HISTOLOGIC STAINING METHODS FOR HEPATITIS B SURFACE ANTIGEN (HBS AG) AND INTERPRETATION OF DYE REACTIONS IN VITRO

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Comparison of five HBs Ag staining methods were carried out in 303 autopsy cases and 398 biopsy cases of various liver diseases, including acute hepatitis, chronic active hepatitis, chronic inactive hepatitis, hepatocellular carcinoma without cirrhosis, hepatocellular carcinoma with cirrhosis, and cirrhosis. The author tried various HBs Ag staining dyes which react with some functional groups and found that all HBs Ag staining dyes react with hydrogen sulfide, disulfide and sulfonic acid residue groups which are formed by the oxidization of disulfide and hydrogen sulfide groups of proteins. Three mechanisms of nonspecific reaction in HBs Ag staining methods were considered.

The histological staining method for HBs Ag is useful for the diagnosis of non-A, non-B hepatitis because the diagnosis of non-A, non-B hepatitis can be made only by ruling out the presence of other viral agents, including hepatitis B virus, hepatitis A virus, cytomegalovirus, herpes simplex virus, and Epstein-Barr virus.

Many methods have been developed for the purpose of staining HBs Ag in paraffin sections, including an orcein method (11), modified orcein method (8, 9), aldehyde fuchsin method (11), aldehyde thionine method (11), Victoria blue method (16), and resorcin fuchsin method (8). Among these Shikata's orcein method was the first one that used dyes, and has been widely employed. This method was compared with the immunofluorescent method (11, 16) and the material stained with orcein and Victoria blue was suggested to be HBs Ag.

The purpose of this paper is to present HBs Ag staining dye reactions in vitro, interpretation of nonspecific reactions which appeared in orcein staining, comparison of five HBs Ag staining methods, and notes of the HBs Ag procedure.

MATERIALS AND METHODS

Materials:

The liver specimens were collected at Nagasaki University Medical School from 303 autopsy cases and 398 biopsy cases of various liver diseases (Tables 1 and
including acute hepatitis (55 cases), chronic active hepatitis (160 cases), chronic inactive hepatitis (63 cases), hepatocellular carcinoma without cirrhosis (46 cases), hepatocellular carcinoma with cirrhosis (132 cases) and cirrhosis (245 cases). The specimens were fixed in formalin, or sometimes in Zenker’s formol and embedded in paraffin.

Comparison of HBs Ag staining methods:

The following five staining methods were tested for staining bile pigment, lipofuscin, ceroid, mast cell and copper-associated proteins: 1. author’s orcein staining method (8), 2. resorcin fuchsin staining method (8), 3. aldehyde fuchsin staining method (11), 4. aldehyde thionine staining method (11), and 5. Victoria blue staining method (16). The bile pigments were stained after Hall’s method (2), and control bile pigment material was used for subacute hepatitis autopsy liver. The lipofuscin and ceroid were stained by AFIP method (2), and a control ceroid material was used for biopsy liver of acute hepatitis, and a control lipofuscin material was used for the aged liver. The mast cells were stained by toluidine blue method (7), and a control mast cell material was used for surgical specimens of stomach. The copper-associated proteins were also stained by the dimethylaminobenzyl-

<table>
<thead>
<tr>
<th>Histologic diagnosis</th>
<th>No.</th>
<th>Orcein: HBs Ag positive No.</th>
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<tbody>
<tr>
<td>Acute hepatitis</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Chronic inactive hepatitis</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Hepatocellular carcinoma without cirrhosis</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>Hepatocellular carcinoma with cirrhosis</td>
<td>121</td>
<td>85</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>105</td>
<td>36</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>303</td>
<td>138</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Histologic diagnosis</th>
<th>No.</th>
<th>Orcein: HBs Ag positive No.</th>
<th>Serum: HBs Ag positive No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute hepatitis</td>
<td>41</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td>141</td>
<td>18</td>
<td>59</td>
</tr>
<tr>
<td>Chronic inactive hepatitis</td>
<td>55</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>Hepatocellular carcinoma without cirrhosis</td>
<td>10</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Hepatocellular carcinoma with cirrhosis</td>
<td>11</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>140</td>
<td>19</td>
<td>44</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>398</td>
<td>46</td>
<td>140</td>
</tr>
</tbody>
</table>
idenerhodanine method (7), and a control copper-associated protein material was used for autopsy liver of Wilson’s diseases.

Studies on the staining mechanism:

In order to examine the reactivity of the dyes to chemical groups, the following solutions of reagents were prepared: 100% ethyl mercaptan for hydrogen sulfide group, oxide ethyl mercaptan for disulfide group, 5% benzen sulfonic acid solution for sulfonic acid group, 37% formaldehyde for aldehyde group, 100% acetone for ketone group, 100% acetic acid for carboxyl group, 100% aniline for amino group. The functional groups were each placed in 5 tubes in an amount of 10 ml and were added respectively with 0.2 ml of the orcein solution, the resorcin fuchsin solution, the aldehyde fuchsin solution, the aldehyde thionine solution and the Victoria blue solution, and a drop of each solution was placed on a slide glass, covered with glass and examined microscopically. Distilled water was used as control. In this paper, the reaction was judged positive when precipitation was observed. The reaction of unformed precipitation is omitted. This decision may be suitable for these examinations.

RESULTS

Comparison of HBs Ag staining methods:

All dyes for staining of the HBs Ag can react with elastic fibers. By the orcein staining method, HBs Ag, elastic fibers, bile pigment and nuclei were stained blue.

![Fig. 1. Inclusion type of HBs Ag are seen in the hepatocytes. Orcein stain, ×400](image1)

![Fig. 2. The arrows indicate copper-associated protein in the hepatocytes. Wilson’s disease of the liver. Orcein stain, ×400](image2)
By the resorcin fuchsin staining method, HBs Ag, elastic fibers, bile pigment and copper-associated protein were stained dark blue to black, and nuclei were stained red. By the aldehyde fuchsin staining method, HBs Ag, elastic fibers, bile pigment, lipofuscin, ceroid, mast cell granules and copper-associated protein were stained deep purple, and nuclei were stained blue. By the aldehyde thionine staining method, HBs Ag, elastic fibers, bile pigment, lipofuscin, ceroid, mast cell granules and copper-associated protein were stained blue, and nuclei were stained red. By the Victoria blue staining method, HBs Ag, elastic fibers, mast cell granules and copper-associated protein were stained blue, and nuclei were stained red. The bile pigment was stained by the orcein staining method, the resorcin fuchsin staining method, the aldehyde fuchsin staining method, and the aldehyde thionine staining method, but was not stained by the Victoria blue staining method. The lipofuscin and the ceroid were stained by the aldehyde fuchsin staining method and the aldehyde thionine staining method, but were not stained by the orcein staining method, the resorcin fuchsin staining method, and the Victoria blue staining method. The lipofuscin was stained by the Shikata’s orcein staining method (17). However, the author's orcein staining method did not stain the lipofuscin and ceroid. The lipofuscin and ceroid were not stained with the Victoria blue dye which often adheres to the tissue sections. The copper-associated proteins were stained with all dyes for staining HBs Ag, and these substances are derived from primary biliary cirrhosis and other cholestatic liver diseases (3-6, 12-14). By the author’s orcein method, most of the copper-associated proteins were stained brown (Fig. 2), a few copper-associated proteins were stained blue to bluish brown.

Fig. 3. The arrow indicates inclusion type of HBs Ag in hepatocellular carcinoma. Orcein stain, ×400

Fig. 4. The arrows indicate HBs Ag in the pancreas. Orcein stain, ×400
However, by Shikata's orcein staining method, most of the copper-associated proteins were stained blue to bluish brown, others were stained brown. Judging by the author's orcein staining method, the orcein-positive granular substances could be copper-associated proteins (Table 3).

**Mechanism of HBs Ag staining dye reactions in vitro:**

All dyes solution for staining HBs Ag can react with hydrogen sulfide, disulfide and sulfonic acid residue groups (Table 4). In all the HBs Ag staining methods using dyes, it is likely that hydrogen sulfide groups and/or disulfide group is oxidized into sulfonic acid residue groups, which then react with the dyes. The orcein, the resorcin fuchsin and the Victoria blue reacted with aldehyde group, but the aldehyde

Table 3. Comparison with results of HBs Ag staining methods

<table>
<thead>
<tr>
<th></th>
<th>HBs Ag</th>
<th>Elastic fibers</th>
<th>Bile pigment</th>
<th>Lipofuscin</th>
<th>Geroid</th>
<th>Mast cell</th>
<th>Copper</th>
</tr>
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<tbody>
<tr>
<td>Orecin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Resorcin fuchsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Aldehyde fuchsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aldehyde thionine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Victoria blue</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+: positive  -: negative
fuchsin and the aldehyde thionine did not react with aldehyde group. All dye solutions for staining the HBs Ag were not able to react with the carboxyl group, amino group and distilled water. The bile pigment was stained with the orcein, the resorcin fuchsin, the aldehyde fuchsin and the aldehyde thionine dyes, but was not stained with the Victoria blue dye. Since the ketone group was contained in bile pigments, these HBs Ag staining dyes which react with the ketone group would stain the bile pigment. However, the Victoria blue dye does not stain the bile pigment, probably because it does not react with the ketone group.

**DISCUSSION**

The staining of HBs Ag with orcein and Victoria blue was illustrated by the comparison with the immunofluorescent methods (11, 16). Therefore, the dye-positive material may be HBs Ag. In view of the results in Table 4, the mechanism of the HBs Ag staining method using dyes may be considered to consist in the oxidization of the hydrogen sulfide and the disulfide groups into sulfonic acid residue groups, and the HBs Ag contained disulfide group by means of biochemistry (15, 18). Therefore, the sulfonic acid residue groups react with the dyes for HBs Ag staining. In the conventional orcein method, it was occasionally experienced that the cytoplasms of hepatocytes were also stained concurrently, and the stained degenerative and necrotic cells made it difficult to determine the HBs Ag (Fig. 6). Moreover, when cytoplasms were stained concurrently, it was difficult to detect the HBs Ag which were stained lightly. However, such disadvantages have been eliminated by addition of sensitizer solution and consequently satisfactory good contrast has become constantly available.

The significance of the sensitizer solution in the orcein method is to prevent concurrent staining. The concurrent staining of hepatocytes in the conventional methods might be caused by entry of the orcein dyes into the small gaps of protein...
molecular structure. When a section is placed in ferric ammonium sulfate solution or uranium nitrate solution prior to the placement in the orcein solution, the gaps of proteins are filled with iron or uranium ions leaving no room for the orcein dyes and consequently it is considered that cytoplasms may not be stained with the orcein dyes. For example, aluminum and chromium ions have no affinity for tissues, and these ions were not useful as sensitizers. In order to examine whether ferric ions are present in the gaps of protein molecule, paraffin sections of the author’s orcein staining and then Prussian blue reaction (2) which is a procedure to identify trihydric iron was performed. As a result, the cytoplasms were stained blue, evenly demonstrating the adsorption of the iron to the cytoplasms.

The orcein dye reacted not only with sulfonic acid residue groups, but also with small gaps in the protein molecular structure in the cell. Therefore, a more satisfactory method of staining HBs Ag by two metal salts in histologic sections was introduced. However, rare nonspecific reactions did occur in histologic sections. Three mechanisms of nonspecific reactions were considered. The first mechanism, in the reaction in which amino groups react with hydrogen in an acidic solution (+NH₃-R-COOH) the electric charge of proteins becomes positive as a result. The orcein can be subdivided into 14 dyes by distribution chromatography (19). If the orcein dyes contain negatively charged dye, protein reacts with it because of coupling of the electric charge. With the same reaction as above, the mechanism of the eosin staining reaction in hematoxylin and eosin staining (10) was considered. The second mechanism, as the sections are placed in the orcein solution for more than 20 min, the cytoplasms begin to be stained though in a slight degree. This is because the larger molecular materials of the orcein expel

Fig. 6. Degenerative hepatocytes are stained with orcein dye. Former orcein stain, ×400
the smaller molecular materials of the iron. The third mechanism, an appropriate
time for the treatment with the ferric ammonium sulfate is approximately one
min. Since the trihydric iron functions as an oxidizing agent by transferring
hydroxyl to substrate (1), the aldehyde group which might be formed by the oxidization
reaction of periodic acid Schiff (PAS) positive materials would be stained with
the orcein dyes. In the biopsy materials as compared with the autopsy materials,
the cytoplasm is often stained slightly with the HBs Ag staining solution. It is
speculated that, since the biopsy materials contain much glycogen, the aldehyde
group is produced by oxidization and it reacts with the HBs Ag staining solution.

\[
\text{Fe(H}_2\text{O)}^{3+} + \text{R} \cdot \rightarrow \text{Fe(H}_2\text{O)}^{2+} + \text{H}^+ + \text{ROH}
\]

The orcein dyes vary considerably in staining properties by brand and lot
number, and it is difficult to obtain constantly stable results. The orcein dyes may
have poor staining properties immediately after purchase, but use after about one
year results in satisfactory staining. As to the amount of hydrochloric acid to be
added to the orcein solution, 1.2 ml gives better staining than the conventional
amount of 1.0 ml. If the staining is excessive, an additional 0.2 ml of the hydro-
chloric acid should provide satisfactory staining.

The use of the ferric ammonium sulfate as the sensitizer solution in the resorcin
fuchsin method results in somewhat blackish staining of the cytoplasm. When
uranium nitrate is used, cytoplasm is not stained and satisfactory preparations of
good contrast are obtained. This is because the larger molecular materials of
resorcin fuchsin expel the smaller molecular materials of the iron ions since treatment
with resorcin fuchsin solution is made for as long as 1–3 hr. Consequently, the use
of uranium nitrate having the uranium atom (atomic weight 238) as compared
with the use of the ferric ammonium sulfate having the iron atom (atomic weight
55.8) is considered appropriate for staining requiring a relatively long time. The
significance of the sensitizer solution in the resorcin fuchsin method may be identical
to that in the orcein method. Treatment with uranium nitrate solution for more
than one min results in poor staining of the nuclei with nuclear fast red (Kernechrot)
solution. The HBs Ag are recognizable in this state but more readily visible upon
yellow counterstain of the cytoplasms with saturated picric acid. However, since
the cytoplasms are also stained with the nuclear fast red somewhat lightly, the
cytoplasms are differentiated with 1% hydrochloric alcohol prior to the contrast
staining, which provide satisfactory results.

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REFERENCES