Complement fixing hepatitis B core antigen immune complexes in the liver of patients with HBs antigen positive chronic disease

M. RIZZETTO, F. BONINO, O. CRIVELLI, M. G. CANESE, AND G. VERME

SUMMARY One hundred and fifty-two biopsies from serologically HBsAg positive and negative patients with liver disease were studied in immunofluorescence for the presence of the surface (HBs) and the core (HBc) antigenic determinants of the hepatitis B virus, of immunoglobulins and complement (C) deposits, and for the capacity to fix human C. Circumstantial evidence is presented suggesting that HBc immune-complexes are a relevant feature in the establishment and progression of chronic HBsAg liver disease. C fixation by liver cells was shown in all HBc positive patients with chronic hepatitis; an active form was present in every case, except two with a persistent hepatitis, an inverse ratio of HBc to C binding fluorescence being noted between active chronic hepatitis and cirrhotic patients. HBc without C fixation was observed in only three patients in the incubation phase of infectious hepatitis. IgG deposits were often found in HBc containing, C fixing nuclei. No C binding or IgG deposits were observed in acute self-limited type B hepatitis, in serologically positive patients with normal liver or minimal histological lesions, with and without HBs cytoplasmic fluorescence in their biopsy, or in serologically negative individuals.

The serological detection of hepatitis B core antibody (Hoofnagle et al., 1973) and the intrahepatic localisation of hepatitis B core antigen (HBc) by immunofluorescence (Hadziyannis, 1973; Gudat et al., 1975) are recent developments with promising clinical applications in the study of HBsAg positive liver disease.

While studying HBc positive liver biopsies (Rizzetto et al., 1976) an attempt was made to set up an indirect immunofluorescence (IFL) test for the detection of core antibodies. This was sometimes impeded by the presence of IgG bound on core positive nuclei, which precluded indirect IFL. When we tried to overcome the problem by using the C fixing capacity of the HBcAg-HBcAb system, it became apparent that many biopsies from HBsAg positive patients were able to stain with anti C3 fluorescent serum independently of HBcAb fixation.

These findings are reported and their serological and clinical implications discussed.

Methods

PATIENTS AND BIOPSIES

One hundred and forty-six percutaneous or laparoscopic needle biopsies and six operative or post mortem specimens were studied.

Eighty patients had no HBsAg in the serum. They included patients with a variety of acute and chronic liver diseases.

Seventy-two patients were HBsAg positive: their serological or clinical diagnosis is shown in Table 1. In the HBsAg acute hepatitis group, 21 biopsies were taken four to 22 days after sudden onset of jaundice and after enzyme levels had reached a peak; in three patients admitted for asymptomatic antigenaemia a biopsy was taken five, seven, and eight days before the onset of acute icteric hepatitis, at a time when transaminase levels were normal in two cases and only slightly raised in the third.

Histological criteria were those of De Groote et al. (1968).

Tissue specimens were divided in two parts, one processed for routine histology, the other frozen, cut
Table 1  HB₄Ag, HBᵥAg, and HB₅Ag associated with HB₄Ag immunofluorescence in 72 serologically HB₄Ag positive patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patients tested (no.)</th>
<th>No. of biopsies positive in IFL for</th>
<th>HB₄Ag</th>
<th>HBᵥAg</th>
<th>HB₅Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy chronic carriers</td>
<td>14</td>
<td></td>
<td>11</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Acute self-limited hepatitis</td>
<td>21</td>
<td></td>
<td>3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Patients incubating acute hepatitis</td>
<td>3</td>
<td></td>
<td>—</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Persistent</td>
<td>10</td>
<td></td>
<td>4</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Active</td>
<td>8</td>
<td></td>
<td>—</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>8</td>
<td></td>
<td>—</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Inactive</td>
<td>6</td>
<td></td>
<td>1</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Hepatic fibrosis</td>
<td>2</td>
<td></td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

with the cryostat in 4 µ sections and fixed with ether at room temperature for five minutes.

**ANTISERA**

HB₄s antiserum was prepared from Behring-Hoescht precipitating antiserum RBB15 by conjugation of the gamma globulin fraction with rodamine isothiocyanate (RITC) (cat. N. 12198, Becton and Dickinson).

HBᵥ antiserum was prepared from the blood of a healthy HB₄Ag chronic carrier; the IgG fraction, isolated after chromatography on DE52 (Whatmann) was conjugated with fluorescein isothiocyanate (FITC) (BDH Isomer I).

The details of preparation and specificities of the antiserum have already been described (Rizzetto et al., 1976). FITC anti human IgA, IgM, C₃, and C₄ were obtained from Behring-Hoechst (TKAO5, TKCO5, TKDO5, TKGO5); FITC anti human C₁q and RITC anti human IgG and anti human C₃ were prepared in the laboratory, by conjugating the immunoglobulin fractions isolated from Behring-Hoechst precipitating antiserum to human C₁q (TNIO5), IgG (TOBO5), and C₃ (TEAO5).

**IMMUNOFLUORESCENCE**

To localise the surface and the core antigens in the liver, each biopsy was double stained with FITC conjugated antiserum against HB₄ and RITC conjugated antiserum against HBᵥ.

To detect immunoglobulins or complement fixed in vivo, biopsies were covered with a drop of FITC conjugated antihuman IgG, A, M, C₁q, C₃, C₄.

To detect the capacity of hepatocytes to fix human complement in vitro, sections were covered for 30 minutes with fresh, AB group human serum, negative for autoantibodies and HBᵥ antibodies in IFL and for HB₄ antibodies in RIA, diluted in Ca²⁺ and Mg²⁺ containing buffer; after 30 minutes, the slides were washed, covered with anti-human C₁q, C₃ and C₄ FITC conjugated sera, washed again and mounted; control slides were covered with the same serum heated for 30 minutes at 56°C, or diluted in EDTA-containing buffers and treated as previously described.

To study the relations between cells containing the HB virus determinants and those with immunoglobulins or with C fixing capacity, three serial slides from each positive biopsy were cut, fixed in ether, covered for 30 minutes with fresh human serum, washed, and double stained—the first with FITC anti HBᵥ and RITC anti human C₃ sera, the second with FITC anti HBᵥ and RITC anti IgG sera, and the third with FITC anti C₃ and RITC anti HBᵥ sera.

Specimens were examined with a Universal Zeiss fluorescence microscope, alternating the fluorescein and rodamine excitation wavelength. Photographs were taken with an automatic camera applied to the microscope.

**ELECTRON MICROSCOPY**

For the ultrastructural study small blocks of tissue from patients nos. 3, 12, and 13 (Table 2) were left in cold phosphate buffered 3% glutaraldehyde for two hours, post-fixed in 1% osmium tetroxide, and embedded in Araldite (Durcupan ACM Fluka). Staining was carried out with uranyl acetate (Watson, 1958) during alcoholic dehydration and lead citrate (Reynolds, 1963) was then applied to the section.

**Results**

**IMMUNOFLUORESCENCE RESULTS**

IFL results are summarised in Tables 1 and 2.

No staining of the liver biopsy was observed in the 80 HB₄Ag negative patients.

In Table 1, HB₄Ag positive patients are divided according to the histological or clinical diagnosis and the results with HBᵥ and HBᵥ antiserum are reported. In 11 healthy chronic carriers without liver disease a diffuse cytoplasmatic localisation of HB₅Ag was observed in many hepatocytes; no other positive reaction occurred in this group of biopsies.

In the acute self-limited hepatitis (AH) group, the majority of biopsies did not react with any antiserum; in three patients HBᵥ was seen in the cytoplasm of a few isolated liver cells. In three individuals incubating infectious hepatitis (nos. 18, 19, 20, Table 2) HBᵥ was found in the nuclei of almost all the hepatocytes in many serial sections, unaccompanied by any other fluorescence.

Reactions with all antisera were negative in 11 patients in the chronic liver disease group (Table 1).
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Table 2  Histological and immunofluorescence data in 20 patients with HBsAg in liver biopsy

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Age (yr.)</th>
<th>Biopsy diagnosis</th>
<th>SGOT (&lt;40)</th>
<th>SGPT (&lt;40)</th>
<th>HBeAg n.</th>
<th>C' fixing capacity</th>
<th>Deposits of C, IgG, IgM, IgA, B2 / C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>35</td>
<td>CAH</td>
<td>265</td>
<td>199</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>62</td>
<td>CAH</td>
<td>110</td>
<td>90</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>48</td>
<td>CAH</td>
<td>124</td>
<td>101</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>11</td>
<td>CAH</td>
<td>85</td>
<td>69</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>29</td>
<td>CAH</td>
<td>80</td>
<td>75</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>46</td>
<td>CAH mild</td>
<td>82</td>
<td>62</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>10</td>
<td>CAH</td>
<td>60</td>
<td>52</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>66</td>
<td>AC</td>
<td>58</td>
<td>23</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>42</td>
<td>AC</td>
<td>51</td>
<td>58</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>35</td>
<td>AC</td>
<td>94</td>
<td>60</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>13</td>
<td>AC</td>
<td>110</td>
<td>105</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>55</td>
<td>CAH</td>
<td>105</td>
<td>55</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>71</td>
<td>AC</td>
<td>320</td>
<td>76</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>62</td>
<td>AC</td>
<td>151</td>
<td>140</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>54</td>
<td>Inactive cirrhosis</td>
<td>14</td>
<td>11</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>34</td>
<td>CPH</td>
<td>92</td>
<td>80</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>36</td>
<td>CPH</td>
<td>22</td>
<td>19</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>46</td>
<td>Normal liver</td>
<td>23</td>
<td>19</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>22</td>
<td>Focal necrosis</td>
<td>178</td>
<td>192</td>
<td></td>
<td>+ + + + + + -</td>
<td>+ +</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>22</td>
<td>Normal liver, a few focal necrosis</td>
<td>39</td>
<td>40</td>
<td>+ + + + + + -</td>
<td>+ + + + + + -</td>
<td></td>
</tr>
</tbody>
</table>

±, +, + +, + + +: density of IFL.
AH: acute hepatitis.
AC: active cirrhosis.
CPH: chronic persistent hepatitis.
CAH: chronic active hepatitis.
c = cytoplasmic.
n = nuclear.
m = month

Scattered HBs cytoplasmic IFL was detected in four individuals with persistent chronic hepatitis, in one with inactive cirrhosis, and one with fibrosis. Nuclear staining with HBs antisera was observed in the other 20 biopsies in this group involving from a few to all the liver cells in the section (Table 2). Occasional simultaneous cytoplasmic HBs IFL was observed in six patients; both determinants were only seldom expressed by the same cells.

In all HBs positive biopsies, except three from patients incubating acute hepatitis, a strong nuclear and sometimes cytoplasmic fluorescence was observed after incubation with fresh human serum and subsequent staining with FITC antisera against human C4, C3, C1q (Fig. 1); this reaction was abolished after heating the serum at 56° for 30 minutes or after adding EDTA to the diluting buffer.

No C deposits were ever detected after direct staining with antisera to C components.

No C binding was observed in any of the biopsies from the 80 HBsAg negative patients or in those from serologically HBsAg positive patients which were negative in IFL or positive only for the cytoplasmic HBs determinant.

After previous incubation with fresh human serum and double staining with FITC anti HBs and RITC anti-human C3 conjugates, HBs positive nuclei were often shown to fix complement; usually, however, C fixing cells outnumbered HBs reacting ones in advanced cirrhotic disease, while more HBs positive than C binding hepatocytes were observed in early active hepatitis (Fig. 2a, b).

In sections from HBs positive biopsies reacting with HBs antisera, double stained with RITC anti HBs and FITC anti C3 sera, many C fixing cells also showed large amounts of HBs in their cytoplasm (Fig. 3a, b).

Nuclear deposits of IgG were observed in 10 HBs positive biopsies; in sections double-stained with RITC anti human IgG and FITC anti HBs conjugates, IgG positive nuclei were always shown to contain the HBs determinant. The relevant histological and serological data of the HBs positive patients in this study are summarised in Table 2).
patients with chronic disease; other liver function tests were as expected from the clinical picture of the individual. Three patients (nos. 1, 3, and 12) were repeatedly biopsied; an increase in C fixing and IgG nuclear deposits was noticed in later biopsies when compared with the first ones. In the post mortem specimen from patient no. 12 only a few nuclei were visualised with anti HB_e serum, while IgG deposits and C binding activity were greatly increased compared with the first biopsy taken nine months earlier.

**ELECTRON MICROSCOPE RESULTS**

In the electron microscope (EM) the nuclei of the liver biopsy from patient no. 3 (Table 2) and of the necropsy specimen from patient no. 12 were filled with numerous particles about 200 Å in diameter.

These particles appeared as isolated or aggregated in clusters of a few units scattered in a rarefied karyoplasm (Fig. 4a); many of them displayed a central clear area, while some others appeared more homogeneously dense. At higher magnification the particles appeared to be composed of globular subunits about 30 Å in diameter (Fig. 4b).

Only a few isolated groups of such particles were detected in the nuclei from patient no. 13 (Table 2); the overall appearance of the karyoplasm was denser than in the previous patients.

**Discussion**

Several serological reactions associated with the hepatitis B virus have been described recently.

The system related to the Dane particle core (Almeida et al., 1971) has proved to be a marker of infectivity and chronic active disease; HB_e antibodies appear to be a sensitive indicator of persistent
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Fig 3 No. 13 (Table 2). Biopsy (a) double stained with RITC anti HBs and (b) FITC anti-human C3 after previous incubation with fresh human serum. The same cells reacting in the cytoplasm with HBs serum, fix complement in their nuclei and weakly in their cytoplasm.

Fig 4 Main figure. Nuclear virus-like particles partly isolated, partly aggregated in clusters (arrows). N = nucleolus. × 48 000.
Inner square. At higher magnification some virus-like particles show a clear area, others (large arrow) appear dense. In some particles globular subunits are present (thin arrow). × 164 000.
viral replication (Hoofnagle et al., 1973) and HBs has been localised by IFL in the liver nuclei of patients with chronic active hepatitis (CAH) (Brzosko et al., 1973; Hadziyannis and Gerber, 1974; Gudat et al., 1975). This determinant was not seen in the liver of healthy chronic carriers (Hadziyannis et al., 1973; Rizzetto et al., 1976) and was rarely observed in acute, self-limiting hepatitis (Gudat et al., 1975); in this study, HBs was found by direct IFL in a high number of patients with chronic liver disease (Table 1), and in three subjects from whom biopsies were taken while incubating acute hepatitis, it was sometimes associated with variable amounts of cytoplasmic HBs IFL.

In all HBs positive biopsies, except three, a strong nuclear and, less frequently, weaker cytoplasmic complement binding activity was observed, sometimes associated with nuclear deposits of IgG; IgG, when present, was always located only in core positive nuclei, as shown by double staining with FITC anti HBs and RITC anti IgG. With the same technique, it was shown that HBs, when present in HBs positive biopsies, was often expressed in C fixing cells (Fig. 1a, b).

None of 80 serologically HBsAg negative patients nor any biopsy reacting only with HBs antiserum, ever bound complement in vitro.

The inverse relationship observed between in vitro C fixation and direct core IFL (Table 2), might be explained by a variable masking by immunoglobulins of HBs determinants that are no longer available to the specific antiserum. There is, therefore, indirect evidence suggesting that core, complement fixing, immune complexes are formed in the liver of patients with chronic HBsAg positive hepatitis, and are somehow linked to the activity and progression of the disease; though the C system cannot enter the intact living cell, as shown by the absence of in vivo C3 binding, it is avidly fixed on the complexes exposed on the slide.

Whether IgG molecules enter an intact or damaged hepatocyte or are taken up as an immune complex with HBs, and how they get across the plasma membrane to the nucleus, is obscure; it has been repeatedly shown that the antinuclear antibodies found in systemic lupus erythematosus cannot penetrate an intact cell (Dubois, 1974) and, to our knowledge, there is no evidence of intranuclear complexes in parenchymal cells in human pathology.

At the ultrastructural level, spherical intranuclear virus-like particles were easily seen in large numbers in two patients with CAH (Fig. 4a, b) and strong HBs IFL and C binding (nos. 3 and 12, Table 2); only a few particles emerging from an electron dense amorphous material could be detected in a patient with cirrhosis (no. 13 Table 2); a similar aggregation of core particles was observed by Huang et al. (1974) and by Deutsch and Spence (1972) and interpreted by the latter author as immune aggregate formations.

Immunocomplexes involving the HB virus have been demonstrated by Nowoslawski et al. (1972) in the liver and in extrahepatic lesions in a significant percentage of subacute and chronic liver diseases and more recently IgG and C3 deposits were seen in HBs positive biopsies by Hadziyannis and Gerber (1974), by Arnold et al. (1975) and by Sarno et al. (1975), the latter authors suggesting that IgG staining was due to an antibody against HBs.

In this study, C fixation was observed in biopsies of untreated and steroid or azathioprine treated patients; in the clinical picture there was nothing specific to this group. A past history of acute hepatitis was recorded in six patients; the majority presented with signs of chronic disease, usually ascites.

The extension of C binding and HBs fluorescence appeared to correlate with the biochemical activity of the disease, not with a single morphological picture; the histological diagnosis varied from a chronic persistent hepatitis to an active cirrhosis.

Though a lesser number of positive cells were found in advanced disease, in these biopsies IgG positive and C fixing cells outnumbered as a rule those reacting with HBs antiserum; it could be argued that a sampling error is possible and might be anticipated as consistent because of the zonal distribution of immunofluorescence, yet in patients repeatedly subjected to biopsy, IgG and cytoplasmic C binding occurred for the first time in the second biopsy, whereas only widespread HBs and scattered nuclear C fixation were observed in the first one (nos. 3 and 12, Table 2).

The different IFL appearances in early or advanced disease might be the result of the progressive, persistent stimulation of the immune system with final complete masking of HBs reactivity by antibody excess in cirrhotic patients; however, whether and how this leads to chronic inflammation and to nodular transformation is at present unknown.

The hypothesis has been put forward that variations in the individual immune response determine the final outcome of exposure to HB virus (Dudley et al., 1972; Gudat et al., 1975). A normoergic reaction leads to sudden and massive destruction of infected hepatocytes and to acute hepatitis, a hyperergic one allows the individual to remove some but not all infected cells thus starting a vicious circle leading to chronic hepatitis. In this study no proof was obtained of a decreased immune response in chronic HBsAg positive liver disease: an abnor-
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mal, yet active response to the virus was always suggested by the IFL appearances and it is doubtful whether HBs is cytotoxic unless an immune reaction against it is mounted, as shown by its presence in the anergic incubation phase of acute hepatitis when no histological damage was yet apparent, and by the absence of aggressive lesions in immuno-suppressed individuals harbouring large amounts of HBs in their liver (Gudat et al., 1975).

These conclusions give rise to considerable therapeutic implications. HBsAg chronic liver disease is currently treated with immunosuppressive drugs; though symptomatic relief is promptly achieved, it is not known whether they help to clear the virus, or, instead, assist its asymptomatic spread and replication by their immune-depressive action: evidence supporting this hypothesis has recently been presented by Galbraith et al. (1975) who reported three HBsAg positive patients with malignant disease in whom fulminant hepatitis followed drug withdrawal.

Clearly, further clinical trials supported by immunofluorescence studies are needed in order to assess the value of immunosuppressive treatment in HBsAg liver disease.

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