Some Properties of Hepatitis B Core Antigen Isolated from Serum of Infected Humans

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SUMMARY

The nucleocapsid of Dane particles (= hepatitis B core antigen; HBcAg) was isolated from human sera either positive or negative for e-antigen (HBeAg) – an apparent marker for the level of infectious hepatitis B virus in serum. HBcAg from the HBeAg-positive serum pool consisted of two distinct populations of particles, one with a buoyant density (d) of 1.358 g/ml and a sedimentation coefficient (s20,w) of ~110, and another with d = 1.28 to 1.30 g/ml and s20,w ~ 70. Only the latter type of particles was isolated from an HBeAg-negative serum pool. HBcAg was labelled with 125I-p-hydroxyphenylpropionic acid N-hydroxysuccinimide ester, dissociated and analysed by polyacrylamide gel electrophoresis. One major and one minor polypeptide with apparent mol. wt. of 16000 ± 500 and 68000, respectively, were detected. Another component having the properties of a glycolipid with a mol. wt. in the order of 10^8 was observed. After isoelectric focusing, HBcAg was recovered in fractions with a pH between 4.0 and 5.8, suggesting heterogeneity in isoelectric points.

INTRODUCTION

The nucleocapsid component of the Dane particle, the putative hepatitis B virus (HBV; Dane et al. 1970) has unique properties which distinguish it from other known small DNA viruses. Its DNA has a mol. wt. of 1.6 × 10^6, is circular, double-stranded (Robinson et al. 1974; Overby et al. 1975; Takahashi et al. 1976) with single-stranded gaps which can be filled by the action of an endogenous DNA polymerase (Kaplan et al. 1973; Summers et al. 1975; Hruska et al. 1977).

Humans persistently infected with HBV have relatively high titres of antibodies to the surface determinants of the capsid (hepatitis B core antigen; HBcAg). The properties of these antibodies (anti-HBc) seem to differ with the level of biosynthesis of HBcAg and the concentration of Dane particles in serum (Neurath et al. 1978).

Characterization of the components of HBcAg may lead to a better understanding of the development of an immune response to HBcAg and of the differences in the host's response to HBV infection, which are decisive for the pathological manifestations and the final outcome of hepatitis B (Blumberg et al. 1970; Edgington & Chisari, 1975).

We describe here some of the properties of HBcAg isolated from Dane particles and of its non-DNA components.
METHODS

Preparation and radiolabelling of HBCaG. Sera positive for e-antigen (HBeAg), a marker for the presence of complete Dane particles, served as a source of HBCaG. Dane particles were concentrated and partially purified as described before (Neurath et al. 1978). HBCaG was released from Dane particles by treatment with 0·1 M-NH₄Cl-0·08 M-MgCl₂-0·25 % mercaptoethanol-0·5 % Nonidet P40, pH 7·6 (disruption buffer) for 1 h at 37 °C. The following control preparations were treated in exactly the same way (including all purification steps) as the HBeAg-positive sera: (1) HBeAg-negative serum containing hepatitis B surface antigen (HBsAg); (2) normal human serum; (3) HBeAg-positive serum to which antiserum to HBsAg was added in sufficient amount to neutralize more than 99 % of HBsAg as determined by a radioimmunoassay (RIA). The mixture was incubated for 1 h at 37 °C, overnight at 4 °C and then clarified by centrifuging at 40000 g for 20 min. (4) Three grams of normal human liver tissue homogenized with 30 ml of 0·1 M-tris-0·14 M-NaCl-0·02 % NaN₃, pH 7·2 (TS), for 2 min at 0 °C using the Brinkman Polytron homogenizer at top speed (Brinkman Instruments, Westbury, New York) and then clarified at 16000 g for 20 min; (5) a similar homogenate from rabbit liver. An additional control consisted of HBsAg treated in the following way: 0·3 ml of purified (Neurath et al. 1973, 1975) HBsAg (absorbance at 280 nm = 0·513) was mixed with 125 µg of pronase, incubated 1 h at 37 °C and chromatographed on a 0·7 × 20 cm column of Sephadex G-25. Fractions corresponding to the void volume of the column and containing 80 % of the original protein were pooled. Nonidet P40 and mercaptoethanol were added to final concentrations of 0·5 % each, and the mixture was incubated 30 min at 37 °C.

Preparations of HBCaG released from Dane particles before or after additional purification by isopycnic centrifugation were extensively dialysed against 0·05 M-borate, pH 8·5, and labelled with ¹²⁵I-ß-hydroxyphenylpropionic acid N-hydroxysuccinimide ester (Bolton-Hunter reagent; 500 Ci/mmol; New England Nuclear, Boston, Massachusetts). The instructions of the manufacturer were followed, except that the labelling was prolonged for 16 h. The labelled components were separated from unbound reagent by gel filtration on 0·7 × 20 cm columns (BioRad Laboratories, Richmond, California) of Sephadex G-75. Phosphate buffer (0·05 M, pH 7·5) containing 0·25 % gelatine was used for elution of the columns. Fractions containing HBCaG (corresponding to the void volume of the column) were dialysed overnight against sodium phosphate, pH 8·0.

In separate experiments HBCaG was reacted with cold Bolton-Hunter reagent at levels similar to those used before for radioactive labelling (approx. 10 µg/µg protein). Such treatment did not affect the immunological activity of HBCaG as indicated by RIA tests.

Rate zonal and isopycnic gradient centrifugation. To determine the size distribution of particles carrying HBCaG determinants, preparations of Dane particles treated with ‘disruption buffer’ were layered on top of a gradient (4·5 ml in centrifuge tubes for the SW 65 rotor, Beckman Instruments, Palo Alto, California) of 12·5 to 35 % (v/v) glycerol in ‘disruption buffer’ diluted 1:1 with H₂O. The tubes were centrifuged for 1 h at 53000 rev/min and fractions of 0·3 ml were collected from the bottom of the tubes. Fifty µl samples were tested for HBCaG. To estimate the sedimentation coefficient (s₂ₐ₀,ₘ; Martin & Ames, 1961; McEwen, 1967) two marker substances, HBsAg subtype ad (s₂ₐ₀,ₘ = 30 to 40; Howard & Burrell, 1976) and horse apoferritin (s₂ₐ₀,ₘ = 16·8; Bjork & Fish, 1971) were centrifuged simultaneously in separate tubes. The relative concentration of these markers in fractions after centrifugation was determined by RIA and by measurements of absorbance at 280 nm, respectively.
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Pre-formed gradients of CsCl (density 1.21 to 1.43 g/ml in 0.06 % Nonidet P40–0.16 % mercaptoethanol) were used for isopycnic centrifugation (40 h at 35000 rev/min in the SW 65 rotor) of HBcAg. A component extracted by chloroform–methanol, 2:1 (v/v), from labelled preparations of HBcAg was applied at the middle of a CsCl gradient (4.5 ml; density 1.16 to 1.40 g/ml) and centrifuged for 120 h at 39000 rev/min in the SW 65 rotor. 125I-labelled insulin was centrifuged simultaneously in a separate tube.

Molecular exclusion chromatography. Components from the labelled preparation of HBcAg and appropriate labelled control preparations and marker proteins were chromatographed on 0.7 x 20 cm columns of Biogel P-60 (BioRad Laboratories). Urea (8 M)-0.1 M-tris (hydroxymethyl) aminomethane (tris)–1 % (w/v) sodium dodecyl sulphate (SDS)–0.25 % gelatine ±1 % mercaptoethanol, pH 7.2, was used for elution of the columns. Samples were boiled for 1 to 2 min in the above buffer and then applied to the column. Fractions (0.25 ml) were collected.

Affinity chromatography. Columns (1.0 to 1.5 ml) of the following antibodies or lectins were used: concanavalin A (Pharmacia, Uppsala, Sweden); anti-IgG (Miles Laboratories, Elkhart, Indiana); antibodies to HBsAg (anti-HBs) and anti-HBc. The preparation of the columns and the conditions for chromatography were similar, as described before (Neurath et al. 1973, 1978). The anti-HBs was from a chimpanzee immunized with purified HBsAg. The anti-HBc was from a rhesus monkey antisera supplied by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases.

Isoelectric focusing. Preparations of unlabelled HBcAg were submitted to isoelectric focusing under conditions described before (Neurath et al. 1975). Ampholytes with a pH range of 3.5 to 10 or 3 to 6 were used. Two ml fractions were collected after completed electrofocusing. They were neutralized by addition of 1 M-tris, pH 7.2 (100 µl). Samples (100 µl) were mixed with 300 µl of sheep serum diluted 1:5 with TS and tested for HBcAg.

A sample of labelled HBcAg components (90 000 ct/min in 33 µl) eluted from an anti-HBc column by 8 M-urea–1 % SDS–0.25 M-phosphate, pH 8.0, were mixed with 33 µl of sheep serum and with 330 µl of 50 % (v/v) methanol. The precipitate containing all radioactivity was dissolved in 0.3 ml of 1 M-tris, pH 7.2, and submitted to isoelectric focusing in a 0 to 50 % glycerol gradient (110 ml) containing 6 M-urea, and 2.5 ml of a pH 3.5 to 10 ampholyte (LKB Instruments, Rockville, Maryland). Electrofocusing was performed for 90 h.

Polyacrylamide gel electrophoresis (PAGE). Preparations of labelled HBcAg were mixed with human anti-HBc IgG (prepared from a serum positive for antibodies to HBcAg) in equivalent proportions determined from titres of the respective reactants (Neurath et al. 1978) before radioactive labelling. The mixtures were incubated for 1 h at 37 °C, at 4 °C overnight and layered on top of 1 ml of 15 % (v/v) glycerol in TS placed in tubes for the SW 65 rotor. The pellets, after centrifugation at 25000 rev/min for 25 min, were dissolved in 100 to 200 µl of either 0.025 M-sodium phosphate–8 M-urea–1 % SDS–1 % mercaptoethanol, pH 8.0 (PAGE buffer I; for continuous PAGE) or in 0.0625 M-tris–8 M-urea–1 % SDS–1 % mercaptoethanol, pH 7.2 (for discontinuous PAGE), boiled for 5 min and applied on top of the polyacrylamide gel [5 to 11 % (w/v) Cyanogum 41, E-C Apparatus Corporation, St Petersburg, Florida].

In one case, HBcAg from a labelled preparation of disrupted Dane particles was purified by affinity chromatography on insolubilized anti-HBc. The labelled preparation (2.5 x 107 ct/min) was divided into two equal portions (of 0.4 ml) and each was applied to a distinct pair of immunosorbent columns (1.2 ml of gel/column) arranged in tandem. The upper columns of each pair contained a mixture of insolubilized anti-human IgG and anti-HBs.
The bottom columns contained either normal human IgG or anti-HBc. The column pairs were washed at 4 °C with 6 ml of 0.5 M NaCl-0.01 M tris, pH 7.2, the upper columns were removed and the bottom columns eluted with PAGE buffer I at room temperature. The labelled material eluted from the anti-HBc column (2.7 × 10⁶ ct/min) was analysed by PAGE and by isoelectric focusing. In comparison, 7.1 × 10⁵ ct/min of radioactive material was eluted from the control IgG column.

PAGE was performed as described before (Neurath et al. 1975) using (1) a gel polymerized in PAGE buffer I and an electrode buffer corresponding to the same buffer without urea and with 10 times less SLS or (2) a gel prepared in 0.375 M tris-HCl-8 M urea-1% SDS, pH 8.8, and an electrode buffer corresponding to 0.025 M tris-0.192 M glycine-0.1% SLS, pH 8.3 (= discontinuous system). After completed electrophoresis, the gels were cut into 2 mm slices and their radioactivity was measured in a γ-counter. The following proteins labelled with Na¹²³I (Hunter, 1974) were used as markers for the estimation of molecular weights: human serum albumin, the heavy and light chains of human IgG, sperm whale myoglobin, lysozyme and bovine insulin.

Immune electron microscopy. Samples containing HBCaAg were mixed with equal volumes of rhesus monkey anti-HBc or human anti-HBc IgG prepared as described (Neurath et al. 1978) or the latter IgG linked to ferritin by glutaraldehyde (Kishida et al. 1975; Goldin et al. 1976). The mixtures were incubated at 37 °C for 1 h and then refrigerated at 4 °C for another hour. The immune complexes that might have formed were pelleted, washed once with distilled water, stained with 2% potassium tungstate, pH 6.4, and deposited on carbon-formvar-coated copper grids as described before (Huang, 1975). Examinations were made with a Philips 300 electron microscope at magnifications of 45000 to 90000.

Other methods. HBsAg and HBCaAg were determined by solid phase RIA as described before (Neurath et al. 1978).

RESULTS

Immunological specificity and cryptic nature of HBCaAg determinants

Only preparations derived from HBsAg-positive sera contained HBCaAg determinants detectable by an RIA test described before (Neurath et al. 1978). Control preparations obtained from normal human and rabbit liver and from purified HBsAg particles approx. 20 nm in diam. were all negative by RIA for HBCaAg. Omission of the detergent Nonidet P₄₀ from the ‘disruption buffer’ resulted in specimens negative for HBCaAg; omission of mercaptoethanol led to a 34 to 77% reduction in radioactive counts with four distinct preparations.

Rabbit and chimpanzee antiserum to purified HBsAg did not have any anti-HBc activity as determined by RIA. Paradoxically, however, about 50% of HBCaAg was absorbed to a column of insolubilized anti-HBs and was subsequently eluted from the column at pH 10.9. Approximately 8% of the HBCaAg from a pool of HBeAg-positive and HBeAg-negative sera, respectively, adsorbed to a column of anti-human IgG, indicating that the major part of HBCaAg particles did not have any attached anti-HBc originating from the serum source for HBCaAg. HBCaAg did not adsorb to insolubilized concanavalin A.

Addition of anti-HBc IgG [also containing antibodies to HBeAg (anti-HBe)] to a preparation which originally contained Dane particles and was treated with the ‘disruption buffer’ resulted in specific agglutination of HBCaAg particles and no other aggregates were seen under the electron microscope (Fig. 1a). Non-agglutinated particles (before addition of anti-HBc) and particles agglutinated with ferritin-conjugated anti-HBc had a smooth surface (Fig. 1b) and no evidence of spikes was found.
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Some physical properties of HBcAg particles

HBcAg prepared from a pool of HBeAg-positive sera consisted of particles with two distinct buoyant densities in CsCl solutions (Fig. 2). A smaller fraction of HBcAg had a density of 1.358 g/ml. The major part of HBcAg was recovered at a density of 1.28 to 1.30 g/ml. Only the less dense particles were found in HBcAg prepared from a pool of HBeAg-negative sera.

Rate zonal centrifugation of HBcAg from HBeAg-positive serum indicated that the particles were heterogeneous with respect to their sedimentation coefficient ($s_{20,w}$). The major part of HBcAg was recovered in fractions corresponding to $s_{20,w} \approx 70$. An additional peak of HBcAg was observed in the region of the gradient corresponding to $s_{20,w} \approx 110$. A minor portion of HBcAg activity was recovered on top of the gradient. Similar results were obtained with HBcAg from HBeAg-negative sera, except that the faster sedimenting particles were absent (Fig. 3).

HBcAg particles have relatively low isoelectric points (Fig. 4, hatched area). The fraction (after isoelectric focusing in a pH 3.5 to 10 gradient) with the highest concentration of HBcAg had a pH = 4.88. Two peaks of HBcAg were observed after isoelectric focusing in a pH 3 to 6 gradient: one at pH 4.40 and another at pH 4.86. Similar results were obtained with HBcAg from HBeAg-positive and negative sera, suggesting that the observed heterogeneity of HBcAg with respect to isoelectric points was not related to the buoyant density of the particles.

Characterization of components obtained after dissociation of HBcAg

PAGE of labelled HBcAg with a density of 1.28 to 1.30 g/ml identified two major components with apparent mol. wt. of 16000 and 5000 respectively (Fig. 5). Additional minor components with mol. wt. of 20000, 22000, 39000 and 68000 could be discerned. The same major constituents were detected in HBcAg with a density of 1.358 g/ml (Fig. 6). However, the 68000 mol. wt. component was much more prominent than in the
Fig. 2. Isopycnic centrifugation of HBcAg prepared from a pool of HBeAg-positive sera. The shaded area corresponds to HBcAg determined by RIA; ●—●, density.

Fig. 3. Rate zonal sedimentation of HBcAg prepared from a pool of HBeAg-negative sera. The shaded area corresponds to HBcAg determined by RIA. Arrows indicate the positions in the gradient of HBsAg (1) and apoferritin (2).

Fig. 4. Isoelectric focusing of intact HBcAg (shaded area; HBcAg was determined by RIA) and of 125I-labelled HBcAg components eluted from an anti-HBc column by 8 M-urea–1 % SLS–0.025 M-sodium phosphate, pH 8.0 (unshaded area). The results obtained from separate isoelectric focusing experiments were superimposed; ●, pH.
Fig. 5. PAGE of $^{131}$I-labelled HBcAg (density 1.28 to 1.30 g/ml). A 10% gel and a continuous buffer system was used. Similar results (except a shift of the peaks to the right) were obtained with a discontinuous buffer system and with 8.5% gels. Numbers on top of the peaks of radioactivity indicate the mol. wt. $\times 10^{-3}$.

Fig. 6. PAGE of $^{131}$I-labelled HBcAg (density 1.358 g/ml). A discontinuous buffer system and an 8.5% gel were used. For further explanations see legend to Fig. 5.

less dense HBcAg. The mol. wt. of the major component was 16000 ± 500 as determined with three distinct HBcAg preparations in a total of 14 PAGE runs. The results obtained with the discontinuous and continuous buffer systems were similar. The resolution of labelled components, except of those near to the tracking dye, was better in the discontinuous system.
Further experiments using either PAGE or chromatography on Biogel P60 indicated that the smallest component was recovered in approximately the same fractions as reduced insulin (mol. wt. = 5700). However, this component, unlike the higher mol. wt. species (presumably polypeptides) discernible by PAGE, was extracted from labelled HBcAg by chloroform–methanol (2:1, v/v) into the organic phase. The labelled material was easily dissolved in various buffers after the organic solvent had been evaporated. After prolonged isopycnic centrifugation the component moved to the top of the CsCl gradient (density = 1.156 g/ml), while the peak of radioactivity corresponding to $^{125}$I-insulin centrifuged simultaneously in a separate tube was observed at a density of 1.303 g/ml.

Adsorption of some of the HBcAg particles to insolubilized anti-HBs suggested incomplete removal of HBsAg determinants and possible contamination of HBcAg with components of the lipoprotein shell of Dane particles. These components might correspond to some of the minor labelled peaks discerned by PAGE (Fig. 5, 6). To investigate this possibility, labelled HBcAg was chromatographed on an anti-HBs column and the unadsorbed portion was further purified by adsorption and elution from an anti-HBc column. PAGE of the eluted radioactive material revealed the presence of the two major components identified before (Fig. 5, 6) and of a minor component with an apparent mol. wt. of 68000.

The radioactive products obtained by dissociation of HBcAg had an average isoelectric point about 0.4 pH units higher than intact HBcAg (Fig. 4).

**DISCUSSION**

Two different populations of particles were distinguished in HBcAg prepared from a pool of HBcAg-positive sera; one with a buoyant density of 1.358 g/ml and a $s_{20,w} \approx 110$, and another with a density of 1.28 to 1.30 g/ml and a $s_{20,w} \approx 70$. Only the latter type of HBcAg was detected in a pool of HBcAg-negative sera. These results agree with previous findings that HBcAg containing DNA and DNA polymerase has a density of 1.358 to 1.360 g/ml in contrast with HBcAg containing very little or no DNA which has a density of about 1.30 g/ml (Gerin et al. 1975; Moritsugu et al. 1975; Kaplan et al. 1976; Howard & Zuckerman, 1977) and the demonstration of a positive correlation between the presence of HBcAg in sera and their levels of HBV-specific DNA-polymerase and DNA (Nordenfelt & Kjellen, 1975; Werner et al. 1977). The $s_{20,w}$ of the denser population of HBcAg agrees with the value determined for HBcAg labelled in its DNA moiety and is similar to the $s_{20,w}$ value of 114 corresponding to bacteriophage $\phi$X174 (Kaplan et al. 1973), which resembles HBcAg with respect to particle size and mol. wt. of DNA. The $s_{20,w}$ of the less dense cores is similar to the value of 70 corresponding to the bacteriophage $\phi$X174 70S component lacking the major portion of DNA (Eigner et al. 1963).

HBcAg prepared from some sera having high levels of anti-HBc was contaminated with bound immunoglobins (Moritsugu et al. 1975). This HBcAg probably corresponds to particles which had a ragged surface and did not react with anti-HBc (Lipman et al. 1973). HBcAg described here had a smooth outer border and had very little if any IgG attached to its surface, as also suggested by the results of affinity chromatography experiments. It seems likely that the treatment of crude preparations of Dane particles with pronase – a standard step in our method for purification of HBcAg – destroyed enough anti-HBc to prevent its reaction with HBcAg released from Dane particles during subsequent treatment with the detergent Nonidet P40. Previous studies indicated that treatment with pronase leads to dissociation of HBcAg–antibody complexes and leaves HBcAg intact (Huang & Groh, 1973; Huang, 1975).
Addition of anti-HBs to HBcAg failed to prevent its attachment to anti-HBc-coated beads used for RIA of HBcAg (Neurath et al. 1978). However, as indicated by the results of affinity chromatography on insolubilized anti-HBs, HBsAg-determinants were apparently not completely stripped off from a subpopulation of HBcAg. This finding had to be taken into account when analysing the results of PAGE. The technique revealed that the major component (presumably a polypeptide) of HBcAg has an apparent mol. wt. of 16000 ± 500, in reasonable agreement with recent results of Budkowska et al. (1977) obtained with HBcAg isolated from nuclei of HBV-infected hepatocytes. However, the larger polypeptide (mol. wt. = 35000) identified by these authors was not observed in our studies, possibly due to the different source (serum instead of liver) of HBcAg. In addition, a minor component with an apparent mol. wt. of 68000 was detected. Since this component (1) was found (unlike the polypeptides with mol. wt. of 20000, 22000 and 39000 – Fig. 5) in HBcAg which did not become absorbed to a column of anti-HBs, and (2) was more abundant in HBcAg with a density of 1.358 g/ml than in the lower density HBcAg, it seems unlikely that it would correspond to one of the polypeptides of HBsAg with a similar mol. wt. (Chairez et al. 1975). Our results also suggest at least quantitative differences between non-DNA components of the two density subpopulations of HBcAg. This seems to agree with the observation that HBcAg particles positive for DNA polymerase had a larger size than particles lacking enzymic activity (Fields et al. 1976), the expression of which depends on the simultaneous presence of endogenous DNA (Kaplan et al. 1976). Therefore, it cannot be excluded that the 68000 mol. wt. component may correspond to DNA polymerase.

In addition to these constituents, labelling of HBcAg with 125I-Bolton-Hunter reagent followed by PAGE identified a component with a mol. wt. in the order of 10^3. This component had a low buoyant density (~< 1.156) and was soluble in both water solutions and chloroform–methanol. Because of these properties and its reactivity with the Bolton-Hunter reagent, reacting with amino groups, this substance probably corresponds to a glycolipid containing amino sugars. Whether it represents an integral constituent of HBcAg or a remnant of the lipoprotein shell of the Dane particle bound tenaciously to HBcAg could not be determined.

Budkowska (1977) suggested that HBcAg contained protein-linked saccharides, since HBcAg was destroyed by lysozyme. We found that prolonged treatment with lysozyme failed to affect HBcAg (unpublished data). Further chemical analyses will be required to determine whether and in what form saccharides are present in HBcAg.

Isoelectric focusing revealed that HBcAg particles were somewhat more heterogeneous than the HBcAg subpopulation positive for DNA polymerase and with an isoelectric point of pH 4.4 (Howard & Zuckerman, 1977). The dissociated components of HBcAg had a higher average isoelectric point than intact HBcAg, suggesting the unmasking of positively charged groups from the interior of HBcAg.

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