Relationship between the Replication of Hepatitis B Virus and the Localization of Virus Nucleocapsid Antigen (HBcAg) in Hepatocytes

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SUMMARY

According to the localization of hepatitis B virus (HBV) core antigen (HBcAg), detected by the avidin–biotin complex method, infected hepatocytes were classified into three types, i.e. those having nuclear (type I), nuclear and cytoplasmic (type II) or only cytoplasmic (type III) antigen. HBcAg-positive hepatocytes of all specimens (three) from non-specific reactive hepatitis and of most (five of seven) from chronic persistent hepatitis (CPH) patients were only type I; the other two CPH samples and all (seven) chronic active hepatitis samples were composed of a mixture of types I, II and III. Linear correlations between the frequency of type I, as well as that of all types (I, II and III) of the HBcAg-positive hepatocytes, and the amount of HBV DNA in serum were found. The relative HBV production of HBcAg-positive hepatocytes (serum HBV DNA amount/frequency of HBcAg-positive cells) was 0.11 in type I and 0.07 in all hepatocytes including types I, II and III. HBV core particles and complete HBV particles were found in type I hepatocytes. On the other hand, these particles were not found in a predominantly type III liver specimen. These results suggest that type I hepatocytes are more involved in the propagation of HBV than types II and III.

Several immunological, histological and biochemical studies on the relationship between the expression of hepatitis B virus (HBV)-related antigens, especially of HBV core antigen (HBcAg), and viral replication have been performed using serum and liver tissues from patients with type B hepatitis. The following connections have been found. (i) When hepatitis B e antigen (HBeAg), which is part of HBcAg (Ohori et al., 1979; Mackay et al., 1981), is detectable in serum, Dane particles, HBV-specific DNA polymerase and DNA are demonstrable in the serum (Nordenfelt & Kjellen, 1975; Imai et al., 1976; Ohori et al., 1980) and HBcAg and HBV DNA in liver tissues (Bonino et al., 1981; Hadziyannis et al., 1983). (ii) The localization of HBcAg in hepatocytes varies even in the same tissue (Gudat et al., 1975; Yamada & Nakane, 1977; Huang & Neurath, 1979). However, because conflicting results have been obtained in studies of the localization of HBcAg and the presence of HBV DNA in hepatocytes, valid conclusions about the correlation between HBV replication and HBcAg expression in hepatocytes can not yet be drawn (Blum et al., 1984; Burrell et al., 1985).

In the present study, we have examined staining conditions for the detection of HBcAg, and classified the HBcAg-positive hepatocytes with respect to the localization of this antigen in liver tissues from patients whose sera were positive for HBeAg. The amount of HBV DNA in serum was determined and electron microscopic observations were made. These results are discussed with regard to virus replication in the three types of hepatocyte.

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A total of 17 patients suffering from type B hepatitis, whose sera were constantly positive for HBeAg, were examined in this study. There were three with non-specific reactive hepatitis (NRH; Gudat et al., 1975), seven with chronic persistent hepatitis (CPH) and seven with chronic active hepatitis (CAH) according to histological findings (Review of the International Group, 1977). All patients were in hospital for 2 to 4 months and serum samples were collected weekly or biweekly during this time. Serum samples were obtained on the day of liver biopsy and kept at -20 °C until use. The titres of HBV surface antigen (HBsAg) and HBeAg were determined by reversed passive haemagglutination (Antihebscell for HBsAg and Anti-e cell for HBeAg; Green Cross Co., Osaka, Japan). For a more precise result, HBeAg was also examined by enzyme immunoassay using an HBe EIA kit (Abbott Laboratories, North Chicago, Ill., U.S.A.). In the present study, 17 paraffin-embedded liver biopsies were examined for the presence of HBeAg by the avidin–biotin complex (ABC) method (Hsu et al., 1981) using a Vectastain ABC kit purchased from Vector Laboratories (Burlingame, Ca., U.S.A.). In order to obtain the best staining conditions for specific and accurate determination of the frequency of HBcAg-positive hepatocytes in tissues, we examined the concentration of antibody to HBcAg (anti-HBc) using rabbit serum as the primary antibody and selected a passive haemagglutination (PHA) titre of 215 (Corecell; Green Cross Co.) and 180 min incubation.

In all liver specimens, there were three types of hepatocytes, differing in HBcAg localization: type I hepatocytes in which HBcAg was localized only in nuclei (Fig. 1a), type II hepatocytes in which HBcAg was detected in both nuclei and cytoplasm (Fig. 1b) and type III hepatocytes in which HBcAg was detected only in the cytoplasm (Fig. 1c). Hepatocytes classified as type II exhibited variation in staining intensity; one pattern was characterized by more intense staining in the nuclei than the cytoplasm (type II-a, single arrowheads in Fig. 1b), whereas the other subtype was stained more uniformly (type II-b, double arrowheads in Fig. 1b).

Table I shows the proportions of the three types of hepatocyte in 17 liver specimens. As shown in the table, it was demonstrated that type I hepatocytes were found in all the liver tissues to some extent. On the other hand, types II and III were not found in every specimen. Specimens of livers showing minimal dysfunctions (three of three NRH and five of seven CPH) contained only type I hepatocytes. In addition to type I hepatocytes, types II and III appeared in all seven CAH liver specimens. These results indicate that type I hepatocytes may be the principal cells in which HBV replicates and that the other types may result from immunological responses to infected cells in accordance with any physiological changes they undergo.

In order to see whether type I hepatocytes shift to types II and III during the course of liver dysfunction, we compared the ratio of subtypes II-a and II-b (Fig. 1b). If the frequency ratios of types II-a and b were proportionate to those of types I and III, respectively, it could be said that type II-a is derived from type I and that type II-b is the precursor of type III. As was expected, type II-a hepatocytes were observed more frequently in tissues in which type I hepatocytes were predominant. This was found without exception in all liver specimens examined (Table 1), suggesting that the type II hepatocyte represents a transition between type I and type III.

The next experiment was performed to see which type is mainly involved in HBV replication. For this purpose we estimated the amount of serum HBV DNA, which reflects the amount of Dane particles (Scott et al., 1983), by spot hybridization in 25 μl of serum, using 32P-labelled HBV DNA probe (2 × 10³ to 4 × 10⁸ c.p.m./μg), according to the method described by Scott et al. (1983). HBV DNA was detectable at 3 pg per 25 μl serum (data not shown). Fig. 2 shows the relation between the frequency of HBcAg-positive hepatocytes and the radioactivity of DNA hybridizing with DNA in the serum. Since types II and III do not occur on their own in liver tissues (Table 1), it is difficult to evaluate the role of each type of hepatocyte in viral replication. Type I hepatocytes, however, appeared independently of types II and III in patients with minimal liver dysfunction. The samples from this group of patients were used to compare the effects of the frequency of all types of HBcAg-positive cells and that of type I hepatocytes to the amount of serum HBV DNA, which is represented by radioactivity (c.p.m.) in Fig. 2. A linear correlation between the above two sets of variables was demonstrated, i.e. correlation coefficient \( r = 0.686 \) (\( P < 0.01 \)) and regression line \( y = 0.11x + 1.93 \) for type I hepatocytes, and \( r = 0.872 \) (\( P < 0.01 \)) and regression line \( y = 0.07x + 1.96 \) for all three types, where
Fig. 1. Localization patterns of HBcAg in paraffin-embedded liver specimens. HBcAg was detected by the ABC method. In this experiment, liver sections were incubated with primary antibody in rabbit anti-HBc serum (PHA titre 215) and incubated at 37 °C for 180 min. (a) Localization of HBcAg only in the nuclei of hepatocytes (type I) from a patient with NRH (no. 7 in Table 1). There is some variation in the staining intensity in the nuclei but no visible reaction in cytoplasmic areas. (b) Localization of HBcAg both in the nuclei and the cytoplasm of hepatocytes (type II) from a patient with CAH (no. 9 in Table 1). Hepatocytes stained more intensely in the nuclei than in the cytoplasm (type II-a) and those stained to the same extent both in nuclei and cytoplasm (type II-b) are indicated with single arrowheads and double arrowheads, respectively. (c) Localization of HBcAg only in the cytoplasm of hepatocytes (type III) from a patient with CAH (no. 17 in Table 1). No counterstain. Bar marker represents 25 μm.
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Fig. 2. Relation between the frequency of HBcAg-positive hepatocytes and the amount of HBV DNA in serum. The amount of serum HBV DNA is plotted as a function of the frequencies of HBcAg-positive hepatocytes of type I (●) and mixed types (○) in all 17 liver specimens. Their regression lines are marked —— and —— respectively. Hepatocyte frequencies are from Table 1.

Table 1. Composition of HBcAg-positive hepatocytes in liver tissues of patients with NRH, CPH and CAH

<table>
<thead>
<tr>
<th>Liver specimen no.</th>
<th>Histological diagnosis</th>
<th>Frequency (%)*</th>
<th>Type of HBcAg-positive hepatocyte (%)</th>
<th>Type II hepatocytes (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I  II  III</td>
<td>a  b</td>
</tr>
<tr>
<td>1</td>
<td>NRH</td>
<td>2-7</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>2</td>
<td>NRH</td>
<td>4-6</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>3</td>
<td>NRH</td>
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<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>4</td>
<td>CPH</td>
<td>0-1</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>5</td>
<td>CPH</td>
<td>2-6</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>6</td>
<td>CPH</td>
<td>5-9</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>7</td>
<td>CPH</td>
<td>13-5</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>8</td>
<td>CPH</td>
<td>9-6</td>
<td>100  0  0</td>
<td>—  —</td>
</tr>
<tr>
<td>9</td>
<td>CPH</td>
<td>12-9</td>
<td>17-2  70-3  12-4</td>
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<tr>
<td>10</td>
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<td>2-0  54-0  44-0</td>
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</tr>
<tr>
<td>11</td>
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</tr>
<tr>
<td>14</td>
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<td>31-8  68-2</td>
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<td>CAH</td>
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<td>10-0  76-0  14-0</td>
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<tr>
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<td>CAH</td>
<td>8-7</td>
<td>2-4  97-6  0</td>
<td>—  —</td>
</tr>
</tbody>
</table>

* Frequency of HBcAg-positive hepatocytes was calculated by counting positive cells in almost 1000 hepatocytes.
† Typing of II-a and II-b depended upon the relative staining intensity of the cytoplasm and nuclei as shown in Fig. 1(b).

\[ x = \text{frequency (\%)} \text{ of HBcAg-positive hepatocytes} \quad \text{and} \quad y = \text{amount of DNA (c.p.m.)}. \]

Comparison of these two regression lines revealed that the relative DNA production (DNA amount/frequency of HBcAg-positive hepatocytes) in HBcAg-positive hepatocytes of type I (0·11) was higher than that in hepatocytes of all three types taken together (0·07).

To confirm that viral propagation takes place more effectively in type I hepatocytes than in those of types II and III, electron microscopic observation was carried out. Ultrathin sections stained with 2\% uranyl acetate and 0·3\% lead citrate were examined in an electron microscope.
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Fig. 3. Ultrastructural localization of HBV-related particles in hepatocytes from a patient with NRH (no. 2, Table 1). HBcAg particles (small arrows) were found in both the nuclear matrix and near the endoplasmic reticulum in the cytoplasm (inset). A Dane particle was found in a cisterna of the endoplasmic reticulum (large arrow, inset). Bar marker represents 500 nm.

(JEM-100S) at a magnification of ×30000 to 50000. For comparison we selected liver specimens in which HBcAg-positive hepatocytes were of only type I (liver specimen no. 2 with NRH) or of predominantly type III (liver specimen no. 17 with CAH in Table 1). Fig. 3 shows the presence of a fairly large number of HBcAg particles of diameter 27 nm (small arrows) in the nucleus of a hepatocyte from the NRH patient. In the same tissue, a clearly double-shelled Dane particle was seen in a cisterna of the endoplasmic reticulum (large arrow in inset, Fig. 3). On the other hand, neither HBcAg particles nor Dane particles were found in the nuclei or cytoplasm of the predominantly type III hepatocytes from the CAH patient, even though the cells were stained strongly by the ABC method. Comparing the overall frequencies of HBcAg-positive hepatocytes in the liver specimens used in this experiment, 4.6% in no. 2 and 8.7% in no. 17, it may be deduced that virus replication occurs effectively in type I but not in type III hepatocytes. These findings agree with those reported by Yamada & Nakane (1977), who also failed to find virus-like particles in the cytoplasm of hepatocytes in which HBcAg was restricted to the cytoplasmic area.

The results obtained in this paper strongly suggest that type I hepatocytes are preferentially involved in active viral replication. This is supported by previous results. First, the DNA polymerase activity in serum from patients with minimal histological changes, whose HBcAg-positive hepatocytes we would classify as type I (Table 1), was usually higher than that of several patients whose HBcAg-positive hepatocytes would be composed of a mixture of types I, II and III (Alberti et al., 1983). Second, in patients whose sera were positive for HBeAg, HBcAg was found only in the nuclei of hepatocytes and viral DNA was detectable in serum by spot hybridization and in liver tissues by Southern and in situ hybridization (Negro et al., 1985). Third, the amounts of viral DNA and HBeAg in serum were higher in patients with NRH than in those with chronic type B hepatitis (Kanno et al., 1987).
In considering which hepatocytes support active viral replication, it may be useful to distinguish the terms 'viral DNA replication' and 'viral replication'. Gowans et al. (1983) found that hepatocytes with cytoplasmic HBcAg contained a large amount of single-stranded DNA in that area suggesting that viral DNA in the cytoplasm may be actively synthesized by reverse transcriptase (Summers & Mason, 1982). Complete HBV replication may not occur in such cells, however, because DNA synthesized in the cytoplasm is not efficiently encapsidated into core particles (Burrell et al., 1982). It is necessary to assess HBV DNA in Dane particles in liver or in serum qualitatively and quantitatively to evaluate the viral replication activity in different types of HBcAg-positive hepatocytes.

It is of interest that type II and III hepatocytes appeared in patients when their diagnoses shifted from NRH to CPH or CAH (Table 1), indicating that virus replication or the expression of viral antigen may be influenced by physiological changes in hepatocytes. The results in Table I strongly suggest that HBcAg-positive hepatocytes may undergo a progressive change of type (I to II to III) according to the extent of liver dysfunction. The later types II and III may be the targets of cytotoxic T cells, as has been reported elsewhere (Trevisan et al., 1982; Dienstag, 1984).

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REFERENCES


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