Steatosis and intrahepatic lymphocyte recruitment in hepatitis C virus transgenic mice

Tonino Alonzi,1 Chiara Agrati,1 Barbara Costabile,2 Carla Cicchini,2 Laura Amicone,2 Claudio Cavallari,1 Carlo Della Rocca,2 Antonella Folgori,4 Cristina Fipaldini,4 Fabrizio Poccia,1 Nicola La Monica4 and Marco Tripodi1,2

Correspondence
Nicola La Monica
nicola_lamonica@merck.com
Marco Tripodi
tripodi@bce.med.uniroma1.it

1Istituto Nazionale Malattie Infettive ‘L. Spallanzani’ IRCCS, Rome, Italy
2Fondazione ‘Istituto Pasteur Cenci-Bolognetti’, Dipartimento di Biotecnologie Cellulari ed Ematologia, Università La Sapienza, Rome, Italy
3Medicina Sperimentale, Sezione Anatomia Patologica, Università La Sapienza, Rome, Italy
4IRBM, P. Angeletti, Pomezia, Italy

Received 16 October 2003
Accepted 3 February 2004

To assess the effects of constitutive hepatitis C virus (HCV) gene expression on liver, transgenic mice carrying the entire HCV open reading frame inserted in the α1 antitrypsin (A1AT) gene were generated. Expression of A1AT/HCV mRNA was found to be mainly limited to perivascular areas of the liver as indicated by in situ hybridization analysis. HCV core protein was detected in Western blots of liver extracts, whereas the expression of E2, NS3 and NS5 proteins was revealed by immunostaining of liver samples using HCV-specific antisera. Histological analysis of HCV transgenic mice showed that these animals develop extensive steatosis, but very little necrosis of liver tissue. Moreover, a consistent T cell infiltrate and a slight hepatocyte proliferation were observed. Phenotypic analysis of cells infiltrating the liver indicated that recruitment and/or expansion of residing CD8+, NK, NKT and CD1d+ T cells occurred in transgenic animals. Among these cells, a large fraction of CD8+ T lymphocytes released mainly IL-10 and, to a lesser extent, IFN-γ upon mitogenic stimulation in vitro. Furthermore, both intrahepatic lymphocytes and splenocytes did not produce cytokines in response to HCV antigens. Thus, these data indicate that constitutive expression of HCV proteins may be responsible for intrahepatic lymphocyte recruitment in absence of viral antigen recognition. This response is likely to be driven by virus-induced cellular factors and may play a significant role in the immunopathology of chronic HCV infection and liver disease.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae and is the major cause of non-A, non-B hepatitis (Choo et al., 1989; Houghton et al., 1991; Kuo et al., 1989). HCV has a single-stranded RNA genome of positive polarity of about 9.5 kb that contains a long open reading frame (ORF) encoding a polyprotein of approximately 3000 amino acids (Choo et al., 1991; Kato et al., 1990; Takamizawa et al., 1991). The polyprotein precursor has the following gene order: NH2-C-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b COOH (Eckart et al., 1993; Grakoui et al., 1993; Hijikata et al., 1993a, b; Lin et al., 1994; Tomei et al., 1993) C is an RNA-binding protein (Santolini et al., 1994) and is considered to be the viral nucleocapsid; E1 and E2 are thought to be the virion glycoproteins; p7 is a protein involved in the formation of ion channels (Griffin et al., 2004) and is inefficiently cleaved from the E2 polypeptide (Mizushima et al., 1994). Processing of the structural polypeptides is mediated by cellular signal peptidase (Hijikata et al., 1993b; Santolini et al., 1994). The HCV-encoded metalloprotease and serine protease located in the NS2 to NS3 region and N-terminal one-third of NS3 protein, respectively, mediate cleavages in the nonstructural region (Clarke, 1997).

More than 170 million people worldwide are infected with HCV, and the complications of liver cirrhosis and hepatocellular carcinoma cause significant morbidity and mortality. The virus is cleared in a minority of patients and 70–80% develop chronic infection that leads to cirrhosis within 20–30 years. Although the current treatment regimens (IFN-x and ribavirin) help to eliminate or reduce the virus load in some patients, these treatments often fail (Armstrong et al., 2000; Lauer & Walker, 2001). Thus, understanding the mechanism responsible for progressive liver disease in chronic HCV infection would be highly beneficial in designing new strategies for therapeutic intervention.
Immune mechanisms are thought to be responsible, as well as for virus clearance (Gonzales-Peralta et al., 1994; Cerny & Chisari, 1999; Bertoletti et al., 2000). Acute self-limited hepatitis C is generally associated with the detection of HCV-specific T cells with a predominant production of Th1 cytokines (Gerlach et al., 1999; Gruner et al., 2000). A prevalent Th2 pattern of cytokine production is instead associated with virus persistence and development of chronic infection (Tsai et al., 1997; Woitas et al., 1997). Patients with long-term chronic HCV infection also display increased intrahepatic expression of Th1-associated cytokines that correlates with progressive liver injury (Napolli et al., 1996). Therefore, recruitment and activation of immunocompetent cells causing hepatocyte necrosis is directly related to the pathogenesis of acute hepatitis C. Moreover, the HCV proteins may be involved in the development of pathogenesis of chronic hepatitis and of hepatocellular carcinoma (HCC) development by modifying hepatocyte gene expression.

The direct role of HCV proteins in hepatitis and liver cancer is supported by the observation that core, NS3, NS4B and NS5A transform fibroblasts, modulate gene transcription and modify the susceptibility of cultured cells to apoptotic signals (Ghosh et al., 1999; Marusawa et al., 1999; Park et al., 2000; Ray et al., 1996; Ruggieri et al., 1997; Sakamuro et al., 1995; Tan et al., 1999; Tanaka et al., 1996). Additionally, as observed in hepatitis B virus-infected patients, the presence of inflammatory cell infiltrates in necrotic lesions of infected livers strongly suggests a pathogenic role of the host immune response (Cerny & Chisari, 1999). The hypothesis that expression of HCV proteins directly injures liver cells triggering an inflammatory response is attractive but difficult to test directly in the absence of a robust tissue culture system or small animal models that mimic HCV infection in humans. As an alternative, transgenic mice represent a powerful tool to investigate the role of chronic HCV gene expression in liver disease. A transgenic mouse line expressing the entire HCV genome under the control of the A1AT promoter was generated to study the direct effect of inflammatory cell infiltrates in necrotic lesions of infected mice. Moreover, impaired interferon-induced intracellular STAT signalling and enhanced susceptibility to lymphocytic choriomeningitis virus was detected in 7–14-week-old animals showing no liver damage (Blindenbacher et al., 2003). In this study, we show that transgenic HCV animals develop with ageing extensive steatosis and limited necrosis in hepatic tissue that are reminiscent of that seen in liver biopsies from patients with hepatitis C infection. More interestingly, a consistent T cell infiltrate made up largely of CD8 T cells secreting Th2-type cytokines and a limited hepatocyte proliferation were observed. The pathogenesis of liver cell injury associated with HCV polyprotein expression is discussed.

METHODS

Generation of HCV transgenic mice. A 9.0 kb cDNA fragment derived from plasmid pCD(38-9.4) (Tomei et al., 1993), covering the HCV ORF (aa 3-3010), was used to generate HCV transgenic mice. By means of homologous recombination in DH1 bacterial cells (Tripodi et al., 1990) the HCV cDNA (nt 339–9416) was inserted downstream of the ATG (exon II) of an A1AT minigene by using the donor plasmid pScull-V ex A1AT (Amicone et al., 1997).

Transgenic C57Bl/6 mice were generated by BRL (Basel, Switzerland) as described (Hogan et al., 1996). Genotype analysis was performed by Southern blot analysis on 10 µg of genomic DNA using an HCV cDNA fragment as probe (nt 1348–2916).

Animals and treatments. Mice were maintained under specific pathogen-free conditions in either IRBM or Charles River facilities under a 12 h light/dark cycle, and provided with irradiated food and autoclaved water ad libitum. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. Bromodeoxyuridine (BrDU; Sigma) was administrated for 1 week [1 mg ml⁻¹ in regular drinking water, supplemented of 1% sucrose (Sigma)]. Mice were euthanized by CO₂ asphyxiation and livers collected immediately.

RNA extraction, RT-PCR and in situ hybridization analysis. RNA was isolated from frozen tissues using Ultraspec RNA reagent (Bioteck) according to the manufacturer’s instructions. RT-PCR with a Promega kit using as oligonucleotides 5'-GGGCGCATGGCTGATCTCC-3' (sense) and 5'-GGCCGCAGGAATTCGTTG-3' (antisense) for HCV (nt 2613–3241) and 5'-ATGGAATGACATGCTGG-3' (sense) and 5'-ATCTTACGAGTTGCACGCGG-3' (antisense) for the β-actin gene.

In situ hybridization was performed according to Bloch (1993). A linearized plasmid containing the region 4041–4840 of HCV flanked by promoter sequences for T7 and T3 RNA polymerases was used to transcribe digoxigenin-labelled sense and antisense riboprobes using Riboprobe Combination System T3/T7 (Promega), according to the manufacturer’s instructions.

Ten µm tissue sections were hybridized overnight at 55 °C in 50% deionized formamide, 4 × SSC, 1 × Denhardt’s solution, 1 µg salmon sperm DNA ml⁻¹, 1 µg rRNA ml⁻¹ and 1–5–3 ng DIG-labelled cRNA probe ml⁻¹. Tissue slides were washed at 37 °C in 2 × SSC, then treated with 20 µg RNaseA ml⁻¹ at 37 °C for 30 min, washed at 65 °C in 2 × SSC and 0.1 × SSC for 1 h, and finally analysed using AP-conjugated anti-digoxigenin Fab fragments (Roche) and NBT/BCIP. Sense cRNA probes were used as control of hybridization specificity.

Western blot analysis. Total protein extracts were prepared by homogenizing livers in buffer A (10 mM HEPES PH 7.9, 10 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 10 µg leupeptin ml⁻¹, 10 µg pepstatin ml⁻¹, 5 µg anti-papain ml⁻¹) containing 0.05% NP40. The nuclei, pelleted by centrifugation, were lysed in buffer C (20 mM HEPES PH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 10 µg leupeptin ml⁻¹, 10 µg pepstatin ml⁻¹, 5 µg anti-papain ml⁻¹): Samples of 100 µg of proteins were used for Western blotting analysis as described (Esposito et al., 1995) using either anti-HCV rabbit antiserum (Tomei et al., 1993), mAbs or serum from HCV-infected patients.

Immunohistological analysis of liver tissues. Liver samples were fixed in 10% (w/v) buffered formalin, embedded in paraffin. For histopathological analysis 4–8 µm thick sections were stained with haematoxylin and eosin. For detection of HCV proteins and BrdU incorporation rabbit polyclonal Abs to E2, NS3 and NS5 (a+b) (Tomei et al., 1993) or a mAb to BrdU (Roche) were used.
respectively. Antigens were retrieved by trypsin treatment (0.1%, w/v, in a solution of 7 mM CaCl₂; 50 mM Tris; 150 mM NaCl; pH 7-8; Dako) and boiling in 10 mM citrate buffer pH 6-0. Sections were incubated with primary antibodies, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG (Dako). Antibody binding was revealed using the Fast Red Substrate System (Dako) on haematoxylin-counterstained sections.

**Isolation of spleen and intrahepatic lymphocytes.** Spleens and livers from wild-type and transgenic mice were weighed, washed with RPMI 1640 (supplemented with 10% heat inactivated FCS and 10 U penicillin/streptomycin ml⁻¹) and manually homogenized using a 70 µm diameter filter (Becton Dickinson). Recovered cells were washed twice and counted to assess the number of lymphocytes per mg of tissue. Mononuclear cells were obtained by Ficoll–Hypaque gradient centrifugation.

**Monoclonal antibodies.** mAbs (Becton Dickinson) coupled to different fluorochromes were combined for simultaneous triple/quadruple staining. Anti-CD3 (17A2), anti-B220 (RA3-6B2), anti-NK1.1 (PK136), anti-IFN-γ (XMGL1.2) and anti-IL-2 (JES6-5H4) were FITC-coupled. Anti-γ/δ (GL3), anti-Pan-NK (DX5), anti-CD25 (3C7), anti-IL-4 (BVD4-1D11) and anti-IL-10 (JE55-16E3) were phycoerythrin (PE)-coupled. Anti-CD8-cy5 (53-6.7) and anti-IL-4 (BVD6-24G2). Intrahepatic lymphocytes were detected by using anti-mouse IL-4 antibodies from Pharmingen (rat anti-mouse IL-4 mAb clone BVD6-24G2). Intrahepatic lymphocytes were detected by using anti-mouse IL-4 antibodies from Pharmingen (rat anti-mouse IL-4 mAb clone BVD6-24G2). Intrahepatic lymphocytes were detected by using anti-mouse IL-4 antibodies from Pharmingen (rat anti-mouse IL-4 mAb clone BVD6-24G2).

**Flow cytometry for surface antigens.** For the analysis of surface antigen expression 5 × 10⁵ splenic or 3 × 10⁵ intrahepatic lymphocytes were incubated for 15 min at 4°C with the mAbs listed above. After washing in 1X PBS containing 1% BSA and 0.1% sodium azide, samples were fixed in 4% paraformaldehyde and acquired by a FACScalibur flow cytometer (Becton Dickinson). Twenty thousand events were acquired for each sample and analysed with the CellQuest software (Becton Dickinson).

**Single-cell analysis of cytokine synthesis.** To measure cytokine production splenic or intrahepatic lymphocytes were stimulated overnight with phorbol 12-myristate 13-acetate (PMA; 50 ng ml⁻¹) and ionomycin (5 µg ml⁻¹). Monensin (10 µg ml⁻¹) was added after 1 hour to stimulate block intracellular transport processes. Cells were stained with anti-CD8-Cy5, anti-CD4–APC mAbs for 15 min at 4°C and then fixed in PBS/1% paraformaldehyde for 10 min at 4°C and incubated with anti-cytokine-specific mAb in the presence of 0.5% saponin to permeabilize the cell membrane. Cells were acquired on a FACScan (Becton Dickinson), using isotype-matched mAbs as control for unspecific staining.

**ELISPOT assays on splenocytes and intrahepatic lymphocytes.** Cells secreting IFN-γ in an antigen-specific manner were detected using a standard enzyme-linked immunospot assay on splenocytes as described (Zucchelli et al., 2000). Cells secreting IL-4 were detected by using anti-mouse IL-4 antibodies from Pharmingen (rat anti-mouse IL-4 mAb clone B11 and biotinylated rat anti-mouse IL-4 mAb clone BVD-264G2). Intrahepatic lymphocytes were prepared as described above and plated at 5 × 10⁴, 2.5 × 10⁵, 1 × 10⁶, 5 × 10⁶ cells per well in the presence of 2.5 × 10⁵ per well of syngeneic antigen-presenting cells. Concanavalin A at 10 µg ml⁻¹ was used as a non-specific stimulus for T cells in all assays. Twenty-amygoid-acid-long peptides, overlapping by 10 residues, were used in the assay at 5 µg ml⁻¹. To facilitate the analysis the peptides were collected in pools: pools C and E encompass, respectively, the sequence of HCV Core and E2 protein from the 1a viral genotype, strain H; pools F, G, H, I and L encompass the sequence of the NS region from NS3 to NS5b (aa 1026–2418) from the 1b viral genotype, BK strain. Immunized mice used as a positive control were electroinjected with 5 µg of plasmid pF78E2 as described (Zucchelli et al., 2000).

**RESULTS**

**Generation of transgenic lineage**

To assess the effects of HCV gene expression on liver pathobiology, a transgenic mouse model of HCV chronic infection was generated, carrying the entire HCV ORF inserted into the human α1-antitrypsin gene (A1AT). The HCV coding sequence derived from an HCV type 1b clone (Takamizawa et al., 1991) was fused to the human A1AT gene to ensure liver-specific expression and high levels of mRNA, as shown for the liver of transgenic mice by Amicone et al. (1997). As shown in Fig. 1(A), the HCV ORF was inserted between the second and fourth exon of the A1AT gene just downstream of the A1AT ATG, resulting in an additional 32 aa fusion to the HCV polyprotein (aa 3–3010).

Northern blot analysis was performed on total RNA from different organs of adult mice to verify A1AT/HCV transgene expression. As previously shown, the predicted 10 kb mRNA spliced transcript derived from the transgene was detected only in the liver (Blindenbacher et al., 2003); no A1AT/HCV mRNA was detected by RT-PCR analysis in the thymus of the transgenic mice (Fig. 1B). The distribution of A1AT/HCV mRNA within the hepatic lobules was assessed by in situ hybridization analysis using an HCV-specific antisense riboprobe. Transgene expression was mostly detected in hepatocytes in the perivascular region reproducing, at least in part, the tonality of A1AT expression (Amicone et al., 1997) (Fig. 1C). However, scattered transgene-expressing hepatocytes were also found. In contrast, no transgene expression was detected in wild-type littermates (Fig. 1D).

High titre anti-HCV-positive sera of chronically infected patients and polyclonal (Tomei et al., 1993) and mAb specific for the individual HCV proteins were used in Western blots and immunohistochemical analysis of transgenic and wild-type liver samples. As shown in Fig. 1(E), a band of 22 kDa was detected in transgenic liver extracts by anti-HCV antisera. A similar protein product was also recognized by a monoclonal antiserum specific for the core protein (data not shown). Moreover, expression of the A1AT/HCV transgene appeared to be fairly constant in mice, as indicated by the presence of similar amount of core protein in mice up to 18 months of age.

Antiseria specific for other HCV proteins did not reveal additional immunoreactive polypeptides by Western blot analysis (data not shown). However, immunohistochemical analysis showed the expression of E2, NS3 and NS5a-b proteins in the transgenic livers (Fig. 2A–B, D–E and G–H, respectively) but not in wild-type littermates (Fig. 2C, F, I). Taken together, these data demonstrate that the transgenic
mouse line carries the entire A1AT/HCV transgene and that translation of the transgenic HCV coding sequence spans the entire ORF.

**Immunohistological analysis of liver tissue**

To verify the effects of constitutive HCV gene expression, histopathological analysis of haematoxylin and eosin-stained liver sections from mice ranging from 1 to 18 months of age was carried out. This histological examination of 86 transgenic animals indicated that the overall architecture of the transgenic livers was always preserved. Three different phenotypes were observed: (i) a diffuse intracellular microvacuolar steatosis observed as the major pathological change in 73% of mice examined (Fig. 3A, B). This phenotype was detected mainly in mice of 3–4 months up to 14 months of age and declined in mice 15–18 months of age. (ii) Microvacuolar steatosis associated with multiple small inflammatory infiltrates detected in 43% of the transgenic animals ranging from 3 to 18 months of age.
Fig. 2. Immunohistochemical detection of HCV proteins in liver of 3-month-old A1AT/HCV transgenic mice. Paraffin-embedded sections of transgenic (panels A, B, D, E, G and H) and wild-type mice (panels C, F and I) were stained with rabbit polyclonal antibodies to E2 (A–C), NS3 (D–F) and mouse polyclonal antibody to NS5a+b (G–I) and revealed by specific secondary antibody–alkaline phosphatase conjugates. Magnification 4× (panel A); magnification 10× (panels B, C, D, F, G and I); magnification 25× (panels E and H). Scale bar, 50 μm.
These infiltrates were mostly lymphocytes distributed in the lobular parenchyma and only occasionally located near the centre-lobular or portal vessels. The liver cells near the infiltrates often showed eosinophilic condensation of the cytoplasm (Fig. 3C, D). (iii) Multiple large inflammatory infiltrates without any significant steatosis were present in 20% of mice examined ranging from 3 to 18 months of age. These large infiltrates were always located in the periportal areas and invaded the lobular parenchyma, often involving centre-lobular perivascular regions. They were nodular in

Fig. 3. Liver histology and hepatocyte proliferation in transgenic mice. Livers of 86 animals ranging from 1 to 18 months of age were analysed by haematoxylin and eosin (H/E) staining; images are of representative liver sections of 14-month-old mice (panels A–F). Hepatocyte proliferation was assessed in five transgenic and four wild-type animals (4–5 months of age). Mice were given BrdU for a week in drinking water and liver sections were then immunostained with an anti-BrdU mAb. At least 3000 hepatocyte nuclei for each section were scored and the percentage of BrdU-positive cells was calculated [(no. of BrdU-positive nuclei/no. of total nuclei) \times 100] (panels G, H). (A) H/E; magnification 10 x. The liver architecture is preserved, and a diffuse steatosis is present. (B) Same as panel (A), H/E, magnification 25 x. (C) H/E, magnification 10 x. Multiple mononuclear inflammatory infiltrates are present in the liver parenchyma not related to portal spaces or centrolobular vessels. (D) Same as panel (C), H/E, magnification 25 x. The lymphocytic infiltration is centred on necrotic liver cells; neighbouring hepatocytes show eosinophilic condensation of the cytoplasm. (E) H/E, magnification 10 x. A large mononuclear infiltrate involving perivascular and lobular areas is evident. (F) Same as panel (E), H/E, magnification 25 x. The mononuclear cells of the extensive infiltrate are almost totally represented by lymphocytes and plasma cells. (G) BrdU treatment, magnification 10 x. A positive signal is present in a large number of hepatocytes. An inflammatory infiltrate shows positive elements also. The steatosis is intracellular and microvacuolar in type. (H) Same as panel (G), BrdU treatment, magnification 25 x. At the higher magnification a percentage of the mononuclear cells of the inflammatory infiltrates are also positive. Scale bar, 50 \mu m.
shape, and mainly made up of plasma cells and lymphocytes (Fig. 3E, F). No lymphocytic infiltrates were noted in mice less than 3 months old (data not shown).

The liver of the transgenic animals was further characterized by measuring hepatocyte proliferation, by BrdU incorporation. Immunohistochemical analysis on control mice showed intense labelling mainly in non-parenchymal cells and only in 0.5 ± 0.49 % of hepatocytes, whereas intense hepatocyte staining (3.72 ± 1.34 %) was clearly detectable in transgenic animals. Additionally, immunostaining of infiltrating foci revealed limited lymphocyte proliferation (Fig. 3G, H).

Thus, expression of HCV proteins in transgenic livers appears to be associated with several histological alterations that are highly indicative of chronic HCV hepatitis in humans. Such alterations, however, were not accompanied by the induction of HCV-specific antibodies or by any significant alteration in the alanine aminotransferase levels (data not shown).

Characterization of intrahepatic lymphocytes

The cell subsets infiltrating the liver of 14 transgenic and 16 wild-type littermate animals were analysed by flow cytometry. Considering that large inflammatory infiltrates were present only in a minority of transgenic animals, mice featuring large nodular perivascular infiltrates (Fig. 3E) were excluded. The total number of lymphocytes was significantly higher in the liver of transgenic mice (2- to 3-fold increase over wild-type; Table 1). Lymphocytes homing significantly higher in the liver of transgenic mice (2- to 3-fold increase over wild-type; Table 1).

Table 1. Phenotypic characterization of lymphocyte subsets in the spleen and liver of wild-type and transgenic mice

The total number of either non-hepatocyte liver infiltrating cells or intrasplenic cells was manually counted from 16 wild-type (WT) mice and 14 transgenic (TG) mice (10–12 months old). The number of cells of different lymphocyte subsets was evaluated by flow cytometry. Results are expressed as mean percentage ± SEM of 10^6 lymphocytes per mg of tissue. Symbols indicate statistically significant differences between the two groups of mice by Student’s t-test: **P<0.002; ***P<0.005, #P<0.0001.

<table>
<thead>
<tr>
<th></th>
<th>Spleen WT (± SEM)</th>
<th>Spleen TG (± SEM)</th>
<th>Liver WT (± SEM)</th>
<th>Liver TG (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cell no.</strong></td>
<td>709 ± 1 ± 111 ± 1</td>
<td>592 ± 2 ± 57 ± 6</td>
<td>4.65 ± 1 ± 13</td>
<td>9.92 ± 1 ± 72 (**)</td>
</tr>
<tr>
<td>CD3</td>
<td>181.6 ± 25.7</td>
<td>129.6 ± 10.6</td>
<td>1.59 ± 0.49</td>
<td>3.56 ± 0.60 (**)</td>
</tr>
<tr>
<td>CD4</td>
<td>111.6 ± 24.7</td>
<td>89.4 ± 8.8</td>
<td>0.99 ± 0.29</td>
<td>1.70 ± 0.30</td>
</tr>
<tr>
<td>CD8</td>
<td>69.3 ± 15.7</td>
<td>55.8 ± 5.8</td>
<td>0.86 ± 0.22</td>
<td>2 ± 0.31 (**)</td>
</tr>
<tr>
<td>NK cells</td>
<td>28.5 ± 3.8</td>
<td>40.3 ± 5.9</td>
<td>0.32 ± 0.04</td>
<td>0.84 ± 0.14 (**)</td>
</tr>
<tr>
<td>NKT cells</td>
<td>15.3 ± 2.2</td>
<td>14.2 ± 1.6</td>
<td>0.25 ± 0.04</td>
<td>0.53 ± 0.04 (**)</td>
</tr>
<tr>
<td>γδ TCR</td>
<td>13.2 ± 1.2</td>
<td>17.3 ± 1.8</td>
<td>0.52 ± 0.15</td>
<td>1.3 ± 0.14 (**)</td>
</tr>
<tr>
<td>B cells</td>
<td>242.9 ± 40.1</td>
<td>248.2 ± 15.7</td>
<td>2.90 ± 0.88</td>
<td>3.98 ± 0.96</td>
</tr>
</tbody>
</table>

Lymphocyte recruitment in HCV transgenic mice

To assess if T cell recruitment to the liver was mediated by an immune response directed against the HCV proteins, antigen-specific release of cytokines by intrahepatic and splenic T cells was measured by IFN-γ and IL-4 ELISPOT assays. Pools of 20-mer peptides spanning 72 % of the entire HCV polyprotein were used to stimulate the production of either IFN-γ or IL-4 by CD4^+ and CD8^+ T cells isolated from wild-type and transgenic mice. As a control, production of cytokines was also measured in mice immunized by electroinjection of plasmid pF78E2, encoding the E2 glycoprotein of HCV and shown to elicit a full T cell response spectrum in mice (Zucchelli et al., 2000). Although significant IFN-γ production was detected in control immunized mice, and upon exposure of the transgenic T cells to Concanavalin A, the assay did not detect any specific cytokine production in intrahepatic lymphocytes and splenocytes of transgenic mice (Table 2). Similarly, no IL-4 secretion by intrahepatic lymphocytes and splenocytes of transgenic mice upon exposure to HCV-derived peptides was observed (data not shown). Thus, these data demonstrate a lack of an immune response directed against the HCV proteins in transgenic animals.

Mouse naive T cells are characterized by the expression of CD44^low and, when activated, by the transition to the CD44^med/high phenotype and CD25 expression. These...
Table 2. IFN-γ ELISPOT assay on intrahepatic lymphocytes and splenocytes

The number of IFN-γ-secreting intrahepatic lymphocytes and splenocytes isolated from four transgenic (Tg) mice and one wild-type (WT) mouse was determined by ELISPOT using pools of overlapping peptides covering HCV core, E2, NS3, NS4, NS5a and part of NS5b proteins (Pools C, E, F, G, H, I and L respectively). DMSO and ConA represent negative and positive controls, respectively. In (A) numbers represent spot forming cells (SFC) per 10⁶ intrahepatic lymphocytes. Mean SFC per 10⁶ splenocytes from mice are reported in the last column (Pos. control). The same negative responses in transgenic mice were obtained with intrahepatic lymphocytes and splenocytes when anti-mouse IL-4 antibodies were used in the assays (not shown). tmtc, Too many to count.

(A) Intrahepatic lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>Tg#1</th>
<th>Tg#2</th>
<th>Tg#3</th>
<th>Tg#4</th>
<th>WT mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool E</td>
<td>8</td>
<td>nt</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Pool C</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pool F</td>
<td>8</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pool G</td>
<td>3</td>
<td>nt</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Pool H</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pool L</td>
<td>5</td>
<td>6</td>
<td>9</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>tmtc</td>
<td>tmtc</td>
<td>tmtc</td>
<td>tmtc</td>
<td></td>
</tr>
</tbody>
</table>

(B) Splenocytes

<table>
<thead>
<tr>
<th></th>
<th>Tg#1</th>
<th>Tg#2</th>
<th>Tg#3</th>
<th>Tg#4</th>
<th>WT mouse</th>
<th>Pos. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool E</td>
<td>8</td>
<td>7</td>
<td>11</td>
<td>7</td>
<td>15</td>
<td>417</td>
</tr>
<tr>
<td>Pool C</td>
<td>13</td>
<td>11</td>
<td>5</td>
<td>11</td>
<td>14</td>
<td>nt</td>
</tr>
<tr>
<td>Pool F</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>17</td>
<td>nt</td>
</tr>
<tr>
<td>Pool G</td>
<td>14</td>
<td>9</td>
<td>5</td>
<td>10</td>
<td>12</td>
<td>nt</td>
</tr>
<tr>
<td>Pool H</td>
<td>14</td>
<td>8</td>
<td>4</td>
<td>13</td>
<td>16</td>
<td>nt</td>
</tr>
<tr>
<td>Pool I</td>
<td>15</td>
<td>8</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>nt</td>
</tr>
<tr>
<td>Pool L</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>14</td>
<td>16</td>
<td>nt</td>
</tr>
<tr>
<td>DMSO</td>
<td>10</td>
<td>13</td>
<td>7</td>
<td>13</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>ConA</td>
<td>tmtc</td>
<td>tmtc</td>
<td>tmtc</td>
<td>tmtc</td>
<td>tmtc</td>
<td>tmtc</td>
</tr>
</tbody>
</table>

markers on intrahepatic and splenic CD4+ and CD8+ T cells were analysed by flow cytometry. Interestingly, wild-type and transgenic T cells reveal no differences in CD25 and CD44 expression by both CD4+ (Fig. 4A, C) and CD8+ (Fig. 4B, D) subpopulations. Since antigen recognition by T lymphocytes induces CD25 expression on the cell surface and a switch from naive (CD44med/med) to memory (CD44med/med) phenotype, this result confirms that the constitutive expression of HCV proteins does not lead to the activation of the lymphocytes recruited to the liver.

Finally, to evaluate the production of cytokines by T lymphocytes both CD8+ and CD4+ T cells isolated from wild-type and transgenic mouse liver and spleen were analysed by intracellular staining. Since we failed to detect any production of cytokines by intrahepatic T cells directly ex vivo (data not shown), we used mitogenic stimulation by PMA and ionomycin. No change in the frequency of cytokine production was observed in the spleen (Fig. 4E, F). Interestingly, in the liver, while no differences were measured among cytokine-releasing CD4+ T cells (Fig. 4G), the number of CD8+ T cells producing cytokines was significantly increased (Fig. 4H). These cells mainly produced IL-10, IL-4 and IFN-γ, albeit to a lesser extent (Fig. 4H). Thus, these data indicate that the liver of transgenic mice is enriched for CD8+ T lymphocytes mainly releasing IL-10.

**DISCUSSION**

The A1AT/HCV transgenic mice display a complex phenotype characterized by steatosis, recruitment and/or expansion of intrahepatic lymphocytes and hepatocyte proliferation. Additionally, hepatic expression of the HCV proteins in these mice leads to inhibition of the interferon-induced Jak-STAT pathway (Blindenbacher et al., 2003), a phenomenon also observed in liver biopsies from patients with chronic hepatitis C (Duong et al., 2004). Thus, this animal model displays some of the pathological features of chronic viral hepatitis in humans, where the above characteristics have been observed (Bach et al., 1992; Bianchi et al., 1987).

The liver of HCV-infected patients is enriched in Th1-type T, NK and NKT cells with cytotoxic activity (Agrati et al., 1995). Thus, the recruitment of hepatic lymphocytes to the liver may be caused by an alteration of hepatocyte gene expression induced by the viral products.

Expression of HCV proteins was noted primarily in the perivascular area, although scattered A1AT/HCV-expressing hepatocytes were also detected. In contrast, lymphocyte recruitment was mainly detected in the parenchyma, suggesting that their localization is not only related to the site of transgene expression but may reflect a more generalized alteration in the microenvironment of the liver that acts as a signal for lymphocyte recruitment. We therefore analysed the gene expression profiles of some
chemokines present in the liver of wild-type and A1AT/HCV mice. No significant differences were found in mRNA levels of Rantes, eotaxin, MIP-1α, MIP-1β, MIP-2, IP-10 and TCA-3, indicating that these chemokines are not responsible for lymphocyte recruitment in transgenic livers (data not shown). The molecular patterns involved in cytotoxic T cell recruitment in the liver of HCV transgenic mice and, in general, in HCV-infected patients remain to be elucidated.

The intrahepatic CD8+ T lymphocytes of transgenic mice were found to produce more cytokines, mainly IL-10, when stimulated with PMA and ionomycin (Fig. 4). IL-10 is a multifunctional cytokine known for its crucial role in regulating immune and inflammatory responses. Among its activities, IL-10 regulates growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells (reviewed by Moore et al., 2001; Akdis & Blaser, 2001). It is tempting to speculate that IL-10 may play a role in the control of immune responses and tolerance against HCV in the liver. In accordance with this hypothesis, Nelson et al. (2003) recently reported that long-term therapy of HCV patients with rIL-10 decreased disease activity and led to increased HCV viral burden via alterations in immunological viral surveillance.

Several transgenic mouse lines expressing portions of the HCV ORF or the entire coding sequence have been reported to be associated with a variety of phenotypes including...
steatosis, necrosis, sensitivity to Fas-induced apoptosis and HCC (reviewed by Koike, 1999; Fimia et al., 2003). The hepatic alterations observed in the A1AT/HCV mice are partially in agreement with these published reports. Although positional effects of the exogenous A1AT/HCV gene cannot be determined based only on results from one mouse line, the data collected support the validity of the A1AT/HCV transgenic mouse line and point to the direct involvement of HCV proteins in liver damage. We cannot exclude the possibility that the fusion between the 32 aa of A1AT and the core protein of HCV may have contributed, at least in part, to the histological alterations noted in the transgenic mice. Nonetheless, the involvement of HCV proteins in liver damage is also supported by the observation that hepatic steatosis and lymphocyte recruitment have not been observed so far with other transgenic mouse lines expressing either the full-length A1AT gene (Ruther et al., 1987) or other genes of interest driven by the A1AT gene (Amicone et al., 1997).

The immunohistochemistry data indicate that expression of the entire ORF takes place in transgenic hepatocytes, although no direct evidence could be provided for correct processing of the nonstructural region. The expression vector utilized for the development of this HCV mouse line is based on a human genomic fragment comprising the A1AT gene, and shown to be suitable for transgenic liver expression (Amicone et al., 1995). Thus, the lack of detection of the NS proteins by Western blot can probably be ascribed to factors such as instability of viral proteins and low antigen affinity of the antisera used for the immunodetection. Additionally, in view of the histological alterations noted in the transgenic mice, it is possible that NS enzymes may be harmful to mouse development and this may allow establishment of mice only with low levels of transgene expression. This conclusion is also supported, at least in part, by the observation that in transgenic mouse lines carrying the full-length HCV cDNA under the control of a mouse albumin enhancer/promoter protein expression could not be confirmed by sensitive immunohistochemical techniques, yet significant hepatic histological alterations were observed (Lerat et al., 2002).

The extensive steatosis noted in the transgenic mice, in agreement with expression of HCV core protein (Moriya et al., 1997), declined with age and no development of neoplastic or cancerous lesions occurred in liver from A1AT/HCV mice by the age of 20 months. Additionally, development of steatosis and liver cancer has been reported in mice carrying the full-length HCV cDNA under the control of a liver-specific promoter (Lerat et al., 2002). Why the extent of steatosis varied over time and neoplastic changes did not appear might be related to the extent of liver injury and/or HCV protein expression in A1AT/HCV mice. Additionally, oxidative stress, associated with steatosis and core gene expression, may determine, at least in part, the onset of hepatocarcinogenesis (Moriya et al., 1997, 2001; Okuda et al., 2002). Although we have not performed any studies to characterize the mechanism of steatosis in the A1AT/HCV mice, the lack of a sustained steatosis phenotype and of neoplasia may be connected with a reduced oxidative stress in these mice. Finally, although the differing phenotypes associated with transgenic mice expressing various portions of the HCV genome may be difficult to reconcile, these observations suggest the possible presence of multiple pathways of liver damage and oncogenesis that are affected by the constitutive expression of the viral proteins.

The A1AT/HCV mice allow the analysis of the effects of HCV genome expression in the absence of humoral or cellular immune responses to the viral polypeptides. The analysis of the molecular mechanisms involved in this animal model may contribute to an understanding of the complex interactions between HCV and the host immune system, with particular focusing on innate pathways.

ACKNOWLEDGEMENTS

We thank G. Ciliberto, M. Heim and A. Nicosia for critical review. We also thank J. Clench for editorial assistance. This work was supported by AIRC (Associazione Italiana Ricerca sul Cancro), by Ministero della Salute (Ricerca Finalizzata) RF02.140 and by Ministero della Ricerca Scientifica e Tecnologica, Italy.

REFERENCES


