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Chronic active hepatitis in transgenic mice expressing interferon-γ in the liver

(viral hepatitis/major histocompatibility complex class II)

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ABSTRACT **Interferon-γ may play an important role in the immune response and in inflammatory diseases, including chronic active hepatitis. To understand the role of interferon-γ in the regulation of inflammation and to establish a mouse model of chronic active hepatitis, we produced transgenic mice in which the mouse interferon-γ gene was regulated by a liver-specific promoter, the serum amyloid P component gene promoter. Four transgenic mouse lines were generated, and two of these lines expressed mRNA of interferon-γ in the liver. Levels of serum transaminases increased gradually as a function of age and were significantly higher than those of interferon-γ-negative littermates after 4 weeks after birth. One transgenic mouse line showed a histology of chronic active hepatitis similar to that found in human patients, although cirrhotic changes such as fibrosis were scarce. Thus, the liver-specific production of interferon-γ is sufficient to induce chronic inflammatory disease and this mouse is a transgenic model of chronic active hepatitis.**

Chronic hepatic diseases, such as chronic active hepatitis and liver cirrhosis, are public health problems of worldwide importance and are major causes of mortality in certain areas of the world. Clinical and epidemiological studies (1–4) have clearly shown the importance of hepatitis B and C viruses (HBV, HCV) in chronic active hepatitis as well as hepatocellular carcinoma (HCC). Furthermore, studies of pathology revealed that HCC arises in a cirrhotic liver and that chronic hepatitis is prerequisite for the development of HCC. The mechanisms responsible for HBV- or HCV-induced hepatocellular injury are not well understood. However, it is generally accepted that HBV is not directly cytopathic and that liver cell necrosis is dependent upon the host's immune response, directed at viral determinants on the hepatocyte membrane (5, 6). This immune response is mainly mediated by cytotoxic T lymphocytes (7).

Several transgenic models of hepatic disease have been described. Chisari *et al.* (8) demonstrated that transgenic mice expressing the HBV large envelope polypeptide under control of the albumin enhancer/promoter develop liver lesions due to accumulation of long filaments composed of HBV surface antigen (HBsAg) in smooth endoplasmic reticulum (ER). Dycaico *et al.* (9) reported that transgenic mice expressing the mutant α_1-antitrypsin gene display intracellular globules within the hepatocyte rough endoplasmic reticulum (RER) that contain mutant protein, leading to neonatal and adult hepatitis. In addition, Sandgren *et al.* (10) showed that transgenic mice expressing the albumin-plasminogen activator gene develop progressive degenerative change in the liver due to accumulation of RER-bounded multivesicular bodies. However, the principal lesion in these models appears to involve the hepatocyte secretory pathway and thus is different from that found in human patients.

A model of acute hepatitis can be produced by administration of chemicals. However, remaining hepatocytes are induced to proliferate until the liver regains its original weight. Shull *et al.* (11) produced transforming growth factor (TGF)-β_1-deficient mice. These mice developed an inflammatory liver disease similar to that found in HBV infection. However, about 20 days after birth these mice died due to a wasting syndrome. Mori *et al.* (12) demonstrated that liver changes histologically mimicking human hepatitis were produced in the mouse liver after repeated immunization with syngeneic crude liver proteins. However, it is not known whether hepatitis continues long after the final immunization. Thus, a mouse model for chronic active hepatitis has never been produced, so far. It is a well-known fact that liver in mice and rats can regenerate and return to normal size even after removal of two-thirds of it. Furthermore, Sandgren *et al.* (10) demonstrated that a small number of liver cells can effectively reconstitute functional liver mass. These suggest the remarkable regenerative capacity of individual liver cells. This might be the reason why a mouse model for chronic active hepatitis is difficult to establish.

Interferon (IFN) seems to be an important regulator of the local immune response in the liver of patients with chronic hepatitis B, because cells expressing IFN-α and -γ are localized in the liver tissue of these patients (13). In addition, two transgenes, the IFN-γ gene and the tumor necrosis factor (TNF)-β gene, have been shown to induce chronic inflammatory lesions in Langerhans islets of the pancreas (14, 15). However, in this case, the expression of TNF-β in islets is shown to be insufficient to cause insulin-dependent diabetes mellitus. On the basis of these facts, we attempted to establish a mouse model of chronic active hepatitis by producing transgenic mice carrying the mouse IFN-γ gene linked to the promoter of serum amyloid P component (SAP) gene. We previously showed that this SAP promoter could direct the liver-specific and developmental expression of the heterologous gene (16, 17). We report here the transgenic mouse model of chronic active hepatitis.

MATERIALS AND METHODS

Construction of SAP–IFN-γ DNA. The SAP gene promoter is derived from the Lm hSAP-8 genomic DNA clone of the

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HBsAg, HBV surface antigen; IFN, interferon; SAP, serum amyloid P component; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; RT-PCR, reverse transcription–polymerase chain reaction; MHC, major histocompatibility complex.

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human SAP gene (18). We used a 0.6-kb *Hin*dIII–*Avr* II fragment. The *Avr* II site is located 14 bp upstream from the start codon. This fragment was inserted into pUC19 digested with *Hin*dIII and *Xba* I to produce the plasmid pUC-SAP. A *Bam*HI–*Hin*dIII fragment from this pUC-SAP was inserted into pLG1-SAP, a plasmid carrying 1.2 kb of the rabbit β-globin gene. The cDNA encoding mouse IFN-γ was inserted into the *Eco*RI site of pLG1-SAP, resulting in construction of the plasmid pLG1-SAP-IFN-γ (Fig. 1). Prior to injection into fertilized mouse eggs, pLG1-SAP-IFN-γ was digested with *Hin*dIII and *Xho* I, and a resulting 2.4-kb fragment, the SAP–IFN-γ gene, was isolated and used for microinjection.

DNA Injection and Screening of Transgenic Mice. BDF_1 (C57BL/6 × DBA/2) mice were used to obtain fertilized eggs, and several hundred molecules of the SAP-IFN-γ fragment (2.4 kb) were microinjected into the pronucleus of fertilized eggs, as described (19). When the mice were 4 weeks of age, total nucleic acid was extracted from a piece of the tail of each mouse and used for Southern blot analysis (20). The *Bam*HI–*Xho* I fragment was used as a probe (Fig. 1).

RT-PCR Analysis. Total RNA was extracted from various tissues by using the guanidine thiocyanate procedure. Five micrograms of total RNA was reverse transcribed into cDNA by using SuperScript RT (BRL SuperScript Preamplification System). To avoid detection of mouse endogenous IFN-γ mRNA, two primers were synthesized for PCR; one corresponded to the IFN-γ gene and the other corresponded to the 3′ untranslated region of rabbit β-globin gene (Fig. 1). The PCR was performed in 20 μl of solution consisting of 1× PCR buffer, 200 μM dNTPs, 5 pmol of primers, and 1.25 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus). Mineral oil was overlaid on the reaction mixtures and the cDNAs were amplified on a heating block (ASTEC PC-700). Cycling parameters were as follows: denaturation at 94°C for 1 min, annealing at 64°C for 2 min, and extension at 72°C for 2 min. RNA samples from the SAP-IFN-γ 5 line or the SAP-IFN-γ 44 line were submitted to 25 or 30 cycles of amplification, respectively. After amplification, the PCR products were electrophoresed on a 5% polyacrylamide gel and stained with ethidium bromide. These gels were subjected to Southern blot analysis using the *Bam*HI–*Xho* I fragment (Fig. 1) as a probe.

Assay of Serum IFN-γ and Transaminase. Blood was obtained from the tail artery and was kept standing for 30 min at room temperature. Sera were separated by centrifugation (3000 rpm in a Tomy MR-150 for 5 min). Each serum (100 μl) was assayed for murine IFN-γ in duplicate using an enzyme immunoassay kit (Genzyme InterTest-γ). Each serum was assayed for plasma glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) by using the Reflotron system (Boehringer Mannheim).

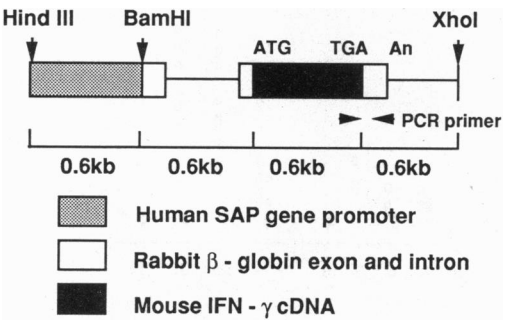

FIG. 1. Structure of the SAP–IFN-γ transgene. An, poly(A). Arrows indicate the sites of restriction endonucleases. Primers that were used in reverse transcription–polymerase chain reaction (RT-PCR) are also represented.

Histological Analysis. Tissues were fixed in buffered 10% formalin and embedded in paraffin, and 4-μm sections were routinely stained with hematoxylin and eosin.

Immunohistochemical Analysis. Tissues were embedded in Tissue-Tek (Miles) and sectioned in a cryostat at −20°C at a thickness of 6–8 μm. Frozen sections of liver were air-dried for 30 min and fixed in acetone/methanol at 4°C for 10 min. After a wash in phosphate-buffered saline, fixed sections were blocked with endogenous avidin and biotin by using a Vector blocking kit. After incubation with 10% normal rabbit serum in phosphate-buffered saline for 20 min, sections were incubated with an appropriately diluted anti-mouse-I-A^b antibody (rat IgG) at 4°C overnight. Sections were then incubated with an appropriately diluted biotinylated anti-rat-IgG antibody (rabbit IgG) for 30 min, with avidin-biotin-peroxidase complex, and subjected to the peroxidase reactions. Sections were counterstained with hematoxylin and mounted.

RESULTS

Establishment of the Transgenic Mouse Line Expressing IFN-γ. Four out of 56 mice, SAP-IFN-γ 5, 27, 30, and 44, were shown to carry the transgene. SAP-IFN-γ 5 was male and the others were female. The numbers of copies integrated were estimated to be 4, 4, 2, and 1, respectively. At 8 weeks of age, these mice were mated with C57BL/6 mice. All lines of transgenic mice transmitted the transgene to their offspring. These offspring were used in the following analysis. As most of SAP-IFN-γ 5 mice that showed the highest plasma transaminase level (see Table 1) died at around 6 months of age under conventional conditions, all mice were transferred to specific-pathogen-free conditions by using *in vitro* fertilization. However, about 80% of transgenic mice died by 1 year of age even under specific-pathogen-free conditions.

Tissue and Developmental Specificity of Transgene Expression. To examine tissue specificity of transgene expression, total RNAs were extracted from various tissues (liver, spleen, stomach, small intestine, pancreas, lung, kidney, and peripheral blood lymphocytes) of transgenic mouse lines

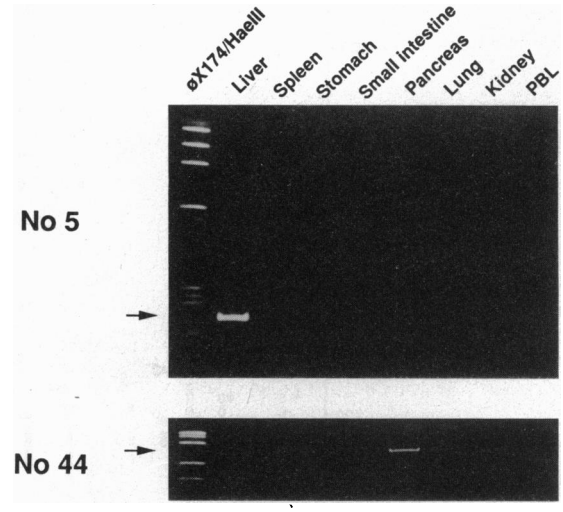

FIG. 2. Tissue-specific transgene expression detected by RT-PCR (SAP-IFN-γ 5 and 44). RT-PCR was performed using PCR primers located on mouse IFN-γ and rabbit β-globin genes (see Fig. 1), and these PCR products were electrophoresed on a 5% polyacrylamide gel. *Hae* III-digested fragments of φX174 DNA were used as DNA size markers. The bands are indicated by arrows. PBL, peripheral blood lymphocytes.

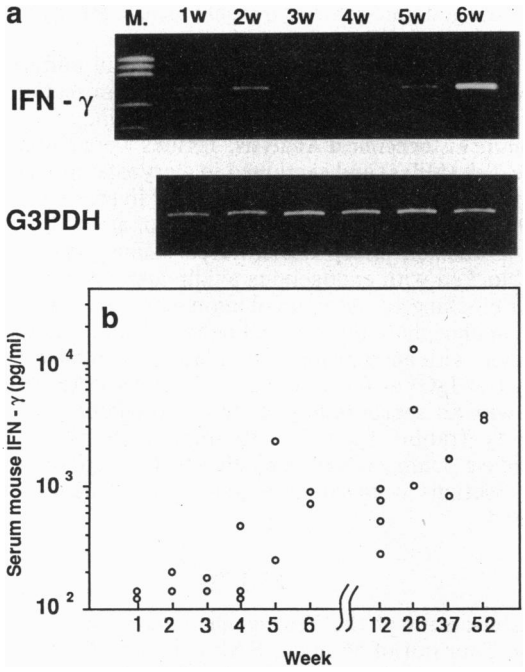

FIG. 3. (*a*) Developmental specificity of transgene expression (1–6 weeks) of the SAP-IFN-γ 5 line measured by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was also amplified as an internal control. Lane M., *Hae* III-digested φX174 DNA. (*b*) Developmental expression of mouse IFN-γ in the SAP-IFN-γ 5 line. Serum IFN-γ was assayed by using an enzyme immunoassay kit (Genzyme InterTest-γ). The negative littermates had less than the detectable level (125 pg/ml) of IFN-γ.

(SAP-IFN-γ 5 and 44) when these mice were 6 weeks of age. Using these total RNAs, we performed RT-PCR. As shown in Fig. 2, the PCR products (265 bp) were detected only in the liver of the SAP-IFN-γ 5 line and in the liver and pancreas of the SAP-IFN-γ 44 line. To confirm that these bands are amplified IFN-γ cDNAs, they were hybridized with the

Table 1. Plasma GOT and GPT at 6 weeks

Line	Activity, units/liter GOT	GPT
SAP-IFN-γ 5 (n = 24)	123 ± 10	69 ± 10
SAP-IFN-γ 27 (n = 5)	71 ± 4	35 ± 2
SAP-IFN-γ 30 (n = 6)	72 ± 5	31 ± 1
SAP-IFN-γ 44 (n = 13)	97 ± 6	46 ± 3
Negative littermates (n = 24)	68 ± 3	25 ± 2

Results are mean ± SEM. The differences between SAP-IFN-γ 5 and 44 and negative littermates were statistically significant by the Student t analysis ($P < 0.001$). n, Number of mice.

*Bam*HI–*Xho* I fragment (see Fig. 1) and autoradiographed. The bands were detected in the same tissues.

To examine developmental specificity of transgene expression, we performed RT-PCR using total RNAs from the livers of 1-, 2-, 3-, 4-, 5-, and 6-week-old SAP-IFN-γ 5 mice. PCR products were detected in the livers at any age, as expected (Fig. 3*a*). No band was detected when PCR was carried out using total RNAs in the absence of reverse transcriptase, suggesting that DNA was not contaminating the samples.

Concentration of Mouse IFN-γ in the Serum. To examine whether murine IFN-γ was present in the serum of each transgenic line, serum IFN-γ was assayed when mice were at 7 weeks of age. The mean concentration of IFN-γ in SAP-IFN-γ 5 mice was 3533 pg/ml, whereas the mean concentrations of other lines and negative littermates were below the detectable level (less than 125 pg/ml). To examine developmental expression of mouse IFN-γ in the SAP-IFN-γ 5 line, we measured serum IFN-γ concentration when mice were 1, 2, 3, 4, 5, 6, 12, 26, 37, and 52 weeks of age. Serum IFN-γ was detected already at 1 week of age, but stayed at a low level until 3 weeks of age. After then, the level of serum IFN-γ increased gradually and reached the maximum at around 6 months of age (Fig. 3*b*). The serum IFN-γ in negative littermates was below the detectable level.

Concentration of Plasma Transaminase. To examine liver cell injury, GOT and GPT were assayed. When mice were 6 weeks of age, blood samples were obtained from the tail

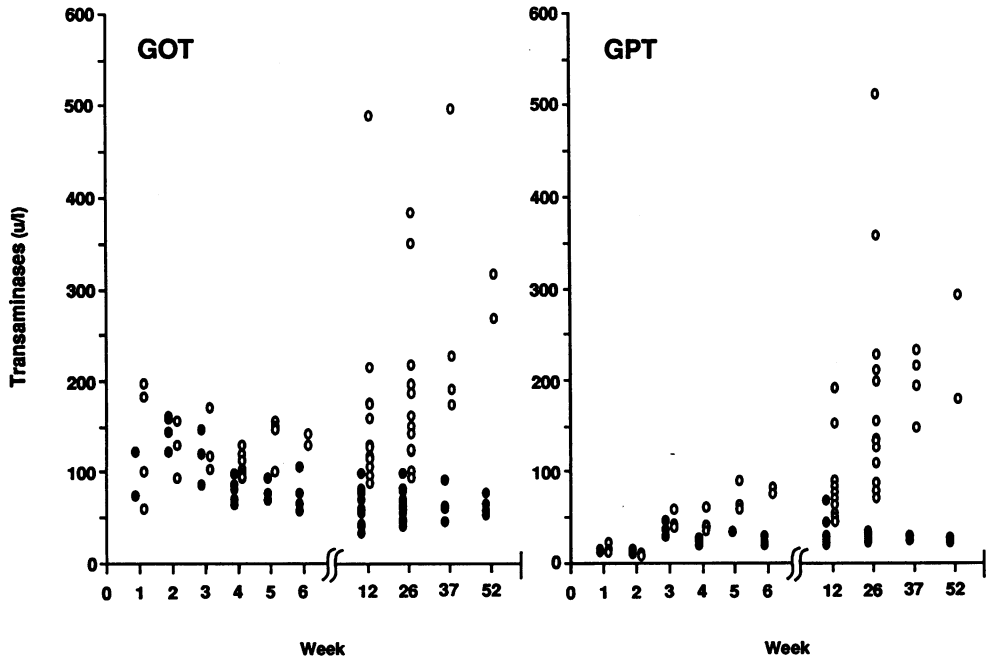

FIG. 4. Concentration of plasma GOT (*Left*) and GPT (*Right*) in the SAP-IFN-γ 5 line at various ages. ○, Transgenic mice; ●, negative littermates. The differences in the levels of transaminases between transgenic mice and negative littermates were statistically significant by Student t analysis after 4 weeks of age ($P < 0.05$). The transaminases were measured by the Reflotron system (Boehringer Mannheim).

artery of each line of transgenic mice. As shown in Table 1, plasma levels of both GOT and GPT were significantly elevated in the SAP-IFN-γ 5 line and were slightly elevated in the SAP-IFN-γ44 line, suggesting the presence of liver cell injury in these transgenic mice. The differences were statistically significant by Student *t* analysis ($P < 0.001$).

We then examined changes of plasma transaminase levels as a function of age in the SAP-IFN-γ 5 line. When the mice were 1, 2, 3, 4, 5, 6, 12, 26, 37, and 52 weeks of age, plasma GOT and GPT were measured. There was no difference in the levels of GOT and GPT between transgenic mice and negative littermates until 3 weeks of age. However, both GOT and GPT levels increased gradually and were considerably higher than those in negative littermates after 4 weeks of age (Fig. 4). The differences in the levels of transaminases between transgenic mice and negative littermates were statistically significant by Student *t* analysis after 4 weeks of age ($P < 0.05$).

Histology. To examine whether pathological changes develop in the liver of each transgenic mouse line, tissue samples were taken at 17 weeks of age. The livers from the SAP-IFN-γ 5 and 44 lines showed histology similar to acute hepatitis, but not the livers of the SAP-IFN-γ27 and 30 lines. Pathological change in the SAP-IFN-γ 44 line was less extensive than in the SAP-IFN-γ 5 line. No pathological change was observed in other tissues.

For sequential morphological observation in the liver of the SAP-IFN-γ 5 line, tissue samples were taken at 1, 2, 3, 4, 5, 6, 12, 26, 37, and 52 weeks of age, from at least two animals at each time point, including both sexes. Negative littermates were analyzed in parallel as controls for the different stages of liver diseases. Up to 2 weeks of age, no recognizable histologic change was observed in the liver of SAP-IFN-γ 5 mice in comparison with negative littermates, although IFN-γ was expressed. During the third week, necroinflammation began to appear randomly in the lobules. This change became prominent in subsequent weeks (Fig. 5*a*), reflected in the serum transaminase level. From 5 weeks onwards, lymphoid cell infiltration appeared at the portal area (Fig. 5*b*). From 26 weeks of age, slight fibrosis appeared, and at 37 weeks of age, ductal proliferation was observed (Fig. 5*c*), reflected in increased serum transaminase level. These sequential observations were consistent in subsequent generations and were not influenced by the sex of the host. We examined more than 30 mice, and all mice older than 3 weeks showed pathological changes in the liver. Thus, penetrance was 100% in transgenic mice. In contrast, negative littermates did not show any significant histologic abnormalities up to at least 52 weeks of age.

Immunohistochemical Analysis of I-A^b Molecules. IFN-γ can induce the expression of major histocompatibility complex (MHC) class II molecules in antigen-presenting cells and other cell types, including pancreatic β cells (14, 21). Thus, we examined the expression of MHC class II molecules. Frozen sections of liver were made from mice of the SAP-IFN-γ 5 line when they were 6, 12, and 26 weeks of age. The expression of I-A^b molecules was observed on the surface of transgenic liver cells at any age examined but was not observed in the livers of negative littermates (data not shown).

DISCUSSION

In this report, we demonstrate that local production of IFN-γ is sufficient to initiate and maintain a liver-specific inflammatory disease in two independent transgenic mouse lines. After 4 or 5 weeks of age, transgenic mice showed continuous elevation of serum transaminases, indicating the presence of persistent liver cell damage. The liver cell damage is more severe in the SAP-IFN-γ5 line than in the SAP-IFN-γ44 line.

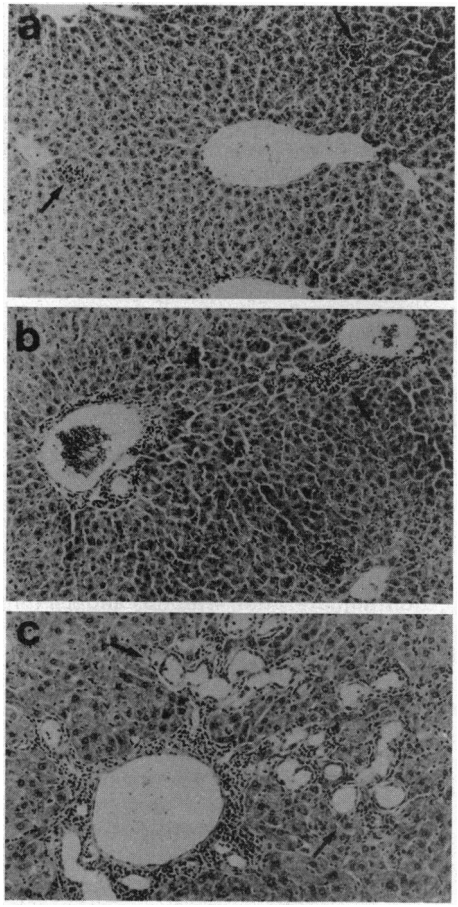

FIG. 5. Sequential morphology in the liver of the SAP-IFN-γ 5 line. (Hematoxylin and eosin; original magnification ×100, final magnification ×70.) (*a*) Necro-inflammation (arrows) is present (male, 4 weeks). (*b*) Lymphoid cell infiltration at the portal area (arrow) is present (female, 12 weeks). (*c*) Ductal proliferation (arrows) is present (male, 52 weeks).

This is consistent with the data that IFN-γ expression is higher in the SAP-IFN-γ5 line than in the SAP-IFN-γ44 line. Histological analysis also revealed the progression of chronic active hepatitis—that is, initial necroinflammatory change followed by lymphoid cell infiltration at the portal area reminiscent of those seen in human viral hepatitis. However, the pathological changes in the mouse liver are modest, and especially cirrhotic changes such as fibrosis are scarce compared with those in human liver. This could be due to the remarkable regenerative capacity of mouse liver cells. In any case, this is a transgenic mouse model for chronic active hepatitis.

It has been reported that IFNs (α, β, and γ) play an important role against viral infection (21). IFN-γ activates cytotoxic T lymphocytes and natural killer cells and blocks viral protein synthesis by inducing (2′-5′)oligoadenylate synthetase. Because of these biological effects, IFNs are beneficial in the treatment of chronic viral hepatitis. On the other hand, Sarvetnick *et al.* (14) reported that the expression of IFN-γ in islets of Langerhans of transgenic mice results in an inflammatory destruction of the islets. They demonstrated that engrafted histocompatible islets were destroyed and that lymphocytes from the transgenic mice are cytotoxic to normal islets *in vitro*. On the basis of these findings, they concluded that islet cell loss was caused by infiltrating lymphocytes, not through deleterious effects of IFN-γ. IFN-γ can induce the expression of MHC molecules in immune cells as well as nonimmune cells. Actually, they

showed that expression of MHC class II molecules is induced in acinar tissue, islets, and epithelial cells lining the ducts. The expression of MHC class II molecules may lead to the presentation of self-antigens, resulting in the activation of quiescent autoreactive cells and loss of pancreatic islet tolerance (22). A similar mechanism may be involved in initiation and development of chronic active hepatitis, because high levels of I-A^b expression are observed in our transgenic mice after birth.

It is of interest that about 80% of our transgenic mice died by 1 year of age. Pathology studies revealed that these mice died of bacteremia, not of liver cell damage. As periodical comprehensive serology and histopathology revealed no indication of pathogens, the bacteria may have been enteric. This is consistent with the data indicating that IFN-γ plays a significant role in the pathogenesis of Gram-negative sepsis (23).

We previously showed that chromosomally integrated HBV genome suffices to allow viral replication in the liver of the transgenic mice (24). But the transgenic mice did not develop hepatitis, because of tolerance to viral gene products. However, these transgenic mice can produce antibody to HBsAg after immunization with this antigen (unpublished data). Double transgenic mice carrying both the SAP-IFN-γ gene and the HBV genome will be a useful mouse model of viral hepatitis, because the release of HBsAg by liver cell damage may enhance the immune response to viral antigen, leading to liver cell injury. On the other hand, it is shown that chromosomal changes linked to HBV DNA integration occur frequently in host DNA of human HCC (25, 26). Furthermore, it was shown that HBV DNA has recombinogenic functions in the hepatitis-related proliferative state (27) and that DNA rearrangement is involved in hepatocarcinogenesis (28). All these results suggest that genomic instability caused by HBV DNA integration to host DNA may play an important role in hepatocarcinogenesis. This possibility can be tested by examining the development of HCC in double transgenic mice carrying both the HBV genome and the SAP-IFN-γ gene.

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