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

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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.

Received for publication 1 July 1976

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

**Antisera**

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

HBc antisera was prepared from the blood of a healthy HBsAg 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 antisera 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 antisera to human C1q (TNIO5), IgG (TOBO5), and C3 (TEAO5).

**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 (Dercupan ACM Fluka). Staining was carried out with uranyl acetate (Watson, 1958) during alcoholic dehydration and lead citrate (Reynolds, 1963) 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 HBsAg negative patients.

In Table 1, HBsAg positive patients are divided according to the histological or clinical diagnosis and the results with HBs and HBc antisera are reported. In 11 healthy chronic carriers without liver disease a diffuse cytoplasmic localisation of HBsAg 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 antisera; in three patients HBc was seen in the cytoplasm of a few isolated liver cells. In three individuals incubating infectious hepatitis (nos. 18, 19, 20, Table 2) HBc 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).
Complement fixing hepatitis B core antigen immune complexes in the liver

Table 2  Histological and immunofluorescence data in 20 patients with HBsAg in liver biopsy

<p>| Case Sex BIO | Histological diagnosis | Transaminases SGOT (&lt;40) | SGPT (&lt;40) | Immunofluorescence | Deposits of |</p>
<table>
<thead>
<tr>
<th>no.</th>
<th>no.</th>
<th></th>
<th></th>
<th>HBsAg c.</th>
<th>HBsAg n.</th>
<th>C fixing capacity</th>
<th>C, IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>B, C</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>35</td>
<td>1</td>
<td>CAH</td>
<td>265</td>
<td>199</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>62</td>
<td>1</td>
<td>CAH</td>
<td>110</td>
<td>90</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>48</td>
<td>1</td>
<td>CAH</td>
<td>124</td>
<td>101</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>4</td>
<td>F</td>
<td>11</td>
<td>1</td>
<td>CAH</td>
<td>78</td>
<td>86</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>29</td>
<td>1</td>
<td>CAH</td>
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<td>69</td>
<td>-</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>46</td>
<td>1</td>
<td>CAH mild</td>
<td>82</td>
<td>62</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>10</td>
<td>1</td>
<td>CAH</td>
<td>60</td>
<td>52</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>66</td>
<td>1</td>
<td>AC</td>
<td>58</td>
<td>23</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>9</td>
<td>M</td>
<td>42</td>
<td>1</td>
<td>AC</td>
<td>51</td>
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<tr>
<td>10</td>
<td>M</td>
<td>35</td>
<td>1</td>
<td>AC</td>
<td>94</td>
<td>60</td>
<td>-</td>
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<tr>
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<td>M</td>
<td>13</td>
<td>1</td>
<td>AC</td>
<td>110</td>
<td>105</td>
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<td>+</td>
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<td>+</td>
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<td>12</td>
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<td>55</td>
<td>1</td>
<td>CAH</td>
<td>105</td>
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<td>F</td>
<td>71</td>
<td>1</td>
<td>AC</td>
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<td>AC</td>
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<td>140</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>54</td>
<td>1</td>
<td>Inactive cirrhosis</td>
<td>14</td>
<td>11</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>16</td>
<td>F</td>
<td>34</td>
<td>1</td>
<td>CPH</td>
<td>92</td>
<td>80</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>36</td>
<td>1</td>
<td>CPH</td>
<td>22</td>
<td>19</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>46</td>
<td>1</td>
<td>Normal liver</td>
<td>23</td>
<td>19</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>22</td>
<td>1</td>
<td>Focal necrosis</td>
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<td>192</td>
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<td>+</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>22</td>
<td>1</td>
<td>Normal liver, a few focal necrosis</td>
<td>39</td>
<td>40</td>
<td>-</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 antigen was observed in the other 20 biopsies in this group involving from a few to all the liver cells in the section (Table 2). Occasionally 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 C3, C4, 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).
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Fig 1 No. 11 (Table 2). Biopsy preincubated with fresh human serum and stained with anti-human FITC C3. C fixation on nuclei and cytoplasms.

No biochemical or histological abnormality specific to this group was observed except an increase in transaminases activity in the majority of 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

Fig 2 No. 1 (Table 2). Biopsy (a) double stained with FITC anti HB$_e$ and (b) RITC anti human C3 after previous incubation with fresh human serum. Not all cells reacting with HB$_e$ fix complement.
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Fig 3  No. 13 (Table 2). Biopsy (a) double stained with RITC anti HB\(_4\) and (b) FITC anti-human C3 after previous incubation with fresh human serum. The same cells reacting in the cytoplasm with HB\(_4\) 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. \(\times 48\,000\).
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). \(\times 164\,000\).
viral replication (Hoofnagle et al., 1973) and HBv 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-limited hepatitis (Gudat et al., 1975); in this study, HBv 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 HBv IFL.

In all HBv 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 HBv and RITC anti IgG. With the same technique, it was shown that HBv, when present in HBv positive biopsies, was often expressed in C fixing cells (Fig. 1a, b).

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

The inverse relationship often observed between in vitro C fixation and direct core IFL (Table 2), might be explained by a variable masking by immunoglobulins of HBv 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 HBvAg 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 HBv, 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 intra-nuclear 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 HBv 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 authors 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 HBv 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 HBv.

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 HBv 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 HBv 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 HBv 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 HBv 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 HBvAg positive liver disease: an abnor-
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 immuno-suppressive 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.

References


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