Hepatitis B virus (HBV) surface antigen (HBsAg) genes were injected directly into the liver of adult rats with non-antistone chromosomal protein high mobility group 1 by the hemagglutinating B virus of Japan (Sendai virus)-liposome method (Kato, K., Nakanishi, M., Kaneda, Y., Uchida, T., and Okada, Y. (1991) J. Biol. Chem. 266, 3361-3364). Immunohistochemical analysis showed that HBV surface antigen was expressed by the hepatocytes in vivo. On successive injections of the HBsAg genes, the antibody to HBV surface polypeptides was produced in the rats, and characteristic pathological changes of lymphocytic focal necrosis and denaturation of hepatic cells were observed in the liver of all the rats. We conclude that hepatitis is caused by the direct injection of HBsAg genes.

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**Direct Injection of Hepatitis B Virus DNA into Liver Induced Hepatitis in Adult Rats**

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Hepatitis B virus (HBV) surface antigen (HBsAg) genes were injected directly into the liver of adult rats with non-antistone chromosomal protein high mobility group 1 by the hemagglutinating B virus of Japan (Sendai virus)-liposome method (Kato, K., Nakanishi, M., Kaneda, Y., Uchida, T., and Okada, Y. (1991) J. Biol. Chem. 266, 3361-3364). Immunohistochemical analysis showed that HBV surface antigen was expressed by the hepatocytes in vivo. On successive injections of the HBsAg genes, the antibody to HBV surface polypeptides was produced in the rats, and characteristic pathological changes of lymphocytic focal necrosis and denaturation of hepatic cells were observed in the liver of all the rats. We conclude that hepatitis is caused by the direct injection of HBsAg genes.
Gene Transfer to Animal Organs

A.

-50 K
-39 K
-27 K

FIG. 1. A, plasmid encoding HBV envelope proteins. pAct-LMS included open reading frames consisting of three subregions (known as preS₁, preS₂, and S) downstream of the chicken β-actin promoter. Kb, kilobases.

B. Western analysis of intrahepatic HBV envelope proteins in rat hepatoma cells. pAct-LMS was transfected into H4-II-E-C3 cells (rat hepatoma; ATCC CRL 1600) by the calcium phosphate precipitation method (16). HBV surface proteins in the transfected cells were analyzed by immunoblotting using anti-preS₂ antibody followed by 125I-rabbit anti-mouse IgG. Lane 1, cytoplasmic fraction derived from H4-II-E-C3 cells transfected with pAct-LMS; lane 2, cytoplasmic fraction derived from H4-II-E-C3 cells transfected with pUC19.

Preparation of HVJ-Liposomes—HVJ-liposomes were prepared as described previously (14). Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4.8:2. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 13 mM Tris-HCl, pH 7.6) containing DNA-HMG1 complex (200 μg:64 μg), which had previously been incubated at 20 °C for 1 h. Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (11 J·m⁻²·S⁻¹) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (64,000 hemagglutinating units) in a total volume of 2 ml of BSS. The mixture was incubated at 4 °C for 10 min and then at 37 °C for 30 min with gentle shaking. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. Volumes of 2 ml of HVJ-liposome suspension (10 mg of lipids and 10–40 μg of encapsulated DNA) in BSS with 1 mg/ml glucose and 1 mM CaCl₂ were injected under the perisplanchnic membrane of the liver of rats (Sprague-Dawley, 7 weeks old). The rat liver was perfused with 4% paraformaldehyde containing 0.1 M phosphate buffer, pH 7.4. After fixation, the tissue was sectioned at 6-μm thickness in a cryostat (Miles). Sections were developed with anti-preS₁ antibody (5520) and examined with a commercial alkaline phosphatase immunoreaction kit (Vectastain, Vector Laboratories, Inc.) and alkaline phosphatase substrate kit (Black; Vector Laboratories, Inc.).

Assay of HBsAg and Anti-HBs Antibody in Rat Serum—After injection of HVJ-liposomes containing pAct-LMS, pAct-MS, and HMG1, the amounts of HBsAg (open symbols) and anti-HBs antibody (closed symbols) in the sera of rats (C-1 (○), C-2 (△), C-3 (□), and C-4 (■)) were determined with a commercial enzyme immunoassay kit (Abbott).
TABLE I

Peak levels of HBsAg and anti-HBs antibody detected in the serum during 7 days after HVJ-liposome injection

HVJ-liposomes containing pAct-LMS, pAct-MS, and HMG1 were injected into adult rat liver on day 0 (1st), day 7 (2nd), day 14 (3rd), and day 21 (4th). In the intervals between injections, the levels of HBsAg (ng/ml) and anti-HBs (µg/ml) antibody secreted into the rat serum were monitored. Maximum levels detected in these intervals are shown. The serum levels of HBsAg and anti-HBs antibody in rats treated with HVJ-liposomes containing pUC19 and HMG1 were <0.2 ng/ml and <0.5 µg/ml, respectively.

| Rat | 1st HBsAg | 1st anti-HBs | 2nd HBsAg | 2nd anti-HBs | 3rd HBsAg | 3rd anti-HBs | 4th HBsAg | 4th anti-HBs |
|-----|------------|-------------|------------|-------------|------------|-------------|------------|-------------|
| A-1 | 0.7        | 0.0         | 0.4        | 0.0         | 0.1        | 0.0         | 0.6        |             |
| A-2 | 0.7        | 0.0         | 0.3        | 0.0         | 0.3        | 0.0         | 0.3        |             |
| B-1 | 0.0        | 0.0         | 0.9        | 0.1         |            |             |            |             |
| B-2 | 0.0        | 1.9         | 0.0        | 0.3         |            |             |            |             |
| B-3 | 0.0        | 0.0         | 6.6        | 0.7         |            |             |            |             |
| B-4 | 0.0        | 0.0         | 2.0        |             |            |             |            |             |
| B-5 | 0.0        | 0.3         | 2.4        |             |            |             |            |             |
| B-6 | 0.0        | 0.0         | 1.2        |             |            |             |            |             |
| B-7 | 0.0        | 0.0         | 0.1        |             |            |             |            |             |
| B-8 | 0.0        | 0.3         | 4.8        |             |            |             |            |             |
| C-1 | 0.5        | 0.5         | 0.0        | 13.3        |            |             |            |             |
| C-2 | 0.5        | 0.3         | 0.0        | 4.8         |            |             |            |             |
| C-3 | 1.0        | 0.2         | 0.0        | 1.5         |            |             |            |             |
| C-4 | 0.8        | 0.8         | 0.0        | 3.1         |            |             |            |             |

HMG1, all rats were bled at the indicated intervals, and the sera were analyzed for the amounts of HBsAg and anti-HBs antibody using a commercial enzyme immunoassay kit (Abbott). Purified HBsAg and anti-HBs antibody (HB7-2, mouse monoclonal antibody, the ChemoSero-Therapeutic Research Institute, Japan) were used as standards.

RESULTS AND DISCUSSION

The outer membrane of HB virus consists of host lipid and HBV major, middle, and large envelope proteins within a large coding region that has three in-phase translation start codons. The mRNAs of large, middle, and major S are transcribed from the presS, preS2, and S initiator codons, respectively. When pBRneo-LMS, carrying the BglII fragment encoding surface proteins of native human HBV (ORF S, nucleotides 2296-0/3215-1856) cloned into the BamHI site of pBRneo, was transcribed into BNL CL.2 cells (normal mouse liver; ATCC TIB 73), H4-II-E-C3 cells, and Huh-7-cl4 cells (human hepatoma) by the calcium phosphate method (16), the level of expression of HBsAg in rodent cells was less than one-twentieth of that in Huh-7-cl4 cells (8). So we constructed pAct-LMS, which includes a large coding region containing the presS, preS2, and S initiator codon, under the control of chicken β-actin promoter (Fig. 1A).

Western blot analysis revealed that rat hepatoma cells
transfected with pAct-LMS by the calcium phosphate precipitation method produced HBsAg in the cytoplasm in vitro (cultured cells). When anti-preS, antibody was used, the large (p35, gp42) and middle (gp35, gp36) HBV envelope proteins were produced (Fig. 1B), while the major surface proteins were not detected by immunoblotting with anti-major S antibody. When pAct-MS (14) was transfected into rat hepatoma cells, major S proteins were expressed (data not shown).

For in vivo gene delivery, we constructed HVJ-liposomes containing pAct-MS, pAct-LMS, and HMG1 for expression of all three HBV envelope proteins in liver cells of rats. As described previously (14), we succeeded in expressing the β-galactosidase gene under the control of the chicken β-actin promoter in hepatocytes of adult rats in vivo by this method. Two days after the injection of HVJ-liposomes containing pAct-LMS, pAct-MS, and HMG1 under the perisinusoidal membrane of rat liver, the liver was fixed, and sections of the tissue were developed with anti-preS antibody. Immunohistochemical analysis showed that in these rats HBsAg was present both on the cell surface and in the cytoplasm of the hepatocytes (Fig. 2A). The immunopositive hepatocytes appeared to be intact.

The level of HBsAg in rat serum was monitored by enzyme immunoassay after introduction of pAct-LMS and pAct-MS. HBsAg was detected during the first 3 days with a maximum of 1 ng/ml on day 2 after the injection (Fig. 3, open symbols). The levels of HBsAg in the serum after injection of pAct-LMS and pAct-MS were lower than that after injection of pAct-MS alone, described previously (14). Next the antibodies to HBV surface proteins in rat serum were assayed. Antibodies were detected on day 4 after injection of HVJ-liposomes (Fig. 3, closed symbols). Seven days after the first injection of pAct-LMS and pAct-MS, both plasmids were again injected into the same rats by the HVJ-liposome method. After the second injection, the secretion of anti-HBs antibody into the serum continued until the time of death with a maximum of 13.3 μg/ml. In the first period (day 0 to day 7 after the first injection), the serum HBsAg levels reached 0.5–1.0 ng/ml in all the rats. After the second period (day 8 to day 28), anti-HBs antibody was detected in most of the serum samples, while HBsAg was not detected in any (Table I). It is likely that anti-HBs antibody functions as a competitor of the detection of HBsAg in rat serum. When HVJ-liposomes containing pUC19 were injected by the same method, no HBsAg nor antibody was detected in the sera.

The pathological changes of liver tissues in the rats were investigated. For this, rats were put to death and perfused with 4% paraformaldehyde 7 days after the second injection (day 14). Liver tissue of rats sectioned and stained with hematoxylin and eosin showed focal necrosis characterized histologically by infiltration of lymphocytes and other mononuclear cells and hepatocytes denaturation (arrowhead) in the parenchyma (Fig. 4, A and B). Infiltration of mononuclear cells was observed in all the rats examined. In liver tissues of rats A-1 and A-2 (Table I) 7 days after the fourth injection, the late phase (day 28) of liver cell injury was characterized mainly by infiltration of Glisson's sheaths (Fig. 4, C and D). These pathological changes in the liver were not observed in rats treated with pUC19 by the same method. Transition from focal necrosis (after two injections) to glissonitis (after four injections) resembled the histological change from acute to chronic inflammation in man.

Thus, without transplantation of spleen cells immunized with HBV surface antigen, the expression of HBsAg in rat liver in situ was able to produce anti-HBs antibody and to induce pathological change of the liver. We think that the difference in the quantities of antibody in the sera of the rats may reflect differences in the level of activation of major histocompatibility complex class I-restricted cytotoxic T lymphocytes. Therefore, we are now examining the activation of cytotoxic T lymphocytes in rats transfected with HBV surface antigen genes.

In these rats, liver inflammation was restricted to the lobules that received injections and was not distributed through total liver. In fact, the serum glutamic-pyruvic transaminase (ALT) activity in the sera of these rats were slightly higher than those in rats treated with pUC19 but lower than those observed in human hepatitis. However, the pathological changes in the liver, such as focal necrosis, inflammation of Glisson's sheaths, and liver cell degeneration, mimicked some changes in human HBV infection. Therefore, these rats treated by the HVJ-liposome method and expressing HBV surface proteins are a potential model of human HBV-induced hepatitis. This system will be useful for elucidating the mechanism of HBV-induced liver injury and for developing suitable therapeutic treatments.

Thus, this direct gene delivery system will provide a new way for studying cellular functions in various organs, establishing animal models of human diseases and postnatal gene therapy in vivo (11).

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