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Production of Recombinant Woodchuck IFNα and Development of Monoclonal Antibodies

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Interferon alpha (IFNα) is the first line treatment for chronic hepatitis B and C. In order to test new IFNα delivery systems and investigate the function of this cytokine in the woodchuck model, the best animal model of chronic hepatitis B, we produced and purified recombinant woodchuck IFNα and used it to produce monoclonal antibodies. wIFNα5 was cloned in a prokaryotic expression system, expressed as His-tagged protein and then purified. The rwIFNα5 protein was found to induce STAT-3 phosphorylation, to enhance 2′,5′-oligoadenylate synthetase mRNA levels and to possess a potent antiviral activity. Two monoclonal antibodies were obtained through immunization of rats with rwIFNα5. Both recognized rwIFNα5 in western blot analysis and one was able to neutralize the antiviral activity of the rwIFNα5 and lymphoblastoid IFNα preparations. Finally, a capture rwIFNα5 ELISA was developed using both antibodies. In summary, the tools generated in this study will allow different forms of IFNα delivery as well as different combination therapies in woodchuck hepatitis virus infection to be tested, thus providing useful information for the design of new strategies to treat chronic hepatitis B in humans.

Introduction

The alpha interferons (IFNα) are members of the type I interferons, a group that includes the IFNα, IFNβ, IFNω, and IFNτ (Roberts and others 1998). Type I interferons are produced by a great diversity of cells in response to viral infections. Although alpha interferons were first identified for their antiviral properties (Isaacs and Lindenman 1957), they have been shown to display a wide variety of biological effects including regulation of the immune system (Biron 1998). IFNα signaling is known to be mediated by the Janus kinase signal transducer and activation of transcription (Jak-Stat) pathway, which is initiated by binding to the cell surface receptors, IFNAR1 and IFNAR2. Ligation of IFNα and its receptors leads to activation of JAK-1 and TYK-2 through tyrosine phosphorylation, which in turn stimulates the phosphorylation of Stats and the subsequent induction of hundreds of genes with antiviral properties (Caraglia and others 2005).

Because of its antiviral, immunoregulatory, and antiproliferative activities (Isaacs and Lindenman 1957; Biron 1998; Fleischmann and others 1998), the recombinant IFNα has been approved for use in a number of indications including chronic hepatitis B, chronic hepatitis C, malignant melanoma, hairy cell leukemia, and AIDS-related Kaposi's sarcoma. IFNα constitutes, together with lamivudine, adefovir, and recently entecavir, the current treatment for chronic hepatitis B virus (HBV) infection, a worldwide illness that affects over 350 millions people and that frequently leads to chronic hepatitis, cirrhosis and liver cancer (Kao and Chen 2002). However, the response rate to recombinant IFNα is only around 40% and the therapy is not devoid of unwanted side effects such as depression, nausea, fever, fatigue, headaches, and muscle aches. To overcome these problems, new ways of IFNα delivery are under development: utilization of biopolymers (Yamagata and others 2000), stabilizing ligands (Zeuzem and others 2000), and the use of gene therapy (Protzer and others 1999; Aurisicchio and others 2005; Berraondo and others 2005). Testing the efficacy of these new treatments requires systematic *in vivo* studies in preclinical animal models. The best animal model to study the pathogenesis, prevention and treatment of HBV infection is the woodchuck hepatitis virus (WHV) infection in woodchuck (*Marmota monax*) (Roggendorf and Tolle 1995; Menne and Tennant 1999). WHV strongly resembles human

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HBV. The replicative cycle, the genomic organization and serologic viral particles are very similar (Feitelson and others 1981; Galibert and others 1982). As is observed with human HBV infections, woodchucks exposed perinatally to WHV develop chronic hepatitis and this condition, similarly to what occurs in HBV infection, may evolve to hepatocellular carcinoma. As has been demonstrated by several groups, WHV infection represents an ideal experimental model to analyze the efficacy of antiviral strategies in the context of chronic illness (Menne and Cote 2007).

In a previous work we cloned and characterized the interferon alpha family of woodchucks (Berraondo and others 2002). The analysis of the IFNα subtypes expressed in the liver showed IFNα5 as the most abundantly expressed subtype. In this study, wIFNα5 was cloned in a prokaryotic expression vector, produced and then purified. The recombinant IFNα was shown to induce STAT-3 phosphorylation, 2′,5′-oligoadenylate synthetase (2′,5′-OAS) expression, and to retain its antiviral activity. Furthermore, the recombinant protein was used to immunize rats to obtain monoclonal antibodies against the protein. Two antibodies were selected for further characterization. Both work in ELISA and immunoblotting, and one, 10A12, showed a strong neutralizing activity against IFNα.

Materials and Methods

Animals and cells

Woodchucks (purchased from Northeastern Wildlife, Ithaca, NY, USA), handled according to the guidelines of the institution (Centro de Investigación Farmacobiológica Aplicada, Pamplona, Spain), were used. Woodchuck peripheral blood mononuclear cells were obtained from blood collected from the saphena vein of the hind legs under anesthesia. Woodchuck hepatoma cells WCH-17 were obtained from the ATCC (ATCC No. CLR-2082) and cultured and maintained in DMEM medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (all from Gibco, Invitrogen, CA, USA) under standard conditions. NS-1 myeloma cells were obtained from the ATCC (ATCC No. TIB-18) and cultured in complete RPMI medium (RPMI 1640 with Glutamax containing 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 5×10^{-5} mol/L 2-mercaptoethanol (all from Gibco, Invitrogen, CA, USA).

Cloning of wIFNα5 for prokaryotic expression

The coding region (without the signal peptide sequence) for wIFNα5 (GenBank Accession No. AF338274) sequence was amplified from woodchuck genomic DNA by PCR. The genomic DNA was obtained from a liver biopsy using QIAamp DNA mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The PCR primers for the amplification were: forward, 5′-TGT GAC CTG CCT CAG ATA CAC-3′, and reverse, 5′-TCA TTC CAT GCT CCT TAG TCT TC-3′. The conditions used were: 95°C for 4 min, 30 cycles of (94°C 15 s, 55°C 15 s, and 72°C 40 s), and a final extension of 72°C 7 min. The PCR products were cloned into pTcrHis-TOPO (Invitrogen, Paisley, UK) to generate the pTcrHis-wIFNα5 and transformed into TOP10F′ *Escherichia coli* cells (Invitrogen, Paisley, UK). Several clones were sequenced to select a wIFNα5-harbouring clone and to determine that the cloned/generated sequence was correct.

Expression and purification

The IFNα proteins that were expressed contained a 6-histidine tag at the N-terminus for purification purposes. TOP10F′ *E. coli* bacteria harboring the expression plasmid pTcrHis-wIFNα5 were grown overnight in 5 mL LB containing 100 μg/mL ampicillin and 1% glucose in a 37°C shaker incubator. The following day, 2 mL of the overnight culture was added to 100 mL LB containing 100 μg/mL ampicillin and grown in a shaker incubator at 37°C for ~2 h until the OD 600 nm reached 0.6–0.8 at which time the culture was induced by 1 mM IPTG (at this time 1 mL of culture was taken for SDS-PAGE analysis-"Uninduced"). Four hours after induction, the cells were harvested by centrifugation at 3000*g* for 10 min at 4ºC. The pellet was resuspended in urea 8M and the protein was purified using the ProBond Purification system (Invitrogen, Paisley, UK) following manufacturer's instructions. The purified protein was filter sterilized and stored at −80°C until further use. The protein concentration of the purified protein was measured by Bradford assay. The purity and size of the recombinant IFNα were examined by SDS-PAGE. In brief, samples were boiled for 3 min in presence of protein loading buffer containing 1% β-mercaptoethanol, were loaded in sodium dodecyl sulfate polyacrylamide 10% gel and then run at 100 V for 1 h. Finally, bands were visualized using Coomassie blue.

Western blot analysis

For STAT phosphorylation analysis, total cell protein (60 μg) was loaded onto SDS-PAGE 7.5% gels. For rIFNα analysis, 15 μg of recombinant protein was loaded onto SDS-PAGE 10% gels. After electrophoresis, the samples were transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were incubated in TBS-T (50 mM Tris-HCl (pH 7.6), 200 mM NaCl, and 0.1% Tween-20) with 5% dry milk. Proteins were detected by incubation with specific antibody in TBS-T. After extensive washing, horseradish peroxidase conjugated antibody was added for 1 h. Protein bands were visualized using the enhanced chemiluminescence detection system (Perkin Elmer, Boston, MA, USA). The first antibodies included a rabbit antiphosphorylated STAT3 (P-Stat3) (Cell Signaling Technology, Danvers, MA, USA), and a rabbit anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as well as the anti-wIFNα monoclonal antibodies 5E8 and 10A12 developed in this study.

2′,5′-OAS stimulation assay

To analyze 2′,5′-OAS expression, 5×10^5 WCH-17 cells were plated in six-well plates and 24 h later, were incubated with 10-fold serial dilutions of the recombinant protein ranging from 10^5 to 0 pg/mL. After 24 h, cells were tripsinized, harvested and RNA was isolated with the Ultraspec-II system following the manufacturer's instructions and finally diluted in 30 μL of RNase free water. Quantitative RT-PCR was performed as described (Berraondo and others 2002). Primers and TaqMan probes (PE Applied Biosystems) for 2′,5′-OAS and β-actin were designed according to the published cDNA sequences of woodchuck genes using the

Primer-Express software. The number of copies of 2′,5′-OAS and β-actin was determined by interpolation, using external RNA standards. For preparation of the standard curves, 2′,5′-OAS and β-actin fragments were cloned into PCR2.1 TOPO, containing a T7 promoter and *in vitro* transcribed with T7 RNA polymerase. RNA concentration was estimated by optical density and copy number was calculated from the concentration, mean molecular weight of nucleotides (330 g/mol) and RNA length.

Antiviral assay

WCH-17 cells were plated in a 96-well plate at 1×10^4 cells/well in DMEM + 10% FBS. After 24 h, IFNα was added to the cells and incubated for 24 h, after which the IFNα was removed and the cells were challenged with Encephalomyocarditis virus (EMCV) (1 pfu/cell) resuspended in DMEM + 2% FBS for 1 h. The virus was then removed and the cells were incubated for 16–24 h in DMEM + 10% FBS. The viable cells were then stained with crystal violet and absorbance at 590 nm was measured with a plate reader. The EC50 and Log [EC50] values were calculated from the dose–response curves by using GraphPad Prism software (GraphPad Software, CA, USA) along with the 95% CI.

Immunization of rats and generation of monoclonal antibodies

The bacterially expressed purified proteins were used to immunize rats. The animals were immunized three times at a 3 week interval using 100 μg of protein per immunization and rat. For the first immunization, the antigen was mixed with the same volume of complete Freund's adjuvant. For the booster immunizations, protein was mixed with the same volume of incomplete Freund's adjuvant. Sera were collected 2 weeks after the third immunization and tested for antibodies against wIFNα5 by Western blot analysis. To discard antibodies that recognize the His-tag or bacterial contaminants, a His-tagged recombinant WHV core protein was used as negative control. To generate hybridoma cell lines producing monoclonal antibodies, we performed a fusion protocol as described previously (Martinez-Anso and others 1994). In brief, a selected rat was i.v. boost immunized and 4 days later, 10^8 spleen cells were extracted and fused with 5×10^7 NS-1 myeloma cells by means of 50% PEG 1500 solution in RPMI as described earlier (Martinez-Anso and others 1994). Fused cells were cultured in selective HAT media containing RPMI as basal media supplemented with 20% FCS, sodium pyruvate, penicillin–streptomycin, HAT, and L-Glutamine (all from Gibco, Invitrogen, CA, USA). Supernatants were tested for the presence of specific antibodies through indirect ELISA assay and western blot using purified recombinant wIFNα. Hybrid cells from positive wells were cloned through limiting dilution in HAT media and used for the expression, characterization and purification of specific antibodies.

Monoclonal antibodies purification and biotinylation

Monoclonal antibodies were purified from the supernatant obtained after culture of the selected hybridomas by affinity chromatography on Protein-A sepharose columns (Pharmacia, Uppsala, Sweden) and stored at −80°C. The mAb 10A12 was biotinylated using Biotin Labelling kit (Roche, Basel, Switzerland) following manufacturer's instructions.

ELISA

Indirect ELISA. Indirect ELISA was performed in 96-well-ELISA plates (Nunc Maxisorb, Roskilde, Denmark) coated overnight at 4°C with purified 0.1 μg of rwIFNα per well diluted in 50 mM carbonate buffer (pH 9.6). Blocking was performed with PBS containing 2% BSA and 0.05% Tween for 1 h at 37°C. Hybridoma supernatant was used undiluted and incubated for 1 h at 37°C. After washing the plates three times with PBS/0.05 % Tween 20, bound antibodies were detected by a polyclonal serum-HRP conjugate against mouse IgG. After extensive washing, 100 μL of TMB chromogen (Abcam, Cambridge, UK) was added and incubated 30 min at RT. The reaction was stopped after 30 min with 50 μL of 2N H_2SO_4 and the OD was read 10 min later at 450 nm.

Capture ELISA. 96-well-ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 100 μL of mAb 5E8 at 7.5 μg/mL diluted in 0.2 M phosphate buffer, pH 6.5 and incubated overnight at 4°C. Plates were washed with wash buffer (PBS/0.05% Tween) five times. Wells were blocked with 300 μL StartingBlock blocking buffer (Pierce, Rockford, IL, USA). After washing, serial dilutions of the recombinant protein were incubated for 2 h at RT. Next, plates were washed and 100 μL of the biotinylated antibody 10A12 was added at a concentration of 4 μg/mL. After incubation 1 h at RT, plates were washed and 100 μL of 1:500 dilution of avidin-HRP was added and incubated 30 min at RT. Protein and antibody dilutions were performed in StartingBlock blocking buffer plus 0.005 % Tween 20. After extensive washing, 100 μL of TMB chromogen (Abcam, Cambridge, UK) was added and incubated 30 min at RT. The reaction was stopped after 30 min with 50 μL of 2N H_2SO_4 and the OD was read 10 min later at 450 nm.

Results

Protein expression and purification

To obtain a biologically active protein, the open reading frame of wIFNα5 without the leader sequence was obtained by PCR using liver genomic DNA as a template. A product of around 500 bp (Fig. 1A) was obtained and this product was then cloned into the pTCR-His plasmid where the IFNα protein was expressed as an N-terminal 6× His tagged fusion protein. After IPTG induction, samples were harvested every hour for 4 h and analyzed on SDS-PAGE. As shown in Figure 1B, IFNα appeared as a band of 22.5 kDa. At the end of the induction period (4 h), the cells were collected by centrifugation, sonicated to disrupt the cellular membranes, and spin down by low-speed centrifugation to collect the insoluble material. The insoluble material was solubilized by the addition of 8M Urea solution and the solubilized protein was subjected to a high-speed centrifugation step to remove any insoluble protein. The supernatant containing partially purified denatured interferon protein was loaded into Ni-NTa affinity chromatography column and the protein was eluted using a pH gradient. All the fractions were analyzed on an SDS-PAGE and those in which the protein appeared were pulled and urea was eliminated by dialyzing

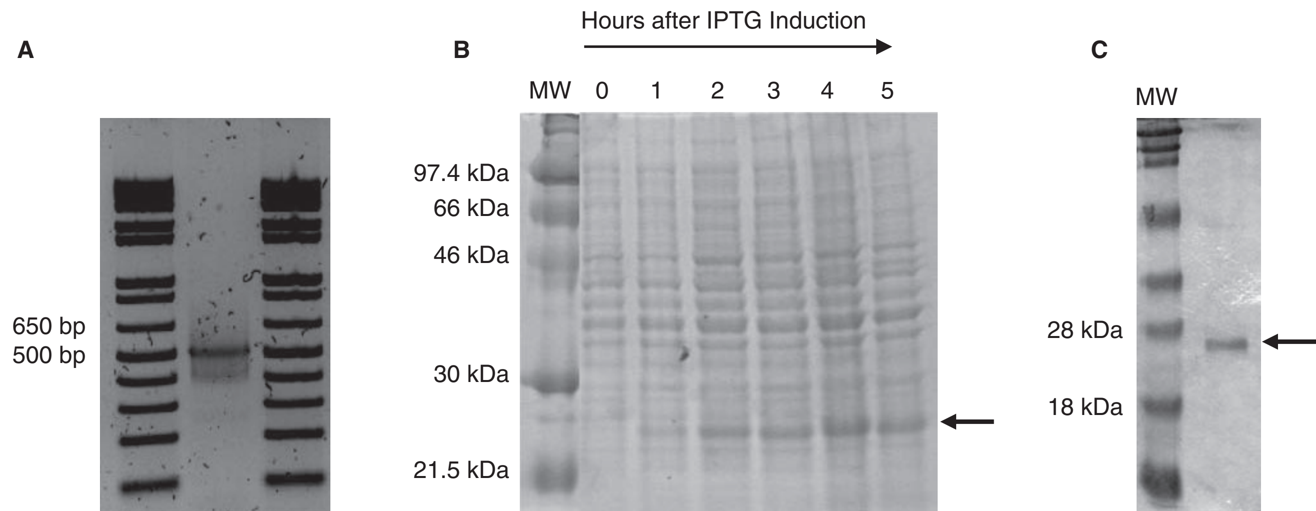

FIG. 1. Cloning, expression, and purification of the His-tagged recombinant wIFNα5. (**A**) Amplification of 502-bp IFNα PCR product from purified genomic DNA. Lane 1 and 3: molecular weight standards. Lane 2: PCR product. (**B**) At 0, 1, 2, 3, and 4 h after induction, samples were taken and run in SDS-PAGE to obtain a more thorough view of the procedure. The induced protein is indicated with an arrow. (**C**) Coomassie stained SDS-PAGE of the purified recombinant IFNα. Lane 1 (MW): molecular weight standards in kDa. Lane 2: 100 ng of purified rwIFNα protein.

it against PBS. The purified wIFNα protein appeared as a single 22.5 kDa protein on SDS-PAGE (Fig. 1C).

Biological activity of recombinant IFNα5

First, we determined if the recombinant wIFNα was able to induce STAT-3 phosphorylation in a woodchuck cell line. For this purpose, 5×10^5 WCH-17 cells were plated in six-well plates and 24 h later the medium was removed and fresh medium containing recombinant IFNα at different doses ranging from 10 to 10^5 pg/mL was added. After 30 min, cells were trypsinized and harvested to analyze STAT-3 phosphorylation status by Western blot. As shown in Figure 2A, recombinant wIFNα induced STAT-3 phosphorylation in a dose dependent manner. To analyze ISGs expression, cells were treated as described for the analysis of STAT-3 activation but in this case, they were incubated with wIFNα for 24 h. After the incubation, cells were harvested and RNA was isolated. 2′,5′-OAS and β-actin expression was determined by RT-quantitative PCR. The levels of 2′,5′-OAS mRNA increased in parallel to IFNα concentration.

Furthermore, we tested if rwIFNα was able to inhibit the cytopathic effect (CPE) induced after ECMV infection. The experiment was performed as described in material and methods. We found that the recombinant cytokine clearly inhibited ECMV mediated CPE. The linearity of the assay was maintained from 2 to 200 pg/mL (Fig. 2C). This result indicates that wIFNα-His is biologically active.

Production and characterization of monoclonal antibodies against wIFNα

Three rats were immunized subcutaneously using 100 μg of the recombinant IFNα protein embedded in complete Freund's adjuvant. Every animal received two more injections of the protein embedded in incomplete Freund's adjuvant. After the third immunization, generation of antibodies against wIFNα was analyzed by indirect ELISA (data not shown). Once it had been determined that the rats had developed antibodies against the recombinant protein, we proceeded to the generation of monoclonal antibodies. The immunized rats were sacrificed, the spleen extracted and splenocytes were fused to NS-1 myeloma cells. Hybridoma cells were screened for the production of antibodies to IFNα by an indirect ELISA using rwIFNα as target antigen and as negative control, a recombinant WHV core protein fused to a His-tag. Two hybridomas were cloned by limiting dilution and the resulting mAbs were termed 5E8 and 10A12. The monoclonal antibodies were tested in western blot against the recombinant protein. As shown in Figure 3A, both antibodies recognized the recombinant protein. To test if the antibodies could neutralize the activity of this cytokine, we performed a CPE assay with decreasing concentrations of both antibodies and a constant amount of 250 pg of rwIFNα. As shown in Figure 3B, 10A12 antibody inhibited woodchuck IFNα antiviral activity. Next we wanted to test if this antibody was able to inhibit naturally produced IFNα protein. For the production of natural IFNα woodchuck PBLs were stimulated with polyinosinic acid-polycytidylic acid (polyIC) complexed with DEAE-dextran and supernatants were harvested. The antiviral activity of the supernatant was analyzed in a CPE assay in the absence (mock) and in the presence of a 1:1000 dilution of the 10A12 and 5E8 antibodies. As shown in Figure 3C, the antibody 10A12 clearly inhibited the antiviral activity of the naturally produce woodchuck IFNα. Overall, these results indicate that the 10A12 antibody recognizes an epitope expressed by different wIFNα subtypes while the 5E8 antibody could recognize an epitope exclusively exposed in the recombinant wIFNα.

wIFNα capture ELISA

Using the mAbs described above, we developed a capture ELISA for rwIFNα. First, we tested a range of dilutions

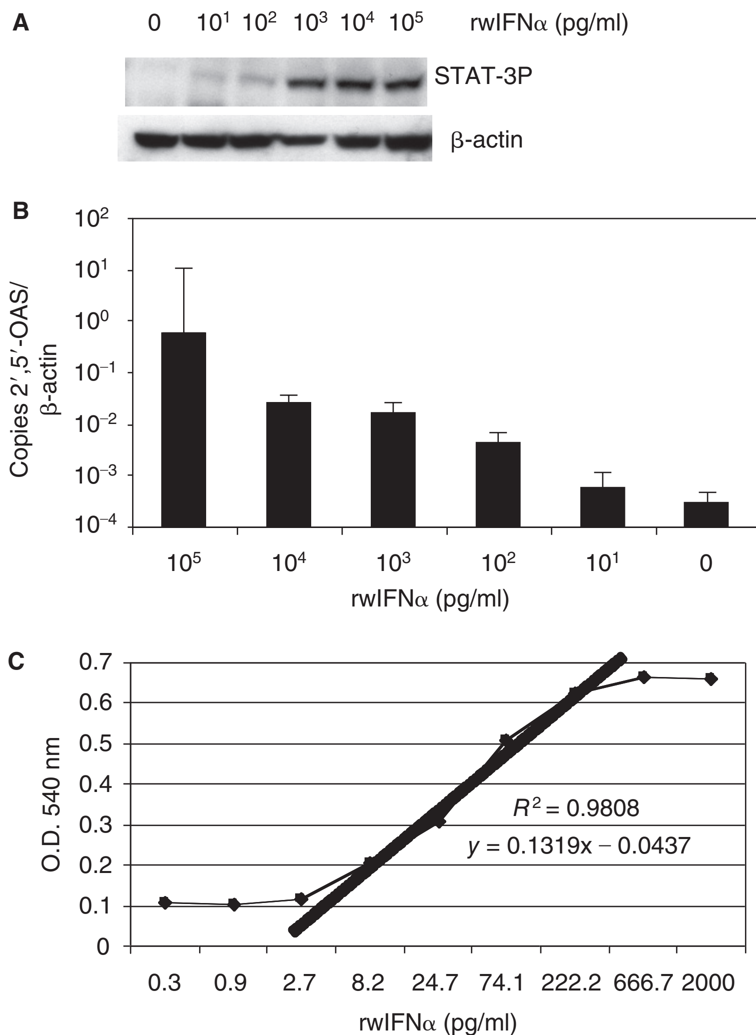

FIG. 2. Biological activity of the rwIFNα in WHC-17 cell line. (**A**) Induction of STAT-3 phosphorylation was analyzed by Western blot 30 min after incubation of the cells with different concentrations of the recombinant protein. (**B**) Expression of 2′,5′-OAS in cells treated with different concentrations of the rwIFNα was analyzed by quantitative RT-PCR. (**C**) Dose dependent inhibition of the cytopathic effect induced by encephalomyocarditis virus (EMCV) on WHC-17 cells after incubation with rwIFNα.

of mAbs to determine the optimal conditions of capture ELISA (data not shown). The optimal coating concentration for mAb 5E8 was 7.5 μg/mL whereas the optimal concentration for the detection antibody the biotinylated-mAb 10A12 was 4 μg/mL. As shown in Figure 4, this protocol resulted in a reliable detection limit of 50 pg/mL and the linearity of the assay was maintained from 50 to 6000 pg/mL.

Discussion

IFNα constitutes the current treatment for chronic HBV infection. However, the low response rate and the side effects associated with systemic administration of recombinant IFNα highlight the need to develop new therapeutic strategies. Among these, new ways of IFNα delivery are under development (Protzer and others 1999; Yamagata and others 2000; Aurisicchio and others 2005; Berraondo and others 2005). Testing the efficacy of these new treatments requires systematic *in vivo* studies in preclinical animal models.

For this purpose in the present study, recombinant woodchuck IFNα5 fused to a histidine tag was expressed by *E. coli*, purified and used as immunogen for production of mAbs against the cytokine. IFNα, upon binding to its receptor, induces STAT phosphorylation and then a number of interferon inducible gene are expressed, including 2′,5′-OAS. Here, we show that the recombinant wIFNα induces STAT-3 phosphorylation as well as 2′,5′-OAS gene expression in a dose dependent manner. Furthermore, rwIFNα showed a strong antiviral activity by inhibiting ECMV mediated infection. Thus, this recombinant protein will allow new IFNα delivery systems as well as their antiviral potential to be tested.

Using the rwIFNα protein as immunogen, we successfully established several monoclonal antibodies, two of which were selected for further characterization. The monoclonal antibodies 10A12 and 5E8 recognized the recombinant protein by western blot and were used to set up an ELISA assay which will allow a fast and easy way to quantify rwIFNα in woodchucks injected with the recombinant protein or transfected with gene therapy vectors expressing rwIFNα5. wIFNα secretion can also be analyzed using an antiviral protection assay which is, in our hands, more time consuming and more variable than an ELISA assay. Moreover, discrimination between the antiviral activity of IFNα and other cytokines such as TNFα or IFNγ is not possible using this assay.

Further characterization of the antibodies revealed that 10A12 has a strong anti-IFNα neutralization activity.

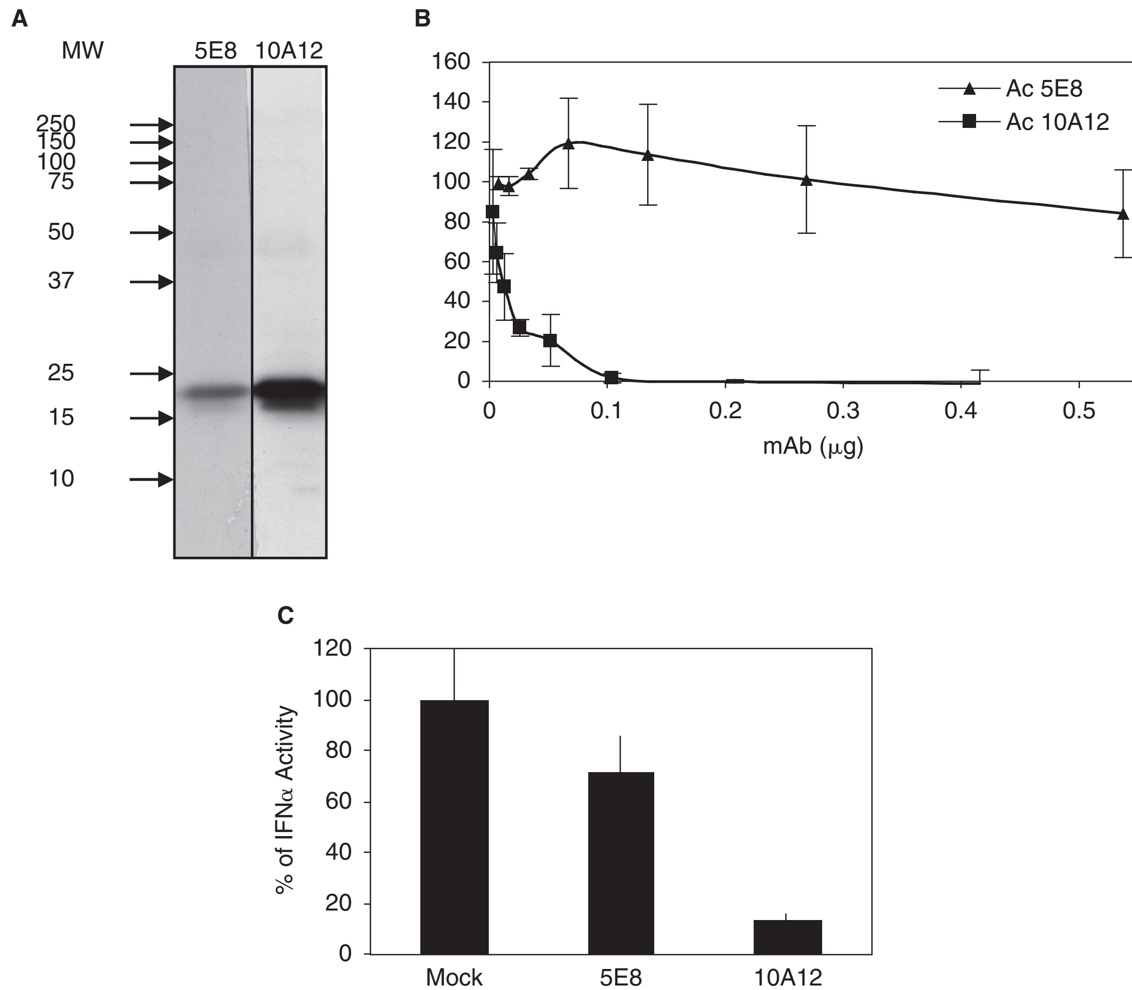

FIG. 3. Characterization of wIFNα monoclonal antibodies. (**A**) Western blot showing the specificity of anti-rwIFNα mAbs 5E8 and 10A12. Secondary antibody was antimouse Ig-HRP and detection was by ECL. (**B**) Analysis of the neutralization activity of both antibodies in a CPE assay using the recombinant protein at a dose of 250 pg. (**C**) Analysis of the neutralization activity of both antibodies in a CPE assay using the IFNα produced by woodchuck PBLs after incubation with polyIC/DEAE-dextran. Values represent the mean of triplicate wells. Errors bars represent SE of the mean.

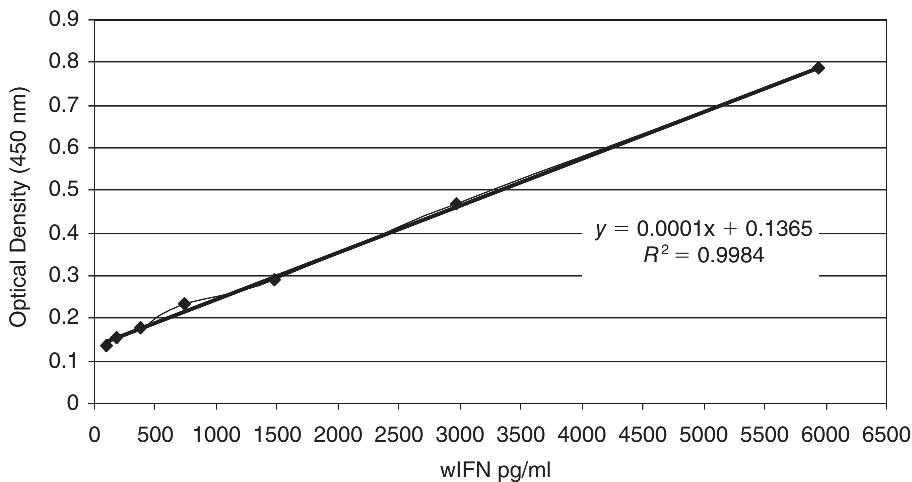

FIG. 4. Detection of rwIFNα in a capture ELISA. The mAb 5E8 was used as capture antibody at a concentration of 7.5 μg/mL and biotinylated 10A12 antibody as detection antibody at a concentration of 4 μg/mL. Bound antibodies were visualized using streptavidin-HRP.

Incubation of the antibody with the recombinant protein and more importantly with supernatants obtained from poli-IC activated PBLs inhibited the antiviral activity of rIFNα and the endogenous protein.

In conclusion, we successfully produced recombinant woodchuck IFNα and monoclonal antibodies against rwIFNα. This protein is biologically active and will be used to test the antiviral activity of new IFNα delivery systems in the best animal model for chronic hepatitis B, the woodchuck chronically infected with the WHV.

Acknowledgments

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