antigenic-integrity, multiple mechanisms are invoked by the infected organism. The uniquely prominent pathways involved in antiviral defense can be classified into two large groups: *(i)* innate (early) and *(ii)* antigen-specific (late) responses. Innate responses constitute the first line of defense, while acquired immunity provides specific protection from each pathogen (reviewed in 2-4).

Early antiviral defense pathways involve the action of several microbicidal substances that can be produced either constitutively or inducibly, including acute-phase proteins, complement factors and interferons (IFNs). The barrier function of skin and mucous membranes also contributes to natural resistance against viral infections. Viruses entering the bloodstream or tissue fluids can be eliminated by phagocytosis. Natural killer cells (NK cells) and subsets of γδ T lymphocytes have also been implicated in early antiviral responses by recognizing and destroying virus-infected cells (17, 18 and reviewed in 2-4, 12-16).

Antigen-specific (late) antiviral responses are mediated by B and T lymphocytes conferring humoral and cellular immunity, respectively. Certain virus-specific antibodies produced by B lymphocytes bind to virions and may exert neutralizing effects, thus may limit spread and restrict the viremic phase of infection. Cytotoxic lymphocytes (CLs) recognize cells infected with viruses, then destroy them by both lytic and apoptotic mechanisms and also by secreting cytotoxic cytokines (5 and reviewed in 6). $CD8^+$ T cells (CTLs) elicit cytoxicity against virus-infected cells in major histocompatibility complex I (MHC I) - restricted manner, while some CLs carry CD4 molecules and are restricted in their antigen-recognition by MHC II (5, 7-9, and reviewed in 2-4).

The concerted action of antiviral defense responses is required for the eradication of the infection or at least to control virus replication (reviewed in 2-4, 6, 12, 15, 16).

I. IFN-α and IFN-β as mediators of early antiviral defense

I.1. Innate cytokine production

Cytokines are polypeptide intercellular signaling molecules that exert their complex and pleiotropic effects by binding to specific high-affinity cell surface receptors. The cytokine nomenclature distinguishes interferons, interleukins, growth factors, colony stimulating factors, chemotactic factors and cytotoxic factors. Cytokine production is transient and regulated by various inducing stimuli at the level of transcription, translation, as well as posttranslationally (reviewed in 10-16). Innate cytokine responses may mediate defense against viral infections by: *(i)* displaying direct antiviral effects *(ii)* inducing apoptosis of the infected cell through triggering the tumor necrosis factor receptor (TNFR)-coupled death pathway, *(iii)* activating innate immune cells, including macrophages, NK cells and lymphokine-activated killer (LAK) cells and *(iv)* shaping antigen-specific (adaptive) immune responses (reviewed in 6, 12-16, 141-143). Among those relatively few cytokines—including interferons (IFNs), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-2, IL-13, transforming growth factor β (TGF-β) and macrophage-specific colony stimulating factor (M-CSF)—which were shown to inhibit virus replication directly, type I IFNs play a prominent role in the first line of antiviral defense (reviewed in 12-16).

I.2. Induction of IFN-α and IFN-β

I.2.1. The structure of the human IFN (HuIFN) genes

HuIFNs are classified into type I and type II superfamilies. Type I, acid-resistant IFNs are further subdivided into four subfamilies, termed IFN-α, IFN-β, IFN-ω and IFN-τ. The type II, acid-labile, superfamily comprises IFN-γ (reviewed in 12, 16).

There are at least 18 HuIFN-α genes, 4 of which are pseudogenes and at least 6 HuIFN-ω genes, 5 of which are pseudogenes (19 and reviewed in 12, 16). HuIFN-β and HuIFN-γ are specified by unique genes (reviewed in 12, 16). All of the type I IFN genes lack introns; are clustered on the short arm of chromosome 9 and evolved from a single common ancestral gene. A duplication of the HuIFN gene occurred about 250 million years ago to give rise to the IFN-α and IFN-β genes. The split between IFN-α and IFN-ω genes occurred 100-120 million years ago. The genes coding for IFN-α subtypes diverged about 85 million years ago (19). The HuIFN-α genes encode mature proteins of 165-166 amino acids. Mature HuIFN-β, HuIFN-ω and HuIFN-τ proteins consist of 166, 172 and 172 amino acids, respectively

(reviewed in 12, 16). All cells of the organism can produce IFN-α, IFN-β and IFN-ω in response to inductive stimuli. However, nonhematopoietic cells tend to synthesize IFN-β together with a variable amount of IFN-α, while hematopoietic cells produce more readily IFN-α and IFN-ω. The most effective inducers of IFN-α/β are viruses and double-stranded (ds) RNA. Many of the studies exploring the molecular mechanism of IFN synthesis have used Sendai virus (SV) and Newcastle disease virus (NDV) belonging to the *Paramyxoviridae* family, since these viruses induce high levels of IFN. Certain bacteria and cytokines were also shown to elicit IFN production (reviewed in 12, 16). Even low molecular weight synthetic compounds, such as the imidazoquinolineamine derivative 1-(2-methyl propyl)-1H-imidazole [4,5-C] qunoline-4-amine (imiquimod) can induce IFN-α in cultures of murine and human leukocytes *in vitro* and in mice, rats, guinea pigs, monkeys or humans *in vivo* (20-22).

There is no obvious nucleotide sequence homology and evolutionary relationship between genes encoding type I and type II HuIFNs. The HuIFN-γ gene is located on chromosome 12; contains three introns and four exons encoding 38, 23, 61 and 44 amino acids, respectively. The HuIFN-γ polypeptide consists of 166 amino acids, 20 of which constitute the signal peptide. The mature 146-amino acid-long protein forms homodimers. IFN-γ is produced only by T lymphocytes ($CD4^+$ T helper type 1 (Th1) and $CD8^+$ cells) in response to stimulation with specific antigens or polyclonal T cell mitogens (23, 24 and reviewed in 12, 16).

I.2.2. The structure of the murine IFN (MuIFN) genes

The general structure of the murine IFN genes is comparable to that of the corresponding human genes. There are at least 12 MuIFN-α genes, 11 of which encode mature proteins of 166-167 amino acids. The single-copy MuIFN-β gene encodes a 182- amino acid-long polypeptide, 21 of which constitute the signal peptide. The mature MuIFN-β protein consists of 161 amino acids. The MuIFN-α and MuIFN-β genes (Ifa and Ifb loci) are clustered on chromosome 4. The MuIFN-γ gene contains three introns and four exons encoding a 136-amino acid-long mature IFN-γ protein. The MuIFN-γ gene (Ifg locus) is located on chromosome 10 (25-27, and reviewed in 12, 16).

I.2.3. The transcriptional regulation of the IFN-β gene

The regulatory region of the human IFN-β gene extends about 200 base pairs (bp) 5' from the transcription initiation site. It contains two negative regulatory elements (NREI and NREII) which are involved in the stable repression of the gene prior to induction. NREI and

NREII are located from -63 to -37 bp and from -210 to -107 bp upstream of the start site of transcription, respectively. The regulatory sequence between positions -104 and -55 corresponds to the virus-responsive enhancer element (VREβ) that contains four positive regulatory domains: PRDI (between -77 and -64), PRDII (between -66 and -55), PRDIII (between -90 and -78) and PRDIV (between -104 and -91). VREβ is involved in the positive control of the IFN-β gene expression and PRDI, as well as PRDII sites are also required for postinduction repression. The DNA elements, located in the regulatory region of the IFN-β gene, serve as binding sites for transactivator and repressor proteins, as well as for architectural transcription factors (reviewed in 12, 16).

The repression of the IFN-β promoter before and after virus induction relies on two distinct mechanisms. Stable repression of the IFN-β gene is mediated by transcription factors that recognize either NREI or NREII and block transcription. NREI functions as a position-independent silencer of PRDII by binding NRF, which designates the NF-κB-repressing factor. NRF belongs to the class of active repressors acting by steric hindrance and also by inhibitory protein-protein interactions targeting NF-κB (28). NREII also exerts a negative regulatory effect on IFN-β transcription, however the proteins binding to this element have not yet been characterized. The repressed state after induction involves the binding of IFN Regulatory Factor-2 (IRF-2) to PRDI/PRDIII. IRF-2 prevents interaction between PRDI/PRDIII elements and positive regulatory proteins, such as IRF-1, IRF-3 and IRF-7 (29). PRDI-Binding Factor 1 (PRDI-BF1) and PRDII-Binding Factor 1 (PRDII-BF1) also play a role in the postinduction repression of the IFN-β gene. PRDI-BF1 decreases transcription of IFN-β by recruiting a complex of non-DNA-binding corepressors belonging to the Groucho protein family, including transducin-like enhancer of split proteins (TLE1 and TLE2) and the protein encoded by the human Groucho-related gene (hGrg), which is a naturally occurring, dominant negative form of Groucho/TLE (30). Recent data showed that the Groucho protein directly interacts with histone deacetylase HDAC1, thereby, decreases the acetylated histones on chromatin, an effect possibly bearing on its repressing effect (31, 32).

Viral infections trigger the derepression of the IFN-β gene and elicit a transient induction of transcription, implicating a complex interplay between constitutive architectural proteins, DNA-binding transcription factors and coactivators. The induction of IFN-β gene expression is transient and does not require an ongoing protein synthesis. Several transactivator proteins recruited to VREβ have been characterized and have been shown to play an important role in the induction of IFN-β (reviewed in 12, 16).

Transcription factor nuclear factor kappa B (NF-κB) is a dimeric complex consisting of different members of the Rel family of proteins, such as p50, p52, p65, c-Rel and RelB. In unstimulated cells, NF-κB is retained in the cytoplasm by binding to its inhibitory subunit IκB (33). NF-κB activation requires the sequential phosphorylation, ubiquitination and degradation of IκB, as well as the consequent translocation to the nucleus (34). Viral infections lead to the phosphorylation and degradation of IκBα, thereby activate NF-κB. NF-κB binds to PRDII element of VREβ and functions as an important mediator of IFN-β induction (35).

The IRF family of transcription factors, including IRF-1, IRF-3 and IRF-7, can also regulate the activity of the IFN-β enhancer. IRF-1 binds to both PRDI and PRDIII and stimulates IFN-β gene expression (29). IRF-1 was also shown to synergize with NF-κB on enhancer elements PRDI/PRDIII and PRDII to activate IFN-β transcription (36). The deletion of the IRF-1 gene does not affect the virus-mediated inducibility of IFN genes, but IFN induction by dsRNA is decreased (37). *In vitro* chromatin precipitation experiments revealed that IRF-1 does not associate with the endogenous IFN-β promoter upon viral infection (38). These data suggest that IRF-1 may be dispensable for virus-mediated induction of IFN-β. The induction of IFN-β also requires the binding of IRF-3 to PRDI/PRDIII (39-41). IRF-3 exists in the cytoplasm of uninfected cells and has several domains, including DNA binding domain (DBD), nuclear export signal (NES), IRF association domain (IAD) and autoinhibitory domain. A cluster of serine residues representing regulatory phosphorylation sites has also been identified within the carboxy-terminal part of the IRF-3 molecule. Virus-mediated phosphorylation events activate IRF-3 leading to nuclear translocation and transactivation of PRDI/PRDIII- and IFN-stimulated response element (ISRE)-containing promoters (39-41 and reviewed in 42). IRF-7 recognizes PRDI/PRDIII and may also be involved in IFN-β induction (38, 43, 44). IRF-7 is regulated at both transcriptional and posttranslational levels. Unlike IRF-3, IRF-7 is expressed at very low levels prior to induction, while in response to viral infections or treatment of cells with IFN, the level of IRF-7 mRNA is highly elevated (43). IRF-7 protein is activated in a serine phosphorylation-dependent manner, as IRF-3, then translocates to the nucleus (45). The kinases responsible for the modification of IRF-3 and IRF-7 are yet to be identified.

It has also been shown that activating transcription factor-2/c-Jun (ATF-2/c-Jun) heterodimer or ATF-2 homodimer recognizes the PRDIV domain of VREβ (46). Mutations that disrupt PRDIV significantly decrease the level of IFN-β gene transcription. The correct

and specific orientation of ATF-2/c-Jun binding is required for the virus-mediated induction of IFN-β (47).

The architectural factor HMG I(Y), belonging to the high-mobility-group (HMG) family of proteins, can also interact with the IFN-β enhancer at five different regions. HMG I(Y) binds to two sites flanking PRDIV, to PRDII, to the region of NREI element located immediately downstream of PRDII and to the TATA box (48-50). The recognition sites of HMG I(Y) are located on the minor groove of DNA. Although HMG I(Y) does not function as a transcriptional activator on its own, it stimulates the interaction of ATF-2/c-Jun heterodimer and NF-κB with VREβ; reduces the binding of other competing factors, such as JunD-FosB; and stabilizes the multiprotein enhancer complex assembled on the IFN-β promoter (48-51).

The higher-order nucleoprotein complex containing NF-κB, IRF-3, IRF-7, ATF-2–c-Jun and HMG I(Y) bound to VRE-β is called enhanceosome. The enhanceosome forms a "pocket" and attracts cAMP responsive element binding protein (CREB) binding protein (CBP) involving direct interactions between CBP coactivator and c-Jun, the p65 subunit of NF-κB, as well as, IRF-3 (38, 40, 41, 44, 47, 51-54). CBP is an integral part of the RNA polymerase II (RNA Pol II) holoenzyme (55, 56). It has been shown that CBP associates with p300/CBP-associated factor (P/CAF) and binds to transcription factor IIB (TFIIB), as well as to TATA-binding protein (TBP), which is a subunit of TFIID (55, 56). Thus, CBP, along with its associated proteins, functions as an adaptor between the enhanceosome and the basal transcriptional machinery, thereby, accelerates the assembly of preinitiation complexes at the IFN-β promoter (41). Both CBP and P/CAF possess intrinsic acetyltransferase activity (57, 58). It has been suggested that on one hand, chromatin remodeling due to localized hyperacetylation of histones H3 and H4 at the IFN-β promoter may facilitate the access of general transcription factors to the DNA (59). On the other hand, acetylation of HMG I(Y) by CBP causing a decrease in its DNA-binding activity leads to enhanceosome disruption and the termination of IFN-β gene transcription (60). Recent data demonstrate that CBP plays an essential role in the virus-induced activation of IFN-β gene expression by mediating the synergistic assembly of IFN-β enhanceosome and represents an additional integration point where several signaling pathways triggered by viral infections converge. Based on the results of extensive work by the Maniatis[30, 38, 46-49, 53, 59], Pitha[27, 39], Hiscott[39, 44, 45], Taniguchi[29, 37, 43], Thanos[34, 41, 49, 50-52, 60] and Fujita[29, 40, 54] groups, the organization of the IFN-β enhancer has been revealed as shown on Fig.1.A. and a model for positive control of IFN-β gene expression has also been proposed as illustrated on Fig.1.B.

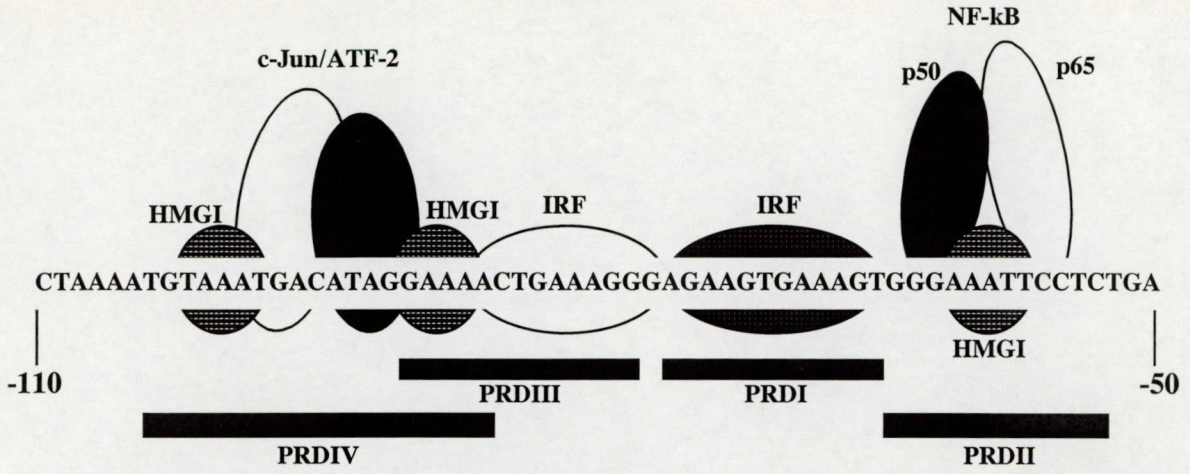

Fig.1.A. The organization of the HuIFN-β gene enhancer (27, 29, 30, 37-54, 59, 60).

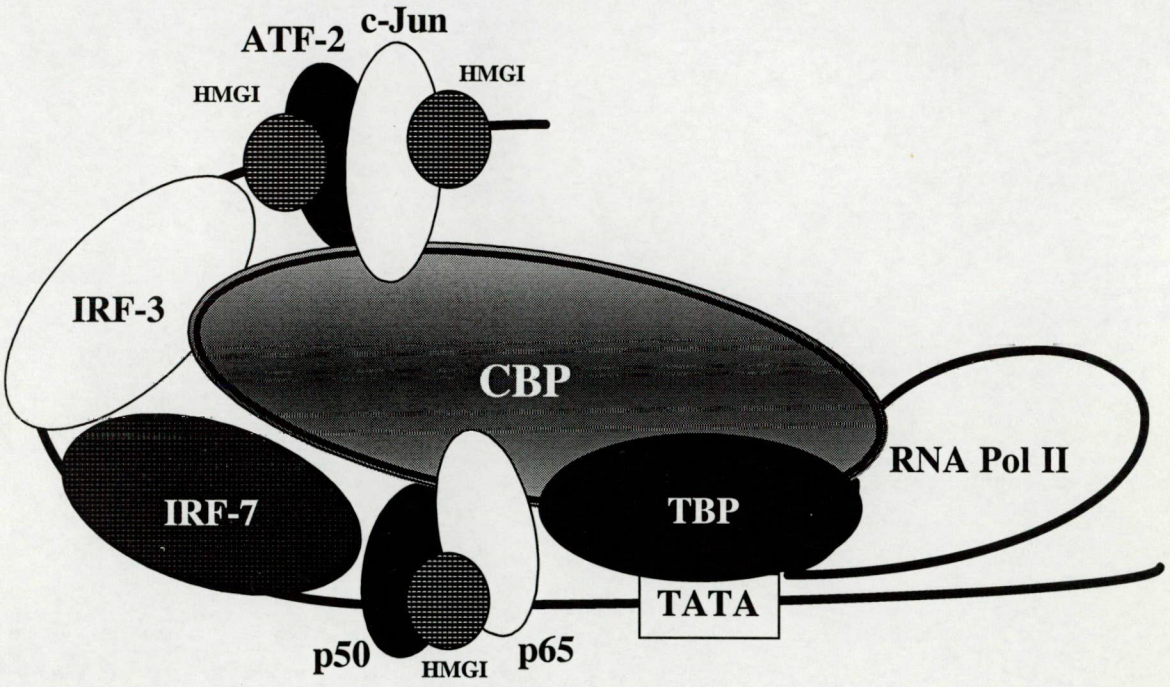

Fig.1.B. A model for positive control of HuIFN-β gene expression (27, 29, 30, 37-54, 59, 60).

I.2.4. The transcriptional regulation of the IFN-α genes

The regulation of the IFN-α genes is less well-defined. The individual IFN-α subtypes show several fold differences in expression that correlates with the transcriptional strength of their promoter regions determined by the number of cooperating enhancer motifs (61, 62). It has also been demonstrated that the expression of individual IFN-α subtypes shows distinct cell type-specific inducibility (63).

The virus-responsive enhancer elements of MuIFN-α4 and HuIFN-α1 subtypes (MuVRE-α4 and HuVRE-α1) contain four enhancer motifs: the αF1 element (A motif), a

PRDI-like site (B motif), the "TG sequence" (C motif) and a second PRDI-like site (D motif) (62-66). Mutations occurring in the promoter regions of MuIFN-α1, 2, 5, 6, 8 and 11 disrupt one or even more enhancer motifs (64-66). Subtype-specific structural differences in the human VRE-α have also been revealed (65, 66). The DNA elements, located in the regulatory region of IFN-α genes, serve as binding sites for transcription factors. The αF1-related and TG-binding proteins recognize A and C motifs, respectively (64). However, these transactivators have yet to be further characterized. Recent data demonstrated that IRF-3 and IRF-7 interact with PRDI-like sites and play critical but different roles in the induction of IFN-α genes (39, 40, 43, 44, 66, 67).

IRF-3 has been identified as the key transcriptional activator of MuIFN-α4 and HuIFN-α1 genes (39, 40). Viral infections elicit a direct and rapid induction of these genes—which does not require ongoing protein synthesis—by triggering activating phosphorylation events targeting IRF-3 expressed constitutively. Based on these recent observations, IFN-β, MuIFN-α4 and HuIFN-α1 have been classified as "early IFN genes"(39, 40).

In contrast, the "late IFN-α subtypes genes" such as MuIFN-α1, 2, 5, 6, 8, 11 (non-MuIFN-α4) and HuIFN-α4, 7, 11 (non-HuIFN-α1) are preferentially activated by IRF-7 only (44, 66). The induction of these subtypes occurs in a delayed, protein synthesis-dependent manner (66). IRF-3 and IRF-7 were shown to display distinct DNA binding properties. The broader DNA binding site specificity of IRF-7 possibly contributes to its capacity to stimulate the expression of "late IFN-α" subtypes (44). It has also been demonstrated that IRF-7 is regulated at both transcriptional and posttranslational levels by IFNs and viral infections, as well (43, 66-68). Experiments using murine fibroblasts have revealed that the synthesis of IRF-7 and subsequent activation "late IFN-α genes" relies on the priming effect of "early IFNs", since the expression of non-MuIFN-α4 genes is undetectable in cells from signal transducer and activator of transcription (STAT)1- and type I IFN receptor (IFNAR)- deficient mice (66). It has been suggested that the induction of "late IFN-α subtypes" occurs in two consecutive stages: *(i)* "early IFNs" evoke the expression of IRF-7, *(ii)* phosphorylation events triggered by viral infections activate IRF-7 protein, which in turn plays an essential role in the transcription of "late IFN-α genes" (66).

Recent studies identified CBP coactivator as an integral component of activation complex recruited to VRE-α (39, 54). Thus, the expression of IFN-α genes requires the synergistic assembly of an "enhanceosome-like" higher-order multicomponent transcription factor-enhancer complex, similar to that of IFN-β (39, 54, 65). However, despite the apparent

similarities, IFN-β, "early and late IFN-α" genes are controlled by different transcription factors and distinct regulatory mechanisms (39-41, 43-45, 65-68).

I.3. The biological effects of type I IFNs

Type I IFNs by binding to specific receptors trigger the activation of the Janus kinase (JAK)-STAT signal transduction pathway that promotes the transcription of IFN-stimulated genes (ISGs) encoding proteins responsible for cellular responses evoked by these cytokines (reviewed in 12). IFN-α, IFN-β, IFN-ω and IFN-τ compete for the same receptor with different binding affinities. The receptor is present on every histological types of cells and has two major subunits (IFNAR1 and IFNAR2c) encoded by genes located on chromosome 21 (69, 70 and reviewed in 12, 71). IFNAR1 binds TYK2 and STAT3, while IFNAR2c associates with JAK1, STAT1 and STAT2. The binding of IFN-α/β to IFNAR induces the dimerization of the receptor and the activation of tyrosine kinases TYK2 and JAK1 by auto- and transphosphorylation, which in turn phosphorylate IFNAR1, STAT1 and STAT2 (72-74 and reviewed in 12, 71, 75, 76). Activated STAT1 and STAT2 form heterodimers, dissociate from the receptor, translocate to the nucleus, where they bind IFN-stimulated gene factor 3γ (ISGF3γ) resulting in the assembly of a multimeric transcription factor, termed ISGF3 (77). STAT1 (p91/p84) and STAT2 (p113) are also termed IFN-stimulated gene factor 3α (ISGF3α) proteins and serve as the regulatory subunit of ISGF3. ISGF3γ (p48) displaying sequence-specific DNA-binding activity directs ISGF3 to a 13-nucleotide conserved cis-acting DNA element (ISRE) located in the promoter region of IFN-driven genes (77 and reviewed in 12, 71, 75). Both STAT1 and STAT2 were shown to interact with CBP suggesting that CBP coactivator may also be involved in the transcriptional activation of ISGs (78). Thus, the signaling mechanism of IFN-α/β relies on the obligatory participation of the JAK-STAT pathway. Type I IFNs induce the activation of other enzymes, including protein kinase C, phospholipase A_2, phosphatidylinositol 3'-kinase and mitogen-activated protein kinase, which may also transduce IFN-α/β signal. Crosstalk between the JAK-STAT and other signal transduction pathways may modulate cellular responses to these cytokines (reviewed in 12 and 75). It has also been shown that certain proteins of the IRF family negatively regulate the expression of ISGs. Members of the STAT-induced STAT inhibitor (SSI) family have recently been identified as negative feedback regulators of JAK-STAT signaling (reviewed in 76, 79). These repressors may prevent the expression of ISGs in the absence of IFNs; to downregulate the induced response; or both. However, a role for SSI proteins in the regulation of IFN-α/β signaling remains to be established.

Experiments using oligonucleotide arrays revealed that the number of ISGs corresponds to 122, while 8 and 12 genes were shown to be downregulated by IFN-α and IFN-β, respectively (80). The physiological function of proteins encoded by several, recently identified ISGs remains to be determined. Table I. is a partial list of the IFN-α/β-inducible proteins and their characteristics.

Table I. A partial list of the IFN-α/β-inducible proteins and their characteristics.

Protein	**Characteristics**	**Principal activity**
(2'-5')$(A)_n$ synthetase/ RNase L system	multienzyme system	synthesis of 2',5'-oligoadenylates and cleavage of ssRNA
PKR kinase	serine-threonin kinase	inhibition of translation of viral mRNA
Mx proteins	GTPases of the dynamin family	inhibition of virus replication
ISGF3γ, STAT1	transcription factors of the IRF family or the STAT family, respectively	signal transduction of IFN-α/β and induction of IFN-α/β gene expression
Fas, MACH-1, HIF-1α, PARP, phospholipid scramblase,	cell surface receptor of the TNFR family, caspase-8, transcription factor enzymes	distinct roles in the biochemical events of apoptosis
IRF-1, IRF-2, IRF-7	transcription factors of the IRF family	antiviral and antiproliferative effect modulation of IFN-α/β gene expression
9-27 protein	unknown	antiviral effect
MHC class I	transmembrane protein of the immunoglobulin supergene family	antigen processing and immune recognition
MHC class II	transmembrane protein of the immunoglobulin supergene family	antigen processing and immune recognition
β_2 microglobulin	light chain of MHC class I	antigen processing and immune recognition
Proteins with poorly characterized roles as mediators of biological effects of IFN-α/β:	GBP-1, Ifi 17/15-kDa protein, ISG15, ISG54, ISG56, ISG58, metallothionein II, IP10, 200 family, 6-16, 1-8, C56, 561, dsRNA adenosine deaminase, RAP46, Bcl-2, hypoxia-inducible factor-1, NF-IL6-β	

The biological effects of IFN-α/β can be classified into three groups: *(i)* antiviral, *(ii)* antiproliferative, and *(iii)* immunomodulatory activities.

I.3.1. Antiviral effects

PKR The IFN-induced double-stranded RNA-activated protein kinase (PKR) is present at low constitutive levels in cells and its expression is enhanced 5- to 10-fold by treatment with IFN-α/β. PKR is activated by dsRNA binding to promote dimerization and subsequent trans-autophosphorylation. PKR is a serine-threonin kinase phosphorylating eukaryotic translation initiation factor-2 on Ser-51 of its α-subunit (eIF-2α) leading to the inactivation of eIF-2 and the inhibition of viral, as well as, cellular protein synthesis. PKR plays a prominent role in the antiviral activities of IFN-α/β and may also be implicated in several diverse cellular functions, including apoptosis, cell cycle regulation, differentiation and signal transduction (81-83 and reviewed in 12, 16, 84).

The (2'-5')(A)$_n$ synthetase/RNase L system The IFN-inducible (2'-5')(A)$_n$ synthetases are activated by dsRNA binding to catalyze the conversion of ATP into (2'-5')(A)$_n$ (where n is 2 to 15) and pyrophosphate. The short series of 2',5'-oligoadenylates bind and activate a second IFN-induced latent protein, the RNase L which cleaves ssRNA after UA, UC, UG and UU residues. The (2'-5')(A)$_n$ synthetase/RNase L-mediated degradation of viral mRNA inhibits the replication cycle at the level of transcription. Furthermore, overexpression of these enzymes was shown to trigger the apoptotic response of cells (85 and reviewed in 12, 16).

The Mx proteins The IFN-inducible Mx proteins are GTPases and inhibit the transcription of viral mRNA and also interfere with the activity or trafficking of viral polymerases, thereby, impair the later stages of viral multiplication (reviewed in 12, 16).

I.3.2. Antiproliferative effects

IFN-α/β lead to arrest of cell cycle progression in G1 and in G2/M phases and also cause S phase slowdown resulting in the inhibition of cell growth and division. The molecular mechanism underlying the growth suppressive action of these cytokines relies on their capability to modulate the expression or activity of proteins involved in cell cycle regulation. IFN-α/β target the protein product of the retinoblastoma tumor suppressor gene (pRb) by negatively regulating the activity of cyclin dependent kinases (cdk), thereby, leaving pRb in an underphosphorylated (active) state required for its G1 checkpoint function (86-88). The cell cycle effects of IFN-α/β have been proved to be highly cell type-specific and the importance of other mechanisms in their antiproliferative effect has also been documented (reviewed in 12).

I.3.3.Immunomodulatory effects

Type I IFNs promote innate and adaptive immune responses mounting host resistance to viral infections by the following mechanisms (reviewed in 2, 4, 12-16):

- IFN-α/β induce the expression of MHC I proteins and β2 microglobulin, thereby, further antigenpresentation and the development of CTL responses.
- IFN-α/β upregulate NK cell activity resulting in the enhanced recognition and subsequent destruction of virus-infected and tumor cells.
- IFN-α/β upregulate their own production, an effect known as "priming" (89, 90).
- IFN-α/β modulate the synthesis of other cytokines including IFN-γ, IL-2, IL-6, IL-10 and IL-12, and promote cell-mediated immunity by means of stimulating Th1 cell differentiation (91).
- IFN-α/β enhance the opsonic phagocytosis of virus-infected and tumor cells by macrophages.
- IFN-α/β modulate B cell development and differentiation, immunoglobulin (Ig) secretion and Ig class switching, thereby, suppress or enhance primary and secondary antibody responses, depending on the dose and time of addition.
- IFN-α/β modulate hypersensitivity reactions.

IFN-α/β inhibit several steps of viral replication, including penetration, uncoating, transcription, RNA stability, translation, maturation, assembly and release, thereby, hinder the initial establishment of infectious foci and restrict viral spread throughout the body. These cytokines also regulate acquired immunity and promote the development of immune effector mechanisms operative against viral infections to aid the eradication of infection. Taken together, IFN-α/β exert complex biological effects and play a pivotal role in keeping viruses at bay.

II.1. Apoptosis as an early antiviral defense mechanism

Viral infections perturb many strictly monitored biochemical processes; thus inhibit cellular protein synthesis, disrupt membrane integrity, modify metabolism, elicit cytokine production, modulate the activity of signaling pathways, alter cellular gene expression and affect cell cycle progression (reviewed in 92). Such perturbations, in turn, frequently trigger apoptosis either of the infected or bystander cells (reviewed in 93-95). Apoptotic cell death is a type of cell deletion characterized by stereotypic cytomorphological changes, such as nuclear compaction, DNA fragmentation to nucleosome-sized fragments, cytoplasmic condensation, membrane blebbing and cell shrinkage resulting in cellular breakdown into membrane-bound apoptotic bodies phagocytosed without evoking an inflammatory response (reviewed in 96).

The biochemical process of apoptosis can be divided into three phases: *(i)* initiation, *(ii)* effector phase and *(iii)* degradation (reviewed in 97), as shown on Fig. 2.

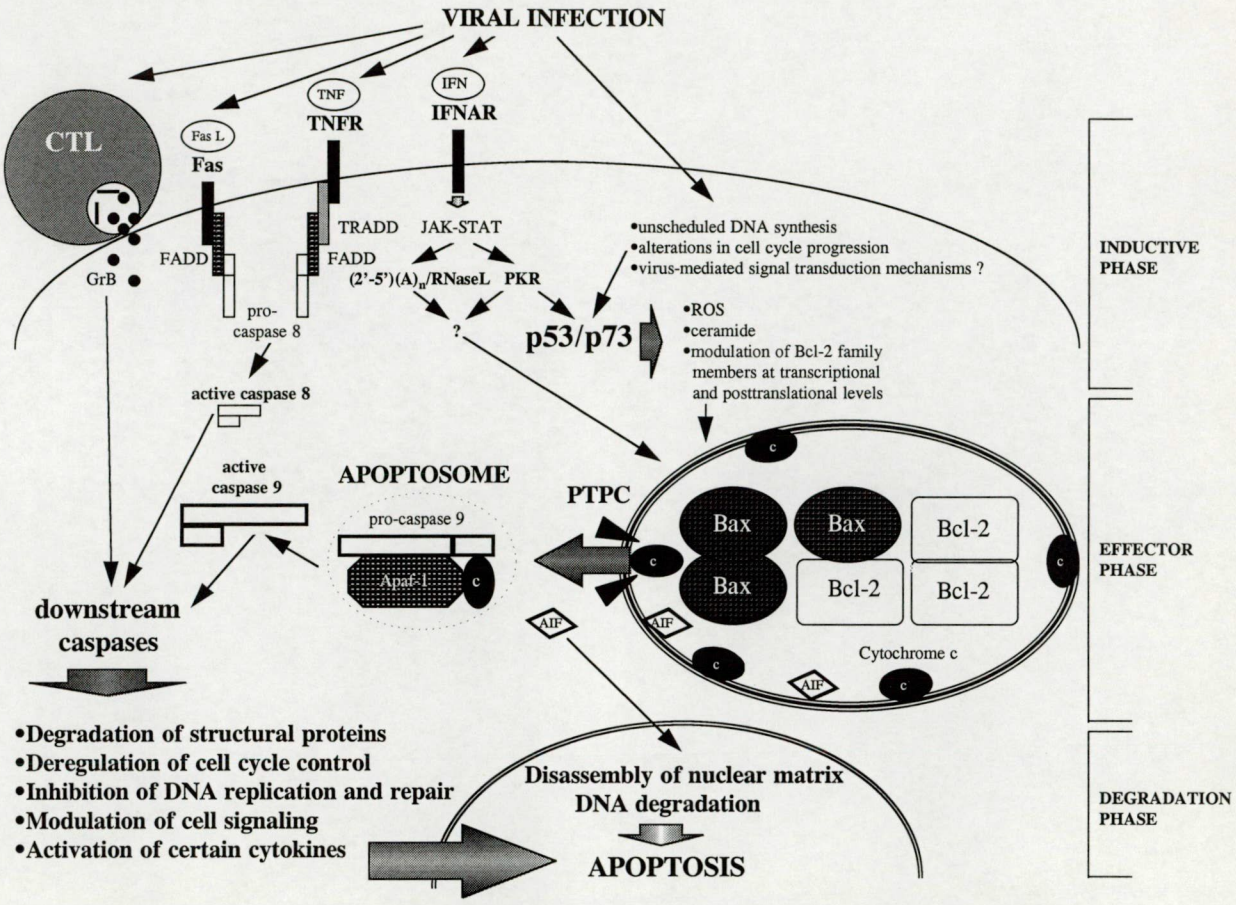

Fig. 2. Schematic overview of the apoptotic process triggered by viral infections.

The inductive phase Human-pathogenic viruses which belong to the *Adenoviridae, Hepadnaviridae, Herpesviridae, Papovaviridae, Parvoviridae, Poxviridae, Coronaviride, Filoviridae, Flaviviridae, Orthomyxoviridae, Paramyxoviridae, Picornaviridae, Reoviridae, Retroviridae, Rhabdoviridae* and *Togaviridae* families were shown to activate the apoptotic death pathway (reviewed in 93-95). The capability of *Arenaviridae, Bunyaviridae* and *Caliciviridae* family members to induce apoptosis has only been proved in animal, but not in human cells. The molecular events constituting the induction phase of apoptosis elicited by different viruses are extremely heterogeneous and can be mediated by p53-dependent and p53-independent mechanisms.

p53-dependent apoptosis was shown to be induced by DNA viruses, which can stimulate cell proliferation in their quiescent host cells, including adenovirus, papillomavirus (HPV), hepatitis B virus (HBV) and Epstein-Barr virus (EBV). E1A protein of adenovirus, E2 and E7 proteins of HPV, HBx protein of HBV and LMP1, EBNA2, EBNA3C proteins of EBV were identified as mediators of p53-dependent apoptotic cell death (98-104).

p53 is a transcription factor having an acidic transcriptional activation domain, a sequence-specific DNA-binding domain, a tetramerization domain and nuclear localization sequences. p53 induces apoptosis or growth arrest following a variety of cellular stresses. The biological consequence (i.e., apoptosis or growth arrest) of p53 accumulation and activation depends on the relative level of p53 protein as well as on the cellular context determined by the histological type of the cell and by the presence of extracellular signals (105-107).

The biochemical events responsible for the activation of p53 in response to viral infections involve distinct mechanisms. Several viral gene products override cell cycle control by inhibiting pRb function that leads to inappropriate cell proliferation and the subsequent activation of the p53-dependent apoptotic pathway (98-104 and reviewed in 92, 95). Alternatively, viral infections may deplete cellular ribonucleotide pools, elicit genotoxic stress, trigger phosphorylation events and, thereby, contribute to the accumulation of p53 (108-111). Furthermore, through the binding of p300/CBP coactivators certain viral proteins may modulate the transactivating effect of p53 and abrogate the mdm2-dependent negative feedback loop that downregulates p53, which, in turn, leads to p53 accumulation (112, 113). There are multiple molecular mechanisms through which p53 promotes apoptosis. It has been demonstrated that in certain cells, gene activation by p53 is indispensable for the induction of apoptosis. Activated p53 forms tetramers, translocates to the nucleus, binds to a response element containing two tandem decamers and modulates the transcription of several downstream target genes, called p53-induced genes (PIGs), including bax and several redox-related genes (114, 115). Simultaneous upregulation of Bax and downregulation of Bcl-2 synthesis by p53 increases the permeability of mitochondrial membranes that triggers the subsequent steps of the apoptotic cascade (116). The transcriptional activation of redox-related genes by p53 leads to the formation of reactive oxygen species (ROS) and to the oxidative degradation of mitochondrial components culminating in cell death (107, 114). The molecular mechanism underlying the development of p53-dependent apoptosis may also involve the transactivation-independent functions of p53 (117).

Numerous studies have shown that p53-dependent growth arrest occurs largely through the transcriptional induction of the cdk-inhibitor $p21^{Waf1/Cip1/Sdi1}$ (p21). p21 regulates the kinase activity of G_1 cyclin–cdk complexes, such as cyclin D–cdk4/cdk6 and cyclin E–cdk2. The inhibition of G_1 cyclin–cdk complexes, in turn, inhibits the phosphorylation of pRb family members, thereby, preventing the activation of E2F-driven G_1/S transition genes such as dihydrofolate reductase, thymidylate synthase and c-myc that culminates in growth arrest. Furthermore, p21 halts cell cycle progression by controlling pathways that operate in the G_2

phase and in mitosis, as well (118). Other uncharacterized factors downstream of p53 may also be involved in the arrest response.

p73 protein belonging to the p53 transcription factor family has recently been shown to activate some of the same downstream target genes as p53 and to induce apoptosis in response to viral infections (119-121). The existence of p53-homologues adds new complexity to the understanding of the p53-dependent apoptotic pathway.

p53-independent mechanisms may also underlie cell death induced by viruses. Apoptosis triggered by respiratory syncytial virus or influenza virus was shown to be mediated by IFN-induced enzymes, including PKR and the (2'-5')$(A)_n$ synthetase/RNase L system (82, 83, 85). The activation of the Fas and TNFR signaling pathways has been implicated in cell death elicited by several viruses, including adenovirus (E1A protein), human herpesvirus 6 and 7 (HHV-6 and HHV-7), HBV (HBx protein), human immunodeficiency virus (HIV; Tat protein), human T-cell leukemia virus (Tax protein) and hepatitis C virus (core protein) (reviewed in 95). Some viruses, including HIV and influenza virus encode proteases which may also promote apoptosis when they cleave their cellular substrates, such as bcl-2 or latent TGF-β, respectively (122). Certain viral proteins activate transcription factors, many of which are involved in cell cycle regulation and apoptosis. The direct contribution of NF-κB to apoptotic cell death has already been demonstrated in infections caused by Sindbis virus (123).

Effector phase Signal transduction cascades triggered by viral infections converge in mitochondria, where pro-apoptotic pathways are integrated and amplified. Several pro-apoptotic second messengers perturb mitochondrial membrane integrity and disrupt transmembrane potential ($\Delta\Psi_m$) leading to the release of intermembrane proteins, such as cytochrome *c* and apoptosis-inducing factor (AIF) into the cytoplasm (124-127). AIF transmits death signal from the mitochondria to the nucleus and causes chromatin condensation and degradation of DNA to fragments of ~50 kb (125). Cytochrome *c* binds to the cytosolic apoptotic protease-activating factor-1 (Apaf-1); the complexes formed are stabilized by Apaf-1-mediated hydrolysis of ATP/dATP (126). Apaf-1 recruits and activates pro-caspase-9, thereby, facilitates the assembly of cytochrome *c*/Apaf-1/pro-caspase-9 complexes, termed apoptosomes (127). The formation of these complexes represents the commitment step in the mitochondria-initiated apoptotic pathway.

The control of cytochrome *c* release plays a pivotal role in the mitochondria-initiated caspase activation pathway. Bcl-2 family member proteins, which may either be pro-apoptotic or anti-apoptotic have already been implicated in the regulation of mitochondrial membrane

permeability and cytochrome *c* release (reviewed in 128). The structure of these proteins can be characterized by variable amounts of Bcl-2 homology (BH) domains and by a transmembrane region restricting their subcellular localization to the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane. The joint effect of Bcl-2 family members on cell fate was shown to be regulated by expression levels, dimerization patterns and by posttranslational modifications, such as phosphorylation. Bcl-2 family member proteins regulate the volume of mitochondria by forming ion channels, as well as control the opening of the permeability transition pore complex (PTPC or megachannel), which is a composite ion channel traversing the inner and outer mitochondrial membrane at sites of contact between them. Accordingly, three theories have been proposed to explain the regulatory role of mitochondria in apoptosis induction: *(i)* the ion flow model (129), *(ii)* the BH3-containing protein model (130), and *(iii)* the permeability transition pore theory (131).

Alternatively, the mitochondrial step can be bypassed and the cell surface death receptor or CTL granule-based apoptotic pathways can also be activated in the course of viral infections (6 and reviewed in 2-5, 93, 94).

Degradation phase Activated upstream (initiating) caspases, such as caspase-2, -8, -9 and -10, cleave and activate downstream (effector) caspases including caspase-3, -6 and -7 responsible for the execution of apoptosis. The caspase family of cysteine proteinases consisting of fourteen members (caspase-1 to -14) can be characterized by its specificity for cleavage after aspartic acid residues (reviewed in 95, 132). Effector caspases trigger the proteolysis of specific subsets of cellular proteins. These caspase substrates include proteins involved in cell structure, signaling, cell cycle, DNA repair or function as cytokines (reviewed in 95, 132). Caspase-mediated cleavage may lead to the degradation and inactivation of several proteins, such as nuclear lamins, actin, STAT1, Raf1, Akt1, NF-κB, pRb, poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK) and the inhibitor of caspase-activated DNase/DNA fragmentation factor-45 (ICAD/DFF-45). Many other proteins, including mitogen-activated protein kinase/ERK kinase kinase1 (MEKK1), cytosolic phospholipase A_2 (cPLA2), protein kinase C (PKCδ and PKCθ), p21-activated kinase 2 (PAK2), IL-1β, IL-16 and IL-18 may become constitutively activated by caspases. Activated signal transducers act to turn on death-promoting pathways and to turn off survival pathways. The proteolytic degradation of structural proteins leads to cytomorphological changes characteristic of apoptosis, including mitochondrial damage, DNA fragmentation and the

formation of apoptotic bodies. Inflammatory cytokines processed on caspase cleavage attract phagocytes and facilitate the removal of apoptotic cells (reviewed in 95, 132).

II.2. Molecular mechanisms viruses use to inhibit apoptosis

Apoptosis had to represent a selective pressure during the evolution of viruses to develop strategies that prevent early host cell death. The inhibition of apoptosis by viruses may have a critical impact on the establishment and outcome of infections.

Inhibition of p53 Different viruses evolved distinct molecular mechanisms to abrogate p53 function. E1B-55K and E4orf6 proteins of adenovirus, HBx protein of HBV, IE1 and IE2 proteins of cytomegalovirus, EBNA-5 and BZLF-1 proteins of EBV and ORF 1 protein of HHV-6 bind to, thereby, inactivate p53 protein. HPV E6 protein interacts with p53 and targets it for degradation by the ubiquitin pathway. Hepatitis C virus (HCV) core protein was shown to block the transcription of p53 gene (reviewed in 93-95).

Bcl-2 mimics In order to suppress the apoptotic response several viruses encode structural or functional homologues of Bcl-2, such as the adenovirus E1B-19K protein, EBV BHRF1 and BALF1 proteins, herpesvirus saimiri (HVS) ORF 16 protein and the HHV-8 KSbcl-2. The LMP1 and EBNA-4 proteins of EBV or the Tat protein of HIV stimulate the transcription of the cellular bcl-2 gene (133 and reviewed in 93-95).

Inhibition of the IFN response Many viruses evolved mechanisms to counteract the apoptotic and antiviral effects of IFNs. NS1 protein of influenza A virus, σ3 protein of reovirus, E3L and K3L proteins of vaccinia virus have the capability of inhibiting PKR action. Herpes simplex virus 1 encodes a protein ($\gamma_1$34.5) which prevents the PKR-mediated inhibition of protein synthesis and the induction of apoptosis by promoting the dephosphorylation of eIF-2α. The E3L protein of vaccinia virus inhibits apoptosis triggered by RNase L and (2'-5')$(A)_n$ synthetase overexpression (reviewed in 93-95).

Inhibition of the Fas and TNF-α responses Viruses may also interact with components of the Fas-, TNFR-associated signaling pathways to block apoptosis initiated by FasL or TNF-α. FLICE-inhibitory proteins (FLIPs) of HHV-8 (K13), HVS (Orf 71) and molluscum contagiosum virus (MC159/MC160) abolish the formation of death-inducing signaling complex (DISC), through their physical interaction with caspase-8 (FLICE). The E1B-19K protein of adenovirus or the LMP-1 of EBV blunt signal transduction from Fas and TNFR1 by binding to Fas-associated death domain protein (FADD) or TNFR-associated death domain protein (TRADD), respectively. Receptor internalization and degradation (RID) complex composed of

E3-14.5K and E3-10.4K proteins encoded by adenovirus was shown to promote the internalization and lysosomal degradation of Fas (reviewed in 93-95).

Caspase inhibitors Certain viral proteins target caspases to inhibit apoptosis. The cowpox virus cytokine response modifier A (crmA) after being cleaved remains firmly bound to caspases thereby blocks their ability to cleave additional substrates. The viral inhibitors of apoptosis (vIAPs), exemplified by other poxvirus proteins, bind and inhibit the activation or enzymatic activity of certain caspases by mechanisms which currently are not yet fully understood (reviewed in 93-95).

The apoptotic self-destruction of infected cells is an important natural defense mechanism that limits virus production and protects the genetic integrity of the invaded organism. Apoptotic mechanisms also contribute to the cytotoxicity elicited by NK cells and CTLs that play an important role in controlling infections partly at the expense of the destruction of infected cells. Viral proteins which counteract the effects of apoptotic signals prevent early cell death, thus may be of survival value by facilitating replication or leading to establishment of persistence. Furthermore, the inhibition of apoptosis by oncogenic viruses is essential for malignant transformation and virus-mediated induction of tumor formation. Several viral gene products served as useful tools to identify the cellular proteins acting in the apoptotic cascade and to gain a better understanding of interrelated cellular functions, as well. However, the precise role of apoptosis modulation (i.e. induction or/and inhibition) in the pathogenesis of certain viral infections is still unclear.

III. Rubella virus and its interaction with the infected cells

RV is the sole member of the *Rubivirus* genus in the family *Togaviridae*. The virion contains three structural proteins, E1, E2, and capsid protein. E1 and E2 are envelope glycoproteins; the capsid protein associates with the genomic RNA to form the nucleocapsid. The genomic 40S RNA serves as a messenger for the nonstructural proteins and as a template for synthesis of a subgenomic 24S RNA that encodes the structural protein precursor, which is processed by host proteases to give rise to the three structural proteins. Capsid morphogenesis occurs in association with membranes coincident with budding (reviewed in 134, 135).

RV is the causative agent of rubella or German measles. The clinical course of natural childhood rubella is usually mild and frequently asymptomatic, whereas establishment of persistent and generally noncytopathic RV infection of the fetus may cause profound damage in

the ontogeny of fetal organs. Histological evidence of cell death, possibly due to a direct cytopathic effect of RV, can also be demonstrated in various organs. The teratogenic effect of RV is manifested in the complex clinical signs of intrauterine growth retardation and organ-specific dysfunctions defined as congenital rubella syndrome (CRS) (reviewed in 134, 136).

Comparative laboratory studies revealed that RV is able to establish persistent, noncytocidal infection in many cell types, while only a limited number of tissue culture systems, including kidney cell lines isolated from different animal species (Vero, RK-13 and BHK-21), human fetal lens cells and rabbit cornea cell line, were shown to be susceptible to the cytopathic effect of this virus. It has also been demonstrated that RV infection slows the growth rate of human diploid fibroblasts, causes mitotic arrest, and ultimately gives rise to genetic alterations, such as chromosomal breaks. RV-induced synthesis of a mitotic inhibitor perturbs physiological cell cycle regulation and thus affects cell growth (137). RV infection also causes cytomorphological alterations in the membrane systems of the endoplasmic reticulum, Golgi complex and mitochondria, depolymerization of actin filaments, and an altered responsiveness to certain cytokines such as epidermal growth factor (reviewed in 134-136).

It has long been suggested that the complex pathophysiological basis of CRS rests in the concerted action of altered cell growth, impaired differentiation and death of certain sensitive cell types. However, the underlying mechanisms involved in the cytopathogenicity of RV remained unclear.

AIMS

I. The examination of molecular mechanisms involved in the production of IFN-α/β and some other cytokines by using viral or synthetic inducers.

Much effort has already been devoted to elucidate the molecular events implicated in the production of cytokines. However, the mechanisms required for the coordinated regulation of certain cytokine genes activated by different viruses currently are not completely understood. Thus, the aim of our study was to investigate the simultaneous induction of IFN-α/β and some other cytokines in an effort to gain more insight into early antiviral defense responses.

I.1. The investigation of IFN and IL-6 production in mouse L929 cells following Sendai virus (SV) infection.

a) The analysis of molecular mechanisms involved in SV-medited induction of mouse IFN-α/β and IL-6.

b) The analysis of the regulatory role of MuIFNs whereby these cytokines modulate their own induction, as well as the synthesis of IL-6.

I.2. The investigation of SV- and imiquimod-induced production of IFN and some other cytokines in human peripheral blood mononuclear cells (PBMC).

a) The comparative analysis of HuIFN-α production of PBMC stimulated either with viral (Sendai virus) or synthetic (imiquimod) inducers.

b) The comparative analysis of the molecular mechanisms involved in SV- and imiquimod-mediated induction of IFN-α, IL-6, IL-8 and TNF-α in PBMC.

II. The investigation of molecular events implicated in rubella virus (RV)-induced apoptotic cell death.

In the light of the important role of apoptotic cell death in the pathogenesis of several viral infections, we asked whether the cytopathogenicity evoked by RV might also involve apoptotic mechanisms. The aim of our study was to investigate the molecular events implicated in RV-induced cell death in an effort to gain more insight into the teratogenic effect of this virus.

a) The analysis of cytopathogenicity evoked by RV in different cell lines, including Vero cells, RK-13 cells and two human embryonic fibroblast cell lines.

b) The investigation of the role of RV replication in the development of cell death.

c) The analysis of the role of p53 in cell death triggered by RV.

MATERIALS AND METHODS

Cell cultures

Human peripheral blood mononuclear cells (PBMC): PBMC were isolated from Leukopack platelets by density gradient centrifugation in lymphocyte separation medium. The cells were further separated on the basis of their adherence to plastic surfaces. B cells, T cells and monocytes were isolated either by sorting with a fluorescence-activated cell sorter (FACS) or by magnetic beads (Dynabeads).

L cells: Two L cell sublines (L929B and L929M) were isolated after serial mutagenesis from a mouse L929 cell line originally provided by G.L. Toms (Department of Microbiology, University of Birmingham, Birmingham, England). The sublines L929B and L929M were established and provided by I. Rosztóczy (Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary).

Vero cells: obtained from the American Type Culture Collection (ATCC)

Viruses

Indiana strain of *vesicular stomatitis virus* (VSV) which was propagated alternately in primary chicken embryo fibroblast cell cultures and L cells

Cantell strain of *Sendai virus* (parainfluenza virus 1) which was propagated in the allantois cavity of 9-days-old embryonated chicken eggs

To-336 strain of *rubella virus* (RV) which was propagated in Vero cell cultures

Cytokine inducers

Inducers of cytokine production used in our studies were the following: *(i)* polyinosinic acid:polycytidylic acid (Sigma), *(ii)* Sendai virus, *(iii)* imiquimod, provided by the Department of Pharmacology, 3M Pharmaceuticals, St. Paul, MN, USA.

Experimental animals

South African *Xenopus laevis* frogs were originally provided by L. Horváth (Agricultural University of Gödöllő, Gödöllő, Hungary) and by L. Kovács (Department of Physiology, University Medical School of Debrecen, Debrecen, Hungary). The further breeding and maintenance of the experimental animals took place in our laboratory.

Cytokine assays

Assay of IFN IFN was measured either on L cells or on human embryo fibroblasts using a cytopathic micromethod, VSV being used for challenge. After visual observation, the viability of IFN-treated cells was also determined by using a tetrazolium dye (MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) that is reduced to a blue formazan by living but not dead cells. The amount of dye formed was quantitated using a microplate spectrophotometer (ELISA plate reader). The titers were calibrated against international reference reagents and expressed in international reference units (IU/ml).

Assay of IL-6 IL-6 was measured on the IL-6-dependent mouse-mouse hybridoma 7TD1 cells provided by J. Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium) using MTT test.

Methods used to detect the hallmarks of apoptosis

DNA fragmentation analysis RV-infected Vero cells were lysed and subjected to phenol-chloroform extraction. After proteinase K and RNaseA treatment, purified DNA samples were electrophoretically separated on 1.8% agarose gels containing ethidium bromide and visualized under UV light.

Terminal deoxynucleotidyltransferase (TdT)-mediated digoxigenin-dUTP end-labelling (TUNEL) assay Cytospin cell preparations were fixed and treated with proteinase K. TdT

enzyme was applied to incorporate digoxigenin-11-dUTP at sites of DNA breaks. Samples were then incubated with FITC-conjugated anti-digoxigenin antibody, stained with propidium iodide and visualized by confocal microscopy.

Quantitation of apoptosis by ELISA The cytoplasmic fractions of RV-infected Vero cell lysates were added to ELISA plates coated with anti-histone MAb and incubated for 90 min at room temperature. After washing, peroxidase-conjugated detecting antibody to DNA was added to each well and incubated for 90 min at room temperature. After the addition of substrate (ABTS) solution, absorbance was measured at 405 and 620 nm. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated as: absorbance of RV-infected cells/absorbance of corresponding mock-infected control cells=enrichment factor.

Cytofluorometric DNA analysis RV-infected Vero cells were pulse-labelled with bromodeoxyuridine (BrdU), trypsinized, fixed and incubated with anti-BrdU MAb. After washing, FITC-conjugated anti-mouse IgG was added to each sample. Cells were then washed, treated with RNaseA, stained with propidium iodide and analyzed using an EPICS XL flow cytometer.

Methods used to identify proteins

Indirect immunofluorescence assay Cytospin cell preparations were incubated with anti-RV antibodies. After washing, samples were reacted with FITC-conjugated species-specific secondary antibodies. The slides were counterstained with 0.01% Evans blue and visualized by confocal microscopy.

Western blot analysis Cell lysates prepared from RV-infected Vero cells were mixed with Laemmli's sample buffer and boiled for 3 min. Aliquots of total protein were resolved by SDS-PAGE and electrotransferred onto nitrocellulose filters. Preblocked blots were reacted with specific antibodies to p53, p21 and bax. Blots were then incubated with species-specific secondary antibodies coupled to peroxidase. Filters were developed using enhanced chemiluminescent detection system and signals were imaged by the exposure of Amersham X-ray films.

Methods used to determine steady-state mRNA levels

Translation of cytokine mRNAs in *Xenopus* oocytes Polyadenylated RNA was prepared from differently treated L cell cultures, and injected into *Xenopus laevis* oocytes using a Leitz micromanipulator. Fifteen oocytes were injected with each preparation and were incubated in 150 µl of Barths' medium for 36 h at 22 °C. The supernatants were then assayed for MuIFN and MuIL-6 activities.

Reverse transcription coupled polymerase chain reaction (RT-PCR) To prepare cDNA, total RNA isolated from human PBMC was reverse-transcribed using oligo(dT) primer and avian myeloblastosis virus reverse transcriptase. In the PCR analysis, two sets of oligonucleotide primers corresponding to sequences of human IFNA genes were used. Reaction mixtures were heated to 94 °C for 4 min and subjected to 30 amplification cycles, each consisting of three rounds at 94, 50 and 72 °C, respectively, for 1 min each followed by a final extension for 5 min at 72 °C. Reactions were performed with an automated thermocycler (Perkin-Elmer). To identify the PCR products, the amplified fragments were either digested with the appropriate restriction endonucleases or ligated to pGEM4 and subjected to DNA sequencing using the dideoxy chain-terminating method.

Northern blot analysis Total RNA prepared from PBMC was size fractionated under denaturing conditions on 0.8% agarose gels containing formaldehyde, subsequently transferred to nitrocellulose filter and hybridized under stringent conditions. After washing, the filters were exposed to Kodak X-ray films. The ^{32}P-labeled DNA and RNA probes were prepared by random primer labelling and either using SP6 or T7 polymerase, respectively.

RNase protection assay Total RNA isolated from RV-infected Vero cells was hybridized with "hCC2" RNA probe set. Single-stranded RNA was digested by RNaseA and RNaseT1. The size fractionation of the protected RNA duplexes was carried out by electrophoresis on 5% polyacrylamide / 8M urea sequencing gels. Dried gels were exposed to Kodak X-ray films and visualized by autoradiography. For the preparation of ^{32}P-labelled RNA probe set, T7 polymerase was used.

Methods used to investigate protein-DNA interactions

Electrophoretic mobility shift assay (EMSA) and *in situ* UV cross-linking

For DNA binding studies, the following double-stranded oligonucleotides were used as probes: *(i)* κB-probe that corresponds to the NF-κB site of human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR), *(ii)* α4IE-probe that corresponds to the virus-inducible element of the IFN-α4 gene promoter region spanning the α4F1 and PRDI-like sequences, *(iii)* α4F1-probe, *(iv)* PRDI-probe. The oligonucleotides were labelled with [α-^{32}P]dCTP using Klenow polymerase. Protein-DNA complexes were formed by the incubation of nuclear extracts prepared from differently treated PBMC with radiolabelled DNA probes and resolved by electrophoresis on 4% nondenaturing polyacrylamide gels. Gels were dried and signals were imaged by exposure of Kodak X-ray films. For *in situ* UV cross-linking, the α4IE-probe was substituted with 5'-bromo-2'deoxyuridine and labelled with Klenow polymerase and [α-

^{32}P]dCTP. Protein-DNA complexes were resolved by gelelectrophoresis and UV irradiated *in situ* for 10 min at 4 °C. The UV-cross-linked protein-DNA adducts were then eluted and separated on SDS-10% polyacrylamide gels.

RESULTS

I. The molecular mechanisms involved in SV- and imiquimod-induced production of IFN and other cytokines

I.1. The induction of IFN and IL-6 in mouse L929 cells

SV-induced MuIFN and MuIL-6 production was studied in two sublines of mouse L929 cells (L929B and L929M), which were originally established and characterized by I. Rosztóczy.

To determine whether the different capacity of IFN and IL-6 production is manifested at the RNA level, the translational activity of IFN and IL-6 mRNAs present in polyA$^+$ RNA isolated from SV-induced and uninfected cells was quantitated by injection into *Xenopus laevis* oocytes. Our data showed that the L929B cells, which produced more IFN and IL-6 in response to SV infection, likewise contained more mRNAs encoding these cytokines than L929M cells (I./Fig.1, II./Fig.1 and Table 2). The results of *in vitro* translation of IL-6 mRNA were confirmed by Northern blot analysis (II./Fig.2). Antiserum to MuIFN-β neutralized ≥90% of the IFN activity present either in the culture media of the infected cells or in the oocyte-translated preparations, indicating the abundance of IFN-β in SV-induced L cells (II./Table 1 and data not shown). These results demonstrate that *(i)* SV induces simultaneous IFN and IL-6 production in L cells and *(ii)* the inherent differences in IFN- and IL-6-producing capacities of the L929B and L929M cells are manifested pretranslationally.

To investigate the regulatory role of IFNs, whereby, these cytokines modulate their own induction, as well as the synthesis of other cytokines, the effect of pretreatment with MuIFN-α/β was analyzed in L929B and L929M cells. The results showed that the SV-mediated induction of IFN- as well as IL-6 synthesis was highly upregulated by IFN and the priming-induced increased production of both cytokines was manifested in the relative levels of the respective mRNAs (I./Fig.1, II./Fig.1, Table 2 and Fig.2). These data indicate that the priming effect of IFN is based on pretranslational control mechanisms.

I.2. The induction of IFN and other cytokines in human PBMC

The induction of IFN and other cytokines was also investigated in human leukocytes in response to SV-infection or treatment with imiquimod. Our IFN-assay revealed a rapid and

transient IFN production, with the highest yields between 3 and 5 h after induction in imiquimod-stimulated PBMC. Maximal response to SV-infection was observed between 10 and 12 h postinduction and synthesis proceeded for about 24 h (III./Table 1). The IFN was identified as human IFN-α by serologic characterization, since ≥98% of the antiviral activity was neutralized by antibodies to human IFN-α. In addition to IFN, both imiquimod and SV induced high levels of IL-6. Thus, in leukocytes, SV and imiquimod stimulates the simultaneous synthesis of IFN-α and IL-6 without detectable production of IFN-β.

To determine the phenotype of the IFN-producing cells in human PBMC, monocytes, B lymphocytes and T lympocytes isolated by FACS sorting or indirect rosetting with magnetic beads (MACS) were induced with imiquimod or SV and culture medium was assayed for IFN activity. Both SV and imiquimod elicited IFN synthesis in B cells and monocytes but not in T lympocytes (III./Table 2). Thus, for the producer cell types, there was no difference between Sendai virus and imiquimod.

To evaluate the effect of SV-infection and imiquimod treatment on the expression of cytokine genes, the relative levels of IFN-α, IFN-β, TNF-α, IL-6, IL-8 and IRF-1 mRNAs were examined by Northern blot analysis. Both SV and imiquimod activated the transcription of all these cytokine genes; however, two major differences between the two inducers were observed: *(i)* SV-induced expression of these genes showed slower kinetics; and *(ii)* SV was a more effective inducer of all cytokine genes, except the IL-8 gene, which was induced more effectively by imiquimod (III./Fig.1). The IFN-α and IFN-β transcript levels were further analyzed with stimulated populations of adherent and nonadherent cells. A significant increase in the levels of IFN-α and IFN-β mRNAs was observed in both Sendai virus-infected and imiquimod-stimulated adherent and nonadherent cells (III./Fig.2). These results are in agreement with those from analyses of biologically active IFNs which were shown to be produced in both cell populations. Further analysis revealed increased levels of all respective cytokine mRNAs in PBMC treated with imiquimod in the presence of cycloheximide (CHX), indicating that the imiquimod-stimulated expression of all these cytokine genes did not require ongoing protein synthesis (III./Fig.1 and 2).

To investigate the signal transduction pathways involved in SV- and imiquimod-mediated induction of IFN-α and IFN-β genes, we analyzed the effects of inhibitors of tyrosine kinase (TK), protein kinase C (PKC) and protein kinase A (PKA) on stimulation of IFN-α and IFN-β gene expression in induced cells. Our data showed that the treatment of PBMC with staurosporin (PKC inhibitor) and genistein (TK inhibitor), before and during induction with

imiquimod, resulted in the inhibition of IFN-α and IFN-β expression, and no IFN-α or IFN-β mRNA could be detected in the cells 2 h postinduction. In contrast, treatment with HA1004 (PKA inhibitor) did not significantly change IFN-α and IFN-β transcript levels in imiquimod induced PBMC (III./Fig.4, lanes 2-9). When the effect of these inhibitors on SV-mediated induction of IFN-α and IFN-β genes was examined, staurosporin and genistein, but not HA1004, were found to inhibit the appearance of IFN-α and IFN-β mRNAs in infected cells. However, while staurosporin was able to completely block the induction of IFN mRNAs, low levels of IFN-α and IFN-β mRNAs could still be detected in genistein-treated cells, suggesting that genistein is a less effective inhibitor of virus-mediated induction than is staurosporin (III./Fig.4, lanes 10-13). These data suggest that both the imiquimod- and SV-activated signal transduction pathways, which mediate the induction of IFN-α and IFN-β genes, require tyrosine kinase and protein kinase C activity.

To identify the IFN-α subtypes, total RNA isolated from PBMC induced by SV or by imiquimod was used as a template for the reverse transcription of IFN-α-specific transcripts. Two sets of pimers were used for the amplification of the cDNAs: *(i)"general"* primers (the sequence was selected from a highly conserved region of the IFN-α genes in order to recognize all of the IFN-α subtypes) and *(ii) "IFN-α2-specific"* primers (III./Fig.5). The PCR-amplified DNA fragments obtained using the *"general"* primers were analyzed by restriction with *Ava II* and *Ava I* endonucleases, which are specific for IFN-α1 and IFN-α4, respectively. After restriction of the amplified PCR product (369 nt) from both SV- and imiquimod-induced cDNAs with *Ava II*, two small fragments of 171 and 188 bp were detected, indicating the presence of IFN-α1 transcripts in these cells. However, the density of these restriction fragments showed that the levels of IFN-α1 transcripts in imiquimod-induced PBMC were much lower than those in SV-induced cells. In contrast, the amplified DNA fragments were not restricted with *Ava I*, indicating the absence of IFN-α4 transcripts in Sendai virus- and imiquimod-induced PBMC (III./Fig.6A). The amplified fragments were then cloned, and 25 transformants were selected for sequencing. The clones of amplified cDNA from SV-induced PBMC were identified as IFN-α8, while from imiquimod-induced cells consisted of IFN-α5 and IFN-α8. Although a restriction analysis (with *Hph I*) of the 369-bp amplified fragment suggested that IFN-α2 mRNA is also present in both the Sendai virus- and imiquimod-induced cells, the IFN-α2 clone was not detected by cloning and sequencing. To verify unequivocally the presence of this subtype, the *"IFN-α2-specific"*primer pair was used. After amplification of

cDNA from SV- and imiquimod-induced cells, we obtained the expected 672-bp product, which was restricted with *Hinc II* into 550- and 122-nt fragments (III./ Fig.6B). The presence of the *Hinc II* site in the amplified region is unique to IFN-α2. The reliability of the *Hinc II*-restriction analysis was verified by sequencing. Restriction analysis indicated that a small portion of the 672-bp PCR product was resistant to *Hinc II* restriction. This unrestricted fragment was isolated and cloned. The sequence analysis showed that this fragment represented IFN-α6 in imiquimod-induced cells, while it was identical to IFN-α2 in SV-infected cells. In summary, we identified IFN-α1, IFN-α2 and IFN-α8 in SV-infected human leukocytes, while IFN-α1, IFN-α2, IFN-α8, IFN-α5 and IFN-α6 were detected in imiquimod-stimulated cells (III./Table 3). These data indicate a higher degree of IFN-α mRNA subtype heterogenicity in response to a synthetic cytokine-inducer than during the course of Sendai virus infection.

Since the promoter regions of all the cytokine genes induced by SV and imiquimod (except for IFN-α) contain an NF-κB binding site, which plays a critical role in the inducible expression of these genes, we examined the binding of nuclear proteins from imiquimod-induced monocytes and controls to DNA probes corresponding to the NF-κB sequences present in the HIV-1 LTR. The results of EMSA showed that imiquimod treatment activated NF-κB-specific binding (III./Fig.7A). Using antibodies specific to p50, p65 and c-rel, we demonstrated the presence of p50 and c-rel but not p65 in the imiquimod-induced NF-κB complexes (III./Fig.7B). In summary, our data showed the enhancement of NF-κB-specific binding in response to imiquimod treatment and suggest that the activation of NF-κB may be required for the induction of IFN-β, TNF-α, IL-6 and IL-8 genes.

To gain insight into the molecular events involved in the transcriptional activation of IFN-α genes, we examined the binding of nuclear proteins from SV-infected and imiquimod-treated PBMC to DNA probes corresponding to the α4F1 sequence using EMSA. The binding of nuclear proteins from untreated cells showed the presence of a strong, slowly moving complex (complex A) and weak, fast-moving complexes (complex B and C) (III./Fig.8A, lane1). In nuclear extracts from SV-infected cells or imiquimod-treated cells, a significant enhancement of complex B formation was detected (III./Fig.8A, lanes 2,3,6 and 7). Competition studies revealed that the formation of complex B was specific.

To analyze the proteins binding to the α4F1 probe, nuclear extracts from SV-infected or imiquimod-treated PBMC were incubated with BrdU-substituted α4IE probe that contains an αF1 sequence, inpartial overlap with a PRDI-like site. The protein-DNA complexes were separated on nondenaturing gels (III./Fig.9A), UV cross-linked *in situ*, then the three

complexes detected were individually eluted and subsequently analyzed by SDS-PAGE (III./Fig.9B). The results showed that four nuclear proteins (96-, 45, 29- and 25-kDa) bind to the IE in both induced and uninduced cells. The α4IE/A complex from uninduced cells showed an additional protein-DNA adduct of about 55 kDa. Induction by SV or imiquimod resulted in *(i)* the disappearance of binding of the 55-kDa protein to α4IE and *(ii)* the increased binding of the 29-kDa protein. Anti-IRF-2 Ab, used to determine whether the p55 and p29 represent IRF-2 and its proteolytical product, did not supershift any of the α4IE complexes, nor did preabsorption of the extracts with this antiserum remove the p55 or p29 protein-DNA adduct (data not shown). These data suggest that the formation of α4F1/complex B, detected in SV-infected and imiquimod-treated PBMC, is not the result of the binding of a novel protein to the inducible region of IFN-α genes, but rather is the result of posttranslational modifications of the constitutively expressed binding proteins, or their interaction with another non-DNA-binding component of the transcriptional complex. The identification of α4F1-binding proteins requires furher experimental work.

II. The molecular events implicated in RV-induced apoptosis

Indirect immunofluorescence assay to determine the frequency of infection in Vero cells infected with the To-336 strain of RV revealed positive staining for the RV E1 envelope glycoprotein on days 1, 3, 5 and 7 (IV./Fig.1, panels B, C, and D, and Fig.2, panel D) in 10, 68, 98 and 98% of the cells, respectively. Thus, the Vero cell line is highly permissive to RV replication, consistent with previous studies (134, 135).

To detect the hallmarks of apoptosis, RV-infected Vero cells were analyzed by different methods and compared to mock-infected cultures.

TdT-mediated digoxigenin-dUTP end-labeling (TUNEL) assay revealed very few positive cells in mock-infected cultures (IV./Figs. 2B and 2C), whereas, 60-70% of cells in RV-infected cultures were positive, although some viable cells were still present even after the 7-day incubation (IV./Figs. 2E and 2F). No cells treated with a UV-inactivated virus preparation were positive for the expression of the RV E1 envelope glycoprotein by immunofluorescence assay (IV./Fig. 2G) and no nuclei displayed positive TUNEL staining (IV./Figs. 2H and 2I). These results demonstrate that *(i)* RV causes DNA damage characteristic of apoptosis in a substantial proportion of cells and *(ii)* productive virus infection is necessary to trigger cell death.

The analysis of cellular DNA indicated DNA fragmentation 3 days after virus inoculation, with highest levels between 5 and 7 days post-infection (IV./Fig.3, lanes 6-8),

whereas no DNA degradation was detected in the mock-infected cultures until 7 days of incubation when a very weak DNA ladder appeared (IV./Fig.3, lanes 1-4). Thus, RV infection promotes the cleavage of cellular DNA into oligonucleosome-length fragments.

A double-parameter cytofluorometric DNA analysis to verify the presence of an apoptotic population in RV-infected cultures and to determine the distribution of proliferating cells within the different compartments of the cell cycle demonstrated that 1.5 and 46% of the total cell population in mock-infected and RV-infected cultures, respectively, had hypodiploid DNA content (IV./Fig.4). Moreover, the values calculated by the exclusion of apoptotic cells from the total number of cells indicated a decrease in active S phase (33%) and an accumulation in G2/M phase (18.5%), as compared with mock-infected cells (41% and 10% in S-phase and G2/M phase, respectively; IV./Fig.4). Thus, RV infection results in the appearance of cells with hypodiploid DNA content, characteristic of apoptosis, and perturbs progression through the cell cycle.

To examine the ability of RV to induce apoptosis in other cell types, the extent of DNA fragmentation was measured by an ELISA in infected RK-13 cells and also in two human embryonic fibroblast cell lines (HEL-17 and HEL-18). The results demonstrated that RV infection elicited apoptosis in RK-13 cells but not in fibroblast cell lines, as compared to the corresponding mock-infected controls (IV./Fig.5). Indirect immunofluorescence assay to determine the frequency of infection revealed positive staining for the E1 antigen in 62% of RK-13 cells or 54% and 58% of HEL-17 and HEL-18 fibroblasts, respectively. RV infection was attended by a visible CPE only in RK-13 cells, but not in the fibroblast cell lines (data not shown). These results indicate that RV causes apoptosis in a tissue-specific manner.

To gain insight into the molecular events implicated in RV-induced cell death, we focused on the role of the p53-dependent apoptotic pathway in Vero cells. The steady-state levels of p53 and p21 was determined by Western blot analysis using the corresponding antibodies (IV./Fig.6). Endogenous p53 protein levels in the mock-infected cells were below the detection limit at the exposure shown in IV./Fig.6. RV-infected cells showed elevated p53 levels 5 days after virus inoculation. Besides the presence of full-length p53 protein, two other products at 49 and 43.5 kDa were also detected in control cultures as well as in RV-infected cells (IV./Fig.6 and data not shown). p21 protein was undetectable in mock-infected cells for about 5 days (IV./Fig.6). During the course of RV infection, p21 protein levels increased with a kinetics similar to that of p53. These results demonstrate that RV infection increases the levels of p53 and p21 proteins in Vero cells.

A multi-probe RNase protection assay was used to evaluate the expression of genes encoding several mediators involved in cell cycle regulation and/or apoptosis, including p53, pRb, pRb-related proteins (p130, p107), and several cyclin-dependent kinase inhibitors (p57, p27, $p21^{Waf1/Cip1/Sdi1}$, p19, p18, p16, p15, p14) (IV./Fig.7). Both mock-infected and RV-infected cells expressed p130, pRb, p53 and p27 transcripts at each time point examined, and no significant alterations were detected in the transcriptional activity of any of these genes. Levels of p57 mRNA gradually increased through the 5 days of culture, but there was no quantitative difference between the mock-infected and RV-infected cultures. p21 gene expression was on average 2.8- and 6.4-fold higher at the 3- and 5-day time points in RV-infected cells as compared to mock-infected cultures in three independent experiments. p107 and p18 mRNA levels gradually decreased in both mock-infected and RV-infected cultures. Levels of p19, p16, p15, and p14 transcripts were very low or undetectable. Together, the results indicate that the level of p21 mRNA is highly increased, while the expression of all the other genes tested, including p53, is unaffected by RV infection.

To obtain further evidence for the importance of the p53-dependent apoptotic pathway we established Vero cell clones expressing dominant-negative p53 mutant ($p53^{W248}$). Immunofluorescence assay using PAb240 MAb (Ab-3) which detects only mutant p53, revealed strong nuclear staining in 98% of the cells in a line designated clone #1 (IV./Fig.8, panel A), while no positive cells were detected with PAb1620 MAb (Ab-5) which recognizes only wild-type 53, (IV./Fig.8, panel B). Similar results were obtained by the analysis of clone #2 (data not shown). These results confirmed that two individual cell clones overexpressed mutant p53 protein. To investigate a potential effect of mutant p53 on RV replication, the expression of RV E1 envelope glycoprotein was examined by immunofluorescence assay. The quantitation of the number of RV-infected cells revealed that 98% of cells were RV E1 antigen-positive in the two $p53^{W248}$ transfectant cell clones, as well as in the mock-transfected control cell line 5 days after infection (data not shown). Infected culture supernatants were also harvested and virus titers were determined by plaque assay. The data of two experiments showed that virus yields of the two Vero cell lines overexpressing $p53^{W248}$ (clone#1: $6.5x10^6$ PFU/ml, clone#2: $6.9x10^6$ PFU/ml) were almost identical to that of the control cell line ($6.6x10^6$ PFU/ml). These results indicate that virus production was unaffected by the presence of the dominant-negative p53 mutant. To determine whether the endogenous p53 in $p53^{W248}$ transfectants was rendered functionally defective, the RV-mediated induction of p21 was also investigated. Western blot analysis revealed a considerably lower expression of p21 protein in

the two cell clones carrying mutant p53 than in the mock-transfected control cell line (IV./Fig.9, lanes 5, 6, and 4, respectively). To assess the functional significance of p53 accumulation in RV-induced cell death, apoptosis was measured in cell clones expressing the $p53^{W248}$ mutant and compared to the mock-transfected control cell line. The results of ELISA detecting the enrichment of nucleosomes in the cytoplasm of apoptotic cells demonstrated that the dominant-negative p53 mutant conferred partial protection from RV-mediated apoptosis (IV./Fig.10). There was no substantial difference between the mock-transfected cells and the non-transfected parental Vero cells in the apoptotic response and virus yields (data not shown). These results indicate that RV induces apoptosis, at least in part, through p53-dependent mechanisms.

DISCUSSION

The molecular mechanisms involved in SV- and imiquimod-induced production of type I IFNs and other cytokines

In light of the important role of cytokines in the pathogenesis of infectious diseases, we investigated the molecular events implicated in the induction of type I IFNs and some other cytokines either in L cells or in PBMC in response to SV infection or treatment with imiquimod.

To analyze MuIFN and MuIL-6 production elicited by SV infection, we used two sublines of mouse L929 cells, established by I. Rosztóczy. Our results demonstrated that the L929B cells displayed a higher IFN production in response to SV than that of the L929M cells, had a higher sensitivity to the antiviral and priming effects of IFN and were more resistant to vesicular stomatitis virus (VSV). Serologic characterization revealed that the vast majority (≥90%) of IFN released by L929B and L929M cells corresponded to MuIFN-β. Our data also showed that SV infection triggered simultaneous production of IFN and IL-6 in both of these two L cell sublines. In good accord with the amount of IFN and IL-6 released, higher mRNA levels of the corresponding cytokines were detected in L929B cells than in L929M cells, indicating that the inherent differences in IFN- and IL-6-producing capacities of these two L cell sublines are manifested pretranslationally. Our findings correspond to observations demonstrating that the synthesis of these cytokines is coordinately regulated at the level of transcription (35, 38, 138, 139).

The priming effect of IFNs plays a prominent role in the early antiviral defense by providing resistance for the uninfected cells that limits replication and restricts spread of

viruses (89, 140 and reviewed in 12). To investigate the regulatory role of IFNs, whereby, these cytokines modulate their own production, as well as the synthesis of other cytokines, we analyzed the effect of SV infection in L929B and L929M cells pretreated with MuIFN-α/β. Our data showed that the SV-mediated induction of IFN, as well as IL-6 synthesis was highly upregulated by MuIFN-α/β. The priming-induced increased production of both cytokines was manifested in the relative levels of the respective mRNAs. These results indicate that: *(i)* the priming effect of IFN is based on control mechanisms, which operate at the level of transcripiton and *(ii)* IFN exerts an enhancing effect on IL-6 production. A primed IFN production may be implicated in the activation of antiviral immune effector mechanisms by promoting Th1 development effectively (141, 142). The fact that the priming effect is not excusive to the IFN system supports the notion that type I IFNs play an important regulatory role in immune responses to viral infections by modulating the production of several cytokines, as well (91, 143). The point of attack of priming in the process of IFN synthesis has been extensively studied. Previous results demonstrating that priming does not change the promoter sequence requirements for IFN induction suggested that the binding of IFNs to cellular receptors, besides triggering the activation of the IFN signal transduction pathway, may also modulate the activity of transcription factors involved in the stimulation of IFN gene expression (90). The role for ISGF3 in the priming effect has already been implied (144, 145). Based on studies showing that IRF-7 is induced by IFN-α/β via ISGF3 activation, it has recently been proposed that ISGF3-induced IRF-7, rather than ISGF3 per se, acts on IFN-α/β genes (43, 66, 145-147). More recent data indicated that ISGF3α proteins (STAT1 and STAT2) interact with CBP (78). This observation raises the possibility that CBP coactivator may also be implicated in the priming effect of IFNs. However, the underlying molecular events required for the priming are not yet fully understood.

Our findings have also demonstrated that SV and imiquimod elicited simultaneous production of IFN and IL-6 in human PBMC. Both SV and imiquimod induced IFN synthesis in B cells and monocytes, but not in T lymphocytes. Serologic characterization revealed that the vast majority (≥98%) of IFN released by leukocytes corresponded to HuIFN-α. We also showed that SV and imiquimod efficiently triggered the expression of genes encoding IFN-α/β, IL-6, IL-8, and TNF-α in PBMC, suggesting that the pattern of cytokine production elicited by the two inducers is similar. However, the comparative analysis of SV and imiquimod revealed substantial differences in the kinetics and level of expression of these cytokine genes. These results suggest that *(i)* SV infection induces the release of IFN-α and proinflammatory

cytokines in PBMC and *(ii)* imiquimod is a potent inducer of IFN-α, IL-6, IL-8, and TNF-α, hence may exert an immunomodulatory effect.

Our data showed that the induction of IFN-α/β and some other cytokine genes by imiquimod was transient and did not require cellular protein synthesis, indicating that imiquimod, similar to viral infection, modifies or activates preexisting latent cellular factors. Our results demonstrating that the induction of IFN-α and IFN-β genes by these two inducers requires TK and PKC activity, further emphasize the importance of protein phosphorylation in the induction of these genes.

To initiate the investigation of the underlying molecular events implicated in imiquimod-induced cytokine production, we analyzed the interaction of nuclear proteins extracted from imiquimod-treated cells with a DNA probe corresponding to the κB enhancer element. By showing a transient increase in the relative levels of NF-κB-specific complexes in imiquimod-induced primary monocytes, our data suggest that NF-κB-specific binding may be required for the imiquimod-mediated activation of IFN-β, TNF-α, IL-6, and IL-8 genes. We have also shown the presence of p50 and c-rel in the imiquimod-induced NF-κB complexes, while virus infection was shown to predominantly activate the binding of the p50-p65 complex (148). These data suggest that the NF-κB-specific proteins induced by Sendai virus and imiquimod may not be identical.

To analyze further the effect of imiquimod stimulation on the expression of IFN-α genes, we identified the individual IFN-α genes expressed in imiquimod-stimulated PBMC and compared them with Sendai virus-induced IFN-α genes in these cells. It was previously shown that IFN-α1 and IFN-α2 are the major mRNAs present in virus-induced leukocytes, while IFN-α8, IFN-α4, IFN-α5, IFN-α7, IFN-α14, IFN-α21, and IFN-α16 mRNAs were identified as minor components (19, 61). By PCR amplification and cloning, we detected IFN-α1, IFN-α2, and IFN-α8 in Sendai virus-induced human leukocytes. Under the same experimental conditions, IFN-α1, IFN-α2, IFN-α5, IFN-α6, and IFN-α8 were detected in imiquimod-induced PBMC. Thus, both inducers elicit the induction of "early (α1) and late (non-α1) IFN-α subtypes", as well. Although we cannot completely eliminate the possibility that another minor IFN-α mRNA is present either in Sendai virus- or imiquimod-induced cells, these results indicate a higher degree of IFN-α mRNA subtype heterogeneity in imiquimod-induced PBMC than in Sendai virus-infected cells.

It has been demonstrated that a 35-nt sequence identified in the promoter of the murine IFNA-α4 gene contains a symmetric sequence, GTAAAGAAAGT (α4F1), in partial overlap with a PRDI-like site (64). This sequence, termed inducible element (IE) is essential for the induction of the MuIFN-α4 promoter, and was shown to be well-preserved in the inducible region of murine and human IFNA genes, including those induced by imiquimod. Our data showed that the induction of IFN-α genes in SV-infected or imiquimod-treated PBMC is associated with an induction-specific transcription factor-DNA complex (αF1/B) formed between the α4F1 enhancer element (A motif) and nuclear extracts from induced cells. Results of UV cross-linking analysis and serologic characterization of proteins binding to the IE element of VRE-α suggest that the formation of α4F1/complex B, detected in SV-infected and imiquimod-treated PBMC, is not the result of binding of a novel protein to the inducible region of IFNA genes, but rather is the result of posttranslational modifications of the constitutively expressed binding proteins or their interaction with another non-DNA-binding component of the transcriptional complex. Since the formation of the α4F1/B complex is abolished in the presence of TK and PKC inhibitors (data not shown), we suggest that imiquimod-induced phosphorylation of the preexisting α4F1 binding proteins is an essential step in the induction process. Virus-mediated phosphorylation events have already been shown to play an important role in the activation of IRF-3 and IRF-7, as well as in the dissociation and degradation of IκB complexes leading to nuclear translocation of NF-κB (34, 39-41, 45, 149, 150). However, the identification of α4F1-binding proteins activated by imiquimod treatment requires further experimental work.

The important role of IFN-α/β, IFN-γ, IL-6, IL-12 and TNF-α, in controlling the growth of many viruses, as well as in shaping the immune responses to viral infections has already been well established (2-4, 12-18). Our results indicate that both SV and imiquimod induce simultaneous expression of IFNα/β, IL-6, IL-8, and TNF-α. Previous and recent observations corroborate and extend our findings by showing that imiquimod is also a potent inducer of IL-12 and IFN-γ, hence enhances innate and cellular immune responses (151-153). *In vivo* studies have also revealed that imiquimod inhibits the replication of certain viruses and growth of several transplantable murine tumors (20-22). Both the antiviral and antitumor effects of this compound are related to its ability to induce cytokine production (20-22, 153). These observations may provide a useful basis for the therapeutic application of imiquimod. Topical imiquimod cream has already been successfully used for the treatment of genital warts (154-158). Furthermore, it has recently been suggested that imiquimod may be an attractive

compound for use as vaccine adjuvant and also in inhibiting pathological responses mediated by Th2 cytokines (151).

The similarity between Sendai virus- and imiquimod-induced expression of IFN and cytokine genes may come as a major surprise to researchers in the IFN field. The requirement for dsRNA has been extensively documented for infections with a large variety of viruses. Our data suggest that activation by both these inducers involves phosphorylation rather than the presence of dsRNA per se.

The molecular events implicated in RV-induced apoptosis

In light of the important role of apoptotic cell death in the pathogenesis of several viral infections, we investigated the molecular events implicated in RV-induced cell death in an effort to gain some insight into the teratogenic effect of this virus.

Our data showed that the To-336 strain of RV replicated efficiently, induced a strong cytopathic effect, and triggered apoptosis in a high proportion of infected Vero cells. The slow time course development of apoptosis was consistent with the extent and kinetics of cytopathic effect. Our experiments also revealed apoptosis in another tissue culture system (RK-13) which is damaged by RV. In contrast, human embryonic fibroblast cell lines, in which RV establishes noncytocidal infection, proved to be resistant to the apoptotic effect of this virus. Thus, the results, we obtained using cells of different histological types, indicate that the cytopathogenicity of RV is linked to apoptotic mechanisms an observation which is consistent with recent findings (159-161).

In accord with other observations we showed that UV-inactivation completely abolishes the capability of RV to elicit an apoptotic response (159-161). This finding indicates that productive infection is necessary to trigger cell death. Recent data demonstrated that the cytopathic effect of RV correlates with the expression of non-structural proteins (NSPs) suggesting that the NSPs mediate apoptosis (162). It has also been demonstrated that overexpression of the structural proteins induces apoptosis suggesting that the capsid protein delivers the death signal (163). Thus, the requirement of productive infection for apoptosis induction possibly reflects the need for RV gene expression to reach sufficient levels of NSPs and/or capsid protein to elicit cell death.

To initiate the investigation of the underlying molecular events implicated in RV-induced cell death, we focused on the role of the p53-dependent apoptotic pathway in Vero cells. We detected a dramatic increase in the steady-state level of p53 protein in response to RV infection. The two additional species (49 and 43.5 kDa) may represent truncated forms of

p53 generated by limited proteolysis, or may correspond to differentially phosphorylated isoforms of this protein. Expression of the p53 gene was unaffected by RV infection, suggesting that posttranscriptional regulatory mechanisms account for the increase in p53 protein levels. Comparison of the kinetics of p53 accumulation with the progression of apoptosis and cytopathic effect provided correlative evidence that RV-induced apoptosis might be mediated by p53. To obtain further evidence for the importance of the p53-dependent pathway in cell death elicited by RV, we established Vero cell clones expressing dominant-negative p53 mutant ($p53^{W248}$). It has been shown before that the single amino acid substitution, Trp248 (W248), which maps to the p53 DNA binding domain confers dominant inhibitory activity to p53 because the mutant protein can oligomerize with wt p53 and abolish the sequence-specific DNA binding activity of the oligomer. Our analysis on the expression of the p53-inducible p21 protein confirmed that the endogenous p53 was rendered functionally defective by this p53 mutant in Vero cells. By showing an impressive reduction of apoptosis in $p53^{W248}$ stable transfectants, our results support the involvement of the p53-dependent apoptotic pathway in RV-mediated cell death. These results also disclose that only p53-independent mechanisms account for the apoptotic cell death of RV-infected cells (160). By showing that RV infection increases the level of p53 protein in Vero cells, these suggestions have recently been confirmed (163). Furthermore, our experiments revealed that the protection conferred by this p53 mutant was only partial, since apoptosis was observed in clonal cell lines overexpressing $p53^{W248}$. Although we can not exclude the possibility that inhibition of endogenous p53 by $p53^{W248}$ was not complete, our results indicate that multiple pathways of apoptosis are implicated in the cellular response to RV infection.

The upstream biochemical processes required for stabilization and activation of p53 highly depend on the inducer. It is well documented that p53 is rapidly stabilized following DNA damage, ribonucleotide depletion, or hypoxia (reviewed in 105). Previous studies have also shown that p53 can be activated by binding of short single-stranded DNA fragments, as well as by interaction with cellular or viral proteins (108 and reviewed in 92, 94, 95). Recent observations emphasize the importance of phosphorylation events in the regulation of p53 activity (109-111). It is possible that RV leads to genotoxic stress, since genetic alterations, including chromosomal breakages, are known to occur following RV infection (reviewed in 134, 135). Virus replication may also deplete cellular ribonucleotide pools and thereby contribute to the accumulation of p53. It has been proposed recently that the capsid protein of RV interacts with a signal cascade which connects events in the endoplasmic reticulum to a nuclear apoptotic response (163). However, a direct link has not been established between the

endoplasmic reticulum-initiated signaling pathway and p53. It is tempting to speculate that p53 represents an integration point where several signal transduction pathways activated by RV converge.

We also showed that RV infection stimulates the expression of p21. Although G1 arrest mediated by p21 can inhibit p53-dependent apoptosis, apoptosis can occur even in the presence of p21 (107). Cytofluorometric DNA analysis revealed no overt cell cycle block in the G1 phase due to RV infection, but instead the presence of a population with hypodiploid DNA content, demonstrating that apoptosis dominates over G1 arrest in a high proportion of cells. Recent data indicate that the biological consequence (i.e., G1 arrest or apoptosis) of the joint effect of p53 and p21 depends on their stoichiometric ratio as well as on the cellular context of their action determined by the histological type of cell and by the presence of extracellular signals (106, 107). Thus, while RV infection causes apoptosis in Vero cells, other cell types that are less susceptible to the cytopathogenic effect of the virus may show predominantly G1 arrest. This combination of CPE and mitotic inhibition is reflected in the pathology of CRS, which exhibits features both of destruction of specific tissues and growth retardation (137). A possible role for p21 might be that of a mediator in the reconciliation of RV replication and cellular functions, promoting the noncytopathogenic form of infection and the establishment of viral persistence rather than mediating apoptosis.

Taken together, our data may bear on CRS, since tissue-specific apoptosis may account for certain dysfunctions observed in CRS patients and point out the importane of the p53-dependent apoptotic pathway in the molecular mechanism of virus-mediated teratogenesis.

SUMMARY

The apoptotic response and cytokine release of virally infected cells can function as early antiviral defense mechanisms. IFNs and some other cytokines display direct antiviral effects and shape antigen-specific immune responses. Apoptosis provides a swift mechanism to eliminate damaged or potentially dangerous virus-infected cells.

In light of the important role of cytokines in the pathogenesis of infectious diseases, we investigated the molecular events implicated in the induction of type I IFNs and some other cytokines.

To analyze IFN and IL-6 production elicited by SV infection, two sublines of mouse L929 cells were used. The L929B cells displayed a higher IFN production in response to SV than that of the L929M cells, had a higher sensitivity to the antiviral and priming effects of IFN and were more resistant to vesicular stomatitis virus (VSV). Serologic characterization revealed

that the vast majority (≥90%) of IFN corresponded to IFN-β. SV infection triggered simultaneous production of IFN and IL-6 in both of these two L cell sublines. In good accord with the amount of IFN and IL-6 released, higher mRNA levels of the corresponding cytokines were detected in L929B cells than in L929M cells, indicating that the inherent differences in IFN- and IL-6-producing capacities of these two L cell sublines are manifested pretranslationally.

Pretreatment of L929 cells with IFN-α/β primed the production of both cytokines. The priming-induced increased production of IFN and IL-6 was manifested in the relative levels of the respective mRNAs. These results indicate that: *(i)* the priming effect of IFN is based on control mechanisms, which operate at the level of transcripiton and *(ii)* IFN exerts an enhancing effect on IL-6 production.

Previous results demonstrated that the imidazoquinolineamine derivative 1-(2-methyl propyl)-1H-imidazole [4,5-C] qunoline-4-amine (imiquimod) induces IFN-α synthesis both *in vivo* and in PBMC *in vitro*. Imiquimod and SV were used to gain more insight into the molecular mechanisms required for the coordinated regulation of certain cytokine genes. Imiquimod stimulated the expression of several IFN-α genes (IFN-α1, IFN-α2, IFN-α5, IFN-α6 and IFN-α8), as well as the IFN-β gene in PBMC. Imiquimod also activated the transcription of TNF-α, IL-6 and IL-8 genes. Expression of all these genes was transient, independent of cellular protein synthesis and was inhibited in the presence of of tyrosine kinase and protein kinase C inhibitors. Infection with SV led to the expression of a similar set of cytokine genes and several of the IFN-α genes (IFN-α1, IFN-α2 and IFN-α8). Imiquimod stimulated the binding of NF-κB-specific complexes that recognize the κB enhancer element present in the promoters of all cytokine genes tested, but not in IFN-α genes. The NF-κB complexes induced by imiquimod were composed of p50 and c-Rel proteins. Both SV and imiquimod induced the formation of nuclear complex(es) that recognize the α4F1 site, 5'-GTAAAGAAAGT-3', conserved in the inducible element of IFN-α genes. These results indicate that imiquimod and SV stimulated the expression of a large number of cytokine genes, including IFNα/β and that the signal transduction pathway induced by both of these stimuli requires tyrosine kinase and protein kinase C activity.

In light of the important role of apoptotic cell death in the pathogenesis of several viral infections, we asked whether the cytopathogenicity evoked by rubella virus (RV) might also involve apoptotic mechanisms.

The To-336 strain of RV induced apoptosis in Vero and RK-13 cells, but not in fibroblast cell lines. UV-inactivated RV virions did not elicit the apoptotic response, indicating that productive infection is required for the induction of cell death. Both p53 and p21 protein levels were highly elevated in RV-infected Vero cells. The level of p21 mRNA was increased, while expression of p53 gene was unaffected by RV infection. A dominant-negative p53 mutant ($p53^{W248}$) conferred partial protection from RV-induced apoptosis. These data implicate a p53-dependent apoptotic pathway in the cytopathogenicity of RV, thereby suggesting a mechanism by which RV exerts its teratogenic effects.

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ANNEX