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Impaired Antiviral Response in Human Hepatoma Cells

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Hepatitis B, C, and D viruses can infect liver cells and in some individuals establish a chronic phase of infection. Presently, relatively little information is available on the antiviral mechanisms in liver cells. Because no good *in vitro* model infection systems for hepatitis viruses are available, we have used influenza A, Sendai, and vesicular stomatitis (VSV) viruses to characterize interferon (IFN) responses and IFN-induced antiviral mechanisms in human hepatoma cell lines. HepG2 or HuH7 cells did not show any detectable IFN-α/β production in response to influenza A or Sendai virus infections. Treatment of cells with IFN-α resulted in upregulation of IFN-α-inducible Mx, 2′,5′-oligoadenylate synthetase (OAS) and HLA class I gene expression but only with exceptionally high levels of IFN-α ($\geq$100 IU/ml). Accordingly, high pretreatment levels of IFN-α, 1000 IU/ml for influenza A and VSV and 100 IU/ml for Sendai virus, were required before any detectable antiviral activity against these viruses was seen. IFN-γ had some antiviral effect against influenza A virus but appeared to be ineffective against VSV and Sendai virus. IFN-γ upregulated HLA class I protein expression, whereas Mx or OAS expression levels were not increased. There was a modest upregulation of HLA class I expression during Sendai virus infection, whereas influenza A virus infection resulted, after an initial weak upregulation, in a clear decrease in HLA class I expression at late times of infection. The results suggest that hepatoma cells may have intrinsically poor ability to produce and respond to type I IFNs, which may contribute to their inability to efficiently resist viral infections. © 1999 Academic Press

INTRODUCTION

Liver is the primary target of several hepatotropic viruses like hepatitis virus A-G. Viral infections caused by hepatitis B and D and especially by hepatitis C virus (HCV) have a great tendency to remain chronic in the liver. Viral hepatoses cause remarkable morbidity and liver-failure-associated mortality throughout the entire world. Especially, the morbidity caused by hepatitis C infection has been steadily increasing during recent years and has been estimated to comprise 2% of the population (Alter, 1995). Chronic disease forms of viral hepatitis predispose the patients to chronic liver failure and cirrhosis and, in some individuals, also to hepatocellular carcinoma (Saito *et al.,* 1990; Shimotohno, 1993). The reason for hepatitis B and C to become chronic is somewhat unclear, but research data suggest that viral as well as host factors are involved. Certain viral genotypes may be more pathogenic than others, but racial, gender-specific, and genetic (*e.g.,* HLA types) factors also may be important (Bruno *et al.,* 1997; Hoofnagle and Bisceglie, 1997; Davis *et al.,* 1998; McHutchison *et al.,* 1998). It is also possible that there are some specific features in the liver that render it susceptible to hepatotropic viral infections and prevent it from being cleared from the virus by innate or specific immune mechanisms. Unavailability of infectious high-titer stock hepatitis virus preparations and inefficiency of *in vitro* hepatocyte culture systems has hampered the research developments.

Interferon-alpha (IFN-α) has initially been the drug of choice for chronic hepatitis B and C infections. However, IFN-α alone is not very effective against chronic hepatitis B or infections caused by genotype 1 of hepatitis C (Poynard *et al.,* 1996; Hoofnagle and Bisceglie, 1997). Therefore combination therapies of IFN-α with other substances, such as nucleoside analogues for hepatitis B and Ribavirin for HCV have shown promising results (Davis *et al.,* 1998; McHutchison *et al.,* 1998). IFNs are classified into type I (IFN-α, -β, and -ω) and type II (IFN-γ) IFNs. Type II IFN is produced by T and NK cells, whereas type I IFNs are produced by leukocytes, fibroblasts, epithelial, and many other cell types. IFNs have antiviral, antiproliferative, and immunomodulatory effects, which are mediated by the gene products of IFN-inducible genes (Vilcek and Sen, 1996). The antiviral effects of IFNs are mediated by several different proteins like Mx proteins, 2′,5′-oligoadenylate synthetase (OAS), and RNAse L and PKR (Staeheli, 1990; Sen and Ransohoff, 1993; Vilcek and Sen, 1996). IFNs also upregulate HLA class I and II expression (Sen and Ransohoff, 1993; Keskinen *et al.,* 1997), which leads to enhanced antigen presentation and stimulation of acquired immunity. MxA protein mediates resistance to several negative-strand RNA viruses like influenza A, VSV, bunya-, and certain paramyxoviruses (Pavlovic *et al.,* 1990; Schnorr *et al.,* 1993; Frese *et al.,* 1996; Kanerva *et al.,* 1996; Zhao *et al.,* 1996) as well as

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to positive-strand Semliki Forest virus (Landis *et al.,* 1998). OAS/RNAse L pathway is involved in resistance to at least HIV, picorna-, reo-, and vacciniaviruses (Sen and Ransohoff, 1993; Zhou *et al.,* 1997; Maitra and Silverman, 1998) and PKR plays a role in restricting the replication of several different viruses by downregulating the initiation of protein synthesis (Sen and Ransohoff, 1993). Biochemical evidence show that the NS5A protein from IFN-resistant HCV genotype 1 virus directly interacts with PKR inhibiting its functions (Gale *et al.,* 1997, 1998). Although this suggests that PKR has a role in inhibiting HCV replication, it does not rule out the possibility that other antiviral proteins are involved in antiviral resistance in the liver. Lack of infectious HCV strains makes it presently impossible to directly study the contribution of each IFN-induced protein in antiviral defense.

In the present work, we have characterized interferon response and IFN-induced antiviral effects in human hepatoma cell lines using three well-established viral infection model systems, namely influenza A, Sendai, and vesicular stomatitis viruses. We demonstrate that human hepatoma cells are not only extremely poor IFN producers, but they also respond to the antiviral actions of IFN-α and IFN-γ very weakly. Poor antiviral activity in hepatoma cells correlates well with a relative weakness of IFNs to turn on IFN-inducible genes. Our results demonstrate that the interferon system in liver cells functions poorly, which could account for the impaired ability of liver to eradicate viral infections.

RESULTS

Viral protein expression in virus-infected hepatocytes

To analyze the susceptibility of hepatoma cells to viral infections, we carried out a kinetic infection experiment with a human pathogenic strain of Influenza A/Beijing/353/89 (H3N2) and with Sendai virus, which is apathogenic to humans. These viruses were chosen as model viruses because they are well characterized and they have been widely used in studies related to IFN and viral pathogenesis research. Virus-infected cells were harvested at different time points after infection, and the expression of viral proteins was analyzed by flow cytometry and Western blotting. In flow cytometric analysis, viral protein levels started to increase already at 4 h after infection with both influenza A and Sendai virus infection. Viral protein expression levels steadily increased throughout the whole 48 h follow-up period (Fig. 1). Based on flow cytometric analysis practically the whole cell population was infected, and the expression level of viral proteins was high. In Western blots viral proteins became detectable at 4–8 h in influenza A (Fig. 2A) and at 4 h in Sendai (Fig. 2B) virus infection. As in flow cytometric analysis, the protein levels continued to increase up to 48 h, suggesting that the cells were well susceptible to infection with both types of viruses.

Lack of IFN-α/β production in virus-infected hepatocytes

Most cells respond to virus infection by producing type I IFNs. To study IFN-α/β production in hepatocytes, we infected HepG2 and HuH7 cells with influenza A or Sendai virus (5-10 pfu/cell, respectively) and collected cell culture supernatants at various time points after infection. We could not observe any measurable (sensitivity limit of the assay was 3 IU/ml) IFN-α/β production in either cell types, suggesting that hepatoma cells were either unable to produce type I IFNs or that the levels produced remained under the detection limit of the assay. RT–PCR analysis also was carried out, but no detectable IFN-β mRNA expression was found in virus-infected hepatoma cells (results not shown). Human primary macrophages (Ronni *et al.,* 1997; Sareneva *et al.,* 1998) used as control cells produced ~500 and 3000 IU/ml of type I IFNs within 24 h in response to influenza A and Sendai virus infection, respectively (data not shown).

Antiviral protein expression is enhanced by Sendai but not by influenza A virus in hepatoma cells

Although hepatoma cells appeared to lack any detectable virus-induced type I IFN production, we wanted to analyze whether viral infection could anyhow turn on the expression of Mx and OAS genes in an IFN-independent manner as has been described to occur in certain cell lines (Goetschy *et al.,* 1989; Bazzigher *et al.,* 1992; Ronni *et al.,* 1995). The expression of MxA, MxB, and OAS proteins in virus-infected cells was studied by Western blotting. Antiviral MxA protein was not detectable in influenza-A-virus-infected cells (Fig. 2A), whereas in Sendai-virus-infected cells (Fig. 2B) MxA protein expression started to increase within 24 h. Similar pattern and kinetics of expression was observed for MxB protein. All four, 100-, 69/71-, 56-, and 40/46-kDa forms of OAS protein (Marié *et al.,* 1990; Rebouillat *et al.,* 1998) were constitutively expressed in HepG2 cells, and no change in expression level was seen in influenza-A-virus-infected cells (Fig. 2A). Sendai virus infection appeared to enhance the expression of all OAS isoforms including the appearance of the 46-kDa protein form (Fig. 2B). The results suggest that in liver cells Sendai virus, but not influenza A, can activate the expression of several IFN-α/β-inducible genes in spite of the lack of measurable IFN-α/β production.

IFN-induced antiviral state in HepG2 cells

Because IFN-α is a drug of choice for viral hepatitis we wanted to analyze the sensitivity of hepatoma cells to the antiviral effects of IFN-α and IFN-γ. HepG2 cells were pretreated with various doses of IFN-α or/and IFN-γ for 24 h followed by infection with influenza A (Figs. 3 and

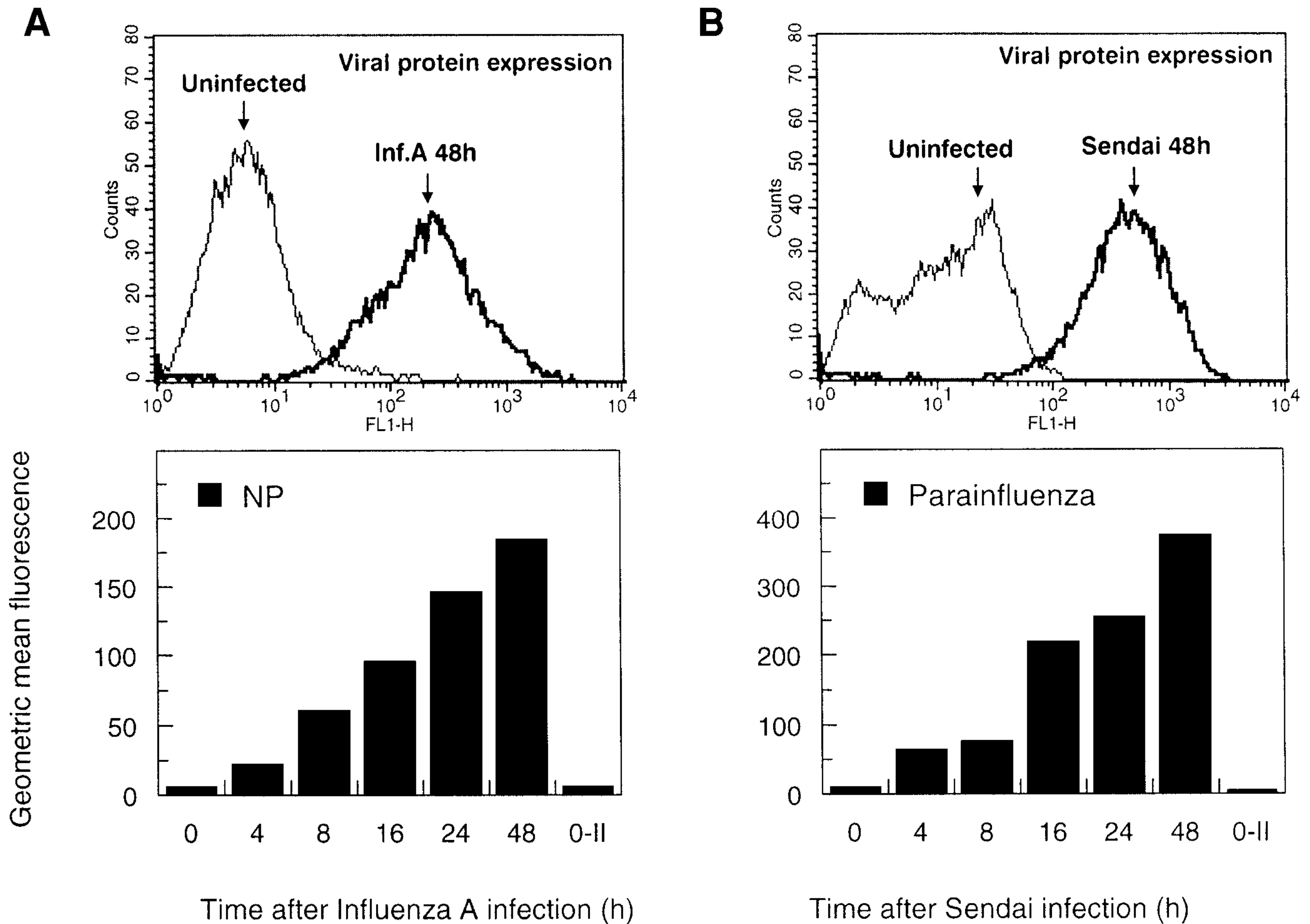

FIG. 1. Kinetics of virus protein expression in influenza-A-virus- or Sendai-virus-infected HepG2 cells. HepG2 hepatoma cells were infected with influenza A (A) or Sendai (B) viruses at a m.o.i. of 5 and 10 pfu/cell, respectively. The cells were harvested at various time points, and the expression of viral proteins were analyzed by flow cytometric analysis after staining with monoclonal anti-influenza A NP antibody and rabbit anti-Sendai virus antibody and secondary FITC-labeled antibodies. Time points and stainings as indicated in the figure.

5A) or Sendai (Figs. 4 and 5B) viruses. The cells were harvested at 20 h after infection, and viral protein expression was monitored by flow cytometry and Western blotting. Based on flow cytometric analysis (Fig. 3) IFN-α-pretreatment, even with the highest dose of 1000 IU/ml, had practically no effect on influenza virus protein expression. A clear upregulation of MxA protein and a weak upregulation of HLA class I expression were seen, indicating that IFN-α was functional in these cells. Surprisingly high levels ($\geq$100 IU/ml) of IFN-α was required for this upregulation. Interestingly, IFN-γ alone or in combination with IFN-α showed a marginal inhibitory effect on influenza A protein expression. In the case of Sendai virus, IFN-α pretreatment with 100–1000 IU/ml had a detectable inhibitory effect on Sendai virus protein expression (Fig. 4). Unlike in the case of influenza A virus, IFN-γ did not seem to contribute to the antiviral activity against Sendai virus. Consistent with the flow cytometric data, Western blot analyses showed upregulation of IFN-α-induced Mx and OAS protein expression only with high doses of IFN-α (Fig. 5). We also tested the effect of virus dose on the IFN-induced antiviral activities. Even with low-infectious virus doses (0.05–1 pfu/cell) the antiviral activities of IFN-α and IFN-γ were poor and similar to the ones described above, suggesting that HepG2 cells show relatively poor antiviral properties.

IFN-induced antiviral state in HuH7 cells

To consider the possibility that HepG2 cell line was exceptionally insensitive to the antiviral effects of IFNs, we used another hepatoma cell line, HuH7 cells, in similar antiviral experiments. HuH7 cells were pretreated with various doses of IFN-α and/or IFN-γ for 24 h, followed by infection with influenza A (Fig. 6A) or Sendai (Fig. 6B) viruses for 20 h. The cells were collected and Western blot analyses were carried out. Similar to HepG2 cells, MxA protein expression was detectable in cells pretreated with $\geq$100 IU/ml doses of IFN-α. Pretreatment of IFN-α or IFN-γ alone had very little or no

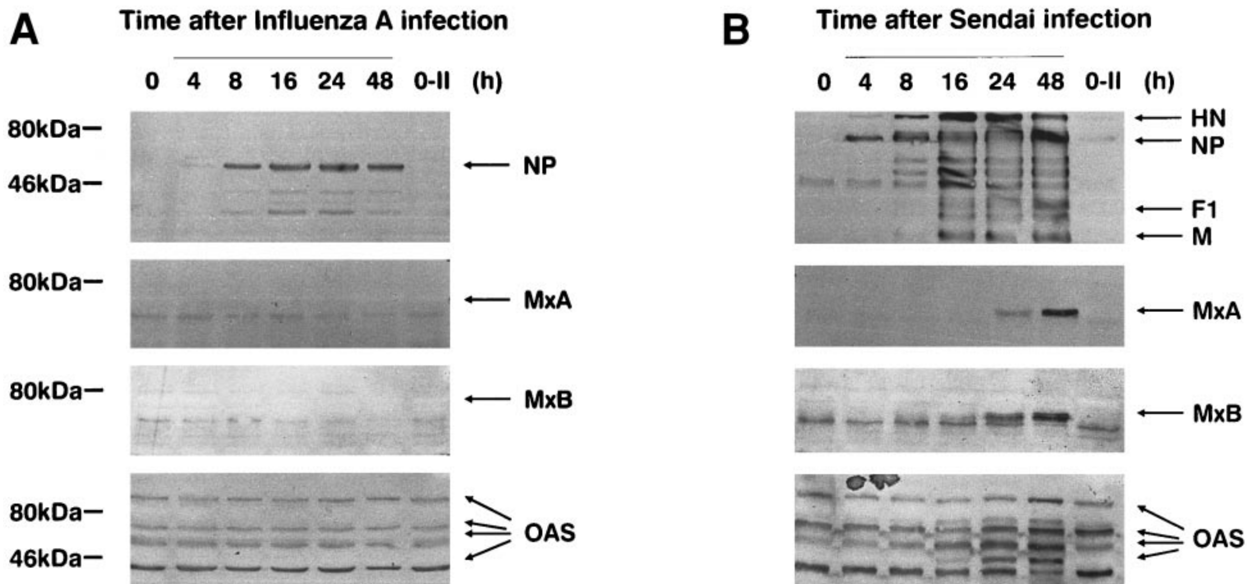

FIG. 2. Kinetics of viral, MxA, MxB, and OAS protein expression in influenza-A- and Sendai-virus-infected cells. HepG2 cells were infected with influenza A (A) or with Sendai (B) virus (m.o.i. of 5 and 10, respectively). The cells were harvested at different time points, and the expression of various proteins were analyzed by Western blotting using virus-, Mx-, and OAS-specific antibodies. Time points and stainings as indicated. Viral proteins marked as described previously (Lamb and Kolakofsky, 1996; Lamb and Krug, 1996).

inhibitory effect, respectively, on influenza A virus HA and NP expression. However, pretreatment of cells with both IFN-α and IFN-γ showed some inhibitory effect on influenza A viral protein expression (Fig. 6A). Sendai virus seemed to be somewhat more sensitive to the antiviral effects of IFN-α than influenza A virus. Doses $>$100 IU/ml of IFN-α alone or combined with IFN-γ showed clear antiviral effects against Sendai virus (Fig. 6B). IFN-γ alone appeared to be ineffective against Sendai virus.

Comparison of IFN-induced antiviral activity in different cell lines

Because infectious progeny viruses are not produced from influenza-A- and Sendai-virus-infected cells, we used VSV to analyze the production of infectious virus in IFN-treated cells. Hepatoma cell lines as well as two other control cell lines, Hep2 and A549, were pretreated with different doses of IFN-α or IFN-γ followed by infection with VSV. Cells and cell culture supernatants were collected, and IFN-induced inhibition of VSV protein synthesis and virus production was analyzed (Fig. 7). VSV protein synthesis as analyzed by Western blotting and flow cytometry (Figs. 7A and 7B) or analysis of infectious virus production (Fig. 7B) was dramatically reduced in Hep2 and A549 control cells compared to hepatoma HepG2 and HuH7 cells. At least 100 IU/ml of IFN-α was required before any inhibition of virus replication was seen in HepG2 or HuH7 cells. There was also a clear difference in the ability of IFN-α to turn on IFN-α-induced genes. In Hep2 (expresses only MxB protein; Melén *et al.,* 1996) and in A549 cells, 10 and 1 IU/ml of IFN-α, respectively, was sufficient to enhance Mx protein expression, whereas $\geq$100 IU/ml of IFN-α was required for any detectable MxA protein expression in both hepatoma cell lines (Fig. 7A). To study whether the differential IFN-α-induced gene expression in different cell lines would be due to variable expression of IFN-α-specific signaling molecules, we quantitated cellular p48, STAT1 and STAT2 levels by quantitative Western blotting. Basal p48 levels in A549 and Hep2 cells were ~3- and 10-fold higher, respectively, compared to hepatoma HepG2 and HuH7 cells whereas STAT1 and STAT2 levels appeared to be equal in all tested cell lines (results not shown).

Regulation of hepatoma cell HLA class I expression by interferons and virus infection

During viral infections, HLA class I antigens present virus specific peptides to effector T cells, and therefore they are an important part of the defense system against viruses. Because HLA genes are also under the control of the interferon system (Sen and Ransohoff, 1993; Keskinen *et al.,* 1997), we tested the efficacy of various doses of IFNs to upregulate HLA class I antigen expression in liver cells. As already shown above (Figs. 3 and 4), very high doses, 1000 IU/ml of IFN-α are needed to stimulate HLA class I expression. Consistent with previous results (Keskinen *et al.,* 1997), IFN-γ was a better stimulant of HLA class I expression compared to IFN-α. No basal or IFN-induced HLA class II expression was seen in hepatoma cells. To study possible virus-induced changes in HLA class I expression, we harvested cells at various time points after influenza A or Sendai infection

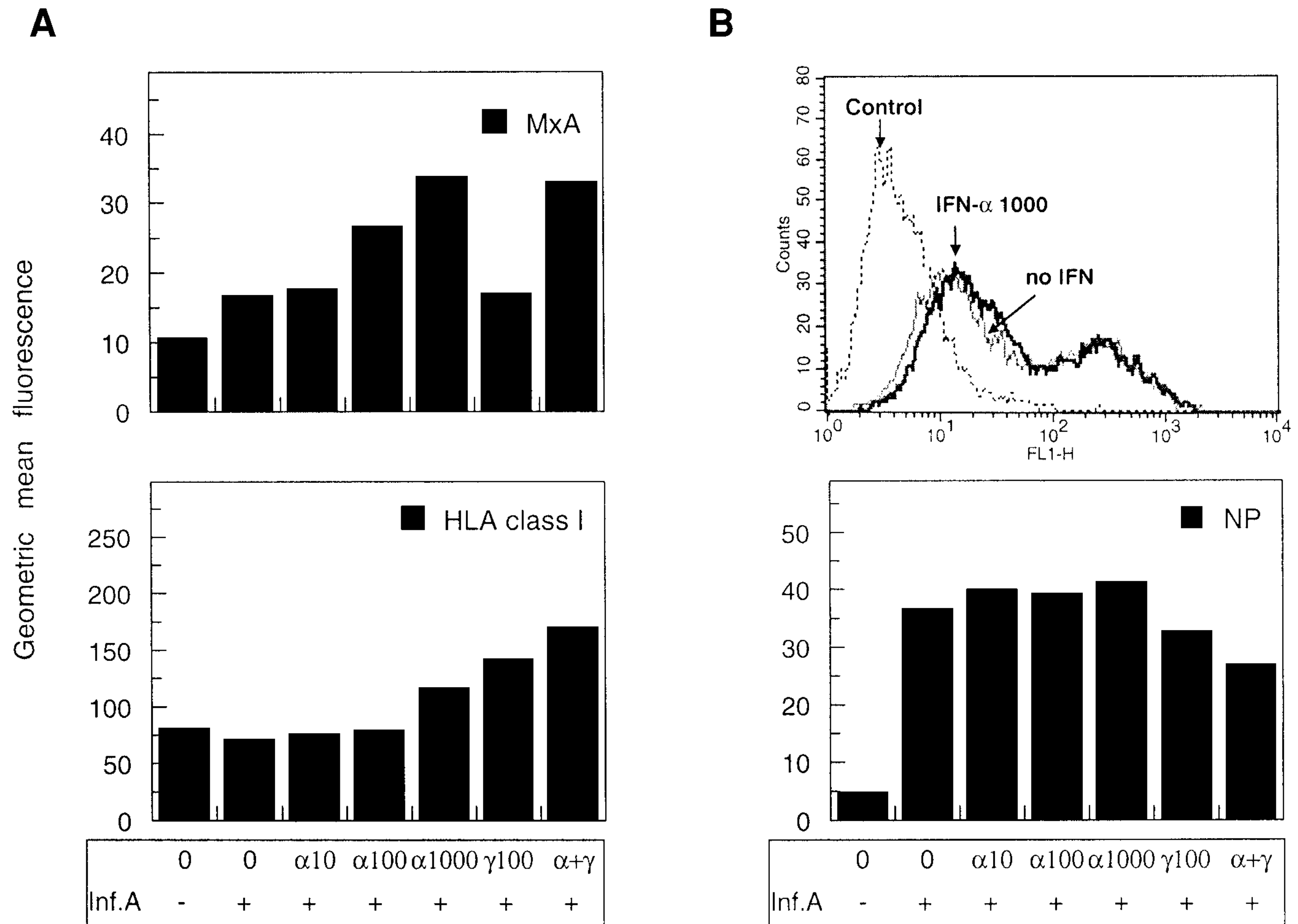

FIG. 3. Flow cytometric analysis of the kinetics of MxA, HLA class I, and virus protein expression in IFN-pretreated and influenza-A-virus-infected HepG2 cells. HepG2 cells were pretreated with various doses of IFN-α and/or IFN-γ for 24 h, followed by infection with influenza A virus (m.o.i. of 0.5). The cells were harvested at 20 h after infection and stained with Abs against MxA, HLA class I, or influenza A NP, followed by flow cytometric analysis.

and analyzed HLA class expression by flow cytometry. In influenza-A-infected cells, HLA class I expression was marginally increased at 8 h after infection, but thereafter its expression started to decrease, being ~50% of that of uninfected cells at the 48 h time point (Fig. 8). In Sendai-virus-infected cells HLA class I expression was different from that of influenza-A-infected cells. HLA class I expression increased slowly, peaking at 24 h after infection.

DISCUSSION

In the present work, we have characterized the interferon system in human hepatoma, namely HepG2 and HuH7 cell lines. These cell lines, especially HepG2, have been widely used as the model system to study liver cell biology. Both of these cell lines are transformed, but they express certain hepatocyte markers and also produce several compounds typical for hepatocytes. *In vitro* studies with primary human hepatocytes, which constitute ~80% of the liver cell population, are difficult to carry out because primary hepatocytes are fragile and they survive poorly in *in vitro* culture conditions. The lack of suitable infectious hepatitis stock viruses and difficulty in cultivating of primary hepatocytes lead us to use well-established virus infection model systems and hepatoma cell lines in our analyses.

Influenza A, Sendai and vesicular stomatitis viruses were found to be infectious to hepatoma cells. The kinetics of virus infection was similar to the ones seen in leukocytes and epithelial cells (Ronni *et al.*, 1995, 1997; Sareneva *et al.*, 1998) with newly synthesized viral proteins starting to accumulate ~4 h after infection (Figs. 1 and 2). Influenza A or Sendai viruses are commonly used virus infection models in IFN research because they are very good inducers of type I IFNs (Cantell *et al.*, 1981; Ronni *et al.*, 1995; Sareneva *et al.*, 1998). Presently the information concerning liver cell IFN production and IFN action has been limited. In our hands, virus-infected

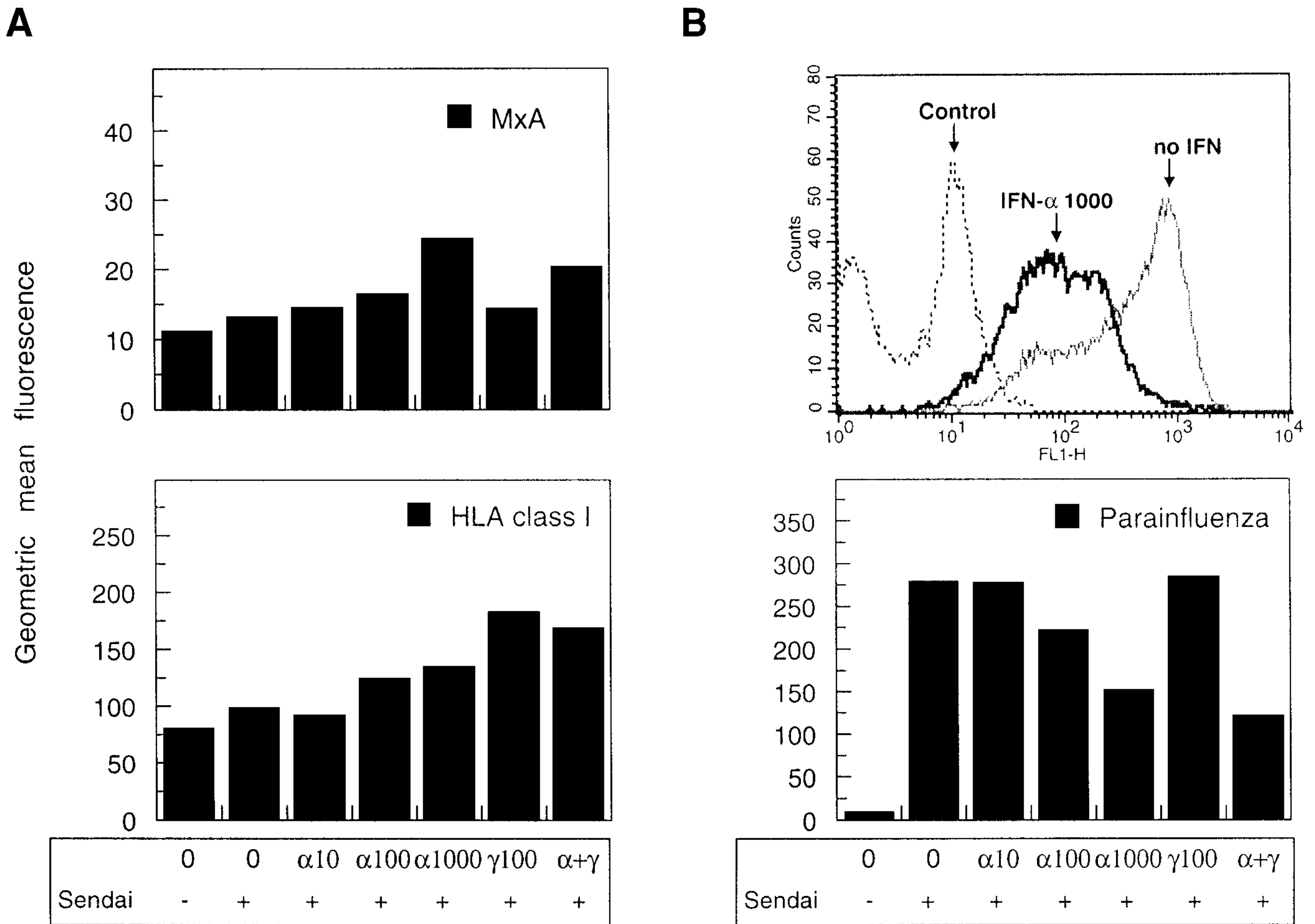

FIG. 4. Flow cytometric analysis of the kinetics of MxA, HLA class I, and virus protein expression in IFN-pretreated Sendai-virus-infected HepG2 cells. HepG2 cells first were treated 24 h with various doses of IFN-α and/or IFN-γ, followed by infection with Sendai virus (m.o.i. of 1). The cells were harvested at 20 h after infection and stained with Abs against MxA, HLA class I, or virus protein followed by flow cytometric analysis.

hepatoma cells were unable to produce any detectable type I IFN activity into the cell culture supernatant. The reason for this remains unknown, but it may well be that some of the crucial transcription factor systems, like interferon regulatory factors (IRF; Nguyen *et al.,* 1997), required for type I IFN gene expression function poorly in hepatocytes. In fact, we observed that in both analyzed human hepatoma cell lines, the constitutive expression of IRF-1 (Melén *et al.,* submitted) and IRF-7 genes (data not shown) were missing. IRF-1 and IRF-7 have been suggested to be important, but perhaps not incompensable, transcription factors regulating the gene expression of type I IFN genes (Nguyen *et al.,* 1997; Au *et al.,* 1998; Marié *et al.,* 1998). In addition to enhancing type I IFN gene expression, certain viruses may also directly upregulate the gene expression of IFN-induced genes (Bazzigher *et al.,* 1992; Goetschy *et al.,* 1989; Ronni *et al.,* 1995). We observed that during influenza A virus infection, MxA, MxB, OAS, or HLA class I expression were not enhanced, suggesting that in hepatoma cells, influenza A virus cannot activate transcriptional systems required for IFN-induced gene expression. Sendai virus, instead, was able to upregulate the expression of all of these genes; this is consistent with the observation that certain paramyxoviruses can efficiently activate transcriptional systems required for IFN and IFN-induced gene expression (Bazzigher *et al.,* 1992; Au *et al.,* 1998; Marié *et al.,* 1998). These results suggest that influenza A and Sendai viruses behaved quite differently in hepatoma cells. Sendai virus, in spite of the lack of detectable IFN production, was able to turn on the expression of IFN-inducible genes, whereas influenza A virus is incapable of doing it. Whether hepatotropic viruses can activate IFN production or IFN-inducible gene expression in liver cells will be of great interest to study. It may be that hepatitis B and C are poor IFN inducers because HBV- or HCV-infected individuals showed no expression of MxA protein in their leukocytes, which provide indirect evidence of the lack of type I IFN production (Jakschies *et al.,* 1994).

Type I IFN-inducible genes Mx, OAS/RNAse L, PKR, and some other less well-studied molecules mediate antiviral actions against different viruses (Staeheli, 1990;

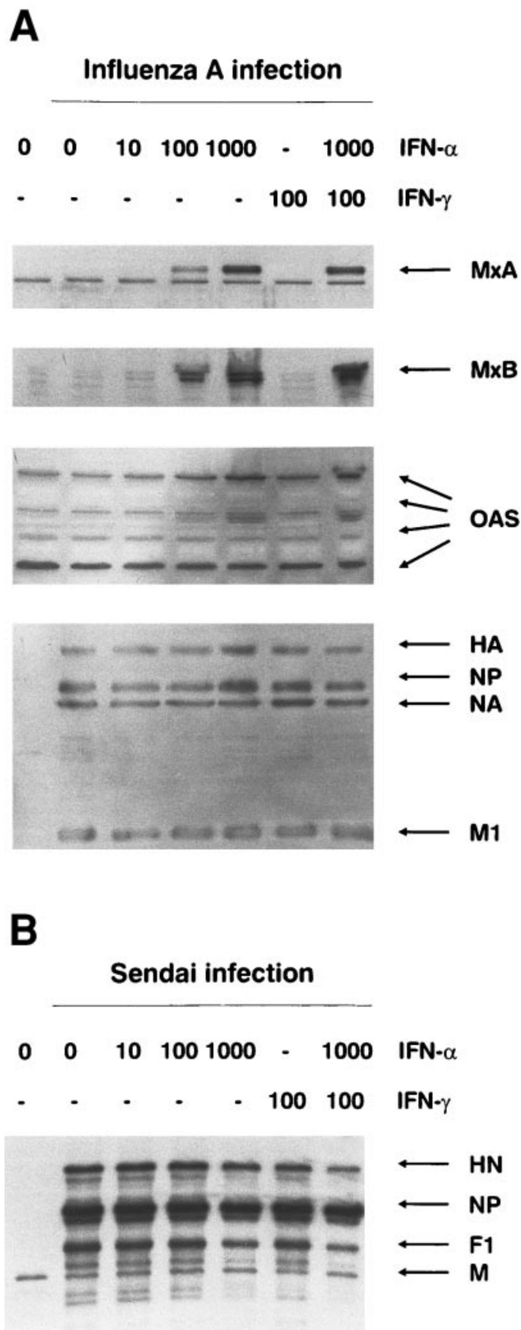

FIG. 5. Kinetics of MxA, MxB, OAS, and viral protein expression in IFN-pretreated virus-infected HepG2 cells. HepG2 cells were pretreated with various doses of IFN-α and/or IFN-γ for 24 h, followed by infection with influenza A (A) or Sendai (B) viruses (m.o.i. of 0.5 and 1, respectively). The cells were harvested at 20 h after infection. Western blots were stained with Abs against MxA, MxB, OAS, and virus protein.

Sen and Ransohoff, 1993). Stimulation of hepatoma cells with IFNs followed by virus challenge showed relatively poor antiviral activity against both influenza A and Sendai viruses. In HepG2 cells, IFN-α pretreatment had practically no effect on influenza A virus protein expression. IFN-γ alone or in combination with IFN-α showed some antiviral activity. The results suggest that hepatoma cells show relatively poor IFN-α-induced antiviral action against influenza A infection. This is surprising because IFN-α turned on the expression of MxA protein, which has previously been shown to mediate resistance to influenza A virus *in vitro* (Pavlovic *et al.,* 1990; Ronni *et al.,* 1995). It is possible that IFN-α-induced expression of MxA protein is not sufficiently high in liver cells to mediate influenza A virus resistance. Alternatively, liver cells are lacking some other factors or antiviral proteins, which contribute to the antiviral actions against influenza A virus. In Sendai infection, IFN-α-pretreatment at doses of 100–1000 IU/ml showed a dose-dependent inhibition in viral protein expression. Unlike in the case of influenza A, IFN-γ alone had no effect on Sendai virus protein expression. The inefficiency of hepatoma cells to resist virus infection appeared to be independent of virus dose, because even the smallest doses of influenza A (0.02 pfu/cell) or Sendai (0.04 pfu/cell) viruses showed requirement of high IFN-α doses for any antiviral activity to be seen. Similarly, in the case of VSV, both viral protein synthesis and infectious virus production were poorly inhibited by IFNs in hepatoma cells. This impaired IFN-induced antiviral activity was not restricted to HepG2 cells because HuH7 cells behaved practically in a similar fashion (Fig. 6). These data match well with the clinical observations that HCV patients benefit from higher IFN-α doses (Poynard *et al.,* 1996).

In HepG2 and HuH7 cells, exceptionally high IFN-α doses ($\geq$100 IU/ml) were needed to turn on the expression of MxA and MxB proteins even though the basal expression levels were undetectable. In other cell types like in human blood mononuclear cells, mature macrophages or epithelial A549 cells MxA gene expression is turned on with IFN-α doses as low as 1 IU/ml (Fig. 7; Ronni *et al.,* 1993, 1997, 1998; Melén *et al.,* 1996). Increased OAS protein expression (100- and 69/71-kDa isoforms only; Fig. 5) also was seen, but, as in the case of Mx proteins, only with high IFN-α doses. It may be that constitutive OAS expression levels were relatively high, and therefore we were unable to detect any clear increase in some OAS isoform expression. The reason for the insensitivity of hepatoma cells to the antiviral actions of IFN-α is unclear, but it may be that hepatoma cells have a relative reduction in p48 protein expression (Melén *et al.,* submitted) that renders the cells weakly responsive to IFN-α.

A crucial factor in the development of virus-specific immune responses is the functionality of the MHC system. MHC class I and II molecules are constitutively expressed either in most cells or in professional antigen presenting cells, respectively, but their expression is also under the regulation of the IFN system (Sen and Ransohoff, 1993). In this manner, IFNs can enhance the presentation of virus-specific peptides by MHC class I molecules, thus leading to more efficient killing of virus-infected cells by cytotoxic T cells. Hepatoma cells readily

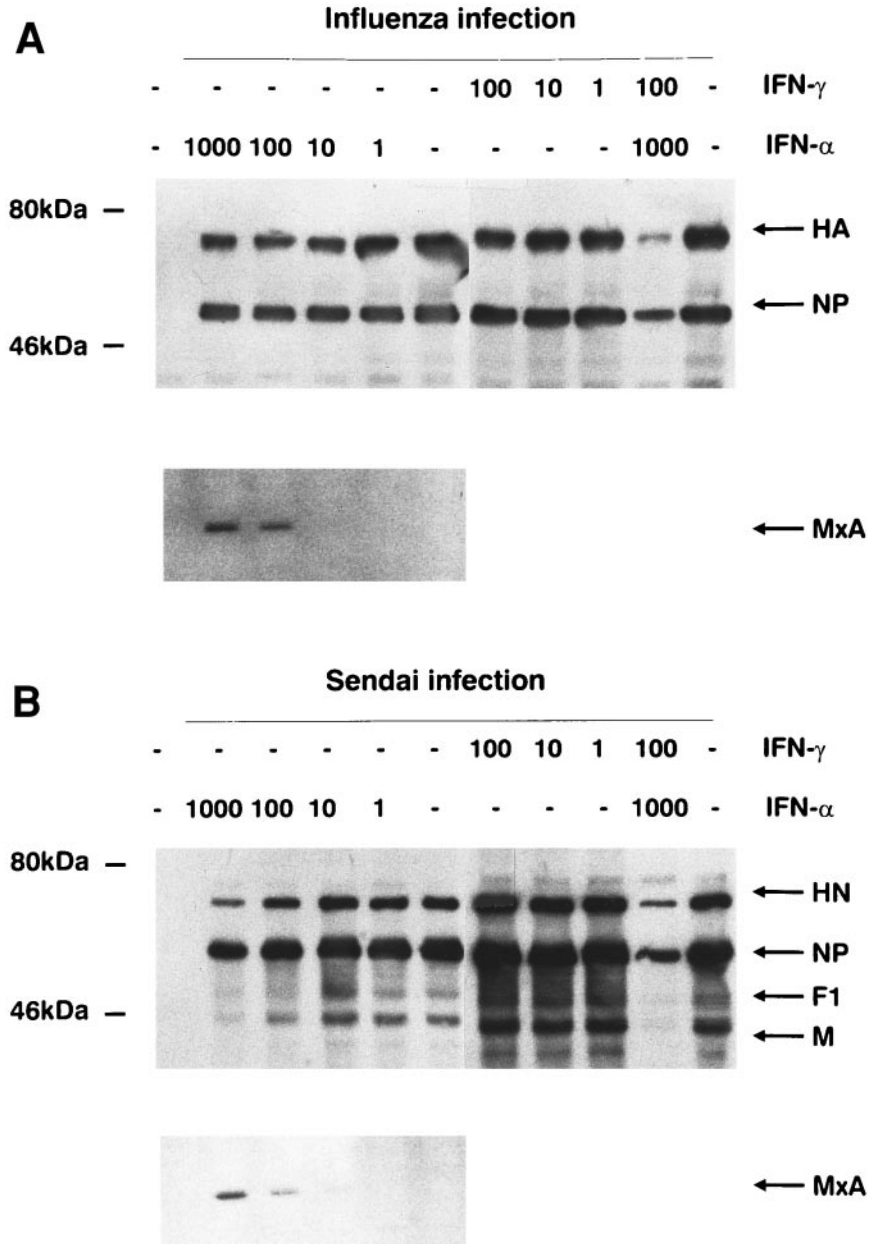

FIG. 6. Kinetics of viral and antiviral MxA protein expression in IFN-pretreated virus-infected HuH7 cells. HuH7 cells were pretreated with various doses of IFN-α and/or IFN-γ for 24 h, followed by infection with influenza A (A) or Sendai (B) viruses (m.o.i. of 0.5 and 1, respectively). The cells were harvested at 20 h after infection. Blots were stained with Abs against MxA and virus proteins.

expressed HLA class I molecules, and their expression was increased, although in a limited fashion, by both types of IFNs. This may indicate that IFN-α can only weakly stimulate the antigen presentation capacity of liver cells. Influenza A infection also marginally increased HLA class I expression at early times of infection, but at later time points its expression was decreased. This may be due to initial virus-induced upregulation of HLA class I expression, but as the infection proceeds, due to a destruction of host premRNA molecules by influenza A virus RNA polymerase complex. The viral polymerase complex uses the so-called "cap-snatching" mechanism in the cell nucleus to cleave a primer for its own mRNA synthesis (Lamb and Krug, 1996). This leads to the degradation of host cell premRNAs including those of HLA mRNAs (Keskinen *et al.,* 1997) followed by inhibition of cellular protein synthesis. Sendai virus, which replicates in the cell cytoplasm, behaved differently, and a steady, although relatively weak increase in HLA class I expression was seen during the infection.

Liver is a specialized organ build-up to synthesize a number of different important molecules like albumin and acute-phase serum proteins. In addition, it is re-

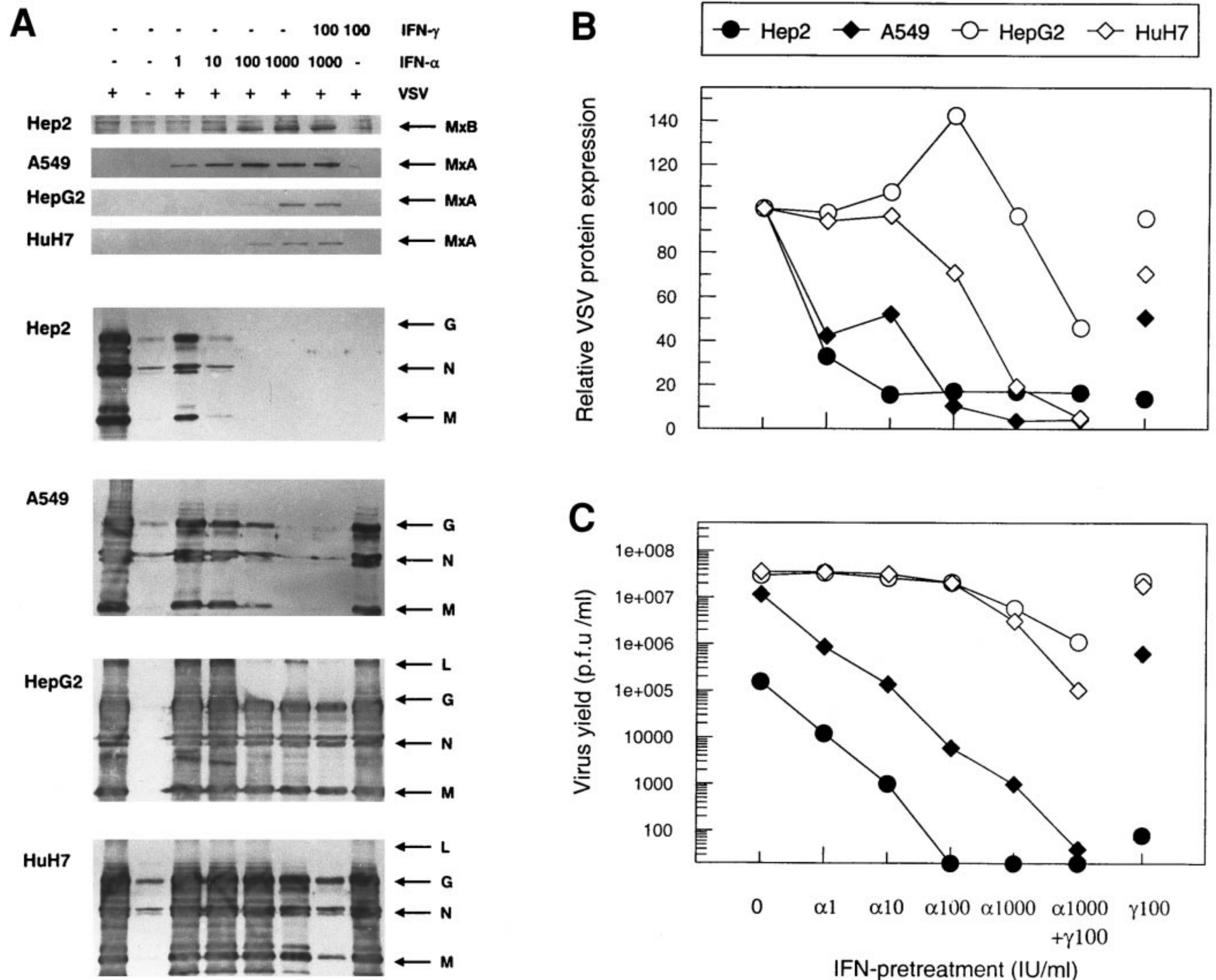

FIG. 7. Interferon-induced antiviral activity against VSV. Larynx epidermoid carcinoma Hep2, lung carcinoma A549 and hepatoma HepG2 and HuH7 cells were pretreated with different doses of IFN-α or IFN-γ for 24 h followed by infection with VSV (1-5 pfu/cell). Virus-infected cells and cell culture supernatants were collected at 20 h after infection. (A) Western blot analysis. Blots (10 μg of protein/lane) were stained with rabbit anti-MxB (Hep2 cells only), anti-MxA, or anti-VSV antibodies as indicated in the figure. (B) Flow cytometric analysis. Cells were stained with rabbit anti-VSV antibodies and FITC-labeled goat anti-rabbit antibodies and analyzed by flow cytometry (FACScan with Cellquest software). Results are shown as relative viral protein expression. (C) Production of infectious VSV. Cell culture supernatants from IFN pretreated and virus-infected cells were subjected to virus plaque assay in Hep2 cells. Virus yields are shown in a logarithmic scale. IFN doses as indicated in the figure.

sponsible for detoxification of potentially hazardous substances in the body. Besides all these important functions, liver cells seemed to be rather poor, at least based on *in vitro* experiments with cultured hepatoma cells, in coping with viral infections. They seem to lack any measurable type I IFN production, and they also responded very poorly to the antiviral activities of IFNs. The reason(s) for this impaired capacity to resist viral infections is presently not known but may at least partially be due to the lack of expression of transcription factors involved in type I IFN gene expression. Another explanation could be a relative reduction in IFN-α-specific signaling molecules such as p48. Taken together, hepatoma cells seem to be weakly responsive to IFNs, which logically would provide one explanation why the liver is the target tissue for several chronic viral infections. However, studies with normal human hepatocytes are urgently needed to characterize the IFN system in normal conditions.

MATERIALS AND METHODS

Cell culture

HepG2 human hepatoma cells (ATCC HB-8065) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (0.6 μg/ml), streptomycin (60 μg/ml), glutamine (2 mM), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH

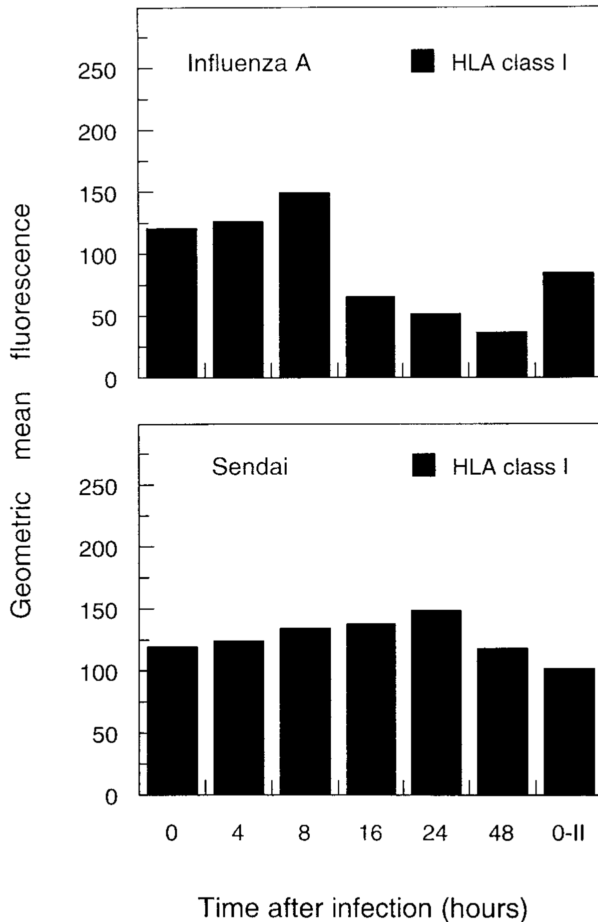

FIG. 8. Kinetics of HLA class I expression in influenza-A-virus- or Sendai-virus-infected HepG2 cells. HepG2 hepatoma cells were infected with influenza A (A) or Sendai (B) viruses (m.o.i. of 0.5 and 1, respectively). The cells were harvested at various time points, fixed and stained for HLA class I expression followed by flow cytometric analysis. Time points and stainings as indicated.

7.4 (20 mM), and 10% fetal calf serum (FCS) (Integro, Zaandam, Netherlands). HuH7 human hepatocellular carcinoma (Nakabayashi *et al.,* 1982), Hep2 human larynx epidermoid carcinoma (ATCC CCL23), and A549 human lung carcinoma (ATCC CCL 185) cells were maintained in minimal essential medium (MEM) with supplements as described above.

Interferons

Type I human leukocyte IFN-α (6×10^6 IU/ml; specific activity of 2×10^8 IU/mg; Cantell *et al.,* 1981) was kindly provided by Dr. Kari Cantell at the National Public Health Institute, Helsinki. Human IFN-γ (1×10^6 IU/ml; specific activity of 1.1×10^7 IU/mg) was obtained from the Finnish Red Cross Blood Transfusion Service, Helsinki, and it was prepared and purified as described elsewhere (Cantell *et al.,* 1986). Interferons were used at concentrations as indicated in each experiment.

Virus infections

The stock of human pathogenic influenza virus strain A/Beijing/353/89 (H3N2) was originally obtained from the National Institute for Medical Research (London). Sendai virus stock (strain Cantell) is from the National Public Health Institute, Helsinki. Virus stocks were cultivated in 8-day-old embryonated hen's eggs and stored at −70°C. The hemagglutination titers of influenza and Sendai viruses were 128 and 6000, respectively, as determined by the standard micro-method (WHO Collaborating Centers for Reference and Research on Influenza, 1982). The egg-infectious dose (EID_{50}) of the influenza A virus stock was 9.6×10^8/ml. Depending on virus infection experiments, we used virus doses of 0.5-5 pfu/cell for influenza A, 1-10 pfu/cell for Sendai virus, and 1-5 pfu/cell for VSV (Indiana strain). VSV stocks were grown in Hep2 and the stock virus had a titer of 4×10^6 pfu/ml.

Detection of IFN-α/β

Culture media from virus-infected cells were treated at pH 2 and assayed for the presence of IFN-α/β activity in Hep2 cells by VSV plaque reduction assay (Cantell *et al.,* 1991). The results are expressed as IU/ml, using an international control IFN-α preparation as the laboratory standard. RT–PCR analysis was carried out with Clontech RT–PCR kit with oligonucleotides 5′ TGT TGT TGA CAT GAC CAA CAA GTG TCT C (upstream oligonucleotide) and 5′ GCA CAG GCT AGG AGA TCT TCA GTT TCG G (downstream oligonucleotide) specific for human IFN-β gene.

Antibodies

The following primary antibodies were used: mouse monoclonal anti-HLA-A,B,C (used at 1:8 dilution, ATCC HB95), mouse monoclonal anti-influenza A nucleoprotein (1:500, IC9 anti-NP, kindly provided by Dr. J. Yeddel, NIH, MD), rabbit anti-NP (1:500, Ronni *et al.,* 1995), rabbit anti-Sendai (1:1000, Julkunen *et al.,* 1983), rabbit anti-VSV (1:500), rabbit anti-MxA (1:1000, Ronni *et al.,* 1993), guinea pig anti-MxA and anti-MxB (1:600 and 1:150, respectively, Melén *et al.,* 1996). Rabbit anti-OAS antibodies (1:500) were prepared against *Escherichia coli*-expressed GST-OAS fusion protein. The cDNA for rat 40- to 46-kDa form of OAS was kindly provided by Dr. E. Truve (Tallinn, Estonia). The following secondary antibodies were used: FITC-conjugated sheep anti-rabbit IgG F(ab′)$_2$ fragment (1:100, Boehringer Mannheim, Mannheim, Germany), FITC-conjugated sheep anti-mouse IgG F(ab′)$_2$ fragment (1:100, Boehringer Mannheim), HRP-conjugated goat anti-rabbit (1:2000, Bio-Rad Laboratories, Richmond, CA), and HRP-conjugated goat anti-guinea pig (1:1000, Dako, Glostrup, Denmark).

Flow cytometric analysis

For flow cytometric analysis, cells were detached with trypsin at 37°C followed by washing with PBS and fixing with Facs lysing solution (Becton Dickinson, San Jose, California) according to manufacturer's instructions. With this fixation process the cells also were permeabilized, which enabled staining for both cell surface and intracellular antigens. After fixation and washing, the cells were suspended in PBS containing 0.5% BSA and 2% normal human serum (NHS). Primary staining was carried out for 30 min at 37°C. After washing with PBS, secondary staining was carried out for 30 min at 37°C. After washings, the cells were suspended in PBS containing 0.5% BSA and 10,000 cells were analyzed by flow cytometry as recommended by the manufacturer (FACScan with Cellquest software, Becton Dickinson, Mountain View, CA).

Gel electrophoresis and Western blotting

SDS–PAGE was carried out using the Laemmli buffer system (Laemmli, 1970). Proteins separated on gels were transferred to Immobilon-P (polyvinylidene difluoride, PVDF) membranes (Millipore, Bedford, MA) with an Isophor electrotransfer apparatur (Hoefer Scientific Instruments, San Francisco, CA) at 200 mA for 1 h. The primary and secondary antibody binding was carried out in PBS containing 5% nonfat milk for 1 h at 37°C. After primary and secondary staining, blots were washed three times for 10 min. The protein bands were visualized on Amersham Hyper-Max film by the ECL chemiluminescence system as recommended by the manufacturer (Amersham, Buckinghamshire, UK). Protein concentrations in the samples were determined with the BioRad protein assay kit.

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