APOBEC3-Independent Interferon-Induced Viral Clearance in Hepatitis B Virus Transgenic Mice[▽]

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Interferon (IFN) has been part of the standard treatment of chronic hepatitis B infection for more than 2 decades, yet the mechanism of action of this antiviral remains poorly understood. It was recently observed that members of the human APOBEC family of cytidine deaminases endowed with anti-hepatitis B virus (HBV) activity are upregulated by type I and II IFNs. However, we demonstrated that, in tissue culture, these cellular enzymes are not essential effectors of the anti-HBV action of these cytokines. Here, we show that murine APOBEC3 (muA3) can also block HBV replication. While expressed at low levels in the mouse liver at baseline, muA3 is upregulated upon IFN induction. However, in HBV-transgenic muA3 knockout mice, IFN induction blocked HBV DNA production as efficiently as in control HBV-transgenic muA3-competent animals. We conclude that APOBEC3 is not an essential mediator of the IFN-mediated inhibition of HBV in vivo.

Acute hepatitis B virus (HBV) infection is rapidly cleared in most immunocompetent adults through a combination of cytotoxic and cytokine-mediated noncytolytic responses (6, 38). In contrast, perinatal and early-childhood contaminations usually lead to chronic infections, which constitute the ground for the development of cirrhosis, liver insufficiency, and hepatocellular carcinoma. In acutely infected chimpanzees, HBV-specific CD8⁺ T cells producing high levels of gamma interferon (IFN- γ) are essential for viral clearance (38, 43), and IFN- α has been the treatment of choice for chronic HBV infection, in spite of its limited efficacy in many patients (15). While the exact mechanisms by which IFN blocks HBV remain unclear, HBV-infected hepatocytes exposed to this cytokine exhibit reduced levels of pregenomic RNA-containing viral cores (27, 44, 45). A remarkably similar effect has been noted upon overexpression of several members of the human APOBEC3 (hA3) family of cytidine deaminases (3, 21, 29, 39, 40), a group of antiviral proteins also active against exogenous retroviruses as well as endogenous retroelements (2, 8, 13, 14, 17, 18, 21, 22, 24, 32). Because several of these enzymes are induced by IFN in various cell types including hepatocytes, it has been suggested that they might be responsible for the antiviral effect of the cytokine (3, 21, 30, 37). However, we recently demonstrated that, at least in tissue culture, IFN efficiently blocks HBV replication even when human APOBEC3F (hA3F), hA3G, and hA3B are inhibited (21). The present study was undertaken to address this point in vivo.

Animal models of HBV infection are limited to chimpanzee

and tupaia, but transgenic mice bearing an integrated terminally redundant HBV genomic construct produce high levels of HBV antigens and DNA in the liver, kidneys, and serum (17). The high levels of HBV production, albeit comparable to those measured in chronically infected humans, are not hepatotoxic in these animals. The HBV transgenic (HBV-Tg) mouse thus represents a suitable system to assess the effect of antiviral agents, at least those acting on viral production. IFN efficiently blocks HBV replication in HBV-Tg mice (5, 12, 16, 27, 35, 45). In this species, there is only one A3 orthologue (9, 19) that can inhibit HIV (1, 11, 22), human T-cell leukemia virus (25, 31) and mouse mammary tumor virus (26). We thus asked whether it acts as the downstream effector of IFN against HBV.

MATERIALS AND METHODS

Cells and DNA. Ear primary fibroblasts were isolated and maintained as previously described (36), while *Mus dunni* tail fibroblasts (MDTF) and human embryonic kidney 293T cells were purchased from the American Type Culture Collection. To induce the IFN pathway, cells in duplicate were treated with 1 μ g/ml murine recombinant IFN- γ (Peprotech) or with 50 μ g/ml double-stranded RNA poly(I · C) for 20 h before RNA was extracted. The pCMV γ HBV plasmid, kindly provided by S. Wieland and F. V. Chisari (Scripps Research Institute), expresses the pregenomic RNA under control of a cytomegalovirus (CMV) promoter. The Δ Vif-defective human immunodeficiency virus type 1 (HIV-1 Δ Vif) proviral clone was previously described (42). The plasmid pCMV4-hA3G-HA expressing a hemagglutinin (HA)-tagged form of hA3G was a kind gift from M. Malim (Imperial College, London, United Kingdom) (33). The pCMV4-muA3-HA was constructed in the same backbone by swapping the hA3G cassette with the murine APOBEC3 (muA3) gene excised from the pCDNA3-muA3-HA plasmid (provided by N. Landau, Salk Institute) (22). The muA3 cDNA contained in this plasmid was originally derived from C57BL/6 mice and corresponds to the most commonly found exon V-deleted form of the transcript.

Virus production, infection, and titration. HBV and HIV-1 Δ Vif viruses were produced by transient transfection in 293T cells using Fugene 6 (Roche). Inhibition of HIV-1 Δ Vif infectivity and of HBV replication by APOBEC proteins was determined by a single-round infectivity assay on HeLa-CD4-LTR Δ LacZ indicator cells and quantitative analysis of cytoplasmic core-associated HBV DNA, respectively, as described previously (7, 21).

Generation and genotyping of HBV-Tg A3^{-/-} double transgenic mice. HBV-Tg mice (kindly provided by U. Protzer) replicate HBV from an integrated terminally redundant HBV genome (HBV 1.3) (17). A3^{-/-} mice were the gen-

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FIG. 1. muA3 is a potent inhibitor of HBV. 293T cells were transfected with HBV or HIV-1 Δ Vif plasmid together with empty vector or with vectors encoding hA3G-HA or muA3-HA. Antiviral activity against HIV-1 Δ Vif was determined by measuring the infectivity of virions produced in the supernatant of transfected cells in a single-round assay by titration on HeLa-CD4-LTR-LacZ indicator cells. Values were normalized for input virus (as determined by RT activity). The effect on HBV was scored by quantitative real-time PCR of core-associated HBV DNA produced from transfected cells. Results obtained in the presence of the empty vector were given the arbitrary value of 100% in both cases. Data are representative of duplicate experiments. Below the graph is a Western blot analysis of extracts from virus-producing 293T cells using antibodies against the HA tag of A3 protein derivatives or PCNA.

erous gift of C. Rada (23). To generate double-transgenic animals, HBV-Tg mice were crossed with muA3-deficient mice, and offspring were further crossed to obtain HBV-Tg-muA3^{-/-} and HBV-Tg-muA3^{+/-} animals. Genomic DNA was extracted from tail samples using a ChargeSwitch genomic DNA mini tissue kit (Invitrogen), adapted for the automated Tecan Evoware station. Aliquots of DNA (50 to 100 ng) were tested by PCR. The wild-type muA3 allele was detected with primers O.mAPO3.5 (5'-GACAACATCCACGCTGAAATCTGC-3') and O.mAPO3.6 (5'-GCGGGAGCTGAAGATGTCCAGGCTC-3'), the knockout muA3 allele was detected with primers O.mAPO3.5 and O.neo.1 (5'-GCGTTG GCTACCCGTGATATTG-3'), and the HBV transgene was detected with primers O.HBV.9 (5'-ACCTGTCTTTAATCTCATTG-3') and O.HBV.2 (5'-AGG CCGATTGTGCTGGCAAAG-3').

Protein analyses. Cells were lysed with radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in phosphate-buffered saline) supplemented with anti-protease cocktail (Calbiochem). Lysates were centrifuged for 30 min at maximum speed in a refrigerated tabletop microcentrifuge; proteins were quantified by bicinchoninic acid assay (Pierce) before separation by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with mouse monoclonal antibodies against HA (anti-HA antibody 3F10 conjugated to peroxidase; Roche Applied Science) and PCNA (Oncogene Research Products).

In vivo analyses. Blood (100 μ l) was harvested by retro-orbital phlebotomy, mixed with 25 μ l of heparin, and centrifuged twice for 10 min at 2,300 rpm in a refrigerated tabletop centrifuge. Aliquots of the upper phase, corresponding to

FIG. 2. Equal levels of HBV production in HBV-Tg muA3-defective and muA3-competent mice. HBV particles were purified from the serum, and core-associated HBV DNA was quantified by quantitative real-time PCR. A total of 15 A3^{-/-}, 7 A3^{+/-}, and 7 A3^{+/+} mice were analyzed. Bars represent the mean values for each group.

serum, were treated with DNase I for 15 min at room temperature to get rid of contaminating transgene DNA. After DNase inactivation, viral capsid-associated DNA was extracted using a DNeasy kit (Qiagen) and subjected to standard PCR with the O.HBV.9/O.HBV.2 primers. For quantification, 2 μ l of DNA was used in a real-time quantitative PCR using primers and a probe specific for HBV (O.HBV1, 5'-AAGGTAGGAGCTGGAGCATTCG-3'; O.HBV2; and O.FAM. HBV.1, 5'-FAM-AGCCCTCAGGCTCAGGGCATAC-3'-TAMRA, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). Each sample was tested in triplicate, and a standard curve with serial dilutions of the HBV-expressing plasmid pCMVayw was included in each TaqMan run. Serum levels of HBV surface antigen (HBsAg) were measured in duplicate using a Monolisa HBsAg Plus enzyme-linked immunosorbent assay kit (Bio-Rad). Poly(I · C) (PO93; Sigma) was injected intraperitoneally once a day for 3 days (5 μ g/g of body weight). For cellular RNA analyses, heparinized blood samples (100 μ l) were resuspended in 500 μ l of erythrocytes lysis buffer (Qiagen) and kept on ice for 15 min. Cells were harvested by two consecutive centrifugations of 10 min each at 2,300 rpm in a refrigerated tabletop centrifuge. A total of 30 mg of liver harvested from sacrificed mice was cut into small pieces, subjected to mechanical homogenization through 18-gauge (0.5-in.) and then 22-gauge (.25-in.) needles, and microcentrifuged for 3 min at maximum speed at 4°C. RNA was then purified from the clarified supernatant. For both lymphocytes and liver cells,

FIG. 3. muA3 is expressed at low levels in the mouse liver. Cytoplasmic RNA was extracted from both liver and PBLs isolated from four different mice. Levels of muA3 gene expression were determined by quantitative real-time RT-PCR and expressed relative to the levels of the 18S normalization gene. To ensure that only RNA was detected by amplification, control reactions were performed with omission of the reverse transcriptase at the RT step (wo RT). Amounts represent means and standard deviations of values obtained from the four animals.

FIG. 4. muA3 expression is induced by IFN in vitro. Primary fibroblasts (fibro) isolated from two different mice and MDTF were treated in duplicate with 1 μ g/ml murine recombinant IFN- γ for 20 h before RNA was extracted. muA3 gene expression was recorded by quantitative real-time RT-PCR and expressed relative to the housekeeping Cox6a1 and Pmp22 genes. Normalized muA3 expression levels obtained from untreated cells were set as 1.

cytoplasmic RNA was extracted using an RNeasy Mini Kit (Qiagen), including an on-column DNase step, and converted into double-stranded cDNA by SuperscriptII reverse transcriptase (Invitrogen) using random hexamers (Roche) as primers. Quantification was performed by real-time PCR using primers and probes specific for muA3 and amplifying an exon junction (O.mA3.1, 5'-CAGA ATCTTTGCAGGCTGGTT-3'; O.mA3.2, 5'-CTTCCAACACTTTTAAATTC GTATAGG-3'; O.FAM.mA3.1, 5'-FAM-AGGAAGGAGCCAGGTGGCTG C-3'-TAMRA). The following housekeeping genes, with the primers and probes, were used for normalization: murine connexin 6a1 (mCox6a1), O.mCox6a1.1

TABLE 1. Characterization of the 20 double-transgenic mice^a

Mouse	Sex ^b	A3 genotype	PCR result from tail DNA ^c			HBsAg level in serum ^d
			A3 WT allele	A3 KO allele	HBV	
A	F	-/-	-	+	+	+
B	F	-/-	-	+	+	+
C	F	-/-	-	+	+	++
D	F	-/-	-	+	+	++
E	F	-/-	-	+	+	+++
F	M	-/-	-	+	+	+++
G	M	-/-	-	+	+	+++
H	M	-/-	-	+	+	+++
I	M	-/-	-	+	+	+++
J	M	-/-	-	+	+	+++
K	F	+/+	+	-	+	+
L	F	+/+	+	-	+	+
M	F	+/+	+	-	+	++
N	F	+/+	+	-	+	+++
O	F	+/+	+	-	+	+++
P	M	+/+	+	-	+	+++
Q	M	+/+	+	-	+	+++
R	M	+/+	+	-	+	+++
S	M	+/+	+	-	+	+++
T	M	+/+	+	-	+	+++

^a Young, age-matched mice genotyped for A3 and positive for the HBV transgene as detected by PCR on tail DNA were included in the experiment. Ten HBV-Tg A3^{-/-} and 10 HBV-Tg A3^{+/+} mice were chosen with a mix of males and females in each group.

^b M, male; F, female.

^c WT, wild type; KO, knockout.

^d Determined by ELISA.

FIG. 5. Induction of cellular IFN-induced mRNAs in the liver of poly(I · C)-injected mice. At day 1 after the last poly(I · C) injection, two A3^{-/-} (E and F) and two A3^{+/+} (M and P) mice were sacrificed, and RNA was extracted from the livers. (a) The IFN-induced MTII mRNA and the Pmp22 control mRNA were detected by RT-PCR. To check for the specificity of the RNA detection, the F and M RNAs were also included in a control reaction where the RT enzyme was omitted (C- wo RT). RT-PCR was also performed without RNA (C-). (b) The induction of muA3 was quantified by real-time RT-PCR in the A3^{+/+} poly(I · C)-treated mice (M and P), in four HBV-Tg untreated mice, and in one C57BL/6 mouse. The mean obtained for the HBV-Tg control mice was set as 1.

(5'-CTCTTCCACAAACCCTCATGTGA-3'), O.mCox6a1.2 (5'-GAGGCCAGG TTCTCTTTACTCATC-3'), and O.FAM.mCox6a1 (5'-FAM-CCCAGTTCGGA CCGGCTATGA-3'-BHQ); murine peripheral myelin protein 22 (mPmp22), O.mPmp22.1 (5'-TTCGTAGTCCACAGTTCCTCTC-3'), O.mPmp22.2 (5'-A CTCGCTAGTCCCAAGGGTCTA-3'), and O.FAM.mPmp22.1 (5'-FAM-CGG TCGGAGCATCAGGACGAGC-3'-BHQ, where BHQ is Black Hole quencher); or the 18S normalization gene (using the 20 $\times$ 18S rRNA mix from Applied Biosystems). Each sample was tested in triplicate, establishing a standard curve with serial dilutions of muA3 expressing plasmid pCDNA3-muA3-HA. For each sample, a normalization factor was obtained by calculating the geometric mean of the values obtained for the housekeeping genes, and this value was subsequently used to normalize the relative amounts of the RNAs of interest. Hepatic levels of metallothionein II (MTII) RNA were measured by reverse transcription-PCR (RT-PCR) using the primers O.mMTII.1 (5'-TTGCGCTCGACCA ATACTC-3') and O.mMTII.2 (5'-CGGAAGCCTCTTTGCAGATG-3').

RESULTS AND DISCUSSION

We first tested whether muA3 is active against HBV. For this, we cotransfected 293T cells with either an HIV-1 Δ Vif- or an HBV-expressing plasmid together with a control vector or vectors encoding HA-tagged versions of muA3 and hA3G. As

FIG. 6. Poly(I · C)-mediated IFN induction inhibits HBV replication as efficiently in A3^{+/+} as in A3^{-/-} mice. Serum levels of HBV-DNA associated with secreted capsids were monitored the day before the first poly(I · C) injection (0) and on days 1 and 4 after the last injection by RT-PCR (a) and further quantified by quantitative real-time PCR (b). Mice that were sacrificed at day 1 after the last poly(I · C) injection are indicated with an asterisk. neg, DNA extracted from serum of mice not transgenic for HBV; pos, DNA extracted from serum of parental HBV^{+/+} mice; C-, PCR performed without DNA; 10 ng, PCR performed with 10 ng of HBV-expressing plasmid; p.i., postinjection.

previously observed (1, 4, 11, 22), at comparable expression levels, muA3 was as efficient as hA3G at blocking HIV-1 ΔVif (Fig. 1, gray bars). Both proteins also had similar antiviral activities against HBV (Fig. 1, white bars).

There is a high degree of variability in serum levels of HBV antigen and DNA among HBV-Tg mice, possibly due to modulation of transgene expression by hormonal and metabolic factors (20, 34). In order to explore the possibility that muA3 might participate in this regulation, we crossed HBV-Tg mice with muA3 knockout (A3^{-/-}) mice (17, 23). We then selected HBsAg- and age-matched male mice differing in their muA3 genotype and quantified capsid-associated HBV-DNA in the serum by real-time PCR. Because the production of HBV DNA-containing capsids initially appeared more heterogeneous among A3^{-/-} mice, we examined 15 of these versus 7 mice each of the A3^{+/-} heterozygous and A3^{+/+} homozygous controls. This analysis revealed no dramatic differences between the three groups, with A3^{-/-}, A3^{+/-}, and A3^{+/+} mice producing on average 32,318, 19,360, and 17,080 HBV DNA molecules per μl of serum, respectively (Fig. 2).

As observed for human APOBEC proteins, muA3 expression is tissue specific (11, 22, 23), but its presence in murine hepatocytes has not been formally documented. We thus first

probed both peripheral blood lymphocytes (PBLs) and liver from four C57BL/6 mice by real-time quantitative RT-PCR. In all four animals, baseline hepatic levels of muA3 RNA were at least 15 times lower than those measured in PBLs (Fig. 3). We then checked whether IFN can stimulate muA3 expression. We treated duplicates of primary fibroblasts freshly isolated from two mice as well as MDTF with murine IFN-γ at 1 μg/ml and measured levels of muA3 mRNA 20 h later by quantitative real-time RT-PCR. We observed a 2.5- to 5-fold induction of muA3 mRNA in IFN-treated cells compare to untreated control cells (Fig. 4) in a dose-dependent fashion (data not shown).

Finally, we examined the IFN sensitivity of viral replication in HBV-Tg mice defective for A3. For this, we first measured serum HBV changes in response to the IFN inducer poly(I · C) in 20 age-matched mice, half A3^{-/-} and half A3^{+/+}, selecting five females and five males in each group. Baseline serum levels of HBsAg were heterogeneous among mice but without a significant difference between A3^{-/-} and A3^{+/+} animals (Table 1). Mice were injected intraperitoneally with the double-stranded RNA poly(I · C) (5 μg/g of body weight) on three consecutive days, a procedure previously demonstrated as optimal for inducing the IFN-α/β pathway (10, 28). The day after

the last injection (day 1), two mice in each group (mice E and F and mice M and P) as well as four HBV-Tg untreated control mice were sacrificed. Induction of the IFN pathway and of muA3 mRNA levels was checked by RT-PCR. As previously described (41), the IFN-responsive metallothionein mRNA was upregulated in mice treated with poly(I · C) (Fig. 5a). The muA3 mRNA was also induced 2- to 2.5-fold in the two HBV-Tg A3^{+/+} poly(I · C)-treated mice as measured by quantitative real-time RT-PCR (Fig. 5b). The efficiency of HBV replication in the 10 A3^{+/+} and 10 A3^{-/-} poly(I · C)-treated mice was monitored by measuring serum levels of HBV DNA-containing capsids either before treatment or 1 and 4 days after the last injection of poly(I · C), first by standard PCR (Fig. 6a) and then by quantitative real-time PCR (Fig. 6b). In all mice for which HBV DNA was detected at baseline, the level decreased after poly(I · C) treatment, irrespective of the A3 genotype. Noteworthy, this response was also seen in A3^{-/-} mice for which HBV DNA quantification yielded an estimate of more than 10⁷ genome equivalents/ml, comparable to levels found in the serum of chronically infected patients.

These experiments unequivocally demonstrate that, in HBV-Tg mice, IFN-mediated inhibition of HBV production occurs independently of A3. This strongly suggests that in humans as well, in spite of similarities between the molecular characteristics of APOBEC- and IFN-induced blockade of HBV replication, the cytokine does not exert its antiviral effect through induction of the cytidine deaminases. The downstream effectors of the anti-HBV effect of IFN thus remain to be identified.

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