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# Sialadenitis histologically resembling Sjögren syndrome in mice transgenic for hepatitis C virus envelope genes

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**ABSTRACT** **Hepatitis C virus (HCV), a major causative agent of non-A, non-B chronic hepatitis, is also suggested to be associated with extrahepatic manifestations such as mixed cryoglobulinemia and glomerulonephritis. Two independent lines of transgenic mice carrying the HCV envelope genes have been shown previously to express the HCV envelope proteins in organs, including the liver and salivary glands, which results in no pathological changes in the liver. Further analysis of these animals now has revealed that they develop an exocrinopathy involving the salivary and lachrymal glands. This pathology resembles Sjögren syndrome, which also is suggested to have a possible association with chronic hepatitis C. These observations suggest that HCV might be involved in the pathogenesis of sialadenitis in humans and that this transgenic mouse system would be a good animal model for the study of HCV infection.**

With discovery of hepatitis C virus (HCV) came the revelation that this virus is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (1, 2). The clinical aspects of HCV infection have been described in detail by the use of simple immunoassays for HCV antibodies or PCR (3–6), and genetic variations have been extensively studied in HCV envelope gene sequences (7–9). Little is known, however, about the role of HCV or its viral proteins in the pathogenesis of HCV infection. One of the major issues regarding the pathogenesis of HCV-associated diseases is whether the HCV proteins have direct effects on pathological phenotypes.

Several strategies have been used to analyze the processing of the polyprotein that is translated from the HCV genome (10, 11) and to characterize individual proteins. Studies also have been made on the expression of HCV envelope proteins (12–15), which revealed that the two putative envelope proteins, E1 and E2, are associated at their N termini in mammalian cells (16). However, very little is known about the role of envelope proteins in virus–cell interactions, particularly about its possible participation in the pathogenesis of chronic hepatitis as well as extrahepatic manifestations in human HCV infection. Such extrahepatic manifestations include mixed cryoglobulinemia, glomerulonephritis porphyria cutanea tarda, and Sjögren syndrome (17–27).

We have previously reported the establishment of transgenic mice that carry the HCV envelope genes (28). These mice show no pathological changes, such as hepatitis or hepatic neoplasia, despite the high level expression of the envelope proteins. Further analysis has disclosed that these mice develop an exocrinopathy in the salivary and lachrymal glands resembling Sjögren syndrome, which is suggested to have a possible association with human HCV infection (23–27).

## MATERIALS AND METHODS

**Production of Transgenic Mice.** Production of HCV envelope gene transgenic mice has been described (28). The E1 and E2 envelope genes of HCV, which are placed downstream of a transcriptional regulatory region from hepatitis B virus, were introduced into mouse embryos from the CD1 strain (Charles River Breeding Laboratories). Mice were fed ordinary chows (Funabashi Farms, Funabashi, Japan) and were maintained in a specific, pathogen-free state.

**Antibodies.** Antibodies used in this study were as follows (28): rabbit anti-E1 serum, mouse anti-E2 mAb, and rabbit anti-E2 serum. mAb was raised against partially purified, recombinant E2 protein expressed by baculovirus. Polyclonal rabbit antibodies were prepared using recombinant vaccinia viruses (16).

**Western Blot Analysis.** Whole tissue homogenates were resuspended in Western blotting sample buffer (5% 2-mercaptoethanol/2% SDS/62.5 mM Tris·HCl/1 mM EDTA/10% glycerol). Appropriate amounts of proteins then were subjected to 10% SDS/PAGE and electrotransferred to nitrocellulose membrane (Schleicher & Schuell) as described (28). The filter was then reacted with primary antibodies, followed by appropriate secondary IgG conjugated with horseradish peroxidase (Vector Laboratories), and visualized by an enhanced chemiluminescence kit (Amersham).

**Histological and Immunohistochemical Methods.** Tissues sections (5 $\mu$m thick) fixed in 10% neutral-buffered formalin or frozen were used for hematoxylin and eosin staining or immunostaining. The HCV E1 or E2 protein was stained with anti-E1 or -E2 rabbit serum. For detection, biotinylated anti-rabbit IgG followed by avidin-biotin peroxidase (Vector Laboratories) was used. Specificity control of immunostaining was carried out as follows: Liver tissues and other organs from a normal littermate mouse were tested with immune serum. Normal rabbit sera were tested with transgenic mouse liver.

## RESULTS

The envelope genes from HCV of genotype 1b (29) were expressed under the control of regulatory elements from hepatitis B virus in these transgenic mice (Fig. 1), which were derived from the CD1 mouse strain. The E1 and E2 envelope proteins were detected in the salivary glands as well as in the liver (28).

**Development of Sialadenitis in HCV Envelope Gene Transgenic Mice.** In the two independent transgenic mouse lines, E101 and E139, lymphocytic infiltration developed in the

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Abbreviations: HCV, hepatitis C virus; E1, envelope glycoprotein 1; E2, envelope glycoprotein 2.

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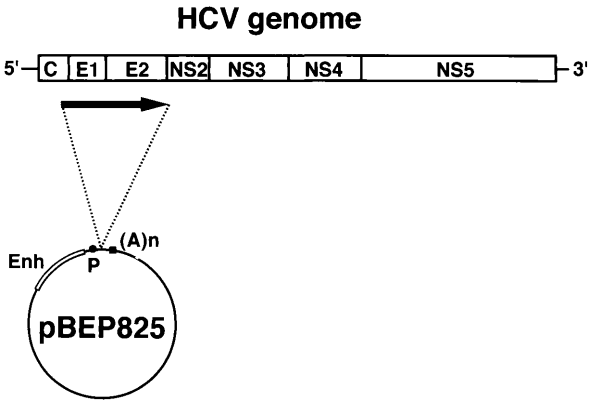


FIG. 1. Schematic representation of the transgene construct. HCV E1 and E2 envelope genes were placed downstream of a transcriptional regulatory region from hepatitis B virus (28).

salivary glands within a few months after birth. Initially, lymphocytes were found around capillaries in the salivary glands (Fig. 2*A*). Soon, focal infiltrates of small lymphocytes appeared [graded 3 and 4 according to the Chisholm and Mason classification of sialadenitis (30)] and aggregated around intralobular ducts (Fig. 2*B*). Lymphocytic infiltrates, which remained focal in general, also were sporadically located as small islands in parenchymal areas. Sialadenitis was slowly progressive in the transgenic mice throughout their life spans. In some mice over the age of 16 months, ductular proliferation (Fig. 2*C*) was observed within lymphocytic infiltrates. Accumulation of fibrous tissues, which was accompanied by the disappearance of acinar cells, was also observed (Fig. 2*D*). No neoplasia was observed in the salivary glands. Nests of lymphocytic infiltrates also were observed in the lachrymal glands although they tended to occur late and were not as extensive as those found in the salivary glands.

The transgenic mice looked healthy and did not spontaneously die, so they were killed at each time point, and the glands were subjected to microscopic examination. Sialadenitis was detected in 58 (84.1%) of 69 HCV envelope gene transgenic mice, with onset at the age of 2 months (Fig. 3). In contrast, only 2 (1.8%) of 113 nontransgenic siblings exhibited these lesions, and no unrelated transgenic mice carrying the hepatitis B viral genome or HCV core gene exhibited them. No sex difference was observed in the incidence of sialadenitis in the HCV envelope gene transgenic mice. Anti-nuclear antibody determined by fluorescence was negative in sera of the transgenic mice ($n = 10$). There was no evidence of infection with the retrovirus that may be associated with Sjögren-like exocrinopathy (31); the transgenic mice survived for more than 24 months without apparent illness, and no lymphadenopathy or hepatosplenomegaly was found. There was no serological evidence of infection with viruses, including murine hepatitis virus.

**Expression of Envelope Proteins.** The degree of pathology in the salivary glands of these transgenic mice corresponded to the expression levels of the HCV envelope proteins in these glands as determined by Western blot analysis of tissue extracts. Both the E1 and E2 proteins were detected in the salivary glands of each founder line, including lines E101 and E139, at levels comparable to those in the liver (Fig. 4, lanes 3–5), and no envelope proteins were detected in the salivary

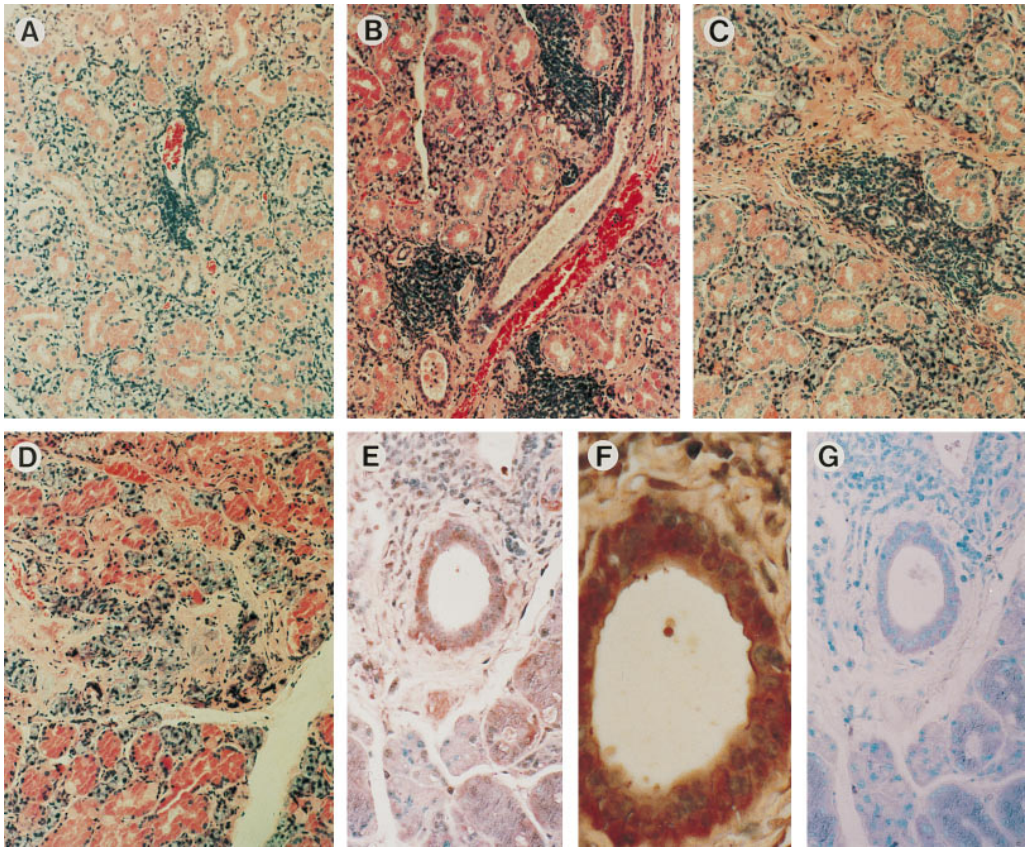


FIG. 2. Microscopic examination of the salivary glands from HCV envelope transgenic mice. (*A*) A 3-month-old male from the E101 line. (*B*) A 6-month-old male from the E139 line. (*C*) A 16-month-old male from the E101 line. (*D*) A 16-month-old female from the E139 line. (*E*) Immunostaining of the E1 protein; a 9-month-old male from the E101 line. (*F*) A higher magnification of *E*. (*G*) A control staining of *E*. Paraffin-embedded sections of salivary glands were stained by hematoxylin and eosin (*A–D*) or immunostained with anti-E1 rabbit serum (*E* and *F*) or preimmune rabbit serum (*G*) by the avidin–biotin complex method (28) and counterstained by methyl green (*E–G*). (*A–D*, ×80; *E* and *G*, ×200; *F*, ×500.)

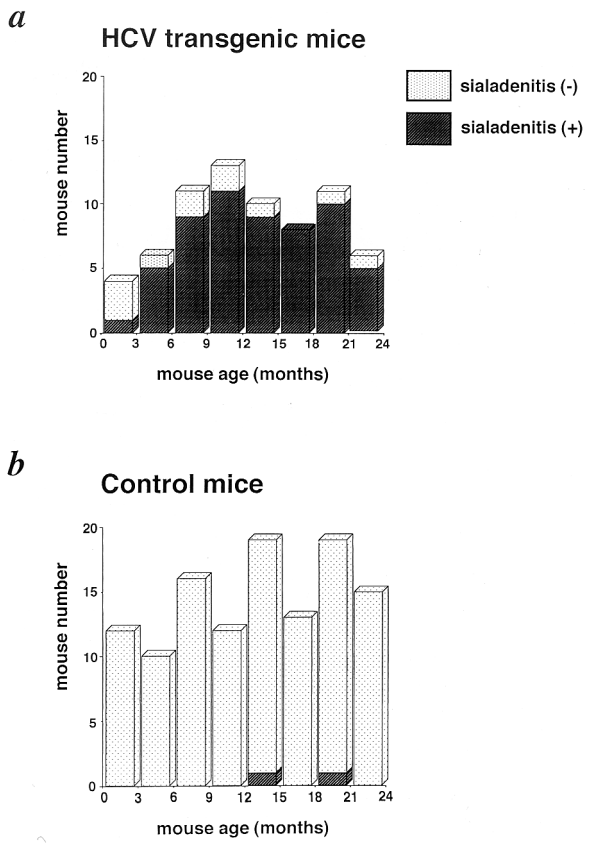


FIG. 3. Incidence of sialadenitis in transgenic mice of the E101 and E139 lines. Mice were killed, and their salivary glands were subjected to microscopic examination. Shaded or dotted bars indicate the number of mice with or without sialadenitis, respectively. (*a*) Transgenic mice. (*b*) Control mice.

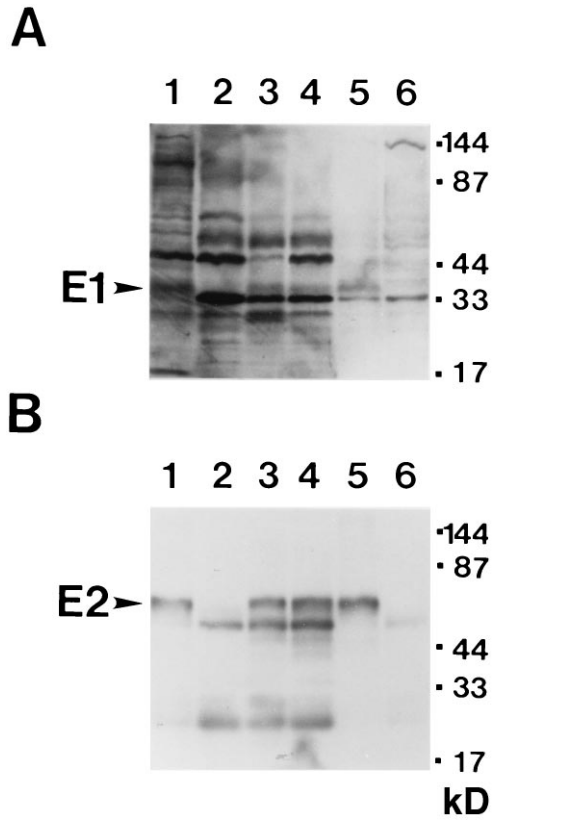


FIG. 4. Western blot analysis. (*A*) Detection of E1 protein. Lanes: 1, positive control pBEP825-transfected HepG2 cells; 2, normal salivary gland; 3, salivary gland, E101 male; 4, salivary gland, E139 male; 5, liver, E101 male; 6, normal liver. (*B*) Detection of E2 protein. Lanes: 1, positive control pBEP825-transfected HepG2 cells; 2, normal salivary gland; 3, salivary gland, E101 male; 4, salivary gland, E139 male; 5, liver, E101 male; 6, normal liver. Arrowheads indicate the positions of E1 or E2 protein. Equivalent amounts of protein were analyzed as described (28).

glands of a control mouse (Fig. 4, lane 2). Low levels of envelope proteins were detected in the salivary glands of 2-month-old mice exhibiting early pathological changes. Immunohistochemistry with specific antibodies (28) revealed that the HCV E1 protein was localized chiefly in the cytoplasm of ductal epithelia (Fig. 2 *E–G*). The E2 protein showed the same distribution pattern.

## DISCUSSION

The changes in the salivary and lachrymal glands that occurred in the HCV envelope gene transgenic mice resembled features observed in Sjögren syndrome. Sjögren syndrome is an exocrinopathy-producing, keratoconjunctivitis sicca and xerostomia with a characteristic sialadenitis involving lymphocytic infiltration of the salivary and lachrymal glands as well as nests of proliferating epithelial cells (32–34).

An association between Sjögren syndrome and chronic HCV infection has been reported (23–27). Haddad *et al.* (23) found lymphocytic sialadenitis resembling Sjögren syndrome in 16 of 28 patients (57%), with HCV infection in only 1 of 20 controls (5%). Pawlotsky *et al.* (24) reported that they found a 14% prevalence of pathological abnormalities resembling Sjögren syndrome in patients with HCV infection compared with 0% in controls without HCV infection. Of interest, they found that nearly 50% of patients with HCV infection had lymphocytic capillaritis in the salivary glands, which was found in all patients with sialadenitis. Lymphocytic capillaritis therefore could represent an early stage of more severe lesions resembling the lymphocytic sialadenitis of Sjögren syndrome. In this sense, it is noteworthy that lymphocytic capillaritis preceded sialadenitis in the HCV envelope transgenic mice, which may reflect the pathological sequence in Sjögren-like syndrome occurring in cases with human chronic HCV infection. However, other researchers refuted such an association by showing an absence of HCV RNA in the sera of patients with Sjögren syndrome (35, 36). Our current results, however, indicate a direct relationship between HCV infection and sialadenitis resembling Sjögren syndrome. HCV infection may account for the pathogenesis of a subgroup of Sjögren syndrome, which is considered to be a multifactorial disease (33, 34). Although the HCV genes were expressed under the control of an exogenous promoter in these transgenic mice, the presence of HCV in human saliva has been reported either through the detection of HCV in human saliva or transmission of hepatitis via saliva (37–39). The pathogenesis of sialadenitis in the transgenic mice is not clear. Possible explanations include (*i*) the induction of cytokines such as interferon-$\gamma$ or interleukin 2 (40, 41) by HCV proteins and (*ii*) the induction of an immunological disturbance by the transgene. The possibility that an immune reaction could be induced against ductal cells expressing viral antigens seems less likely; only 1 out of 20 transgenic mice showed a weak antibody reaction to the E1 protein. Absence of sialadenitis in HCV core transgenic mice in which the core protein of HCV is expressed under the control of the same promoter (K.M., H.Y., Y.S., H.F., K.I., Y.M., T.M., and K. Koike, unpublished data) supports the idea that the envelope protein(s) may function to recruit lymphocytes in the salivary glands.

Our current results support the existence of a relationship between chronic HCV infection and Sjögren syndrome-like

sialadenitis and suggest a direct role of the viral protein(s) in the pathogenesis of sialadenitis that develops in chronic HCV infection. This mouse system might be a good animal model for studying the pathogenesis of sialadenitis and other HCV-associated extrahepatic manifestations.

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