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Stimulation of Interferon and Cytokine Gene Expression by Imiquimod and Stimulation by Sendai Virus Utilize Similar Signal Transduction Pathways†

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The imidazoquinolineamine derivative 1-(2-methyl propyl)-1*H*-imidazole [4,5-*c*]quinoline-4-amine (imiquimod) has been shown to induce alpha interferon (IFN-α) synthesis both in vivo and in peripheral blood mononuclear cells in vitro. In this study, we show that, in these cells, imiquimod induces expression of several *IFNA* genes (*IFNA1*, *IFNA2*, *IFNA5*, *IFNA6*, and *IFNA8*) as well as the *IFNB* gene. Imiquimod also induced the expression of interleukin (IL)-6, IL-8, and tumor necrosis factor alpha genes. Expression of all these genes was transient, independent of cellular protein synthesis, and inhibited in the presence of tyrosine kinase and protein kinase C inhibitors. Infection with Sendai virus led to expression of a similar set of cytokine genes and several of the *IFNA* genes. Imiquimod stimulates binding of several induction-specific nuclear complexes: (i) the NF-κB-specific complexes binding to the κB enhancer present in the promoters of all cytokine genes, but not in *IFNA* genes, and (ii) the complex(es) binding to the A4F1 site, 5′-GTAAAGAAAGT-3′, conserved in the inducible element of *IFNA* genes. These results indicate that imiquimod, similar to viral infection, stimulates expression of a large number of cytokine genes, including IFN-α/β, and that the signal transduction pathway induced by both of these stimuli requires tyrosine kinase and protein kinase activity.

The low-molecular-weight imidazoquinolineamine derivative 1-(2-methyl propyl)-1*H*-imidazole [4,5-*c*]quinoline-4-amine (imiquimod) inhibits replication of herpes simplex virus type 2 and cytomegalovirus in infected guinea pigs (6, 24). Imiquimod-mediated inhibition of virus replication is related to its ability to induce interferon (IFN). Oral administration of imiquimod induces IFN-α in mice, rats, guinea pigs, monkeys, and humans. (New approved nomenclature for IFN genes [9a] is used throughout this paper.) In addition to having antiviral activity, imiquimod was shown to inhibit growth of several transplantable murine tumors, including MC-26 colon carcinoma and Lewis lung carcinoma (71). The induction of IFN-α plays a major, but not exclusive, role in this growth inhibition, since an antiserum to mouse IFN-α and IFN-β significantly reduced the antitumor effect of imiquimod but was not able to abolish it completely.

IFN-α proteins are represented by a large family of structurally related genes which show about 94% homology at the nucleotide level, while IFN-β is encoded by a single gene. *IFNA* genes are expressed preferentially in cells of lymphoid lineage, and the individual subtypes show cell-type-specific differences in expression (2, 29, 35), while *IFNB* is expressed in a large variety of cells. The biological significance of the large abundance of *IFNA* genes is not clear; all IFN-α and IFN-β subtypes show antiviral and antitumor properties and seem to bind to a common receptor (39); however, some differences between their immunomodulatory effects have been reported (21, 56).

Virus-induced expression of *IFNA* and *IFNB* genes is mediated by a virus-responsive element (VRE) present in the promoters of *IFNA* and *IFNB* genes that, by itself, functions as a virus-specific enhancer and can confer inducibility in infected cells (3, 14, 19, 20, 58, 65). At least two families of transcriptional factors were shown to play a role in induction of IFN genes that have binding sites within the VRE. One family is the set of IFN-responsive factors IRF-1 and IRF-2, which function as activator and repressor, respectively (22, 23). Overexpression of these two factors can regulate activity of both *IFNA* and *IFNB* promoter regions in a transient expression assay; a single nucleotide mutation in the IRF-1 binding site of the murine *IFNA4* gene promoter decreased inducibility by about 100-fold (2), and cells expressing IRF-1 antisense mRNA were unable to express *IFNB* genes (62). However, the role of IRF-1 in induction of the IFN gene has been questioned, since deletion of the IRF-1 gene did not affect virus-mediated inducibility of IFN genes either in mice in vivo or in cultured cells in vitro (53, 64). The second family of transcriptional factors are the κB-specific binding proteins that play a role in activation of the *IFNB* gene (16, 19, 28, 43, 77), and recently, a direct role of IFN-induced double-stranded RNA (dsRNA)-dependent kinase in activation of NF-κB has been shown (37, 49). Furthermore, it was shown that HMG I(Y) and ATF-2 can bind to the VRE (48, 75) of the *IFNB* gene, where HMG I(Y) proteins appear to bend the DNA and facilitate binding of both NF-κB and ATF-2.

Factors involved in the regulation of *IFNA* genes other than IRF-1 are less well-defined. Two proteins of 68 and 96 kDa were found to bind the A4F1 regulatory element in the VRE of the murine *IFNA4* gene promoter (1, 2), and MacDonald et al.

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(47) described a factor (named TG) that binds to the VRE of the human *IFNA1* gene. These proteins, however, have not yet been characterized.

The signal transduction pathway, triggered by viral infection that activates these factors and leads to the induction of IFN genes, is not known. Earlier studies have suggested that the dsRNA formed as an intermediate product during replication of RNA viruses is essential for the stimulation of expression of IFN genes (32, 50). This assumption was based on the following observations. First, there is a difference in the IFN-inducing capacities of plus- and minus-stranded RNA viruses. Infectivity of plus-stranded viruses is essential for IFN induction, while the ability of UV-irradiated minus-stranded viruses to induce IFN correlated with their ability to transcribe their genome and produce complementary RNA (26, 46). Second, D1 particles of vesicular stomatitis virus containing covalently linked complementary message and antimessage RNA are very effective IFN inducers (51) and dsRNA, such as poly(rI)·poly(rC), alone is able to induce expression of the *IFNB* gene (11). Third, DNA viruses that do not form dsRNA during the replication cycle are not effective inducers of IFN (30). However, in lymphocytes, expression of the Sendai virus C gene was sufficient to induce IFN (74), and viral glycoproteins or nucleic acid-free viral envelopes were also able to induce IFN production in peripheral blood mononuclear cells (PBMC) (12, 33, 40, 42, 81). Thus, the role of dsRNA as an essential trigger for IFN induction remains questionable.

The aim of the proposed study was to analyze the molecular mechanism by which imiquimod (R-837) stimulates IFN synthesis in PBMC in vitro, to determine the responsive cell type(s), and to identify the expressed IFN subtypes. The results show that imiquimod stimulates expression of *IFNA1*, *IFNA2*, *IFNA5*, *IFNA6*, and *IFNA8* genes as well as *IFNB* in PBMC and that IFN synthesis can be detected in B cells and monocytes/macrophages. In addition to inducing expression of IFN genes, imiquimod also induced expression of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-8 genes; expression of all these genes was transient, independent of cellular protein synthesis, and inhibited by tyrosine kinase (TK) and protein kinase C (PKC) inhibitors.

MATERIALS AND METHODS

Isolation of different populations of leukocytes. Human PBMC were isolated from Leukopack platelets by density gradient centrifugation in lymphocyte separation medium (LSM) (Organon Teknika Corp., Durham, N.C.) (4). The cells were further separated on the basis of their adherence to plastic surfaces (36). To determine the type of cells responsible for IFN production, B cells, T cells, and monocytes were isolated either by sorting with a fluorescence-activated cell sorter (FACS) or by indirect rosetting with Dynabeads. T and B lymphocytes were separated by FACS from the nonadherent population of human PBMC after being stained with anti-Leu-4 (CD3) and anti-Leu-12 (CD19) monoclonal antibodies, respectively (Simultest T and B Cell Test; Becton-Dickinson). For separation by magnetic beads, T cells and B cells were first isolated from the nonadherent fraction of PBMC by rosetting with sheep erythrocytes as described elsewhere (13). T cells were then isolated by positive selection with anti-CD3 monoclonal antibody and incubated with anti-mouse-immunoglobulin G (IgG)-coated Dynabeads. B cells consisted of a population of a nonadherent, E-rosette-negative fraction of PBMC that did not form rosettes with the anti-mouse-IgG-coated dynabeads after treatment with anti-CD3 and anti-CD14 murine monoclonal antibodies. Monocytes were isolated from a fraction of human PBMC that adhered (after 15 min) to plastic by staining with anti-CD14 mouse monoclonal antibody and selection with anti-mouse-IgG-coated Dynabeads. For induction, 5×10^6 PBMC, monocytes, or nonadherent cells per ml and 5×10^4 separated cells per ml were used. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

PCR analysis. Two sets of oligonucleotide primers corresponding to sequences of human *IFNA* genes were used (25). A pair of primers designed to recognize all of the *IFNA* subtypes had the following sequences: 5′ primer, GTACTGCAGAATCTCTCCTTTCTCCTG (nucleotides [nt] +1067 to 1086); 3′ primer, GTGTCTAGATCTGACAACCTCCCAGGGCACA (nt +1415 to 1435). The second set of primers was specific for *IFNA2* and had the following sequences: 5′ primer, GTACTGCAGCATCTGCAACATCTAC (nt +911 to 930); 3′ primer, GTGTCTAGAGTCTTTGAAATGGCAG (nt +1565 to 1582). The general primers, selected from a highly conserved region of the *IFNA* genes (see Fig. 4), amplified a 369-bp fragment, while the *IFNA2*-specific primers amplified a 672-bp product corresponding predominantly to *IFNA2* mRNA. Each primer contained either a *Pst*I (5′ primer) or an *Xba*I (3′ primer) restriction sequence that facilitated its cloning. The sequences of the restriction sites are underlined; a single line represents the *Pst*I site, and a double line represents the *Xba*I site.

In the reverse transcription (RT)-PCR analysis, first-strand synthesis (cDNA) was carried out in 50 mM Tris-HCl (pH 8.3)–140 mM KCl–10 mM $MgCl_2$–4 mM dithiothreitol (DTT)–1 mM each deoxynucleoside triphosphate (dNTP mix)–20 U of RNase inhibitor (Life Technologies, Inc.)–1.25 U of avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.)–1 μg of oligo(dT) (Sigma). The reaction mixture (25 μl) was incubated at 42°C for 2 h. Next, 1/10 of the cDNA mixture was amplified in 50 mM KCl–10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$–0.01% (wt/vol) gelatin–200 μM dNTP mix–200 μg of each primer–2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.) in a volume of 50 μl by use of an automated thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). 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. As a control, amplifications were done in a reaction mixture without first-strand cDNA. Amplified fragments were either digested with the appropriate restriction endonucleases to identify the fragment amplified or ligated to pGEM4.

DNA sequencing. Sequencing of dsDNA was done by the dideoxy chain-terminating method (68) with a Sequenase kit (United States Biochemical Corp.) and SP6 and T7 primers.

RNA and DNA probes. *IFNA* riboprobe was prepared by insertion of a 764-bp *Eco*RI-*Bgl*II fragment of human *IFNA2* cDNA (55) into pSP64 followed by cloning. The plasmid was linearized with *Eco*RI, and RNA was transcribed by SP6 polymerase. The *IFNB* probe was prepared by insertion of a 520-bp fragment of human *IFNB* cDNA (60) into pSP64, and the plasmid was linearized with *Eco*RI. The IL-6 probe was prepared by insertion of a 550-bp *Pst*I fragment of human IL-6 cDNA into pSP64 vector, followed by linearization with *Eco*RI. IRF-1 probe was prepared by ligation of a 460-bp *Bgl*I-*Eco*RI fragment of human IRF-1 cDNA into the *Hin*cII site of pSP65 and linearization with *Hin*dIII. TNF-α probe was represented by an 800-bp *Eco*RI fragment of human TNF-α cDNA in Bluescript SK vector; after linearization with *Bam*HI, RNA was transcribed with T7 polymerase. Human γ-actin cDNA in pSP64 vector was linearized with *Hin*tI. All transcribed RNAs were radiolabelled with [α-^{32}P]GTP as described previously (1). pTZ/IL-8 plasmid was digested with *Pst*I, and the ~400-bp cDNA insert was purified and labelled with [α-^{32}P]dCTP (Pharmacia) by random priming.

Northern (RNA) blot analysis. Human PBMC (10^7 per cell sample) were stimulated with 3 μg of imiquimod per ml or infected with Sendai virus for various times; control cultures incubated in parallel were left untreated. Total cellular RNA was prepared by the guanidine thiocyanate method (7), and 10 μg was denatured in 50% deionized formamide–2.2 M formaldehyde–20 mM MOPS (morpholinepropanesulfonic acid) for 15 min at 60°C and chilled on ice. RNA was then fractionated by electrophoresis in 0.8% agarose gels containing 2.2 M formaldehyde and 20 mM MOPS and subsequently blotted onto nitrocellulose filters with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization and hybridization with single-stranded riboprobes or dsDNA probes were performed at 60 and 45°C, respectively, in a buffer containing 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 5× SSC, 1× Denhardt's solution, 50 mM potassium phosphate buffer (pH 6.6), and 0.25 μg of denatured salmon sperm DNA per ml. After hybridization, filters were washed with 0.1% SDS–0.5× SSC at 60 or 45°C and exposed to Kodak XAR-5 film with intensifying screens at −70°C. Blots were stripped for sequential hybridization by boiling with 0.1× SSC–0.1% SDS for 15 min.

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously (2), and specific protein-DNA complexes were detected by a gel mobility shift assay (2). Briefly, each cell pellet was resuspended in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.3 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5% Nonidet P-40) and incubated at 4°C. Nuclei were collected by centrifugation, resuspended in buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 500 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), and incubated for 30 min at 4°C. After centrifugation at 15,000 rpm in a Tomy MTX-150 microcentrifuge, the supernatant fraction was dialyzed against buffer D (20 mM HEPES [pH 7.9], 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF) and stored at −70°C.

Preparation of radiolabelled oligonucleotides and electrophoretic mobility shift assays. Double-stranded oligonucleotide spanning the NF-κB site of human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (5′-TCAAGGGACTTTCCGCTGGGACTTTCCCTCTCCTT-3′) (78) was used for DNA binding studies. The underlined sequence was used for annealing of a primer, GGGAGAGGAA, and synthesis of the complementary strand with Klenow polymerase labelled with [^{32}P]dCTP. Protein-DNA complexes were formed by incubation of 5 to 10 μg of nuclear protein with 1 to 10 pg of radiolabelled DNA probe at 4°C for 5 min in a 25-μl reaction volume containing 12 mM Tris (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$ 2 μg of poly(dI)·poly(dC) per ml, and 7% glycerol. For supershift experiments,

TABLE 1. Induction of IFN synthesis in R-837-stimulated and Sendai virus-infected PBMC

Cell type	No. of Sendai virus-infected cells at 16 h	Amt of IFN (U/ml) induced by imiquimod at:						
		0 h	2 h	3 h	4 h	5 h	9 h	24 h
Unseparated[a]	5×10^3	<2	<2	160	ND[b]	1,280	192	2
Monocytes	8×10^3	<2	<2	8	ND	16	6	16
Nonadherent	6×10^3	<2	16	2,048	640	256	128	28
$CD14^+$ monocytes[c]	256						6	6
$CD18^+$ B cells[a]	128						32	
$CD3^+$ T cells[a]	<4						<4	

[a] 5×10^6 cells per ml were used for stimulation.
[b] ND, not done.
[c] Cells were separated by FACS; separation with Dynabeads gave similar results. 5×10^4 cells per 100 μl were used for stimulation.

serum containing antibodies against p65, p50, or c-rel was added to the preincubated DNA-protein binding mixture and then the mixture was incubated for 1 h on ice.

The sequences of A4F1 (2) and PRDI (48) used as probes were 5′-GCGTA AAGAAAGTCCCTCTCCTT and 5′-GAGAAGTGAAAGTGGGAACCCTC TCCTT, respectively; the primer-annealing sequence is underlined. The ^{32}P-labelled double-stranded probes were prepared by extension of the primer with Klenow polymerase and [^{32}P]dCTP. The binding mixture was identical to that described above.

Protein-DNA complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels in 0.4× Tris-borate-EDTA, pH 8.3, at 150 V for 2 h at room temperature. Gels were dried and exposed to Kodak X-ray film.

UV cross-linking in situ. Nuclear proteins were bound to a probe (A4IE) that corresponds to the virus-inducible element of the *IFNA4* gene promoter region containing the A4F1 and IRF-1 binding sequences (2) substituted with 5′-bromo-2′-deoxyuridine (BrdUrd) (Boehringer-Mannheim) and labelled with Klenow polymerase and [^{32}P]dCTP. The DNA-protein complex was resolved on a 4% nondenaturing polyacrylamide gel and UV irradiated in situ for 10 min at 4°C (2, 78). The UV-cross-linked DNA-protein adduct was then eluted and separated on an SDS–10% acrylamide gel.

RESULTS

Induction of IFN in human PBMC. To determine the kinetics of IFN production in human PBMC, cells were induced with 3 μg of imiquimod per ml for different periods of time. The applied dose was found to be optimal in a dose-response experiment (data not shown). Imiquimod induced rapid and transient IFN production, with the highest yield between 3 and 5 h after induction (Table 1). In contrast, the maximal response to Sendai virus infection was observed between 10 and 12 h postinduction (data not shown) and synthesis proceeded for about 24 h (Table 1 and data not shown). 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 inducing IFN, both imiquimod and Sendai virus induced high levels of IL-6 (6,400 U at 4 h).

Identification of the cell types producing IFN-α. To evaluate the phenotype of the IFN-producing cells in human PBMC, we initially separated PMBC into a population of cells that adhered to plastic (the majority of cells are monocytes) and those that were nonadherent (predominantly B and T cells) and found that both these cell populations produced IFN-α after exposure to imiquimod. We therefore further purified B cells, T cells, and monocytes by FACS sorting or indirect rosetting with dynabeads as described in Materials and Methods. Separated cells were induced with imiquimod or Sendai virus, and medium was assayed for IFN activity (Table 2). Both Sendai virus and imiquimod induced IFN synthesis in B cells and monocytes but not in T cells. Thus, for the producer cell types, there was no difference between Sendai virus and imiquimod.

Imiquimod-induced expression of *IFNA*, *IFNB*, TNF-α, IL-6, IL-8, and IRF-1 mRNAs. We further examined the effect of imiquimod treatment on expression of IFN genes and analyzed the relative levels of *IFNA* and *IFNB* mRNAs in stimulated cells. The results of Northern blot analysis are shown in Fig. 1. Stimulation with imiquimod led to a significant increase in the relative levels of *IFNA* and *IFNB* mRNAs as early as 2 h after induction, while at 6 h poststimulation *IFNA* and *IFNB* mRNAs were undetectable. Although we did not detect IFN-β protein, Northern blot analysis revealed the presence of *IFNB* mRNA in induced cells. *IFNB* mRNA was stabilized in the presence of cycloheximide (CHX) as described previously (60, 61) (Fig. 1). Expression of the *IFNB* gene without detectable secretion of the IFN-β protein has previously been found to occur in Sendai virus-induced Namalwa cells (59, 70).

Imiquimod stimulation of PMBC also increased the relative levels of TNF-α, IL-6, and IL-8. Expression of all these genes was transient, reaching a maximum between 2 and 4 h after induction and decreasing to baseline levels at 24 h. Expression of the transcription factor IRF-1, which is induced by viral infection, showed slower kinetics; maximal levels of IRF-1 mRNA were detected at 4 h after induction, and expression did not return to baseline levels over 12-h period. It has been shown that both TNF-α and IFNs induce expression of IRF-1 mRNA (15); thus, the prolonged increase in IRF-1 levels observed may be mediated by the cytokines induced by imiquimod. Imiquimod-stimulated expression of all these cytokine genes did not require ongoing protein synthesis, and increased levels of the respective cytokine mRNAs could be detected in CHX-treated cells. CHX was previously shown to increase the rate of *IFNB* transcription by interfering with synthesis of the

TABLE 2. Induction of IFN-α in different cell types

Selection method and cell type[a]	Amt of IFN (U/ml)[b] induced by:	
	Imiquimod	Sendai virus
FACS selection		
Monocytes ($CD14^+$)	14	64
B cells ($CD19^+$)	16	128
T cells ($CD3^+$)	<2	<2
Dynabead selection		
Monocytes ($CD14^+$) (adherent)	6	256
B cells ($CD3^-$ $CD14^-$) (nonadherent, E-rosette negative)	32	ND
T cells ($CD3^+$) (nonadherent, E-rosette positive)	<4	<4

[a] 5×10^4 separated cells were used for induction; uninduced cells produced less than 4 U of control activity per ml.
[b] Antiviral activity was abolished by treatment with antibodies to human IFN-α.

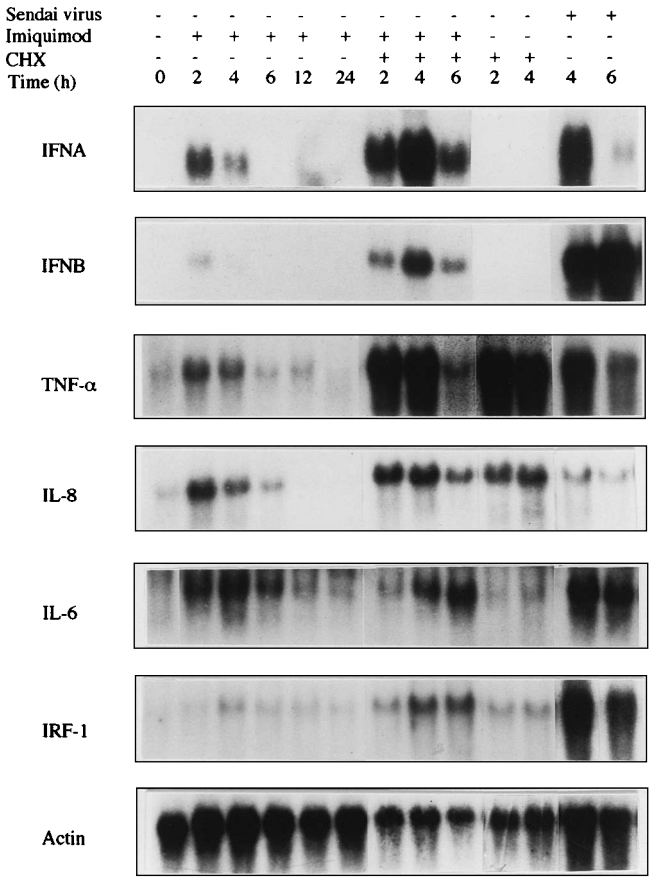

FIG. 1. Expression of IFN-α/β, TNF-α, IL-6, IL-8, and IRF-1 genes in imiquimod-stimulated PBMC. PBMC isolated on a Ficoll gradient were induced (2×10^7 cells per sample) with imiquimod (3 μg/ml) in the presence (+) and absence (−) of CHX (30 μg/ml) for the indicated times. Isolated total RNA (10 μg) was analyzed by Northern hybridization with the respective probe as described in Materials and Methods. As a control, cells were induced with 200 hemagglutinin units of Sendai virus per ml for 4 and 6 h. Films were exposed for 16 h. Hybridization with γ-actin probe is shown as a control for RNA loading.

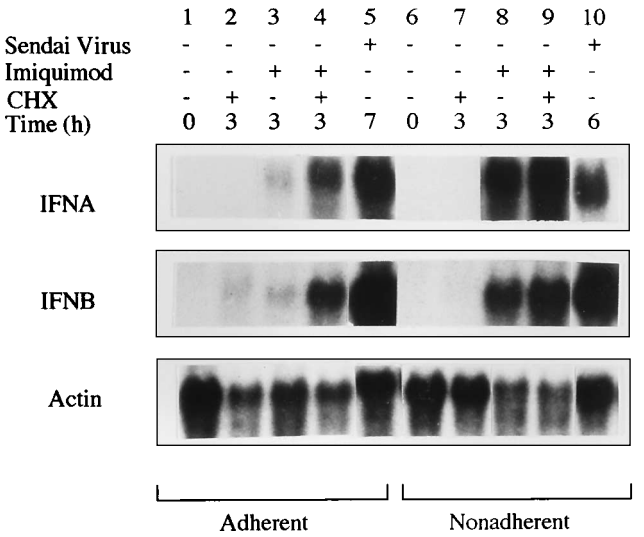

FIG. 2. Expression of *IFNA* and *IFNB* genes in induced adherent and nonadherent PBMC. PBMC were allowed to adhere to plastic for 50 min; nonadherent cells were collected, and adherent cells were washed with medium containing 1% fetal bovine serum. Induction was done for 3 h with imiquimod-stimulated cells (lanes 3 and 8) and for 6 to 7 h with Sendai virus-infected cells (lanes 5 and 10). Cells treated with CHX alone or CHX in combination with imiquimod are shown in lanes, 2, 4, 7, and 9. Isolation and analysis of total RNA were done as described in the legend to Fig. 1. A 16-h exposure of the membranes is shown. Lanes 1 to 5 represent adherent cells, and lanes 6 to 10 represent nonadherent cells. +, present; −, absent.

transcriptional repressor (34) as well as activating NF-κB (p50-p65 complex) (27, 69), which may lead to increased transcription of TNF-α, IL-6, and IL-8 genes, as well as *IFNB*. Furthermore, the posttranscriptional effect of CHX, which occurs at the level of mRNA stabilization, was demonstrated for *IFNB* and TNF-α (45, 60).

Induction of IFN genes and various cytokine genes in Sendai virus-infected PBMC was analyzed as a control. Sendai virus induced expression of all these cytokine genes; however, two major differences between imiquimod and Sendai virus were observed: (i) Sendai virus-induced expression of these genes was slow; maximal increases in the *IFNA* and *IFNB* mRNA levels occurred at 4 and 6 h postinfection, respectively; and (ii) Sendai virus was a more effective inducer of all cytokine genes, except the IL-8 gene, which was induced more effectively by imiquimod.

The levels of *IFNA* and *IFNB* mRNAs were further analyzed with stimulated populations of adherent cells enriched with monocytes and macrophages and nonadherent cells containing mostly T and B cells. A significant increase in the relative levels of *IFNA* and *IFNB* mRNAs after imiquimod treatment was observed to occur in both imiquimod-stimulated and Sendai virus-infected adherent and nonadherent cells (Fig. 2). These results are in agreement with those from analyses of biologically active IFNs which were shown to be produced in both adherent and nonadherent cells (Table 1).

Imiquimod does not stimulate expression of *IFNA/B* and IL-6 genes in murine monocyte line Raw but alters the NDV-mediated stimulation of these genes. We have not been able to induce expression of IFN-α/β genes by imiquimod in any fibroblast, B-cell, T-cell, or monocyte line tested (data not shown). However, we have been able to induce effectively the expression of various cytokine genes in the murine macrophage line Raw 264, both by viral infection and by treatment with lipopolysaccharide. We have, therefore, tested whether imiquimod induces expression of *IFNA/B* and cytokine genes in these cells. While no stimulation of *IFNA*, *IFNB*, or the IL-6 gene could be induced by imiquimod in these cells, treatment with imiquimod during virus infection significantly inhibited the Newcastle disease virus (NDV)-mediated induction of *IFNA* genes (Fig. 3A). In contrast, treatment with imiquimod superinduced NDV-induced *IFNB* mRNA levels by about 2- to 3-fold and IL-6 mRNA levels by about 10-fold. These results show that, in the absence of detectable stimulation of *IFNA/B* and IL-6 genes, imiquimod differentially altered the expression of *IFNA* and *IFNB* or IL-6 genes.

To determine whether these effects can also be seen in cells where imiquimod induces expression of cytokine genes, PBMC were infected with NDV in the presence of imiquimod. The relative levels of *IFNA* mRNA were lower in cells treated with both inducers than in those induced with NDV alone. The presence of *IFNA* mRNA could also be detected in cells treated with imiquimod alone (Fig. 3), although the levels were much lower than those induced by NDV, since maximal levels of *IFNA* mRNA in imiquimod-treated cells are induced at 2 h postinfection (compare Fig. 1). These data indicate that imiquimod has a noticeable inhibitory effect on the NDV-stimulated expression of *IFNA* genes in both human PBMC and the

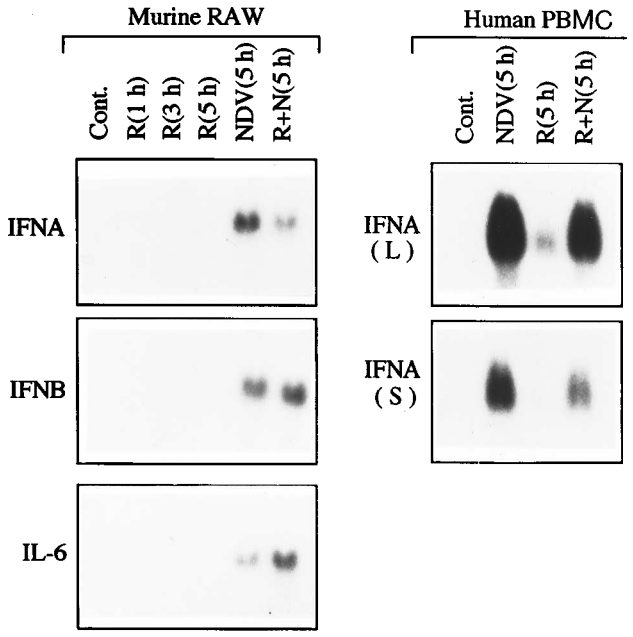

FIG. 3. Imiquimod modulates NDV-mediated induction of IFN and IL-6 genes. Murine Raw 264 cells at 80% confluency and human PBMC were induced with imiquimod (R) (3 μg/ml) for the indicated periods of time, after which total RNA was isolated. Alternatively, these cells were induced with NDV (N) (multiplicity of infection of 5) in the presence and absence of imiquimod (3 μg/ml) for 5 h. Total RNA (10 μg) was analyzed by Northern hybridization as described in Materials and Methods. A 20-h exposure of the films is shown in panel IFNA (L), and a 4-h exposure is shown in panel IFNA (S). Cont., control; L, long; S, short.

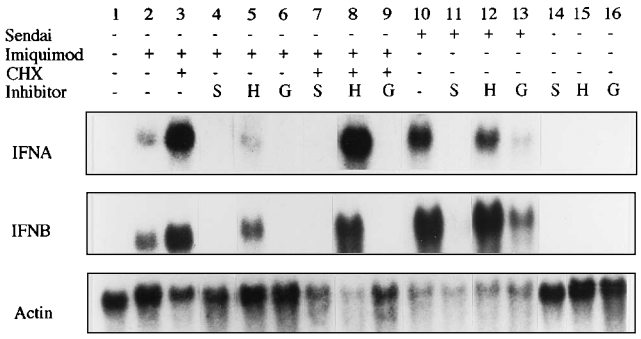

FIG. 4. Inhibition of imiquimod-mediated induction of *IFNA* and *IFNB* genes by genistein (G) and staurosporin (S). PBMC were pretreated with genistein (100 nM), staurosporin (80 nM), or HA1004 (H) (30 μM) for 30 min and then induced with imiquimod or Sendai virus in the presence or absence of an inhibitor. RNA was isolated at 3 h postinduction with imiquimod (lanes 2 to 9) or at 6 h postinfection with Sendai virus (lanes 10 to 13) and analyzed by Northern hybridization with *IFNA* and *IFNB* probes as described in Materials and Methods. Hybridization with γ-actin probe was used to estimate the amounts of RNA on filters. +, present; −, absent.

murine macrophage line Raw. Similar to what was observed with murine Raw cells, no inhibition in the *IFNB* and IL-6 mRNAs was seen after treatment with virus and imiquimod (data not shown). These data suggest that the virus-induced signal transduction pathways that lead to the induction of *IFNA* and *IFNB* genes are not identical.

Effect of kinase inhibitors on expression of IFN genes. To determine whether induction of *IFNA* and *IFNB* genes by imiquimod and induction by Sendai virus occur through similar signal transduction pathways, we analyzed the effects of inhibitors of TK, PKC, and protein kinase A (PKA) on stimulation of *IFNA* and *IFNB* gene expression in induced cells. Figure 4 shows that treatment of the cells with staurosporin (PKC inhibitor) and genistein (TK inhibitor), before and during induction, resulted in inhibition of *IFNA* and *IFNB* expression, and no *IFNA* or *IFNB* mRNA could be detected in cells 2 h postinduction (lanes 4, 6, and 7). Additional experiments in which we used calphostin C, a more specific inhibitor of PKC, also showed that pretreatment with 50 nM calphostin C completely inhibited induction of *IFNA* (data not shown), thus indicating that PKC activity is required for the induction process. In contrast, treatment with HA1004 (PKA inhibitor) did not significantly change levels of *IFNA* and *IFNB* mRNAs induced by either Sendai virus or imiquimod (Fig. 4, lanes 5 and 12). These results suggest that both TK and PKC activity, but not PKA, are part of the imiquimod-mediated transduction signal. When the effect of these inhibitors on Sendai virus-mediated induction of *IFNA* and *IFNB* genes was examined, staurosporin and genistein, but not HA1004, were found to inhibit the appearance of *IFNA* and *IFNB* mRNAs in infected cells (Fig. 4, lanes 11 to 13). However, while staurosporin was able to completely block induction of *IFNA* mRNAs, low levels of *IFNA* and *IFNB* mRNAs could still be detected in genistein-treated cells, suggesting that genistein is a less effective inhibitor of virus-mediated induction than is staurosporin.

Similar results were observed when the effect of these inhibitors on expression of IL-6, IL-8, and TNF-α genes was examined (data not shown). Both genistein and staurosporin, but not HA1004, inhibited the imiquimod- and virus-induced expression of these genes. In contrast, there was little variation in the relative levels of actin mRNA induced in the presence and absence of inhibitors, showing that the effect is specific and is not due to toxicity or variations in RNA loading. These data, together with lack of dependence of induction of *IFNA* and *IFNB* genes on cellular protein synthesis, suggest that induction of IFN genes by both virus infection and imiquimod involves phosphorylation of preexisting cellular factors.

Determination of *IFNA* subtypes induced by imiquimod. The high degree of homology among *IFNA* genes prevents identification of various *IFNA* mRNA subtypes by Northern blot hybridization. We and others have previously used S1 and RNase protection analyses for identification of various *IFNA* mRNAs in infected murine and human cells (29, 35). In the present study, we used a more sensitive RT-PCR assay. Total cellular RNA isolated from PBMC induced by imiquimod or by Sendai virus was used as a template for reverse transcription of *IFNA*-specific transcripts. The IFN cDNAs were then amplified by PCR, and the amplified DNA was characterized by restriction analysis, cloned, and sequenced.

Two sets of primers were used for amplification of the cDNAs (Fig. 5). Initially, primers containing highly conserved sequences (general primers) were used (see Materials and Methods) and PCR-amplified DNA fragments were analyzed by restriction with *Ava*II and *Ava*I endonucleases, which are specific for *IFNA1* and *IFNA4*, respectively. After restriction of the amplified DNA fragment (369 nt) from both Sendai virus- and imiquimod-induced cDNAs with *Ava*II, two small fragments of 171 and 188 bp were detected, indicating the presence of *IFNA1* transcripts in these cells (Fig. 6A). However, the density of these restriction fragments indicated that the levels of *IFNA1* transcripts in imiquimod-induced cells were much lower than those in Sendai virus-induced cells. In contrast, the amplified DNA fragments were not restricted with *Ava*I, indicating the absence of *IFNA4* transcripts in Sendai virus- and imiquimod-induced cells. The amplified fragment from both

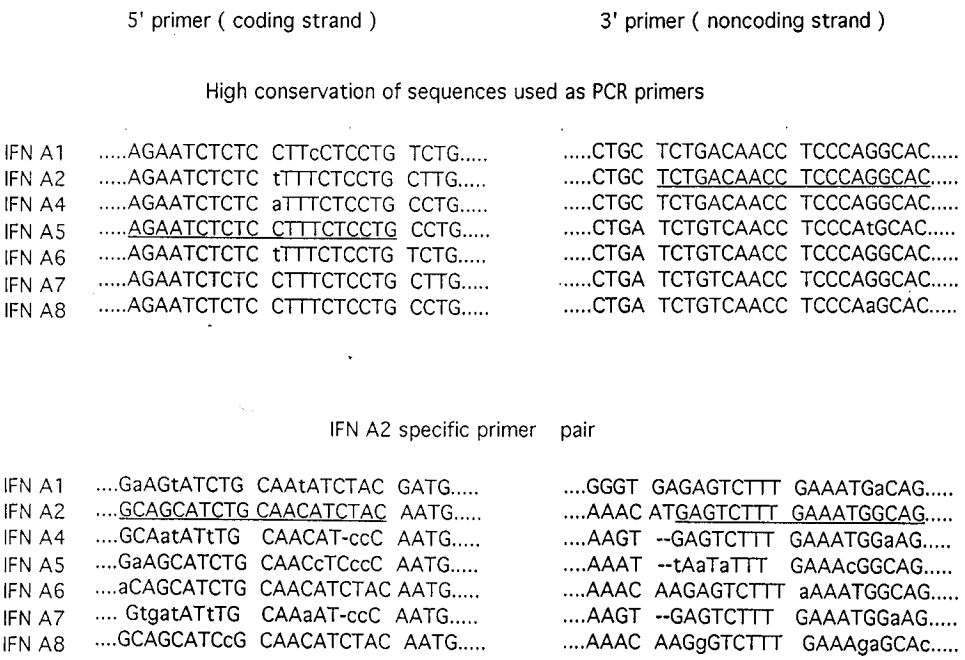

FIG. 5. General and *IFNA2*-specific primer sequences present in various human *IFNA* genes (25). The underlined sequences were used for PCR amplification. Lowercase letters indicate nucleotides distinct from those in the corresponding region of the primer. The 5′ and 3′ conserved primers correspond to nt +1067 to 1086 and nt +1415 to 1435, respectively. The *IFNA2*-specific primers correspond to nt +911 to 930 (5′) and nt +1565 to 1582 (3′).

Sendai virus- and imiquimod-induced cDNAs could also be restricted with *Hpa*I, suggesting the presence of *IFNA2* mRNA in induced cells (data not shown).

The amplified fragments were then cloned, and 25 transformants were selected for sequencing. As shown in Table 3, all the colonies amplified from the cDNA of Sendai virus-induced cells were identified as *IFNA8*, while the clones of amplified cDNA from imiquimod-induced cells consisted of *IFNA8* and *IFNA5*. The relative proportions of *IFNA8* and *IFNA5* were 77 and 23%, respectively. The preferential cloning of the *IFNA8* subtype may be due to the 100% homology of the selected primers with the *IFNA8* cDNA. Although the general primer pair was designed to recognize highly conserved regions of the *IFNA* genes, there were small differences in the sequences corresponding to the primer regions in *IFNA1*, *IFNA2*, *IFNA6*, *IFNA5*, and *IFNA4* cDNAs (Fig. 5). The conserved 5′-end primer selected shows 100% homology with the *IFNA8*, *IFNA7*, and *IFNA5* coding region, while all other corresponding coding regions show a single nucleotide difference. The 3′ primers selected show 100% homology with all the *IFNA* genes except *IFNA5* and *IFNA8*, which show a single nucleotide difference.

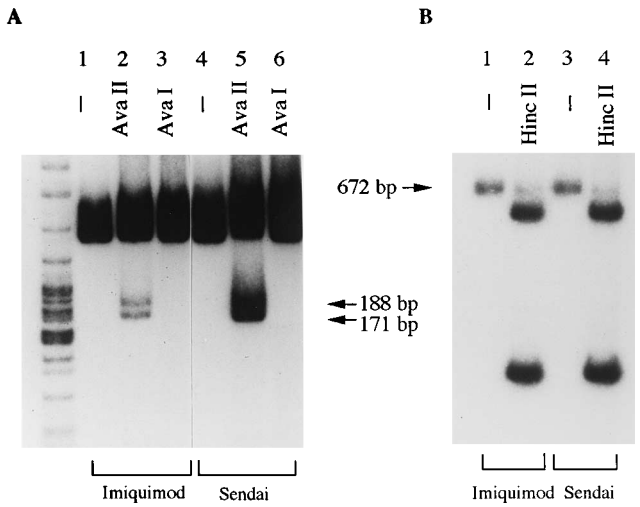

FIG. 6. Restriction analysis of DNA fragments amplified with a general and an *IFNA2*-specific set of primers. The RT-PCR was done as described in Materials and Methods with the end-labelled general and *IFNA2*-specific primers. General primers (A) amplified a 369-bp DNA fragment from imiquimod-induced (lanes 1 to 3) and Sendai virus-induced (lanes 4 to 6) cDNAs. Lanes 1 and 4 represent the uncut fragments; restriction with *Ava*II and the appearance of two fragments of 171 and 188 bp are shown in lanes 2 and 5. Lanes 3 and 6 show a lack of restriction with *Ava*I. The *IFNA2*-specific primer (B) amplified a 672-nt DNA fragment (lanes 1 and 3) that was restricted with *Hinc*II (lanes 2 and 4). Lanes 1 and 2 show amplification of a fragment from imiquimod-induced cDNAs, and lanes 3 and 4 show amplification of a fragment from Sendai virus-induced cDNAs. The amplified 672-bp fragment and the 171- and 188-bp fragments are marked.

Although a restriction analysis (with *Hph*I) of the amplified fragment suggested that *IFNA2* mRNA is present in both the imiquimod- and Sendai virus-induced cells, the *IFNA2* clone was not detected by sequencing. Therefore, we used a second *IFNA2*-specific primer pair (Fig. 5) to verify unequivocally the presence of this subtype. After amplification of cDNA from imiquimod- and Sendai virus-induced mRNAs, we obtained, as expected, the 672-bp fragment, which was restricted with *Hinc*II into 550- and 122-nt fragments (Fig. 6B). The presence of the *Hinc*II site in the amplified region is unique to *IFNA2*.

This 672-nt fragment was then cloned, and 50 colonies were selected and analyzed by restriction with *Hinc*II restriction endonuclease; all clones but one could be restricted with *Hinc*II and thus were identified as *IFNA2*. The undigested clone was identified as *IFNA6* by sequencing. All the clones obtained by cloning of an amplified fragment from Sendai virus-induced cells were characterized as *IFNA2* cDNA by *Hinc*II digestion. The reliability of the analysis with *Hinc*II was verified by se-

TABLE 3. *IFNA* subtypes expressed in imiquimod- and Sendai virus-stimulated PBMC

Stimulation	Result[a] for:								
	IFNA1	*IFNA13*	*IFNA2*	*IFNA4*	*IFNA5*	*IFNA6*	*IFNA7*	*IFNA8*	*IFNA14*
Sendai virus	+[b]	—	+[b,c]	—	—	—	—	+[d]	—
Imiquimod	+[b]	—	+[b,c]	—	+[d]	+[c]	—	+[d]	—

[a] —, not detected; +, present.
[b] Determined by restriction analysis of the IFN cDNA amplified by using general primers.
[c] Determined by restriction analysis and cloning of the IFN cDNA amplified with *IFNA2*-specific primers.
[d] Determined by cloning and sequencing of the IFN cDNA amplified with general primers.

quencing; sequences of two clones derived from imiquimod- and Sendai virus-induced samples were identical to *IFNA2*. Restriction analysis (Fig. 6B) indicated that a small portion of the 693-bp fragment was resistant to *Hin*cII restriction. This unrestricted fragment was isolated and cloned. The sequence analysis showed that this fragment represented *IFNA6* in imiquimod-induced cells, while it was identical to *IFNA2* in Sendai virus-infected cells. Thus, in summary, this analysis indicates that whereas imiquimod induces expression of *IFNA1*, *IFNA2*, *IFNA5*, *IFNA6*, and *IFNA8* in PBMC, in the same cells Sendai virus infection stimulates expression of *IFNA1*, *IFNA2*, and *IFNA8* (Table 3).

Induction of NF-κB-specific binding activity. Since the promoter regions of all the cytokine genes induced by imiquimod (except for *IFNA* genes) contain an NF-κB binding site, which plays a critical role in the inducible expression of these genes, we examined binding of nuclear proteins from imiquimod-induced cells and controls to DNA probes corresponding to the NF-κB sequences present in the HIV-1 LTR (78). Binding of nuclear proteins from CHX-treated cells and Sendai virus-infected cells was used as a positive control since both CHX and virus infection stimulate binding of the p50-p65 complex (16, 69, 76). Relatively high levels of constitutive nuclear NF-κB activity were observed in uninduced PBMC, since NF-κB is constitutively nuclear in mature B cells (54, 69). We therefore analyzed induced NF-κB binding in separated primary monocytes. Figure 7 shows the transient increase in the relative levels of NF-κB-specific complexes (A and B) that could be detected as early as 25 min after imiquimod induction, followed by a rapid decrease to basal levels. The formation of complex A was specific and could be completely inhibited by a 10-fold excess of the unlabelled probe (Fig. 7), while formation of complex B was slightly inhibited only by a 50-fold excess of unlabelled probe. In contrast, treatment with CHX induced a complex with mobility slightly faster than that of complex A. These data show that imiquimod treatment, similar to viral infection (16, 76), activates NF-κB-specific binding. Using antibodies specific to p50, p65, and c-rel, we have demonstrated, by mobility shift assay, the presence of p50 and c-rel but not p65 in the imiquimod-induced κB complexes (Fig. 7B).

Induction of α4F1-specific binding activity. The A4F1 element plays a critical role in the induction of murine *IFNA* genes (1), and the transcriptional activation of murine *IFNA* genes in virus-infected cells is associated with formation of a novel complex, A4F1/B (1, 2). Since the consensus of A4F1 sequence is also present in the inducible region (VRE) of human *IFNA* genes (Fig. 8C), we examined the binding of nuclear proteins from imiquimod-treated and virus-infected cells to DNA probes corresponding to the A4F1 sequence.

In the gel retardation assay, the binding of nuclear proteins from untreated cells shows the presence of a strong, slowly moving complex (complex A) and weak, fast-moving complexes (complexes B and C) (Fig. 8A, lane 1). In nuclear extracts from Sendai virus-infected cells or imiquimod-treated cells, a significant enhancement of complex B formation was detected (Fig. 8A, lanes 2, 3, 6, and 7). The formation of this complex was transient: enhancement was detected as early as 30 min after virus infection or imiquimod treatment (data not shown), and the levels of the A4F1/B complex returned to basic levels at 3 to 4 h postinduction. The formation of the A4F1/B complex was specific; it could be inhibited either with the unlabelled A4F1 probe or with the 35-nt-long inducible element (A4IE) of the *IFNA4* promoter region that contains the A4F1 sequence (Fig. 8B) but not with a nonspecific DNA such as pGEM plasmid or NF-κB probe (data not shown). The unlabelled A4F1 fragment also inhibited the formation of complex C, present in both the induced and uninduced nuclear proteins, but not complex A. In contrast, unlabelled A4IE effectively inhibited the formation of all complexes (A, B, and C). These results suggest that, similar to what occurs with murine cells (2), the formation of complex B is related to transcriptional activation of *IFNA* genes and that a similar complex(es) is induced in Sendai virus-infected and imiquimod-treated cells. However, it is unlikely that complex B

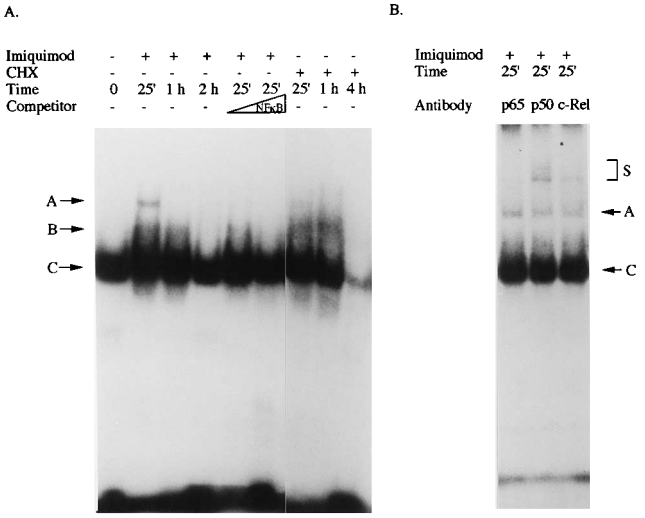

FIG. 7. Induction of NF-κB binding activities in imiquimod-induced monocytes. Monocytes were prepared from the adherent fraction of PBMC. Nuclear extracts were prepared as described in Materials and Methods from imiquimod-treated (3 μg/ml) cells at different times postinduction and analyzed for the presence of NF-κB activity by using oligonucleotides corresponding to the HIV-1 LTR NF-κB site (see Materials and Methods). All reaction mixtures contained 1 μg of a nonspecific competitor, poly(dI) · poly(dC). (A) The unlabelled probe was used as a specific competitor at 5- and 50-fold molar excesses. CHX-treated and uninduced cells were used as controls. (B) A supershift experiment with the indicated antibodies was done as described in Materials and Methods. +, present; −, absent; S, supershift.

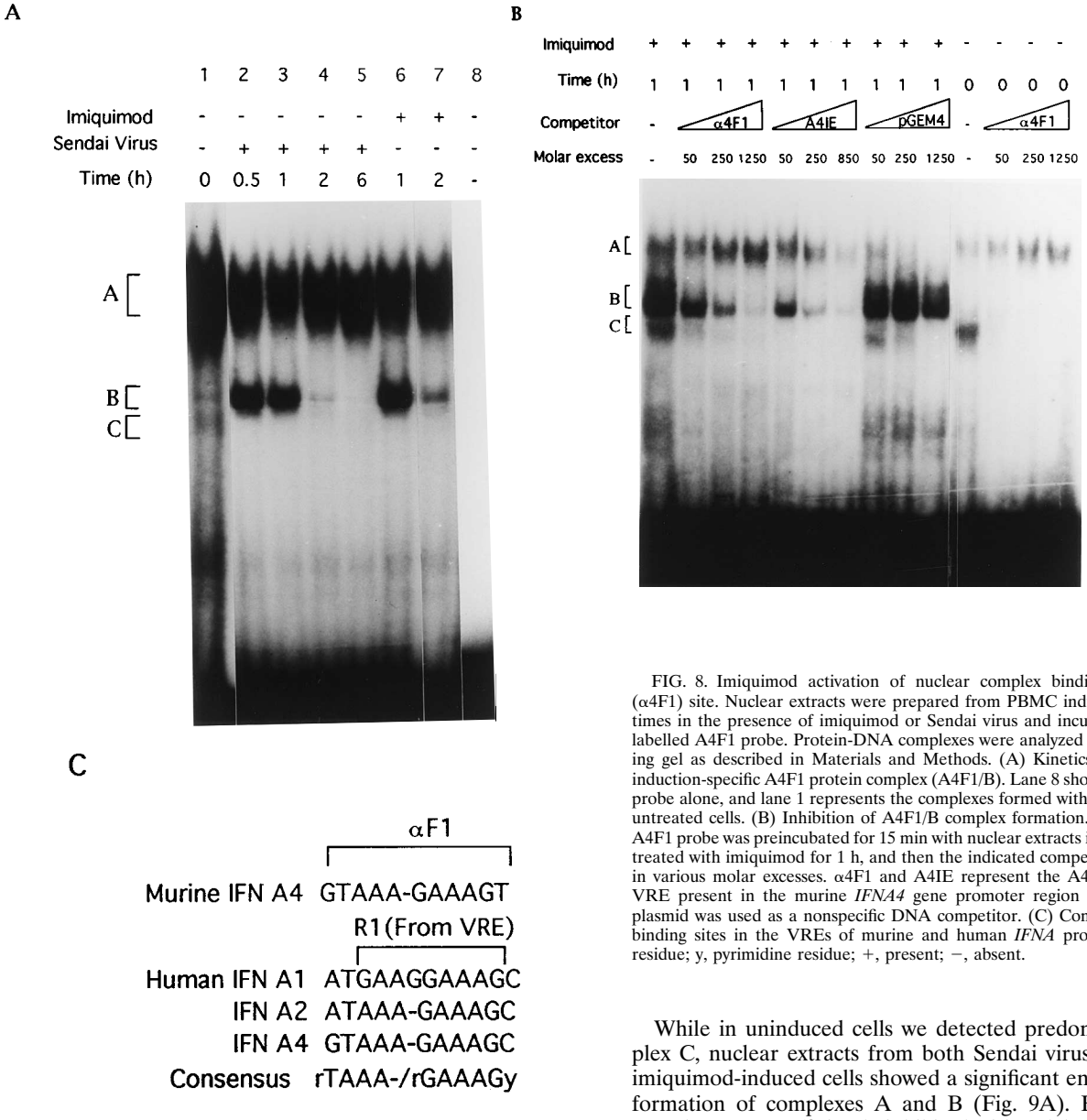

FIG. 8. Imiquimod activation of nuclear complex binding to the A4F1 (α4F1) site. Nuclear extracts were prepared from PBMC induced for different times in the presence of imiquimod or Sendai virus and incubated with a ^{32}P-labelled A4F1 probe. Protein-DNA complexes were analyzed on a nondenaturing gel as described in Materials and Methods. (A) Kinetics of formation of induction-specific A4F1 protein complex (A4F1/B). Lane 8 shows mobility of the probe alone, and lane 1 represents the complexes formed with extracts from the untreated cells. (B) Inhibition of A4F1/B complex formation. The ^{32}P-labelled A4F1 probe was preincubated for 15 min with nuclear extracts isolated from cells treated with imiquimod for 1 h, and then the indicated competitors were added in various molar excesses. α4F1 and A4IE represent the A4F1 sequence and VRE present in the murine *IFNA4* gene promoter region (2), and pGEM4 plasmid was used as a nonspecific DNA competitor. (C) Comparison of A4F1 binding sites in the VREs of murine and human *IFNA* promoters. r, purine residue; y, pyrimidine residue; +, present; −, absent.

represents binding of IRF-1 or NF-κB-specific proteins, since it could be supershifted neither with IRF-1-, p50-, p65-, nor c-rel-specific antibodies (data not shown).

Characterization of proteins binding to A4F1 by UV cross-linking. We have previously shown (2) that, in murine cells induced with virus, the A4F1/B complex contains at least two DNA-binding proteins, p96 and p68, but not the IRF-1 protein. To analyze the proteins binding to the A4F1 probe in Sendai virus-infected and imiquimod-treated PBMC, nuclear extracts prepared from Sendai virus-infected or imiquimod-treated cells at 1 h posttreatment were incubated with the BrdUrd-substituted A4IE probe. We used this probe rather than the A4F1 probe because the BrdUrd substitution in A4F1 altered the mobility of A4F1/B and A4F1/C complexes. The DNA-protein complexes were separated on nondenaturing gels (Fig. 9A) and UV cross-linked in situ, and the three complexes detected were individually eluted and subsequently analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 9B).

While in uninduced cells we detected predominantly complex C, nuclear extracts from both Sendai virus-infected and imiquimod-induced cells showed a significant enhancement in formation of complexes A and B (Fig. 9A). Both of these complexes were efficiently inhibited with unlabelled A4IE oligonucleotides (data not shown). Cross-linking of the A4IE/A complex shows the presence of two DNA-protein adducts of 96 and 45 kDa present in both induced and uninduced cells (Fig. 7B). However, the A4IE/A complex from uninduced cells shows an additional DNA-protein adduct of about 55 kDa that is absent in induced cells. The A4IE/B complex shows the presence of 29- and 25-kDa DNA-protein adducts in both induced and uninduced cells (Fig. 9B). The fast-moving complex, A4IE/C, contains three DNA-protein adducts of 45, 29, and 25 kDa in induced cells, while in uninduced cells, only 29- and 25-kDa adducts were detected. The 28- and 25-kDa proteins are present in both induced and uninduced cells; however, the levels of the 29-kDa DNA-protein adduct detected in induced cells were much higher than those in uninduced cells, indicating that induction increased DNA binding of the 29-kDa protein. Thus, these data show that induction by Sendai virus or imiquimod results in (i) disappearance of binding of the 55-kDa protein to A4IE and (ii) increased binding of a 29-kDa protein. To determine whether the p55 and p29

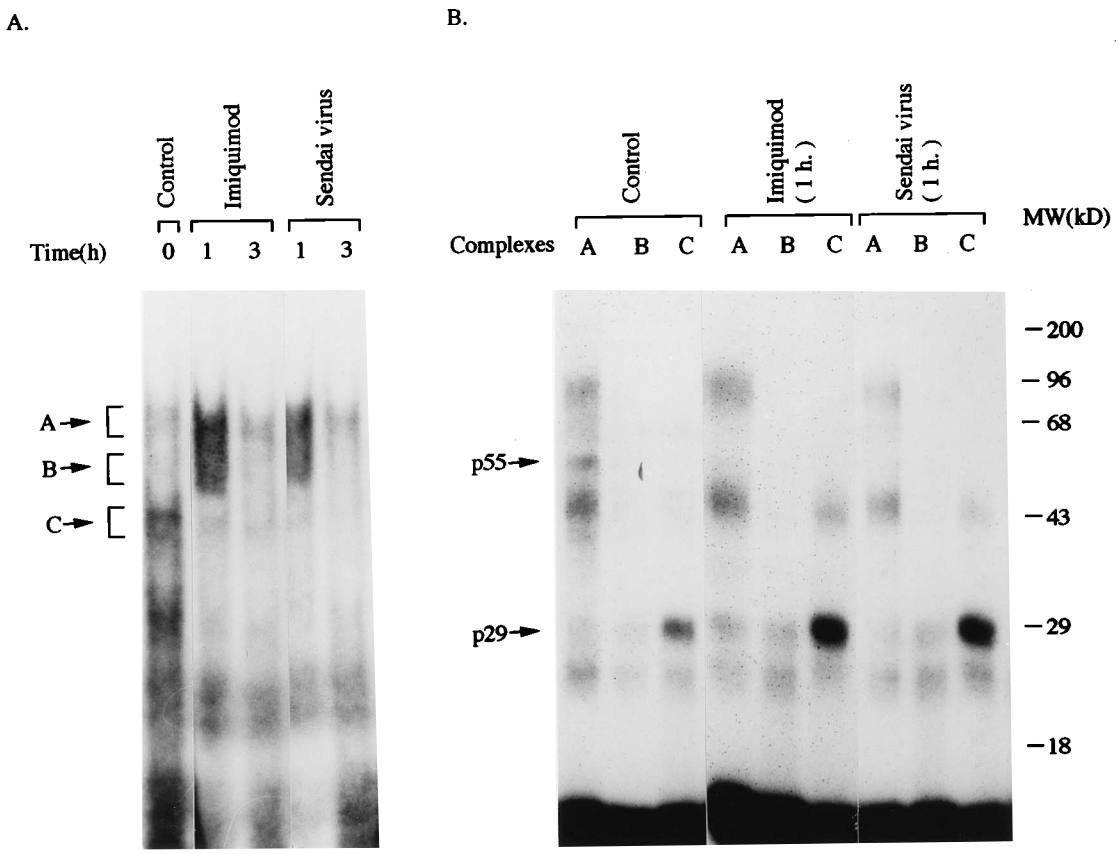

FIG. 9. UV cross-linking analysis of DNA-protein adduct formed between the IE sequence and nuclear extracts from imiquimod-treated and Sendai virus-infected cells. (A) Nuclear extracts prepared from induced cells at 1 and 3 h postinduction and from uninduced controls were incubated with the BrdUrd-substituted A4IE probe and resolved on a 4% acrylamide gel. (B) DNA and proteins were UV cross-linked in situ; gels were UV irradiated as described previously (78); bands corresponding to the A, B, and C complexes were eluted; and protein-DNA complexes were analyzed on an SDS–10% polyacrylamide gel. The molecular mass (MW) markers and the p55 and p29 DNA-protein adducts are indicated.

present in the uninduced cells represent IRF-2 and its proteolytic product (8, 57), we used the IRF-2 antibodies to analyze the presence of IRF-2 in the A4IE complexes. However, the IRF-2 antibodies did not supershift any of the A4IE complexes, nor did preabsorption of the extracts with these antibodies remove the p55 or p29 DNA-protein adduct (data not shown).

Imiquimod does not stimulate the appearance of new PRDI binding complexes. Virus-mediated induction of IFN genes has been associated with the induction of binding IRF-1 to PRDI. The same region can also bind IRF-2, which can repress IRF-1 activity (22). Induction-specific complexes PRDI-BFi and TH3 have been shown to contain cleaved IRF-2 or a 14-kDa N-terminal fragment of IRF-2 (8, 57). To determine whether imiquimod induces PRDI-specific binding, nuclear extracts from uninduced and imiquimod-induced cells were incubated with PRDI probe and DNA-protein complexes formed were analyzed by a gel retardation assay. Two DNA-protein complexes (A and B) were detected with extracts from uninduced cells (Fig. 10). These complexes were also observed after treatment with imiquimod; however, a small, transient increase in complex A formation was observed at the early stages of induction. The binding of complex A was easily inhibited by the unlabelled PRDI, while the decrease in binding of complex B required at least a 50-fold excess of the unlabelled probe. The unlabelled NF-κB probe did not complete the formation of these two complexes when used in a 5-fold excess, while a 50-fold excess decreased binding of both of these complexes. These results indicate the PRDI-specific binding of complex A. The identities of the proteins present in these complexes were examined by testing the abilities of various antibodies to modulate their formation. However, antiserum to neither IRF-1 nor IRF-2 could abolish formation of complex A or B (when added before formation of the complexes) or supershift these complexes (when added after complex formation) (data not shown). Since these antibodies were shown to interact with IRF-1 and IRF-2 on Western blot (immunoblot) analysis and by a supershift assay (27a), these data suggest that complexes A and B do not contain either IRF-1 or IRF-2.

DISCUSSION

We have shown in this study that imiquimod induces in PBMC expression of *IFNA/B* genes and cytokine genes, including IL-6, IL-8, and TNF-α genes. In contrast to viral infection, which effectively induces IFN genes in a large variety of primary cells and established cell lines, imiquimod was able to induce these genes only in primary human monocytes or in mouse spleen cells, and not in established lines of fibroblasts, B cells, or monocytes. Moreover, occasionally some PBMC did not respond to imiquimod stimulation and no IFN synthesis could be detected. The reasons for this unresponsiveness are presently unknown.

Induction of IFN and cytokine genes by imiquimod is transient and does not require cellular protein synthesis, indicating

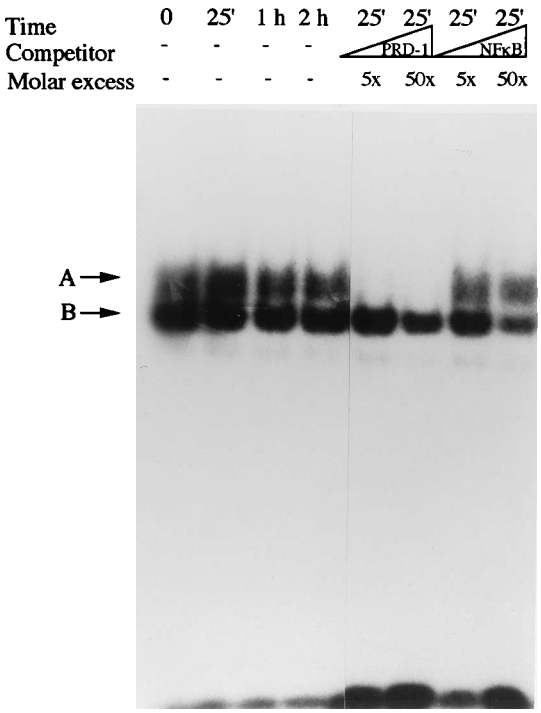

FIG. 10. Imiquimod does not stimulate PRDI-specific binding activity. Nuclear extracts prepared from imiquimod-induced cells and uninduced controls were incubated with ^{32}P-labelled PRDI probe, and the DNA-protein complexes were analyzed by a gel shift assay. Competition for complex formation was done with the unlabelled PRDI and NF-κB probes at 5- and 50-fold excesses.

that imiquimod, similar to viral infection, modifies or activates preexisting latent cellular factors. The fact that induction of *IFNA* and *IFNB* genes by these two inducers requires TK and PKC activity further emphasizes the importance of protein phosphorylation in the induction of these genes. The fact that virus and imiquimod induce similar sets of cytokine genes indicates that activation may involve a common *cis* element(s) conserved in the promoter regions of all these cytokine genes, such as an NF-κB binding site preserved in the promoter regions of all genes examined, except the *IFNA* genes. Stimulated transcription of the TNF-α gene was shown to involve interaction between NF-κB and AP-1 binding proteins, and transcription of IL-6 and IL-8 genes required interaction synergism between NF-IL6 and NF-κB (38, 44, 52); transcriptional activation of the *IFNB* gene depends on interaction between IRF-1, ATF-2, HMGI(Y), and the NF-κB complex (10, 75). Our data showed enhancement of NF-κB-specific binding in imiquimod-treated primary monocytes and suggest that NF-κB-specific binding may be required for the imiquimod-mediated activation of *IFNB*, TNF-α, IL-6, and IL-8 genes. Interestingly, imiquimod induced expression of the IL-8 gene more efficiently than did viral infection. It was recently shown that the NF-κB-like site in the IL-8 promoter region is activated by the c-rel/p65 complex (38), suggesting that the NF-κB-specific proteins induced by Sendai virus and imiquimod in PBMC may not be identical. Indeed, we have shown in this study 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 (76).

To analyze further the effect of imiquimod stimulation on the expression of *IFNA* genes, we identified the individual *IFNA* genes expressed in imiquimod-stimulated PBMC and compared them with Sendai virus-induced *IFNA* genes in these cells. It was previously shown (18, 29) that *IFNA1* and *IFNA2* are the major mRNAs present in virus-induced leukocytes, while *IFNA8*, *IFNA4*, *IFNA5*, *IFNA7*, *IFNA14*, *IFNA21*, and *IFNA16* mRNAs were identified as minor components. By PCR amplification and cloning, we detected *IFNA1*, *IFNA2*, and *IFNA8* in Sendai virus-induced human leukocytes. Under the same experimental conditions, *IFNA1*, *IFNA2*, *IFNA8*, *IFNA5*, and *IFNA6* were detected in imiquimod-induced PBMC. Although we cannot completely eliminate the possibility that another minor *IFNA* mRNA is present either in Sendai virus- or imiquimod-induced cells, these results indicate a higher degree of *IFNA* mRNA subtype heterogenicity in imiquimod-induced PBMC than in Sendai virus-infected cells.

The region (VRE) required for maximal inducibility of the human *IFNA1* and *IFNB* genes contains sets of GAAAGT and GAAATG repeats (14, 47, 48) that are also preserved in the inducible region of the murine *IFNA* gene promoters (58). When multimerized, these repeats serve as the binding site for IRF-1 and IRF-2 (22, 47), and IRF-1 was shown to activate these sequences in the yeast system (67) as well as in a transient expression assay (1). Nevertheless, our analysis did not show a significant increase in IRF-1-specific binding in imiquimod-treated PBMC. A virus-inducible, 35-nt sequence identified in a promoter of the murine *IFNA4* gene contains a symmetric sequence, GTAAAGAAAGT (A4F1), essential for the induction of the *IFNA4* promoter (2), that partially overlaps the putative IRF-1 binding site. This sequence is well preserved in the inducible region of murine and human *IFNA* genes, including those induced by imiquimod (Fig. 8C). In this study, we have shown that induction of *IFNA* genes in virus-infected and imiquimod-treated cells is associated with an induction-specific complex (A4F1/B) formed between the A4F1 sequence and nuclear extract from induced cells. The mobility of this complex could not be supershifted with either anti-IRF-1 or anti-IRF-2 antibodies or anti-STAT p91 antibodies (data not shown). The UV cross-linking analysis showed that at least four nuclear proteins bind to the 35-nt VRE. While the majority of DNA-protein adducts were identified in both induced and uninduced cells, two major differences in the binding profiles were observed. First, extracts from the control cells, but not from induced cells, show the presence of a 55-kDa protein that corresponds in size to the proteins of the IRF-1 family. The down-regulation of expression of *IFNA* and *IFNB* promoter regions by IRF-2 has been well documented in cotransfection experiments. Although the binding of IRF-2 to the inducible element could contribute to the negative regulation of this promoter (22, 23), we have not detected the presence of IRF-2 in the AF-1 complexes. Another PRDI zinc family binding protein has been shown to function as a repressor in cotransfection experiments (80); however, this protein is 88 kDa and therefore differs from the 55-kDa protein identified in the present study. The second difference is the increase in the relative levels of the 28-kDa DNA-protein adduct in induced cells, suggesting an increase in binding capacity or a more efficient cross-linking of the 28-kDa protein in induced cells. The possibility that this protein represents a processed form of IRF-2 (8, 57) cannot be eliminated, since the antibodies against IRF-2 used do not recognize the N-terminal region of this protein. These results, however, indicate that, similar to what has been observed with the murine system (2), the formation of an induction-specific complex B is not the result of binding of a novel protein to the inducible region 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 A4F1/B complex is abolished in the presence of TK and PKC inhibitors (data not shown), we suggest that imiquimod-induced phosphorylation of the preexisting A4F1 binding proteins is an essential step in the induction process.

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 (50). The present data suggest that activation by both these inducers involves phosphorylation rather than the presence of dsRNA per se. Phosphorylation was shown to play a role both in the dissociation or degradation of IκB complexes, leading to translocation of the NF-κB1 complex to the nucleus (5, 17), and in the activation of IRF-1 (79).

Recently, it has been shown that IFNs activate tyrosine kinases of the Jak family (9). These kinases directly phosphorylate proteins Stat91 and Stat113, which are part of the ISGF-3 complex that binds to the IFN-responsive element (ISRE) of the IFN-activated genes and activates transcription of these genes (31, 66). We have shown (63) that a priming with IFN (73) enhances virus-induced expression of IFN-α/β, as well as the other cytokines, e.g., IL-6 and TNF-α. Accordingly, priming with IFN enhanced imiquimod-stimulated expression of *IFNA* and *IFNB* genes (data not shown). It is likely, therefore, that IFN provides a costimulation signal both in the imiquimod- and virus-induced phosphorylation pathways. Activation of a signal transduction by a synthetic ligand able to aggregate membrane receptors was used to study the T-cell receptor-mediated signalling pathway (72). The membrane-permeable imiquimod may, therefore, function as a ligand, alter intramembrane interactions, and initiate intracellular signalling. Furthermore, the direct binding of pyridinyl-imidazole compounds to mitogen-activated protein kinase (CSBP) with a consequent inhibition of kinase activity reported recently (41) raises the possibility that imiquimod interacts directly with a kinase modulating the transduction pathway leading to the transcriptional activation of the cytokine genes.

The fact that imiquimod inhibits virus-mediated induction of *IFNA* genes while it superinduces induction of *IFNB* and IL-6 genes indicates that induction of *IFNA* may proceed by a pathway distinct from that for the other cytokines examined and adds to the complexity of both the pleiotropic effects of these inducers and the mechanisms by which these genes are activated. Finally, the use of imiquimod and its analogs may provide a useful tool for dissecting the role of kinase pathways in the virus-mediated signal transduction inducing inflammatory cytokines.

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