Priming of cytotoxic T cell responses to exogenous hepatitis B virus core antigen is B cell dependent

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INTRODUCTION

The hepatitis B virus (HBV) core antigen (HBcAg) has a unique ability to bind a high frequency of naïve human and murine B cells. The role of HBcAg-binding naïve B cells in the immunogenicity of HBcAg is not clear. The HBcAg-binding properties of naïve B cells were characterized using HBcAg particles with mutated spike region (residues 76–85) sequences. Deletion of residues 76–85 (HBcD76–85) destroyed naïve B cell binding, whereas deletion of residues 79–85 did not. HBcAg particles with an Ile instead of the natural Ala at position 80 did not bind naïve B cells, whereas reversion of Ile80→Ala restored B cell binding. Destroying the B cell-binding ability of HBcAg had a marginal effect on the overall B cell immunogenicity of the different particles, suggesting that they were equally efficient in priming T helper cells. Therefore, the importance of HBcAg-binding B cells is studied with relation to the priming of HBcAg-specific cytotoxic T cells (CTLs). The role of HBcAg-binding B cells in the priming of HBcAg-specific CTLs was evaluated by immunization with endogenous HBcAg (DNA immunization) and exogenous recombinant HBcAg particles. Endogenous HBcAg primed HBcAg-specific CTLs in wild-type and B cell-deficient mice, whereas exogenous HBcAg primed HBcAg-specific CTLs only in wild-type mice. Importantly, HBcD76–85 did not prime CTLs despite the presence of B cells. Thus, the ability of exogenous HBcAg particles to prime specific CTLs is B cell dependent, suggesting a possible role for HBcAg-binding B cells in HBV infections.
class I MHC molecules, they could potentially act as APCs for CD8+ T cells (Bennett et al., 1998). Therefore, it is of interest to determine the importance of B cells in priming HBCAg-specific CTLs.

METHODS

Mice. C57BL/6 (B6, H-2b) mice were obtained from B&K Universal Sollentuna, Sweden. B cell-deficient H-2b mice (µMT.B6) (Kitamura et al., 1991) were kindly provided by Karin Sandtstedt and Anna Berglof, Huddinge University Hospital, Stockholm, Sweden. Mice were maintained at the animal facility at Huddinge University Hospital. All mice were used at 6–8 weeks of age and the local committee on animal ethics approved all experiments.

Recombinant proteins, peptides and plasmid DNA vectors. Recombinant particulate HBCAg (ayw subtype) encompassing residues 1–183 were produced in Escherichia coli and purified as described previously (Schodel et al., 1993).

Recombinant HBCAg (subtype ayw) encompassing residues 1–183 and with an Ile at position 80 and a Gly at position 74 was designated HBcAg-Ala57/8 (Pushko et al., 1991; Schumacher et al., 1993) by solid phase ELAs. In brief, recombinant proteins were coated overnight on microplates at 1 µg ml−1 in sodium bicarbonate buffer (pH 9.6) at 4°C. Plates were incubated with primary antibodies 57/8 or 35/312 diluted in PBS containing 1 % BSA, 2 % goat sera and 0·05 % Tween 20 for 1 h. Thereafter plates were incubated with anti-mouse immunoglobulin peroxidase conjugate (P250) (Dako). All incubation steps were carried out at room temperature. Plates were developed using O-phenylenediamine substrate for 30 min and the reactions were stopped using 2 M H2SO4. Optical density measurements were determined at 490 nm.

Immunizations. To study humoral responses, groups of C57BL/6 mice were immunized intraperitoneally with 20 µg HBCAg, HBcAg-Ala57/8, HBcAg79–85 and HBcAg76–85 in PBS. Mice were boosted 4 weeks later with the same dose of antigen. Sera were collected at weeks 2 and 6, pooled and antibody titres were determined by ELA.

To prime CTLs, groups of C57BL/6 and µMT.B6 mice were immunized with a single injection of the 100 µg HBCAg93–100 peptide in complete Freund’s adjuvant (CFA) (Sigma), or 20 µg of recombinant protein in incomplete Freund’s adjuvant (IFA), subcutaneously at the base of the tail. Mice were sacrificed 9–13 days later.

For DNA immunizations, mice were immunized by needle injections of 100 µg of plasmid DNA encoding HBCAg. Plasmid DNA reconstituted in PBS was given intramuscularly to the tibialis anterior (TA) muscle (Lazdina et al., 2001b). At 5 days prior to DNA immunization, mice were injected intramuscularly with 50 µl per TA muscle of 0·01 mM cardiotoxin (Latoxan) in 0·9 % sterile saline (Lazdina et al., 2001b). Mice were boosted at intervals of 4 weeks.

B cell enrichment. To prepare enriched naive B cells, spleens were removed from non-immune C57BL/6 mice. Single cell suspensions were prepared and depleted of red blood cells using Red Blood Cell Lysing Buffer (Sigma). T cells were depleted using a 1 : 1 : 1 ratio of supernatants from hybridomas 31M (anti-CD8), RL172.4 (anti-CD4) and AT83 (anti-Thy1.2) (kindly provided by Eva Severinson and Lena Ström, CMB, Karolinska Institutet, Sweden) plus low-toxicity rabbit complement (Saxon Europe). The cells were

Table 1. Sequence within residues 71–90 of HBCAg, subtype ayw, aligned with mutant HBcAg

<table>
<thead>
<tr>
<th>Amino acid position…</th>
<th>71 74 80 85 90</th>
</tr>
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<tbody>
<tr>
<td>HBcAg</td>
<td>WVGNNLEDPSRLDVLYNVY</td>
</tr>
<tr>
<td>HBcAg-Ile</td>
<td>WVGNNLEDPSRLDVLYNVY</td>
</tr>
<tr>
<td>HBcAg79–85</td>
<td>WVGNNLED----YLYNVY</td>
</tr>
<tr>
<td>HBcAg76–85</td>
<td>WVGNNdKK-----YLYNVY</td>
</tr>
<tr>
<td>HBcAg-Ala</td>
<td>WVGNNLEDPSRLDVLYNVY</td>
</tr>
</tbody>
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ClonC1 (5′-AATTTAAGCTTATGGAATCAGACTGAC-3′) and ClonCE1 (5′-AAAGGCCCGTTAAGCACGATTGC-3′). The resulting ampli-
copon was ligated into the HindIII/Apal-digested pVAX vector (Invitrogen). The HBCAg DNA plasmid was sequenced to ensure the correct reading frame of the insert. In vitro translation of the HBCAg DNA plasmid was performed using the T7-Coupled Reticulocyte Lysate system (Promega) and expression of the 21 kDa HBCAg protein was confirmed (data not shown). The plasmid DNA used for in vivo injections was purified using DNA Purification Columns (Qiagen), according to the manufacturer’s instructions.

Recombinant soluble dimeric mouse H-2Kb–Ig fusion protein used for flow cytometry analysis was purchased from Becton Dickinson. Ionomycin and phorbol myristate 13-acetate were purchased from Sigma.

Antibodies. Traditional anti-Hbc mouse monoclonal antibodies (mAbs) 32/312, recognizing amino acid residues 78–83, and HBE/ CAg-specific mAb 57/8, recognizing an epitope at residues 128–133, have been described in detail previously (Pushko et al., 1994; Salberg et al., 1991, 1993).

The following anti-mouse mAbs were used for flow cytometry analysis: anti-CD19 (clone 1D3), anti-B220 (clone RA3-68B) and anti-CD8 (clone 53-6.7) (all purchased from Becton Dickinson).

Enzyme immunoassays (EIAs). The panel of HBCAg recombinant proteins was tested for recognition by previously characterized mAbs 35/312 (specific for residues 76–85 of HBCAg) and 57/8 (specific for residues 128–133 of HBc/eAg) (Pushko et al., 1994; Salberg et al., 1993) by solid phase EIAs. In brief, recombinant proteins were coated overnight on microplates at 1 µg ml−1 in sodium bicarbonate buffer (pH 9.6) at 4°C. Plates were incubated with primary antibodies 57/8 or 35/312 diluted in PBS containing 1 % BSA, 2 % goat sera and 0·05 % Tween 20 for 1 h. Thereafter plates were incubated with anti-mouse immunoglobulin peroxidase conjugate (P250) (Dako). All incubation steps were carried out at room temperature. Plates were developed using O-phenylenediamine substrate for 30 min and the reactions were stopped using 2 M H2SO4. Optical density measurements were determined at 490 nm.
incubated for 1 h at 37°C. Resulting T cell-depleted cell populations consisted of ≥85% CD19-positive cells, as determined by flow cytometry.

**Direct binding of HBcAg to B cells.** A total of 1 × 10⁶ T cell-depleted spleen cells from non-immune C57BL/6 mice were incubated on ice for 20 min with Fc-receptor blocking reagent (Fc-block) (Becton Dickinson) diluted in PBS containing 1% foetal bovine serum (hereafter referred to as FACS buffer). Cells were then incubated with 1 μg of recombinant HBcAg, HBcAg-Ile, HBcΔ79–85, HBcΔ76–85, HBcAg-Ala or the control antigen HBeAg for 30 min on ice. Cells were washed and incubated with HBeAg-specific mAb 57/8 conjugated with sulfo-NHS-LC-biotin (Pierce). Biotinylation was done according to the protocol provided by the manufacturer. After two washes, cells were incubated on ice for 30 min with allophycocyanin-conjugated streptavidin (Becton Dickinson) and phycoerythrin (PE)-conjugated CD19 antibody (Becton Dickinson).

**Detection of HBcAg-specific CTLs.** Spleen cells from mice immunized with recombinant protein or peptide were harvested 13 days after immunization. DNA-immunized spleens were obtained 2 weeks after the second boost (6 weeks after the first injection). Single cell suspensions were prepared as described and 2 × 10⁶ splenocytes were restimulated with 25 × 10⁶ syngeneic irradiated (20 Gy) splenocytes pulsed with 0.05 μM of peptide, as described previously (Sandberg et al., 2000). Restimulation cultures were set in 12 ml complete RPMI medium. After 5 days, effector cells were harvested and washed twice. RMA-S target cells (Karre et al., 1986) were pulsed with 50 μM peptide for 90 min at 5% CO₂ and 37°C. Serial dilutions of effector cells were incubated with 5 × 10⁶ chromium-labelled peptide-pulsed RMA-S target cells in a final volume of 200 μl per well in 96-well plates. After a 4 h incubation at 5% CO₂ and 37°C, 100 μl of supernatant was collected and the radioactivity was determined using a γ-counter. The percentage of specific release was calculated according to the formula: (experimental release – spontaneous release/total release – spontaneous release) × 100. Results are shown as the mean of triplicate values.

**Quantification of HBcAg₉₃–100-specific CTLs by flow cytometry.** The frequencies of HBcAg-specific CD₈⁺ T cells was analysed by ex vivo staining of spleen cells from HBcAg₉₃–100 peptide, HBcAg protein and HBcAg DNA-immunized mice using the recombinant soluble dimeric mouse H-2Kb—Ig fusion protein (Becton Dickinson). Spleen cells (1–2 × 10⁶) were suspended in 50 μl FACS buffer and pre-incubated with 1 μg of Fc-block per 10⁶ cells on ice for 15 min. After a single wash, cells were incubated on ice for 90 min with 3 μg of H-2Kb–Ig fusion protein per 1 × 10⁶ cells pre-loaded for 48 h with a 640 M excess of HBcAg₉₃–100 peptide (MGLKFRQL) (Kuhober et al., 1996). H-2Kb–Ig fusion protein unloaded or loaded with irrelevant peptide served as control for unspecific staining. In some experiments, naive spleen cells were also included as a control for non-specific staining. After incubation with the H-2Kb–Ig fusion protein, cells were washed twice in FACS buffer and resuspended in 50 μl FACS buffer containing a 1:5 dilution of PE-conjugated rat anti-mouse IgG₁ antibody (Becton Dickinson) and incubated on ice for 30 min. Cells were washed twice and incubated with 1 μg of FITC conjugated-mouse CD8 antibody per 10⁶ cells for 30 min. After incubation, cells were washed twice and resuspended in 0.5 ml FACS buffer for immediate FACS analysis. Approximately 50–100 000 events from each sample were counted on a FACS Calibur.

**RESULTS**

**Characterization of mutant HBcAg particles**

The HBcAg-Ile, HBcΔ79–85 and HBcΔ76–85 proteins were examined by electron microscopy for particle formation. All three E. coli-derived proteins spontaneously formed virus-like particles of a comparable size (Fig. 1).

To characterize the particles further, all were tested for binding to the previously characterized mAbs 35/312, recognizing the HBc or HBe1 epitope LEDPASRDLV at residues 76–85 (Salfeld et al., 1989; Sallberg et al., 1991), and 57/8, recognizing the HBe2 epitope TPPAYR at positions 128–133 of HBcAg, by EIA (Pushko et al., 1993). As expected, mAb 35/312 was highly reactive against HBcAg and HBcAg-Ile but not reactive against particles HBcΔ79–85 and HBcΔ76–85 lacking the complete epitope region at positions 76–85 (Fig. 2). Also as expected, mAb 57/8 was reactive to all HBcAg particles (Fig. 2).

To characterize the immunogenicity of HBcAg particles in vivo, C57BL/6 mice were immunized with the different particles. C57BL/6 (H-2b) mice were used since none of the deletions affects CD4⁺ T cell recognition. In general, the
immunized mice developed the highest antibody titres against the respective immunogen, suggesting that the particles are antigenically distinct (Fig. 3). Sera from all immunized groups were analysed by the competitive anti-HBc AXSYM Core assay (Abbott) for competition with human anti-HBc antibodies. This confirmed that HBcAg and HBcAg-Ile primed anti-HBc antibodies that compete with human antibodies (data not shown). In contrast, particles HBcΔ79–85 and HBcΔ76–85 were unable to prime antibodies with a true anti-native HBcAg specificity (data not shown). In conclusion, the three mutant HBcAg proteins form virus-like particles, have the predicted antigenicity and have a comparable overall immunogenicity. These particles were therefore suited for the ability to bind naive B cells.

Mapping of the binding site on the HBcAg particle for naive B cells

We have demonstrated previously that HBcAg binds to BCRs through a common motif expressed by germ line Ig receptors on naive B cells (Lazdina et al., 2001a). We were therefore interested to map the region on the HBcAg particle responsible for binding slg. The panel of recombinant HBcAg particles was assayed for direct binding to B cells by flow cytometry, as described previously (Lazdina et al., 2001a). T cell-depleted naive splenocytes were incubated with the recombinant particles on ice to prevent endocytosis. The cells were then double stained with the biotinylated HBc/e-specific mAb 57/8 and the B cell marker CD19. Consistent with previous data (Lazdina et al., 2001a), we noted that HBcAg of the ayw subtype bound to approximately 5% of the naive B cell population (Fig. 4).

**Fig. 2.** Reactivities of (a) mAb 57/8 and (b) mAb 35/312 against a panel of solid-phase bound HBcAg particles in EIA. Microplates were coated with recombinant HBcAg, HBcAg-Ile, HBcΔ79–85 and HBcΔ76–85 proteins and incubated with serial dilutions of mAb 35/312 or mAb 57/8. The amount of bound mAb was determined as described in Methods. Values shown are the optical density measurements at 490 nm.

**Fig. 3.** Immunogenicity of the HBcAg particles in vivo. C57BL/6 (H-2b) mice were immunized intraperitoneally with HBcAg, HBcAg-Ile, HBcΔ79–85 or HBcΔ76–85 in PBS and boosted 4 weeks later. Sera were collected on week 6 and analysed for reactivity against solid-phase antigens in EIA, as described in Methods. Values shown are optical density measurements at 490 nm.

**Fig. 4.** Mapping of the binding site on HBcAg particles by direct B cell-binding assays. Enriched B cells were incubated on ice with HBcAg derivatives, HBeAg or medium only and stained with CD19 mAb and anti-HBc 57/8, as described in Methods. The percentage of HBcAg-binding B cells is shown on the x-axis and respective antigen is indicated on the y-axis. Results are the mean values from three different experiments (±SEM).
Interestingly, the HBcAg-Ile particle did not bind naive B cells (Fig. 4). This suggests that the amino acids at positions Val74 or Ala80 in the original HBcAg particle could be important for B cell binding (Table 1). This was confirmed further by the HBcAg-Ala particle in which region 67–89 is replaced with the spike sequence from HBcAg of adw subtype, with a Gly74 and an Ala80 (Table 1). Using this particle, B cell binding was restored (Fig. 4), indicating that the Ala at position 80 is important for binding sIg. The importance of the tip of the spike region of HBcAg was evaluated further using particles with deletions in the spike region. Deletion of residues 79–85 (HBcAD79–85) of HBcAg only slightly affected B cell binding, whereas a deletion of residues 79–85 and an inversion of positions 76–78 (HBcAD76–85) completely destroyed the ability to bind naive B cells (Fig. 4). This suggests that residues 76–78 also affect B cell binding and that the new loop sequence LEDVS, contained in particle HBcAD79–85, allows B cell binding. These experiments show that binding of HBcAg to the naive B cell is dependent on residues 76–80, present at the tip of the spike. Thus, we confirmed that mutant HBcAg proteins formed virus-like particles but had different abilities to bind naive B cells.

The role of HBcAg-binding B cells in the priming of HBcAg-specific CTL responses by endogeous and exogenous HBcAg

B cells are efficient APCs when presenting HBcAg to CD4+ T cells (Milich et al., 1997). However, it is unknown whether B cells play any role in the priming of HBcAg-specific CTL responses. To address this question, wild-type and B cell-deficient mice (μMT.B6) (Kitamura et al., 1991) were immunized with endogenously or exogenously produced forms of HBcAg. Immunization with HBcAg as a peptide CTL epitope or an endogenously produced antigen (i.e. DNA-based immunization), efficiently primed HBcAg-specific CTLs in both wild-type and B cell-deficient mice.
thus independently of B cells (Fig. 5). DNA immunization did prime HBcAg-specific antibodies at titres of $10^3$–$10^4$. Surprisingly, HBcAg-specific CTL activity could be primed in wild-type but not in B cell-deficient mice immunized with recombinant HBcAg particles. Splenocytes from naive mice restimulated with the HBcAg-derived CTL peptide did not showCTL activity (data not shown). In additional control experiments, mice were immunized with irrelevant recombinant protein (HCV NS3) and no detectable HBcAg-specific CTL activity was observed (data not shown). Also, restimulation with an irrelevant H-2Kb-restricted peptide from SeV NP failed to activate HBcAg-specific CTLs in vitro (data not shown).

**Enumeration of HBcAg-specific CD8$^+$ T cells by direct ex vivo staining with dimeric H-2Kb–Ig fusion protein**

To confirm the results obtained by the lytic assays, the frequency of HBcAg$_{93–100}$-specific CD8$^+$ T cell precursors after HBcAg immunization was determined directly ex vivo. Spleen cells were stained with recombinant soluble dimeric mouse H-2Kb–Ig fusion protein loaded with the HBcAg$_{93–100}$ peptide. Although a single immunization with exogenous HBcAg particles primed HBcAg-specific lytic CTL responses comparable to that primed by HBcAg peptide or HBcAg DNA immunizations, the frequency of HBcAg$_{93–100}$ peptide-specific CD8$^+$ CTLs was very low (0–0.4%; mean value 0.1%, n=4) in wild-type mice (Fig. 6). Importantly, after a short (5 days) restimulation in vitro with HBcAg$_{93–100}$ peptide, the frequencies of HBcAg peptide-specific CD8$^+$ T cells increased (mean value 0.7%).

No HBcAg-specific CTL precursors could be detected in B cell-deficient mice immunized with exogenous HBcAg particles and they were still undetectable after restimulation in vitro. These data are fully consistent with the cytotoxicity data (Fig. 5). Altogether, HBcAg as an endogenously produced antigen primes CTLs efficiently in wild-type and B cell-deficient mice at high frequencies. In contrast, exogenous HBcAg primes low frequencies of HBcAg-specific CTLs and requires the presence of B cells.

**Exogenous HBC particles unable to bind naive B cells do not prime specific CTLs**

To test further the importance of HBcAg-binding naive B cells in the priming of CTLs using exogenous particles, groups of wild-type mice were immunized with recombinant HBcAg and HBcA76–85 particles. Fully consistent with previous data, native HBcAg did prime CTLs as an exogenous antigen (Fig. 7). In contrast, HBcA76–85 particles, unable to bind naive B cells, did not prime HBcAg-specific CTLs as an exogenous antigen (Fig. 7). Thus, despite the presence of B cells, the HBcA76–85 particle was unable to prime CTLs. This is fully consistent with the
inability of HBCAg particles to prime CTLs in the B cell-deficient mice and confirms a key role of B cells in priming CTLs using exogenous HBCAg.

**DISCUSSION**

It has been shown that HBCAg is a unique B cell immunogen binding to a high frequency of naive murine as well as human B cells. This interaction with naive murine B cells is mediated through conserved motifs encoded by some germ line gene families (Lazdina et al., 2001a). Previous experiments suggested that the spike region of HBCAg was involved in the binding of naive B cells (Lazdina et al., 2001a). The present studies confirmed that residues 76–80 located at the tip of the spikes on the HBCAg particle are indeed involved in binding the naive B cell receptor. Interestingly, immunization of mice with mutant HBCAg particles revealed no significant difference in the ability to elicit high levels of antibodies. Thus, B cell immunogenicity of HBCAg seems to be independent of its ability to bind to a high frequency of naive B cells and is possibly more related to the ability of the particle to prime CD4+ T cells.

It has been shown that exogenous antigen is taken up by endocytosis and can reach the MHC I compartment rendering B cells susceptible to lysis by specific class I-restricted CD8+ T cells (Ke & Kapp, 1996). HBsAg-specific B cells are able to present exogenous HBsAg to HBsAg-specific MHC class I-restricted CD8+ T cells (Barnaba et al., 1990). To study the role of B cells in priming CTLs by endogenously and exogenously produced HBCAg, we immunized wild-type and B cell-deficient mice. HBcAg-specific CTLs were detected by in vitro cytotoxicity assays quantified directly ex vivo by flow cytometry. These experiments showed that B cells were not required for CTL priming by the HBCAg-derived CTL peptide or endogenously produced HBCAg. In contrast, exogenous HBCAg particles were only able to prime HBcAg-specific CTLs in the presence of B cells. Exogenous HBCAg primed in vitro active CTLs at very low precursor frequencies in wild-type mice. Importantly, the mutant HBCAg particle lacking residues 76–85 at the spike region and unable to bind naive B cells could not prime HBCAg-specific CTLs when used as an exogenous immunogen in the presence of B cells. This clearly shows that the priming of HBcAg-specific CTLs by exogenous HBCAg is B cell dependent.

There are several mechanisms by which exogenous HBcAg may prime CTLs in a B cell-dependent fashion. First, the role of HBCAg-binding B cells could be as the primary APC for CD8+ T cells through a leakage between the class I and class II antigen-presenting pathways. Alternatively, B cells may produce HBcAg-specific antibodies that form immune complexes with HBcAg. These immune complexes could then be taken up by macrophages or dendritic cells via Fc-R-mediated endocytosis and thereafter processed and presented to CD8+ T cells (Schuurhuis et al., 2002). Since we know that immunization with the HBcA76–85 particle, which is unable to prime CTLs, elicits antibodies in wild-type mice, we would favour the first hypothesis. Additional explanations may be that other cells, such as CD4+ T cells, are participating in CTL priming and the priming of these cells may be insufficient in the absence of B cells or when the particle is unable to engage naive B cells effectively. However, further experiments are needed to fully understand this.

In conclusion, endogenously produced HBcAg primes HBcAg-specific CTLs effectively at a high precursor frequency independently of the presence of B cells. However, binding of particulate HBcAg by naive B cells is pivotal in priming HBcAg-specific CTLs using exogenous HBcAg. These findings have clear implications for vaccine design using recombinant HBcAg particles and suggest that exogenous HBcAg released from infected hepatocytes could possibly regulate the CTL response through B cells.

**ACKNOWLEDGEMENTS**

The study was supported by grant #3825-B99-04XAC from the Cancer Foundation.

**REFERENCES**


