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Specificity of an Anti-Capsid Antibody Associated with Hepatitis B Virus-Related Acute Liver Failure

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Abstract

Previously, the livers of patients suffering from acute liver failure (ALF), a potentially fatal syndrome arising from infection by Hepatitis B Virus (HBV), were found to contain massive amounts of an antibody specific for the core antigen (HBcAg) capsid. We have used cryo-electron microscopy and molecular modeling to define its epitope. HBV capsids are icosahedral shells with 25Å-long dimeric spikes, each a 4-helix bundle, protruding from the contiguous “floor”. Of the anti-HBcAg antibodies previously characterized, most bind around the spike tip while one binds to the floor. The ALF-associated antibody binds tangentially to a novel site on the side of the spike. This epitope is conformational. The Fab binds with high affinity to its principal determinants but has lower affinities for quasi-equivalent variants. The highest occupancy site is on one side of a spike, with no detectable binding to the corresponding site on the other side. Binding of one Fab per dimer was also observed by analytical ultracentrifugation. The Fab did not bind to the e-antigen dimer, a non-assembling variant of capsid protein. These findings support the propositions that antibodies with particular specificities may correlate with different clinical expressions of HBV infection and that antibodies directed to particular HBcAg epitopes may be involved in ALF pathogenesis.

Keywords

cryo-electron microscopy; three-dimensional image reconstruction; monoclonal antibody; conformational epitope; fulminant hepatitis

INTRODUCTION

Acute liver failure (ALF; also known as fulminant hepatitis) associated with HBV infection is a dramatic clinical syndrome that is rarer but more severe than the classic form of acute hepatitis B (Hollinger, 1996). In patients with ALF, clinical deterioration is much more

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rapid and drastic in its outcome, resulting in a mortality rate of about 80% in patients for whom a liver transplant is not possible (Lee, 1993). Recently, the opportunity arose for a comprehensive study of the immunological status and the patterns of gene expression in ALF-infected livers (Farci et al., 2010). This analysis revealed what was primarily a strong B cell response, unlike the T cell-based pathology of classic acute hepatitis. In the diseased liver tissue, massive production of IgG and IgM antibodies specific for the HBV core antigen (capsid; HBcAg) was observed. Furthermore, a predominant heavy chain allele in germline configuration was detected in antibody libraries. In that study, RNA extracted from liver and phage display technology were used to select specific anti-HBcAg Fab clones; the most common and highest affinity clone was E1 (hereafter called E1 Fab). In the present study, we sought to characterize its interactions with the capsid surface.

HBV capsids are icosahedrally symmetric protein shells of two sizes, corresponding to triangulation numbers of T=4 and T=3 respectively (reviewed by (Steven et al., 2005)). Accordingly, they have 4 and 3 quasi-equivalent versions of the capsid protein subunit and of each epitope. As most anti-HBcAg antibodies recognize conformational epitopes (Milich and McLachlan, 1986), conventional immunochemical assays such as Western blots or Pepscanning are inapplicable. However, a combination of cryo-electron microscopy, three-dimensional image reconstruction, and molecular modeling may be used to define such epitopes (Gurda et al., 2012; Smith et al., 1993; Wang et al., 1992). Capsids are decorated with Fabs and cryo-electron micrographs recorded of the resulting complexes. These data are used to calculate a three-dimensional reconstruction of the labeled structure. By comparing it with an unlabeled control, the points of contact of the Fabs with the capsid surface may be identified. The surface loops that make up the epitope can then be identified by molecular modeling, if a high resolution structure of the capsid is available. In determining anti-HBcAg epitopes we have used the crystal structures of the T=4 capsid (Wynne et al., 1999) and of surrogate Fabs taken from the Protein Data Bank (Belnap et al., 2003; Conway et al., 1998; Harris et al., 2006). In addition, we used analytical ultracentrifugation and nickel-chelate chromatography to investigate the reactivity of E1 Fab with two dimeric forms of the HBV capsid protein: Cp149.G123A, in which a Gly at position 123 is replaced with Ala, rendering HBcAg assembly-incompetent; and e-antigen (HBeAg), which differs in retaining a 10-residue propeptide. (HBeAg is initially synthesized with a 29-residue N-terminal extension, which is subsequently processed proteolytically, leaving the propeptide - Standring et al., 1988).

MATERIALS AND METHODS

Preparation and characterization of E1 Fabs

The expression and purification of E1 Fabs were carried out as described previously (Chen et al., 2006). In brief, the histidine-tagged Fabs were expressed in *E. coli* and were initially affinity-purified on a nickel column and then on a cation-exchange SP column (GE Healthcare). The purity of the Fabs was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was determined by optical density measurements at 280 nm, assuming that 1.4 A₂₈₀ corresponds to 1.0 mg/ml. The affinity between E1 Fab and immobilized HBcAg was measured by surface plasmon resonance (SPR) using a ProteOn XPR36 Protein Interaction Array System (Bio-Rad, Hercules, CA), following a standard procedure (Bronner et al., 2010).

EM of Fab-labeled capsids

E1 Fabs in 10 mM Hepes buffer (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20 were mixed with Cp149.3CA capsids, prepared as described (Watts et al., 2010), in 50 mM Tris (pH 7.5), 150 mM NaCl, and incubated overnight at 4°C. (Cp149.3CA refers

to a construct consisting of residues 1 – 149 (the “assembly domain”) in which the three Cys residues were exchanged for Ala). Capsids were at a protein concentration of 15 μ M (of capsid protein dimers = 0.5 mg/ml), and the molar ratio of Fab : HBcAg dimer (2:1) was intended to give an excess of Fabs and saturating binding. The reaction mix was checked for capsid decoration by negative staining (2% uranyl acetate; sample diluted 10-fold), using a Philips CM120 electron microscope. Cryo-EM data were then recorded as described (Cheng et al., 1999) on a Philips CM200-FEG instrument, using the undiluted sample.

Image Reconstruction

52 films were digitized on a Nikon Super Coolscan 9000 scanner with a 6.35 μ m step size and binned 2-fold, giving a sampling rate of 2.54 Å/pixel. EMAN (Ludtke et al., 1999) and EMAN2 (Tang et al., 2007) were used for image processing. T=4 and T=3 capsids were boxed semi-automatically with *e2boxer.py*. Particles were then extracted using *batchboxer* and screened using *boxer*, yielding totals of 3,787 T=4 capsids and 493 T=3 capsids. The contrast transfer function (CTF) for each micrograph was estimated from curves obtained by combining the rotationally averaged power spectra of all boxed T=4 capsids from a given micrograph. These curves were fitted automatically using *fitctf.py* and fine-tuned manually using *fitctf*. Phase-flipped images were used in the refinement. Amplitude correction was applied to the reconstructions by “sharpening”, whereby a structure factor curve was calculated by rotationally averaging the combined spectra of all particles in several micrographs and used as reference to boost the high spatial frequency terms in the Fourier transform of the reconstruction.

Reconstructions were calculated by iterative projection matching in EMAN, using previously solved T=4 and T=3 HBV capsid structures (WW et al, unpublished results) as starting models. Due to low Fab occupancy on the T=3 capsids, a tight mask had to be applied in the 2D alignment step (control capsid radius ~ 68 pixels, mask radius ~ 80 pixels) but was not used in the 3D reconstruction step. Resolution was estimated by the Fourier shell correlation (FSC) criterion with a threshold of 0.5, giving ~10 Å for T=4 and ~15 Å for T=3 capsids (Supp. Figure 1s). To quantitate Fab binding in the low-occupancy region around the 5-fold axis of the T=4 capsid, the reconstruction was filtered to 12 Å resolution, using a soft-edged low-pass Gaussian filter.

Molecular modeling

First, a crystal structure of the T=4 capsid (PDB: 1QGT) was fitted into the density map of control T=4 (undecorated) capsids by hand, or the triplet of chains A, B, C taken from that structure was fitted into the density map of control T=3 capsids. The fitted dimers were then loaded, together with the Fab-decorated density maps, into Chimera and the surrogate Fab structure (see Results) was docked manually into the Fab-related density. The high-occupancy region around a 3-fold axis on the T=4 capsid was used for fitting. Because of Fabs’ pseudo-2-fold symmetry around their long axis, two orientations related by a 180° rotation about that axis were tested, and the solution chosen as the one that gave the higher correlation. Automated fitting was also performed for both labeled density maps, using *colores* in the SITUS program (Wriggers et al., 1999). In the high-occupancy region around the 3-fold axes of the T=4 capsid, automated fitting gave a unique solution that was closely consistent (RMSD ~ 0.8 Å) with the result obtained manually. To identify the correct orientation of the Fab, fitting was also done after rotating the Fab by 180° around its long-axis but this gave a poorer result, judged both by visual inspection of the simulations and in terms of FSC curves calculated between the reconstruction and the two simulations (data not shown). The curve from the correct solution systematically overlay the other curve. In lower occupancy regions on both capsids, the automated procedure performed erratically; however, when the capsid density was subtracted, leaving Fab-related density, the automatic

fitting performed more reliably and gave solutions consistent with the results from manual fitting.

Compared with the sequence of the surrogate Fab, there are four extra residues AMHL (in red in Supp. Figure 2s) in the CDR3 loop of the E1 heavy chain. To estimate the contribution of these four residues, a model of the E1 heavy chain was built in the I-TASSER server (Zhang, 2007), using 1VGE-H and 1Y01-H as templates. In this model, the CDR3 comes even closer to the α 4a helix of capsid protein, suggesting a stronger interaction.

Fab-binding occupancies were estimated initially in terms of the ratios between the highest densities in the variable domains of bound antibodies to the highest capsid densities, after background subtraction. The results were fine-tuned by visually comparing grayscale sections of the density maps with the corresponding sections from a series of simulations with differing occupancies. We estimate the uncertainty to be at the $\pm 10\%$ level. The latter were converted from sets of coordinates to density maps using *pdb2mrc* in EMAN, and band-limited to the same resolution as the corresponding reconstructions. No symmetry was further applied when using *pdb2mrc* because the coordinates covered all the asymmetric units on the icosahedral surface using the *sym* command in Chimera (Pettersen et al., 2004).

Analytical Ultracentrifugation

A Beckman Optima XL-I analytical ultracentrifuge, absorption optics, an An-60 Ti rotor and standard double-sector centerpiece cells were used. Equilibrium measurements were taken at 20°C and concentration profiles recorded after 16–20 hours at 18,000 rpm. Baselines were established by over-speeding at 45,000 rpm for 3 hours. Data (the average of five scans collected using a radial step size of 0.001 cm) were analyzed using the standard Optima XL-I data analysis software. Protein partial specific volumes were calculated from the amino acid compositions (Cohn and Edsall, 1943) and solvent densities estimated using the program SEDNTERP (<http://www.rasmb.bbri.org/>).

Ni-chelate chromatography

E1 Fab, bearing a carboxy-terminal 6His-tag, was mixed with either Cp149.G123A dimer or Cp(–10)149.G123A dimer, then applied to Ni-NTA agarose, washed with 500 mM NaCl, 30 mM imidazole.HCl (pH 7.5), and step-eluted with 500 mM NaCl, 500 mM imidazole.HCl (pH 7.5).

RESULTS

Binding of E1 Fabs to T=4 and T=3 capsids

Labeling experiments were conducted with purified recombinant capsids obtained by expressing a core domain construct in *E. coli*. Capsids were mixed with E1 Fabs at a 2:1 molar ratio of Fab to HBcAg dimer and incubated overnight. Vitrified samples were then prepared and observed. Compared with controls, the labeled capsids are seen to be densely covered with Fabs (Figure 1). The two sizes of capsids, T=3 and T=4, are readily distinguished and reconstructions of both labeled capsids were calculated. As T=4 capsids predominate with this HBcAg construct, the resolution of this reconstruction is higher, 10 Å vs. 15 Å. In both cases, Fab-associated density was lower than in the capsid shell and varied markedly between quasi-equivalent sites, reflecting differing occupancies of these sites (Figure 2e).

To quantitate the occupancies, both reconstructions were modeled, starting with crystal structures of the T=4 capsid (PDB: 1QGT) and a surrogate Fab (see below) and band-

limiting the models to the resolution of the reconstructions. Occupancies were estimated by matching the Fab-related density in the simulation to that in the reconstructions. The results are illustrated in Figure 2 in central sections of the T=4 capsid as viewed along a 2-fold axis of symmetry. The cryo-EM density map (Figure 2e) of the labeled capsid is compared with the simulation (Figure 2f) and the unlabeled control (Figure 2d). Fab E1 binds to subunit C with the highest occupancy (~ 60%), compared with ~ 20% for subunit A and ~ 0% for subunits B and D - Figures 2d & e. (Subunits A, B, etc are marked on a capsid model in Figure 2a, as are the E1 binding sites). On T=3 capsids, Fab E1 binds to subunits B and C at the same occupancy ~30%, but not (~ 0%) to subunit A (Suppl. Figure 3s).

In Figure 2b is shown a surface rendering of the Fab-labeled capsid. The three densities around the 3-fold axis, which represent labeling of CD dimers, match the expected shape of Fabs, indicating that there is little or no mutual occlusion of neighboring epitopes at these sites. (Neighboring epitopes occlude if they cannot be simultaneously occupied because of steric blocking). In contrast, the densities in the 5-fold region (labeling of AB dimers) do not have Fab-like shapes, indicating that they are built up from overlapping contributions from Fabs bound to mutually occluding epitopes. On the T=3 reconstruction, no Fab density is detected in the 5-fold region and the shape of Fab-related densities grouped around the 3-fold region again points to mutual occlusion between neighboring sites (Suppl. Figure 3s). We conclude that binding affinities of quasi-equivalent versions of this epitope vary markedly; and that there are, on average, 48 Fabs bound per T=4 capsid (60 sites at 60% occupancy and 60 sites at 20% occupancy). A similar calculation yielded an average of 36 Fabs bound per T=3 capsid. In retrospect, therefore, the labeling experiment was carried out with a 5-fold rather than a 2-fold excess of Fabs over occupiable binding sites.

Localization of the E1 epitope

The Fab-related densities around the 3-fold axis are the best defined and they have the right shape for a Fab molecule. Moreover, the central section through the labeled T=4 capsid shows significant substructural detail in this Fab (Figure 2e). This is indicative of tight binding (Conway et al., 2003; Watts et al., 2010) because it means that Fabs cannot rock around their binding sites, smearing the reconstructed density. The reconstruction shows that the E1 Fab binds to the side of the spike (Figure 2c), with its long axis lying nearly tangential to the underlying floor. For these reasons, we focused on these Fabs in the modeling experiments intended to identify the motifs on the capsid surface that make up the E1 epitope.

To obtain the most suitable Fab structure for modeling, we searched the Protein Data Bank for Fabs whose variable domain sequences most closely matched those of E1. These turned out to be the Fab in the Ppl-Fab complex (PDB: 1HEZ) for the light chain with 87% identity and Fab TR1.9 (PDB: 1VGE) for the heavy chain with 77% identity. Their CDRs were identified as described (<http://www.bioinf.org.uk/abs/>). In principle, an optimal surrogate would combine the light chain of 1HEZ and the heavy chain of 1VGE, but the difference between the relative positions of the light and heavy chains in 1HEZ and 1VGE ruled against this solution. However, the full 1VGE structure fitted readily into the Fab densities at the 3-fold site on the T=4 capsid and also faithfully reproduced the other Fab-related densities on both reconstructions. These fittings were done by hand but the same results were obtained when an automated procedure was used (Wriggers et al., 1999). We conclude from these experiments that CDR3 on the E1 heavy chain and CDR1 on the light chain are the major contributors to antigen binding, and the latter has a somewhat smaller interacting area.

The interaction between the surrogate E1 Fab and subunit C epitope on the T=4 capsid is illustrated in Figure 3. It involves two major interfaces: one is between the heavy chain

CDR3 and four residues 82R.83D.86V.87S on the $\alpha 4a$ helix of subunit C; the other is between the light chain CDR1 and three residues 70T.73G.74T on the $\alpha 3$ helix of subunit. These interactions hold the Fab firmly in place, extending out from the C subunit side of the C-D spike.

Interactions of E1 Fab with soluble dimeric forms of the HBV capsid protein

A priori, it is unclear to what extent the variations in occupancy that we observe among quasi-equivalent variants of the E1 epitope reflect conformational distinctions and to what extent they reflect accessibility. To address this question, we examined the binding of E1 to two soluble dimeric forms of the capsid protein. Cp149.G123A has a substitution at position 123 which is in the part of the HBcAg dimer that engages in their clustering in rings of five and six around the symmetry axes of capsids. eAg is a variant of Cp149 that retains a 10-residue portion of its 29-residue propeptide at its N-terminus. *In vivo*, eAg is secreted in soluble form into the infected person's serum and is a major viral antigen. For these experiments, we used a recombinant eAg construct (Watts et al., 2010) that also had the Gly-to-Ala substitution at position 123.

E1 Fab and Cp149.G123A dimers were mixed in a 2:1 molar ratio and applied to a Ni-chelate column, washed with 500 mM NaCl, 30 mM imidazole.HCl (pH 7.5) and then step-eluted with 500 mM NaCl, 500 mM imidazole.HCl (pH 7.5). The eluate, containing the resulting immune complex and any unbound E1 Fab, was analyzed by sedimentation equilibrium. The average molecular mass of the mixture was ~ 68,000 Da with a systematic increase in mass with radial distance. The equilibrium data were best fitted as a two-species mixture of E1 Fab /Cp149 dimer (82,250 Da) and E1 Fab alone (48,800 Da) in a 0.7 : 1 ratio (Figure 4). The composition of the mixture corresponds closely to the original 1 : 2 molar ratio of E1 Fab to Cp149 dimer in the sample used for affinity binding (and is also consistent with densitometry of Coomassie-stained SDS-PAGE of column eluate). Further analysis of the affinity column eluate by sedimentation velocity centrifugation confirmed the absence of any high molecular mass species corresponding to higher order complexes such as (E1 Fab)₂/Cp149 dimer (130,050 Da). These data clearly support the conclusion that E1 Fab binds in a monovalent manner to Cp149 dimer. Once one site on Cp149 is occupied, binding to the other is disfavored, most likely by an induced change in conformation in the spike's 4-helix bundle. We can exclude the alternative explanation that the complex, (E1 Fab)₂/Cp149 dimer, is unstable and aggregates, as this is not supported by recoveries from the affinity column.

To determine the reactivity of E1 Fab with eAg (i.e. Cp(-10)149.G123A), we mixed eAg with His-tagged E1 Fab and assayed for complex formation in terms of retention of eAg on Ni-chelate resin. Cp149.G123A was run in the same assay as a positive control. The mixtures were applied to Ni-NTA agarose, washed, and then eluted with imidazole. Analysis by reducing SDS-PAGE (Figure 5) showed eAg eluting in the wash whereas Cp149.G123A was eluted with E1 Fab by the imidazole. These results demonstrate that E1 Fab does not bind to eAg.

DISCUSSION

In previous studies, the reactivities of a number of anti-HBcAg and anti-HBeAg antibodies have been characterized by electron microscopy, mutational analysis, and surface plasmon resonance (Steven et al., 2005; Watts et al., 2010). These include several mouse monoclonals originally developed in the 1980's to discriminate between HBcAg and HBeAg and later employed in diagnostic assays; a mouse monoclonal derived from a naïve B-cell line that recognizes HBcAg (Watts et al., 2008); and polyclonal HBcAg-specific antibodies obtained from a chronic HBV patient (Kandiah et al., 2012). These antibodies are compared

in Table 1, where their epitopes are assigned to four areas: spike tip, floor, C-terminal, and spike side. Some examples, including E1, are mapped on the capsid surface in Figure 6. Most antibodies bind to the spike tip, a region that includes two copies of the so-called “immunodominant loop”. The floor is another major site for epitopes, including that of the high-affinity antibody 3120. The HBeAg-specific e6 epitope is in a site that associates with other dimers around the axes of symmetry in the capsid, and is buried upon assembly. E1 binds to the side of a spike in a region where no epitope has previously been localized.

Properties of the E1 epitope

Several features of the way in which E1 Fab interacts with capsids are noteworthy.

- i. The number of residues involved in the interface is larger than for any other HBcAg Fab characterized to date except Mab 3120 ($K_d \sim 10^{-12}$ M (Watts et al., 2010)). This is consistent with Fab E1 being a tight binder (the K_d is $\sim 5 \times 10^{-9}$ M).
- ii. Its epitope combines patches on both spike helices, $\alpha 3$ and $\alpha 4a$, on the same subunit. As the two helices should be correctly juxtaposed for binding to occur, it is expected to be a conformational epitope. Consistent with this inference, we see major differences in occupancy - and hence, in affinity - among the four quasi-equivalent versions of the epitope on the T=4 capsid. On the T=4 capsid, binding is observed on the C-subunit and the A-subunit. Although there is an approximate dyad running down the center of each spike, no binding is detected at the symmetry-related positions on the other side of these spikes, i.e. on the B- and D-subunits. Binding of one E1 Fab may either induce a conformational change in the four-helix bundle that disallows binding of the second Fab, or the Fab may detect preexisting conformational differences between the two sides of dimer. While both the X-ray structure of capsids (Wynne et al., 1999) and the NMR structure of free dimers (Freund et al., 2008) do not reveal such asymmetry, the X-ray structure of free dimers (Packianathan et al., 2010) does show pronounced asymmetry near the apex of the four-helix bundle. Thus it may be that Fab E1 is sensitive to subtle conformational changes in its epitope not previously detected in capsid structures.
- (iii) Its long axis is oriented almost tangentially to the capsid surface whereas previously characterized Fabs tend to point approximately radially outwards. Of the eight other monoclonal anti-HBcAg Fabs characterized to date (Table 1), 9c8 is the only one other than E1 to have an approximately tangential binding aspect. This antibody, which is closely related to membrane-bound HBcAg-specific antibodies on B cells, i.e. B-cell receptors, interacts in a bipartite manner with the capsid, primarily through a canonical interaction with a conformational epitope on one spike and secondarily through a non-canonical interaction of the same Fab with an adjacent spike (Watts et al., 2008). HBcAg is particularly immunogenic, apparently because it presents a multivalent array of its epitopes that efficiently cluster B-cell receptors. In this context, the tangential binding aspect of Fab E1 may be significant in that it should be conducive to clustering receptors, whereas Fabs with radial binding aspects more readily form immuno-precipitates with capsids (Figure 7).

Implications in relation to the immunological response

HBeAg, a secreted soluble (i.e. un-assembled) form of essentially the same polypeptide chain as HBcAg, can suppress seroconversion to anti-HBcAg antibody production in some mouse strains: thus, it may serve as a regulatory protein (Chen et al., 2004). HBeAg is normally present along with HBcAg in HBV infections, but a mutation can render it inoperative (a so-called pre-core mutation). In these circumstances, HBeAg is not synthesized and the lack of its immune modulation is thought to lead to an unregulated and

potentiated innate immune response to HBcAg, in some cases associated with ALF in patients (Fagan et al., 1986; Liang et al., 1991). It is of note that E1 does not bind HBeAg (this study) and the two original ALF patients were sero-negative for this antigen (Farci et al., 2010).

Although convincingly demonstrated in various mouse models, the pathogenesis of the immune response to HBcAg has been difficult to confirm in humans. However, in this study and its predecessor (Farci et al., 2010), it has been possible to compare results pertaining to human HBV-associated ALF with data previously obtained in mice. Notably, 1) the patients had pre-core mutations that prevented the synthesis of HBeAg; 2) probably as a result, there was a massive and apparently uncontrolled anti-HBc response; 3) monoclonal anti-HBcAg antibodies produced from the RNA of B cells in the patients' livers had high affinity despite the fact that they had undergone virtually no maturation; 4) the heavy chain germ lines used by the B cells for the anti-HBcAg antibodies recovered from the patients were restricted to the same families as those recovered previously from mice: HV1 and HV3.

Previous studies have shown that the prevalence of certain antigens and classes of antibodies in the sera of infected individuals correlates with disease status (Hollinger, 1996). For instance, anti-HBcAg IgMs first appear in the mid-acute phase of HBV hepatitis and are followed, months later, by anti-HBcAg IgG molecules (*loc. cit.*). However, these analyses have only distinguished between antibodies of different families, IgG and IgM. The possibility exists that there may be correlations at a more detailed level (Sällberg et al., 1990) - e.g. the incidence of antibodies directed against particular HBcAg epitopes - that can serve as markers of disease status. The present observations are consistent with that idea.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cryo-electron micrographs of HBV capsids: (a) unlabeled control; and (b) decorated with E1 Fabs. Examples of T=4 (T=3) capsids are marked with arrows (dashed arrows). Scale bar = 100 nm.

Figure 2.

(a) Model of HBcAg dimer packing in the T=4 capsid with Fab-E1 binding sites labeled with light blue dots. The four quasi-equivalent subunits A, B, C, and D are colored as shown. (b) Surface rendering of the reconstruction of the E1-decorated T=4 capsid. Three Fab-related densities around a 3-fold axis are shaded red and Fab-related densities around a 5-fold axis are shaded green. (c) Model at 10 Å resolution of a portion of the T=4 capsid surface with two Fabs, one (green) bound to the side of an A-subunit in the spike closest to the 5-fold axis, and the other (red) to the side of a C-subunit in a spike adjacent to a 3-fold axis. (d–f) are central cross-sections through T=4 capsids viewed along a 2-fold axis: (d) control capsid, cryo-EM reconstruction; (e) E1-decorated capsid, cryo-EM reconstruction; (f) band-limited simulation of E1-decorated capsid, with 60% occupancy of C-related sites and 20% occupancy of A-related sites.

Figure 3.

Modeling of the interaction between an E1 Fab surrogate and the HBV capsid. The Fab binds to both α -helices on the C subunit on one side of the C-D spike. The C-subunit residues engaged in major interactions with the Fab are colored black: they are 70T, 73G and 74T in $\alpha 3$ and 82R, 83D, 86V, and 87S in $\alpha 4a$.

Figure 4.

Sedimentation equilibrium of the E1 Fab/Cp149.G123A complex. The lower panel shows the absorbance gradient (open circles) and the solid line the calculated fit for an ideal two-species model (a mixture of two proteins which do not interact). The best fit was with E1 Fab (48,800 Da) and E1 Fab:Cp149, 1:1 (82,250 Da). Determination of the composition required input of the respective molar extinction coefficients at 280 nm which were calculated from the amino acid compositions. The upper panel shows the residuals, which are the differences between the fitted and experimental values as a function of radial position.

Figure 5.

E1 Fab binds to HBcAg dimer but not to eAg dimer. E1 Fab, bearing a C-terminal 6-His-tag, was mixed with either HBcAg dimer or eAg dimer and analyzed by Ni-chelate chromatography followed by reducing SDS-PAGE. eAg appeared in the low-imidazole wash whereas E1 Fab and HBcAg eluted with the high-imidazole buffer. Two additional wash cycles to completely remove unbound material prior to the high-imidazole elution are not shown. These results demonstrate the specificity of E1 Fab for HBcAg versus eAg.

Figure 6. Mapping the E1 epitope and those of three other anti-HBcAg antibodies. Top: the peptides involved in these epitopes are marked on a ribbon diagram of three adjacent dimers. E1 and 312 (Conway et al., 1998) each bind to a single subunit within a dimer. Whereas the 312 epitope is continuous and linear, the E1 epitope is discontinuous and conformational, combining patches on both spike helices on the same subunit. The 3105 epitope has contributions from both subunits in a given spike (Belnap et al., 2003). The 3120 epitope bridges the interface between subunits in different dimers in the floor region and involves a total of five loops (Conway et al., 2003). Bottom: mapping of these peptides on the core domain sequence.

Figure 7. Diagram illustrating the relative propensity of membrane-bound B-cell receptors that bind tangentially to spikes to be aggregated by HBcAg capsids, compared with radially binding antibodies which, if soluble, are more likely to aggregate. As there is likely to be considerable flexibility about the Fab-Fc junction, the diagram is meant to suggest only a general trend.

TABLE 1

Comparison of E1 with other antibodies.

Antibody ^a	HBcAg ^b		HBcAg Dimer	Epitope ^c	Reference
	Capsid	Dimer			
m-312	+	ND	ND	Spike tip	(Conway et al, 1998)
m-88	+	ND	ND	Spike tip	(Harris et al, 2006)
m-842	+	ND	ND	Spike tip	(Harris et al, 2006)
m-9c8	+	±	ND	Spike tip	(Watts et al, 2008)
m-3105	+	+	+	Spike tip	(Belnap et al, 2003)
m-F11A4	+	+	+	Spike tip	(Belnap et al, 2003)
m-3120	+	-	-	Floor	(Conway et al, 2003)
m-e6	-	+	+	C-terminal	(Watts et al, 2010)
h-polyclonal ^d	+	ND	ND	Spike tip, floor	(Kandiah et al, 2011)
h-E1	+	+	-	Spike side	This study

^aAntibodies are of mouse (m) or human (h) origin. Antibody 9c8 was derived from a naïve B-cell line reactive with HBcAg. In all cases the Fabs were obtained from the corresponding antibody by proteolytic digestion, except Fab E1, which was generated from patient-derived RNA by phage-display.

^bBoth HBcAg and HBcAg proteins were expressed in *E. coli*. HBcAg capsids were assembled from purified dimeric protein and non-assembling dimers were obtained by introducing a G123A mutation. Soluble dimeric HBcAg was obtained from protein with the amino-terminal propeptide oxidized to form an intra-molecular disulfide bond. Binding of Fab to antigen was determined by electron microscopy and surface plasmon resonance. ND, not determined.

^cLocations of the epitopes were determined primarily by cryo-EM and molecular modeling.

^dThe binding sites on the capsid of the unfractionated set of polyclonal antibodies were deduced by *in silico* modeling, employing a maximum of five classes of antibodies that together accurately reproduced the experimental electron density.