Demonstration of Hepatitis B e Antigen in Hepatitis B Core Particles Obtained from the Nucleus of Hepatocytes Infected with Hepatitis B Virus

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SUMMARY

Liver tissue infected with hepatitis B virus was homogenized and nuclei were separated by centrifugation. Hepatitis B core particles were obtained from the nucleus by the digestion with pronase followed by ultracentrifugation in a sucrose density gradient. Hepatitis B core particles were then treated with sodium dodecyl sulphate and 2 mercaptoethanol and tested for hepatitis B e antigen (HB\textsubscript{e}Ag) by the haemagglutination method. The antigenicity of HB\textsubscript{e}Ag was clearly demonstrated in hepatitis B core particles so treated, although untreated core particles did not reveal any detectable HB\textsubscript{e}Ag activity. The localization of HB\textsubscript{e}Ag in hepatitis B core particles was further supported by the results of a fluorescent antibody technique. When a frozen section of the liver infected with hepatitis B virus was stained with the specific rabbit antibody against HB\textsubscript{e}Ag labelled with fluorescent isothiocyanate, only nuclei of hepatocytes were stained, in a similar distribution to hepatitis B core antigen visualized by fluorescent antibody against hepatitis B core antigen in a nearby section.

INTRODUCTION

Three morphologically different forms of microparticles are identified in the serum of persons infected with hepatitis B virus (HBV). They are 42 nm double-shelled Dane particles (Dane \textit{et al.} 1970) and 20 nm spherical and tubular particles. They all share a surface antigen designated as hepatitis B surface antigen (HB\textsubscript{s}Ag). The core of Dane particles has distinct antigenicity which has been referred to as hepatitis B core antigen (HB\textsubscript{c}Ag) (Almeida \textit{et al.} 1971). Hepatitis B e antigen (HB\textsubscript{e}Ag) occurs as a soluble protein separate from Dane or 20 nm particles, and is antigenically different from HB\textsubscript{s}Ag or HB\textsubscript{c}Ag (Magnius & Espmark 1972).

In the liver of individuals infected with HBV, HB\textsubscript{s}Ag is localized in the membrane or cytoplasm of hepatocytes. In contrast, HB\textsubscript{c}Ag is localized in the nucleus of hepatocytes (Gudat \textit{et al.} 1975; Huang 1975; Ray \textit{et al.} 1976). It has not yet been settled, however, where and how HB\textsubscript{s}Ag exists in the hepatocytes infected with HBV, nor has it been established whether or not HB\textsubscript{e}Ag is carried by hepatitis B core particles, an accepted hepatitis B virion nucleocapsid. We have found that HB\textsubscript{e}Ag was an integral constituent of hepatitis
B core particles which had been isolated from the nucleus of infected hepatocytes. The presence of HB\textsubscript{A}g in the nucleus of hepatocytes in association with HB\textsubscript{V}Ag was further reinforced by the results of immunofluorescent staining.

**METHODS**

*Liver tissue.* A block of liver tissue obtained at autopsy from a patient who died of severe diabetes mellitus and who carried HBV without hepatic dysfunction, had been stored at $-20^\circ$C, and used as a source to prepare hepatitis B core particles. When a portion of liver tissue was cut in a cryostat and stained for HBV-associated antigens by immunofluorescent methods, HB\textsubscript{A}g was stained diffusely in the cytoplasm and HB\textsubscript{V}Ag in the nucleus of hepatocytes.

*Preparation of hepatitis B core particles from liver tissue.* Liver tissue weighing 17 g was minced by scissors and suspended in tris-HCl buffer ($0.01 \text{M, pH 7.2}$) containing $0.001 \text{M-EDTA}$ and $0.1 \text{M-NaCl}$ to make a total volume of 100 ml. The liver fragments were homogenized in a Silverson homogenizer (Silverson Ltd., England) for 5 min and filtered through two layers of gauze. The homogenate was centrifuged at 5000 rev/min for 15 min in a Beckman SW27 rotor. The supernatant was discarded and the precipitate was homogenized in 85 ml of the buffer for 5 min. In order to separate them from the contaminating antibody to HB\textsubscript{A}g, anti-HB\textsubscript{A}g, in the blood and subcellular cytoplasmic microorganisms, nuclei were further purified by centrifugation. Ten ml of suspension was overlaid on to 26 ml of the buffer containing 30% sucrose in a nitrocellulose tube (capacity 36 ml). After centrifuging the tube at 5000 rev/min for 20 min in a Beckman L5-75 centrifuge, anti-HB\textsubscript{A}g, immunoglobulins and cellular debris stayed at the interface and only nuclei were spun down to the bottom. Nuclei were suspended in the buffer and washed three more times by the same procedure, to give a preparation containing purified nuclei. When the smear of the preparation was stained with haematoxylin and eosin, all the particulate components were identified as nuclei of hepatocytes.

The pellet consisting of purified nuclei was finally suspended in an equal volume of the buffer. Five ml of the nuclear suspension were mixed with an equal volume of the buffer containing 2% of pronase E (Kaken Chemical Co., Japan), and incubated at 37 $^\circ$C for 2 h with constant agitation. The digest was centrifuged at 10000 rev/min for 20 min in a Beckman SW27 rotor and the supernatant, rich in hepatitis B core particles, was harvested. Core particles in the supernatant were purified by ultracentrifugation in a discontinuous sucrose gradient. In a Beckman SW27 rotor tube (capacity 16 ml), 1 ml of 60% sucrose was placed at the bottom and 12 ml of 30% sucrose was overlaid. Three ml of the sample containing core particles were centrifuged at 26000 rev/min for 18 h. The bottom was pierced and 1 ml fractions were collected. Each fraction was assayed for HB\textsubscript{A}gAg and fractions rich in core particles were pooled.

*Determination of HB\textsubscript{V}-associated antigens and antibodies.* HB\textsubscript{A}g and antibody to HB\textsubscript{A}g (anti-HB\textsubscript{A}g) were determined by the haemagglutination method (Vyas & Shulman 1970). HB\textsubscript{A}g and antibody to HB\textsubscript{V}Ag (anti-HB\textsubscript{V}Ag) were determined by the immune adherence haemagglutination method (Tsuda et al. 1975). HB\textsubscript{A}g was determined by the inhibition of specific antibody to HB\textsubscript{V}Ag (anti-HB\textsubscript{V}Ag) to agglutinate sheep erythrocytes fixed by glutaraldehyde and coated with purified HB\textsubscript{V}Ag (Takahashi et al. 1977). The test sample was diluted in an acryl microtitre plate and 2 haemaggulinating units of anti-HB\textsubscript{A}g were added. After incubation, fixed sheep erythrocytes coated with purified HB\textsubscript{V}Ag were delivered to the well and the pattern of haemagglutination was observed. Anti-HB\textsubscript{A}g was determined by
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The passive haemagglutination method utilizing fixed erythrocytes coated with purified HBsAg (Takahashi et al. 1977). The results were expressed by the highest dilution of the test sample (2^n) that inhibited haemagglutination.

Fluorescent antibody technique. HBsAg was purified from the plasma of asymptomatic carriers of HBsAg by an affinity column of anti-HBs followed by gel filtration on Sephadex G-200 (Takahashi et al. 1977). The small molecular HBsAg was separated from the large molecular HBsAg (Takahashi et al. 1978) and injected into the rear foot-pads of male albino rabbits and the antiserum was harvested. After the antiserum was absorbed with insolubilized normal human serum, it contained anti-HBs with a haemagglutination titre of 1:512, but anti-HBc, anti-HBx or antibodies to human immunoglobulins were not detectable by the haemagglutination method. Gamma globulin fraction was obtained from the antiserum, and labelled with fluorescein isothiocyanate (FITC) at an F/P ratio of 1:63. FITC-labelled anti-HBs was diluted twofold with phosphate buffer (0.01 M, pH 7.2) containing 0.15 M-NaCl and used as a working reagent.

Liver tissue was biopsied from a 33-year old male patient with liver cirrhosis. His serum titres were 1:4096 for HBsAg, 1:128 for HBsAg, and 1:16384 for anti-HBs. Biopsied liver was snap-frozen in n-hexane cooled in a dry ice/acetone bath. Frozen sections of 4 μm thickness were cut in a cryostat, fixed in acetone and stained with FITC-labelled rabbit anti-HBs, human anti-HBs, as well as with rabbit anti-HBc, and observed in a Leitz Orthoplan microscope operating by incident-light excitation. The positive fluorescence of HBsAg was confirmed by an inhibition test. FITC-labelled anti-HBc (undiluted) was incubated with an equal volume of a solution containing purified HBsAg (haemagglutination titre 1:1024) at 37 °C for 1 h. After centrifuging the labelled antibody at 25000 rev/min for 30 min in a Beckman Type 40 rotor, the supernatant was evaluated for its ability to stain liver sections.

RESULTS

Cryptic nature of HBsAg in hepatitis B core particles

The preparation containing purified hepatitis B core particles obtained from nuclei of hepatocytes was tested for HBsAg and HBsAg by the haemagglutination methods. It was also tested after it had been incubated in the presence of 0.1% sodium dodecyl sulphate (SDS) and 0.1% 2 mercaptoethanol at 37 °C for 2 h. As shown in Table 1, HBsAg was not detected on the surface of the intact hepatitis B core particles. After the treatment with SDS and 2 mercaptoethanol, however, HBsAg activity was clearly demonstrated with a complete loss of HBsAg.

Localization of hepatitis B antigens in hepatocytes infected with HBV

Frozen liver sections of a patient with HBsAg-positive cirrhosis were stained with antibodies to HBV-associated antigens as shown in Fig. 1 (a to c). HBsAg was stained along the cellular surface in a membranous pattern; HBsAg was localized exclusively in the nucleus and HBsAg was stained in the nucleus without any distribution in the cytoplasm. The nuclei positive for HBsAg were somewhat less abundant than those stained by anti-HBs. The specificity of the staining for HBsAg was ascertained by the inhibition of positive fluorescence when the section was stained with FITC-labelled anti-HBs, which had been absorbed with purified HBsAg (Fig. 1 d).
### Table 1. Demonstration of HB<sub>e</sub>Ag in hepatitis B core particles obtained from the nucleus of hepatocytes after treatment with SDS and 2-mercaptoethanol*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HB&lt;sub&gt;e&lt;/sub&gt;Ag titre†</th>
<th>HB&lt;sub&gt;e&lt;/sub&gt;Ag titre‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>&lt;2</td>
<td>64</td>
</tr>
<tr>
<td>After treatment with SDS and 2-mercaptoethanol</td>
<td>64</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* A preparation containing purified hepatitis B core particles was incubated in the presence of 0.1% SDS and 0.1% 2-mercaptoethanol at 37°C for 2 h.
† Hepatitis B e antigen was determined by the inhibition of passive haemagglutination.
‡ Hepatitis B core antigen was determined by the immune adherence haemagglutination method.

### DISCUSSION

A number of lines of evidence strongly indicate an intimate relationship between HB<sub>e</sub>Ag and Dane particles, the presently accepted hepatitis B virions. In epidemiological studies, HB<sub>e</sub>Ag has been demonstrated to signal a high infectivity both in vertical and horizontal transmission of HBV, especially where small doses of inocula are concerned (Alter et al. 1976; Okada et al. 1976). Furthermore, HB<sub>e</sub>Ag is closely associated with morphological, immunological and biochemical markers of Dane particles. Both ourselves and others have observed that HB<sub>e</sub>Ag-positive serum samples containing HB<sub>e</sub>Ag revealed the morphology of Dane particles, the antigenicity of the core of Dane particles and also HB<sub>e</sub>Ag-associated DNA polymerase activity (Nordenfelt & Kjellen 1975; Imai et al. 1976; Takahashi et al. 1976). In contrast, HB<sub>e</sub>Ag positive serum samples containing anti-HB, rarely revealed any detectable markers of Dane particles. Despite this overwhelming evidence to connect HB<sub>e</sub>Ag and Dane particles, no data have been obtained to prove the presence of HB<sub>e</sub>Ag in Dane particles. HB<sub>e</sub>Ag was not detected on the surface of Dane particles conducted independently in at least two laboratories (Gerin et al. 1978; Takahashi et al. 1978). These backgrounds gave rise to queries concerning the exact nature of HB<sub>e</sub>Ag. It has not been possible to decide whether HB<sub>e</sub>Ag is a virus antigen coded by HBV genome or whether it is a host factor raised in response to HBV infection (Neurath & Strick 1977).

We have tried to find the relation of HB<sub>e</sub>Ag and HBV by looking for HB<sub>e</sub>Ag activity in hepatitis B core particles which had been found abundantly in the nucleus of hepatocytes infected with HBV. Hepatitis B core particles were isolated from the nucleus of hepatocytes and tested for HB<sub>e</sub>Ag by the haemagglutination method. We were unable to detect HB<sub>e</sub>Ag on the surface of core particles. When the cores were treated with 2-mercaptoethanol and SDS, however, HB<sub>e</sub>Ag activity disappeared whilst HB<sub>e</sub>Ag activity became detectable by the immunological method.

On the basis of these results, it has been concluded that HB<sub>e</sub>Ag exists in hepatitis B core particles in a cryptic form, covered with a surface layer of molecules bearing HB<sub>e</sub>Ag activity. By treating the cores with 2-mercaptoethanol and SDS, the surface layer was disrupted and the internal HB<sub>e</sub>Ag could be exposed.

Intrahepatic localization of HB<sub>e</sub>Ag has been reported to be either cytoplasmic (Trepo et al. 1976), or nuclear (Arnold et al. 1977). The results of our immunofluorescence study lend support to the nuclear localization of HB<sub>e</sub>Ag in hepatocytes. This view is strengthened by the finding of HB<sub>e</sub>Ag activity in hepatitis B core particles obtained from the nucleus of liver tissue infected with HBV, taken together with the established intranuclear distribution of HB<sub>e</sub>Ag. The possibility remains, however, of cytoplasmic localization of HB<sub>e</sub>Ag.
Fig. 1. Localization of hepatitis B antigens in the biopsied liver specimen of a patient with liver cirrhosis by the fluorescent antibody technique. Immunofluorescent micrographs of (a) a liver section stained with FITC-labelled anti-HBc, (b) a liver section stained with FITC-labelled anti-HBd, (c) a liver section stained with FITC-labelled anti-HBz, and (d) a liver section stained with FITC-labelled anti-HBz which had been absorbed with purified HBzAg.
at a level undetectable by the present immunofluorescent antibody technique. Considerable amounts of HB,Ag are apparently secreted from hepatocytes into the blood-stream in a free form to allow detection by immunodiffusion methods. The presence of HB,Ag in cytoplasm can be reasonably assumed; even though it may be produced in the nucleus, it must go through the cytoplasm to be shed into the circulation.

Although both HB,Ag and 'HbAg were stained in the nucleus, nuclei stained positive for HB,Ag were more abundant than those stained for HB,Ag. Arnold and co-workers (1977) also noted the difference in the staining of nuclei with fluorescent anti-HB, and anti-HB, reagents. In order to stain HB,Ag, core particles in the nucleus would have to be uncovered to expose the internal HB,Ag sites. In the conditions used for the fluorescent antibody technique in the present study, hepatitis B core particles would not have been stripped to allow the staining of HB,Ag in all the nuclei positive for HB,Ag. Alternatively, HB,Ag stained in the nucleus may represent excessively produced molecules bearing HB,Ag activity, which had not been assembled into hepatitis B core particles.

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REFERENCES


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