Immunoelectron microscopic observation of hepatitis B surface antigen on the surface of liver cells from patients with hepatitis B virus infection.

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Abstract

A recently modified method using peroxidase labeled antibodies for light and electron microscopic demonstration of hepatitis B virus (HBV) was applied to the evaluation of hepatitis B surface antigen (HBsAg) on the surface of liver cells in biopsy specimens from 24 HBsAg chronic carriers. Membranous distribution of HBsAg was demonstrated in diffuse or scattered hepatocytes in all 4 asymptomatic carriers and in 3 of the 20 patients with HBsAg-positive chronic active hepatitis or liver cirrhosis. In these patients with membranous expression of HBsAg, hepatitis B e antigen, Dane particles and DNA polymerase were often detected in sera, and large amounts of hepatitis B core antigen appeared in the liver. These results suggest that membrane-bound HBsAg may be expressed by the HBV genome. The ultrastructural study of liver cells showing membranous expression disclosed dense deposits of reaction product indicative of HBsAg on the cell membrane and/or on assembled particles within the extracellular space. In some hepatocytes showing both diffuse cytoplasmic and membranous expression of HBsAg, HBsAg-positive membrane of cisternae open to the intercellular space was connected with the liver cell membrane. These findings supported the conjecture that HBV associated antigens are integrated into the liver cell membrane.

KEYWORDS: type B hepatitis, membrane-bound HBsAg, immunoelectron microscopy, peroxidase-labeled antibody method.

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IMMUNOELECTRON MICROSCOPIC OBSERVATION
OF HEPATITIS B SURFACE ANTIGEN ON THE
SURFACE OF LIVER CELLS FROM PATIENTS
WITH HEPATITIS B VIRUS INFECTION

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Many studies employing histochemical and immunohistologic techniques
have defined the intrahepatic distribution of hepatitis B surface antigen (HBsAg)
and hepatitis B core antigen (HBcAg) in various types of hepatitis B virus
(HBV) infection (1, 2). HBV is observed more frequently in liver tissue from
both healthy carriers and immunosuppressed HBsAg carriers than from patients
with acute and chronic hepatitis (3, 4). These studies suggest that the pathologic manifestations of hepatitis B infection have been attributed to the outcome of different host-virus interactions. In considering viral antigens, HBsAg expressed at the hepatocyte surface has been postulated to play a major role in the initiation or perpetuation of hepatic injury, since a membranous pattern of HBsAg appears in the early stage of acute hepatitis (5, 6) and in the stage prior to exacerbation of chronic active hepatitis (7, 8). Nevertheless, the details of membrane expression of HBsAg and pathogenesis remain to be clarified.

Our recently modified method of using peroxidase labeled antibodies has allowed precise localization of HBsAg and HBeAg in the liver and identified a possible mode of intracellular HBV synthesis (9, 10). In this study (9) membrane-bound HBsAg was found in hepatocytes of an asymptomatic carrier. The studies described now apply this method for the further evaluation of membrane expression of HBsAg on the surface of hepatic cells in various forms of hepatitis B. These findings strongly suggest that HBsAg is incorporated into the cell membrane of liver cells, as described by Edgington et al. (1).

MATERIALS AND METHODS

Patients. This study included 24 patients in whom hepatitis B viral infection was diagnosed by the reversed passive hemagglutination test of HBsAg. Four chronic HBsAg carriers with normal liver tests and, histologically, with portal fibrosis, variable degrees of fat or focal cellular pleomorphism without significant portal tract inflammation, were classified as asymptomatic carriers. Twenty additional carriers had histologic findings of chronic active hepatitis or liver cirrhosis in their liver biopsies.

Serologic tests. Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) in the serum were concentrated three fold by the addition of 5 mg per milliliter of Lyphogel (German Instrument Company, Ann Arbor, Michigan) and tested by double Ouchterlony immunodiffusion with a hexagonal arrangement (11).

Dane particles in the serum had been centrifuged at 150,000 g for 3 h and recentrifuged with 0.01 M phosphate buffered saline, pH 7.1. The pellet from each serum was observed by using negative staining under an electron microscope (12).

DNA polymerase activity in the serum was determined according to Kaplan et al. (13) with a few modifications. From each patient, one ml serum was centrifuged and 25 μl sample from the pellet was used for preparations.

Preparation of antisera. Antiserum to HBsAg (anti-HBs) was produced in rabbits using HBsAg purified on a large scale from plasma of asymptomatic carriers (14). The IgG was prepared by precipitation with Na2SO4, followed by diethylaminoethyl (DEAE)-Sephadex (Sigma Chemical Company) column equilibrated with 0.05 M tris (hydroxymethyl)-aminomethane (Tris)-HCl buffer, pH 7.0 (9).
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Antiserum to HBCAg (anti-HBc) was raised in guinea pigs by the immunization of core particles isolated from Dane particles purified on a large-scale from plasma of asymptomatic carriers (15). Anti-HBs was absent as determined by radioimmunoassay ("Ausab", Abbott Laboratories). The IgG fraction was isolated by DEAE-Sephadex column chromatography.

Preparation of conjugates. Fab' of IgG fractions of specific antisera was prepared by pepsin digestion (16). Five mg of Fab' was conjugated to horseradish peroxidase (HRPO) (Sigma Chemical Company), HRPO type VI with sodium m-periodate using the procedure of Nakane and Kawai (17). The conjugates were isolated from unconjugated Fab' or free HRPO by column chromatography on Sephadex G-100 (9).

Fixation of liver tissues. Liver biopsy specimens were fixed for both light and electron microscopy with periodate-lysine-parafomaldehyde (PLP) (18) for 4 hr overnight, and washed in 0.05 M sodium phosphate buffer, pH 7.2 with 7.5 per cent sucrose overnight, phosphate buffer with 15 per cent sucrose for 12 hr, and phosphate buffer with 20 per cent sucrose and 10 per cent glycerol for 2 hr (9).

Light microscopic study for immunohistochemical localization. Liver specimens fixed with PLP were embedded in Ames OCT compound, quick-frozen, sectioned 6 μm, mounted on albumin-coated glass slides, and air-dried for 30 min. Then, tissue sections were washed in PBS, incubated with normal sheep serum, washed, incubated with HRPO-labeled Fab' and washed. They were reacted with diaminobenzidine (DAB)-peroxide for 10 min, washed, dehydrated and mounted. In liver tissues containing excessive endogenous peroxidase activity, periodic acid and sodium borohydride (NaBH₄) treatment was used to inactivate endogenous peroxidase (9). Controls included staining with the conjugates devoid of specific activity by absorption with purified HBsAg or HBCAg (15), the conjugates absorbed with acetone-dried liver powder, and replacement of the conjugates with PBS for the evaluation of endogenous peroxidase activity.

Immunoelectron microscopy. The 6 μm sections were mounted on glass slides, coated with gelatin and egg albumin, and air-dried. The sections were rehydrated in PBS, blocked with normal sheep serum, and incubated in HRPO-Fab' (HRPO-anti-HBs, HRPO-anti-HBc, the neutralized HRPO-anti-HBs, or the neutralized HRPO-anti-HBc) for 4 hr. They were then fixed for 20 min with 2 per cent glutaraldehyde, reacted with DAB for 30 min and with DAB-peroxide for 2 min, and again fixed in osmium tetroxide for 30 min. After dehydration in graded ethanol, they were embedded in Epon-Araldide. Ultrathin sections were cut and observed without additional staining under a Hitachi H-700H electron microscope at magnifications of ×6,000 to 30,000.

RESULTS

Light Microscopic Findings

A characteristic staining of HBsAg was observed in biopsy specimens in all 4 asymptomatic carriers, in 6 out of 10 cases with chronic active hepatitis and in
5 out of 10 cases with liver cirrhosis. The patients showing intrahepatic HBsAg are summarized in Table 1. HBsAg was localized in variable amounts in the cytoplasm of hepatocytes in all 15 cases. The staining of HBsAg on the cell surface was found in diffuse or scattered liver cells of all 4 asymptomatic carriers. That staining appeared prominently along the sinusoidal pole and between hepatocytes. Such a distinctive membranous distribution was focally detected in 2 out of 10 patients with chronic active hepatitis. In cirrhosis, membrane-bound HBsAg was not visible. A large amount of nuclear and/or cytoplasmic HBeAg was demonstrated in 5 of 6 cases with membranous expression of HBsAg, although the quantity of HBeAg in the liver parenchyma was variable in each case as illustrated in Table 1. Of the 6 patients with membrane staining, 4 showed HBeAg and 5 had many Dane particles in their sera. DNA polymerase activity was also found in 4 of them.

Excessive endogenous peroxidase was blocked by 0.01 M periodic acid and

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**Table 1. HBeAg, Dane particles and DNA polymerase activity in sera, and light microscopic observation of HBsAg and HBeAg in the liver of patients with intrahepatic HBsAg**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Serum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liver&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBeAg</td>
<td>Dane particle</td>
</tr>
<tr>
<td>W. R.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. S.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. Y.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. H.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T. M.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>I. I.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T. O.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T. K.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R. P.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. Y.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. Y.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T. T.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K. T.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H. K.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H. W.</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> - Negative; +, Positive; /, Not estimated. <sup>b</sup> Immunoperoxidase staining
N, Negative; +1, Slightly positive; +2, Positive; +3, Very positive.
0.1 mg per ml of NaBH₄ treatment prior to antibody staining. The specificity of anti-HBs and anti-HBc was ascertained by the staining controls. The specific staining of HBsAg or HBcAg was completely blocked by adsorbed specific HRPO-Fab′ conjugates. The staining patterns of the conjugates adsorbed with normal human liver powder were indistinguishable from those of the same non-adsorbed specific HRPO-Fab′ conjugates.

**Immunoelectron Microscopic Findings**

**Cytoplasmic expression of HBsAg.** The dense reaction products indicative of HBsAg were demonstrated in the cisternae of the endoplasmic reticulum (Fig. 1), in which spherical particles and tubular forms were heavily stained (Fig. 2). Furthermore, occasional large particles, probably representing Dane particles, were also positively stained. The reaction deposits were also localized on membranes of its enclosing cisternae and a part of nuclear membrane. Most hepa-
Fig. 2. Higher magnification of cytoplasmic HBsAg in a hepatocyte of a patient with chronic active hepatitis. The electron dense reaction products of HBsAg are localized on the particles within cisternae and on the cisternal membranes. Endogenous peroxidase is strongly reacted in the granules of a neutrophil (NE) in Disse space. Hepatocyte (H), microvilli of the cell membrane (mv), mitochondria (m), bundles of fibers (f). No counterstain. ×18,000.

tocytes showing diffuse cytoplasmic staining revealed no membranous staining on the cell surface. In a few of them, however, there were focal reaction deposits on the cell membrane. On the surface of a hepatocyte showing diffuse cytoplasmic staining, some cisternae appeared to be open to the intercellular space (Fig. 3-A, B). HBsAg particles in a cluster being released from a cisterna were observed in that space, and fusion of the HBsAg-positive cisternal membrane with the plasma membrane was demonstrated on the cell surface (Fig. 3-A, B). No positive staining was found in hepatocytes incubated with the neutralized HRPO-anti-HBs (Fig. 1-B).
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Fig. 3. Higher magnification of HBsAg localized on the surface of a hepatocyte showing diffuse cytoplasmic HBsAg in an asymptomatic carrier. Microvilli of the cell membrane (mv), intercellular space (ics). No counterstain. A. HBsAg-positive cisterna is open to the intercellular space. HBsAg-positive particles are present in that cisterna. $\times 40,000$. B. Two cisternae containing HBsAg particles are open to the intercellular space. The HBsAg-positive membrane of the cisterna (1) is connected with the plasma membrane (arrow), and HBsAg-positive particles are going out from cisterna (2) to intercellular space. $\times 40,000$. C. The clusters of HBsAg-positive particles are observed in the intercellular space. Hepatocyte (H). $\times 50,000$.

Membranous expression of HBsAg. In the ultrastructural study of hepatocytes showing membranous distribution by light microscopy, the reaction products were found on the cell membrane and in the extracellular space where spherical particles were positively stained (Fig. 4). Diffuse or partial membrane staining appeared independently from HBsAg-positive particles (Fig. 5), though staining particles were occasionally near or minimally attached to portions of the cell.
surface. HBsAg-positive particles within the intercellular space appeared often as clusters (Fig. 3-C), and those in the Disse space were dispersed. In almost all of these liver cells there was little or no intracytoplasmic HBsAg.

In all asymptomatic carriers, in 2 out of 6 patients with chronic active hepatitis and in 1 out of 5 patients with liver cirrhosis there were hepatocytes showing mainly membranous expression of HBsAg by electron microscopy (Table 2), and in these cases membrane-bound HBsAg was also found on the surface of occasional hepatocytes showing diffuse cytoplasmic HBsAg. In other cases, membranous expression was rarely found, although HBsAg-positive cisternae were present near the cell membrane of some liver cells showing cytoplasmic HBsAg (Table 2).
Fig. 5. High power view of electron-dense HBsAg on the plasma membrane of hepatocytes (H) showing membranous expression in an asymptomatic carrier. HBsAg is located on the plasma membrane facing intercellular space (ics), but free HBsAg-positive particles are not found in this space. Microvilli of the cell membrane (mv), mitochondria (m). No counterstain. \( \times 76,000 \).

<table>
<thead>
<tr>
<th>Patients</th>
<th>Plasma membrane-bound HBsAg</th>
<th>HBsAg-positive particles in extracellular space</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. R.</td>
<td>+3</td>
<td>+3</td>
</tr>
<tr>
<td>L. S.</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>S. Y.</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>S. H.</td>
<td>+2</td>
<td>+1</td>
</tr>
<tr>
<td>T. M.</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>I. I.</td>
<td>N*</td>
<td>+1</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. O.</td>
<td>N*</td>
<td>+1</td>
</tr>
<tr>
<td>T. K.</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>R. P.</td>
<td>N</td>
<td>+1</td>
</tr>
<tr>
<td>A. Y.</td>
<td>+2</td>
<td>+2</td>
</tr>
</tbody>
</table>

Table 2 continued
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<th>HBsAg-positive particles in extracellular space</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Y.</td>
<td>+1</td>
<td>N</td>
</tr>
<tr>
<td>T. T.</td>
<td>N*</td>
<td>N</td>
</tr>
<tr>
<td>K. T.</td>
<td>N*</td>
<td>N</td>
</tr>
<tr>
<td>H. K.</td>
<td>N*</td>
<td>N</td>
</tr>
<tr>
<td>H. W.</td>
<td>N*</td>
<td>N</td>
</tr>
</tbody>
</table>

N, no specific reaction products; +1, specific reaction deposits on a few cells; +2, specific reaction deposits on scattered cells; +3, specific reaction deposits on diffuse cells. * HBsAg-positive cisternae adjacent to cell membrane in a few hepatocytes.

DISCUSSION

HBsAg on the cell membrane of liver cells in the tissue or of isolated hepatocytes was observed in an early phase of acute hepatitis and in asymptomatic carriers by using the immunofluorescent antibody technique (5, 6). Of chronic hepatitis, divergent opinions were noted. Albert et al. could not demonstrate surface membrane expression of HBsAg in liver cells in patients with chronic hepatitis and cirrhosis (5). On the other hand, Ray et al. showed HBsAg expression in liver cell membranes to be the most prominent in active forms of chronic hepatitis, and suggested the close relationship between membranous expression of HBsAg and the activity of the disease (8). Similar results were reported recently by Huang et al. (19). Further study of both the structural and nonstructural forms of HBsAg with respect to membranous expression has been conducted in our laboratory employing the peroxidase labeled antibody method. In all asymptomatic carriers, and a few cases with chronic active hepatitis, a light microscopic study revealed diffuse or focal membranous staining, and an ultrastructural study, furthermore, demonstrated HBsAg on the surface of these hepatocytes. Surface HBsAg was present as a nonstructural form on the plasma membrane and/or as small particles in the extracellular space. This suggests that membrane-bound HBsAg may represent both protein constituents incorporated into the plasma membrane and the binding of circulating HBsAg particles on it. In the cytoplasmic staining, HBsAg was present on intracisternal particles and on the cisternal membrane of endoplasmic reticulum, as previously reported (9). Particularly, in some hepatocytes showing both cytoplasmic and membranous HBsAg, the HBsAg-positive cisternal membrane is incorporated into the plasma membrane when the HBsAg particles are released from cytoplasm to extracellular space by exocytosis.

A high correlation between membranous distribution of HBsAg and positive data in the tests for HBeAg, DNA polymerase and Dane particles in sera
was observed. Intrahepatic HBeAg was strongly correlated with a membranous pattern of HBsAg. This is in agreement with the fluorescent studies by Ray et al. (8) and recently by Bianchi et al. (20). However, the abundance of HBsAg on the cell surface in our asymptomatic carriers differs from less surface HBsAg in near-normal liver reported by Ray et al. (8). The discrepancy of the results may be due to the difference between HBeAg-positive carriers and HBeAg-negative ones. These findings suggest that membranous HBsAg is expressed by a complete HBV genome.

There is good evidence that a large amount of HBsAg can be present in liver cells without producing hepatocellular necrosis; suggesting that a direct cytolytic effect of HBsAg is unlikely and that the host immune reaction is responsible for the tissue damage. In the previous study (10) we demonstrated that the pattern of HBsAg localization in a liver transplant recipient was changed from intracytoplasmic in the native liver cirrhosis to membrane associated in the new liver after three months of pharmacologic immunosuppression. In the new liver, acute or chronic hepatitis was not found by light microscopy (10). An opposite change in the pattern of HBsAg distribution from membrane associated to cytoplasmic has been observed in carriers with chronic hepatitis who subsequently developed acute exacerbation (7, 8). In these studies the interaction between immune responses and viral antigens expressed at the hepatocyte surface has been closely related to hepatic cell lysis. In the present series membranous expression of HBsAg was observed in some cases with chronic active hepatitis or liver cirrhosis, though the extent of it was slight in comparison with that of asymptomatic carriers. In the elimination of HBV, hepatocytes with surface expression of HBsAg are susceptible being affected by host immune responses. In carriers with chronic active liver disease, the specific immune responses may fluctuate and become high enough to eliminate some, but not all, infected cells. However, it has not been established whether chronic active inflammation may be caused only by host immune responses against HBV antigens or not. The specific immune responses against HBV induced neo-antigens or liver membrane proteins altered by HBV infection have been also suspected as the cause of liver cell necrosis (21, 22). Further ultrastructural studies for the evidence of cellular and humoral immune reactions in liver tissues will be necessary to elucidate upon the mechanism of HBV mediated liver injury.

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REFERENCE


HBsAg on Liver Cell Surface
