Host Components in Hepatitis B Antigen

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(Accepted 6 January 1975)

SUMMARY

Purified [1\(^{25}\)I]-labelled 20 to 25 nm hepatitis B antigen particles were found to give low affinity immunoprecipitation reactions with antisera to several normal serum components, which were immunologically distinct from the reaction due to the classical hepatitis B antigen surface determinant. These additional antigenic determinants were acid-stable and tightly bound to the particles; they could not be released by treatment with Tween 80 or ether, but were removed by protease digestion with the preservation of particle integrity. It was not possible to distinguish whether they were due to the presence of trace amounts of partly denatured serum components, or to a weak cross-reaction with antigens present in normal serum. The implications of this finding for hepatitis B antigen and antibody detection in sensitive assays are discussed. No evidence was found for native antigenic material, present in normal serum or normal liver cells, being integral to the structure of these particles.

INTRODUCTION

The three main species of hepatitis B antigen (HBAg) particles (20 to 25 nm sphere; filamentous form; 42 nm Dane particle) all bear the surface antigen (HB\(\dot{A}\)g) considered to be a marker of the virus of hepatitis B (HBV; Dane, Cameron & Briggs, 1970). In addition, the 42 nm particle contains a 27 nm inner core of unrelated antigenic specificity (HB\(\dot{A}\)g; Almeida, Rubenstein & Stott, 1971). These two antigens are assumed to be virus-coded. However, the role played by host components in the structure of these particles has been a matter of some controversy. In particular, it is unclear whether the 20 to 25 nm particles are intrinsically composed of normal serum components, modified by HBV infection in the host to express a unique virus specific antigenic character (HB\(\dot{A}\)g), or whether these particles represent primarily virus coded material, associated in some preparations with contaminating serum components.

Millman et al. (1971) reported that purified 20 nm HBAg particles, free from known serum components as judged by immunodiffusion against specific antisera, produced precipitin lines with antisera to several human serum components after treatment with 1 % Tween 80; specific HB\(\dot{A}\)g immunoreactivity was weakened or abolished by this treatment. The authors suggested that serum components were integral parts of the HBAg particle and responsible for specific immunoreactivity. Blumberg (1973) has suggested that HBAg particles may be modified serum proteins and discussed the view that host protein on an infecting agent may function as an isoantigen when infection is transmitted from one host to another. Further evidence that HBAg in unfractionated serum may contain host material has been reported by Neurath et al. (1973b), using antiserum to apolipoprotein C, which is related...
to the protein moiety of LP-X (an abnormal serum lipoprotein found in patients with obstructive jaundice). The possibility that host-coded antigens might be incorporated into HBAg particles from the membranes of infected cells has been previously discussed (Burrell et al. 1973).

On the other hand, Gerin (1972) and Dreesman et al. (1972) have reported that antisera from animals hyperimmunized with purified HBAg showed no reaction with normal human serum by immunodiffusion and complement fixation. Howard & Zuckerman (1974) suggested that HBAg in unfractionated serum was in close association with normal serum components, since after purification antigen activity could be focused into much sharper bands in isoelectric gradients. Adequate removal of such material is essential in purification for structural studies.

Clarification of these points is desirable for three reasons: (1) If host-derived serum or cell membrane constituents are found to be integral components of the particles, further understanding could be gained about their site and mode of synthesis. (2) More information about the antigenic complexity of HBAg is essential to interpret the sensitive serological techniques currently used for HBAg detection. (3) The recent report of double-stranded DNA of mol. wt. 1·6 × 10^6 in the Dane particle, the candidate hepatitis B virus (Robinson, Clayton & Greenman, 1974) suggests that the coding potential of the virus genome may be remarkably limited; host material may therefore provide essential components involved in the assembly of HBAg particles.

This report demonstrates that 20 to 25 nm HBAg particles have tightly bound acid-stable antigenic determinants or antibody-binding sites, distinct from HBsAg, that react at a low affinity with antisera to a number of normal human serum components. It was not possible to distinguish whether these reactions were due to the presence of trace amounts of partly-denatured serum components, or to a weak cross-reaction with antigens present in normal serum. No evidence was found for native antigenic material present in normal serum or normal liver cells being integral to the structure of HBAg particles.

METHODS

**Source of HBAg.** HBAg was purified from the serum of blood donors who had been found to be antigen-positive on routine screening by counter-current immunoelectrophoresis (CIE) (Prince & Burke, 1970). All antigen-positive sera were irradiated for 25 min with a 30 W u.v. germicidal lamp at a distance of 15 cm before purification.

**Radioactive labelling of purified HBAg.** Samples of the antigen peak (100 µl, containing approx. 25 µg protein) after two discontinuous sucrose/caesium chloride equilibrium gradients (see Results), were labelled directly with [125I] as previously described (Burrell et al. 1973). The [125I]-labelled HBAg was then isolated by fractionation through Sepharose 6B followed by sucrose velocity gradient sedimentation (5 to 20 % sucrose in PBS containing 0·5 % BSA at 42000 rev/min for 2 h in a Spinco SW50 L rotor). Centrifuge tubes were routinely examined after sedimentation for the presence of a pellet of aggregated labelled material. Labelling efficiency was from 5 to 10 %, giving a sp. act. of 2 to 4 µCi/µg of protein.

**Radioimmunoprecipitation.** All dilutions and assays were performed in phosphate buffered saline (PBS) containing 0·5 % BSA and 0·02 % sodium azide (RIP buffer). Generally, 50 µl of the first antibody was incubated at 4 °C for 1 to 3 days with 50 µl of [125I]-labelled HBAg diluted to contain approx. 50 cts/s of [125I] and 1 ng of HBAg protein. To precipitate immunoglobulins, 100 µl of the second antibody was added at the appropriate dilution, and after a further 16 h at 4 °C the percentage of [125I]-HBAg precipitated was determined as
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Fig. 1. Optical density profile at 280 nm after the second equilibrium density centrifuging of a HBAg containing preparation (••••). The antigen titre of the fractions by CIE is superimposed (○—○). Fraction 10 was used for subsequent radioactive labelling.

previously described (Burrell et al. 1973). Carrier non-immune serum was included in the first antibody dilutions when necessary. For antigen blocking assays, 50 µl first antibody was incubated for 3 h at 37 °C with 100 µl of RIP buffer with or without the antigen being tested, before the addition of labelled HBAg. To allow comparison of radioimmunoassays, samples of labelled antigen treated in various ways and re-isolated on sucrose gradients were diluted to give 50 ct/s [125I] in 50 µl before reacting with the appropriate first antibody.

Antisera and reagents. The following sera were used as first antibodies: anti-HBv, anti-human serum albumen, anti-α, lipoprotein, anti-β lipoprotein, anti-whole human serum, all of rabbit origin and purchased from Hoechst Pharmaceuticals, Hounslow, Middlesex; anti-human IgG (Fc fragment) of rabbit origin (Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands).

Donkey anti-rabbit IgG (generously provided by Dr W. M. Hunter, M.R.C. Radioimmunoassay Team, Edinburgh) was used as the second antibody to precipitate rabbit IgG.

RESULTS

Purification of HBAg

After u.v. irradiation (see Methods), 15 ml samples of antigen-positive serum were diluted with an equal volume of PBS and passed through a 90 × 3.2 cm column of Sepharose 6B. The HBAg containing fraction was identified by CIE, concentrated tenfold by dialysis against polyethylene glycol (mol. wt. 20000), and banded twice in discontinuous sucrose (0 to 50 %) gradients containing 14.3 % CsCl throughout at 40000 rev/min for 18 h in a Spinco SW50 L rotor (considerable antigen losses occurred in gradients containing 28 % CsCl alone). The yield of antigen, estimated by CIE titre, was at least 60 % using this procedure; the optical density profile at 280 nm of the second gradient usually showed a sharp peak coinciding with the peak of antigen activity, at a density of 1.20 to 1.22 g/ml, depending on the antigen source (Fig. 1). This peak usually contained only densely packed
Table 1. Immunoprecipitation of $^{125I}$-labelled HBAg

Percentages of labelled antigen precipitated after pre-incubation shown below

<table>
<thead>
<tr>
<th>Antiserum*</th>
<th>Buffer</th>
<th>Normal serum</th>
<th>Albumen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HB</td>
<td>95.8%</td>
<td>95.1%</td>
<td>N.D.</td>
</tr>
<tr>
<td>Anti-whole serum</td>
<td>48.9%</td>
<td>8.4%</td>
<td>32.5%</td>
</tr>
<tr>
<td>Anti-albumen</td>
<td>41.9%</td>
<td>4.2%</td>
<td>8.2%</td>
</tr>
<tr>
<td>Anti-α lipoprotein</td>
<td>24.8%</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Anti-β lipoprotein</td>
<td>27.1%</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>18.1%</td>
<td>3.2%</td>
<td>17.9%</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>6.1%</td>
<td>3.3%</td>
<td>6.4%</td>
</tr>
</tbody>
</table>

* Each antiserum (50 μl of a 1/100 dilution) was incubated for 3 h at 37 °C with 100 μl of RIP buffer, or buffer containing normal human serum at a dilution of 1/6 or 5 μg of human serum albumen. Labelled HBAg (50 μl) was added and after 16 h at 4 °C, donkey antiserum to rabbit IgG (100 μl) was added.
† Not done.

20 to 25 nm HBAg particles, but occasional filamentous forms and Dane particles were found in some preparations. A very faint precipitin line could be produced by immunodiffusion against rabbit antiserum to whole human serum, but the degree of purity at this stage was adequate for radioactive labelling, before further purification steps.

Homogeneity of radioactively labelled antigen preparations

More than 95% of such preparations was precipitated by excess rabbit antiserum to HBAg in a radioimmunoprecipitation assay. To examine whether the simultaneous precipitation of contaminating normal serum components was contributing to this reaction, a similar assay was done in the presence of unlabelled normal serum diluted 1 in 6; no competitive inhibition of the precipitation of labelled material was found, indicating the absence of free contaminating material. Labelled HBAg preparations of both antigenic subtypes, ‘ad’ and ‘ay’ gave a reproducible peak on sucrose velocity gradients. Comparison with ribosomal markers gave S values of approx. 35 to 45, depending on the antigen source. Preparations from some donors gave a shoulder on sucrose gradients, which on occasions could be resolved into a separate 55 to 60S peak. This faster sedimenting peak was indistinguishable from the main peak in serological and disrupting experiments; its finding supports the observation of Dreesman et al. (1972) that two distinct sub-populations of the 20 to 25 nm HBAg particle exist. Most radioimmunoassays used labelled antigen containing particles of both sizes.

Labelled antigen gave a single peak in discontinuous sucrose/CsCl equilibrium gradients of density 1.225 to 1.25 g/ml. In general, iodination produced an increase in density of 0.01 g/ml which increased further on storage in RIP buffer, possibly due to adsorption of BSA.

Reaction of $^{125I}$-HBAg with antisera to human serum components

Significant immunoprecipitation of $^{125I}$-HBAg preparations of both subtypes occurred in radioimmunoassays using 1/100 dilutions of rabbit antisera to HBAg, whole serum, and several human serum components (Table 1). The reaction with anti-HB, was unaffected by prior incubation of the antibody with normal human serum, whereas the reaction with anti-human serum was reduced to an insignificant level, establishing that the respective antigenic determinants involved were immunologically distinct. Prior incubation of the antisera with 5 μg of crystalline human serum albumen (Sigma Chemical Co., London) abolished
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Fig. 2. Standard antiserum dilution curves for rabbit anti-HB, (○–○), rabbit anti-whole human serum (●–●), and rabbit anti-human albumen (▲–▲). Dilutions of first antibody containing carrier non-immune rabbit serum (50 μl) were incubated with 50 μl of [125I]-labelled HBAg at 4 °C for 1 day (anti-HB) or 3 days (anti-whole human serum and anti-albumen). Donkey anti-rabbit IgG was added, and the percentage of radioactivity bound was calculated.

the precipitation by anti-albumen and reduced the precipitation by anti-whole human serum. In an attempt to dissociate loosely bound contaminating material, labelled antigen was centrifuged for 2 h at 42,000 rev/min through a 5 to 20 % sucrose gradient containing 0.05 M-glycine, pH 2.5, and 0.5 % BSA. No additional labelled material was released to the top of the gradient and the peak of labelled antigen, after isolation and adjustment to pH 7.2, reacted similarly in radioimmunoassays to the starting preparation.

Standard antiserum dilution curves were determined by radioimmunoassay, using different [125I]-HBAg preparations and rabbit antiserum to HBAg, whole human serum or human serum albumen (Fig. 2). The curve obtained with anti-HB, was typical of a specific high affinity reaction, whereas those obtained with anti-human serum and anti-albumen suggested low affinity or cross-reacting antibodies.

When the radioimmunoassay supernatant fluids containing [125I]-HBAg non-reactive with antisera to whole serum, albumen and IgG, were re-incubated with a further 5 μl of the undiluted corresponding antiserum, little further precipitation of the remaining labelled HBAg occurred. This demonstrated that a more reactive sub-population of HBAg particles had been precipitated initially by these antisera and that not all particles were equally reactive.

Some information about the chemical nature of these additional determinants was gained by reduction and alkylation experiments. [125I]-labelled HBAg in 0.6 M-tris buffer, pH 8.3, containing 0.5 % BSA, was treated with 0.1 M-dithiothreitol for 1 h at room temperature, followed by 4 h at 4 °C in the presence of excess recrystallized iodoacetamide. The preparation was then dialysed in PBS before radioimmunoassays similar to those in Table 1. A control preparation was treated similarly, but with the omission of dithiothreitol. Immunoprecipitation of labelled antigen with the various antisera was unaffected by alkylation alone. However, reduction and alkylation abolished the precipitation due to anti-albumen
Fig. 3. Radioactivity profiles after centrifuging of [¹²⁵I]-labelled HBAg through 5 to 20 % linear sucrose gradients in RIP buffer for 3½ h at 39000 rev/min in a Spinco SW40 rotor. Samples of 200 µl were applied after (a) no treatment (●—●), (b) treatment with Tween 80 (○——○), and (c) treatment with ether (▲——▲). Gradient (b) contained 0.05 % Tween 80, and gradient (c) contained 0.05 % NP40.

and lowered the precipitation due to anti-HB, and anti-whole serum, but had no effect on the reaction with anti-IgG. This provided additional evidence that the determinants reacting with anti-albumen and anti-IgG were separate, and suggested that the determinant reacting with anti-albumen was a protein dependent on disulphide bonds.

The above findings indicated that antigenic determinants reacting with low affinity with antisera to normal serum components were present on some of the [¹²⁵I]-labelled material in the labelled HBAg preparations. These determinants were acid stable, and could not easily be dissociated from the particles. This could occur if a certain proportion of the labelled HBAg particles contained tightly bound contaminating material from serum. Alternatively, structurally distinct but immunologically cross-reacting determinants could have been an integral part of the surface of most particles.

Disruption studies

In an attempt to release antigenically active components from HBAg, the labelled antigen preparations were treated with Tween 80, ether, or proteolytic enzymes, followed by velocity gradient sedimentation, and radioimmunoassays using the peaks of labelled material isolated were performed.

After treatment with 1 % freshly prepared Tween 80 in RIP buffer for 2 h at room temperature (Millman et al. 1971), no labelled material was released to the top of the gradient,
Table 2. Immunoprecipitation of $[^{125}I]$-labelled HBAg after protease treatment

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>None</th>
<th>Bromelain</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HB,</td>
<td>96.5</td>
<td>91.6</td>
<td>11.1</td>
</tr>
<tr>
<td>Anti-whole serum</td>
<td>69.8</td>
<td>23.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Anti-albumen</td>
<td>53.7</td>
<td>11.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>25.4</td>
<td>15.3</td>
<td>13.0</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>4.6</td>
<td>6.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Samples of labelled HBAg (200 μl) were incubated at 37 °C for 4 h in RIP buffer containing 0.1 % of the proteolytic enzyme indicated. The radioactive HBAg peak isolated on sucrose velocity gradients was used in radioimmunoassays similar to those in Table 1, with the omission of the initial pre-incubation at 37 °C.

no alteration in S value was seen (Fig. 3), and the peak of radioactive antigen (density in discontinuous sucrose/CsCl 1.22 g/ml) reacted similarly to untreated antigen (density 1.25 g/ml) in radioimmunoassays.

Attempts to remove lipid from the labelled antigen with ether produced variable results. When antigen was treated at 4 °C for 4 to 16 h with a tenfold excess of ether with occasional gentle mixing, less than 1 % of the radioactivity partitioned in the ether phase. Addition of 0.05 % Nonidet P40 (NP40) after ether treatment was necessary to prevent aggregation of the labelled components on storage. Considerable adsorption of labelled material to glass-walled test tubes could not easily be overcome; however, subsequent recovery of this material by repeated rinsing yielded labelled antigen with an identical sucrose gradient profile to material that did not adsorb. Ether treatment increased the density of labelled antigen by 0.02 g/ml; a broader labelled antigen peak of slightly higher S value than the control was reproducibly obtained in sucrose gradients (Fig. 3), and release of up to 20 % of the total radioactivity to the top of the gradient occurred with antigen from some sources but not with others. The released material was not precipitated by any antisera in radioimmunoassays while the broad antigen peak gave similar reactions to the control preparations.

These results indicated that the Tween 80 treatment did not destroy the integrity of the particle or its antigenic determinants, or release any radioactively labelled material reacting with the antisera used. Gentle ether treatment, resulting in partial removal of lipid from the antigen particle, produced more heterogeneous particles of higher S values and higher density. No further exposure of antigenic determinants was found in either case.

When antigen preparations in RIP buffer were treated at 37 °C from 1 to 4 h with 0.1 % trypsin or 0.1 % bromelain, no alteration was seen in the sedimentation value of the labelled antigen in 5 to 20 % sucrose velocity gradients containing RIP buffer (2 h at 40000 rev/min in a SW 50 L rotor), and less than 10 % of the radioactivity was released from the particles in each case. The antigen peak after 4 h trypsin digestion was not precipitated significantly by any of the antisera tested in a radioimmunoassay, whereas bromelain digestion reduced the precipitation occurring with some antisera to normal serum components while leaving the HBsAg determinant unaffected (Table 2). These findings are compatible with the release of external antigenic determinants by proteolytic digestion, while leaving the bulk of the protein and the basic architecture of the particle intact.
Reaction of HBAg with antisera to liver homogenates

The reaction of labelled HBAg with antisera to human liver was examined, in order to detect any host cell antigenic material incorporated into HBAg particles during synthesis. This might be expected since HBAg contains significant amounts of carbohydrate (Burrell et al. 1973) and many membrane maturing enveloped animal viruses contain host derived carbohydrate which, in the case of influenza virus at least, can retain host antigenic specificity (Laver & Webster, 1966).

A sample of adult liver obtained post mortem from a patient negative for HBsAg by radioimmunoassay was homogenized in an equal vol. of PBS; the pellet obtained by low speed sedimentation was washed three times in PBS to remove soluble serum components, and hyperimmune rabbit antisera to this liver cell extract were prepared by repeated subcutaneous injections in Freund's incomplete adjuvant at fortnightly intervals. Such antisera produced a faint precipitin line by immunodiffusion with liver cell extract containing 0.5% NP40, and no reaction with normal human serum.

In radioimmunoassays, 1/100 dilutions of such antisera from 2 rabbits precipitated approx. 40% of labelled HBAg with or without prior treatment of the antigen with Tween 80 or ether. This precipitation could be totally blocked by pre-incubation of the antiserum with a 1/6 dilution of normal human serum as described above. It was thus concluded that the immunoprecipitation was due either to the presence of antigenic serum components in the original liver cell extract, or to cross-reacting antigens between the liver material and normal serum. Thus, no new antigenic determinants, present in human liver cells, were detected in association with the antigen particles.

DISCUSSION

Using a range of antisera to normal serum components and a liver cell extract, no specific high affinity reaction with purified [125I]-labelled HBAg preparations was found; no release or exposure of labelled antigenic material was found after treatment of the antigen with excess of a nonionic detergent (Tween 80), or after removal of lipid with ether. Removal of lipid from β lipoproteins under similar conditions has been reported to expose additional antigenic sites (Goldstein et al. 1971), and treatment of purified HBAg with ether increased the susceptibility of the antigen to proteolytic digestion (Kim & Bissell, 1971), presumably due in each case to the partial removal of surface lipid in close association with portions of polypeptide chains.

Unlabelled antigenic material released from the particles would not have been detected in the above assays. However, comparison of SDS-polyacrylamide gel profiles of HBAg polypeptides detected by radioactivity or Coomassie blue staining has indicated that the major polypeptides of HBAg are all radioactively labelled with [125I] to a similar extent (P. Mackay, personal communication). It remains likely that the lipid and carbohydrate of HBAg may be host-coded, but no native antigenic determinants of host origin were detected in this work.

The low affinity serological reaction observed between purified HBAg and antisera to normal serum components may be due to traces of tightly bound serum components, perhaps having undergone some denaturation. It has been proposed that serum proteins, adsorbed to HBAg, are necessary to preserve antigenic activity (Gerlich & May, 1973). However, the several lines of evidence reported here suggest that such proteins are unlikely to be intrinsic components of all HBAg particles. In a possibly analogous example, Rifkin,
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Compans & Reich (1972) have recently concluded that purified influenza virus, grown in medium containing serum, retains contaminating serum proteins on the outer surface of the virus particle, which are less abundant in virus grown in serum free medium. Alternatively, some cross-reaction with antigens in normal serum, in particular involving the superficial carbohydrate moieties of HBAg (Neurath, Prince & Lippin, 1973a), would not be surprising in the sensitive assay used here. False positive reactions have been described in a direct solid phase radioimmunoassay in current use for HBAg detection (Prince et al. 1973), which employs tubes coated with anti-HB, and [125I]-labelled anti-HB. These could in part be due to the above mechanism, where anti-HB, preparations containing such low affinity anti-human serum activity were used. The above finding is of particular relevance in the detection of anti-HB, in radioimmunoassays using purified [125I]-labelled HBAg.

During the course of this study, Neurath, Prince & Lippin (1974) reported serological reactions between purified 20 to 25 nm HBAg particles and insolubilized antisera to a number of normal human serum components by affinity chromatography. Since these determinants were distinct from HBAg and were tightly bound to the particles, the authors suggested that determinants related to host proteins were located on integral components of HBAg. However, the data reported above favour the conclusion that, in describing the essential structure of the HBAg particle, such reactions are likely to be of doubtful importance.

In conclusion, the demonstration that antigenic material can be removed from HBAg by proteolytic enzymes, with the preservation of particle integrity and the bulk of the labelled protein, provides a useful tool for the exploration of the architecture of the particle.

I wish to thank Mrs P. Mackay and Professor B. P. Marmion for helpful discussions, and Mrs E. Proudfoot and Miss G. Leadbetter for skilled technical assistance. This work was supported by a grant from the Scottish Home and Health Department.

REFERENCES


(Received 6 August 1974)