Antigenic Cross-reactions between Woodchuck Hepatitis Virus and Human Hepatitis B Virus Shown by Immune Electron Microscopy

By LINDA M. STANNARD,1 * O. HANTZ2 AND C. TREPO2

1 Virus Unit, U.C.T. Medical School, Observatory, Cape Province, South Africa 7925 and
2 INSERM, Edouard Herriot Hospital, Lyon, France

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SUMMARY

Using immune electron microscopy (IEM), low-level cross-reactions could be demonstrated between the surface antigens of hepatitis B and woodchuck hepatitis. However, immune complex formation was greatly enhanced by pre-exposure of the antigens to 0.5% deoxycholate. Cross-reaction between the core antigens and e antigens of both viruses was also confirmed by IEM as well as radioimmunassay. It appears that the woodchuck sera used in this study may well contain an anti-immunoglobulin akin to rheumatoid factor.

Great interest was shown in the report in 1978 (Summers et al., 1978) of a virus, closely resembling hepatitis B virus, which was associated with hepatitis and hepatoma in the North American woodchuck, Marmota monax. Until then, the human hepatitis B virus (HBV) was unlike any other known virus and considered to be unique. More recently, other similar viruses have been discovered in the Beechey ground squirrel (Marion et al., 1980) as well as the Pekin duck (Mason et al., 1980).

An animal model would undoubtedly provide valuable information regarding the pathogenesis of the human hepatitis B virus which, as yet, has not been successfully cultivated in vitro. It is therefore necessary to establish the degree of relatedness between HBV and those viruses from different species.

In this paper, we have used immune electron microscopy (IEM) to study cross-reactions between the various antigens of HBV and the woodchuck hepatitis virus (WHV). Three main antigenic determinants are associated with HBV: surface antigen (HBsAg), which is found on the surface of the 42 nm virions (also called Dane particles) as well as on the spherical and filamentous particles 20 nm in diameter; core antigen (HBcAg) which is present on the core or nucleocapsid of the virus particles; e antigen (HBeAg) which is commonly present in sera which contain numerous virus particles. The corresponding antigens for WHV are named WHsAg, WHcAg and WHeAg respectively.

Serological relationships between HBV and WHV have been studied previously using passive haemagglutination and immunoprecipitin methods (Werner et al., 1979) as well as radioimmunoassays (Millman et al., 1982) and peptide mapping (Feitelson et al., 1981). Galibert et al. (1981) performed nucleotide sequence analysis to compare the genes coding for the surface antigens of WHV and HBV. These studies have indicated partial cross-reactivity of varying degrees.

For our experiments, we obtained sera from four woodchucks which were carriers of WHV and displayed high DNA polymerase activity in their sera, as well as from one woodchuck which had no WHV in its blood. Specific antiserum to WHV was obtained from a woodchuck initially found to possess antibodies to WHsAg. This animal was given three 'booster' inoculations of purified WHV in the form of intramuscular injections at 4-week intervals, and then bled after the 12th week.

Human sera were obtained from two chronic carriers of HBsAg; neither had circulating immune complexes or detectable anti-HBs by radioimmunoassay (RIA; Abbott Ausab) and one of these patients, who was immunosuppressed, had no detectable anti-HBc by RIA (Abbott
Table 1. Serological reactions by RIA (Abbott Laboratories)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ausria II</th>
<th>Ausab</th>
<th>HBeAg</th>
<th>Anti-HBe</th>
<th>Corab</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV (CsCl)</td>
<td>+ + + *</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND†</td>
</tr>
<tr>
<td>Human serum 304</td>
<td>+ + +</td>
<td>-</td>
<td>+ +</td>
<td>-</td>
<td>+ +</td>
</tr>
<tr>
<td>Human serum 8</td>
<td>+ + +</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Human serum 10</td>
<td>-</td>
<td>+ + +</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>WHV (CsCl)</td>
<td>+ + +</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Woodchuck serum 334</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Woodchuck serum 38</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Woodchuck serum 61</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Woodchuck serum 417</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Woodchuck serum 0‡</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Woodchuck serum 90</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

* +, Positive, -, negative.
† ND, Not done.
‡ This serum contained no WHV detectable by electron microscopy.

Corab). As a source of anti-HBs, use was made of a commercial hyperimmune rabbit anti-HBs (Behringwerke) as well as human serum with a high titre of anti-HBs by RIA but no detectable HBsAg.

Some experiments were also performed using CsCl density gradient-purified WHV and HBV. These preparations were free of any antibody activity and were rich in virus particles. Results of the RIA for HBV markers are shown in Table 1.

All viral preparations were initially examined by direct negative staining electron microscopy to ascertain the distribution of the viral components and assess their suitability for IEM experiments. Gradient-purified antigen was applied directly to the electron microscope grid, washed with distilled water and stained with 2% phosphotungstic acid pH 6.1 (PTA). Serum samples were diluted 1/25 with phosphate buffer pH 7.2 (PB) and centrifuged at 50000 g for 1.5 h in a Spinco SW50.1 rotor. Pellets were 'washed' twice by resuspension in PB followed by recentrifugation. The final pellets were examined by negative staining using 2% PTA.

Morphologically, WHV preparations (Fig. 1 a) were closely similar to HBV preparations (Fig. 1 b). Both contained 40 to 45 nm double-shelled virus particles and smaller spherical and filamentous structures approximately 20 to 24 nm in diameter. Although total amounts of antigen in each of the three WHV-positive woodchuck sera varied, all three contained virus particles. The virions were frequently 'intact', i.e. not penetrated by the negative stain, but when stain penetration occurred, an inner core 27 nm in diameter could be seen. The tubular components of WHsAg frequently displayed a well-defined striation pattern of very regular periodicity. In addition to the usual 22 nm tubules and spheres, and the 42 nm virus particles, one of the woodchuck sera also contained large round particles varying in diameter from 55 nm to 120 nm (Fig. 1 c). These large particles were frequently 'dimpled', suggesting an internal cavity but at no stage could an intact core be shown in the centre. On a few occasions there appeared to be evidence of incomplete core structures similar to those described for HBV (Stannard & Hodgkiss, 1979). The large 'pseudo-virions' could be linked to other surface antigen components by specific antibody.

Circulating immune complexes were present in two of the four WHV-positive sera, but for all IEM experiments we made use of a serum with no naturally occurring immune complexes. Antigen was concentrated from the serum by centrifugation at 50000 g for 4 h and pellets resuspended in PB to a vol. equalling 1/5 the original vol. of serum. This concentrated antigen was used as a stock suspension for IEM experiments.

Standard methods of IEM were employed whereby 0.2 ml of concentrated antigen was mixed with 0.2 ml of antiserum at appropriate dilutions and incubated for 2 to 6 h at 37 °C. The
Fig. 1. (a) WHV; the tubules display distinct striations. (b) HBV. (c) WHV showing three 42 nm virus particles and one large 'pseudo-virion' (arrow). (d) Immune complex of WHsAg and anti-WHs. (e) Immune complex of HBsAg and rabbit anti-HBs showing antibody excess. (f) Small complexes formed by mixing untreated WHsAg and high titre anti-HBs. Bar marker on (a) represents 100 nm for (b) and (d) to (f) also; that on (c) represents 50 nm.

mixture was then centrifuged at 50000 g for 1-5 h and pellets examined by negative staining electron microscopy. In addition, we devised a more rapid method of IEM which proved to be equally effective. Equal volumes of antigen and antibody (approx. 20 µl amounts) were mixed in a droplet on a sheet of dental wax. Formvar–carbon-coated grids were floated face down on the droplet for 30 min to 1 h at 36 °C, then washed with distilled water and stained with PTA.

Strong reactions (determined by the degree of immune complex formation) were obtained with either HBsAg or WHsAg and their respective homologous antisera (Fig. 1d, e), but
complex formation of WHsAg with anti-HBs was not readily achieved. Very few complexes were formed and then only using high concentrations of antibody (Fig. 1f). The reverse IEM test, i.e. HBsAg mixed with anti-WHs gave somewhat stronger reactions, but the degree of complex formation was, nevertheless, only slight in comparison with the homologous antigen–antibody systems. The WHV-containing sera also reacted positively on RIA (Ausria II, Abbott) for human HBsAg. These three sera, each diluted 1:4 for the test, gave counts 20-3-, 17-6- and 16-6-fold greater than the negative control value, whereas the WHV-free woodchuck serum gave a negative result (Table 1).

These findings correlate well with previous observations by Werner et al. (1979) who used passive haemagglutination to establish serological relationships between HBsAg and WHsAg and found that about 0.1 to 1.0% of the surface antigen was common to both viruses. The low degree of cross-reactivity, however, appeared at first to be at variance with the findings of Galibert et al. (1981) who demonstrated a high degree (75%) of homology between those portions of the genome of the respective HBV and WHV virions that code for the surface antigens. Some explanation of the apparent inconsistency may well be found in the observed serological cross-reactions seen in the present study after pretreatment of the surface antigen with 0.5% deoxycholate. Deoxycholate, which was used to release cores from the virus particles, had a surprising effect on the cross-reactions of the heterologous surface antigen system. Immune complex formation was greatly enhanced using treated HBsAg and anti-WHs as well as treated WHsAg and anti-HBs (Fig. 2a, b). No increase in reaction was noted when combining deoxycholate-treated surface antigen with the homologous antiserum, and deoxycholate alone did not cause aggregation. It would appear that exposure to the bile salt helps to unmask antigenic sites common to HBsAg and WHsAg. These sites may normally be concealed, whereas the species-specific antigenic determinants are possibly predominantly on the outer surface of the particles.

By IEM, cross-reactions between HBeAg and anti-WHe, as well as WHcAg and anti-HBc, were strong (Fig. 2c). Similar findings were shown by Werner et al. (1979) who examined complexes of cores extracted from gel-precipitin lines. The WHV-positive woodchuck sera used in this study all gave low positive results in the RIA assay for HBeAg (Abbott Laboratories) and the anti-WHV serum was positive for anti-HBe. Since the RIA assays are designed to detect HBeAg and anti-HBe from human sera there appears to be antigenic cross-reactivity between the e antigens of HBV and WHV. Similar findings were shown by Millman et al. (1982) in some of their woodchuck sera.

Of special interest to us was the observation that complexes resembling e antigen and antibody (as described by Stannard et al., 1982) could be formed by mixing e antigen-positive samples with either homologous or heterologous antiserum. These distinctive complexes (Fig. 2b, c) were produced with either anti-WHV serum or human anti-HBV serum (Both of which were anti-HBe positive by RIA) but were not formed with the commercial rabbit anti-HBs, which was negative for anti-HBe by RIA. The appearance of e antigen-like complexes was strikingly obvious when anti-WHV was mixed with the Dane particle-rich CsCl-purified HBsAg after deoxycholate treatment, but not before. The reason for this is unclear but warrants further investigation. Although it is tempting to speculate that the HBeAg may have been released from the disrupted Dane particles, no positive proof of this exists as yet.

IEM experiments using woodchuck antiserum and deoxycholate-treated HBV or WHV frequently produced mixed aggregates containing immune complexes of a variety of different antigens: surface antigen, core antigen and e antigen. These mixed aggregates (Fig. 2c) closely resembled those seen when immune complex formation occurred in the presence of rheumatoid factor (RF) (Stannard et al., 1980). A specific test for woodchuck RF was not available, but all five woodchuck sera (including the WHV-negative serum) gave positive agglutination using latex beads coated with human IgG (Wellco-test, Wellcome Reagents). If RF activity is not species-specific, it could, under certain circumstances, produce false positive serological results. Electron microscopy, however, enables one to identify visually the precise antigen–antibody reaction which is occurring, and moreover, just one IEM test can determine the reaction or cross-reaction of multiple antigens as well as assess the effect of RF.
Fig. 2. (a) Large immune complex formed by the admixture of deoxycholate-treated WHV and human anti-HBs. (b) Deoxycholate-treated HBV (CsCl-gradient purified) mixed with anti-WHV, producing large complexes containing HBsAg and ruptured Dane particles as well as finely stippled masses resembling immune complexes of HBeAg. (c) Deoxycholate-treated HBV from serum from an immunosuppressed hepatitis B carrier mixed with woodchuck anti-WHV. Mixed aggregates containing complexes of cores, HBsAg and HBeAg were formed suggesting RF activity. Arrows indicate e-antigen-like complexes. All bar markers represent 50 nm.
Short communication

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REFERENCES


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