Woodchuck Gamma Interferon Upregulates Major Histocompatibility Complex Class I Transcription but Is Unable To Deplete Woodchuck Hepatitis Virus Replication Intermediates and RNAs in Persistently Infected Woodchuck Primary Hepatocytes

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Received 18 May 2001/Accepted 19 September 2001

Gamma interferon (IFN- γ) is an important mediator with multiple functions in the host defense against viral infection. IFN- γ , in concert with tumor necrosis factor alpha (TNF- α), leads to a remarkable reduction of intrahepatic replication intermediates and specific mRNAs of hepatitis B virus (HBV) by a noncytolytic mechanism in the transgenic mouse model. Thus, it is rational to evaluate the potential value of IFN- γ for the treatment of chronic HBV infection. In the present study, we expressed recombinant woodchuck IFN- γ (wIFN- γ) in *Escherichia coli* and mammalian cells. wIFN- γ protected woodchuck cells against infection of murine encephalomyocarditis virus in a species-specific manner. It upregulated the mRNA level of the woodchuck major histocompatibility complex class I (MHC-I) heavy chain in permanent woodchuck WH12/6 cells and regulated differentially the gene expression. However, the level of the replication intermediates and specific RNAs of woodchuck hepatitis virus (WHV) in persistently WHV-infected primary woodchuck hepatocytes did not change despite a treatment with 1,000 U of wIFN- γ per ml or with a combination of wIFN- γ and woodchuck TNF- α . Rather, hepatocytes derived from chronic carriers had an elevated level of the MHC-I heavy-chain mRNAs, most probably due to the exposure to inflammatory cytokines *in vivo*. Treatment with high doses of wIFN- γ led to an abnormal cell morphology and loss of hepatocytes. Thus, wIFN- γ regulates the gene expression in woodchuck hepatocytes but could not deplete WHV replication intermediates and mRNAs in persistently infected hepatocytes. The cellular response to wIFN- γ may be changed in hepatocytes from chronically WHV-infected woodchucks. It should be clarified in the future whether the continuous exposure of hepatocytes to inflammatory cytokines or the presence of viral proteins leads to changes of the cellular response to wIFN- γ .

Gamma interferon (IFN- γ) plays an important role in host defense against infections and pathogenesis (reviewed in references 1 and 2). IFN- γ shows various functions, e.g., activation of macrophages and upregulation of the major histocompatibility complex classes I and II (MHC-I and -II) expression. It shows direct antiviral actions against a number of viruses (12, 38). The role of IFN- γ in the virus clearance during hepadnavirus infection has been investigated intensively in recent years (4, 5, 13, 14, 16, 35). In acute hepadnavirus infection, IFN- γ was detectable in the intrahepatic compartment and may contribute to the upregulation of MHC-I expression (15, 16, 28, 29, 32). This is necessary for the recruitment of virus-specific T cells and the recognition of virus-infected hepatocytes by antigen-specific T cells. A direct nonlytic mechanism of inhibition of the hepatitis B virus (HBV) gene expression and replication by IFN- γ was proposed (4, 5, 13). In transgenic mice with liver-specific expression of HBV genes, a transfer of activated cytotoxic T lymphocytes (CTLs) to HBV surface antigen (HBsAg) led to the suppression of HBV replication (14). The HBV-specific transcripts were destabilized by a nonlytic mech-

anism at the posttranscriptional level. IFN- γ and tumor necrosis factor alpha (TNF- α), which were released by activated CTLs, were found to be necessary for the inhibition of HBV-specific gene expression in transgenic mice. Guidotti et al. demonstrated that the clearance of HBV from liver of acutely infected chimpanzees mainly occurred without cell destruction (15). In the duck model, IFN- γ was active to inhibit the duck HBV (DHBV) replication in primary duck hepatocytes. However, the formation of DHBV cccDNA was not prevented by duck IFN- γ (35).

The woodchuck model is an excellent animal model for studying hepadnavirus infection (10, 24, 27, 33, 36). Recently, several groups worked on T-cell responses to woodchuck hepatitis virus (WHV) during acute and chronic WHV infection in this model (6, 7, 16, 25, 26, 30). Particularly, woodchuck IFN- γ (wIFN- γ) has been characterized by molecular cloning and sequencing previously (20). wIFN- γ has sequence similarities to human IFN- γ and mouse IFN- γ of 60 and 40%, respectively. It contains an amino-terminal hydrophobic sequence that corresponds to a putative signal sequence. Its intrahepatic expression in liver tissues from acutely and chronically WHV-infected woodchucks was detected by RNA protection assays or reverse transcription-PCR (16, 29, 41). In acute-resolving WHV infection, the intrahepatic expression of wIFN- γ was correlated with the appearance of CD8, a specific marker of

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FIG. 1. (A) Construction of peWHIG and pQE-WHIG. An *Eco*RI fragment containing the complete coding region of wIFN- γ (nt 1 to 501) was cut out of a plasmid pWHIG described previously (20) and inserted into the *Eco*RI site of pcDNA3 to create peWHIG. (B) The coding region of mature wIFN- γ (nt 70 to 501) was cloned into pQE30. (C) His-wIFN- γ with an aminoterminal His₆ tag was expressed by *E. coli* harboring pQE-WHIG, purified on an Ni-NTA column, and stained with Coomassie blue after electrophoresis on a 15% polyacrylamide gel. M, molecular weight marker; Kd, kilodalton(s).

CTLs, and had the peak level at the time of recovery. In the WHV-infected neonatal woodchucks, an acute-resolving infection was associated with increased intrahepatic expression of wIFN- γ and woodchuck TNF- α (wTNF- α) mRNAs in the mid-acute phase (16). The exact role of IFN- γ in the clearance of WHV, however, is not clear yet.

It is an important issue whether IFN- γ can be used for the therapy of chronic hepatitis B. In the present study, we expressed recombinant wIFN- γ in *Escherichia coli* and mammalian cells in biologically active forms. wIFN- γ led to a strong upregulation of the expression of the woodchuck MHC class I heavy chain and inhibited specifically the gene expression under the control of viral promoters. However, the amounts of the WHV replication intermediates and mRNAs in persistently infected primary woodchuck hepatocytes were not reduced by a treatment with wIFN- γ and wTNF- α . Rather, hepatocytes derived from chronic carriers expressed an elevated mRNA level of the woodchuck MHC class I heavy chain as a result of continuously in vivo exposure to inflammatory cytokines. A treatment of a high dose of wIFN- γ led to an abnormal cell morphology and loss of hepatocytes. Our results indicate that IFN- γ is unable to achieve the clearance of hepadnaviruses in persistently infected hepatocytes by a direct antiviral action.

MATERIALS AND METHODS

Cells and reagents. A baby hamster kidney (BHK) cell line, murine L929 cell line, and a permanent woodchuck cell line WH12/6 (kindly provided by P. Banasch in German Cancer Research Center DKFZ Heidelberg, Germany) were used. The following recombinant cytokines were purchased from R&D system (Wiesbaden-Nordenstadt, Germany): recombinant mouse IFN- γ (rmIFN- γ), recombinant mouse interleukin-2 (rmIL-2) and rmIL-12, and recombinant human IL-12 (rhIL-12) and rhIL-15. Recombinant woodchuck TNF- α (wTNF- α) with a His tag was expressed in *E. coli* and purified by using a Ni-nitrilotriacetic acid (NTA)-Superose column (Pharmacia Biotech, Freiburg, Germany) as described previously (21). Purified wTNF- α was refolded into the bioactive form by removing imidazole and urea by using a ultrafiltration cell.

Expression of wIFN- γ in *E. coli* and mammalian cells. A DNA fragment containing the coding region for wIFN- γ was cut by *Hind*III and *Xho*I from pWHIG described previously (20) and was placed into pcDNA3 predigested with

same restriction enzymes, resulting in the mammalian expression plasmid peWHIG (Fig. 1A). The expression of wIFN- γ in peWHIG is under the control of the cytomegalovirus (CMV) promoter. To express wIFN- γ in BHK cells, peWHIG (4 μ g) was incubated with 10 μ g of Lipofectamine (Gibco-BRL, Eggenstein-Leopoldshafen, Germany) in 100 μ l of medium for 45 min and was given to cells in 1 ml of Opti-Media (Gibco-BRL) for 5 h at 37°C and 5% CO₂. Transfected cells were maintained for 48 h at 37°C and 5% CO₂. wIFN- γ released into medium by transfected cells was detected by virus protection assays described below.

A DNA fragment comprising the coding region for the putative mature wIFN- γ (nucleotides [nt] 70 to 501) was amplified by using the primers Winf-BamHI (GAC GGA TCC TGT TAC TCC CAG CAC AC [nt 70 to 86]) and Winf-HindIII (GGC AAG CTT TTA TTT GGA TGC TCT CCG [nt 484 to 501]) containing tags with restriction sites for *Bam*HI and *Hind*III, respectively (Fig. 1B). The PCR fragment was restricted with *Bam*HI and *Hind*III and ligated into pQE30-vector, resulting in the plasmid pQE-WHIG for the expression in *E. coli*. A His tag was located at the N-terminal end of recombinant wIFN- γ . His-wIFN- γ was expressed at a high level in *E. coli* after 4 h of culture with 2 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The His-wIFN- γ formed inclusion bodies and can be enriched from *E. coli* lysats by ultracentrifugation at 30,000 rpm in an SW40 rotor for 4 h. After being dissolved in 8 M urea, His-wIFN- γ could be purified further by affinity chromatography through a Ni-NTA-Superose 6 column (Pharmacia) according to the manufacturer's instructions (Fig. 1C). Fractions containing IFN- γ were diluted with phosphate-buffered saline and centrifuged in an amicon cell to reduce the amount of urea and imidazol. The purified His-wIFN- γ had a specific activity of 30,000 U per mg and was rather unstable. Sera of three rabbits immunized with His-wIFN- γ were reactive to wIFN- γ in Western blotting. However, only one rabbit produced sera with the ability to neutralize wIFN- γ generated by transfection with peWHIG and His-wIFN- γ in viral protection assays (see below).

Virus protection assay. Virus protection assay was carried out to measure amounts of IFN- γ . Briefly, woodchuck WH12/6 or murine L929 cells were seeded into 96-well microtiter plates and cultured in 100 μ l of Ham's F-12 medium supplemented with 10% fetal calf serum at 37°C and 5% CO₂ until 100% it reached confluence. After the culture medium was discarded, 100 μ l of F-12 medium containing appropriate dilutions of samples containing wIFN- γ was added to the cells for an additional incubation of 24 h. Afterward, murine encephalomyocarditis virus (EMCV) was added to cells and incubated for further 24 h. Cells were stained and fixed with 0.1% crystal violet in 20% ethanol. A unit of wIFN- γ was defined by its ability to protect 50% of the cells in a well.

Luciferase assay. Three reporter plasmids expressing firefly luciferase were used to assess the gene expression in woodchuck WH12/6 cells under the treatment with wIFN- γ . pTA-Luc vector (Clontech Laboratories, Inc., Heidelberg, Germany) contains a TATA box. The IFN- γ activation sequence was added to TATA box in pGAS-TA-Luc vector (Clontech). PWHpreSs-Luc contains the promoter region for the WHV s gene (nt 2611 to 295 according to the numbering

of reference 8). The fragment was amplified with the primers 5'-ATC GGT ACC ATG CAA TTA CAG GTC TTT-3' (nt 2611 to 2628) and 5'-GTC GGT GAC CAT AGT TAA GTG GGG GTG-3' (nt 115 to 99), restricted with *Kpn*I and *Bst*EII, and ligated into the predigested plasmid pSP-Luc +NT fusion vector (Promega, Mannheim, Germany). WH12/6 cells were cultured in 24-well plates to 70% confluence. One microgram of the plasmids was transfected into woodchuck cells by using Lipofectamine. wIFN- γ was added to transfected cell after 24 h. After 48 h of incubation at 37°C and 5% CO₂, the luciferase activity in transfected cells was measured by using the LucLite reporter gene assay kit (Packard BioScience Company, Meriden, Conn.) according to the manufacturer's instructions. Briefly, woodchuck cells were washed four times with phosphate-buffered saline and lysed with 500 μ l of lysis buffer containing the substrate. The luminescence was measured with a TopCounter NTX (Packard).

Culture of wPBMCs and measurement of proliferation of wPBMCs. Woodchuck peripheral blood mononuclear cells (wPBMCs) were isolated by Ficoll-Paque density gradient centrifugation and cultured in AIM V medium (Gibco-BRL) supplemented with 4 mM L-glutamine (Sigma, Deisenhof, Germany) and 10% fetal calf serum (Cytogen, Berlin, Germany) at 37°C in a humidified atmosphere containing 5% CO₂ (25, 26). Then, 10⁶ wPBMCs were cultured in 1 ml of medium supplemented with phytohemagglutinin (PHA) at concentrations of 1, 2, or 5 μ g per ml. Different heterologous cytokines rmIL-2, rmIL-12, rhIL-12, or rhIL-15 were added to wPBMC cultures with 2 μ g per ml of PHA. Supernatants of wPBMC cultures were collected every day for 4 to 5 days for the determination of wIFN- γ concentrations in virus protection assays. The determination of wPBMC proliferation at different culture conditions was performed as described previously (25, 26).

Immunofluorescence (IF) staining of woodchuck MHC-I with a specific monoclonal antibody. WH12/6 were seeded into eight-well chamber slides and cultured in 400 μ l of F-12 medium supplemented with 10% fetal calf serum at 37°C and 5% CO₂ until reaching 80 to 90% confluence. Then, 40 μ l of supernatant of peWHIG-transfected BHK cells containing wIFN- γ (1,000 U/ml) was added to WH12/6 cells for an additional incubation of 48 h. Afterward, cells were fixed with 50% methanol for 20 min at 4°C. IF staining was done with a MHC-I specific monoclonal antibody (kindly provided by T. Michalak) and fluorescein isothiocyanate-labeled anti-mouse-immunoglobulin G antibodies (Sigma).

Liver perfusion in woodchucks and primary woodchuck hepatocyte cultures. Liver perfusion was carried out according to the protocol described previously (23). Anesthetized woodchucks received an intravenous injection of 2 ml of heparin (10⁴ U/ml). After the peritoneum was opened, 400 ml of calcium-free preperfusion solution (Spinner minimal essential medium supplemented with 2 mM glutamine, 0.05% glucose, 20 mM HEPES [pH 7.4], 1.4 IU of insulin per ml, 5 mM sodium pyruvate, and 50 IU of penicillin-streptomycin per ml) were pumped into liver through portal vein. Next, 400 ml of collagenase medium (Williams medium supplemented with 0.4 mg of collagenase per ml, 3 mM CaCl₂, 2 mM glutamine, 0.05% glucose, 20 mM HEPES [pH 7.4], 12 IU of insulin per ml, 5 mM sodium pyruvate, and 50 IU of penicillin-streptomycin per ml) was pumped through the portal vein with a flow rate of 20 ml/min. Liver tissues were dissected from the abdominal cavity. Hepatocytes were separated from the liver with a forceps and scalpel and stirred in 100 ml of collagenase medium for additional 30 min at 37°C and 5% CO₂. Cell suspensions run through a 70- μ m (pore-size) filter to remove tissue fragments. Hepatocytes were separated from other cells by repeated centrifugation at 50 $\times$ g.

Primary woodchuck hepatocytes were seeded in 60-mm plates at a density of 10⁶ per well. Plates were coated with collagen type 1 before use. Hepatocytes were maintained for 11 days in Williams medium supplemented with 2 mM glutamine, 0.05% glucose, 20 mM HEPES (pH 7.4), 5 μ g of hydrocortisone per ml, 12.5 μ g of inosine per ml, 12 IU of insulin per ml, 5 mM sodium pyruvate, 50 IU of penicillin-streptomycin per ml, and 1% dimethyl sulfoxide. The medium was changed every 2 days.

Analysis of WHV replication intermediates in woodchuck primary hepatocytes. Total DNA was extracted from cultured hepatocytes by using a QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. WHV replication intermediates were analyzed by Southern blot hybridization with a full-length WHV8 genome as probe, as described previously (11).

Detection of mRNAs of woodchuck MHC-I, β -actin, and WHV. Total RNA was purified from woodchuck cells by using an RNeasy kit (Qiagen) and subjected to denaturing agarose gel electrophoresis with formamide. After transfer of the RNA to a nitrocellulose sheet by vacuum blotting, the mRNAs of the woodchuck MHC-I heavy chain, β -actin, or WHV were detected by specific ³²P-labeled probes, respectively. A full-length clone of cDNA of woodchuck MHC-I heavy chain MamoA01 was used hybridization (39). The relative signal strength of bands on the Northern blots was quantified by a phosphor image (Cyclone; Packard Instrument Company).

RESULTS

Protection of woodchuck liver cells against EMCV by recombinant wIFN- γ . EMCV, when added to woodchuck WH12/6 cells, led to cytopathic effect (Fig. 2A). By using serial dilutions of EMCV, the sensitivity of WH12/6 cells to EMCV was determined and was comparable with this of murine L929 cells. Virus titers which led to a complete lysis of L929 cells within 24 h caused also a complete lysis of woodchuck 12/6 cells in the same time interval. To test whether wIFN- γ is able to confer protection against EMCV infection, WH12/6 cells were preincubated with serial dilutions of culture supernatants of BHK cells transfected with peWHIG for 24 h. An EMCV stock resulting a complete lysis of cells after 24 h of incubation was added to cells. Woodchuck cells treated with wIFN- γ were protected against EMCV infection, since no cytopathic effect on cells was observed after 24 h (Fig. 2B). The culture supernatant of transfected BHK cells was able to protect against EMCV at a dilution of 1:526, corresponding to a wIFN- γ concentration of 1,024 U/ml (Fig. 2C). The antiviral action of wIFN- γ on WH12/6 was abolished by incubation with a specific neutralizing antibody raised against His-wIFN- γ .

wIFN- γ , when incubated with L929, did not protect L929 cells against EMCV infection (Fig. 3A). In reverse, rmIFN- γ , even at a concentration of 1,000 U/ml, had no protective effect on WH12/6 cells (Fig. 3B). rhIFN- γ also was not active on WH12/6 (data not shown). These results indicate that wIFN- γ acts in a species-specific manner, as known for IFN- γ of other species.

Production of wIFN- γ by wPBMCs stimulated by PHA and heterologous interleukins. IFN- γ is a cytokine that can be produced by activated T lymphocytes. We measured the wIFN- γ production of PHA-activated wPBMCs by using the virus protection assay. wPBMCs were cultured at a density of 10⁶/ml. Culture supernatants of wPBMCs were collected every day for a week and were serially diluted to determine the relative amount of wIFN- γ in virus protection assay. The concentration of wIFN- γ in supernatants of PHA-activated wPBMCs increased to the highest level at day 3 and fell thereafter (Fig. 4A). The production of wIFN- γ by wPBMCs from individual woodchucks showed slight differences (data not shown). The peak level of wIFN- γ produced by wPBMCs cultured with 5 μ g of PHA per ml ranged between 160 and 1,000 U/ml. No IFN- γ was detectable in culture supernatants of unstimulated wPBMCs.

IFN- γ release by activated lymphocytes can be influenced by cytokines such as IL-12 and IL-15 (3, 37). Several recombinant mouse and human cytokines were added to the PHA-activated wPBMCs to examine their effect on wIFN- γ production. rmIL-2 did not significantly change the IFN- γ production by wPBMCs, though rmIL-2 was known to stimulate the wPBMC proliferation (Fig. 4B). rmIL-12 enhanced the wIFN- γ production of PHA-activated wPBMCs, whereas it had only minor effects on proliferation (Fig. 4B). The IFN- γ concentration had the peak level at day 4. The effect of rhIL-12 had an optimum concentration of 50 U/ml. These results indicate that rmIL-12 and rhIL-12 are able to stimulate wPBMCs to release IFN- γ . Interestingly, the release of wIFN- γ was also enhanced by rhIL-15 (Fig. 4B). Like IL-2, rhIL-15 was able stimulate the

A. -IFN- γ , 24h/+EMCV, 24h **B. +IFN- γ , 24h/+EMCV, 24h** **C.**

FIG. 2. (A) The susceptibility of woodchuck WH12/6 cells to EMCV infection. WH12/6 cells were incubated with EMCV for 24 h. The cytopathic effect of EMCV on WH12/6 was visible by light microscopy. (B) Protection of WH12/6 cell against EMCV by wIFN- γ . WH12/6 cells were pretreated with the supernatant of BKH cells transfected with pWHIG for 24 h before the incubation with EMCV. WH12/6 cells retain the normal cell morphology. (C) Titration of

proliferation of PHA-activated woodchuck lymphocytes (data not shown).

Preparations containing a high concentration of wIFN- γ were produced by culturing wPBMCs at 4×10^6 to 5×10^6 per ml with 5 μ g of PHA and 50 U of IL-12 per ml for 3 or 4 days. These preparations had concentrations of wIFN- γ of >10,000 U/ml and of wTNF- α of <100 U/ml (Fig. 4C). For further experiments, these preparations were diluted to 1,000 U of wIFN- γ /ml for the treatment of woodchuck cells.

Effects of wIFN- γ on the gene expression in woodchuck cell lines. Further, we tested the effects of wIFN- γ on the gene expression in woodchuck cells. Woodchuck WH12/6 cells were treated with wIFN- γ at 0, 100, or 1,000 U/ml for 48 h. The expression of woodchuck MHC-I heavy chain and β -actin was examined by detection of the respective mRNAs on Northern blots (Fig. 5A). In treated WH12/6 cells, the mRNA level of woodchuck MHC-I heavy chain was upregulated up to nine-fold over the basal level. Concomitantly, the expression of β -actin was slightly decreased to 60% of the basal level. It appears that wIFN- γ at high concentrations acts antiproliferatively on WH12/6 cells and influences the expression of house-keeping genes (2). The upregulation of the transcription of woodchuck MHC-I heavy chain in treated WH 12/6 cells was abolished or strongly reduced when wIFN- γ preparations were preincubated with antiserum to wIFN- γ (Fig. 5B). The expression of woodchuck MHC-I could be further demonstrated by IF staining with a monoclonal antibody to the woodchuck MHC-I (Fig. 5C). Without the treatment with wIFN- γ , WH12/6 cells expressed only a low level of MHC-I. A strong expression of MHC-I was detected by IF staining in WH12/6 cells after incubation with 100 U of wIFN- γ per ml for 48 h. The regulation of gene expression by wIFN- γ was tested with reporter plasmids expressing firefly luciferase under different regulatory elements (Fig. 5D). wIFN- γ had only marginal effects on the luciferase expression in WH12/6 cells transfected with pTA-Luc. The expression of luciferase in WH12/6 cells transfected with the pGAS-TA-Luc vector was upregulated to 4.2-fold at a wIFN- γ concentration of 100 U/ml, while a concentration of 1,000 U of wIFN- γ per ml had little effect. In addition, WH12/6 cells were transfected with the reporter vector pWHpreS-Luc which express luciferase under the control of the WHV pres/s-promoter. The expression of luciferase in WH12/6 cells transfected with pWHpreS-Luc was significantly reduced to 52.6 and 41.7% by wIFN- γ at concentrations 100 and 1,000 U/ml, respectively. Even a low concentration of wIFN- γ of 1 or 10 U/ml led to an inhibition of the luciferase expression. A prolonged incubation with wIFN- γ at the low concentrations for 48 h led to a stronger reduction to more than 70% of the luciferase expression (not shown). Further, the luciferase expression under the simian virus 40 promoter or CMV immediate-early promoter in transiently transfected

wIFN- γ . The supernatant of BKH cells transfected with pWHIG was diluted and added to WH12/6 cells. EMCV was added to WH12/6 cells for a further incubation of 24 h. The Survival of WH12/6 cells was judged after being stained with crystal blue (■). To prove the specificity of wIFN- γ action, the supernatant of transfected BHK cells was incubated with a neutralizing antibody to wIFN- γ before it was applied to WH12/6 cells (◆).

FIG. 3. The species specificity of the action of woodchuck IFN- γ . WH12/6 cells or murine L929 cells were pretreated with different dilutions of wIFN- γ preparations (A) or different concentrations of rmIFN- γ (B) for 24 h and then incubated with EMCV. Dilutions of the supernatant of BKH cells transfected with pWHIG containing wIFN- γ were used for these experiments. The protection against EMCV was only seen on wIFN- γ -treated WH12/6 cells and rmIFN- γ -treated L929 cells.

WH12/6 cells was inhibited by wIFN- γ , as tested with the commercially available reporter plasmids pGL3 and pRL-CMV (Promega). Thus, wIFN- γ regulated selectively the cellular gene expression.

Effects of woodchuck IFN- γ on the MHC-I heavy-chain transcription in primary woodchuck hepatocytes. Woodchuck primary hepatocytes were prepared from naive and chronically WHV-infected woodchucks and cultured with or without wIFN- γ . Naive woodchuck primary hepatocytes showed an absent or low expression level of MHC-I heavy-chain mRNAs during culture without wIFN- γ . In the presence of wIFN- γ , the level of MHC-I heavy-chain mRNAs in naive woodchuck hepatocytes was upregulated after incubation with wIFN- γ (Fig. 6A). At day 3, the level of the woodchuck MHC-I heavy-chain mRNAs was ca. seven- and ninefold over the basal expression level for wIFN- γ concentrations at 100 and 1,000 U/ml, respectively. In contrast, hepatocytes from chronically WHV-infected woodchucks had a higher level of MHC-I heavy-chain mRNAs (ca. threefold of the basal level in naive woodchuck hepatocytes) without a preincubation with wIFN- γ , most probably due to the intrahepatic expression of inflammatory cytokines, including wIFN- γ in chronic carriers. These results are consistent with the data published by Michalak et al. (29). The amount of MHC-I heavy-chain mRNAs in these hepatocytes decreased gradually during culturing in the absence of wIFN- γ (Fig. 6B). The upregulation of woodchuck MHC-I heavy-chain expression occurred also in WHV-infected primary hepatocytes from chronic carriers upon the treatment with wIFN- γ . However, a decrease of the mRNA amount of the MHC-I heavy chain was observed at day 9, probably due to the cytotoxicity of high concentrations of wIFN- γ (see below).

WHV replication and gene expression in persistently WHV-infected woodchuck primary hepatocytes under treatment with

wIFN- γ . WHV replication intermediates were detected in woodchuck primary hepatocytes from chronically WHV-infected hepatocytes for at least 7 days. Obviously, no reduction of the WHV replication intermediates was achieved by the incubation with wIFN- γ compared to untreated woodchuck hepatocytes (Fig. 7A). Similarly, the amount of WHV 2.1-kb transcripts was the same in wIFN- γ -treated and untreated woodchuck hepatocytes (Fig. 7B). The amount of WHV 3.5-kb transcripts was even slightly increased in wIFN- γ -treated hepatocytes. Thus, incubation with wIFN- γ did not result in the degradation of WHV-specific transcripts in woodchuck hepatocytes. Further, a combination of 1,000 U of wIFN- γ and 100 U of wTNF- α per ml did not show any effect on WHV replication in woodchuck primary hepatocytes.

A concentration of wIFN- γ at 10 U/ml did not lead to any microscopic recognizable change of primary hepatocytes. However, primary hepatocytes derived from chronically WHV-infected woodchucks showed abnormal cell morphology with large vacuoles after incubation with higher concentrations 100 or 1,000 U of wIFN- γ per ml (Fig. 8A). An extensive cell loss occurred at day 11. Hepatocytes treated with medium controls were morphologically normal during culturing at least for 14 days (Fig. 8B). These results indicate that high concentrations of wIFN- γ were cytotoxic for woodchuck primary hepatocytes.

DISCUSSION

In the present study we expressed wIFN- γ in *E. coli* and mammalian cells and characterized its antiviral properties. We were able to demonstrate that wIFN- γ shares a wide spectrum of the biological activities of its mammalian homologues: (i) the ability to protect woodchuck cells from the cytopathic effect of EMCV infection; (ii) species specificity; (iii) the ability to up- or downregulate specific gene expressions; and (iv) its

FIG. 4. The production of wIFN- γ by activated wPBMCs. (A) WPBMCs stimulated by different concentrations of PHA: 0 ($\blacksquare$), 1 ($\blacklozenge$), 2 ($\bullet$), and 5 ($\blacktriangle$) μ g/ml. (B) WPBMCs stimulated with 2 μ g of PHA per ml ($\blacksquare$) and in the presence of heterologous cytokines 10 U of rmIL-2 per ml ($\blacklozenge$), 50 U of rhIL-12 per ml ($\blacktriangle$), or 50 U of rhIL-15 per ml ($\bullet$). (C) The production of high concentrations of wIFN- γ by WPBMCs at a density of 5×10^6 per ml, stimulated with 5 μ g of PHA alone per ml or in the presence of 50 U of rmIL-12 per ml.

production by activated woodchuck lymphocytes, which was regulated by IL-12 and IL-15.

wIFN- γ induces an enhanced transcription of the woodchuck MHC-I heavy-chain mRNAs in both naive and persistently WHV-infected woodchuck hepatocytes. However, wIFN- γ was not able to inhibit the WHV replication in primary WHV-infected woodchuck hepatocytes at a concentration of 1,000 U/ml. A combination with wTNF- α did not improve the antiviral effect on WHV replication in primary woodchuck hepatocytes. The high concentration of wIFN- γ led to an extensive loss of hepatocytes during culture. Thus, an increase of wIFN- γ concentrations would not be beneficial because of its cytotoxicity for woodchuck hepatocytes.

The failure of the inhibition of WHV replication in primary hepatocytes from chronically WHV-infected woodchucks by wIFN- γ may be explained in different ways. In vivo, inflammatory cytokines including wIFN- γ and wTNF- α has been detected in the intrahepatic compartment in chronically WHV-infected woodchucks (16, 29; unpublished data). Therefore, the hepatocytes of these woodchucks are continuously exposed to wIFN- γ for long periods of chronic WHV infection and may be adapted to the presence of inflammatory cytokines in a

specific way. In addition, Michalak et al. demonstrated that the expression of the woodchuck MHC-I is blocked at the post-transcriptional step (29). This finding suggests that woodchuck hepatocytes from chronic carriers are functionally changed in the response to wIFN- γ . Early reports indicate that cells expressing HBV proteins showed a defective response to IFN (31). Further studies are needed to determine whether such functional changes were due to the action of viral proteins or a result of the adaptation of hepatocytes to the continuously elevated intrahepatic wIFN- γ expression. In the HBV-transgenic mouse model, a transfer of HBsAg-specific CTLs into transgenic mice lead to the destabilization of HBV-specific mRNA and the reduction of the HBV replication. The effect of CTLs is mediated by IFN- γ and TNF- α , as demonstrated under different experimental conditions. Though HBV-transgenic mice show liver-specific HBV-gene expression and replication, no specific T-cell response to HBV protein is present before the immune transfer. Therefore, hepatocytes of HBV-transgenic mice are naive to IFN- γ and may be functionally different from those adapted to the long-term presence of IFN- γ .

Our results imply that an enhancement of the intrahepatic

FIG. 5. Influence of wIFN- γ on gene expression in woodchuck WH12/6 cells. WH12/6 cells were incubated with dilutions of the supernatant of pWHIG-transfected BKH cells (1, 10, and 100 U of wIFN- γ per ml) or wPBM culture supernatant (1,000 U of wIFN- γ per ml). For the control (–), the supernatant of BKH cells transfected with the control vector pcDNA3 was used at a dilution of 1:10. (A) Northern blotting for detection of woodchuck MHC-I and β -actin mRNA. Total RNAs were purified from WH12/6 cells after treatment with wIFN- γ for 48 h. Then, 1 μ g of total RNA from WH12/6 cells per lane was subjected to Northern blotting. (B) Inhibition of the enhanced MHC-I heavy-chain transcription in treated WH12/6 cells by neutralization of wIFN- γ . Samples containing wIFN- γ were incubated with a neutralizing antiserum to wIFN- γ (at 1:100) or a unrelated rabbit serum for 30 min at 37°C before incubation with WH12/6 cells. (C) Detection of woodchuck MHC-I expression by IF staining with a monoclonal antibody. (Upper panels) Pictures obtained by phase-contrast microscopy (P) from cells incubated with 0 or 100 U of wIFN- γ per ml for 48 h. (Lower panels) IF staining of corresponding cells. Magnification, $\times 40$. (D) Expression of the reporter gene luciferase in woodchuck WH12/6 cells treated with woodchuck IFN- γ . The luciferase activities were measured as luminescence by using a Lucite reporter gene assay kit. The basal luminescence levels of three reporter vectors in a given experiment were 4,480 U per ml for pTA-luc, 68,880 U per ml for pGAS-TA-luc, and 18,920 U per ml for pWHpreS-luc.

IFN- γ expression may not be effective for suppressing hepadnavirus replication. Pilot experiments with adenoviral vector-mediated transfer of the wIFN- γ gene into chronically carriers were not successful to suppress the WHV replication (our unpublished data; M. Nassal et al., unpublished data). Zhou et al. showed that a combination of lamivudine treatment and adenovirus superinfection led to a transient suppression of chronic WHV infection in woodchucks (41). The mRNA level for wIFN- γ and wTNF- α was elevated about twofold. However, the relative contribution of noncytolytic mechanisms via

antiviral cytokines and cytolytic mechanisms to the transient suppression of WHV replication could not be assessed. The WHV replication resumed after the clearance of adenovirus, indicating that non-virus-specific mechanisms are not sufficient for virus clearance.

The expression of MHC-I heavy chain is mainly regulated by IFN- γ . In chronic carriers the elevated mRNA level of the woodchuck MHC-I heavy chain appears to be a result of the exposure to intrahepatic expressed wIFN- γ . MxA, an IFN- α -

FIG. 6. Upregulation of MHC-I expression in woodchuck cell lines and primary hepatocytes by incubation with wIFN- γ . (A) mRNA levels of woodchuck MHC-I heavy chain in naive and WHV-infected hepatocytes at day 3. Hepatocytes were treated with 0, 100, and 1000 U of wIFN- γ per ml. Dilutions of the supernatant of peWHIG-transfected BKH cells (100 U of wIFN- γ per ml) or WPBMC culture supernatant (1,000 U of wIFN- γ per ml) were used for experiments. For control (-), the supernatant of BKH cells transfected with the control vector pcDNA3 was used at a dilution of 1:10. Then, 1 μ g of total RNA from WH12/6 cells per lane was subjected to Northern blotting. "fold*" refers to the relative level of mRNA, defined as digital units measured by a phosphorimager. (B) mRNA level of the woodchuck MHC-I heavy chain in WHV-infected woodchuck hepatocytes during the culturing without or with wIFN- γ . "fold*" refers to the relative level of mRNA, defined as digital units measured by a phosphorimager.

inducible protein, was not detectable in hepatocytes from chronic carriers, indicating that wIFN- α was not responsible in the elevated MHC-I heavy-chain mRNA level (unpublished results). Previous publications suggested that viral proteins, e.g., HBV x-protein, may influence the expression of MHC-I (40). However, the mRNA level in WHV-infected woodchuck hepatocytes decreased during culture unless wIFN- γ was added. These results indicate that the WHV proteins are not responsible for the upregulation of woodchuck MHC-I.

There are few apparent differences between the results from

FIG. 7. WHV replication and gene expression in persistently WHV-infected primary woodchuck hepatocytes treated with 0, 100, and 1,000 U of wIFN- γ per ml or in combination with 100 U of wTNF- α per ml at day 7. Dilutions of the supernatant of peWHIG-transfected BKH cells (100 U of wIFN- γ per ml) or WPBMC culture supernatant (1,000 U of wIFN- γ per ml) were used for experiments. For control (-), the supernatant of BKH cells transfected with the control vector pcDNA3 was used at a dilution of 1:10. (A) WHV replication intermediates in primary woodchuck hepatocytes detected by Southern blotting. (B) WHV-specific mRNAs detected by Northern blotting. An RNA sample from WHV-infected woodchuck liver tissues was used as a standard.

experiments performed with the permanent cell line WH12/6 and primary hepatocytes. While the luciferase expression under the WHV promoters was inhibited by wIFN- γ , WHV mRNAs in woodchuck primary hepatocytes were not reduced by treatment with wIFN- γ . The amount of WHV prec/c tran-

A. with IFN- γ 1000 units per ml **B. without IFN- γ**

FIG. 8. Woodchuck primary hepatocytes treated with 10^3 U of wIFN- γ per ml for 9 days (A) or with medium control (B). The woodchuck primary hepatocytes treated by wIFN- γ show abnormal cell morphology with large vacuoles. A dilution of the wPBM culture supernatant containing 1,000 U of wIFN- γ per ml was used to treat woodchuck hepatocytes. The pictures were obtained by light microscopy at a magnification of $\times 400$.

scripts was even increased in woodchuck hepatocytes treated with a high concentration of wIFN- γ . As discussed previously, this may be due to the changed response of woodchuck hepatocytes to wIFN- γ . Alternatively, the regulation of the WHV genes in context of the whole genome may be different from the regulation by an isolated promoter. Sequence motifs such as GAS and ISRE were identified by screening the WHV genome sequence (8, 11). Further studies will reveal whether such sequence motifs are functional and therefore lead to the differences in the gene regulation by isolated WHV promoters or in context of the whole WHV genome.

The molecular characterization of wIFN- γ provides a basis for further experiments on the T-cell functions in the woodchuck model. The role of specific CTLs to hepadnaviral proteins for the virus clearance during acute self-limiting hepadnavirus infection remains to be elucidated. Particularly, it has to be clarified whether noncytolytic mechanisms mediated by antiviral cytokines operate during acute-resolving hepadnavirus infections (16, 18). Schultz et al. demonstrated that duck IFN- γ is able to inhibit DHBV replication in duck primary hepatocytes though the formation of covalently closed circular DNA is unaffected by IFN- γ (35). Therefore, IFN- γ may at least reduce the extent of viral replication and contribute to the limitation of viral infection. It has also been shown that IFN- γ can inhibit hepadnavirus replication in transfected hepatoma cells (17, 19). Our preliminary results showed that wIFN- γ is able to suppress WHV infection on naive primary woodchuck hepatocytes. The molecular mechanisms of antihepadnaviral actions of IFN- γ will be analyzed further. Additional antiviral functions of IFN- γ in vivo need to be elucidated. IFN- γ may be included for the improvement of vaccines to hepadnaviruses. It was shown in the mouse model that a coadministration of plasmids expressing of IFN- γ with HBsAg DNA vaccines significantly enhanced the specific immune response to HBsAg (9). In the woodchuck, genetic immunizations with WHcAg- and WHsAg-expressing plasmids alone only induced very low titers of specific antibodies and weak T-cell responses (22).

The coadministration of wIFN- γ did enhance the WHcAg-specific lymphoproliferative response and protected immunized woodchucks against subsequent WHV challenge (34).

We found in the present work that heterologous cytokines rhIL-12 and rhIL-15 are able to support the functions of wPBMCs, as demonstrated for wIFN- γ production. These cytokines also promote the proliferation of wPBMCs in vitro (Lu et al., unpublished results). These findings allow us to assess directly the values of these cytokines for treatment of chronic hepatitis B in the woodchuck model.

ACKNOWLEDGMENTS

We thank Hans Will for helpful discussions and critical reading of the manuscript, Thomas Michalak for providing the antibody to woodchuck MHC-I and for helpful suggestions, and Dirk Bauer, Ulrike Protzer, and Ulla Schultz for helpful advice.

This work is supported by grants of Deutsche Forschungsgemeinschaft to M.R. and M.L. (Ro 687/6-1).

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