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RESEARCH ARTICLE

Regulated and prolonged expression of mIFNα in immunocompetent mice mediated by a helper-dependent adenovirus vector

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A major goal in gene therapy is to develop efficient gene transfer protocols that allow tissue-specific, long-term and tightly regulated expression of the desired transgene. This objective is becoming more attainable through the co-evolution of gene transfer vectors and regulation systems. The ideal vector should efficiently transduce non-dividing cells with minimal toxicity, thus endowing the system with persistent transgene expression. The helper-dependent adenovirus vectors meet these requirements, as demonstrated in various studies in the literature. The most promising regulation system is the tet-on system, which has low basal transcriptional activity and high inducibility. To explore the regulated transgene expression in the context of a helper-dependent vector, we constructed the HD-TET-IFN vector, containing the mIFNα gene under the control of the tetracycline inducible transactivator rtTA2^s-S2. Mice injected with HD-TET-IFN showed high levels of serum mIFNα only upon transcriptional activation. The transgene expression was reinducible to the same high level up to 3 months p.i., and the amount of expressed cytokine could be regulated by dosing doxycycline. Transcriptional activation of mIFNα induced by doxycycline resulted in prolonged survival and reduced liver damage in HD-TET-IFN-injected mice challenged with a lethal dose of coronavirus. Activation of antiviral genes mediated by doxycycline-dependent mIFNα expression was also observed at low HD-TET-IFN doses. The possibility of controlling gene expression by the combination of HD vectors and the latest tet-on transactivator also holds promise for studying gene function in other animal models. Gene Therapy (2001) **8,** 1817–1825.

Keywords: *tetracycline; helper-dependent adenovirus vector; gene regulation; antivirals*

Introduction

To date, various strategies including viral and nonviral vectors have been developed for gene transfer. Among the viral vectors, adenovirus (Ad) vectors deliver genes to a wide variety of cell types and tissue independently of their proliferative state (reviewed in Ref. 1). These vectors have been modified by introducing deletions in the early genes to increase cloning capacity and reduce cellular toxicity.[2] However, these modifications have not lead to a prolonged transgene expression in rodents and non-human primates, indicating that residual low level expression of Ad genes is responsible, at least in part, for the short-term persistence. The 'gutless' or helper-dependent adenovirus vector (HD) allowed transgene expression to persist for almost the entire lifetime of mouse as shown in liver, brain and muscle.[3–5] These vectors lack all viral coding sequences and are produced by the co-infection of a suitable cell line with an Ad helper virus, which provides in *trans* the proteins required for replication and packaging.[6,7] In light of the extensive cloning capacity (35 kb) and the persistence of expression in many cell types, HD vectors are ideal vectors for studying gene functions in the absence of interfering viral promoters, undesired expression of potentially toxic Ad genes, and reduced immune response against Ad proteins.

To achieve safe and effective protein expression, pharmacological control over the level of gene transcription is required. Placing gene activity under control from outside via an effector molecule allows the activity to be limited within a 'desired window'. Thus expression can be adjusted according to the evolution of induced biological effects, and treatment to be terminated by drug withdrawal. Different systems based on small-molecule control of transcription have been developed and proven to be effective in mice. The rapamycin-regulated system (RRS) uses a 'dimerizer' drug to bring together the functional units of a bipartite transcriptional factor.[8] A different chimeric system is based on the progesteron antagonist, RU486. An HD vector expressing human growth hormone (hGH) under control of the RU486-inducible system was very effective both in controlling hGH expression and in inducing a biological response for a limited period of time.[9] The Tet systems, widely applied both *in vitro* and *in vivo*, have been derived from elements of the tetracycline (Tc) resistance operon of *Escherichia coli* which were converted into eukaryotic transcription activation systems.[10] In one version a Tet-controlled trans-

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activator (tTA), a fusion between the Tet repressor protein (TetR) and a eukaryotic transcription activation domain, binds to tet operator sequences fused to a minimal promoter and activates transcription in the absence of Tc.[11] Addition of Tc, or one of its derivative such as doxycycline (Dox), abolishes transcription. In a second version, a mutated form of tTA, rtTA, binds and activates transcription only in the presence of Dox.[12] Both Tet systems were used in gene therapy models whereby a variety of vectors were applied: retroviruses, adeno-associated virus (AAV) binary systems, first generation adenoviruses, and electroporated DNA.[8,13–15] Despite numerous successful applications, the Tet regulatory systems originally described show some limitations. rtTA exhibits some residual affinity to *tetO* in the absence of Dox and requires relatively high levels of Dox to achieve full activation. In addition, rtTA and possibly its mRNA are rather susceptible to degradation, at least in certain cell types. These limitations were overcome by novel rtTA versions, which were identified via mutagenesis and genetic selection in yeast. The new transactivator species such as rtTA^s-S2, which were subsequently embedded in synthetic sequences designed to optimize expression in mammals, showed drastic improvements in all parameters mentioned above.[16]

Pharmacological control of gene expression may be critical in those applications utilizing pleiotropic cytokines such as IFNα, which is currently used in the treatment of viral hepatitis. A more effective antiviral treatment against HBV infection was obtained by introducing asialoglycoprotein binding sites in the recombinant IFNα, which in turn led to higher intrahepatic concentrations.[17] IFNα gene transfer may offer the possibility of limiting IFNα expression to the liver,[18,19] with a potential reduction of side-effects in other tissues, such as the central nervous system, provided that transgene expression is kept within safe and therapeutic levels. The antiviral IFNα action is thought to be mediated by transcriptional activation of many genes including 2′,5′-oligoadenylate synthetase (2′5′OAS) and GTPase such as TGTP.[20]

In this report, the features of regulated mIFNα expression are explored utilizing the novel rtTA2^s-S2 transactivator carried in a single HD vector, HD-TET-IFN. The efficacy of a Dox-regulated expression of mIFNα was verified in a murine model of acute hepatitis. Liver protection was dependent only on activation of mIFNα expression induced by Dox treatment. Secretion of the cytokine was repeatedly re-induced to the same high level for a period of 3 months, and controlled by the amount of Dox delivered. In addition, C57/B6 mice injected at low HD-TET-IFN doses resulted in Dox-mediated regulation of liver restricted mIFNα expression, which was associated with induction of antiviral genes. These results indicate that desired levels of mIFNα expression can be achieved and maintained by controlling both the vector dosage and the transcriptional activity.

Results

Description of HD-TET-IFN

To control mIFNα expression an inducible cassette based on the Tet system was constructed (Figure 1a). The tetracycline-sensitive transactivator rtTA2^s-S2 was cloned under the liver-specific TTR promoter and enhancer followed by the SV40 polyadenylation signal. mIFNα2 gene was inserted in the opposite orientation under the control of the P_{tet-1} promoter,[10] followed by the bGH polyadenylation signal. To increase mRNAs stability, two introns were inserted upstream of the transactivator and of the mIFNα gene, respectively. The expression cassette was inserted in the helper-dependent backbone vector C4HSU generating pHD-TET-IFN plasmid and the HD-TET-IFN vector was rescued and amplified in 293cre cells using the H14 helper virus.[21] To compare *in vitro* the potency of mIFNα expression mediated by HD-TET-IFN or HD-IFN, which expresses mIFNα2 directly under the TTR promoter, Hep3B cells were treated with 50 pp/cell and mIFNα2 released in the medium was measured at day 2 p.i. (Figure 1b). In HD-TET-IFN-treated cells 200-fold Dox-dependent induction of mIFNα production was observed. Lower values were observed in HD-IFN-treated cells. To visualize the cells which expressed mIFNα as a function of the vector dose, Hep3B were treated with increasing amounts of HD-TET-IFN and mIFNα expression revealed by an immunohistochemistry assay. A dose–response correlation was observed between the vector dose and the cells stained with an

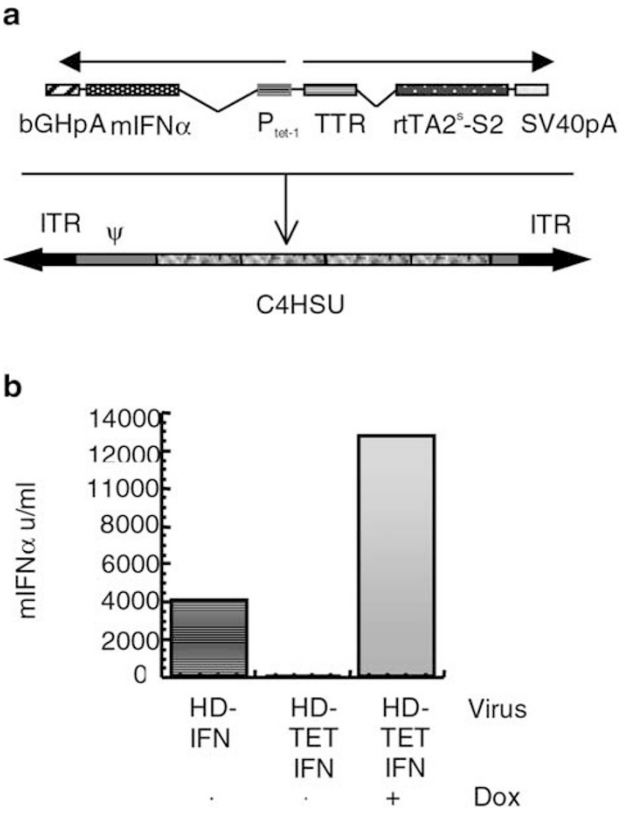

Figure 1 *(a) Structure of HD-TET-IFN vector. The inducible transactivator rtTA2^s-S2 was inserted under the control of the liver-specific TTR promoter/enhancer. In the opposite orientation, the tetracycline responsive element (TRE)/CMV minimal promoter drives the expression of mIFNα2. To increase the level of expression, introns were added downstream of the promoters. The expression cassette was cloned in the NotI site of C4HSU backbone.[21] (b) Comparison between mIFNα2 expression vectors. Human hepatoma cells, Hep 3B, were transduced with 50 pp/cell of HD-IFN[18] or HD-TET-IFN, 48 h after the mIFNα present in the cell supernatant was measured in VSV cytopathic inhibition assay described in Materials and methods. Dox was added at 1 μg/ml.*

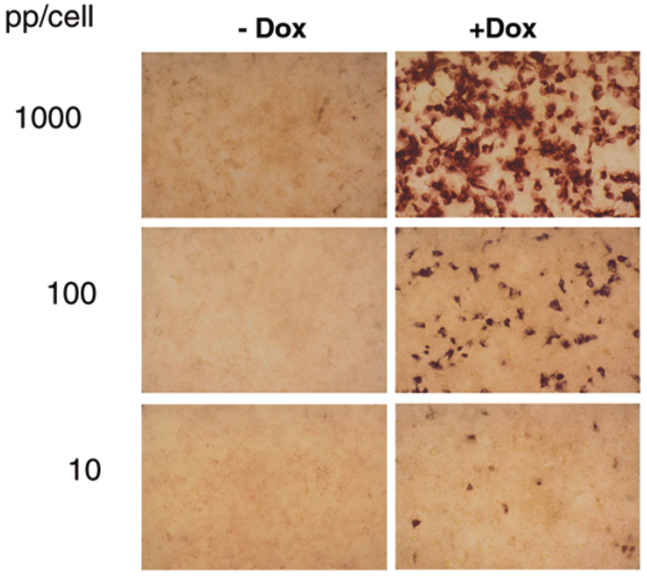

Figure 2 *Detection of HD-TET-IFN transduced cells by immunocytochemistry. Hep3B cells were transduced with different pp/cells and treated with or without Dox (1 μg/ml). Forty-eight hours after transduction, mIFNα-positive cells were revealed with an anti-mIFNα as described in Materials and methods.*

anti-mIFNα antibody only in Dox-treated cells, whereas no positive staining was observed in the absence of Dox even in cells treated with HD-TET-IFN at the highest doses of 1000 pp/cell (Figure 2). These results indicate that HD-TET-IFN allowed a significant expression of mIFNα controlled *in vitro* by Dox.

Regulated and prolonged secretion of mIFNα in mice injected with HD-TET-IFN

To verify the efficiency and persistence of mIFNα secretion, C57/B6 mice were injected i.v. with 1.4×10^{10} pp of HD-IFN or HD-TET-IFN and the mIFNα released in the serum was measured over time. As previously reported, circulating mIFNα was not detected at this dosage in HD-IFN-treated mice. Differently, in HD-TET-IFN-injected mice serum mIFNα was observed at this dosage starting from day 14 p.i. after 3 days of Dox treatment (Figure 3). An average of 4000 U/ml was detected in all

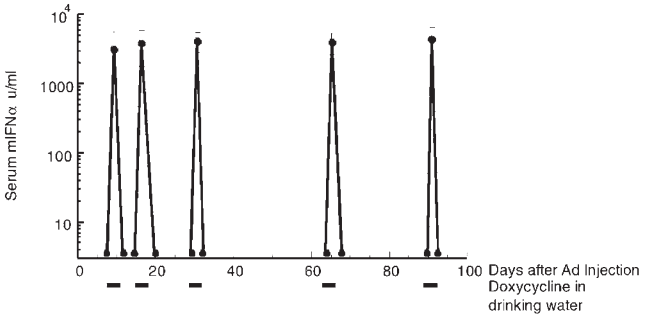

Figure 3 *Repetitive induction of mIFNα expression. Five C57/B6 mice were i.v. injected with 1.4×10^{10} pp of HD-TET-IFN vector. Dox (200 μg/ml) was administered in the drinking water for 3 days starting from day 14 p.i. at different time-points, as indicated in the graph. mIFNα levels present in the serum, expressed as units per milliliters, were measured in the VSV inhibition assay as described in Materials and methods. The assay detection limit was 20 U/ml.*

treated mice ($n = 5$). Removal of Dox resulted in a rapid decrease of serum mIFNα to undetectable levels in 3 days (below 20 U/ml). Therefore, expression was increased at least 200-fold. The same expression kinetics as a function of the Dox treatment was observed throughout the 3 months analyzed. HD-TET-IFN injection in the muscle did not lead to detectable serum mIFNα present in the serum, indicating that liver specificity was maintained in this expression vector (data not shown).

To verify the level of mIFNα released in the blood stream as a function of Dox added in the drinking water, mice were treated for 3 days with different Dox concentrations. As shown in Figure 4, a clear correlation was observed between the concentration of Dox in the drinking water and the serum mIFNα measured in both VSV and ELISA assay. At a concentration of 200 μg/ml, serum mIFNα was in the range of 4000 U/ml, whereas at 20 μg/ml it was 10–30 U/ml. At the lowest Dox concentration, serum mIFNα was detectable only with the more sensitive, albeit not quantitative ELISA assay. These results are in line with observations in transgenic mice where a 10-fold reduction in the amount of Dox added in the drinking water resulted in 100-fold lower amount of luciferase detected in the liver.[22] To further characterize the kinetics of expression induced by a single oral dose of Dox, C57/B6 mice were injected with 4×10^{10} pp and serum mIFNα measured over time (Figure 5). At a dose of 20 mg/kg of Dox, rapid induction of mIFNα expression was observed as early as 6 h after induction. Maximal expression was observed between 6 and 24 h with mIFNα values ranging from 200 to 1000 U/ml. Serum mIFNα levels returned to background within 48 h. The rapid kinetic of expression did not allow the precise measurement of the peak value for each mouse and may explain, at least in part, the five-fold variation in maximal mIFNα expression. At 200 mg/kg of Dox, higher levels were observed, indicating that maximal induction may not be achieved with a single dose of 20 mg/kg of Dox (data not shown).

Overall, these results show that in the context of HD-Ad vector rtTA2^s-S2 allows both long-term and dose-dependent Dox regulation of the mIFNα gene expression in the liver.

Liver protection in acute hepatis model by transcriptional activation of mIFNα

To investigate the therapeutic potency of a regulated expression of mIFNα2 gene, we assessed the antiviral strength of HD-TET-IFN in an acute hepatitis model. For this purpose, we examined the effects of HD-TET-IFN on the infection of susceptible mouse strain C57/Bl6 with mouse coronavirus MHV-3. Treatment with recombinant mIFN-β type I was shown to prolong survival following MHV-2 exposure, particularly when the treatment was initiated before viral infection.[23,24]

Mice were injected i.v. with 1×10^{10} pp of HD-TET-IFN and mIFNα induction measured at day 14 and 21 p.i. As expected, in all injected mice treated with Dox in the drinking water at a concentration of 200 μg/ml for 3 days, circulating mIFNα2 was observed in the range of 1900 U/ml and returned to basal level 3 days after Dox withdrawl. At day 40 p.i., a subgroup of mice were re-induced with Dox in the same condition described above, and all mice were infected at day 42 with an i.p. injection of 200 p.f.u. of MHV-3. To verify the impact of mIFNα2

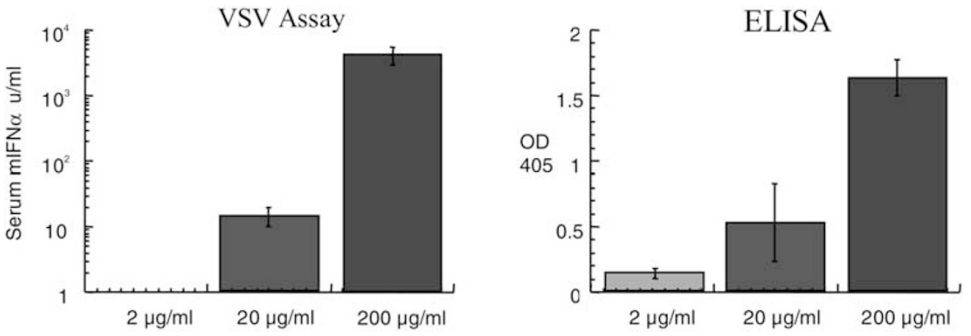

Figure 4 *Dox dose-dependent mIFNα expression at a systemic level in mice injected with HD-TET-IFN. C57/B6 mice were i.v. injected with 1.4 × 10^{10} pp and divided into three groups of three mice. At day 30 p.i. Dox was added to the drinking water at the concentrations indicated. After 3 days, mIFNα levels in the serum, expressed as units per milliliter, were measured in the VSV inhibition assay and by ELISA, and expressed as units per ml or OD_{405} respectively.*

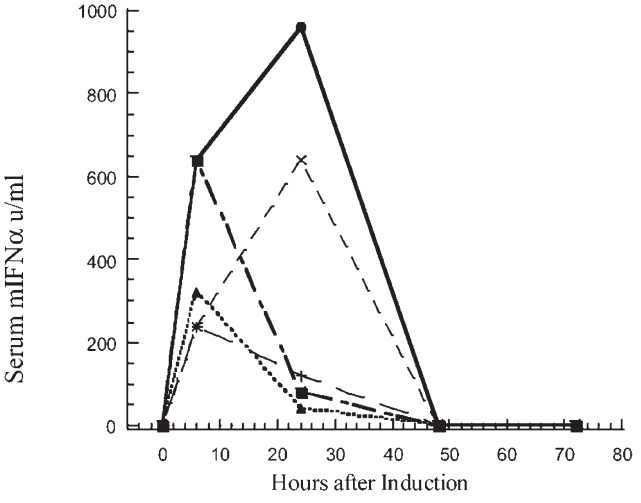

Figure 5 *Kinetics of mIFNα induction after a single dose of Dox. C57/B6 mice were i.v. injected with 4 × 10^{10} pp of HD-TET-IFN and treated with 20 mg/kg of Dox 2 weeks after vector injection. Blood was drawn at 6, 24, 48 and 72 h after Dox induction. Serum mIFNα levels were measured in the VSV inhibition assay as described in Materials and methods. Serum mIFNα levels of individual mice are shown in units per ml.*

induction on MHV-3-mediated liver damage, transaminase (ALT) present in the serum were measured 3 days after MHV-3 infection (Figure 6a). Hepatic protection was observed in mice pre-induced with Dox, where serum transaminase levels remained at basal levels. However, a sharp rise in serum ALT levels was observed in mice injected with HD-TET-IFN and not treated with Dox or in control mice (mock). An additional effect, observed only in Dox-treated mice, was a slight but significant prolonged mean survival time ($P < 0.008581$). As shown in Figure 6b, control mice and those not treated with Dox died between day 3 and 4 after MHV-3 infection, whereas in the Dox-treated group one out of six survived till day 21 when the experiment was terminated (mean survival time ± standard deviation: mock 4 ± 0 days; no Dox 3.8 ± 0.37; Dox 5.8 ± 0.83).

Therefore, these experiments demonstrate that Dox-dependent mIFNα expression results in liver protection and prolonged survival in MHV-3 infected mice.

Liver-restricted and Dox-dependent mIFNα expression in mice injected with low doses of HD-TET-IFN

Previously, we have shown that injection of low doses of HD-IFN vector gives rise to intrahepatic production of the cytokine without any detectable mIFNα in circulation.[18] Therefore, we examined whether such a result could also be obtained with a Tet-regulated vector. C57/B6 and Balb/C mice were i.v. injected with different doses of HD-TET-IFN and mIFNα measured at day 21 p.i. after a treatment for 3 days with Dox, 200 µg/ml, present in the drinking water (Figure 7a). In C57/B6 mice injected with low vector doses (1.4 × 10^9 pp and 2.8 × 10^9 pp) and not treated with Dox, mIFNα was detected neither in the liver, nor in the serum. However, liver-restricted mIFNα in the range of 60–120 U/g was observed upon induction depending on the vector dose. Higher HD-TET-IFN dose (4.4 × 10^{10} pp), in the absence of Dox treatment, resulted in 100 U/g of mIFNα present in the liver, but not in the serum. After Dox treatment mIFNα levels increased 100-fold in the liver and 1000-fold in the serum. These levels returned to background upon Dox withdrawal 3 days later (data not shown). In Balb/C mice, where Ad is less persistent,[25] liver-restricted mIFNα was induced only at a virus dose of 4.4 × 10^{10} pp, whereas at lower vector doses the cytokine was undetectable. The HD-TET-IFN doses that resulted in liver-restricted mIFNα expression were further analyzed for the induction of mIFNα responsive genes (Figure 7b). In agreement with the presence of mIFNα protein, Northern blot analysis showed a clear signal for the mIFNα mRNA in both C57/B6 and Balb/C only in Dox-treated mice (lane A). As a consequence of mIFNα expression, 2′5′OAS and TGTP transcripts were elevated, as indicated by the RNase protection assay (lanes C and D). A 7.6- and three-fold induction of 2′5′OAS signal was observed at the highest HD-TET-IFN dose indicated in Balb/C and C57/B6, respectively. When compared with 2′5′OAS, the induction of TGTP over the background was less pronounced. To examine the reversal of induction of antiviral gene activities, 2′5′OAS and TGTP transcripts were examined 3 days after Dox withdrawal. At this time point, in C57/B6 mice injected with 2.8 × 10^9 pp previously induced with Dox, mRNA synthesis from antiviral genes had returned to basal level. To evaluate the cell types which express mIFNα upon Dox treatment,

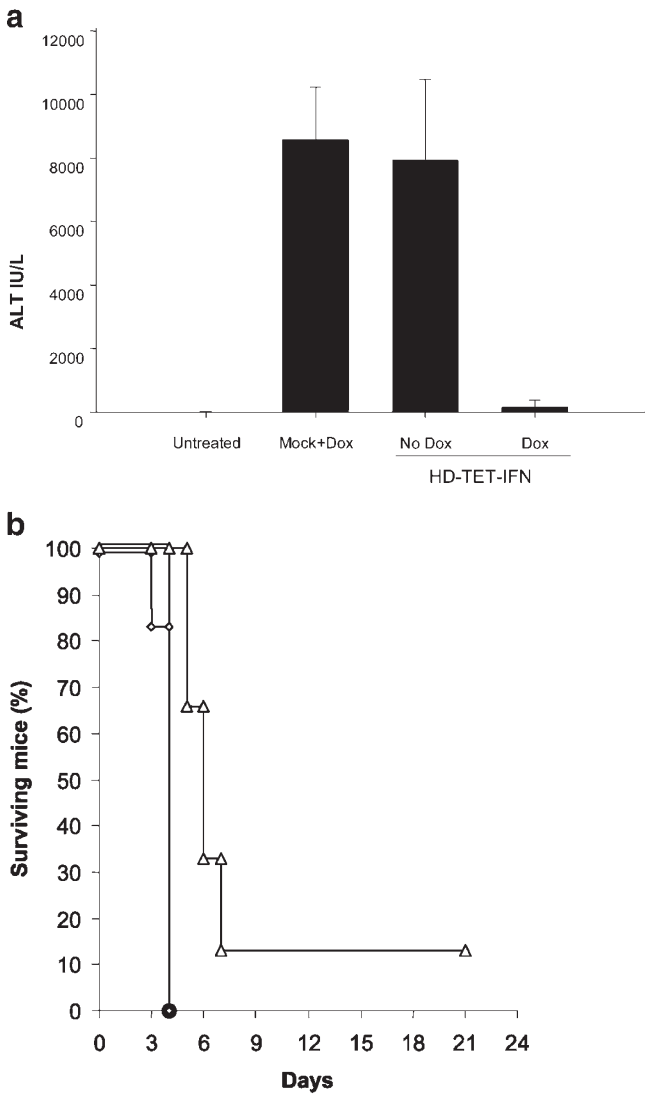

Figure 6 *MHV-3 challenge in HD-TET-IFN-treated mice. Groups of C57/B6 mice were injected i.v. with HD-TET-IFN (1×10^{10} pp) or mock injected. At day 40 p.i. six out of 13 mice injected with HD-TET-IFN were subjected to Dox treatment present in the drinking water at the concentration of 200 μg/ml. At day 42 all mice were infected with an i.p. injection of MHV-3 (200 p.f.u.). (a) Hepatic protection revealed as transaminase (ALT) levels (expressed as units per liter) present in the serum measured 3 days after MHV-3 infection. (b) Effect of mIFNα induction on survival time in the same group of mice of panel (a) after MHV-3 infection. HD-TET-IFN injected mice induced with Dox (Δ); HD-TET-IFN injected mice not induced with Dox (◇); mice not injected with HD-TET-IFN (●).*

immunohistochemistry with an antibody against mIFNα was performed in the liver of C57/B6 (Figure 7c). Positive hepatocytes were observed in the proximity of vessel only upon Dox induction. Lastly, inflammation was evaluated by RNase protection assay for mRNA of infiltrating lymphocytes, eg CD4, CD8, CD3ϵ and macrophages. These markers were not elevated in HD-TET-IFN-injected mice independently of the Dox treatment (data not shown).

These experiments indicate that liver-restricted mIFNα expression can be induced in both mouse strains without apparent liver damage by controlling the dose of the vector and by regulating, via Dox, the expression of the mIFNα gene.

Discussion

In this study we show the features of Dox-dependent mIFNα expression delivered through an HD vector. Among the different vectors used in gene transfer, adenovirus vectors have emerged as one of the more efficient in transducing liver cells.[26] To limit transgene expression in the liver further, the rtTA^s-S2 transactivator was cloned downstream of the liver-specific promoter/enhancer TTR and the mIFNα gene was inserted under the tet response promoter in a head-to-head configuration on the same vector, HD-TET-IFN (Figure 1). A tight control of mIFNα expression was observed in Hep3B cells using different amounts of vector (Figure 2).

The ability to control the expression of a therapeutic gene within a relevant and safe window is a major prerequisite for a variety of regimens envisioned in gene therapy. The rapid adjustment of the activity of a therapeutic gene to the progression of the diseases is of particular importance. The HD-TET-IFN vector meets several of these requirements. Injecting large doses (1.4×10^{10} pp) in C57/B6 mice resulted in undetectable serum levels of mIFNα in the absence of Dox (Figure 3). Upon induction with Dox, mIFNα was detected in the serum and its expression correlated with the amount of Dox delivered to the animal (Figure 4). High levels of secreted mIFNα were observed at the largest dose of Dox, with only slight variations over a period of 3 months. Although the rapid induction of secreted cytokines has already been widely reported, the rapid reversal of mIFNα induction represents a major improvement over the previously described systems. The rapamycin system, which was explored in the context of Ad or AAV vectors, demonstrated rapid induction of hGH and controlled expression over a wide range of doses.[27] However, hGH remained above the basal levels for up to 12 days after a single injection of the inducer. Similar slow de-induction kinetics were observed in the brains of rats using the Tet-off system delivered by a first generation Ad vector.[8] Improved reversal of induction was reported,[9] with the RU486 regulation system carried on a HD vector and driven by the same TTR promoter used in this study. Interestingly, Burcin *et al*[9] observed a similarly inefficient induction of their reporter gene at an early stage after HD injection, as did our system. Further experiments are required to understand why 2 weeks appear to be required to reach steady state expression. Since full induction of Tet-controlled genes is achieved within less than 8 h in the liver of transgenic mice,[28] other factors such as the immuno-response against Ad particles may have a detrimental effect on transcription at this early stage. One limitation of the actual rtTA2^s-S2 based system is its poor sensitivity to Dox, which in our experimental conditions was not saturated using 20 mg/kg (Figure 5). However, in parallel with rtTA2^s-S2, the rtTA2^s-M2 transactivator was developed, which shows a 10-fold higher sensitivity to Dox in HeLa cells. It will be interesting to explore this additional transactivator in the context of HD vectors, which may bring the Dox required within the dose range of human antibiotic therapy.

Apparently, the long persistence and sustained re-inducibility of mIFNα expression (Figure 3) in immuno-

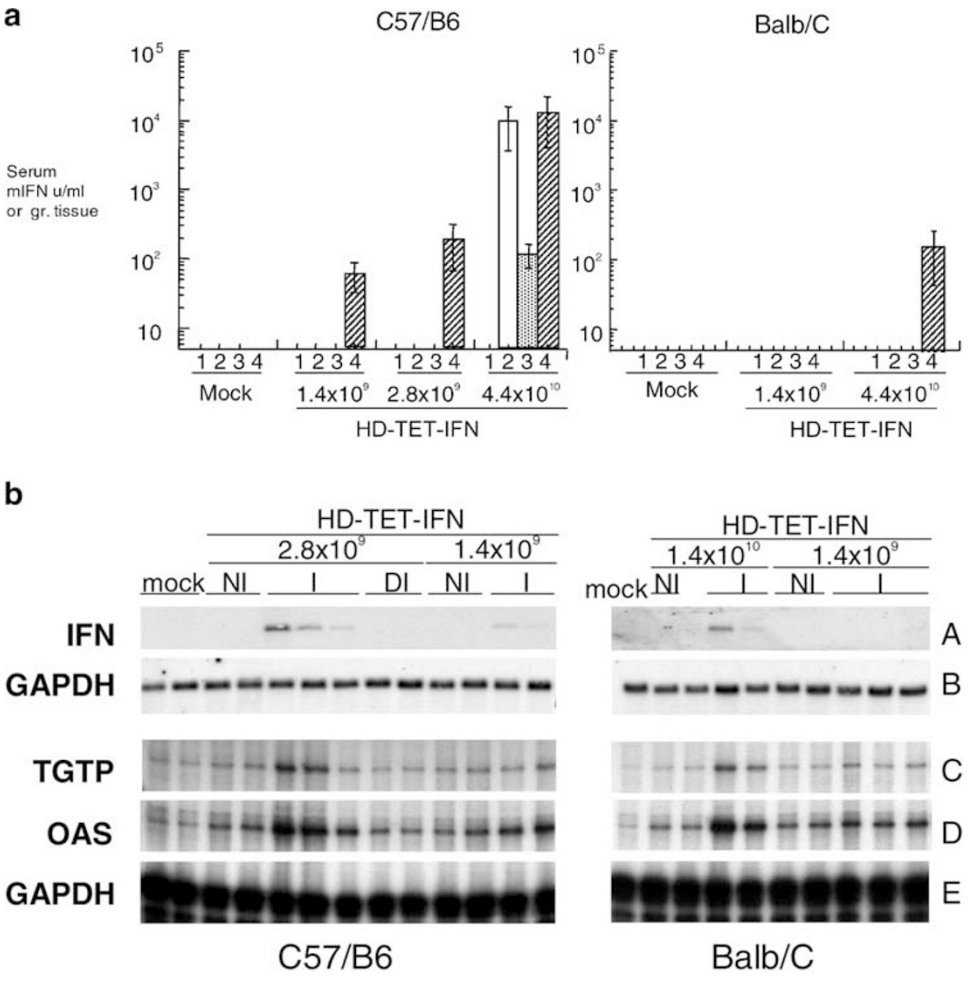

Figure 7 *Mice injected with HD-TET-IFN at low doses. C57/B6 and Balb/C mice were injected i.v. with HD-TET-IFN at different doses and mIFNα expression induced at day 21 p.i. for 3 days with 200 μg/ml od Dox added in the drinking water. (a) Expression of mIFNα. C57/B6 and Balb/C mice were i.v. injected at the indicated vector doses expressed as pp. Bars represent from the left, mIFNα present in: (1) serum without Dox induction; (2) serum after induction; (3) liver not induced; (4) liver induced. (b) Antiviral genes induced in the liver of HD-TET-IFN injected mice. NI, animals not treated with Dox; I, animals treated with Dox for 3 days; DI, Dox treated animals after withdrawal of the antibiotic for 3 days. (A) Northern blot analysis was performed with 20 μg of total liver RNA from two representative mice per group. Membrane was hybridized with a radiolabeled mIFNα2 probe. (B) Northern blot with the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH) used to normalize loading in each land. (C) Total liver RNA was analyzed for induction of TGTP expression in an RNase protection assay. (D) RNase protection assay for expression of 2′5′OAS. (E) RNase protection assay with the control GAPDH gene. (c) Immunohistochemistry with anti-mIFNα antibody: (A) injected with 2.8×10^9 pp HD-TET-IFN and not treated with Dox; (B) injected with the same vector dose and treated with Dox; (C) mock-injected mice; (D) injected with the same vector dose and treated with Dox at a higher magnification. Bars, 35 μm panel A, B, C; and 15 μm in panel D.*

competent mice indicates that the rtTA2^s-S2 transactivator is not immunogenic, although we presume that the use of a liver-specific promoter such as TTR, which expressed the transactivator only in hepatic cells, and not in professional antigen presenting cells, may have contributed to this persistence. This hypothesis is in agreement with previous reports showing long-term transgene expression in the context of first generation Ad vector only in the presence of a liver-specific promoter.[29]

In this study, liver protection against MHV-3 challenge was observed only upon Dox-mediated induction of mIFNα expression (Figure 6a). The striking difference observed in association with the Dox treatment is very significant in view of the fact that the same viral preparation was injected in both groups, eliminating experimental bias such as difference in the transducing particles per unit volume, purity of vector preparation and dilution volume. In addition, liver protection was achieved 6 weeks after HD-TET-IFN injection, in line with the notion that therapeutic effects mediated by helper-dependent vectors persist for a long period of time. Previously we showed that lower levels of serum mIFNα were associated with partial hepatic protection, but had no effects on survival rate.[18] In this study, all mice treated with Dox showed liver protection and one out of six survived the MHV-3 infection (Figure 6b). The higher level of circulating mIFNα may have elicited stronger local and peripheral effects such as induction of antiviral genes or peritoneal macrophage activation, which may have limited MHV-3 replication in a more efficient manner. Although we showed that injection of HD-TET-IFN resulted in Dox-dependent hepatic protection, the impact of this gene therapy approach on chronic hepatitis remains to be established, as no mouse model for this disease exists, unlike HBV (woodchuck model) or HCV infection (non-human primates, such as chimpanzees).

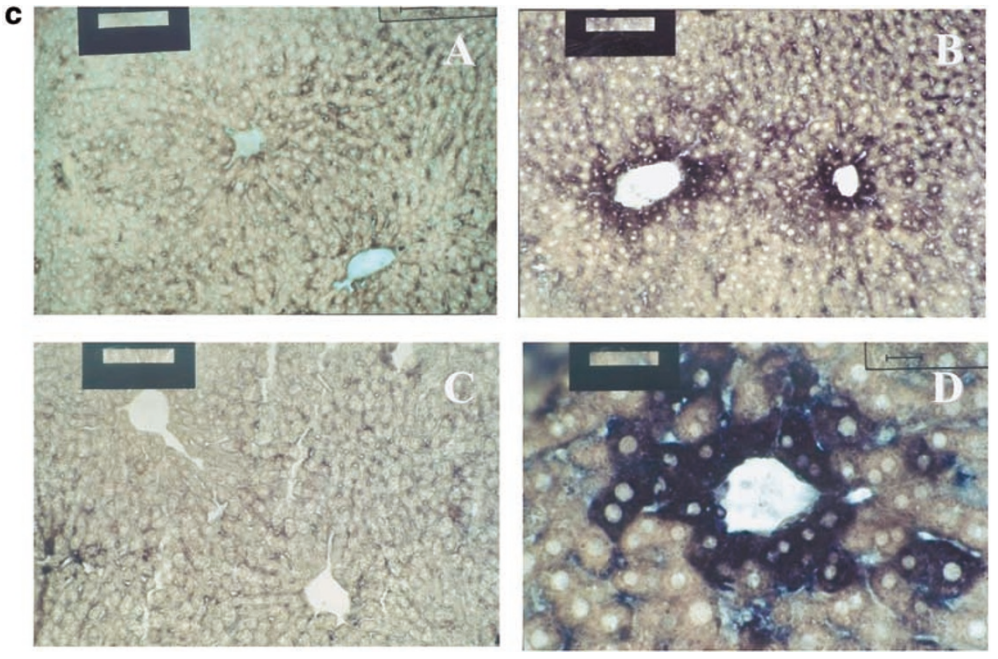

Figure 7 *Continued.*

However, the pharmacological control of IFNα expression is also required in the context of these animal models in light of safety concerns such as toxicity, side-effects and adjusting the IFNα level as a function of disease progression.

Recombinant IFNα is widely used in the treatment of human hepatitis B and C. However, systemic injection is associated with side-effects, particularly on the neuronal system, which cause the withdrawal of a significant number of patients from the therapy. Therefore, it was of interest to evaluate the possibility of limiting the biological response induced by IFNα expression to the liver as a function of the Dox treatment and the vector dosage. In a previous study, we observed that in mice injected with the HD-IFN, which express mIFNα directly under the constitutive TTR promoter, liver-restricted mIFNα expression, as well as the induction of antiviral genes last for 10 months (data not shown). This observation is in agreement with recent reports where gene transfer mediated by HD vectors resulted in long persistence of transgene expression.[3,5,21] This long period of IFNα expression could, nonetheless, be considered 'over-therapeutic' in light of the current treatment for HCV, which lasts for 6 months. Using the HD-TET-IFN vector, however, allows a controlled timing and potency of expression on the basis of Dox treatment even at low vector doses. Indeed, low doses of HD-TET-IFN resulted in mIFNα being detectable in the liver and not in the bloodstream only upon Dox induction (Figure 7a). As a consequence of liver-restricted mIFNα expression, antiviral genes were induced (Figure 7b) in the absence of liver damage or inflammation response. Also in the liver, the reversal of mIFNα induction was fairly rapid, as indicated by the reduction of antiviral gene mRNAs 3 days after Dox withdrawal. Lastly, the expression of mIFNα was highlighted in the hepatocyte cells upon Dox treatment (Figure 7c). As expected on the basis of previous experiments with marker genes, Dox-dependent mIFNα expression distributed in hepatocytes, which are in the proximity of the vessel.

The use of HD vectors and the rtTA^s-S2 transactivator allows the expression of transgenes to be regulated for a long period of time within an expression window, which can be either restricted to the liver or to circulating protein. The lack of a detectable immunoresponse against the HD-TET-IFN in association with its prolonged reinducibility candidate these vectors and regulation system not only for gene therapy purposes, but, at least in some applications, as an alternative option to the use of transgenic animals for tissue-specific expression. While germline transgenesis can generate useful animal models for genetic studies, it can be costly and time-consuming. Additionally, it requires the use of a large number of animals and can be limited by toxicity during embryogenesis. The features of the system described in this paper allow the study of gene function within specific cells in a variety of animal models including non-human primates, where the HD vector-mediated gene transfer is very efficient.[30]

Materials and methods

Cell lines

293 and 293Cre4.15 cells were grown in MEM supplemented with 10% heat-inactivated FCS. L-929 (mouse fibroblasts) HuH-7 and Hep3B (human hepatoma) cells were grown in DMEM supplemented with 10% FCS.

Mouse strains

Mice used in this study were immunocompetent, 6 to 8 weeks old (at the time of injection) C57/B6 and Balb/C females purchased from Charles River (Lecco, Italy). Groups of between four and five mice received injections in the tail vein of adenovirus vectors diluted in physiologic solution in volumes of 200 μl. Dox (2 to 200 μg/ml,

in 5% sucrose, pH 6.0) was added in the drinking water at the indicated time-points. Blood was obtained by retroorbital bleeding and serum was stored at −80°C. At the indicated time, mice were killed and organs were rapidly frozen in liquid nitrogen, and stored at −80°C.

Tet-inducible mIFNα2 expression cassette

The rtTA2^s-S2 transactivator gene was recovered from the plasmid pHUD52.1 as a *Eco*RI/*Bam*HI fragment and subcloned in the vector pTTR-bGH, containing the liver-specific transthyretin gene minimal enhancer and promoter and the bovine growth hormone poly-A site (bGH). To improve the level of expression, an artificial intron was amplified by PCR from pCAT3basic (Invitrogen, Carlsbad, CA, USA) and subcloned *Hin*dIII/*Eco*RI between the TTR promoter/enhancer and the mIFN cDNA, generating pTTR-intr-rTA2-bGH. The intron A from the vector pVIJ-nsA as a *Sac*II/*Eco*RI fragment was subcloned in the vector pHUD10.3 between the TRE (Tet responsive element) and the SV40 polyadenylation site. A *Pac*I site and a *Not*I site were inserted, respectively, upstream and downstream of the SV40 polyA by PCR. At this point the TRE-intron A-SV40 fragment was excised as a *Xho*I/*Kpn*I fragment and inserted in pTTR-intr-rTA2-bGH, thus generating pTet−/−. The mIFNα2 gene was amplified by PCR adding a *Bam*HI site at 5′ and a *Pac*I site at the 3′ and subcloned in pTet−/−, generating pTet-mIFNα2.

Adenoviral vectors

To construct pC4-Tet-mIFN, the mIFNα2 expression cassette was excised with *Not*I and subcloned in the *Not*I site of pC4-HSU.[21]

To rescue the HD-TET-IFN vector, the pC4-Tet-mIFN plasmid was cleaved by *Pme*I and transfected into 293Cre4.15 cells.[21] Subsequently, the cells were infected with the helper adenovirus H14. The titer of the HD vector during the amplification passages on 293Cre4.15 cells was followed by infecting HuH-7 cells with lysates from each passage and determining the amount of mIFNα in cells supernatants with and without Dox at 48 h after infection by VSV inhibition assay. Multiple viral passages were performed to reach a high titer and vector was purified by double CsCl gradient. Physical particles were measured by optical density of DNA.

Cytopathic inhibition assay for interferon

The viral cytopathic inhibition assay using vescicular stomatitis virus (VSV) has been described elsewhere. mIFNα2 activity is expressed in units/ml. The mIFNα2 activity was calibrated against a standard recombinant mIFNα (Calbiochem, San Diego, CA, USA).

ELISA for mIFNα

Ninety-six-well plates were coated overnight at 4°C with 0.25 μg/ml Rat ascite monoclonal Ab to mIFNα (clone 4E-A1, Yamasa Corporation, Japan) in carbonate buffer 50 mM pH 9.6. After blocking with 3% BSA, 1 × PBS, 0.05% Tween 20 for 1 h at 37°C, samples were added and incubated for 2 h at RT in 1% BSA, 1 × PBS, 0.05% Tween 20. Subsequently, plates were washed three times with 1 × PBS, 0.05% Tween 20 and incubated for 2 h at RT with 100 μl/well polyclonal Ab to mIFNα/β (RDI) diluted at 1 μg/ml in 1% BSA, 1 × PBS, 0.05% Tween 20. Wells were washed again and incubated with anti-sheep IgG AP conjugate (Sigma, St Louis, MO, USA) diluted 1:10 000 in 1% BSA, 1 × PBS, 0.05% Tween 20 for 1 h at RT. Final detection was done adding 100 μl/well *p*-nitrophenyl phosphate, disodium, 1.0 mg/ml in 10% diethanolammine buffer, pH 9.8 containing 0.5 mM $MgCl_2$ and reading at OD 405. A standard mIFNα was used as reference in each experiment.

Intra-hepatic mIFNα2 measurement

Livers were weighed and homogenized in PBS using a Polytron homogenizer and lysates were centrifuged for 30 min at 4°C at 14 000 r.p.m. to eliminate cell debris. Since IFNα is acid stable, HCl 0.5 N was added to reach pH 2.0 and extracts were incubated overnight at 4°C. Neutral pH was reached by adding NaOH and centrifuged for 30 min at 4°C at 14 000 r.p.m. Clarified extracts were then analyzed by VSV inhibition assay.

Northern blot analysis and RNase protection assay

Frozen tissues were mechanically pulverized and RNA was isolated from tissues using Ultraspec RNA reagent (Biotecx Laboratories) according to the manufacturer's instructions. Total RNA (20 μg) was used in Northern blot analysis. The intensity of bands was quantified by phosphorimager analysis. The RNase protection assay for quantification of mRNA was performed using the RiboQuant Multi-Probe RPA Assay System (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. The probe set for mIFNα pathway activation was kindly provided by Iain Campbell (SCRIPPS, San Diego, CA, USA).

Biochemical and immuno-histochemical analysis

The extent of hepatocellular injury induced by adenovirus injection was monitored by measuring serum alanine aminotransferase (ALT) activity at the indicated time-points. ALT activity was measured in a SPOTCHEM model SP-4410 according to the manufacturer's instructions.

For cell staining, 24 h after infection Hep3B cells were washed with PBS and fixed in 1 × PBS, 3.7% formaldehyde for 20 min at RT. For tissue staining, at the indicated time-points livers were harvested from HD-TET-IFN-injected mice and fixed in 1 × PBS, 4% paraformaldehyde for 4 h at 4°C, then incubated overnight in 1 × PBS, 30% sucrose. Livers were then included in OCT and stored at −80°C. Cells or liver sections were washed twice with PBS and incubated with PBS/0.1 M glycine for 15 min at RT. After washing with PBS, samples were permeabilized with PBS/0.1% Triton-X-100 for 10 min and treated with PBS, 0.1% Triton-X-100, 10% goat serum for 10 min at RT. Samples were incubated for 1 h at RT with rat ascite monoclonal Ab anti-mIFN α (clone 4E-A1, Yamasa Corporation) diluted 1:200 in PBS, 10% goat serum. After washing five times with PBS, a second incubation with anti-rat IgG AP conjugate (Sigma, St Louis, MO, USA) antibody diluted 1:200 in PBS/10% goat serum was performed for 1 h. After subsequent washings, samples were incubated with AP substrate (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl, pH 9.5, 330 μg/ml NBT, 165 μg/ml BCIP) until sufficient staining was reached, then washed with PBS and mounted with Aquatex.

Induction of acute hepatitis

As described previously,[18] mouse hepatitis virus type 3 (MHV-3) was amplified on mouse fibroblast DBT cells, and the titer was measured in a standard plaque assay. A total of 200 p.f.u. was injected intraperitoneally (i.p.) in 100 μl of physiologic solution.

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