Factors affecting the presentation of exogenous Hepatitis B virus core antigen

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Hepatitis B virus core antigen (HBcAg) plays a critical role in terminating acute Hepatitis B virus infection and may be used as a potential vaccine candidate. The cell surface major histocompatibility complex (MHC) class 1 molecules are thought to be involved in the presentation of HBcAg. Surface MHC class 1 HLA A2 heavy chain (HC) and trimeric molecules were characterized on transfected Hela cells used as antigen presenting cells (APC) for the presentation of HBcAg. The results show that antibodies against HC HLA A2 and trimeric HLA-A2 molecules resulted in increased activation of HBcAg 18-27 minimal peptide specific cytotoxic T lymphocytes (CTLs), while the addition of exogenous β2-microglobulin decreased the activation of HBcAg specific CTLs. Further, specific CD8+ T cells were activated only when Hela cells as APCs were primed with HBcAg (peptide, soluble or embedded on virosomes) at pH 6.5.

Keywords: Hela, Hepatitis B virus, MHC, TAP, Virosomes

Hepatitis B virus (HBV) has infected 350 million people worldwide with 1 million deaths annually1. While the available vaccine comprising of the Hepatitis B surface antigen (HBsAg) provides protection against the development of chronic Hepatitis B infection, it cannot be used as a prophylactic vaccine2. An obstacle to the development of an efficacious vaccine in chronic Hepatitis B is the weakened immune response against Hepatitis antigens by T cells of infected patients3. The development of an efficacious vaccine requires the induction of specific CD8+ cytotoxic T cells3-6.

Professional antigen presenting cells (APC), in particular dendritic cells process cell associated antigens for presentation by major histocompatibility complex (MHC) class 1 molecules7. CD8+ cytotoxic T lymphocytes (CTL) are cross-primed during viral and bacterial infection and by many vaccination approaches8. Processing and presentation to MHC class 1 restricted CTL has been exploited for the development of vaccines against intracellular infections9,10. In Acute Hepatitis B infection, class-1 restricted CTL response to HBV core antigens (HBcAg) has been detected in patients who cleared the infection successfully unlike in chronic HBV infection3. Thus the HBV core antigen plays a critical role in terminating HBV infection and can be used as a potential vaccine candidate11.

Exogenous antigens in soluble form, as immune complexes, protein coated beads or embedded on virosomes can be used as carrier molecules to facilitate endocytosis and/macropinocytosis12-16. MHC class 1 presentation of exogenous antigen by APCs occurs through at least two distinct pathways12-15,17. In the cytosol antigens are degraded by the proteosome and transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) for presentation by MHC class 1 molecules12-15. Alternatively, antigens can be processed to oligopeptides in endolysosomal compartments where they bind to recycling MHC class 1 molecules and are transported to the cell surface for presentation16.

TAP independent cross presentation of HBV surface antigen (HBsAg) has been shown in mouse18. Cell surface MHC class 1 heavy chain (HC) do not bind exogenous β2-microglobulin involved in the presentation of HBsAg in mouse unless internalized18,19. This HC surface subset of mouse Ld molecules was recognized by the mab 64-3-718,19. The mab 64-3-7 binds to the sequence of amino acids including residues 48Q and 50P present on the hinge connecting the β-strand with the α1 helix above the ligand binding site in Ld molecules, a conserved structure across class 1 molecules18-20. MHC HLA A2 HC molecules with this epitope, when expressed on

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the surface of transfected cells were recognized by the monoclonal antibody 64.3.7.

It is hypothesized that cell surface MHC class 1 HC molecules play a role in the presentation of exogenous antigen. In this study the role of HC and trimeric HLA-A2 were analyzed for the presentation of HBCAg using HLA A2 containing the mouse Ld epitope and wild type MHC class 1 HLA A2 molecule transfected into Hela and C1R cells. The study demonstrates that cell surface MHC class 1 heavy chain molecules do not bind HBCAg minimal peptide 18-27 but has to be internalized to form the functional trimeric complex.

Analysis of the factors affecting the presentation of HBCAg by Hela and C1R cells show the importance of pH for the activation of specific CTLs during the presentation of exogenous molecules to specific protective CD8\(^+\) T lymphocytes.

**Materials and Methods**

**Culture medium and reagents**—Culture medium RPMI 1640 and modified DMEM (GIBCO, BRL) was supplemented with 1mM HEPES (GIBCO, BRL) and 5% bovine calf serum (GIBCO, BRL). β2-microglobulin, dimethy amiloride, brefeldin A, hygromycin B, G418 and interferon-γ were purchased from Sigma. Anti-human CD8, OKT8, anti-free HLA-A2 antibody 64.3.7 and anti trimeric HLA-A2 antibody BB7.2 stocks, pCDNA3.1 encoding altered HLA-A2, the free HC was detectable using mab 64-3-antibody BB7.2 stocks, pCDNA3.1 encoding altered HLA-A2 antibody 64.3.7 and anti trimeric HLA-A2 microglobulin, dimethy amiloride, brefeldin A, hygromycin B, G418 and interferon-γ microbeads (Miltenyl Biotech, according to manufacturers' instructions) using bb7.2 at 10 μg/ml to select for transfected cells. Cells were frozen in FBS containing 10% DMSO and stored in liquid nitrogen.

**FACS analysis of transfected cells**—Appropriate number of cells was washed in PBS and stained for MHC- class 1 trimeric and HC HLA A2 molecules with monoclonal antibodies b.b.7.2 and 64.3.7 (10μg/ml) respectively by incubation at 4°C for 45 min. Secondary antibody used was FITC or PE conjugated anti-mouse antibody (10 μg/ml). The cells were washed twice in FACS buffer (1% FBS, 0.01% NaN₃ in PBS) and analyzed by FACS. Dead cells were excluded by using 2-amino actinomycin D.

**Scatchard analysis of HC and trimeric HLA A2 molecule**—Total binding was calculated by incubating 50μl of cells (4.0×10⁶ cells/ml) with 50μl of labeled antibody (1.28 to 75nM) for 2 hr on ice. The cells were further incubated with 100 fold excess of cold antibody for 2 hr on ice. Non-specific binding was calculated using mock transfected Hela cells incubated with labeled antibody. The cells were washed thrice with 1% FBS in PBS and counted using a γ-counter. Analysis was done using GraphPad software, Prism 4.

**Western blot analysis of transfectants**—Cells (50×10⁶) were re-suspended in 1ml of lysis buffer (1% Triton x-100, 50 mM Tris HCl, pH 7.6, 300 mM NaCl, 1mM benzamidine, 0.04 mg/ml DNAse) and vortexed for 3 sec at medium speed followed by incubation on ice for 30 min. The cell debris was removed at 19,000 g and the supernatant carefully transferred to a fresh tube. The supernatant was incubated with 4 μg of antibody (b.b.7.2 and/ 64.3.7 / control antibody DC-SIGN 117) and 30 μl of 50% bead slurry (γ bind™ plus Sepharose) on ice for 30 min. The beads were washed twice in lysis buffer and re-suspended in 20 μl 1× SDS-PAGE sample buffer. The samples were boiled and run on a 7.5% SDS-PAGE gel. After electrophoresis, transfer was done using Hybond ECL (Amersham, according to manufacturers’ instructions). Non-specific sites were blocked by immersing the membrane in 5% skimmed milk, 0.1% Tween 20 in TBS (20 mM Tris HCl, pH 7.6; 150 mM NaCl). First antibody used for detection of HLA A2 molecules was R5996 (anti α
chain antibody at 1:25000 dilutions). The membrane was washed twice in 0.1% Tween TBS and incubated with the second antibody, goat anti-rabbit HRP antibody (1:50000 dilution) for 45 min on a shaker at room temperature. The membrane was washed twice for 5 min and analyzed by chemiluminescence. Detection of β2-microglobulin was using rabbit anti β2-microglobulin (Dako) at 1:1000 dilution.

**Intracellular cytokine staining**—Hela (pretreated with IFN-α at 500 units/ml) and C1R transfected cells (pretreated with IFN-α at 500 units/ml) used as APCs were incubated with peptide at 1 μM for 30 min at 37°C in 1X DMEM medium adjusted to pH 6.5. The cells were washed and incubated with approximately 10,000 CTLs for 4 hr at 37°C in presence of 10μg/ml BFA. The cells were stained with anti-human CD8, OKT8 (10 μg/ml in 1% FBS PBS) for 10 min at room temperature. The secondary antibody used was goat anti-mouse PE conjugated antibody at room temperature for 10 min. The cells were washed and 100 μl of cytofix/cytoperm (BD) was added to each well and incubated on ice for 30 min. The cells were washed and FITC conjugated anti-human IFN-γ antibody (BD) was added at 2.5 μg/ml and incubated at room temperature for 30 min. The cells were washed twice in 1% FBS PBS and re-suspended in 50μl PBS for analysis by FACS. Priming of Hela with peptide 1-124 (soluble) and peptides 1-144 and 1-183 embedded on virosomes to CTLs was for 18 hr in presence of BFA (5 μg/ml).

**Results**

**Expression of MHC class IHC and trimeric HLA A2 molecules**—Hela and C1R cells both MHC class 1 HLA A2 negative, were transfected with vector pCDNA 3.1 expressing native type HLA A2 (w) or mutated HLA A2 (m). The altered HLA A2 gene contained the epitope recognized by mab 64.3.7 when present as free HC molecules.

The number of HC and trimeric HLA A2 molecules in Hela A2 mutant (Hela A2m) cells were estimated by saturation binding assays. The Kd and Bmax values were calculated using non-linear regression with a one site binding equation. The Bmax obtained for the HC and trimeric HLA A2 molecules were 126730 and 970481 respectively with standard errors less than 9% (Fig. 1). By scatchard analysis the Kd values were similar for the antibody b.b.7.2 (an antibody that binds the trimeric HLA A2/β2-microglobulin and peptide complex) binding to both the altered and native HLA A2 trimeric molecules at 15.75 nM and 16.6 nM respectively (Fig. 1a,c).

**Cell surface free HLA-A2 HC molecules do not bind β2-microglobulin**—When Hela A2m cells were immunoprecipitated from total cell lysates of Hela A2m cells with mab 64.3.7, a specific band was detected at 48 kDa implying a small proportion of HC (Fig. 2a: lanes 4-6) when compared to Hela A2w cells (Fig. 2: lanes 7-9). As shown mab 64.3.7 could not detect native HLA A2 HC (Fig. 2: lane 7). Similarly, by immunoprecipitation β2-microglobulin was detected in Hela A2m and Hela A2w cells immunoprecipitated with trimer specific antibody b.b.7.2 (Fig. 2b: lanes 5 & 6 and 8 & 9). β2-microglobulin was

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**Fig. 1**—Estimation of HC and trimeric HLA A2 molecules by saturation binding assay: Hela A2m and Hela A2w cells were incubated with radioactive 125I-b.b.7.2 or 125I-64.3.7 followed by incubation with unlabelled antibody to determine total binding and non-specific binding as described in Methods. (a) scatchard plot of Hela A2m cells with mab b.b.7.2; (b) scatchard plot of Hela A2m cells with mab 64.3.7; (c) scatchard plot of Hela A2w cells with mab b.b.7.2. Analysis was done using GraphPad software Prism 4, the standard errors (SE) are shown.
absent when precipitated with HLA A2 HC specific mab 64.3.7 (Fig. 2b: lanes 4,7). In Hela control cells neither HLA A2 nor β2-microglobulin was detectable (Fig. 2a,b: lanes 1-3).

**Activation of cytotoxic T lymphocytes by C1R A2w and C1R A2m cells**—Activation of cytotoxic CD8+ T cells was assessed by the production of intracellular IFN-γ. Presentation of HBV core antigen by C1R A2m cells was very inefficient with low levels of IFN-γ production when incubated with the peptide 1-124 or peptides 1-144 (V 0047) and 1-183 (V 0048) embedded on virosomes unlike in C1R A2w cells (Fig. 3a). Lowering the pH of the media did not affect the presentation of the peptide as no activation of CTLs was observed (data not shown).

**Activation of cytotoxic T lymphocytes by Hela A2m and A2w cells**—Presentation of HBcAg peptide 1-124 by Hela A2m and Hela A2w cells showed 1 and 11% intracellular IFN-γ production respectively (Fig. 3b). The lower activation seen by Hela A2m cells was at the same level as C1R A2w transfected cells (Fig. 3a,b). However, activation of CTLs by Hela cells was observed only when Hela cells were pulsed with antigen at pH 6.5 (data not shown). Presentation of HBcAg embedded on virosomes was observed in Hela A2m and Hela A2w cells (Fig. 3c) but not in C1R A2m cells (Fig. 3a). Virome preparation V 0047 containing HBcAg 1-144 showed higher activation of CTLs than virome V 0048 (peptide 1-183).

**Factors affecting activation of CD8+ cytotoxic T lymphocytes**—When Hela A2m cells were pulsed with HBcAg 18-27 minimal peptide in presence of HLA A2 specific trimer and HC specific antibodies b.b.7.2 and 64.3.7 respectively, activation of CTLs increased 3-fold and 0.5-fold respectively (Fig. 4a). A
similar increase in IFN-γ production was observed in
Hela A2m cells pulsed with peptide and MHC class 1
HLA A2 specific antibodies b.b.7.2 and 64.3.7 (Fig.
4a).

When Hela A2m cells were pulsed with the HbcAg
minimal peptide in presence of exogenous β2-
microglobulin there was a decrease in activation of
CTLs (Fig. 4b). If Hela A2m cells were pulsed with
the minimal peptide in presence of exogenous β2-
microglobulin with either mab bb7.2 or 64.3.7, a
proportional decrease in IFN-γ production was
observed demonstrating that exogenous β2-
microglobulin inhibited the presentation of HbcAg
and subsequent activation of specific CTLs.

Discussion

Chronic Hepatitis B infection—The development of
an efficacious vaccine against chronic Hepatitis
infection requires the induction of specific CD8+
cytotoxic T cells. In acute Hepatitis B infection a
MHC class I restricted cytotoxic T cell response to
HbcAg has been detected in patients who cleared
the infection unlike in chronic Hepatitis B virus infection
and can be used as a potential vaccine candidate.
Hepatitis B core antigen used as a DNA vaccine for
immunization of Balb/c mice induced strong humoral
and cellular responses. In C57BL/6 mice an increase
in CD8+ activation accessed by CTL cytolysis and IFN-
γ secretion was seen when a DNA vaccine of HbcAg
minimal peptide 18-27 was fused with an ER targeting
sequence. Further, endogenous HbcAg was able to
induce a specific CTL response in both wild and B cell
deficient mice while exogenous HbcAg induced a
specific CTL response only in wild type mice implying
a possible presentation of exogenous HbcAg by B
cells.

MHC class 1 presentation of Hepatitis B specific
antigens—Cross presentation consists of the loading
onto MHC class I molecules of peptides derived from
endocytosed exogenous antigen by antigen presenting
cells. There are various pathways by which antigen
can be presented to cytotoxic CD8+ T cells.
Conventional TAP dependent MHC class I peptide
loading occurs in the ER. Processing of exogenous HBsAg
occurs through cell surface-associated MHC class I Ld HC molecules. Processing of exogenous Hepatitis B surface antigen (HBsAg)
VLP occurs in endolysosomal compartments, where
antigens bind MHC class I Ld molecules and
transported to the cell surface in a TAP independent
manner for presentation to cytotoxic CD8+ T
lymphocytes.

MHC class 1 HC exists in different proportion on
different cell types which is sensitive to the levels of
cytokines, presence or absence of serum and peptide.
In presence of IFN-α and IFN-γ the level of cell
surface HC increases more than 100% as seen in Hela
cells and to approximately 4% in C1R cells (data not
shown). While in a dendritic like cell line (KG-1), there
was no effect of cytokines on the levels of cell surface
HC/trimeric HLA A2 complexes (unpublished data).
Hence for the presentation of an exogenous antigen
involving recycling MHC class I molecules an
appropriate APC should be selected.

Endocytosis of antigen using various carrier
systems—Exogenous antigens in soluble form, as
immune complexes, opsonised on beads or embedded
on virosomes can be used for uptake of antigen by

Fig. 4—Activation of CTLs by Hela A2m cells preincubated with antibodies b.b.7.2 or 64.3.7 with/without β2-microglobulin: (a) Hela A2m and Hela A2w cells were incubated with 1 μM HbcAg peptide [p] and mab b.b.7.2 [p+bb72] or 64.3.7 [p+6437] at 10 μg/ml for 30 min at 37°C. (b) Hela A2m cells were incubated with
and without β2-microglobulin [β2] at 6 μg/ml [p+β2] with/without antibody as indicated for 30 min at 37°C. Hela A2m primed with
HbcAg peptide without antibody was used as a control as shown
for both (a) and (b). The cells were washed and incubated with
approximately 10,000 CTLs for 4 hr at 37°C in the presence of 10
μg/ml BFA and analyzed by intracellular staining of IFN-γ, as
described under Materials and Methods.
endocytosis and/macropinocytosis. Stimulating macropinocytosis by phorbol esters increased presentation by dendritic cells, similarly as shown, presentation by Hela cells is higher at an acidic pH. It has been shown that ovalbumin coupled to latex beads accessed the alternative MHC class 1 pathway in dendritic cells and elicited a specific cytotoxic T cell response. Similarly particle associated antigen have an increased efficiency at being presented by the alternative MHC class I pathway to cytotoxic T cells. Virosomes have been used successfully in the delivery of Hepatitis A and Influenza vaccine. In this article antigen presentation by Hela cells, an epithelial cell line using virosomes was higher compared to C1R cells (a B cell line) an APC, which could be due to the higher endocytic capacity of the Hela cell. Since endocytosis was the preferred mode of internalization by Hela cells, the activation of specific CTLs by soluble HBCag was higher than HbcAg embedded on virosomes. Hence the efficiency of presentation of an exogenous antigen is dependent on the antigen presenting cell.

Effect of acidic conditions on the presentation of exogenous antigen—Presentation of HBCag to CTLs was more efficient by Hela cells an epithelial carcinoma cell line, at a low pH of 6.5. Hela cells have a 3-fold higher endocytic and/macropinocytosis activity of FITC-dextrane than C1R cells (data not shown) and KG-1 cells (unpublished data). This perhaps accounts for the higher level of activation of CTLs observed by Hela cells at low pH. Processing of the long peptides at neutral pH by Hela and C1R HLA A2 transfectants did not activate HBCag specific CTLs (unpublished data). Although acidic conditions destabilize the cell surface MHC class 1 molecule, it has been known to facilitate peptide exchange.

Effect of antibody and β-2 microglobulin on presentation of HBCag—Hela cells pulsed with the minimal HBCag 18-27 peptide in presence of antibody specific for HLA HC and trimeric HLA A2 molecules showed a significant increase in CTL activation (Fig. 5a). The activation by antibody specific for the trimer complex was higher than when HLA HC specific antibody was used. This was presumably due to an increase in concentration of MHC class 1 molecules HLA A2 molecules and β2-microglobulin. Thus implying the binding of the HBCag peptide to the MHC class 1 molecules occurs in a sub-cellular compartment. Further, presentation of HBCag minimal peptide by C1R A2w cells was not affected by Brefeldin A, an inhibitor of the ER Golgi traffic (data not shown). Presentation of HBCag does not require newly synthesized MHC class 1 molecules or transport from the ER. While dimethylamiloride an inhibitor of macropinocytosis causes a reduction in activation of CTLs by C1Rw cells (data not shown). Hence presentation of HBCag does not follow the conventional MHC class-1 presentation pathway but occurs through an ER independent pathway.

In mouse, addition of exogenous β2-microglobulin increased cross presentation of HBsAg. A decrease in the presentation of minimal HBCag peptide 18-27 by Hela/C1R cells was observed in presence of β2-microglobulin. This was not surprising as it is known that exogenous β2-microglobulin on monocyte derived dendritic cells down-regulates MHC class I molecules, inactivates the Raf/MEK/ERK cascade and results in a diminished ability to activate T cells. Hence the ability of β2-microglobulin to enhance or decrease activation of specific CD8+ T cells is dependent on the exogenous antigen.

In conclusion, the data presented clearly demonstrates that (i) presentation of HBCag is independent of the conventional MHC class 1 TAP dependent pathway and is presented through an alternative TAP independent MHC class 1 pathway and (ii) a change in the pH increases the presentation of an immunodominant epitope i.e., the presentation of HBCag long peptide (whether soluble form or embedded on virosomes). Further, an increase in CTL activation in the presence of MHC class 1 specific antibody was observed indicating an increased endocytosis of the cell surface MHC class 1 molecules. These observations may be important in the design of preventive/prophylactic vaccine reagents against infectious diseases.

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