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Interleukin-12 Inhibits Hepatitis B Virus Replication in Transgenic Mice†

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Interleukin-12 (IL-12) is a heterodimeric cytokine produced by antigen-presenting cells that has the ability to induce gamma interferon (IFN-γ) secretion by T and natural killer cells and to generate normal Th1 responses. These properties suggest that IL-12 may play an important role in the immune response to many viruses, including hepatitis B virus (HBV). Recently, we have shown that HBV-specific cytotoxic T lymphocytes inhibit HBV replication in the livers of transgenic mice by a noncytolytic process that is mediated in part by IFN-γ. In the current study, we demonstrated that the same antiviral response can be initiated by recombinant murine IL-12 and we showed that the antiviral effect of IL-12 extends to extrahepatic sites such as the kidney. Southern blot analyses revealed the complete disappearance of HBV replicative intermediates from liver and kidney tissues at IL-12 doses that induce little or no inflammation in these tissues. In addition, immunohistochemical analysis demonstrated the disappearance of cytoplasmic hepatitis B core antigen from both tissues after IL-12 treatment, suggesting that IL-12 either prevents the assembly or triggers the degradation of the nucleocapsid particles within which HBV replication occurs. Importantly, we demonstrated that although IFN-γ, tumor necrosis factor alpha, and IFN-α/β mRNA are induced in the liver and kidney after IL-12 administration, the antiviral effect of IL-12 is mediated principally by its ability to induce IFN-γ production in this model. These results suggest that IL-12, through its ability to induce IFN-γ, probably plays an important role in the antiviral immune response to HBV during natural infection. Further, since relatively nontoxic doses of recombinant IL-12 profoundly inhibit HBV replication in the liver and extrahepatic sites in this model, IL-12 may have therapeutic value as an antiviral agent for the treatment of chronic HBV infection.

During hepatitis B virus (HBV) infection, the cellular immune response to HBV antigens plays a pivotal role in eradication of the infection (7). In acutely infected patients who successfully clear HBV, the class I- and class II-restricted T-cell responses to the virus are vigorous, polyclonal, and multispecific. In contrast, these T-cell responses are weak and narrowly focused in chronically infected patients who do not clear the virus.

We have previously demonstrated that HBV-specific cytotoxic T lymphocytes (CTL) cause a necroinflammatory liver disease in HBV transgenic mice (1, 2, 30). Recently we have shown that the same CTL can profoundly inhibit viral gene expression and replication in the liver by a noncytopathic mechanism that is mediated by gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (15, 19). Based on these observations, we have suggested that the dominant cytokine profile of the antiviral CTL response may determine the ultimate outcome of HBV infection by influencing the extent to which the CTL kill infected hepatocytes and cause liver disease or suppress viral replication and mitigate the infection (17).

Interleukin-12 (IL-12) is a pleiotropic cytokine that is produced by antigen-presenting cells (6, 26, 39) and is required for the optimal generation of Th1 $CD4^+$ T cells and $CD8^+$ CTL (39). Several studies have demonstrated a critical role for IL-12 during viral, bacterial, parasitic, and fungal infections (reviewed in references 4 and 39). In many of these reports, protective immunity and survival mediated by IL-12 reflect its ability to induce endogenous IFN-γ (12, 23, 31, 34, 37, 40, 43). For example, exogenously administered murine IL-12 protects wild-type but not IFN-γ receptor knockout mice from a lethal infection with encephalomyocarditis virus (34). Similarly, in vivo treatment with IL-12 protects normal mice from murine leukemia virus-induced murine AIDS but is not effective in the same mice treated with anti-IFN-γ or in IFN-γ knockout mice (12).

Collectively, the foregoing results indicate that IL-12 plays a critical role in the clearance of many intracellular pathogens. They do not, however, establish whether IL-12 enhances viral clearance by biasing the cellular immune response to develop along the Th1 differentiation pathway or whether the cytokines produced by these T cells have direct antiviral effects themselves. Since we have recently demonstrated that Th1-like cytokines released by HBV-specific CTL can abolish viral gene expression and replication in the livers of HBV transgenic mice, in the current study we determined whether IL-12 could exert the same antiviral effect in the absence of a specific immune response in these animals.

MATERIALS AND METHODS

HBV transgenic mice. The HBV transgenic mice used in this study, lineage 1.3.46 (official designation, Tg [HBV 1.3 genome] Chi46), have been described previously (21). These animals were produced by microinjection of a terminally redundant viral DNA construct (HBV 1.3) into inbred B10.D2 embryos. High-level viral replication occurs in liver parenchymal cells (i.e., hepatocytes) and in the proximal convoluted tubules in the kidneys of these transgenic mice at levels comparable to those observed in the infected livers of patients with chronic HBV hepatitis, without any evidence of cytopathology (21).

Lineage 1.3.46 was expanded by repetitive backcrossing against B10.D2 and then backcrossed one generation against BALB/c to produce the F_1 hybrids used in all experiments. Experiments were performed with mice that were matched for age (6 to 10 weeks), sex (male), and levels of hepatitis B surface antigen (HBsAg) in serum (using a commercially available kit from Abbott Laboratories, Abbott Park, Ill.).

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IL-12. Recombinant murine IL-12 was kindly provided by Maurice Gately (Hoffmann-La Roche, Nutley, N.J.). In all experiments, groups of three to four transgenic mice were injected intraperitoneally with IL-12 (doses ranging from 1 ng to 1 μg, in a 300-μl volume) daily for 1, 3, 5, 7, or 14 consecutive days. IL-12 was diluted in 0.9% NaCl solution (saline) containing 1% serum from 1.3.46 × BALB/c F_1 hybrids. Control animals were injected with saline diluent (saline containing 1% serum) only. Animals were sacrificed 16 h after the last injection of IL-12, and their livers and kidneys were harvested for analysis. Tissues obtained at autopsy were processed either for histological analysis or snap frozen in liquid nitrogen and stored at −80°C for subsequent DNA and RNA analyses.

HBV DNA analysis. Frozen liver and kidney tissues were mechanically pulverized, and total DNA was isolated by standard methods (35). HBV replication was analyzed by Southern blot analysis of 30 μg of *Hin*dIII-digested DNA as previously described (21). Before electrophoresis, all DNA samples were digested with RNase A (Boehringer Mannheim, Indianapolis, Ind.) at 10 μg/ml for 1 h at 37°C. Nylon filters (Amersham Life Sciences, Arlington Heights, Ill.) were hybridized with a ^{32}P-radiolabeled HBV-specific DNA probe. In lineage 1.3.46, the transgene is integrated at a single site within the genome (21) and since it does not contain restriction endonuclease sites for *Hin*dIII, digestion with this enzyme produces a single slowly migrating band which can be used to normalize the amount of DNA bound to the membrane. Replicative forms of HBV DNA migrate as a broad smear spanning viral single-stranded (SS) linear, double-stranded (DS) linear, and relaxed circular (RC) forms at 1.6, 3.2, and 3.5 kbp, respectively (21).

Serum HBV DNA analysis. Quantitation of serum HBV DNA was performed by dot blot analysis, as previously described (21), using 400 μl of serum pooled from animals within the same treatment group. Pooled serum from age- and sex-matched saline-injected HBV transgenic mice was used as a positive control, and pooled serum from B10.D2 × BALB/c nontransgenic mice was used as a negative control.

RNA analyses. (i) Northern blot analysis. Frozen tissues were mechanically pulverized, and total RNA was extracted by the acid guanidium isothiocyanate-phenol-chloroform method (9). RNA (20 μg) was analyzed for HBV, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 2′,5′-oligoadenylate synthetase (2′,5′-OAS) expression by Northern blotting as previously described (16).

(ii) RNase protection assay. The RNase protection assay for quantitation of cytokine mRNA was performed exactly as described previously (24), using a pool of linearized subclones as templates for T7 polymerase-directed synthesis of ^{32}P-labeled antisense RNA probes. The mouse (m) IFN-γ, mTNF-α(A), and ribosomal protein light 32 [mL32(A)] probes subcloned in the pGEM-4 transcription vector have been previously described (24), as have the mCD4(IC), mCD3γ(IC), and mCD8α(DM) probes (19).

Immunohistochemical analysis. The intracellular distribution of hepatitis B core antigen (HBcAg) was assessed by the labeled avidin-biotin detection procedure exactly as described elsewhere (20). This method sequentially uses rabbit anti-HBcAg (Dako, Carpinteria, Calif.) primary antiserum, a secondary antiserum of biotin-conjugated goat anti-rabbit immunoglobulin G (IgG) F(ab′)$_2$ (Sigma Chemical Co., St. Louis, Mo.), streptavidin-horseradish peroxidase conjugate (Extravidin; Sigma), and 3-amino-9-ethyl carbazole (Shandon-Lipshaw, Pittsburgh, Penn.) as a coloring substrate.

Biochemical and histological analysis of disease. Hepatocellular injury was monitored by measuring serum alanine aminotransaminase (sALT) activity at various times after IL-12 injection. sALT activity was measured in a Paramax chemical analyzer (Baxter Diagnostics Inc., McGaw Park, Ill.) exactly as previously described (8), and results were expressed as mean sALT activity in units/liter. For histological analysis, liver and kidney tissue samples were fixed in 10% zinc-buffered formalin (Anatech, Battle Creek, Mich.), embedded in paraffin, sectioned (3 μm), and stained with hematoxylin and eosin.

Anticytokine antibodies. Anticytokine monoclonal antibodies (MAb) were administered intraperitoneally 6 h before the first IL-12 injection. Hamster MAb H22 specific for mIFN-γ (36), hamster MAb TN3 19.12 specific for mTNF-α (38), and neutralizing sheep Ig to mIFN-α/β (14) were used in this study. Purified hamster IgG (Jackson ImmunoResearch, West Grove, Penn.) and normal sheep Ig were used as control antibodies. The hamster antibodies were diluted to 250 μg/200 μl/mouse with nonpyrogenic phosphate-buffered saline (GIBCO BRL, Gaithersburg, Md.) immediately prior to administration. Sheep antiserum was used undiluted (300 μl/mouse).

RESULTS

IL-12 inhibits HBV replication in the livers and kidneys of HBV transgenic mice. To examine the effects of IL-12 on HBV replication, transgenic mice were treated with doses of recombinant murine IL-12 which are known to enhance resistance to various viral infections in other murine models (3, 12, 32–34). In a preliminary experiment, groups of age-, sex-, and serum HBsAg-matched animals were injected intraperitoneally with 1, 10, or 100 ng of IL-12 daily for 3 consecutive days and their livers were harvested 16 h after the last injection for Southern analysis of hepatic HBV DNA replicative forms. The results were compared with total hepatic DNA pooled from 10 matched HBV transgenic control animals injected with saline diluent only. As shown in Fig. 1, a dose-dependent antiviral effect was observed, with nearly complete disappearance of HBV DNA replicative intermediates at an IL-12 dose of 100 ng once a day for 3 days (lanes 6 and 7). At higher doses, hepatic HBV DNA replicative forms were further reduced and eventually completely abolished by increasing the daily dose to 1 μg for 5 and 14 days, respectively (Fig. 2). Serum HBV DNA was also reduced to undetectable levels in the same animals (Fig. 2), demonstrating that IL-12 induces the disappearance of HBV viremia as well. These results indicate that the minimal effective IL-12 dose required to inhibit hepatic HBV replication and viremia in this model is 100 ng daily for 3 days. Importantly, the antiviral effect of IL-12 was accompanied by a dose-dependent increase in sALT activity (Fig. 1 and 2), reflecting the ability of IL-12 to induce a mild necroinflammatory liver disease in these animals (see below).

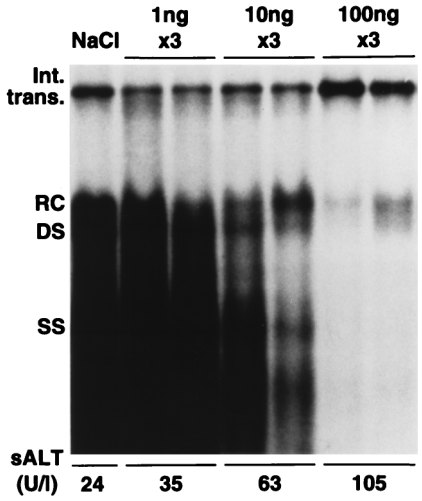

FIG. 1. IL-12-induced inhibition of hepatic HBV replication. Three groups of four HBV transgenic mice were injected intraperitoneally with 1, 10, or 100 ng of recombinant murine IL-12 daily for 3 consecutive days, and total liver DNA was isolated 16 h after the last injection. Southern blot analysis was performed with 30 μg of total hepatic DNA from two representative mice per group, and HBV DNA content was compared with total DNA pooled from the livers of 10 age-, sex-, and serum HBsAg-matched transgenic saline-injected controls (lane NaCl). All DNA samples were RNase treated before gel electrophoresis. Bands corresponding to the integrated transgene, RC, DS linear, and SS linear HBV DNA replicative forms are indicated. The integrated transgene can be used to normalize the amount of DNA bound to the membrane. The filter was hybridized with a ^{32}P-labeled HBV-specific DNA probe. The mean sALT activity, measured at the time of autopsy, is indicated for each group and is expressed in units/liter.

We have recently reported that the antiviral effects of CTL administration in HBV transgenic mice include the disappearance of HBV RNA due to posttranscriptional degradative mechanisms (41). Therefore, the effects of IL-12 administration on HBV gene expression were evaluated by Northern blot analysis of total hepatic RNA extracted from the livers of the same animals as described above (Fig. 2). Even at the highest dose of 1 μg of IL-12 daily for 14 days, there were no changes observed in the 3.5-kb pregenomic and 2.1-kb envelope HBV mRNAs that are abundantly expressed in the livers of mice of this lineage. Thus, despite the dramatic effects on HBV replication and serum HBV DNA, there was no effect of IL-12 on hepatic HBV gene expression in this model.

Members of the transgenic mouse lineage used in this study

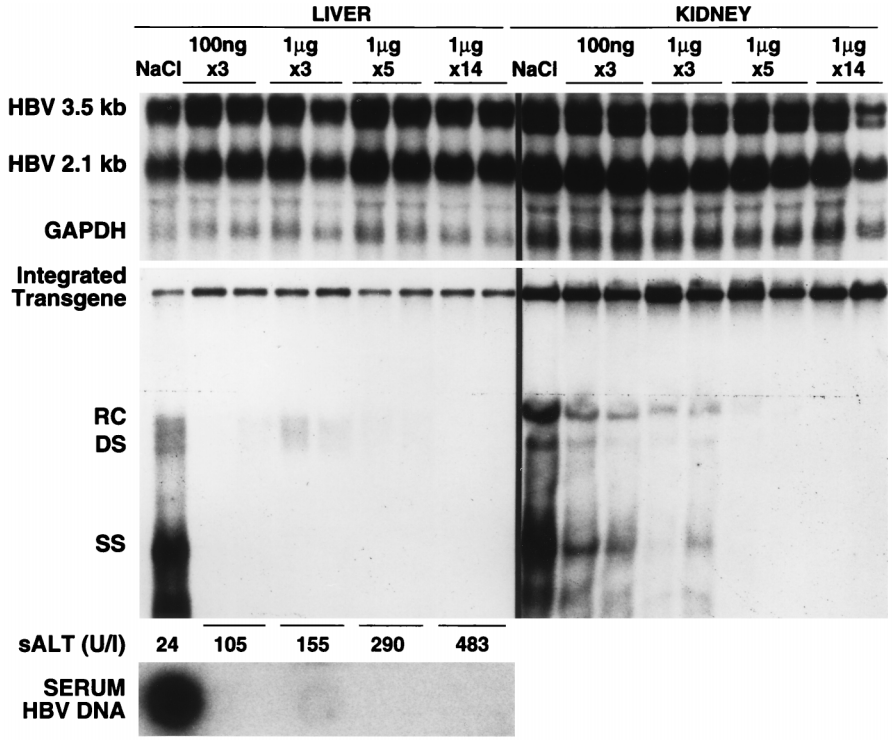

FIG. 2. IL-12-induced inhibition of HBV replication, but not gene expression, in the livers and kidneys of HBV transgenic mice. Groups of three HBV transgenic mice were injected intraperitoneally with 100 ng or 1 μg of recombinant murine IL-12 daily for 3, 5, or 14 consecutive days, as indicated, and their livers and kidneys were harvested 16 h after the last injection. (Top panel) Northern blot analysis was performed with 20 μg of total liver and kidney RNA from two representative mice per group. The filter was cohybridized with ^{32}P-labeled HBV- and GAPDH-specific DNA probes. The steady-state HBV and GAPDH mRNA content was compared with total RNA pooled from the same saline-injected controls (lanes NaCl) described in the legend to Fig. 1. Bands corresponding to the 3.5- and 2.1-kb viral mRNAs are indicated. (Middle panel) Southern blot analysis of 30 μg of total liver and kidney DNA isolated from the same mice. Control mice were injected with saline (lanes NaCl). The mean sALT activity for each group is expressed as units/liter. (Bottom panel) Dot blot analysis of HBV DNA in serum collected at the time of autopsy. Serum (400 μl) was pooled from each group, and DNA was extracted and spotted onto a filter which was then hybridized with a ^{32}P-labeled HBV-specific DNA probe.

replicate HBV to very high levels in the kidney as well as the liver (21). To determine if the administration of IL-12 also influenced HBV replication in extrahepatic sites, Southern blot analysis was performed with total DNA isolated from the kidneys of the same mice as described above. As shown in Fig. 2, mice treated with IL-12 completely cleared HBV DNA replicative forms from both tissues, although somewhat higher doses were required to inhibit the formation of HBV replicative intermediates in the kidney than in the liver. Despite these minor tissue-specific differences, it is clear that the antiviral effect of IL-12 on HBV replication extends to extrahepatic sites such as the kidney. Like the results found in the liver, there was also no effect of IL-12 on renal HBV gene expression (Fig. 2).

Cytopathic effect of IL-12 in the liver and kidney. Because IL-12 is known to be hepatotoxic at high doses (11), liver disease was measured biochemically as sALT activity in IL-12-treated animals at the time of autopsy (shown at the bottom of Fig. 1 and 2). At the minimal effective IL-12 dose (100 ng daily for 3 days), sALT levels at the time of autopsy were only slightly elevated (105 U/liter) above normal values (25 to 50 U/liter), indicating that the loss of HBV replicative forms was not due to hepatocyte destruction. Greater sALT levels were observed at the highest IL-12 dose (1 μg) given for 5 or more days (290 U/liter for 5 days and 483 U/liter for 14 days). However, even under these conditions, sALT elevations were only modest compared with previously reported sALT values observed during CTL-mediated liver disease in members of this same mouse lineage (19). For example, the sALT activity observed in HBV transgenic mice receiving 1 μg of IL-12 daily for 14 days (490 U/liter) represents less than one-fifth the activity measured in mice of the same lineage at the peak of disease severity after the adoptive transfer of 10^7 HBV-specific CTL (2800 U/liter), which was estimated to kill only a small fraction of the hepatocytes (19).

Since IL-12 is a proinflammatory cytokine, liver and kidney tissue sections were examined 16 h after the last injection for histological evidence of inflammation (Fig. 3). Foci of inflammatory cells were detected only at the highest dose of IL-12 (1 μg) administered for 5 or more days. In the liver, these foci consisted of clusters of lymphomononuclear cells infiltrating the parenchyma (Fig. 3C), with rare, scattered areas of necrosis containing dead and degenerating hepatocytes. In animals receiving 100 ng or 1 μg of IL-12 daily for 3 days, treatments which are capable of abolishing hepatic HBV replication, the livers remained histologically normal (data not shown). These results are compatible with the sALT levels shown in Fig. 1 and 2. These data indicate that at doses sufficient to inhibit HBV replication, IL-12 does not cause a histologically detectable inflammatory liver disease. More importantly, these data demonstrate that, in the liver, doses of IL-12 above the minimal effective dose (100 ng daily for 3 days) provide little additional benefit in inhibiting HBV replication (see Fig. 2) and in fact induce the appearance of hepatotoxicity.

Histological analysis of kidney tissue sections for evidence of inflammation revealed a mild interstitial nephritis consisting of infiltrating mononuclear cells in those animals receiving 1 μg of IL-12 for 7 or more consecutive days (Fig. 3F). In mice

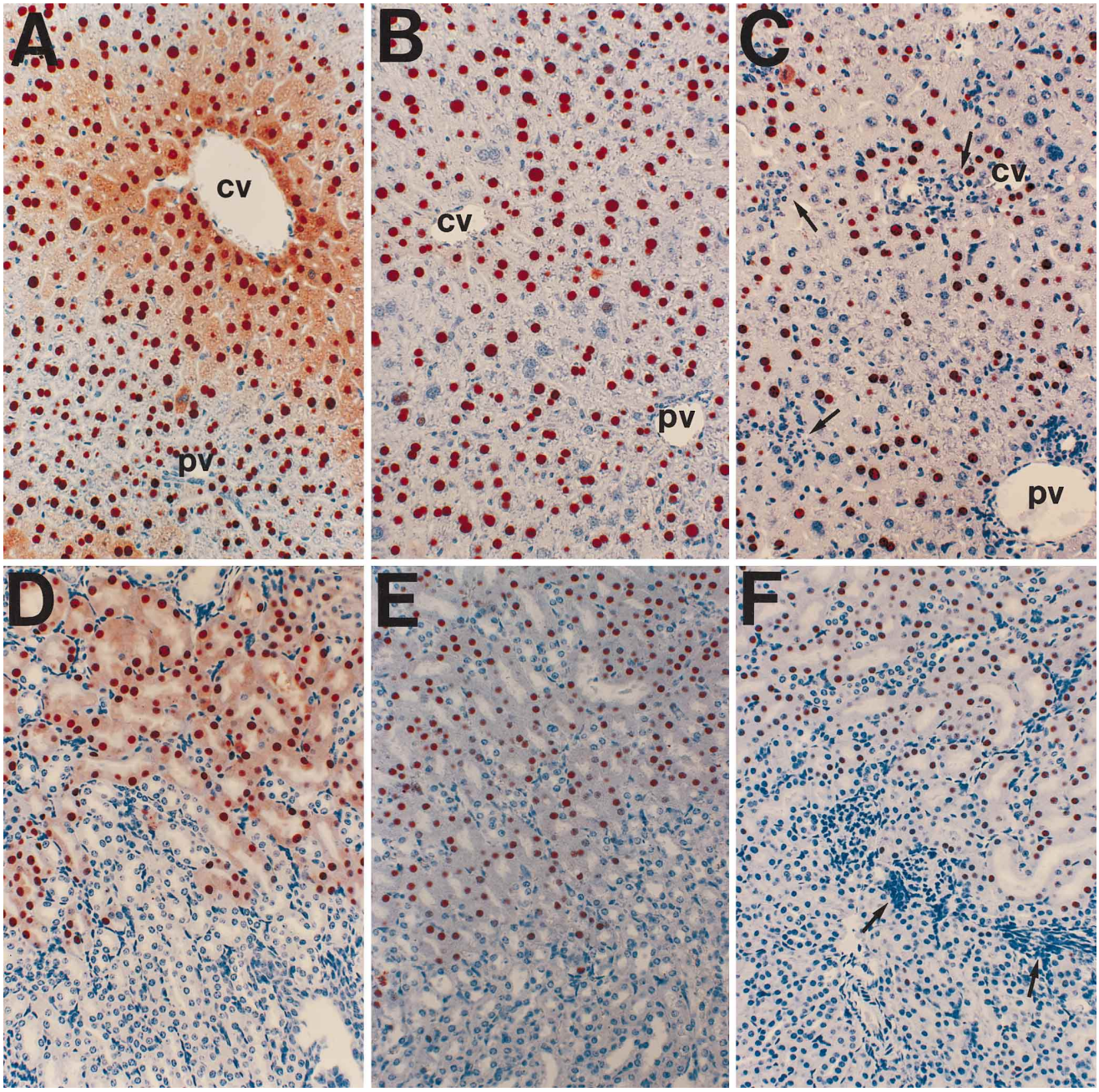

FIG. 3. IL-12-induced disappearance of cytoplasmic HBcAg in the livers and kidneys of HBV transgenic mice. Groups of three age-, sex-, and serum HBsAg-matched HBV transgenic mice were injected intraperitoneally with saline or with 1 μg of recombinant murine IL-12 daily for 3 or 7 consecutive days, and their livers and kidneys were harvested 16 h after the last injection and placed in 10% zinc-buffered formalin. Immunohistochemical analysis of HBcAg expression in liver (A to C) and kidney (D to F) sections from a saline-injected control animal (A and D) and animals receiving IL-12 treatment for 3 (B and E) or 7 (C and F) days. The arrows indicate foci of mononuclear cells. Portal veins (PV) and central veins (CV) are indicated. Immunoperoxidase stain for HBcAg; original magnification, ×200.

receiving 1 μg of IL-12 daily for 5 days, the formation of HBV replicative intermediates in the kidney was nearly abolished (Fig. 2) in the absence of histologically detectable kidney disease (data not shown), indicating that the loss of HBV DNA replicative forms from the kidney, like the liver, is not due to tissue destruction.

Cytoplasmic HBcAg disappears from the liver and kidney in IL-12-treated HBV transgenic mice. HBV DNA replication occurs inside viral nucleocapsid particles, which are detected immunohistochemically as HBcAg, in the cytoplasm of centrilobular hepatocytes and proximal renal tubular epithelial cells in these animals (21) (Fig. 3A and D). Nucleocapsid particles are also detectable in the majority of nuclei; however, these intranuclear nucleocapsid particles are empty (21). After IL-12 treatment, cytoplasmic HBcAg disappeared from both the liver (Fig. 3B) and the kidney (Fig. 3E), concomitant with the disappearance of HBV replicative forms (shown in Fig. 2). This correlation was observed with all IL-12 doses that inhibited HBV DNA replication (data not shown), suggesting that these two events are closely linked. Consistent with the lack of any

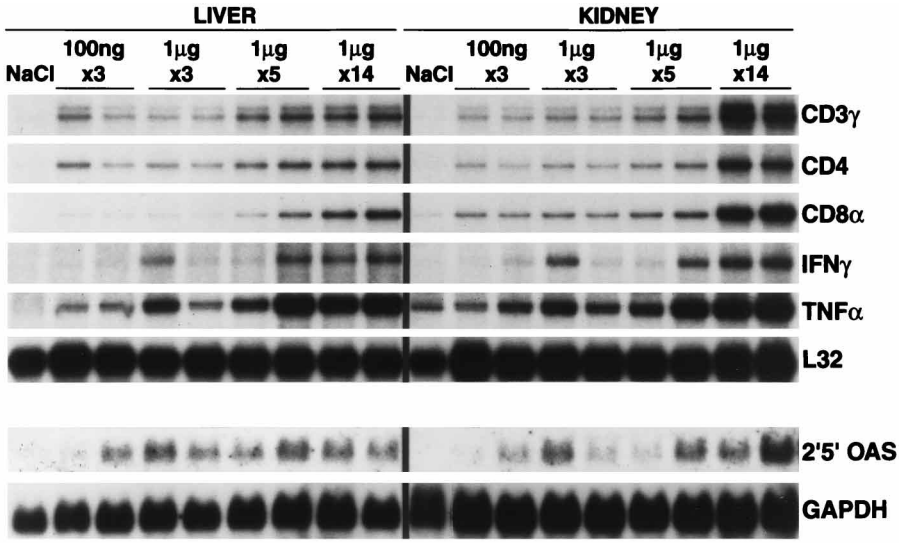

FIG. 4. Cytokine gene expression and T-cell markers induced in the livers and kidneys of HBV transgenic mice after IL-12 administration. (Top panels) Total RNA (10 μg) extracted from the livers and kidneys of the mice described in the legend to Fig. 2 was analyzed by RNase protection for the expression of IFN-γ and TNF-α cytokine transcripts and for the expression of CD3γ, CD4, and CD8α, as indicated. The mRNA encoding the ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane. The gel was exposed onto X-ray film for 20 h. (Bottom panels) The same RNA (20 μg) was analyzed by Northern blot analysis for the expression of 2′,5′-OAS, a marker of IFN-α/β induction. The housekeeping enzyme GAPDH was used to normalize the amount of RNA loaded in each lane.

effect of IL-12 on HBV RNA expression (Fig. 2), however, nuclear HBcAg was unchanged in either the liver or the kidney after IL-12 treatment (Fig. 3), suggesting that the translation of viral mRNA is unaffected by IL-12.

Cytokine gene expression induced by IL-12 administration. Because IL-12 is known to induce several cytokines, particularly IFN-γ (reviewed in reference 39), we monitored cytokine mRNA expression in the same tissues as those shown in Fig. 1 to 3. As shown in Fig. 4, total RNA was examined for the expression of TNF-α and IFN-γ by RNase protection analysis (top panels) and for the expression of 2′,5′-OAS, a marker of IFN-α/β induction, by Northern blot analysis (bottom panels). In animals receiving IL-12 doses below 100 ng, there was little or no induction of cytokine expression (data not shown). In mice treated with 100 ng of IL-12 daily for 3 days, a dose sufficient to inhibit HBV replication, cytoplasmic HBcAg expression, and viremia (Fig. 1 to 3), transcripts for TNF-α and 2′,5′-OAS were induced relative to the transcripts for housekeeping gene products (L32 and GAPDH) in both the liver and kidney (Fig. 4). At higher doses, the expression of TNF-α mRNA was further induced and messages for IFN-γ became detectable, probably reflecting the recruitment and activation of inflammatory cells, such as monocytes and lymphocytes (Fig. 3C and 3F), with the subsequent production of additional cytokines by these recruited immune cells. Consistent with these observations, we also observed a dose-dependent induction of mRNAs encoding the CD3γ, CD4, and CD8α T-cell markers in both the livers and kidneys of IL-12-treated animals. The production of IFN-α/β, indicated by the expression of 2′,5′-OAS transcripts, was not detectable at IL-12 doses of less than 100 ng (data not shown); however, low-level induction of IFN-α/β was observed at higher doses (Fig. 4). Collectively, these results demonstrate that IL-12 administration induced the expression of mRNAs for TNF-α, IFN-γ, and IFN-α/β in both the liver and kidney tissues of HBV transgenic mice at doses which induced little or no inflammation.

Suppression of HBV replication by IL-12 is mediated by IFN-γ. Presumably, the induction of cytokine mRNAs in IL-12-treated mice results in increased amounts of these proteins. To determine the extent to which the IFNs and TNF-α were responsible for the IL-12-mediated inhibition of HBV replication in these animals, we monitored the ability of neutralizing antibodies specific for these cytokines to modulate the effects of IL-12. Groups of age-, sex-, and serum HBsAg-matched HBV transgenic mice were injected intraperitoneally either with antibody specific for murine TNF-α (38), IFN-γ (36), or IFN-α/β (14) or with control antibody. In all animals, IL-12 treatment (100 ng daily for 3 days) was initiated 6 h after antibody administration. Sixteen hours after the last IL-12 injection, the livers were harvested, total hepatic DNA was extracted, and a Southern blot analysis was performed (Fig. 5). The administration of MAb specific for IFN-γ completely blocked the ability of IL-12 to inhibit hepatic HBV replication, and it also blocked the IL-12-induced clearance of cytoplasmic HBcAg from the hepatocytes in the same livers (data not shown) and the clearance of circulating virions from the serum (Fig. 5). In contrast, the antibodies against TNF-α or IFN-α/β were much less efficient and only partially blocked the effects of IL-12. These results indicate that the antiviral effect of IL-12 in the liver is mediated primarily by its ability to induce hepatic IFN-γ production in this transgenic mouse model. Although in Fig. 4 the expression of IFN-γ and TNF-α mRNAs is barely detectable at a dose of 100 ng of IL-12 daily for 3 days, they become clearly visible at this same dose upon a longer exposure of the gel to X-ray film (Fig. 5).

In addition, the induction of hepatic mRNAs encoding TNF-α, IFN-γ (Fig. 5), and IFN-α/β (data not shown) by IL-12 was greatly reduced by the anti-IFN-γ MAb, suggesting that TNF-α and IFN-α/β are both induced by IFN-γ after IL-12 administration. In addition, the TNF-α-specific antibody slightly reduced the expression of IFN-γ mRNA, suggesting that the induced TNF-α reciprocally induces IFN-γ. Finally, the IFN-α/β-specific antiserum suppressed the induction of 2′,5′-OAS mRNA (data not shown) without having any effect on messages for the other cytokines (Fig. 5).

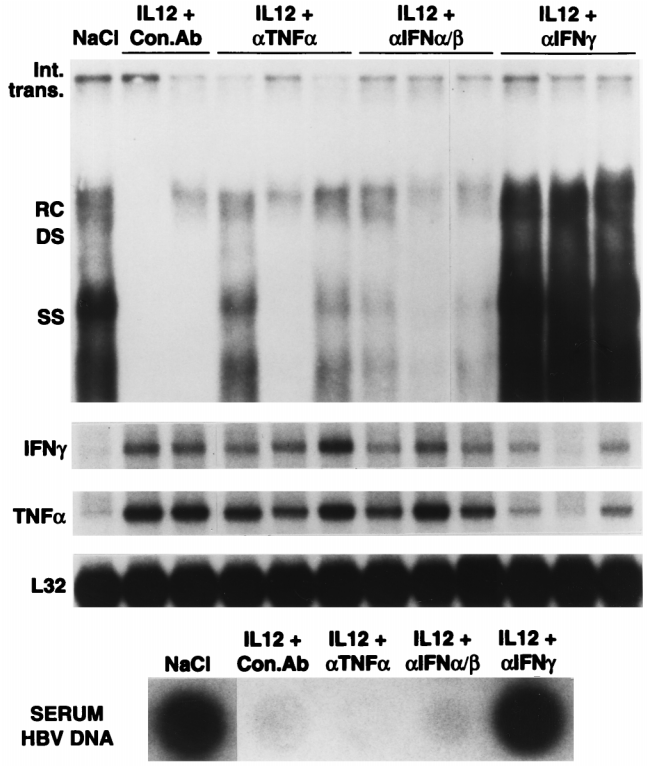

FIG. 5. Suppression of hepatic HBV replication by IL-12 is mediated principally by IFN-γ. Southern blot analysis (top panel) of total liver DNA isolated from groups of three age-, sex-, and serum HBsAg-matched HBV transgenic mice that were injected intraperitoneally with antibodies to TNF-α, IFN-α/β, or IFN-γ or with control antibody 6 h before treatment with 100 ng IL-12 daily for 3 consecutive days. The mice were sacrificed 16 h later. Total RNA (10 μg) from the same livers was analyzed by RNase protection assay (middle panels) for the expression of IFN-γ and TNF-α. The ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane. The gel was exposed to X-ray film for 7 days. Included is a dot blot analysis (bottom panel) of HBV DNA in 400 μl of serum pooled from each group at the time of autopsy. The results were compared with those for mice that were injected with saline (NaCl).

Duration of the antiviral effect of IL-12 in HBV transgenic mice. To determine the duration of the IL-12 effect, groups of transgenic animals were treated with 1 μg of IL-12 daily for 7 days and HBV replication was monitored at several times following the cessation of treatment. As shown in Fig. 6, hepatic HBV replication was profoundly inhibited for as long as 7 days after the discontinuation of IL-12 treatment. Subsequently, in keeping with the natural replication cycle of HBV, the SS linear DNA form reappeared first, followed by the appearance of the DS linear and RC forms on days 10 and 14. Consistent with these results, hepatic expression of TNF-α and IFN-γ mRNAs was induced by IL-12 and remained detectable, at diminishing levels, for up to 7 days after cessation of treatment (data not shown).

In the kidneys of the same animals as described above, HBV replication was also profoundly inhibited by IL-12 treatment. However, unlike the results in liver tissues, HBV replication in the kidney returned to pretreatment levels by day 3 after the discontinuation of treatment (data not shown). These results suggest that the kidney is more resistant to the antiviral effects of IL-12 than the liver.

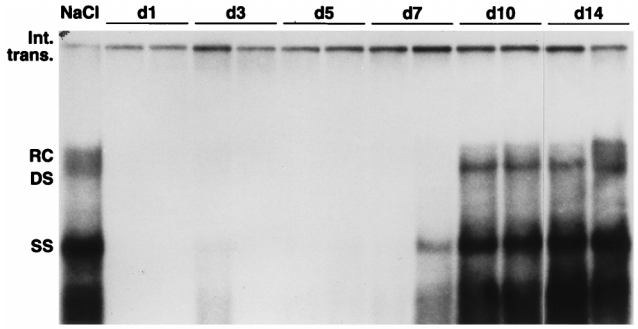

FIG. 6. Duration of the antiviral effect of IL-12. Southern blot analysis of total liver DNA isolated from groups of age-, sex-, and serum HBsAg-matched HBV transgenic mice that were injected intraperitoneally with 1 μg of IL-12 daily for 7 days and which were sacrificed at various times after the cessation of this treatment, as indicated at the top. Control mice were injected with saline (NaCl).

DISCUSSION

We have previously reported that HBV gene expression and replication are abolished in the livers of HBV transgenic mice, noncytopathically, by HBV-specific CTL that secrete IFN-γ and TNF-α upon antigen recognition (19). We have also shown that hepatic HBV replication is profoundly inhibited in these animals during lymphocytic choriomeningitis virus infection, again noncytopathically, due to the secretion of IFN-α/β and TNF-α by infected intrahepatic macrophages (16).

In the current study we show that noncytopathic doses of IL-12 cause HBV nucleocapsids to disappear (Fig. 3) and viral replication to cease in the livers and kidneys of HBV transgenic mice (Fig. 2), ultimately clearing HBV virions from their blood (Fig. 2). We also show that IL-12 induces IFN-γ, IFN-α/β, and TNF-α in both of these tissues (Fig. 4) and that the principal mediator of the antiviral effect of IL-12 is IFN-γ (Fig. 5). Finally, we show that IL-12 inhibits HBV replication in extrahepatic sites, such as the proximal convoluted tubular epithelial cells in the kidney (Fig. 2 and 3). This finding is very important because it is the first evidence that HBV can be inactivated intracellularly by antiviral cytokines in tissues other than the liver.

The pleiotropic immunostimulatory effects of IL-12, including its ability to induce IFN-γ and other inflammatory cytokines (10, 11), are well known. The cytopathic effects of IL-12, particularly its ability to cause a focal lymphomononuclear inflammatory reaction in many tissues, including the liver and kidney, have also been described (5). Importantly, the antiviral activity of IL-12 described in this report was not due to its cytopathic potential because it was easily detectable at doses that did not cause any evidence of liver or kidney disease or an increase in the number of inflammatory cells in these tissues. Collectively, these data suggest that IL-12 can induce cytokines and suppress HBV replication in the liver and kidney simply by activating resident lymphomononuclear cells, without recruiting circulating inflammatory cells into the tissues and without injuring parenchymal cells in these tissues.

In order to define the sequence of cellular and regulatory events initiated by IL-12 in these animals, we attempted to suppress the effects of IL-12 with antibodies to murine IFN-γ, TNF-α, or IFN-α/β. Anti-IFN-γ reduced the induction of all three cytokines by IL-12, strongly suggesting that IFN-γ induction is the initial event in the IL-12-activated cytokine cascade. This is compatible with reports that IFN-γ induces TNF-α gene expression in macrophages and TNF-α receptor gene expression in many cell types (10). The reciprocal is also true, as TNF-α is known to trigger its own production and release as well as the production of other inflammatory cytokines by lymphocytes and macrophages. Based on these results, we suspect that the initial effect of IL-12 is to induce IFN-γ produc-

tion by resident natural killer cells and T cells in the liver and kidney. In turn, IFN-γ probably activates resident tissue macrophages to release additional cytokines, especially TNF-α and IFN-α/β, which could further increase local cytokine synthesis and synergize with IFN-γ, thereby inhibiting HBV replication in neighboring hepatic and renal parenchymal cells as described in this report.

In addition to their ability to suppress HBV replication, IFN-γ, TNF-α, and IFN-α/β are known to inhibit HBV gene expression (13, 18, 19) by a posttranscriptional process that destabilizes the viral RNA in the nucleus of the cell and accelerates its degradation (22, 41). Despite its ability to suppress HBV replication, however, IL-12 did not inhibit hepatic or renal HBV mRNA content at any dose used in this study (Fig. 2). The reason for this is unclear at this time. Perhaps HBV replication is more susceptible to cytokine-mediated suppression than HBV gene expression and IL-12 did not induce the cytokines in sufficient quantities or in the proper ratio to inhibit viral gene expression in the treated animals. Further studies are needed to understand this unresolved question.

Since IL-12 causes cytoplasmic viral nucleocapsids and replicative DNA intermediates to disappear from the liver and kidney with similar kinetics, the two processes could be linked. Precedent for this hypothesis exists since IFN-γ and TNF-α drastically alter the morphogenesis of murine cytomegalovirus nucleocapsids, with greater than a 1,000-fold reduction in virus yield (27). Since HBV replication occurs inside of viral nucleocapsids, the current data suggest that IL-12 either accelerates the degradation of or prevents the formation of viral nucleocapsid particles as the essential event in this process (19). Both alternatives are compatible with the observation that mature RC replicative DNA intermediates appear to be more resistant to the antiviral effects of IL-12 than the less mature DS and the least mature SS linear forms (Fig. 1 and 2). Reduced capsid assembly would affect the immature SS and DS DNA forms first, allowing their preexisting counterparts to develop into more mature RC forms, and without being replaced, these less mature replicative forms would be rapidly depleted. Alternatively, the preferential degradation of immature cytoplasmic HBV nucleocapsids would have the same effect, but this would require selective recognition of immature capsids by the presumptive cytokine-inducible degradative pathway. Additional experiments are needed to resolve this very interesting issue.

The relative resistance of the kidney to the inhibitory effects of IL-12 is worthy of comment. Perhaps the tubular epithelial cells of the kidney produce less of the putative cytokine-induced cellular factors that mediate the antiviral effect than hepatocytes. The higher doses of IL-12 required to inhibit HBV replication in the kidney and the shorter duration of the antiviral effects of IL-12 in the kidney are compatible with this hypothesis. Further studies are needed to clarify this issue.

Absolute clearance of HBV from infected cells requires the elimination of the covalently closed circular (ccc) HBV DNA species that serves as the template for transcription of all viral RNAs (42). Since the HBV transgenic mice used in this study do not produce cccDNA (21), we do not know if it is also susceptible to cytokine-mediated control. However, woodchuck hepatitis virus (WHV) cccDNA disappears from the livers of massively infected woodchucks during acute WHV hepatitis in the absence of massive hepatocellular destruction or regeneration but in the presence of intrahepatic inflammation and Kupffer cell hyperplasia (25). In light of these results, it is conceivable that HBV cccDNA might be eliminated from infected cells by cytokine-dependent pathways. If this is correct, IL-12 may not only play an important role in viral clearance during acute HBV infection; it might also function as an antiviral agent for the treatment of chronically infected patients. Furthermore, IL-12 is known to enhance the development of Th1 cells, which mediate cellular immunity (39). Since it has been suggested that a predominant Th2 response to HBV may contribute to chronicity in HBV infection (28), treatment with IL-12 may favor viral clearance in chronic HBV infection because of its immunoregulatory activity (29) as well as its direct antiviral effects.

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