Construction and Immunological Evaluation of Multivalent Hepatitis B Virus (HBV) Core Virus-Like Particles Carrying HBV and HCV Epitopes

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A multivalent vaccine candidate against hepatitis B virus (HBV) and hepatitis C virus (HCV) infections was constructed on the basis of HBV core (HBc) virus-like particles (VLPs) as carriers. Chimeric VLPs that carried a virus-neutralizing HBV pre-S1 epitope corresponding to amino acids (aa) 20 to 47 in the major immunodominant region (MIR) and a highly conserved N-terminal HCV core epitope corresponding to aa 1 to 60 at the C terminus of the truncated HBcΔ protein (N-terminal aa 1 to 144 of full-length HBc) were produced in Escherichia coli cells and examined for their antigenicity and immunogenicity. The presence of two different foreign epitopes within the HBc molecule did not interfere with its VLP-forming ability, with the HBV pre-S1 epitope exposed on the surface and the HCV core epitope buried within the VLPs. After immunization of BALB/c mice, specific T-cell activation by both foreign epitopes and a high-titer antibody response against the pre-S1 epitope were found, whereas an antibody response against the HBc carrier was notably suppressed. Both inserted epitopes also induced a specific cytotoxic-T-lymphocyte (CTL) response, as shown by the gamma interferon (IFN-γ) production profile.

Genetically engineered virus-like particle (VLP)-based vaccines are one of the most promising tools in modern vaccinology. VLPs from almost all classes of viruses are being evaluated now or have just been adopted to use as carriers for presentation of foreign immunological epitopes (for a review, see references 29 and 31). VLP technologies possess obvious advantages for the generation of safe and efficacious vaccines. First, the repetitive antigenic structure of VLPs makes them highly immunogenic. Second, VLPs lack viral genomes or genes and are noninfectious, although they mimic infectious viruses in their structural and immunological features. Third, VLPs are generated by highly efficient heterologous expression of the cloned viral structural genes with subsequent quantitative in vivo or in vitro self-assembly of their products. Fourth, VLPs can be obtained by simple and efficient purification procedures. VLPs can be used for a broad range of applications, but the generation of vaccines against hepatitis B virus (HBV) and hepatitis C virus (HCV) infections is of special interest.

The HBV core (HBc) protein was first reported as a promising VLP carrier in 1986 and was published in 1987 (6, 10, 24). In many ways, HBc occupies a unique position among the VLP carriers because of its high-level synthesis and efficient self-assembly in virtually all known homologous and heterologous expression systems, including bacteria (for a review, see references 29 to 31). The major HBc B-cell epitopes (c and e1) are localized within the major immunodominant region (MIR), whereas the next important epitope, e2, is localized around amino acid position 130, close to the C-terminal histone-like region (for a review, see reference 30).

The high-resolution spatial structure of HBc icosahedrons (11, 43) shows that the MIR is located on the tip of the spike, around the most protruding region between amino acids (aa) 78 and 82. For this reason, the MIR is generally accepted as the target site of choice for insertion of foreign epitopes (30). The other widely accepted site for insertions is C-terminal position 144, a short stretch after the e2 epitope. For C-terminal insertions, so-called HBcΔ vectors lacking a 39-aa-long positively charged C-terminal histone-like fragment are preferred for their high insertion capacities (up to 741 amino acid residues) (30).

Here, we present the construction and preliminary immunological characterization of a first multivalent HBV and HCV vaccine candidate. As an HBV epitope, we chose the pre-S1 sequence aa 20 to 47, which alone is able to elicit HBV-neutralizing and protective antibodies (23), for insertion into the HBc MIR. Concurrently, we inserted at the C terminus of the HBcΔ vector the N-terminal 60-aa fragment of the HCV core, which is highly conserved among various HCV genotypes with amino acid homology exceeding 95% (12, 14) and therefore is an attractive target for the generation of an HCV vaccine (19, 41). Such a combination of foreign epitopes did not prevent correct self-assembly of chimeric HBc-based particles and provided them with specific HBV and HCV antigenicity and immunogenicity in mice.

MATERIALS AND METHODS

Construction of recombinant plasmids. Escherichia coli strains R1 [F− rK− mK− leuB6 proA2 thi-1 araC14 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Str−) glnV44 Δ(mcrC-mrr)] and DH5α [λ− e80d lacZAM15 ΔlacZYA-argF)U169 recA1 endA1 hsdR17 (rK− mK− supE44 thi-1 proA A1)] were used for cloning and selection of recombinant plasmids; K802 (F− rK− mK− e14 MetA metB1 lacY1 [or lacI-Y6])
Casamino Acids (Difco) and 0.2% glucose with a final optical density at 540 nm 750-ml flasks containing 300 ml of M9 minimal medium supplemented with 1% purity of the HCV core (1-98) protein according to Coomassie blue staining of recombinant HCV core antigen (His-tagged protein 1-98) and its purification moter, which allowed a high expression level without induction. The construction clonal anti-HCV core 34-7 antibody (1), were used in this work.

The stability of VLPs was determined by evaluation of fluorescence-based diameter measurement. Mice and immunization schemes. Female BALB/c (H-2d) mice, 6 to 8 weeks of age and weighing 18 to 20 g, were obtained from the Animal Breeding Centre of the Institute of Virology, Riga, Latvia, and held at the Latvian Biomedical Research and Study Centre. Five mice in each group were immunized subcutaneously with 25 µg of the appropriate protein, HBCa, HBcα-pre-S1, HBcα-HCV core, or HBcα-pre-S1-HCV core, in 200 µl of sterile PBS. No adjuvants were used for immunizations. On day 14 after the first immunization, the second 25-µg boost was performed. For monitoring of the humoral response, immune serum was collected 14 days after the first and second immunizations. For T-cell proliferation tests, mice were sacrificed and spleens were obtained at day 28 after the first immunization. The ethics of the mouse immunization experiments for the Latvian Biomedical Research and Study Centre (registration number 025413) were approved in compliance with the Latvian Animal Protection Law (certificate no. 4 from the Food and Veterinary Service, 8 January 2009) on the basis of the decision of the Council for Ethical Treatment of Animals in Latvia.

ELISAs. Direct and competitive enzyme-linked immunosorbent assays (ELISAs) were performed, in general, as described in reference 36. Recombinant HBC, HCV core (1-98), and pre-S1 peptide aa 20 to 47 were adsorbed overnight at 4°C to 96-well plates (Nunc) at 10 µg/ml in 50 mM sodium carbonate buffer, pH 9.6. After being blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h at 37°C, serial dilutions of mouse sera were added to the plates and incubated for an additional 1 h at 37°C. After being washed 3 times with PBS containing 0.05% Tween 20, an anti-mouse IgG horseradish peroxidase-conjugated anti-body (Sigma) was applied at a 1:1,000 dilution. Following 1 h of incubation at 37°C, the plates were washed, and substrate OPD (Sigma) was added for color development. The results were checked in an automatic reader (Multiscan, Sweden) at 492 nm. The endpoint titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of negative-control sera derived from nonimmunized mice.

Detection of IgG isotypes. Detection of total IgG1 and IgG2a from sera of immunized mice was performed using a murine-antibody-isotyping ELISA kit (Sigma).

T-cell proliferation assay. Murine splenocytes were harvested using red blood cell lysing buffer (Sigma). Single-cell suspensions were prepared (5 × 10^6 cells/ml) and cocultured in RPMI 1640 (Gibco, Germany) with the appropriate recombinant protein or pre-S1 (20-47) peptide with three consecutive 10^−dilutions of carrier VLPs or peptide starting from 10^−14 g/ml in 50 mM sodium carbonate buffer, pH 9.6. After 18 h of incubation at 37°C, the plates were washed, and substrate OPD (Sigma) was added for color development. The results were presented as stimulation indexes (SI), which were calculated as a ratio of the mean cpm values obtained in the presence and absence of a stimulator.

Cytokine test. For detection of interleukin 2 (IL-2), gamma interferon (IFN-γ), and IL-10 cytokines in cell cultures, the supernatants from the wells were removed at 24 h and at 48 h for IL-2, IL-10, and IFN-γ measurements using the T-cell proliferation tests were started. Analysis for the presence of cytokines in cell supernatants was performed using the commercial tests OptEIA Mouse IFN-γ, IL-2, and IL-10 ELISA Sets from BD Bioscience Pharmingen, according to the manufacturers’ instructions.

RESULTS

Structure and self-assembly of VLPs carrying the pre-S1(20-47) and HCV core (1-60) epitopes. The HBcα-pre-S1 fusion protein was constructed by insertion of the pre-S1(20-47) fragment between positions 78 and 79 of the HBcα protein with the intention of exposing the pre-S1 fragment on the surface of the HBc particle. The HBcα-HCV core fusion protein was constructed by the addition of HCV core fragment (1-60) downstream of amino acid residue 144 to displace the arginine-rich histone-like C terminus of the HBc molecule. The HBcα-pre-S1-HCV core fusion protein is HBcα with both pre-S1(20-47) and core (1-60) fragments inserted as described above in HBcα-pre-S1 and HBcα-HCV core constructs, respectively (Fig. 1).
Chimeric HBcΔ-pre-S1, HBcΔ-HCV core, and HBcΔ-pre-S1–HCV core proteins were purified and evaluated by Western blot analysis with mouse monoclonal anti-HBc and anti-pre-S1 antibody (C), and rabbit polyclonal anti-HCV core antibody (D). Lanes 1, HBcΔ; 2, HBcΔ-pre-S1; 3, HBcΔ-HCV core; 4, HBcΔ-pre-S1–HCV core. Lanes M, Page Ruler Plus Prestained Protein Ladder and Prestained Protein Molecular Mass Marker (both from Fermentas, Lithuania) were used as molecular mass markers on the stained gel and on Western blots, respectively.

The expression level of chimeric proteins was around 5% of the total intracellular protein. The presence of VLP-like structures was detected by double radial immunodiffusion using polyclonal rabbit anti-HBc antibodies. Chimeric proteins were precipitated by anti-HBc antibodies with the same efficiency as nonchimeric HBcΔ VLPs, suggesting efficient formation of correctly folded VLPs (data not shown). The morphology of particles in the chimeric VLP preparations did not differ significantly from that of the particles in the nonchimeric HBcΔ VLP preparation (Fig. 3). The HBcΔ, HBcΔ-HCV core, and HBcΔ-pre-S1–HCV core preparations demonstrated similar levels of the T=3 particles: 23, 20, and 16%, respectively.

It is surprising that HBcΔ-pre-S1 showed a much higher content of T=3 particles (35%).

The stability of VLPs was determined by comparing the melting points of VLPs in starting buffer and in the presence of 0.5 M NaCl and 20 mM DTT (not shown). Whereas HBcΔ, HBcΔ-pre-S1, and HBcΔ-HCV core demonstrated similar melting points (77 to 80°C), double-inserted HBcΔ-pre-S1–HCV core VLPs were a bit less stable, with a melting point of 67 to 69°C. For all VLP variants, the melting points did not depend on the presence of 0.5 M NaCl and 20 mM DTT.

Surface exposure of foreign sequences on VLPs was checked by competitive ELISA (Fig. 4), which showed that the pre-S1(20-47) epitope was well exposed but the HCV core (1-60) epitope was buried within the particles.

The presence of nucleic acids within chimeric VLPs was determined by native agarose gel electrophoresis of VLPs (Fig. 5). Nucleic acids were present in the HBcΔ-HCV core and HBcΔ-pre-S1–HCV core preparations, but not in the HBcΔ-pre-S1 and nonchimeric HBcΔ preparations.

Humoral response in mice. Animals were bled on days 14 and 28 after immunization. The boost on day 14 led to a significant increase of specific antibody response (compare Fig. 6A and B). Control groups were immunized with the initial HBcΔ without any insertions, which induced anti-HBc antibody response at a titer of 1:15,000.

Insertion of the pre-S1 epitope into the MIR led to a substantial decrease of anti-HBc response (HBcΔ-pre-S1 and HBcΔ-pre-S1–HCV core), whereas C-terminal addition of the
HCV core in the HBcΔ-HCV core construct did not affect the anti-HBc response. Surprisingly, the multivalent HBcΔ-pre-S1-HCV core VLPs induced a higher anti-pre-S1 antibody response than HBcΔ-pre-S1 with a single epitope insertion. The humoral anti-HCV core response remained low in both HBcΔ-HCV core and HBcΔ-pre-S1–HCV core VLP immunizations (Fig. 6).

**Isotyping of IgG antibodies.** We analyzed specific isotype profiles of observed antibodies of vaccinated mice. Chimeric VLPs containing HCV core induced a predominantly IgG2a antibody response against the HBc carrier, whereas HBcΔ and HBcΔ-pre-S1 induced a predominantly IgG1 antibody response (Fig. 7). In contrast, the antibody response against pre-S1 remained predominantly IgG1. Levels of anti-HCV antibodies were too low to confirm the apparent predominance of IgG2a. These observations were further confirmed by the results of the T-cell proliferation assay, in which all immunized groups of animals showed high T-cell proliferation.

**T-cell proliferative responses and cytokine profile.** Lymphocytes of mice immunized with all studied VLPs demonstrated detectable proliferation in vitro by HBc carriers, with the high-
est level for HBcΔ-pre-S1–HCV core induced by recombinant HBc at day 28 after the first immunization (Fig. 8). Proliferation in vitro with the pre-S1 peptide (20-47) resulted in SI levels similar to the appropriate SI levels for proliferation with HBc. Proliferation in vitro with HCV core protein (1-98) demonstrated the highest SI levels in the case of the HBcΔ-HCV core.

Figure 9 shows direct measurements of the Th1 cell-derived cytokines IL-2, IFN-γ, and IL-10 after appropriate stimulation. In general, these data follow the proliferation response data. The highest production of IFN-γ was detected for all VLP variants after stimulation with HBc. Lower but highly specific stimulation of IFN-γ production was achieved after stimulation with the pre-S1 peptide (20-47) or HCV core protein (1-98).

Production of IL-2 was very low, whereas production of IL-10 was detectable in all cases. We also performed direct measurement of the Th2 cell-derived cytokine IL-4. Due to the low sensitivity level of the test, not all VLP variants demonstrated IL-4 production (data not shown).

DISCUSSION

VLPs carrying foreign epitopes gave one of the first impulses to the idea of universal, self-assembling, and noninfectious carriers for foreign epitopes, which are now widely used in VLP technology (29, 31). HBc VLPs have been used as the carrier of choice for the construction of many successful vaccine candidates against foot and mouth disease, malaria, human papilloma, hantavirus infection, and influenza, but first of all against HBV and HCV infections (for references, see reference 31).

The target of the present work was to combine HBV and HCV epitopes within the same VLP for the first time with the aim of developing a novel multivalent vaccine. HBcΔ (1-144) vector carrying all HBc T-helper cell and cytotoxic T-lymphocyte (CTL) epitopes but lacking the C-terminal arginine-rich region was chosen for epitope insertions as an efficient vaccine scaffold. The HBc MIR region and C terminus of such short-
36 (5). Numerous variants of the pre-S1 (21-47) insertions into the MIR with and without internal MIR deletions in HBc and HBcΔ were studied (25, 38). Further, three tandem copies of pre-S1 (21-47) were inserted at the MIR (44). Moreover, practically full-length pre-S1, with deletion of the inner hydrophobic membrane-targeting fragment, was exposed on the surfaces of the HBc and HBcΔ VLPs (35). In addition, numerous variants of mosaic HBc particles carrying the complete pre-S sequence at the MIR have been constructed with and without MIR deletions (16).

In regard to the immunogenicity of the pre-S1 epitope in our present study, the surprising thing is that the bivalent chimera HBcΔ–pre-S1–HCV core induces stronger anti-pre-S1 antibody response than the monovalent HBcΔ–pre-S1. At the same time, the anti-pre-S1 antibody responses gained here are definitely not lower than those in the long list of previous studies, especially taking into account differences in the lengths and compositions of pre-S1 fragments, immunization schemes and doses, adjuvants, and methodologies of titer calculations.

A strong therapeutic potential was shown with the HBcΔ (aa 1-105) variant bearing the C-terminally fused pre-S1 (aa 3-55) peptide, which was found to be surface accessible (9), although C-terminally added epitopes are mostly buried within HBc VLPs (for a review, see references 29 and 31). It encouraged us to try C-terminal addition of the HCV epitope within the planned multivalent vaccine candidate.

The N-terminal part of the HBc core was chosen, since it was found to contain three distinct antibody binding sites, including the previously reported site at residues 7 to 18 (7). The other two are located at residues 19 to 26 and residues 29 to 34 (33). A highly conservative T-cell epitope (HLA-A2 type) of the HCV core region is located within aa 35 to 44 (2). Attempts to insert the epitope into the HBc MIR and to use it as a DNA vaccine were made earlier (8).

Nevertheless, in our multivalent vaccine candidate, C-terminally added HBc core epitope (1-60) was neither exposed on the VLP surface nor able to induce remarkable antibody response, in contrast to the pre-S1 epitope (20-47). The latter showed good accessibility on the VLP surface and high-titer induction of specific antibodies. HBc core is known to be a relatively weak inducer of antibody response after insertion into chimeric VLPs (for a review, see reference 31). Remarkably, in this study, the immunogenicity of the HCV core epitope within the bivalent chimera HBcΔ–pre-S1–HCV core was not lower than that within the monovalent HBcΔ–HCV core. Moreover, HCV epitope (1-60) showed sufficient T-cell immunogenicity, which supports the idea of further elucidation of the multivalent construction.

Elimination of the C terminus of the HBc in the HBcΔ particles drastically reduced the Th1-biased immunogenicity of the HBc (32). This finding can be explained by the presence of CpG-like sequences in the bacterial RNA, which is packaged within the full-length HBc-derived particles. Synthetic oligodeoxynucleotides containing CpG motifs (CpG ODNs) are known as strong Th1 cell and CTL activity inducers when administered together with HBs (42) and HBc (39, 40) VLPs. According to our data, HBcΔ VLPs carrying HCV core epitope (1-60) restored the ability to package nucleic acids and switched the Th2 type of anti-HBc immune response to the Th1 type. In earlier work, we showed that HCV core (1-98) His-tagged protein was able to induce Th1-mediated response (1).

Therefore, bivalent VLPs carrying various foreign epitopes at different places are able to induce an immunological response to inserted epitopes that is higher, or at least not lower, than the response to individual epitopes within the corresponding monovalent VLP chimeras. One of the further activities in the application of the multivalent VLP constructions could be connected to a combination of DNA prime and protein boost vaccination schemes.

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