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Modulation of the immunogenicity of virus-like particles composed of mutant hepatitis B virus envelope subunits

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ABSTRACT

Virus-like particles (VLPs) are non-infectious subviral protein complexes, which possess structural features identical or closely related to infectious virions. They are utilized as delivery tools for immunologically relevant antigenic sequences. In order to investigate whether mutant subunits can modulate the VLP immunogenicity, comparative immunization studies with wild-type and non-native VLPs were performed. To determine whether disulfide bonding impacts on the immunogenicity of hepatitis B virus envelope proteins (HBsAg), mutant HBsAg subunits with single, double and triple cysteine residue substitutions were generated. The mutant proteins were expressed in cell culture, secretion competent non-native VLPs generated, followed by immunization studies in mice to measure the cellular immune response. The reduced ability of mutant HBsAg proteins to form disulfide bonds does not interfere with their ability to assemble into secretion competent VLPs. Depending on specific cysteine to alanine changes, VLPs could be generated with or without an increased ratio of monomeric versus dimeric/oligomeric subunits compared to wild-type VLPs. The utilization of non-native VLPs resulted in enhanced cellular immune responses and does not seem to depend on the ratio between monomeric or dimeric/oligomeric subunits. Comparative immunization studies strongly indicate that changes in the disulfide bonding modulate the VLP immunogenicity most likely due to structural changes. We hypothesize that structural features have evolved with reduced immunogenicity to evade the constraints imposed by the immune system. Altering VLP conformation may represent an attractive strategy to modulate antigen processing resulting in an enhanced immune response and/or a changed hierarchy of epitope presentation.

1. Introduction

Hepatitis B viruses comprise a group of small, enveloped, partially double-stranded DNA viruses replicating via reverse transcription. The human hepatitis B virus (HBV) is a major pathogen of global importance that causes acute and chronic infections. The genome contains four open reading frames encoding core (capsid), surface (envelope), polymerase and X proteins. The DNA containing nucleocapsid is packaged by a lipoprotein envelope containing three related transmembrane proteins (Liang, 2009). The synthesis of the envelope proteins is initiated at three different in-frame translation start sites resulting in the expression of HBsAg-large (HBsAgL), HBsAg-middle (HBsAgM) and HBsAg-small (HBsAgS), which have the S-domain in common. HBsAgS is composed only of the S-domain consisting of 226 amino acids (aa); HBsAgM and HBsAgL contain additional N-terminal extensions, the preS2 and preS1 domains (Bruss, 2007; Liang, 2009). The ability of HBsAgS to self-assemble in the presence of lipid results in the formation of secretion competent, highly compact virus-like particles (VLPs) with a size of 22–25 nm in diameter. The first step in the morphogenesis of the HBsAgS VLPs is the cotranslational insertion of the protein into the membrane of the endoplasmic reticulum with a short luminal exposed N-terminal sequence, two transmembrane regions separated by a 57 aa cytosolic loop, a luminal external 70 aa domain containing the major B-cell epitopes ('a'-determinant) and a hydrophobic C-terminal end of 56 aa. Each HBsAgS subunit contains 14 cysteine residues, which contribute to the formation of intramolecular and intermolecular disulfide bonds (Bruss, 2007; Mangold et al., 1997). Most of the HBsAgS cysteine residues and disulfide bonds are not essential for generating the structural features involved in VLP formation (Mangold et al., 1995, 1997; Mangold and Streeck, 1993). Specific cysteine to alanine changes can generate VLPs containing HBsAgS subunits with a reduced ability to form dimeric or oligomeric subunits (Mangold et al., 1995,

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1997; Wunderlich and Bruss, 1996) indicating that the HBsAgS assembly requirements can tolerate disruptions of disulfide bonds without compromising particle formation. HBsAgS VLPs are the sole antigenic components of a widely used protective vaccine with the ability to induce neutralizing anti-HBV antibodies, and therefore indicating that antigenicity and structural features are shared with the infectious virion.

VLPs in general represent also an effective vaccine modality for the delivery of immunologically relevant antigenic sequences. Both antibody and cellular immune responses can be induced by different types of VLPs, which are distinguished by size, structure and the presence or absence of lipids (Buonaguro et al., 2002; Jeong et al., 2004; Leggatt and Frazer, 2007). The application of VLPs to present antigens to the immune system offers a great promise for the treatment of various infectious diseases and cancer (Ludwig and Wagner, 2007; Ramqvist et al., 2007). In addition to being an essential vaccine component, HBsAgS VLPs hold great potential as delivery platforms for foreign epitopes or protein domains (Agnandji et al., 2010; Cheong et al., 2009; Delpeyroux et al., 1986; Gonzalez et al., 2009; Patient et al., 2009; Phogat et al., 2008; Vietheer et al., 2007). Based on the importance of VLPs as medical tools and platforms for presentation of native viral structures, it is critical to understand their immunogenicity in relation to antigen structure in order to enhance their immunogenic potential.

VLPs are delivered as exogenous antigens and taken up by antigen-presenting cells, followed by proteolytic processing to peptides in an endosomal–lysosomal compartment. The peptides are subsequently loaded onto major histocompatibility complex (MHC) class II molecules, or onto MHC class I molecules after cross-presentation events (Lin et al., 2008). Conformational flexibility of protein regions seems to be an important factor for proteolytic sensitivity where local instability can increase immunogenicity in adjacent sequences (Carmicle et al., 2007; Dai et al., 2002; Landry, 2008). Studies have shown that subtle changes in antigen structure can modulate helper T cell responses due to qualitative and quantitative differences in protein processing (Li et al., 2002; Mirano-Bascos et al., 2010). Disulfide bonding can regulate antigen processing and epitope selection by changing the conformational flexibility (Li et al., 2002). Distinct helper T cell epitope profiles emerged from human immunodeficiency virus type 1 (HIV-1) gp120 molecules after destabilizing the three-dimensional structure as a consequence of deleted cysteine residues (Mirano-Bascos et al., 2010). Antigen three-dimensional structure guides processing and presentation of helper T cell epitopes (Carmicle et al., 2007). Similarly, Prato et al. (2006) showed that the immune response specific for a defined malaria-specific cytotoxic T lymphocyte (CTL) epitope is conformation-dependent. *In vitro* studies showed that a malaria antigen with two disulfide bridges induced a stronger CTL recognition in its reduced form compared to the oxidized form. The immunization outcomes obtained with modified immunogens in mice is possibly relevant to the human system as "hot spots" of epitopes are shared between mouse and human (Sealy et al., 2008; Surman et al., 2001; Zhan et al., 2007) and are likely the result of antigen degradation by similar intracellular enzymes and processing pathways (Landry 2008; Sealy et al., 2008).

In order to determine whether the immunogenicity of native viral structures can be enhanced to design potent immunization tools with increased effectiveness, cysteine residues within HBsAgS subunits were mutated, and comparative immunization studies with wild-type and non-native VLPs were performed. We hypothesize that destabilized conformational structures may permit the induction of enhanced and/or modulated immune responses targeting different epitope profiles.

2. Materials and methods

2.1. In vitro site-directed mutagenesis (SDM)

Expression plasmids based on the pCI vector (Promega, Madison, WI) were constructed encoding for HBsAgS proteins (genotype D, serotype ayw) with cysteine to alanine changes at the amino acid positions 121 and 147. The plasmids p121-HBsAgS, p147-HBsAgS, p121/147-HBsAgS and p121/124/147-HBsAgS encode HBsAgS proteins with single, double or triple mutations at the indicated positions. SDM was carried out with pairs of complementary primers introducing the cysteine to alanine changes in the HBsAgS open reading frame (ORF) under the following conditions: *Pfu* DNA polymerase (1 μl, 10 U/μl) in the recommended buffer (Promega), 200 μM each dNTP, 0.4 μM primer pair, and 100 ng template in a total volume of 50 μl. The extension reaction was initiated by a pre-heating step at 95 °C for 2 min, then followed by 18 cycles with each cycle 95 °C for 50 s, 62 °C for 1 min, and 68 °C for 15 min, then followed by a final step at 68 °C for 7 min. The reaction sample was treated with 1 μl of *Dpn*I (10 U/μl) restriction enzyme (Promega) for 1 h at 37 °C, then the DNA products used for transformation of *Escherichia coli* DH5α cells. Colonies were grown in the presence of ampicillin (100 μg/ml) on Luria–Bertani agar plates, plasmids were isolated and verified by sequencing.

2.2. Cell lines

HEK293-T cell line was grown in Dulbecco's modified Eagle's medium, DMEM (Gibco-BRL, Grand Island, NY) supplemented with GlutaMax-1 (Gibco-BRL), 10% fetal calf serum (FCS), penicillin and streptomycin (Gibco-BRL). For production of VLPs, HEK293-T cells were transfected using polyethylenimine (MW 25000, linear) (PEI) (Polysciences, Warrington, PA). After transfection, the VLP-producing cells were kept in FCS-free HEK293 medium (Gibco-BRL) for 5 days, then the VLPs harvested.

2.3. Purification and quantitation of VLPs

Tissue culture supernatant was collected 5 days after transfection, centrifuged at 3,500 rpm for 15 min in a benchtop centrifuge (Labofuge 400, Heraeus, Hanau, Germany), the supernatant (25–28 ml) was transferred to an ultracentrifuge tube (Sorvall, Newtown, CT), and underlaid with a 5 ml 20% sucrose cushion (20% sucrose in STE buffer, 100 mM NaCl, 10 mM Tris (pH8), 1 mM EDTA). Particles were pelleted by ultracentrifugation at 25,000 rpm for 16 h at 4 °C in a Sorvall AH629 rotor (Sorvall). The supernatant was discarded, and the pelleted VLPs resuspended in HEPES/NaCl buffer for vaccination purposes. The presence of HBsAgS was assessed using Monolisa Ultra assay according to the manufacturer's instructions (BioRad, Hercules, CA).

2.4. Gradient analysis and electron microscopy (EM)

Partially purified VLPs were further purified through a 10–40% (w/v) sucrose gradient in STE for EM analysis or through a 10–40% (w/v) cesium chloride (CsCl) gradient for density measurement. Fractions were collected and quantitated using a HBsAg-specific enzyme immunosorbent assay (EIA) (Monolisa, BioRad). The refractive index of each fraction was quantitated using an Abbe refractometer NAR-1T (Atago, Tokyo, Japan). Fractions containing HBsAgS at a sucrose density of approximately 1.2 g/ml were dialyzed against phosphate buffered saline (PBS). The purified particles were concentrated to approximately 500 ng/μl. Ten microliters of sample was applied to a carbon grid,

blotted and negatively stained with phosphotungstate acid (PTA). Images were analyzed on a Hitachi H7500 electron microscope (Tokyo, Japan) operating at 120 keV.

2.5. Immunoprecipitation and gel electrophoresis

Cells were seeded into 6-well plates at 5×10^5 cells/ml and transfected using PEI (Polysciences) 2 days prior to the isotopic labeling. Cells were aspirated and incubated for 40 min in 1.5 ml of methionine-free minimal essential medium supplemented with 10% FCS. Two hundred μCi of ^{35}S-labeled cysteine/methionine was then added to the media and incubated for 3 h, washed twice in PBS and incubated with 2 ml of supplemented DMEM for 18 h. Supernatant was collected and iodoacetamide was added to a final concentration of 20 mM followed by incubation with rabbit anti-HBsAg antibodies (Biodesign, Saco, ME) (final dilution 1:100) for 2 h on ice, followed by 1 h incubation with 20 μl protein A Sepharose CL-4B (GE Healthcare, Piscataway, NJ) at 4 °C with rotation. The mixture was washed three times in RIPA buffer (10 mM Tris–HCl (pH7.5), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and once with 0.1 M Tris–HCl, pH6.8. Samples were prepared for SDS-15% polyacrylamide gel electrophoresis (PAGE) by adding loading buffer (250 mM Tris–HCl, pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol) in the presence or absence of 20% 2-mercaptoethanol, then boiled for 5 min. After electrophoresis, the gel was destained in 7.5% acetic acid, 5% methanol and dried before exposure to a BioMax film (Kodak, Rochester, NY) or a phosphoimager (Molecular Dynamics Inc., Sunnyvale, CA).

2.6. Immunoblot

For the detection of the large hepatitis delta antigen (dAgL), supernatant and cell lysate were collected from human hepatoma cells (HuH-7) 5 days after transfection. Supernatant was concentrated by ultracentrifugation for 4 h at 39,000 rpm hours at 4 °C in a Sorvall TH641 rotor (Sorvall) over a 20% sucrose cushion in STE. Pellet and cell lysate were then resuspended in loading buffer in the presence of 2-mercaptoethanol, then separated on a 15% SDS PAGE. After transfer onto a Hybond-P membrane (Amersham Biosciences, Piscataway, NJ), the membrane was blocked with 5% skim milk powder in water, then incubated with a patient anti-delta antigen serum (1:1000). After incubation, the membrane was washed twice in PBS-Tween (0.1%), twice with PBS and then incubated with a rabbit anti-human immunoglobulin G antibody conjugated to horseradish peroxidase (1:1000) (Dako, Glostrup, Denmark). The membrane was then washed and incubated with ECL Plus Western blotting detection substrate (GE Healthcare), exposed to a Amersham Hyperfilm ECL (GE Healthcare).

2.7. Animals

BALB/c mice were used at 6–10 weeks of age. Within a given experiment, the mice were age- and sex-matched. Mice were housed under specific pathogen-free conditions. Groups of four mice were immunized subcutaneously at the base of the tail with 20 μg wild-type or recombinant HBsAgS VLPs. Ten days after injection, spleens were collected, and used for *in vitro* restimulation in CTL and Elispot assays.

2.8. Interferon (IFN) γ Elispot assay

To measure the IFNγ levels in mouse splenocyte cultures, MAIPS4510 plates (Millipore, Billerica, MA) were coated with 50 μl/well (8 μg/ml) IFNγ-specific capture monoclonal antibody (mAb) (BD Pharmingen, San Diego, CA) and incubated overnight at 4 °C. Splenocytes (10^6/50 μl) were then added to each well and stimulated in the presence of 40 μg/ml of cognate peptide or in the absence of peptide. As positive control, cells were incubated in medium containing 2 μg/ml concanavalin A (ConA) (Sigma, St. Louis, MO). Plates were cultured for 16–17 h at 37 °C, then incubated with biotinylated anti-cytokine mAb (BD Pharmingen), followed by ExtraAvidin Alkaline Phosphatase conjugates (BD Pharmingen). The presence of IFNγ-producing cells was visualized using Fast TM BCIP/NBT conjugate kit (Sigma). Plates were dried and spots were quantitated with AID-Elispot Reader System (Autoimmun Diagnostika GmbH, Straßberg, Germany).

2.9. CTL assay

For *in vitro* restimulation, the splenocyte suspension was adjusted to 5×10^6/ml and transferred into T25 or T75 cell culture flasks depending on the total cell number available, then cultured with a mixture of H-2^d restricted peptides (Sette et al., 2001) at a final concentration of 1 μg/ml each (PQS-29 [NH2-PQSLDSWWTSL-H, L^d-restricted], WYW-199 [NH2-WYWGPSLYSI-H, K^d-restricted] and WGP-201 [NH2-WGPSLYSIL-H, D^d-restricted]) for 5 days at 37 °C by maintaining a CO_2 level of 5%. The mastocytoma P815 cell line (ATCC TIB-64) displaying the peptide mixture was utilized as target cells for H-2^d restricted CTL lysis. The cells were maintained in supplemented RPMI 1640 medium (JRH Biosciences, Lenexa, KS) in the presence of 400 μg/ml G418 (Gibco-BRL). Target cells (1.5×10^6) were sensitized at 37 °C for 1½ hour with 1 μg/ml cognate peptide or incubated in the absence of peptide, and labeled with 100 μCi ^{51}Cr (MP Biomedicals, Irvine, CA). *In vitro* stimulated effector cells (splenocytes) were added at various effector to target (E:T) ratios in triplicate in 96 well microtiter plates (Nunc, Roskilde, Denmark). Controls included target cells but no effector cells (minimum ^{51}Cr release) and target cells incubated with 10% SDS (maximum ^{51}Cr release). A volume of 100 μl of supernatant was collected from each well and transferred into disposable 1.2 ml microtiter tubes (QSP, Petaluma, CA). The tubes were wax-sealed for counting in the gamma counter 1470 Wizard (LKB Wallac, Turku, Finland). The percentage specific lysis was calculated as indicated by Woo et al. (2006).

2.10. Analysis of database

A total of 1556 DNA sequences encoding the overlapping HBV surface and polymerase genes were extracted from a large HBV database (SeqHepB) (Yuen et al., 2007), and were analyzed using BioEdit (Hall, 1999) and MS Excel. The sequences used in the analysis were not stratified, containing sequences of all HBV genotypes and variants that occur naturally and due to selection pressures such as antiviral drug therapy and complete hepatitis B immune globulin (HBIg). The occurrence of each amino acid at a particular codon was counted, the amino acid with the highest frequency was defined as the consensus, and the level of conservation of this consensus amino acid reported as a percentage. If mixed amino acids were detected at a particular codon and one of the amino acids was the same as the consensus, it was counted as a consensus amino acid. If a mixed population did not include the consensus amino acid, it was counted as non-consensus. For the analysis, the overlapping HBV polymerase sequence was included. The first two nucleotides in each surface codon correspond to the last two nucleotides in the overlapping polymerase codon (s1/p2 and s2/p3 or −1 frameshift). The last nucleotide of the surface codon corresponds to the first nucleotide in the next overlapping polymerase codon (s3/p1 or +2 frameshift, Table 1).

Table 1
Database analysis (SeqHepB) (Yuen et al., 2007) of in total 1556 DNA sequences encoding the overlapping HBV surface (envelope) and polymerase genes (reverse transcriptase, RT (rt) domain). The sequences were analyzed using BioEdit (Hall, 1999) and MS Excel. The first two nucleotides in each surface codon corresponding to the last two nucleotides in the overlapping RT codon (−1 frameshift, f.s.), the last nucleotide of the surface codon corresponds to the first nucleotide in the next overlapping RT codon (+2 frameshift). Color code: dark gray: 100% conserved, no variations detected, light gray:>99% conserved, aa substitutions can occur, White:<99% conserved. s: surface, aa: amino acid.

Surface protein			Reverse transcriptase			
Functional region	aa	Conservation	f.s.	aa	Conservation	Functional region
Internal loop	s48_C	≥99%	-1	rt56_V	100%	F-A interdomain
			+2	rt57_S	≥99%	
	s65_C	100%	-1	rt73_L	67%	
			+2	rt74_S	≥99%	
	s69_C	≥99%	-1	rt77_L	≥99%	A domain
			+2	rt78_S	≥99%	
	s76_C	95%	-1	rt84_V	≥99%	
			+2	rt85_S	≥99%	
Second transmembrane helix	s90_C	≥99%	-1	rt98_M	≥99%	A-B interdomain
			+2	rt99_P	100%	
Major hydrophilic region ('a' determinant)	s107_C	100%	-1	rt115_L	95%	
			+2	rt116_S	>99%	
	s121_C	100%	-1	rt129_M	94%	
			+2	rt130_Q	94%	
	s124_C	100%	-1	rt132_L	≥99%	
			+2	rt133_H	100%	
	s137_C	≥99%	-1	rt145_M	49%	
			+2	rt146_L	100%	
	s138_C	100%	-1	rt146_L	100%	
			+2	rt147_L	100%	
	s139_C	100%	-1	rt147_L	100%	
			+2	rt148_Y	≥99%	
	s147_C	100%	-1	rt155_L	100%	
			+2	rt156_H	100%	
	s149_C	100%	-1	rt157_L	≥99%	
			+2	rt158_Y	100%	
C-terminal hydrophobic region	s221_C	96%	-1	rt229_L	94%	C-D interdomain
			+2	rt230_S	≥99%	D domain

3. Results

3.1. Generation of VLPs comprised of different mutant HBsAgS subunits

To determine whether disulfide bonding impacts on the immunogenicity of HBsAgS VLPs, VLPs comprised of different mutant HBsAgS subunits were generated. Cysteine to alanine changes at HBsAgS amino acid (aa) positions 121, 124 or 147 (C121A, C124A, C147A) were introduced. The residues C121 and C147 were selected as they are involved in the formation of intermolecular disulfide bonds and hence, are essential for the formation of dimeric/oligomeric subunits (Mangold et al., 1997). The cysteine residue at position 124 is probably involved in the formation of intramolecular disulfide bonds and was selected as it blocks the accessibility of a trypsin cleavage site. Trypsin sensitivity after a cysteine 124 substitution is an indicator of structural changes (Mangold et al., 1997). The HBsAgS proteins with single cysteine mutations (C121A-HBsAgS and C147A-HBsAgS), double mutations (C121/147A-HBsAgS), or triple mutations (C121/124/147A-HBsAgS) were expressed in HEK293-T cells, and VLP formation was determined. The cell culture supernatant was harvested, concentrated and analyzed via CsCl density gradient centrifugation. HBsAgS activity in samples with wild-type (wt) and mutant proteins was detected in fractions with a density of approximately 1.21 g/ml, which is indicative of particle formation (Mangold et al., 1997; Dubois et al., 1980) (data not shown). Electron microscopy studies verified particle formation with particle sizes in the range of 22–25 nm (Fig. 1). To determine whether the VLPs are composed of monomeric and dimeric/oligomeric subunits, ^{35}S-labeled VLPs were immunoprecipitated and analyzed by SDS–PAGE analysis under reducing and non-reducing conditions. The reducing gel shows that the presence of the cysteine to alanine changes did not change the ratio of glycosylated and unglycosylated subunits in non-native VLPs compared to wt-VLPs (Fig 2A). However, the non-reducing gel revealed that the VLPs composed of C121/147A-HBsAgS contain an increased proportion of monomeric subunits at a ratio of monomers versus dimers/oligomers at approximately 1:1. In contrast, VLPs composed of mutant HBsAgS proteins with single cysteine to alanine substitutions did not contain an increased proportion of monomeric subunits (Fig. 2A), possibly, the unpaired cysteine residues generated intermolecular disulfide bonds with random cysteine partners. Consistently, possibly due to fortuitous intermolecular disulfide bonds, VLPs composed of C121/124/147A-HBsAgS with three cysteine to alanine residue changes contain a reduced proportion of monomeric subunits compared to C121/147A-VLPs. Beside the differences in the presence of monomeric versus dimeric/oligomeric subunits, the C124A mutation in HBsAgS results in structural differences indicated by an increased sensitivity to trypsin (Fig. 2B; Mangold et al., 1997). The functionality of the VLPs was tested by their ability to co-package and secrete large hepatitis delta antigen (dAgL) (Chang et al., 1991; Ryu et al., 1992). Wt or mutant HBsAgS proteins supported the release of dAgL into the cell culture medium with similar efficacy. The reduced ability of non-native HBsAgS

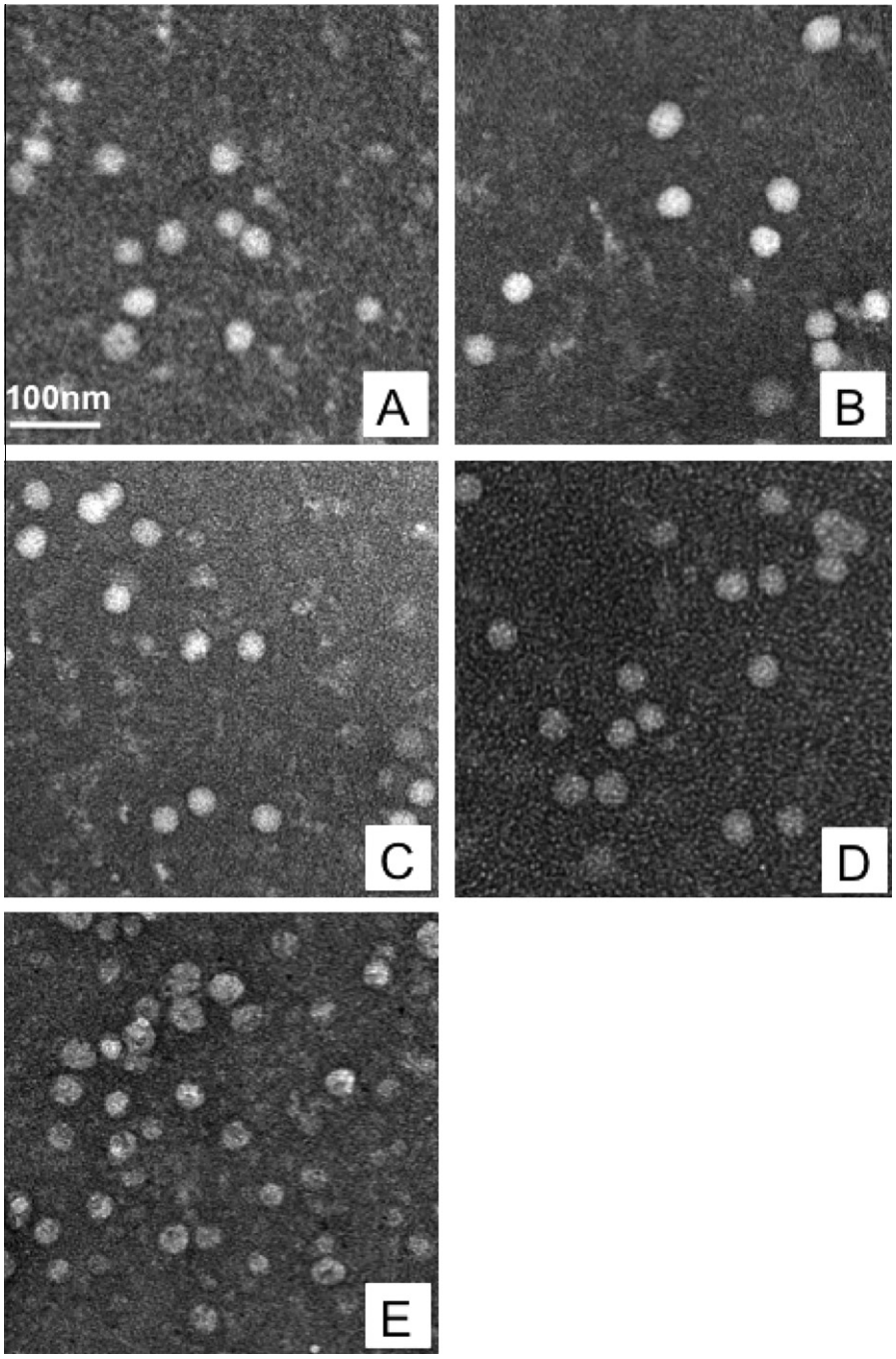

Fig. 1. Electron microscopy on VLP samples. VLPs were purified by sucrose density gradient centrifugation, dialyzed against PBS, and visualized by negative staining with phosphotungstate acid (PTA). (A) Wild-type HBsAgS VLPs, (B) C121A-VLPs (C) C147A-VLPs (D) C121/147A-VLPs (E) C121/124/147A-VLPs.

proteins to form intermolecular disulfide bonds and the presence of monomeric subunits did not interfere with the particle forming capability of the envelope proteins and their ability to package and secret dAgL (Fig. 2C).

3.2. Immunogenicity of C121/147A-HBsAgS VLPs containing an increased proportion of monomeric subunits

HBsAgS-specific CTL epitopes presented by murine MHC-I molecules have been identified (Sette et al., 2001). Three different H-2^{d}-restricted CTL-epitopes, PQS-29, WYW-199 and WGP-201, were selected to assess the VLP immunogenicity. The epitopes are located at distant positions from the substituted cysteine residues. To test whether the presence of monomeric HBsAgS subunits has an impact on the VLP immunogenicity, comparative immunization studies were performed. BALB/c mice were subcutaneously immunized in the absence of an adjuvant with 20 μg of wt-VLPs, or non-native VLPs composed of mutant C121/147A-HBsAgS proteins (C121/147A-VLPs). At day 10 post immunization, the presence of HBsAgS-specific IFNγ production by *ex vivo* splenocytes was quantified by an Elispot assay by stimulating the splenocytes overnight with the PQS-29, WYW-199 or WGP-201 peptides. Immunizations with VLPs containing monomeric subunits showed an equal immune response compared to the wt-VLPs with a slight difference

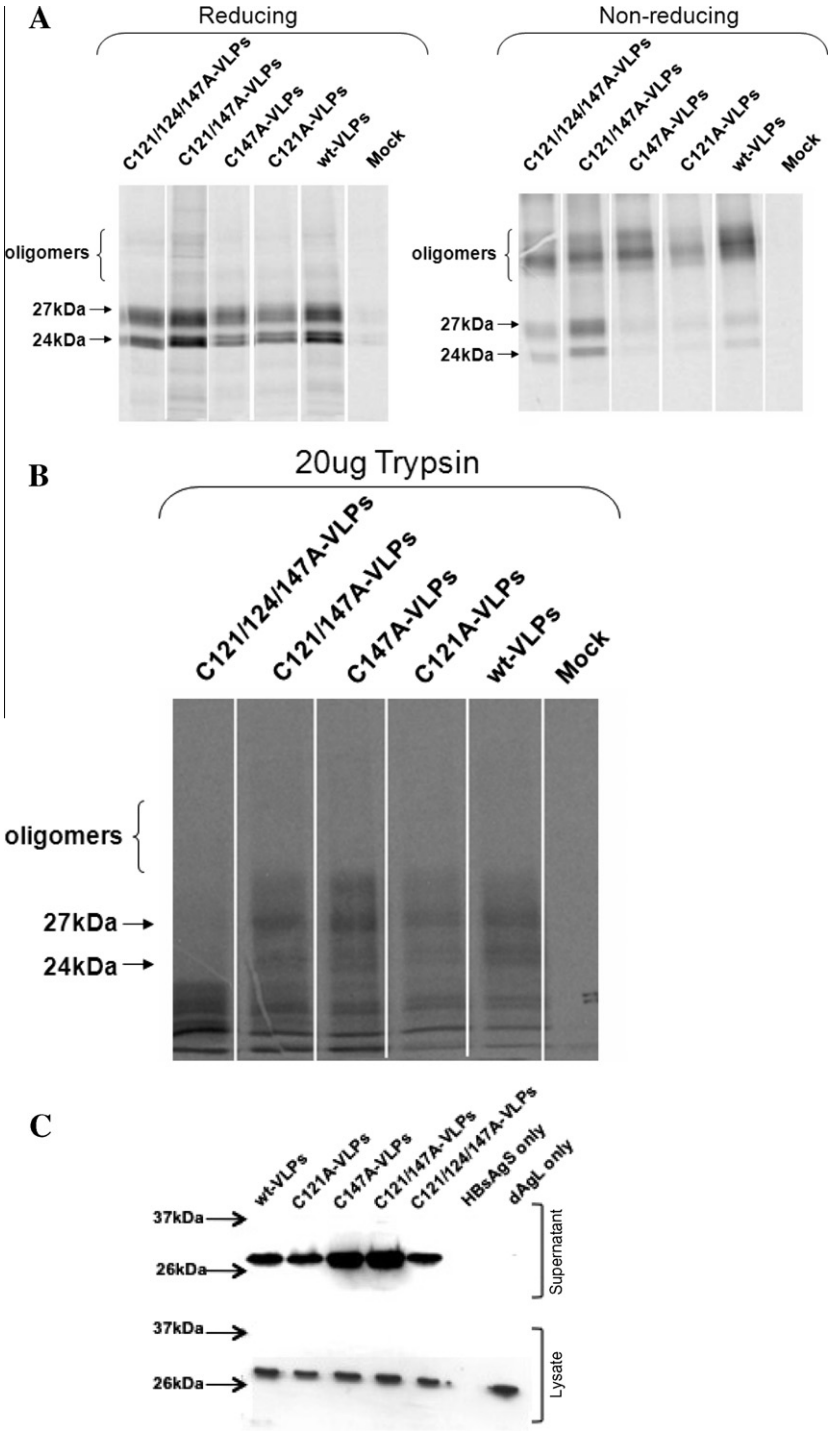

Fig. 2. (A) Immunoprecipitation of HBsAgS VLPs followed by the analysis of HBsAgS subunits under reducing or non-reducing conditions. HEK293-T cells were transfected with plasmids expressing wild-type (wt), C121A, C147A, C121A/C147A, C121A/C124A/C147A mutant HBsAgS proteins. Cells were pulse-labeled with [^{35}S] cysteine/methionine for 3 h and then chased for 18 h. Supernatants were immunoprecipitated with anti-HBsAg antibodies followed by 15% SDS–PAGE. Arrows indicate the glycosylated (27 kDa), non-gylcosylated (24 kDa) and oligomeric HBsAgS proteins. (B) Trypsin digestion with the different sets of [^{35}S] cysteine/methionine labeled VLPs after immunoprecipitation with anti-HBsAgS antibodies. (C) Western blot analysis of delta antigen large (dAgL) in the cell culture supernatant in the presence of wt, C121A-, C147A-, C121A/C147A- or C121A/C124A/C147A-HBsAgS proteins. Human hepatoma cells (HuH-7) were transfected with plasmids encoding wt or mutant HBsAgS proteins. At day 5 post transfection, the supernatant (upper panel) and cell lysate (lower panel) were collected, and separated by 15% SDS–PAGE, then assayed for the presence of dAgL using anti-hepatitis delta virus serum. Control: transfections with plasmids expressing HBsAgS or dAgL.

in epitope selection with an enhanced response against the WGP-epitope (Fig. 3A). To test whether differences in the CTL activity can be measured, splenocytes were restimulated *in vitro* with a mixture of PQS-29, WYW-199 and WGP-201 peptides for 5 days, then incubated with ^{51}Cr-labeled and peptide-pulsed P815 target cells or P815 cells expressing HBsAgS. The cytolytic activity of lymphocytes derived from mice immunized with the non-native VLPs was increased compared to mice immunized with wt-VLPs as indicated by an enhanced killing of peptide-pulsed target cells or HBsAgS-antigen expressing target cells (Fig. 3B).

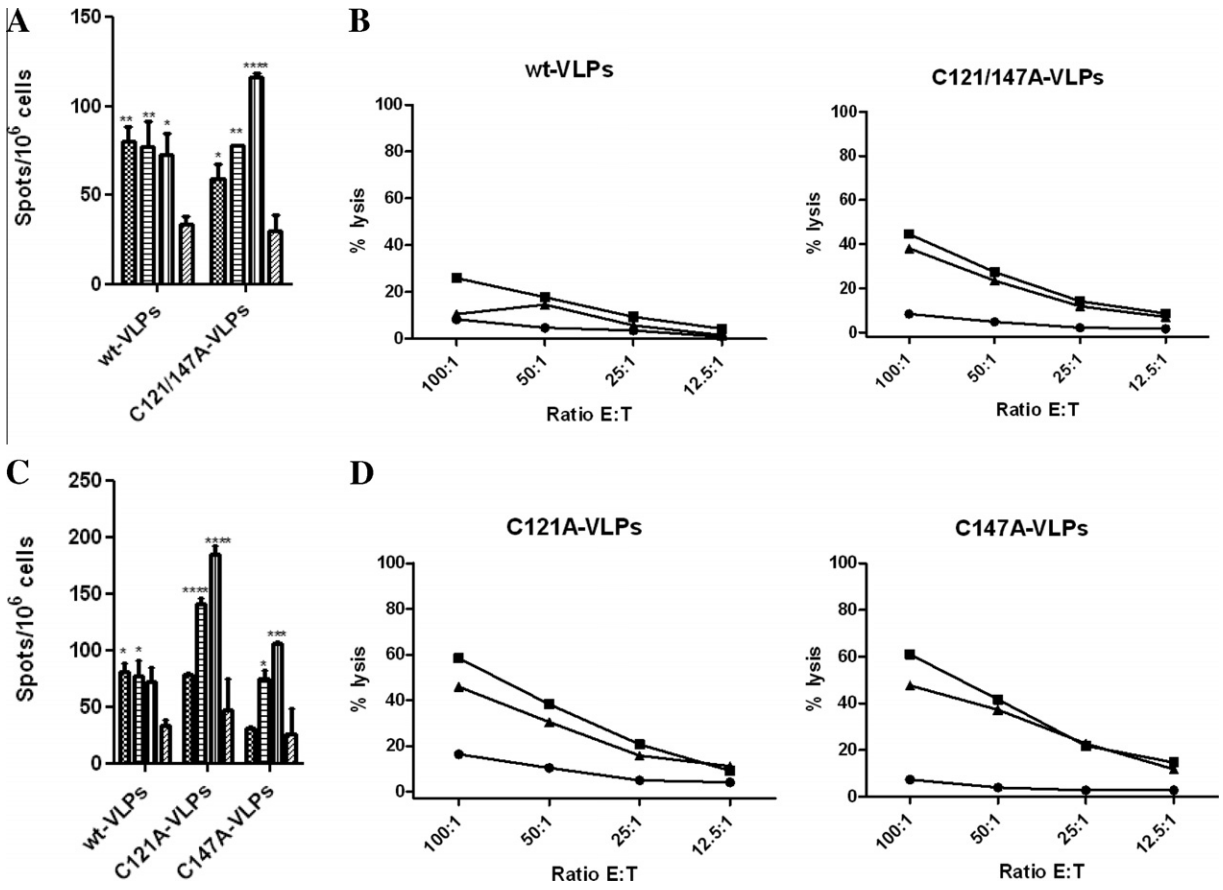

Fig. 3. Measurement of immunization outcomes with wild-type (wt), C121A-, C147A-, C121/147A-VLPs by Elispot assay (A, C), and 51-chromium release assay (CTL-assay) (B, D). Splenocytes derived from BALB/c mice immunized with 20 μg VLPs were *in vitro* cultured for one day in the absence (no stimulation) or presence of HBV-specific peptides (PQS-29, WYW-199 and WGP-201) and then the number of IFNγ-producing cells was determined in the presence of the individual peptides. Histogram bars represent means and standard deviations of samples derived from four mice at 10^6 cells per well, ▩ PQS-, ▤ WYW-, ▥ WGP-peptide stimulation, and ▨ no stimulation (A, C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{***}P<0.0001$ (ANOVA). Percentage cytotoxicity obtained by splenocytes (effectors) restimulated in the absence and presence of HBV-specific peptides was measured using P815 target cells (●), HBV-specific peptides-pulsed P815 target cells (■) or P815/S target cells expressing HBsAg-S endogenously (▲). Splenocytes derived from BALB/c mice immunized with wt-VLPs, C121/147A-VLPs, C121A-VLPs, and C147A-VLPs were *in vitro* restimulated with a mixture of the PQS, WYW, and WPG peptides for 5 days, then mixed with different effector to target ratios as indicated (*x*-axis). Each value represents the mean of four mice in triplicates. The values vary less than 10% within one group. As control, the Elispot outcome of the immunization with wt-VLPs is shown in graph A and C.

3.3. Immunogenicity of HBsAgS VLPs in the absence of a substantial proportion of monomeric subunits

To further confirm that the presence of monomeric subunits or possibly introduced structural changes facilitate the slightly enhanced immune response, immunization studies were performed with VLPs composed of subunits with single cysteine substitutions, C121A-HBsAgS or C147A-HBsAgS. Immunizations with both C121A-VLPs and C147A-VLPs resulted in stronger immune responses than immunizations with wt-VLPs as documented by a higher level of IFNγ producing lymphocytes (Fig. 3C) and an increased level of cytotoxicity (Fig. 3D). The immune response has shifted towards the epitopes WYW-199 and WGP-201 indicating a change in the hierarchy of epitope processing. To utilize VLPs with more profound structural changes, the cysteine residue 124, which contributes to the formation of intramolecular disulfide bonds, was replaced with alanine in combination with the C121A and C147A mutations. The C121/124/147A-VLPs induced a prominent cellular immune response with an enhanced number of PQS-29, WYW-199 and WGP-201 specific IFNγ producing lymphocytes compared to the immune response induced by wt-VLPs (Fig. 4A). The outcome is confirmed by the presence of a strong cellular cytolytic immune response in BALB/c mice immunized with C121/124/147A-VLPs (Fig. 4B). Weakening a disulfide stabilized bonding pattern seems to influence the immunogenicity of the VLP immunogen by destabilizing structural features, and is not necessarily determined by the presence of monomeric subunits.

3.4. Conservation of HBsAgS cysteine residues

To support the hypothesis that the disulfide bonding pattern provides a structure to minimize epitope processing, the HBsAgS cysteine residues were analyzed regarding their level of conservation. A total of 1556 DNA sequences encoding the overlapping HBV surface and polymerase genes were extracted from a large HBV database (SeqHepB) (Yuen et al., 2007) and the conservation of the individual amino acids was determined. The overall conservation of each HBsAg codon ranged from 48–100%, the mean variation across the HBsAgS protein was 94%. Each of the fourteen consensus cysteine residues in the HBsAg was analyzed in more detail (Table 1). The cysteines were highly conserved, ranging between 95–100% conservation. Eight of these fourteen cysteines were 100% conserved with no variation detected across the entire 1556 sequences analyzed. Four of the cysteines were between 99–99.99% conserved, and the remaining two cysteines were 95–96%

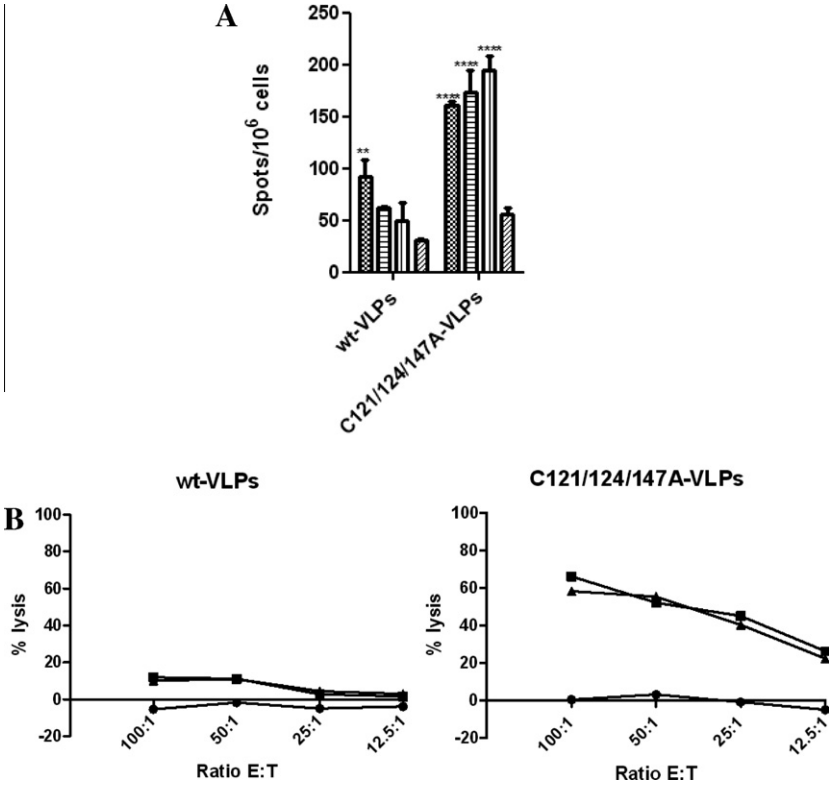

Fig. 4. Measurement of immunization outcomes with wild-type (wt) VLPs and VLPs composed of HBsAgS subunits with triple cysteine to alanine changes (C121/124/147A-VLPs) by Elispot assay (A), and 51-chromium release assay (CTL-assay) (B). Splenocytes derived from BALB/c mice immunized with 20 μg VLPs were *in vitro* cultured for 1 day in the absence (no stimulation) or presence of HBV-specific peptides (PQS, WYW and WGP) and then the number of IFNγ-producing cells determined in the presence of the individual peptides. Histogram bars represent means and standard deviations of samples derived from four mice at 10^6 cells per well, histogram fills are as described in legend of Fig. 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (ANOVA) (A). Percentage cytotoxicity obtained by splenocytes (effectors) restimulated in the absence and presence of HBV-specific peptides was measured using P815 target cells (●), HBV-specific peptides-pulsed P815 target cells (■) or P815/S target cells expressing HBsAg-S endogenously (▲). Splenocytes derived from BALB/c mice immunized with wt- or C121/124/147A-VLPs were *in vitro* restimulated with a mixture of the PQS, WYW, and WPG peptides for five days, then mixed with different effector to target ratios as indicated (*x*-axis). Each value represents the mean of four mice in triplicates. The values vary less than 10% within one group.

conserved (Table 1). For a further analysis, the reverse transcriptase (RT) domains of the HBV polymerase protein were included as they are encoded by an alternate reading frame, which overlaps with the HBsAg-S reading frame. The level of conservation of the two overlapping RT amino acids was also determined. With relevance to the cysteine residues in the external hydrophilic loop region of HBsAgS, 6 of the 8 strictly conserved cysteines of the 'a'-determinant (s107, s121, s124, s137, s139, s149), had a lower level of conservation in the overlapping RT. The other two 'a'-determinant cysteines with 100% conservation (s138, s147) also had 100% conservation in the overlapping RT. In contrast, for cysteines in the internal loop (s48, s65, s69, s76), second transmembrane (s90) and C-terminal hydrophobic regions (s221), only sC65 has a higher level of constraint than the overlapping RT. The outcome of the analysis clearly defines the cysteine residues especially in the 'a'-determinant region as highly conserved possibly contributing to a structure, which guides antigen processing to minimize immunogenicity.

4. Discussion

Antigen processing is dictated by a series of different factors such as structural stability of the external antigen, proteolytic digestion, and the delivery of external antigens to endosomal and lysosomal compartments. Disulfide bonds are important in conferring structural stability of the protein, and hence disruption of the native disulfide bonding pattern can result in different antigen processing pathways due to an altered protease susceptibility, and hence leading to a shift in epitope selection for presentation (Li et al., 2002). Comparative immunization studies with different sets of VLPs were performed to address whether the presence of monomeric subunits and/or structural changes in a particulate multiprotein complex can modulate the immunogenicity of the immunogen. Three different sets of non-native VLPs were generated in addition to wt-VLPs. VLPs composed of HBsAgS subunits with single amino acid (C121A or C147A) changes contain predominantly dimeric/oligomeric subunits similar to wt-VLPs, and are resistant to trypsin digestion. VLPs composed of HBsAgS subunits with two amino acid (C121A and C147A) changes contain a higher proportion of monomeric subunits compared to wt-VLPs, and are resistant to trypsin digestion. VLPs with triple substitutions (C121A, C124A and C147A) contain a reduced proportion of monomeric subunits compared to C121/147A-VLPs, and are trypsin-sensitive. The different sets of VLPs have retained at least partially their antigenicity as they are detected by a HBsAgS-specific EIA showing that HBsAgS-specific epitope structures have been retained. The mutant HBsAgS subunits are assembly- and secretion-competent, and have retained the capability to co-package dAgL indicating that they have retained functionality. The immunization outcomes strongly suggest that the introduced structural changes by substituting cysteine residues modulated the cellular immune response (Figs. 3 and 4). VLPs composed of subunits with single or triple cysteine residue substitutions have the tendency to induce more prominent immune responses than wt-VLPs or C121/147A-VLPs, possibly due to the presence of unpaired cysteine residues facilitating structural changes as a result

of non-native disulfide bonding patterns. The weakening of disulfide-stabilized structures and changes in the local flexibility of stably folded antigen structures may have caused differences in VLP processing and hence in the generation of epitope sets, which is not necessarily dependent on the presence of monomeric subunits.

Twelve of the fourteen HBsAgS cysteines had very high levels of constraint (>99%), of these, eight were 100% conserved, with no variation detected across the 1556 patient-derived sequences analyzed. Six of these cysteines had higher levels of conservation than the overlapping HBV polymerase, suggesting that the constraint at these codons is likely imposed by vital functionality of the surface proteins, not the overlapping RT. This is consistent with the findings of Zaaijer et al. (2007) which showed that the overlapping polymerase and surface protein genes of HBV evolved independently. Most of the strictly conserved HBsAg cysteines are located in the 'a'-determinant region (Table 1), which overlaps with the A–B interdomain of the HBV RT. The A–B interdomain is not part of the active site of the RT, and possibly act as a spacer between the A and B functional domains. The presence of highly conserved HBsAgS cysteine residues in a region overlapping with the A–B RT interdomain further supports the hypothesis that the cysteine residues are of functional importance. HBsAgS cysteine residues located in the 'a'-determinant region are not necessarily essential for generating structural features involved in viral assembly but in infectivity. Sureau and coworkers (Abou-Jaoudé and Sureau, 2007; Le Duff et al., 2009; Salisse and Sureau, 2009) have shown that cysteine and non-cysteine residues located in the external loop region are important for conferring infectivity. In addition, this study indicates that the cysteine residues and disulfide bonding pattern preserves a structure which influences VLP processing, and may facilitate immune escape. The findings in the mouse model may have direct relevance to the human system as antigenic "hot spots" are shared between mouse and human (Sealy et al., 2008; Surman et al., 2001; Zhan et al., 2007) possibly due to proteases with similar specificity and similar processing pathways (Landry, 2008; Sealy et al., 2008).

The design of a vaccine based on modified antigens can have practical applications. The induction of a strong immune response against epitopes which are less efficiently generated during a natural infection and therefore fail to induce an immune response allowing viral escape, may control the progression of infections. Vaccines based on structurally modified antigens inducing immune response against different epitope profiles can potentially overcome chronic viral infections or may complement a vaccine based on native proteins.

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References

Abou-Jaoudé, G., Sureau, C., 2007. Entry of hepatitis delta virus requires the conserved cysteine residues of the hepatitis B virus envelope protein antigenic loop and is blocked by inhibitors of thiol–disulfide exchange. J. Virol. 81, 13057–13066.

Agnandji, S.T., Asante, K.P., Lyimo, J., Vekemans, J., Soulanoudjingar, S.S., Owusu, R., et al., 2010. Evaluation of the safety and immunogenicity of the RTS, S/AS01$_E$ malaria candidate vaccine when integrated in the expanded program of immunization. J. Infect. Dis. 202, 1076–1087.

Bruss, V., 2007. Hepatitis B virus morphogenesis. World J. Gastroenterol. 13, 65–73.

Buonaguro, L., Racioppi, L., Tornesello, M.L., Arra, C., Visciano, M.L., Biryahwaho, B., et al., 2002. Induction of neutralizing antibodies and cytotoxic T lymphocytes in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A. Antiviral Res. 54, 189–201.

Carmicle, S., Steede, N.K., Landry, S.J., 2007. Antigen three-dimensional structure guides the processing and presentation of helper T cell epitopes. Mol. Immunol. 44, 1159–1168.

Chang, F.L., Chen, P.J., Tu, S.J., Wang, C.J., Chen, D.S., 1991. The large form of hepatitis delta antigen is crucial for assembly of hepatitis delta virus. Proc. Natl. Acad. Sci. USA 88, 8490–8494.

Cheong, W.-S., Reiseger, J., Turner, S.J., Boyd, R., Netter, H.J., 2009. Chimeric virus-like particles for the delivery of an inserted conserved influenza A-specific CTL epitope. Antiviral Res. 81, 113–122.

Dai, G., Carmicle, S., Steede, N.K., Landry, S.J., 2002. Structural basis for helper T-cell and antibody epitope immunodominance in bacteriophage T4 Hsp10. Role of disordered loops. J. Biol. Chem. 277, 161–168.

Delpeyroux, F., Chenciner, N., Lim, A., Malpièce, Y., Blondel, B., Crainic, R., et al., 1986. A poliovirus neutralization epitope expressed on hybrid hepatitis B surface antigen particles. Science 233, 472–475.

Dubois, M.F., Pourcel, C., Rousset, S., Chany, C., Tiollais, P., 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. Proc. Natl. Acad. Sci. USA 77, 4549–4553.

Gonzalez, M.C., Kostrzak, A., Guetard, D., Pniewski, T., Sala, M., 2009. HIV-1 derived peptides fused to HBsAg affect its immunogenicity. Virus Res. 146, 107–114.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Jeong, S.H., Qiao, M., Nascimbeni, M., Hu, Z., Rehermann, B., Murthy, K., et al., 2004. Immunization with hepatitis C virus-like particles induces humoral and cellular immune responses in nonhuman primates. J. Virol. 78, 6995–7003.

Landry, S.J., 2008. Three-dimensional structure determines the pattern of CD4+ T-cell epitope dominance in influenza virus hemagglutinin. J. Virol. 82, 1238–1248.

Le Duff, Y., Blanchet, M., Sureau, C., 2009. The pre-S1 and antigenic loop infectivity determinants of the hepatitis B virus envelope proteins are functionally independent. J. Virol. 83, 12443–12451.

Leggatt, G.R., Frazer, I.H., 2007. HPV vaccines: the beginning of the end for cervical cancer. Curr. Opin. Immunol. 19, 232–238.

Li, P., Haque, M.A., Blum, J.S., 2002. Role of disulfide bonds in regulating antigen processing and epitope selection. J. Immunol. 169, 2444–2450.

Liang, T.J., 2009. Hepatitis B: the virus and disease. Hepatology 49, S13–21.

Lin, M.L., Zhan, Y., Villadangos, J.A., Lew, A.M., 2008. The cell biology of cross-presentation and the role of dendritic cell subsets. Immunol. Cell Biol. 86, 353–362.

Ludwig, C., Wagner, R., 2007. Virus-like particles–universal molecular toolboxes. Curr. Opin. Biotechnol. 18, 537–545.

Mangold, C.M.T., Streeck, R.E., 1993. Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. J. Virol. 67, 4588–4597.

Mangold, C.M.T., Unckell, F., Werr, M., Streeck, R.E., 1995. Secretion and antigenicity of hepatitis B virus small envelope proteins lacking cysteines in the major antigenic region. Virology 211, 535–543.

Mangold, C.M.T., Unckell, F., Werr, M., Streeck, R.E., 1997. Analysis of intermolecular disulfide bonds and free sulfhydryl groups in hepatitis B surface antigen particles. Arch. Virol. 142, 2257–2267.

Mirano-Bascos, D., Steede, N.K., Robinson, J.E., Landry, S.J., 2010. Influence of disulfide-stabilized structure on the specificity of helper T-cell and antibody responses to HIV envelope glycoprotein gp120. J. Virol. 84, 3303–3311.

Patient, R., Hourioux, C., Vaudin, P., Pagès, J.C., Roingeard, P., 2009. Chimeric hepatitis B and C viruses envelope proteins can form subviral particles: implications for the design of new vaccine strategies. New Biotechnol. 25, 226–234.

Phogat, S., Svehla, K., Tang, M., Spadaccini, A., Muller, J., Mascola, J., et al., 2008. Analysis of the human immunodeficiency virus type 1 gp41 membrane proximal external region arrayed on hepatitis B surface antigen particles. Virology 373, 72–84.

Prato, S., Fleming, J., Schmidt, C.W., Corradin, G., Lopez, J.A., 2006. Cross-presentation of a human malaria CTL epitope is conformation dependent. Mol. Immunol. 43, 2031–2036.

Ramqvist, T., Andreasson, K., Dalianis, T., 2007. Vaccination, immune and gene therapy based on virus-like particles against viral infections and cancer. Expert Opin. Biol. Ther. 7, 997–1007.

Ryu, W.S., Bayer, M., Taylor, J., 1992. Assembly of hepatitis delta virus particles. J. Virol. 66, 2310–2315.

Salisse, J., Sureau, C., 2009. A function essential to viral entry underlies the hepatitis B virus "a" determinant. J. Virol. 83, 9321–9328.

Sealy, R., Chaka, W., Surman, S., Brown, S.A., Cresswell, P., Hurwitz, J.L., 2008. Target peptide sequence within infectious human immunodeficiency virus type 1 does not ensure envelope-specific T-helper cell reactivation: influences of cysteine protease and gamma interferon-induced thiol reductase activities. Clin. Vaccine Immunol. 15, 713–719.

Sette, A.D., Oseroff, C., Sidney, J., Alexander, J., Chesnut, R.W., Kakimi, K., et al., 2001. Overcoming T cell tolerance to the hepatitis B virus surface antigen in hepatitis B virus-transgenic mice. J. Immunol. 166, 1389–1397.

Surman, S., Lockey, T.D., Slobod, K.S., Jones, B., Riberdy, J.M., White, S.W., et al., 2001. Localization of CD4+ T cell epitope hotspots to exposed strands of HIV envelope glycoprotein suggests structural influences on antigen processing. Proc. Natl. Acad. Sci. USA 98, 4587–4592.

Vietheer, P.T., Boo, I., Drummer, H.E., Netter, H.J., 2007. Immunizations with chimeric hepatitis B virus-like particles to induce potential anti-hepatitis C virus neutralizing antibodies. Antivir. Ther. 12, 477–487.

Woo, W.P., Doan, T., Herd, K.A., Netter, H.J., Tindle, R.W., 2006. Hepatitis B surface antigen vector delivers protective cytotoxic T-lymphocyte responses to disease-relevant foreign epitopes. J. Virol. 80, 3975–3984.

Wunderlich, G., Bruss, V., 1996. Characterization of early hepatitis B virus surface protein oligomers. Arch. Virol. 141, 1191–1205.

Yuen, L.K., Ayres, A., Littlejohn, M., Colledge, D., Edgely, A., Maskill, W.J., et al., 2007. SeqHepB: a sequence analysis program and relational database system for chronic hepatitis B. Antiviral Res. 75, 64–74.

Zaaijer, H.L., van Hemert, F.J., Koppelman, M.H., Lukashov, V.V., 2007. Independent evolution of overlapping polymerase and surface protein genes of hepatitis B virus. J. Gen. Virol. 88, 2137–2143.

Zhan, X., Hurwitz, J.L., Brown, S.A., Slobod, K.S., 2007. HIV-1 envelope T cell epitope "hotspots" among mice and humans and among CD4+ and CD8+ T cell subpopulations. AIDS Res. Hum. Retroviruses 23, 471–476.