Chimeric derivatives of Hepatitis B core particles carrying major epitopes of the Rubella virus E1 glycoprotein

Running title: Hepatitis B core particles carrying Rubella virus E1 epitopes

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Abstract

Three variants of major Rubella virus (RV) E1 protein virus-neutralizing epitope 214-285 were exposed on the hepatitis B virus (HBV) C-terminally truncated core (HBcΔ) in a virus-like particle (VLP) vector and were produced in Escherichia coli (E. coli). All three chimeras demonstrated VLPs in bacterial cell lysates, but only HBcΔ-E1 (245-285) demonstrated the correct VLP structure after purification. The other chimeras HBcΔ-E1 (214-285) and HBcΔ-E1 (214-240) appeared after purification as non-VLP aggregates of 100 - 900 nm in diameter according to dynamic light scattering data. All three variants possessed the intrinsic antigenic activity of RV E1, since they were recognized by natural human anti-RV E1 antibodies, and induced an anti-RV E1 response in mice. HBcΔ-E1 (214-240) and HBcΔ-E1 (245-285) can be regarded as prototypes for a putative RV vaccine because they were able to induce antibodies recognizing natural RV E1 protein in RV diagnostic kits.
1. Introduction

Rubella virus (RV) is an enveloped, positive single-stranded RNA virus and a member of the genus Rubivirus, which belongs to the Togaviridae family. Rubella is normally a mild, self-limited disease but may cause fetal damage if it is acquired during the first trimester of pregnancy. In this case, congenital rubella syndrome could be generated in infants after birth (for review see (1)). One of the most widely used RV vaccines, Meruvax, is a live attenuated vaccine, which was propagated using the human cell line WI-38 that was derived from embryonic lung tissue in 1961 (2,3), and is used as a component of the mixed MMR (measles, mumps, and rubella) vaccine (for review see (4)). Because of the drawbacks of human cell line-derived vaccines, there is an urgent need for the construction of recombinant RV vaccine candidates.

RV consists of three structural proteins: a capsid protein and two membrane-spanning glycoproteins, E1 and E2, localized in the virus envelope (5). E1 is the dominant surface molecule of the virus particle: it represents the main target for the detection and subsequent elimination of RV by the host’s immune system (6,7). Immunoprecipitation or immunoblot techniques have shown that most of the anti-RV immunoglobulin response seems to be induced by the E1 glycoprotein. Although both E1 and E2 provide lifelong immunity, the hemagglutination activity and viral neutralization activity have been attributed to the E1 protein at amino acid positions 208-239 (7,8), 213-239 (9), and 214-240 (10). Three additional neutralizing and hemagglutination epitopes have been identified within the E1 glycoprotein between residues 245-285 (11). Therefore, these E1 protein epitopes may have potential not only in diagnostics but also in vaccine development against RV infection (12).
The Hepatitis B virus (HBV) core (HBc) protein was first reported as a promising VLP carrier in 1986 (13) and was published in 1987 (14,15). In many ways, HBc maintains a unique position among other VLP carriers because of its high-level synthesis; efficient self-assembly in virtually all known homologous and heterologous expression systems, including bacteria and yeast; and high capacity for foreign insertions (for review see (16-19)).

HBc protein spontaneously forms dimeric units (20,21), which self-assemble in HBV-infected eukaryotic cells by allosterically controlled mode (22). Natural, as well as recombinant HBc particles are represented by two isomorphs with triangulation numbers T=4 and T=3 (23) consisting of 120 and 90 HBc dimers and possessing 35 and 32 nm diameters (23,24), respectively.

The high-resolution spatial structure of HBc (23,25) shows that the region maximally protruding on the HBc surface, the major immunodominant region (MIR), is located on the tip of the spike between aa positions 78 and 82. Therefore, the MIR is generally used for the insertion of foreign B-cell epitopes that are expected to be maximally exposed on the outer surface of VLPs (for review see (16-19)). HBcΔ particles lacking the 39 aa, positively charged C-terminal histone-like fragment are often the preferred HBc carrier because of their high-level synthesis efficiency using well-established purification schemes from bacteria (for review see (16-19)).

Here, we selected the RV E1 protein fragment 214-285, encompassing a major RV-neutralizing epitope, for insertion into the MIR of the HBcΔ vector. In addition to the insertion of the full-length E1 fragment, the latter was divided into two parts for separate insertions into the MIR consisting of aa 214-240 and aa 245-285. Although all three fragments allowed VLP self-assembly in bacteria, only HBcΔ-E1 (245-285) was able to retain correct VLP structure after purification. HBcΔ-E1 (245-285)
induced high titers of anti-RV E1 antibodies. Although the other fragments are less efficient in induction of anti-RV E1 antibodies than HBc\(\Delta\)-E1 (245-285), purified HBc\(\Delta\)-E1 (214-285) and HBc\(\Delta\)-E1 (214-240), which appeared as non-VLP aggregates of the appropriate HBc\(\Delta\)-E1 dimers, induced significant anti-RV E1 antibody levels in immunized mice.

2. Materials and methods

2.1. Construction of recombinant HBc\(\Delta\)-E1 genes

The general scheme for the HBc\(\Delta\)-E1 gene structures is shown in Fig. 1. The amino acid sequences for the RV E1 insertions, and the insertion-carrier junction regions are listed in Table 1.

*E. coli* strain RR1 (F\(^{-}\), r\(_B\)-m\(_B\)-, leuB6 proA2 thi-1 araC14 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Str\(^r\)) glnV44 \(\Delta\) (mcrC-mrr)) was used for selection of transformants, and BL21 (F\(^{-}\), hsdS\(_R\)-m\(_B\)-, dcm ompT lon gal) was used for expression of recombinant HBc-derived genes. The HBc\(\Delta\)-E1 fusions, encoding RV E1 aa residues 214-240, 245-285 and 214-285 flanked with GGSGG spacers and inserted into the HBc\(\Delta\) MIR, were constructed by amplifying the appropriate RV E1 gene fragments from the plasmid pTopoXL-E1 (obtained from Dr. L. Jin, Health Protection Agency, London, UK) using the following primer pairs: (1) RV E1 aa 214 5’ (5’-CTGGATCCAGGTGGATCTG GTGGACAGCAGTCCCGGTGGGGC-3’) and RV E1 aa 240 3’ (5’-GATATTGGATCC CCTCCAGATCCACGGGAATGGCGTTGCA) for amplification of the RV E1 (214-240) fragment; (2) RV E1 aa 245 5’ (5’-CTGGATCCAGGTGGATCTG GTGGACAGCAGTCCCGGTGGGGC-3’) and RV E1...
aa 285 3’ (5’-CTGGATCCCCTCCAGATCCACCGCGCGCCTGAGAGCCTAT-3’) for amplification of the RV E1 (245-285) fragment; and (3) RV E1 aa 214 5’ (5’-CTGGATCCAGGTGGATCTGGTGGACAGCAGTCCCGGTGGGGC-3’) and RV E1 aa 285 3’ (5’-CTGGATCCCCTCCAGATCCACCGCGCGCCTGAGAGCCTAT-3’) for amplification of the RV E1 (214-285) fragment (MWG-Biotech, Ebersberg, Germany).

The obtained fragments were ligated into the BamHI (Fermentas, Lithuania) site in the I-832 vector. The I-832 vector is a modification of the pT31 polylinker vector described earlier (26). In the I-832 vector the HBcΔ encoding fragment was transferred into the better expression plasmid based on the pBR327 vector. After ligation and transformation, plasmids carrying the HBcΔ-E1 (214-285) and HBcΔ-E1 (245-285) genes were identified by analytical restriction and sequencing. A plasmid carrying the HBcΔ-E1 (214-240) gene was obtained by inserting the PCR fragment carrying E1 (214-240) sequence with adjusted BamHI sites from plasmid with the HBcΔ-E1 (214-285) gene into the BamHI-cleaved I-832 vector. All HBcΔ-derived genes were expressed under the control of the E. coli Trp promoter, which allowed a high expression level without induction.

2.2 Expression and purification of HBcΔ-E1 fusions

Transformed E. coli BL21 cells were grown overnight on a rotary shaker at 25°C or 37°C in 750-ml flasks containing 300 ml of M9 minimal medium supplemented with 1% Casamino Acids (Difco, USA) and 0.2% glucose to a final optical density of 4 to 6 (OD₅₄₀). The cells were sedimented by low speed centrifugation (10 min at 4,000 x g) and incubated on ice in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X-100.
Then, the cells were ultrasonicated at 22 kHz 7 times for 30 s each time. After ultrasonication, urea was added to 0.5 M, and incubation on ice was continued for 30 min. After clarification (30 min at 10000 x g), the supernatant was collected. The sediment was extracted step by step with 1 M urea and 2 M urea in PBS (with 50 μg/ml PMSF and 0.1% Triton X-100) and centrifuged (30 min at 10000 x g). The obtained supernatants were collected and loaded on a 20-60% (w/w) discontinuous sucrose gradient (in PBS with 0.05% Triton X-100). After centrifugation at 25,000 rpm for 20 h at 4°C (Beckman, rotor SW 28), fractions of 2 ml were collected and analyzed for protein content by SDS-PAGE. The presence of recombinant protein in the fractions was detected by SDS-PAGE and the standard Western blot procedure with monoclonal murine anti-HBc 13C9 antibody (27). To remove sucrose by buffer exchange, chimeric HBcΔ containing fractions were pooled and loaded on a Sephadex G-25 column. The presence of HBcΔ-derived VLPs was detected by Ouchterlony double radial immunodiffusion using polyclonal rabbit anti-HBc antibodies.

2.3. Construction and expression of the C-terminally truncated RV E1 (1-441) gene lacking hydrophobic domain sequences

The RV E1 protein aa 1-441 sequence was PCR-amplified by Pfu DNA polymerase using primers RV E1 5′ (5′-AGTACTAGTACAATGGAAGAAGCTTTCACCTACCTCTCTGCACCTGCAC-3′) and RV E1 3′ (5′-AGCAGCAGTTACTACCTCAGCCAGGTCTGCGGAGGTCT-3′) (MWG-Biotech, Ebersberg, Germany) and plasmid pTopoXL-E1 as a template. The resulting PCR fragment was inserted into the yeast expression plasmid pFX7-6His (28,29). The resulting expression plasmid pFX-RV-E1 was re-transformed into the S.
cerevisiae haploid strain AH22 derivative 214 (ura3 leu2 his4). Yeast transformation and cultivation was performed essentially as previously described (28,30,31). Yeast cells carrying the RV E1 (1-441) gene within the pFX7-6His plasmid were inoculated in YEPG growth medium (1% yeast extract, 2% peptone and 2% glucose) supplemented with 3 mM formaldehyde at 30°C for 24 h in a shaking incubator. Protein expression was induced by adding an equal volume of YEPG medium with 6% galactose. After a subsequent 18 h incubation at 30°C in a shaking incubator, yeast cells were harvested by centrifugation at 2,500 x g for 5 min, 8 grams of wet cells were resuspended in 24 ml of disruption buffer (20 mM PBS, pH 7.5, 2 mM EDTA, 1 mM PMSF), and an equal amount (32 grams) of glass beads (Sigma, G9268) were added. The yeast cells were disrupted by vortexing 8 times for 1 min at 4°C. The supernatant was decanted, and the glass beads were washed with disruption buffer and cleared by centrifugation at 2,500 x g for 5 min. The obtained supernatants were centrifuged at 10,000 x g for 30 min. The pellet was washed 4 times with 25 ml of washing buffer (PBS, pH 7.4, 1 mM PMSF and 1% Tween-20). After the final centrifugation (10,000 x g 20 min), the sediment was suspended in 20 ml of extraction buffer (6 M Guanidine-HCl, 100 mM Tris-HCl, pH 8.0 and 20 mM DTT) by shaking at 4°C overnight on a rotary shaker. After extraction, the insoluble material was separated by centrifugation at 10,000 x g for 30 min. Before loading onto the IMAC Ni-Superflow agarose (Qiagen, Hilden, Germany), buffer exchange was performed with a Sephadex G-25 column to replace 20 mM DTT with 5 mM β-ME and 6 M Guanidine-HCl with 8 M urea. The recombinant protein was purified by IMAC in denaturing conditions (8 M urea, 5 mM β-ME and 100 mM Tris-HCl, pH 8.0), according to the manufacturer’s instructions. The main portion of recombinant RV E1 was eluted in the IMAC elution buffer (8 M urea, 5 mM β-ME, 100 mM Tris-HCl, pH
8.0, linear gradient with 30-500 mM imidazole). Protein samples were analyzed in 12% SDS-PAGE gels. The RV E1 protein containing fractions were pooled, and purified protein was diluted to final concentration of 0.5 mg/ml. The proteins were subsequently dialyzed and refolded two times (overnight and 4-6 h) using refolding buffer 1 (RB1: 2 M urea in PBS, 0.5 M Arginine, 5 mM glutathione (reduced form), 0.5 mM glutathione (oxidized form)) and refolding buffer 2 (RB2: 1 M urea in PBS, 0.5 M Arginine, 5 mM glutathione (reduced form), 0.5 mM glutathione (oxidized form)) and then 1 M urea in PBS. The protein concentration was determined using the Bradford method.

2.4. Electron microscopy

VLPs in suspension were adsorbed to carbon-Formvar coated copper grids and were negatively stained with a 1% uranyl acetate aqueous solution. The grids were examined with a JEM-1230 electron microscope (Jeol Ltd., Tokyo, Japan) at 100 kV.

2.5. Detection of the particle size in solution

The size of particles was detected by a dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments Ltd, UK) instrument and by gel filtration on a self-packed Omnifit column 6.6MM/400MM with Sepharose 4 Fast Flow and Superdex 75 HR 10/30 columns in a Shimadzu Prominence HPLC instrument with a PDA detector. On the Sepharose 4 Fast Flow column (12 ml) with a flow rate of 0.3 ml/min, aggregates, VLPs, and dimers appeared in 19-23, 26-32, and 35-50 min,
respectively. On the Superdex 75 column (24 ml) with flow rate of 0.5 ml/min, aggregates and VLPs appeared in 15-18 min, and dimers appeared in 19-24 min.

2.6. Mice and immunization schemes

Six groups of 6-8-week-old female BALB/c (H-2d) mice, 4 mice per group, weighing 18-20 g were obtained from the Latvian Experimental Animal Laboratory of Riga Stradins University and held at the Latvian Biomedical Research and Study Centre. Three immunization schemes were used: (i) 25 μg of the appropriate antigen diluted in PBS (0.2 ml) inoculated subcutaneously; (ii) 25 μg of the appropriate antigen diluted in PBS and Alhydrogel (250 μg; Brenntag Biosector, Denmark) in a total volume of 0.5 ml inoculated intraperitoneally; and (iii) 25 μg of the appropriate antigen diluted in PBS and CFA for the 1st injection and IFA for the 2nd and 3rd injections, at a 1:1 ratio (Sigma, USA) in a total volume of 0.2 ml, were inoculated subcutaneously. Immunization was performed at days 0, 14 and 28. Animals were bled 42 days after the first immunization. Control animals were immunized with the RV E1 (1-441) protein using the same time and dose schedule as the experimental groups.

2.7. Direct ELISA for detecting the antigenicity and immunogenicity of chimeric proteins

To assay for protein antigenicity, HBcΔ-E1 (214-285), HBcΔ-E1 (214-240), HBcΔ-E1 (245-285), HBc and RV E1 (1-441) were adsorbed overnight at 4°C to 96-well plates (Nunc, USA) at 10 μg/ml in 50 mM sodium carbonate buffer, pH 9.6. After blocking with phosphate buffered saline (PBS) containing 1% BSA for 1 h at 37°C,
serial dilutions of the anti-RV E1 (1-441) Ab mouse sera or human serum containing IgG antibodies to the RV Antigen (Enzygnost Anti-Rubella-Virus/IgG (Rub/IgG), OWBF 15, Siemens Healthcare, Marburg, Germany) were added to the plates and were incubated for an additional 1 h at 37°C. After washing 3 times with PBS containing 0.05% Tween-20, a horseradish peroxidase-conjugated anti-mouse antibody (Sigma, USA) or anti-human IgG/POD conjugate was applied, according the manufacturer’s instructions. Following 1 h of incubation at 37°C, the plates were washed and the substrate OPD (Sigma, USA) or the chromogen working solution was added for color development. Absorbance values were measured using an automatic reader (Multiscan, Sweden) at 492 nm or 450 nm, depending on the substrate used.

To assay for protein immunogenicity, full length HBc (1-183) or RV E1 (1-441) were adsorbed to the plates as described above, and serial dilutions of the sera from the immunized mice were investigated. All of the ELISA steps were performed as described above. The optical density was checked using an automatic reader at 492 nm. The end-point titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of the negative control sera derived from preimmunized mice.

2.8. Commercial anti-RV test

The enzyme immunoassay for the qualitative detection of IgG antibodies to the inactivated RV antigen was performed using the human antibody ELISA kit (Enzygnost Anti-Rubella-Virus/IgG (Rub/IgG), OWBF 15, Siemens Healthcare, Marburg, Germany). The results were calculated as described above in the paragraph 2.7.
2.9. Detection of IgG isotypes

The IgG1 and IgG2a subsets in the sera of immunized mice were detected using an isotype-specific ELISA with the mouse monoclonal antibody isotyping reagent (ISO-2, Sigma, USA) and anti-goat/sheep IgG peroxidase conjugate (Sigma, USA). The data were expressed as the antibody titer representing the highest dilution of the immunized animal sera that yielded three times the OD of the preimmunization sera.

3. Results

3.1. Structure and self-assembly of VLPs carrying the RV E1 epitopes

The RV E1 fragment, 214-285, overlapping with the RV-neutralizing epitope and the RV E1 fragment halves 214-240 and 245-285 were inserted into the MIR of the HBcΔ carrier using insertions with flanking spacers (Table 1). All three variants expressed VLPs in bacterial cell lysates (Fig. 2A, C and E). Stepwise extraction of the lysates with urea showed that the HBcΔ-E1 (245-285) variant appeared in the 0.5 M urea extract, whereas the HBcΔ-E1 (214-285) and HBcΔ-E1 (214-240) variants required 1 M urea to begin solubilization and were maximally extracted in 2 M urea. The appropriate urea extract fractions were loaded onto a sucrose gradient, which were prepared in PBS with 0.05% Triton X-100 (Fig. 3). Using a sucrose gradient in the same buffer with 2 M urea did not improve the resolution of proteins (data not shown).

In the sucrose gradient, only HBcΔ-E1 (245-285) in 0.5 M urea accumulated in a band corresponding to the VLP position in the gradient (Fig. 3C). HBcΔ-E1 (214-
HBcΔ-E1 (214-240) and a 2 M urea fraction of HBcΔ-E1 (245-285) accumulated primarily in dimer fractions (Fig. 3A, B and D, respectively). Presence of the appropriate covalent and non-covalent dimeric forms of the HBcΔ derivatives in the above-mentioned dimer-containing fractions was confirmed by SDS-PAGE in the absence of β-mercaptoethanol and by size exclusion chromatography on a Superdex 75 column (not shown). As confirmed by electron microscopy, only HBcΔ-E1 (245-285) retained correct VLPs after purification (Fig. 2F), whereas the HBcΔ derivative dimer-containing fractions contained mostly aggregated non-symmetric irregular structures (Fig. 2B, D and G).

To maintain the consistency of proteins within solutions over time, the samples, which have been stored in 50% glycerol at -20°C after purification, were tested by SEC (size-exclusion chromatography) and DLS (dynamic light scattering) techniques at immunization day (Fig. 4), and tested again at after 14 days of storage when needed for boosters. The latter results did not differ significantly (not shown). Recombinant HBc VLPs, which were produced in E. coli cells, were used as a control.

An SEC analysis of the purified samples was performed using Sepharose 4 Fast Flow (Fig. 4A-E) and Superdex 75 columns (data not shown). The analysis confirmed the expected predominance of dimers in the HBcΔ-E1 (214-285) and HBcΔ-E1 (214-240) preparations and the predominance of aggregates and VLPs in the HBcΔ-E1 (245-285) VLP fraction. However, the HBcΔ-E1 (245-285) dimer fraction contained a remarkably high percentage of VLPs, which strongly suggests that the HBcΔ-E1 (245-285) dimers are organized into VLPs during storage. The HBc preparation showed only one major peak with a retention time of 25 min, which corresponds to HBc VLPs (Fig. 4E).
Direct measurement of the particle size in solution using the DLS instrument revealed particles with high mean diameters from 150 to 900 nm for all four preparations (Fig. 4F-I). Two different aggregated non-VLP associations occurred in the protein sample obtained from the HBcΔ-E1 (214-285) dimer fraction: a larger 800-900 nm aggregate and a smaller 140-150 nm aggregate. Similarly, a larger 500-600 nm aggregate and a smaller 100-120 nm aggregate were observed using the protein sample obtained from the HBcΔ-E1 (214-240) dimer fraction. Both HBcΔ-E1 (245-285) VLP and dimer preparations demonstrated in solution aggregated VLP material with a mean diameter of 280 nm and 150 nm, respectively. Contrary, the HBc preparation showed diameters of 32-35 nm, which are close to the predicted HBc \( T=3 \) and \( T=4 \) values (Fig. 4J).

To determine whether temperature affects the stability of HBcΔ-derived VLPs in vivo, cultivation of appropriate bacterial producers was performed not only at 37°C but also at 25°C. No increase in VLP production was observed (data not shown).

3.2. Purification and refolding of the C-terminally truncated RV E1 (1-441) protein

The C-terminally truncated RV E1 (1-441) protein was purified from yeast and appeared as insoluble aggregates. After the addition of 8 M urea without DTT, the bulk of the recombinant protein remained insoluble. Decreasing the concentration of urea from 8 M by stepwise dialysis (8 M – 4 M – 2 M – 1 M, in PBS without urea) was unsuccessful because the solution became turbid at 2 M urea and the recombinant protein precipitated. Because the recombinant RV E1 (1-441) protein contained 20 Cys residues and at least 16 of them are likely linked by forming intramolecular Cys-Cys bonds (32-34), protein refolding with a glutathione red/ox pair in the presence of
0.5 M arginine was performed (35). Stepwise dialysis and refolding resulted in recombinant RV E1 protein that was soluble in PBS supplemented with 1 M urea.

3.3. Antigenicity of chimeric proteins: recognition of the inserted epitopes by anti-RV antibodies

Direct ELISA was used to determine the antigenicity of the inserted RV E1 epitopes on the surface of chimeric proteins. Microtitration plates were coated with the recombinant HBcΔ-E1 proteins, and human IgG sera to RV (Fig. 5A) or polyclonal murine anti-RV E1 antibodies (Fig. 5B) were used as detection agents. All of the chimeric proteins were recognized by both anti-RV sera. There was a clear difference between the negative control and the positive control using HBc particles coated on plates. In both ELISA variants, antigenicity was evident for all of the chimeras including HBcΔ-E1 (214-240), which demonstrated the lowest level of anti-RV recognition.

3.4. Humoral response in mice

3.4.1. Anti-HBc antibodies

First, the antibody response to the HBc carrier was manifested. The presence of anti-HBc antibodies in the serum was monitored using a direct ELISA test on two available HBc antigens produced in S. cerevisiae and E. coli to control for the possible effect of E. coli protein contamination of antigens used for immunization and adsorption on ELISA plates. Nevertheless, titration on E. coli and S. cerevisiae HBc-coated plates showed very similar results (data not shown). Two weeks after the 3rd
immunization, the anti-HBc antibody response (Fig. 6A) demonstrated a clear dependence first, on the ability to form VLPs, and second, on the method of formulation of the chimeric proteins. HBc\(\Delta\)-E1 (245-285) (Fig. 6A, column 3) was more immunogenic in correct VLP form than the corresponding capsomeric HBc\(\Delta\)-E1 (245-285) fraction (Fig. 6A, column 4) or the HBc\(\Delta\)-E1 (214-285) and HBc\(\Delta\)-E1 (214-240) non-structured aggregates formed by dimers in all three adjuvant formulations. All three proteins induced relatively low anti-HBc antibody response after immunization in PBS buffer without adjuvant formulation (Fig. 6A). HBc\(\Delta\)-E1 (245-285) in the VLP form demonstrated a much higher immunogenicity using a CFA/IFA formulation than the other two non-structured aggregates formed by dimers (Fig. 6, columns 1 and 2). Compared to the CFA/IFA formulation, Alhydrogel formulation resulted in only a 1.2-1.5 times lower anti-HBc antibody response, except for HBc\(\Delta\)-E1 (214-240), which demonstrated the same immunogenicity in both Alhydrogel and CFA/IFA formulation variants.

3.4.2. Anti-RV antibodies

To detect anti-RV antibodies, two independent test systems were used: (i) a direct ELISA using plates coated with recombinant RV E1 (1-441) protein purified by our lab from yeast and (ii) the commercial ELISA-based RV diagnostic test containing all of the structural RV proteins. Because the commercial ELISA test was developed to test for human antibodies in patients’ sera, we substituted the detecting anti-human IgG antibodies with anti-mouse IgG antibodies in our test. In all cases, the ELISA test using the recombinant RV E1 (1-441) protein was more sensitive than the commercial one.
As shown in Fig. 6B, a remarkably high anti-RV response was obtained in BALB/c mice immunized with HBcΔ derivatives harboring the RV E1 epitope 214-240 (column 2). However, the titers of anti-RV antibodies induced by the HBcΔ-E1 (245-285) protein were much higher and were dependent on the structural state of the chimeric protein: VLPs showed much higher activity than the corresponding dimer aggregates (Fig. 6B, columns 3 and 4) in all three formulation variants. Surprisingly, the HBcΔ-E1 (214-285) protein carrying the full-length RV E1 fragment 214-285 elicited a comparatively low level of specific antibodies (Fig. 6B, column 1), which was comparable with the anti-RV E1 antibody level induced by the recombinant RV E1 (1-441) protein from yeast (Fig. 6B, column 5).

CFA/IFA formulation led to the highest anti-RV E1 titers with essentially all of the immunized proteins. Nevertheless, Alhydrogel formulation also significantly improved the anti-RV E1 response and level of induced antibodies. The latter was only slightly different from the CFA/IFA formulation.

In general, the anti-RV titers obtained after immunization with HBcΔ-E1 (214-240) were high compared to the anti-carrier titers when proteins were formulated with adjuvants but not diluted in PBS. When immunized in PBS, the titer of anti-RV antibodies was lower than that of the anti-HBc antibodies (1:3,617 and 1:11,500, respectively). In Alhydrogel and CFA/IFA formulations, the anti-RV E1 titers were very similar to the anti-HBc titers, e.g., 1:31,538 and 1:50,000 of anti-RV compared to 1:41,000 and 1:40,000 of anti-HBc, respectively.

In contrast to HBcΔ-E1 (214-240), the HBcΔ-E1 (214-285) chimera carrying the full-length E1 fragment 214-285 did not induce a high anti-RV E1 titer with either adjuvant formulation. The anti-RV E1 titers were 1:7,788 and 1:10,000 when
formulated in Alhydrogel and CFA/IFA, respectively, whereas the anti-HBc antibody titers were much higher at 1:28,000 and 1:54,225, respectively. HBcΔ-E1 (245-285) displayed a difference in immunogenicity between VLPs and non-structured aggregates formed by dimers. Chimeric VLPs induced not only a higher anti-HBc antibody response but also a higher anti-RV E1 response with all formulation variants. The observed anti-HBc and anti-RV E1 antibody titers showed similar levels of 1:86,667 and 1:95,000, respectively; therefore, the CFA/IFA formulation induced both titers to $10^4$. HBcΔ-E1 (245-285) VLPs in PBS induced a titer of anti-RV E1 antibodies that was similar to the highest titer of the corresponding non-structured aggregates formed by dimers and formulated in CFA/IFA, 1:26,000 and 1:31,000, respectively (Fig. 6B, columns 3 and 4). These results showed the critical importance of the VLP structure for the induction of maximal antibody titers.

Surprising results were obtained using the commercial ELISA diagnostic test containing all structural RV proteins to test the ability of our murine antibodies to recognize native RV proteins (Fig. 6C). HBcΔ-E1 (214-240) induced more anti-RV E1 antibodies than the other chimeras. The anti-RV E1 antibody titer induced by HBcΔ-E1 (214-240) in CFA/IFA was 3 times higher than the titer in Alhydrogel formulation, 1:6,000 and 1:1,800, respectively (Fig. 6C, column 2). In contrast, the HBcΔ-E1 (214-285) protein, which contained the same 214-240 epitope within a longer E1 sequence, did not induce a substantial anti-RV antibody response (Fig. 6C, column 1). HBcΔ-E1 (245-285), even in the VLP form, induced a remarkably high level of anti-RV E1 antibodies, although lower than the HBcΔ-E1 (214-240) protein (Fig. 6C, column 3).

3.4.3. Immunization with recombinant RV E1 (1-441) protein
To assay the effect of the HBcΔ carrier on the anti-RV E1 response, BALB/c mice were immunized with purified recombinant RV E1 (1-441) protein as a control (Fig. 6B, column 5). The observed anti-RV antibody titers were relatively low in all immunized mice groups, including PBS, Alhydrogel, and CFA/IFA formulations. The observed anti-RV E1 antibody titers were 1:273, 1:9,025, and 1:11,000, respectively. Therefore, the titers of anti-RV E1 antibodies induced by the recombinant RV E1 (1-441) protein were approximately 5-10 times lower than the titers induced by the most active HBcΔ-carried RV E1 fragments injected into mice with PBS or Alhydrogel. Protein HBcΔ-E1 (214-285) formulated in Alhydrogel and CFA/IFA was the exception; the titers of anti-RV E1 antibodies were the same as for recombinant RV E1 (1-441), e.g., 1:7,788 and 1:9,025, and 1:10,000 and 1:11,000, respectively.

3.4.4. IgG1/IgG2a isotype ratio

The chimeric proteins induced different distributions of IgG1 and IgG2a antibody isotypes. The anti-HBc IgG isotype distribution, predominantly the IgG1 isotype, was similar within each protein immunization group in all three formulation variants (Fig. 7A), whereas the distribution differed for the anti-RV E1 antibodies (Fig. 7B). After immunization in PBS or Alhydrogel, IgG1 was the predominant isotype with a very low level of accompanying IgG2a. During formulation in CFA/IFA, the IgG1/IgG2a ratio was lower. As shown in Fig. 7A, the IgG1/IgG2a ratio ranged from 3.2 (HBcΔ-E1 (214-285)) to 10.5 (HBcΔ-E1 (214-240)). The Alhydrogel adjuvant induced more IgG1 isotype antibodies with an IgG1/IgG2a ratio from 7.0 (RV E1 (1-441)) to 22.0
(HBcΔ-E1 (214-240) and HBcΔ-E1 (214-285)). Therefore, although IgG1 isotype was predominant in all three adjuvant formulations, CFA/IFA stimulated more IgG2a isotype antibodies than PBS and Alhydrogel formulations.

4. Discussion

It is generally accepted that VLPs are one of the most preferred modern carrier candidates for the construction of advanced genetically engineered vaccines (for review see (17,36-38)). VLP-based vaccines against hepatitis B and human papilloma viruses are currently accepted and widely used, whereas VLP-based vaccine candidates against malaria, HIV/AIDS, hepatitis C, human and avian influenza, and other infectious and non-infectious diseases are situated at different evaluation stages. Repetitive and symmetric exposition of epitopes on the surface of VLPs, which makes them highly immunogenic, is regarded as a major advantage for the VLP approach to vaccine construction. To present foreign epitopes in a native fashion on the outer surface of VLPs, two major strategies are currently used: (1) insertion of DNA copies of the epitopes at selected sites in the VLP genes and (2) chemical coupling of epitope peptides to the VLP surface (for review see (17,36-38)). VLPs are likely to provide the inserted foreign protein fragments with a high induction capacity for B- and T-helper cell activity during immunization. Peptides exposed on the VLP surface far surpass the immunogenicity induced by the same
sequences located in the non-assembled peptides or the low-molecular-mass carrier proteins (for review see (16,36-38)).

However, due to the different stabilities of the chimeric VLPs, the latter may be purified as dimers, which are converted into non-symmetric high-molecular-mass aggregates. Here, we observed the spontaneous formation of such aggregates from dimers after purification of some of the chimeric HBe-based proteins.

The RV E1 fragment 214-285, which is associated with virus-neutralizing activity (7-11), and each half, 214-240 and 245-285, were inserted into the HBe MIR, which is located on the tips of the HBe spikes and represents the most protruding region on the HBe VLP surface (23,25). HBeΔ-E1 (245-285) produced a VLP form, and HBeΔ-E1 (214-285) and HBeΔ-E1 (214-240) produced dimers that converted spontaneously into large aggregates after purification. Both of the latter constructs produced small amounts of the correct VLP structures in bacterial lysates but were purified by sucrose gradient centrifugation as dimers. However, electron microscopy and DLS analysis revealed a constant aggregation process in dimer fractions, including dimers aggregating into VLP-forming HBeΔ-E1 (245-285) products. Moreover, the HBeΔ-E1 (245-285) VLPs aggregated into 280 nm particles in solution unlike the initial HBe VLPs.

VLP-forming HBeΔ-E1 (245-285) products demonstrated a high anti-RV immunogenicity. Surprisingly, the 214-240 epitope within the HBeΔ-E1 (214-240) aggregates induced relatively high anti-RV E1 antibody titers. In contrast, the immunization of mice with recombinant carrier-less RV E1 (1-441) from yeast, where the 214-240 epitope is in its endogenous location, resulted in low titers of anti-RV antibodies. A possible explanation for this phenomenon may be due to features of the recombinant RV E1: the recombinant RV E1 (1-441) protein contains 20 Cys
residues, and at least 16 are likely form intramolecular Cys-Cys bonds (32-34).

Although, we had no direct evidence that, after the refolding process, the obtained soluble RV E1 (1-441) protein was organized with the correct intramolecular Cys-Cys bonds. Therefore, the HBcΔ carrier was able to provide foreign epitopes with a high immunological capacity not only in the form of VLPs but also as the high-molecular-mass aggregates.

The remarkably high capacity of protein aggregates to enhance immune responses to the monomeric form of proteins is described (for review see (39)). For example, the high immunologic activity of recombinant dengue 2 virus envelope glycoprotein aggregates in combination with Alhydrogel adjuvant was demonstrated (40). However, surprisingly little is known about the nature of the aggregate species responsible for such effects. Like HBc VLPs, aggregates may retain elements of arrayed structure and ability to induce antibody responses, even in the absence of T-cell activity. Their potency may be due to the ability of multivalent protein species to extensively cross-link the B-cell receptor, which (1) activates B-cells proliferation via Bt kinases and (2) targets protein to class II major histocompatibility complex (MHC)-loading compartments, efficiently eliciting T-cell activity for antibody responses (39). As a result, early and rapid IgM responses may be generated against aggregates before T-cell activation, similar to VLPs. Unlike polysaccharide antigens, which are not capable of recruiting T-cells, B-cell receptor aggregation would be expected to accelerate the recruitment of T-helper cells (39).

The role of the HBc carrier in the induction of a strong anti-RV humoral response is unknown, but the carrier contains a set of major and minor strong T-cell epitopes, which may specifically recruit T-cells to inserted RV E1 B-cell epitopes and strongly enhance the anti-RV response, as suggested by Bachmann and Dyer (36). Our results
show that this carrier enhances the activity of not only VLPs but also aggregates. The possibility that neo-epitopes appearing in the chimeric proteins as a combination of the carrier and insertion epitopes may enhance the generation of the immune response to aggregates cannot be excluded (39).

In addition to the VLP or aggregate state, the structure and correct composition of epitopes may also play a role in the induction of immunological response. When the 214-240 epitope was prolonged with further E1 sequence to position 285 within the full-length 214-285 fragment, the anti-RV antibody titers dropped significantly despite the aggregation of the HBcΔ-E1 (214-285) products into structures similar to the HBcΔ-E1 (214-240) proteins.

On the other hand, the phagocytic activity of antigen-presenting cells toward complex antigens depends on antigen size. High-molecular-mass aggregates could be less efficient than smaller VLPs during APC processing, but the HBcΔ-E1 (214-240) harboring potential virus-neutralizing epitope (7-10) is processed well, regardless of its aggregate status.

Although the VLP form of the chimeric protein HBcΔ-E1 (245-285) can induce the immune system to produce large amounts of anti-RV E1 antibodies that recognize the recombinant RV E1 (1-441) antigen, less recognition was observed on microtitration plates coated with native RV antigens. In contrast, the 214-240 epitope within the HBcΔ-E1 (214-240) aggregates induced lower levels of anti-RV E1 antibodies, but recognition of the latter by the native RV antigen was higher. Nevertheless, the ability of these anti-RV antibodies to be recognized in the native RV test is a good indication of the potential use of HBc-derived chimeras in vaccine development.

After choosing the correct epitope and location of the chimeric product, the success of a putative vaccine lies in the choice of the best adjuvant for immunization. Although
HBcΔ-E1 (245-285) VLPs were able to induce an anti-RV antibody response without an adjuvant, a better effect was achieved with Alhydrogel, and the highest anti-RV E1 antibodies were obtained by the CFA/IFA formulation. Because Alhydrogel is approved for human immunization (41), HBc-based RV E1 (245-285) VLPs are a prospective candidate for the generation of a novel RV vaccine. Moreover, chimeric protein can be obtained from *E. coli* by simple and efficient purification procedures, as described here. Nevertheless, direct evaluation of virus-neutralizing efficacy of the induced anti-E1 antibodies is needed to aid in the selection of the optimal vaccine candidate for further evaluation.

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References


highly pure nucleocapsid proteins of different hantaviruses can be generated in the yeast *Saccharomyces cerevisiae*. J. Biotechnol. 111:319-333.


Table 1. Detailed structure of recombinant RV E1 fragments-containing proteins. RV E1 epitopes selected for insertion into HBc vectors are marked by highlighting (214-285) and underlining (214-240 and 245-285) on the RV E1 (1-441) sequence. Cys residues are highlighted red within the inserted RV E1 sequences. HBcΔ is derived from the HBV320 genome, genotype D, subtype ayw [42].

<table>
<thead>
<tr>
<th>Designation of</th>
<th>Elements of the protein primary structure: from the N- to C-terminus</th>
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<tbody>
<tr>
<td>proteins</td>
<td>Hbc vector</td>
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<tr>
<td>HbcΔ-E1 (214-285)</td>
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<tr>
<td>HbcΔ-E1 (214-240)</td>
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<tr>
<td>HbcΔ-E1 (245-285)</td>
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<tr>
<td>RV E1 (1-441)</td>
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Figure Legends

Fig. 1. General construction scheme for the chimeric HBcΔ-derived RV E1 fragment-containing protein-encoding genes. Gene boxes are drawn to scale (in aa residues). The aa numbers are shown for the HBcΔ vector with the RV E1 fragment aa residues in italics. Spacers around the insertions are shown as bold black lines.

Fig. 2. Electron microscopy of the chimeric HBcΔ-E1 proteins before and after purification by sucrose gradient centrifugation: HBcΔ-E1 (214-285) in lysates (A), fractions 16-18 (B), HBcΔ-E1 (214-240) in lysates (C), fractions 16-17 (D) and HBcΔ-E1 (245-285) in lysates (E), fractions 6-9 (F) and 16-18 (G). Bar scale, 50 nm.

Fig. 3. Distribution of chimeric HBcΔ-E1 proteins after sucrose gradient centrifugation. The sucrose gradient fractions were separated on SDS-PAGE and stained with Coomassie Blue R-250. The fractions were numbered from the bottom to the top of centrifuge tubes. The location of collected VLP- and dimer-containing fractions is depicted by brackets. (A) HBcΔ-E1 (214-285) (fractions 16-18 correspond to the immunization group 1); (B) HBcΔ-E1 (214-240) (fractions 16-17 correspond to the immunization group 2); (C) HBcΔ-E1 (245-285) (fractions 6-9 correspond to the immunization group 3) and (D) HBcΔ-E1 (245-285) (fractions 16-18 correspond to the immunization group 4). In A, B and D, 2 M urea extracts were loaded on sucrose gradients, whereas a 0.5 M urea extract was loaded on a sucrose gradient in C. (E) SDS-PAGE of purified proteins used for immunization: 1) HBcΔ-E1 (214-285), 2) HBcΔ-E1 (214-240), 3) HBcΔ-E1 (245-285), VLPs; 4) HBcΔ-E1 (245-285), purified as dimers, which then aggregated into non-VLP aggregates after purification; and 5) RV E1 from yeast, refolded.
Fig. 4. The size of particles in the purified samples measured by SEC (size-exclusion chromatography) and DLS analysis. In the left panel (A-E), SEC Sepharose 4 Fast Flow was used. The retention time is indicated on the x-axis and mAU (milli-absorbance unit) is indicated on the y-axis. In the right panel (F-J), the results of the DLS size distribution are shown with the particle diameter in nm on the x-axis and the number of particles in % on the y-axis: (A and F) HBcΔ-E1 (214-285); (B and G) HBcΔ-E1 (214-240); (C and H) HBcΔ-E1 (245-285), VLPs; (D and I) HBcΔ-E1 (245-285), purified as dimers and (E and J) HBcAg. Brackets indicate SEC elution times of aggregates, VLPs, and dimers.

Fig. 5. Comparison of the antigenicity of chimeric HBcΔ-derived proteins by direct ELISA. Recombinant proteins (1-5) were coated on the plates, and Human serum containing IgG antibodies to the RV antigen (A) or mice serum containing anti-RV E1 antibodies (B) were added, as described in Materials and Methods. Recombinant HBc (1-183) (6) was used as a negative control. Human IgG sera to the RV antigen from a commercial kit (see in 2.8) was used as positive control and the absorbance at 450 nm was 0.91.

Fig. 6. The anti-HBcΔ carrier and anti-RV E1 antibody responses induced by chimeric HBcΔ-derived proteins and by recombinant RV E1 (1-441) protein at day 42, after BALB/c mice immunizations. Serum IgG antibodies were tested using ELISAs for specific binding to HBc (A) and recombinant RV E1 (1-441) (B) and were tested using the commercial anti-RV test (C). Formulation of the chimeric proteins for immunization in PBS, Alhydrogel, or CFA/IFA was distinguished by different colored bars. The following proteins were used for immunization: HBcΔ-E1
(214-285), sucrose gradient fractions 16-18 (1); HBcΔ-E1 (214-240), sucrose gradient fractions 16-17 (2); HBcΔ-E1 (245-285) VLPs, sucrose gradient fraction 6-9 (3) and purified as dimers 16-18 (4); and refolded recombinant RV E1 from yeast (5).

Fig. 7. IgG1/IgG2a isotype ratios for antibodies induced by the chimeric HBcΔ-derived proteins and recombinant RV E1 (1-441) proteins at day 42, after the BALB/c mice immunization with the appropriate proteins, as shown in Fig. 6.