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*J Immunol* 2004; 172:6453-6459; doi: 10.4049/jimmunol.172.10.6453

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Antiviral Cytokines Induce Hepatic Expression of the Granzyme B Inhibitors, Proteinase Inhibitor 9 and Serine Proteinase Inhibitor 6

Mahmoud B. Barrie,2 Heather W. Stout,2 Marwan S. Abougergi, Bonnie C. Miller, and Dwain L. Thiele3

Expression of the granzyme B inhibitors, human proteinase inhibitor 9 (PI-9), or the murine orthologue, serine proteinase inhibitor 6 (SPI-6), confers resistance to CTL or NK killing by perforin- and granzyme-dependent effector mechanisms. In light of prior studies indicating that virally infected hepatocytes are selectively resistant to this CTL effector mechanism, the present studies investigated PI-9 and SPI-6 expression in hepatocytes and hepatoma cells in response to adenoviral infection and to cytokines produced during antiviral immune responses. Neither PI-9 nor SPI-6 expression was detected by immunoblotting in uninfected murine or human hepatocytes. Similarly, human Huh-7 hepatoma cells were found to express only very low levels of PI-9 relative to levels detected in perforin- and granzyme-resistant CTL or lymphokine-activated killer cells. Following in vivo adenoviral infection or in vitro culture with IFN-α or IFN-γ, SPI-6 expression was induced in murine hepatocytes. Similarly, after culture with IFN-α, induction of PI-9 mRNA and protein expression was observed in human hepatocytes and Huh-7 cells. IFN-γ and TNF-α also induced 4- to 10-fold higher levels of PI-9 mRNA expression in Huh-7 cells, whereas levels of mRNA encoding a related serine proteinase inhibitor, proteinase inhibitor 8, were unaffected by culture of Huh-7 cells with IFN-α, IFN-γ, or TNF-α. These findings indicate that cytokines that promote antiviral cytopathic responses also regulate expression of the cytoprotective molecules, PI-9 and SPI-6, in hepatocytes that are potential targets of CTL and NK effector mechanisms. The Journal of Immunology, 2004, 172: 6453–6459.

Cytotoxic T lymphocyte and NK cells kill virally infected and malignant cells by two major pathways. The concerted actions of cytotoxic granule molecules, perforin and granzymes A and B represent the major pathway responsible for rapid killing of most target cell lines (1–3). An alternative pathway for target cell killing is mediated by cytotoxic lymphocyte engagement of Fas and/or other TNFR death receptor family members expressed on target cells (1–3). A number of cellular and viral mechanisms have evolved to limit killing by these mechanisms. Such mechanisms include negative regulators of apoptosis such as FLIP and various Bcl-2 family members (4, 5) which are very effective in limiting cytotoxicity mediated through TNF death receptors, and the serine proteinase inhibitors (serpin), inhibitors of granzyme B (6–9), that selectively block perforin- and granzyme B-mediated cytotoxicity.

Virally infected hepatocytes are resistant to killing by perforin- and granzyme-dependent cytotoxic effector pathways (10, 11). This appears to explain the more prominent role of Fas- and TNFR-mediated apoptosis in killing of virally infected liver cells and in clearance of hepatic viral infections (10–13). Expression of human proteinase inhibitor 9 (PI-9) (6, 14–16), or the murine homologue serine proteinase inhibitor 6 (SPI-6) (6, 17), by leukocytes or tumor cells affords selective resistance to perforin- and granzyme-dependent killing mechanisms and allows PI-9 or SPI-6-expressing cells to evade killing by activated CTL or NK cells. Initial surveys of human or mouse tissues for PI-9 or SPI-6 expression revealed high levels of expression in dendritic cells and immune-privileged sites, but did not reveal high levels of PI-9 or SPI-6 expression in normal liver (18, 19). However, PI-9 has been observed to be an estrogen-inducible gene in human hepatoma cells and hepatic sections (20). The present studies were designed to assess whether factors present during hepatic viral infections, and associated antiviral immune responses, might also induce expression of PI-9 or SPI-6 in human or murine liver cells and thereby explain resistance of virally infected liver cells to perforin- and granzyme-dependent hepatotoxicity.

Materials and Methods

Cytokines

Recombinant human IFN-α-2b/INTRON A was obtained from Schering-Plough (Kenilworth, NJ), recombinant mouse IFN-αβ was obtained from Sigma-Aldrich (St. Louis, MO), recombinant human IL-2 was obtained from the Biological Resources Branch, National Cancer Institute, Frederick Cancer Research Development Center (Frederick, MD), and recombinant human IFN-γ, recombinant human TNF-α, and recombinant mouse IFN-γ were obtained from R&D Systems (Minneapolis, MN).
and 200 U/ml penicillin G. PBMC cultures were supplemented with 0.5 lymphokine-activated killer (LAK) cells. Anti-H-2q-specific T lymphocytes or with 100 U/ml rIL-2 for 2 days to generate LAK cells.

Protein levels were assayed and 40 μg of protein were loaded per lane before SDS-PAGE separation of proteins and Western blotting with affinity-purified anti-PI-9211-260.

FIGURE 1. SPI-6 expression in AML-12 hepatocytes and in spleen and liver of control and adenovirus-infected mice. Lysates were prepared as detailed in Materials and Methods from AML-12 cells or from spleen or liver isolated from control mice or mice infected for 7 days with AdCMV-lacZ. Protein levels were assayed and 40 μg of protein were loaded per lane before SDS-PAGE separation of proteins and Western blotting with affinity-purified anti-PI-9211-260.

Mice

C57BL/6 (B6), C57BL/ Ppom1Sld4 (pfp−/−), gld, and FVB/NJ (H-2b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Adenovirus vector

The E1-deleted, replication-deficient, β-galactosidase-encoding recombinant adenovirus (AdCMV-lacZ) was propagated in 293 cell cultures, purified on a cesium chloride gradient, and titers of infectious virus were determined by plaque assay as previously described (21). Mice were infected via the tail vein with 109 plaque-forming units (PFU) of AdCMV-lacZ.

Cells and culture conditions

Human PBMC were isolated from heparinized venous blood of healthy donors by centrifugation over sodium diatrizoate/polyacryl serum gradients (Histopaque-1077; Sigma-Aldrich). Human PBMC, human Huh-7 hepatoma cells (22), and mouse AML-12 cells (23) were cultured in 25 cm2 tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified, 5% CO2 atmosphere in RPMI 1640 medium (Huh-7 cells; BioWhittaker, Walkersville, MD) or equal proportions of DMEM and F12 medium (AML-12 cells; BioWhittaker), supplemented with 10% FBS (Life Technologies), 1 mM pyruvate, 100 μM streptomycin, and 200 U/ml penicillin G. PBMC cultures were supplemented with 0.5 μg/ml PHA (Roche Molecular Biochemicals, Indianapolis, IN) for 3 days to activate T lymphocytes or with 100 μM IL-2 for 2 days to generate lymphokine-activated killer (LAK) cells. Anti-H-2b-specific CTL were generated in 5-day MLC containing 12 × 106 responder spleen cells from C57BL/6 background (H-2b) mice and equal numbers of irradiated FVB-NJ (H-2b) stimulator spleen cells in 6 ml of RPMI 1640 medium supplemented with 10% FBS as previously described (10, 12).

Primary human hepatocytes prepared from fresh human tissue were purchased from Centest (Woburn, MA). Only hepatocytes from donors free of serological evidence of CMV, HIV, hepatitis B, and hepatitis C infection were used. Following isolation, hepatocytes were cultured in BD Hepatostim Hepatocyte Culture Medium (BD Biosciences, Woburn, MA) for 48 h and had achieved >85% confluency in BD Biocoat Collagen I, 6-well microplates (BD Biosciences) before addition of varying concentrations of IFN-α during an additional 24 h of culture.

Primary marine hepatocytes were isolated as previously described (10), and then were cultured in plates coated with 1% collagen type I (Sigma-Aldrich) in William’s medium supplemented with FBS (10%), HEPES (25 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), fungosine (5 μg/ml), insulin (10 μg/ml), transferrin (10 μg/ml), selenious acid (10 μg/ml), dexamethasone (1 μM), epidermal growth factor (5 μg/ml), glucagon (0.1 μg/ml), somatostatin (10 μg/ml), and prolactin (20 μg/ml), all purchased from Sigma-Aldrich and added to the medium immediately before use.

Chromium release assay

Targets were labeled with 150 μCi of Na2CrO4 for 90 min at 37°C and washed twice before incubation with effector cells at different E:T ratios in 200 μl of cultures. After 12 h, 100 μl of supernatant was harvested from experimental and control wells and the percentage of specific lysis calculated from the formula: percent specific lysis = (maximal release (cpm) − spontaneous release (cpm)) / (maximal release (cpm) − spontaneous release (cpm)). All assays were performed in triplicate and results shown are mean ± SEM.

RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies) or RNA STAT 60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. Total RNA was treated with RNase-free DNase (Promega, Madison, WI), and first-strand cDNA was generated with random hexamer priming using the SuperScript II RNase H-Rt kit (Life Technologies). In some experiments, relative SPI-6 or PI-9 mRNA levels were determined by standard RT-PCR using previously described primers (17) for human GAPDH (GGTCCGAGTCAACCGAGTTGG and ATAGGC CCCACCCTTCTCAT; annealing temperature, 61°C), mouse GAPDH (ACACCAGCTCACTGACATGCCG and CCAACACCCCTTGTGCGATGCC; annealing temperature, 58°C), mouse SPI-6 (TGTTATTTCTCGTGGGAGACATC and TTCTGATGCAAGCCGCC; annealing temperature, 42°C), IL-9 (TCTGGCTGCGCCTGATCTT and CTGGCC TTTGCTCCTCTGGTCTT; annealing temperature, 58°C), and vimentin, and varying numbers of cycles as indicated. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time RT-PCR was used in other experiments. Real-time RT-PCR primer sets for PI-9 and PI-9 were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA). Real-time PCR were performed in a final volume of 10 μl containing cDNA from 20 ng of reverse transcribed total RNA, 150 μM forward and reverse primers and SybrGreen Universal PCR master mix (Applied Biosystems). PCR were conducted in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate. Melting curve analysis was performed to identify primer sets and conditions yielding specific products. Primers validated by this technique and used in the present studies were GAPDH: 5′-GCCCATGCTTCAATCAGCTG and 5′-GCCACACCTGTTGCGGATGCC; annealing temperature, 42°C, PI-9: 5′-TCTGGCTGCGCCTGATCTT and CTGGCC TTTGCTCCTCTGGTCTT; annealing temperature, 58°C, and vimentin, and varying numbers of cycles as indicated. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Generation of PI-9- and SPI-6-specific Abs

Using standard techniques, rabbits were immunized with synthetic peptides corresponding to aa 160–182 of human PI-9 or aa 221–260 of murine SPI-6 (21) that were coupled to keyhole limpet hemocyanin by an N-terminal cysteine residue (Pierce, Rockford, IL). SulfoLink coupling gel and reagent kit (Pierce) was used to immobilize the PI-9 synthetic peptide and affinity purify anti-PI-9-specific Abs from whole rabbit serum.

Western blotting

Cells were washed, suspended in 10 mM Tris-Cl (pH 7.8) lysis buffer containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, and protease inhibitors (10 μg/ml E64, 7.5 μg/ml pepstatin A, 40 μg/ml 3,4-dichloroisocoumarin, 5 μg/ml benzamidine, 20 μg/ml apro- tinin, and 50 μg/ml PMSF) as previously described (24), and lysed by

FIGURE 2. SPI-6 expression in hepatocytes from control and adenovirus-infected mice. Hepatocytes were isolated from control mice or AdCMV-lacZ-infected mice (day 7). Hepatocyte lysates were prepared as detailed in Materials and Methods, protein levels were assayed, and 20 or 40 μg of protein were loaded per lane as indicated before SDS-PAGE separation of proteins and Western blotting with the same lot of affinity-purified anti-PI-9211-260 as in the experiment depicted in Fig. 1.

~42 kDa
repeated freeze-thawing and centrifuged for 10 min at 10,000 × g to remove debris. Protein content was assayed by the bicinchoninic acid method as previously described (25). Equal amounts of total protein (20 μg per lane) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose in pH 9.9 carbonate buffer (24). Immunodetection was performed using primary rabbit anti-human PI-9 or primary anti-SPI-6, and secondary HRP-conjugated anti-rabbit Ig and the ECL Western Blotting Analysis System from Amersham Pharmacia Biotech (Piscataway, NJ). Biotinylated protein markers (Cell Signaling Technology, Beverly, MA) detected by HRP-linked anti-biotin Abs were used for molecular mass estimation. In some experiments, the relative levels of PI-9 were determined by densitometry of the scanned images by using IMAGEQUANT software (Amersham Pharmacia Biotech).

**Results**

In initial studies, AML-12 cells, a mouse hepatocyte line that is resistant to perforin- and granzyme-mediated cytotoxicity (10, 12), and tissues from control and AdCMV-lacZ-infected mice were assessed for SPI-6 expression. As illustrated in Fig. 1, affinity-purified anti-PI-9\_{169–182} detected a single ~42-kDa protein band in lysates of AML-12 hepatocytes and in lysates of spleens isolated from control or AdCMV-lacZ-infected mice. In contrast to the similar levels of splenic SPI-6 expression in virally infected and uninfected mice, liver SPI-6 expression was only detected in AdCMV-lacZ-infected mice (Fig. 1). Immunoreactive SPI-6 expression also was detected in hepatocytes isolated from virally infected animals (Fig. 2) but not in uninfected hepatocytes demonstrating that the increased liver expression of SPI-6 observed during adenoviral infection is not merely related to infiltration by SPI-6-expressing leukocytes, but rather reflects induction of SPI-6 expression in hepatocytes.

To extend these observations to human cells, immunoreactivity of serum from a PI-9\_{169–182} immune rabbit was assessed by Western blotting of human Huh-7 hepatoma cells and human PHA-activated lymphocytes. As displayed in Fig. 3, unfractionated PI-9\_{169–182} immune serum identified two protein bands in PHA-activated human PBMC. Immunodetection of an ~42-kDa band (second lane, left panel) was eliminated by the presence of high concentrations (1 mg/ml) of competing PI-9\_{169–182} during primary Ab incubation (second lane, right panel) confirming the specificity of this reaction. Immunodetection of a second higher molecular mass band was not altered by the presence of competing PI-9\_{169–182}. Affinity-purified anti-PI-9\_{169–182} detected only the ~42-kDa PI-9 bands in both PHA-activated T cells and LAK cells (Fig. 4). Both the unfractionated anti-PI-9\_{169–182} and the affinity-purified anti-PI-9\_{169–182} detected only trace quantities of ~42-kDa PI-9 immunoreactivity in Huh-7 cells (see Figs. 3 and 4). Low levels of PI-9 gene transcription in Huh-7 cells maintained under standard culture conditions were confirmed by RT-PCR assays of PI-9 mRNA using multiple PI-9-specific primer sets (data not shown).

In additional studies, primary human hepatocytes were assessed by immunoblotting and no PI-9 expression was detected (data not shown). However, 24 h after addition of 250-1000 U/ml human IFN-α to primary hepatocyte cultures, ~42-kDa anti-PI-9\_{169–182} immunoreactive proteins could be detected in human hepatocyte lysates (data not shown). To verify IFN induction of PI-9 gene expression, RNA was isolated from hepatocytes cultured in the absence or presence of IFN-α and assessed by RT-PCR with human GAPDH- and PI-9-specific primers over a range of cycles for

**FIGURE 3.** Specificity of anti-PI-9\_{169–182}. Lysates were prepared from the indicated cell lines as detailed in Materials and Methods, protein levels were assayed, and 20 μg of protein were loaded per lane before SDS-PAGE separation of proteins and Western blotting. Where indicated, 1 mg/ml PI-9\_{169–182} or an unrelated 22-aa fragment of human dipeptidyl peptidase I (control peptide), was added during the primary Ab incubation step in Western blotting. The location of an ~42-kDa protein band is shown.

**FIGURE 4.** PI-9 expression in Huh-7, LAK cells, and PHA-activated lymphocytes. Lysates were prepared from the indicated cell lines as detailed in Materials and Methods, protein levels were assayed, and 20 μg of protein were loaded per lane before SDS-PAGE separation of proteins and Western blotting with affinity-purified anti-PI-9\_{169–182}.

**FIGURE 5.** Induction of PI-9 mRNA expression in human hepatocytes cultured with IFN-α. Primary human hepatocytes obtained 48 h after isolation were cultured for an additional 24 h in the absence or presence of 1000 U/ml IFN-α. RNA was isolated and equal quantities were reverse transcribed. Portions of the same reverse-transcribed RNA were subjected to PCR with PI-9-specific (top panel) and GAPDH-specific (middle panel) primers for the indicated number of cycles. Densitometric quantitation of the PI-9 products at all cycles was performed and normalized to densitometric volume of GAPDH products obtained after 22 cycles of amplification of the same reverse-transcribed primer (bottom panel).
PI-9 and GAPDH mRNA expression. As shown in Fig. 5, while PI-9 mRNA could be detected by RT-PCR in control hepatocytes after 30 amplification cycles, >10-fold higher levels of PI-9 mRNA expression were observed in hepatocytes cultured for 24 h with IFN-α. Similarly, as illustrated in Figs. 6 and 7, significant induction of SPI-6 mRNA expression was observed after culture of primary mouse hepatocytes for 24 h with IFN-γ or IFN-α2b.

To further investigate the effect of cytokine stimulation on serpin expression, Huh-7 cells were cultured for varying time intervals with IFN-α, IFN-γ, or TNF, and PI-9 mRNA levels were monitored by quantitative real time RT-PCR assays. In addition, mRNA levels of GAPDH and PI-8, another serpin previously observed to be expressed in the liver (26), were assessed in parallel. Concentration-dependent induction of PI-9 mRNA by IFN-α2b was apparent 20 h after addition of this antiviral cytokine to Huh-7 cultures, whereas no significant effects of IFN-α on PI-8 gene transcription were detected (Fig. 8). In additional experiments using semiquantitative immunoblot assays (data not shown), ~3-fold increases in levels of PI-9 protein expression in Huh-7 cells were detected within 24 h after addition of IFN-α to the cultures. In the experiments detailed in Fig. 9, culture of Huh-7 cells with IFN-γ or TNF, two additional cytokines expressed during antiviral immune responses, also were found to induce significantly increased expression of PI-9, but not PI-8 mRNA. Initial induction of PI-9 mRNA by each cytokine was apparent within 12 h after cytokine stimulation (data not shown) and achieved maximal levels of PI-9 mRNA induction within 20 h that were sustained for at least the next 24 h (Fig. 9).

Additional experiments were conducted to assess the effects of antiviral cytokines on sensitivity of hepatocytes to CTL killing by various effector mechanisms. In these experiments, anti-H-2d-specific CTL with selective deficiencies in each of the two major CTL cytotoxicity pathways were generated in in vitro MLC and found to have comparable efficiency in killing H-2d expressing 3T3 fibroblast target cells (Fig. 10). These allospecific CTL were then used to assess the sensitivity of the TNF-resistant AML-12 hepatocyte cell line (12) to killing by either the perforin- and granzyme-mediated killing mechanism that remains intact in Fas ligand defective gld CTL or to the Fas ligand-mediated killing mechanism that remains intact in pfp−/− CTL. As illustrated by the results of the experiment detailed in Fig. 10 and as noted in previously published work from our laboratory (10, 12), the SPI-6-expressing AML-12 hepatocyte line is much less sensitive to killing by gld CTL with intact granzyme-dependent CTL effector mechanisms than to killing by the alternate, Fas ligand-dependent killing pathway preserved in pfp−/− CTL. As in the case of primary murine or human hepatocytes, severalfold higher levels of SPI-6 mRNA and protein expression (additional data, not shown) are induced by culture of AML-12 hepatocytes with either type I or II IFNs. As illustrated by results displayed in Fig. 10, AML-12 hepatocytes cultured with IFN-γ are even more resistant to perforin- and granzyme-dependent cytotoxicity mediated by gld CTL. This increased resistance occurs despite the fact that IFN-γ, a known inducer of class I MHC molecules, cell surface adhesion molecules, and other cell surface receptors that enhance susceptibility to a host of Ag-specific T cell responses (27), dramatically increases sensitivity of AML-12 hepatocytes to alternative killing pathways mediated by pfp−/− CTL. Thus, following exposure to IFN-γ, AML-12 hepatocytes are even more resistant to granzyme-dependent CTL effector mechanisms despite increased capacity to trigger alternative CTL responses.

**Discussion**

The results of the present study indicate that expression of PI-9 and SPI-6, selective serpin inhibitors of granzyme B (6, 8, 19), is induced in hepatocytes and hepatoma cells by any of several antiviral cytokines known to be induced in the liver during hepato- viral infection (10, 28). Furthermore, these studies indicate that SPI-6 expression is induced in mouse livers during hepatic adenoviral infection at time points previously noted to correlate with peak intrahepatic, anti-adenoviral CTL activity (10). Moreover, IFN-treated, SPI-6-expressing mouse hepatocytes exhibit resistance to perforin- and granzyme-mediated cytotoxicity mechanisms despite enhanced ability of these IFN-induced cells to trigger other T cell responses, such as the alternative Fas ligand-mediated cytotoxicity pathway. The lack of concurrent cytokine regulation of expression of PI-8, a serpin not known to modulate lymphocyte cytotoxicity (29), suggests that such cytokine-induced expression of PI-9 and SPI-6 may have evolved specifically as part of a hepatocyte cellular defense mechanism designed to avoid excess liver cell injury by granzyme-dependent mechanisms during...
viral hepatic infections or other inflammatory states while leaving these cells capable of triggering alternative T cell immune responses.

The two most common viral causes of chronic hepatitis in humans, hepatitis B and hepatitis C virus, are both largely noncytopathic viruses that induce significant hepatocellular injury only after generation of host antiviral NK and CTL responses (30). During the course of acute hepatitis B or hepatitis C virus infection in humans, antiviral cytokine responses typically precede induction of prominent CTL responses (30). This sequence of host antiviral responses has been postulated as important in avoidance of acute liver failure secondary to overly robust CTL responses directed at virally infected hepatocytes. Since both IFN responses and enhanced intrahepatic TNF expression during viral hepatitis appear to significantly suppress viral replication via noncytopathic mechanisms, the fraction of hepatocytes susceptible to killing by antiviral CTL at any one point in time is thus reduced (28, 30–32). The results of the present study identify another mechanism whereby early type I and II IFN and TNF responses to hepatic viral infection might limit hepatotoxicity via induction of expression of murine SPI-6 or human PI-9, selective inhibitors of the major perforin- and granzyme-dependent pathway of lymphocyte-mediated cytotoxicity (3, 33). Of note, as previously reported (34), the induction of PI-9 by TNF, a prominent component of innate responses to endotoxin and other bacterial stimuli, may also provide liver cells with a mechanism to avoid excess bystander toxicity from hepatic NK cell activation (35) during abdominal or systemic bacterial infections in which the liver reticuloendothelial system plays a prominent role in clearance of invading organisms.

The dichotomy between the apparently constitutive expression of PI-8 and the regulated expression of PI-9 in hepatoma cells is not entirely unexpected as, despite structural homology, these two molecules are encoded by genes residing on different chromosomes as part of different serpin gene clusters (36, 76). Others have

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**FIGURE 7.** Induction of SPI-6 mRNA expression in mouse hepatocytes cultured with IFN-αβ. Primary murine hepatocytes were cultured for 24 h in the absence or presence of the indicated concentrations of IFN-αβ. RNA was isolated and equal quantities were reverse transcribed. Portions of the same reverse-transcribed RNA were subjected to PCR with SPI-6-specific (top panel) and GAPDH-specific (middle panel) primers for the indicated number of cycles. Densitometric quantitation of the SPI-6 products was performed and normalized to densitometric volume of GAPDH products obtained after 16 cycles of amplification of the same reverse-transcribed primer (bottom panel).

**FIGURE 8.** Induction of PI-9 mRNA expression in Huh-7 cells by IFN-α. RNA was isolated from Huh-7 cells after 20 h of culture with the indicated concentrations of IFN-α. RNA was isolated and equal quantities were reverse transcribed and assessed for PI-8, PI-9, and GAPDH mRNA by real-time PCR techniques as detailed in Materials and Methods.

**FIGURE 9.** Induction of PI-9 mRNA expression in Huh-7 cells by IFN-α, IFN-γ, and TNF-α. RNA was isolated from Huh-7 cells after the indicated time in culture with the indicated cytokines (500 U/ml). RNA was isolated and equal quantities reverse transcribed and assessed for PI-8, PI-9, cyclophilin, and GAPDH mRNA by real-time PCR techniques as detailed in Materials and Methods. Similar results were obtained when PI-8 and PI-9 mRNA levels were normalized to GAPDH expression (not shown).
also noted that PI-8, in contrast to PI-9, appears to be constitutively expressed at relatively high levels in the liver (26) and thus appears more likely to be involved in regulation of proteases expressed during normal physiologic function of the liver rather than during pathologic immune responses as has been postulated for PI-9 function (14, 38).

The similar patterns of IFN-induced hepatocyte expression of the human serpin, PI-9, and the mouse serpin, SPI-6, provides additional evidence that SPI-6 is a close murine orthologue of the human PI-9 gene (39). Furthermore, the present studies reveal that SPI-6 is expressed in both AML-12 hepatocytes and adenovirally infected mouse hepatocytes, two types of target cells previously found to be highly resistant to CTL killing mediated by perforin- and granzyme-dependent mechanisms (10, 12). Moreover, in hepatocyte targets in which SPI-6 expression is enhanced by IFN stimulation, resistance to granzyme-dependent cytoxicity is augmented following IFN-γ exposure despite enhanced capacity to trigger alternative CTL effector mechanisms. Thus, the present observations regarding induction of PI-9 and SPI-6 expression in epithelial cells of hepatic origin extends the range of cell types in which PI-9 and SPI-6 expression has been observed and suggests that this cytoprotective molecule is involved not only in regulation of immune responses (38) and in prevention of bystander cell injury during CTL responses (14) but also is likely to play a role in modulating the mechanisms, whereby NK and CTL kill virally infected parenchymal cells in the liver.

References


