Three variants of the major rubella virus (RV) E1 protein virus-neutralizing epitope from position 214 to 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. 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 to 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.

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 a review, see reference 1). One of the most widely used RV vaccines, Meruva, is a live attenuated vaccine that was propagated using the human cell line WI-38, which was derived from embryonic lung tissue in 1961 (2, 3), and is used as a component of the MMR vaccine (for a review, see reference 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 to 239 (7, 8), 213 to 239 (9), and 214 to 240 (10). Three additional neutralizing and hemagglutination epitopes have been identified within the E1 glycoprotein between residues 245 and 285 (11). Therefore, these E1 protein epitopes may have potential not only in diagnostics but also in the development of vaccines against RV infection (12).

The hepatitis B virus (HBV) core (Hbc) protein was first reported as a promising virus-like particle (VLP) carrier in 1986 (13), and this 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 reviews, see references 16, 17, 18, and 19).

Hbc protein spontaneously forms dimeric units (20, 21), which self-assemble in HBV-infected eukaryotic cells by an 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 with diameters of 35 and 32 nm, respectively (23, 24).

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 amino acids (aa) 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 surfaces of VLPs (for reviews, see references 16, 17, 18, and 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 reviews, see references 16, 17, 18, and 19).

Here, we selected the RV E1 protein fragment from aa 214 to 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 to 240 and aa 245 to 285. Although all three fragments allowed VLP self-assembly in bacteria, only HBcΔ-E1(245-285) was able to retain the 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 HBcΔ-E1(245-285), purified HBcΔ-E1(214-285) and HBcΔ-E1 (214-240), which appeared as non-VLP aggregates of the appropriate HBcΔ-E1 dimers, induced significant anti-RV E1 antibody levels in immunized mice.

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MATERIALS AND METHODS
Construction of recombinant HBcΔ-E1 genes. The general scheme for the HBcΔ-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.

Escherichia coli strain RR1 [F− rpsL− mcrC-mrr− lacY1 galK2 xyl-5 mtl-1 rpsL20] was used for expression of recombinant HBc-derived genes.

The HBcΔ-E1 fusions, encoding RV E1 residues 214 to 240, 245 to 285, and 214 to 285 flanked with GSGG spacers and inserted into the HBcΔ MIR, were constructed by amplifying the appropriate RV E1 gene fragments from the plasmid pTopoXL-E1 (obtained from L. Jin, Health Protection Agency, London, United Kingdom) using the following primer pairs: (i) RV E1 aa 214 5′-CTGGATCCAGGTGGATCTGGTGAGACAAGCATCGGTTGCGG-3′ and RV E1 aa 240 3′-GGTGAGACACCGCGAGTACCGTGGACTTGTGGGAGCTACGCCA-5′ for amplification of the RV E1(214-240) fragment, (ii) RV E1 aa 245 5′-CTGGAGACACCGCGAGTACCGTGGACTTGTGGGAGCTACGCCA-3′ and RV E1 aa 285 3′-GGTGAGACACCGCGAGTACCGTGGACTTGTGGGAGCTACGCCA-5′ for amplification of the RV E1(245-285) fragment, and (iii) RV E1 aa 214 5′-CTGGATCCAGGTGGATCTGGTGAGACAAGCATCGGTTGCGG-3′ and RV E1 aa 285 3′-GGTGAGACACCGCGAGTACCGTGGACTTGTGGGAGCTACGCCA-5′ for amplification of the RV E1(214-285) fragment.

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 at 540 nm (OD540) of 4 to 6. The cells were sedimented by low-speed centrifugation (10 min, 4,000 × 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. The cells were then ultrasonicated at 22 kHz 7 times for 30 s each time. After ultrasonication, urea was added to 9.5 M, and incubation on ice was continued for 30 min. After clarification (30 min at 10,000 × g), the supernatant was used for the next step.

TABLE 1 Detailed structure of recombinant RV E1 fragment-containing proteins

| Protein designation | Elements of the protein primary structure (from the N to the C terminus) | HBcΔ vector | Left spacer | Inserted RV E1 sequence | Right spacer | HBcΔ vector | C-terminal linker |
|---------------------|---------------------------------------------------------------------|-------------|-------------|------------------------|-------------|-------------|-----------------|
| HBcΔ-E1(214-285)    | GGSGG                  | 1-79        | GSGG        | QSQRWGLGSNPCCHGPWDASPVQHRHSPDCSRLVGATPER | PRLRLVDADDPLLLRTAPGPGEVWVTIPvGpQaR | GSGG        | 78-144          | ISLGPGLEKLEFG |
| HBcΔ-E1(214-240)    | GGSGG                  | 1-79        | GSGG        | QSQRWGLGSNPCCHGPWDASPVQHRHSP | PRLRLVDADDPLLLRTAPGPGEVWVTIPvGpQaR | GSGG        | 78-144          | ISLGPGLEKLEFG |
| HBcΔ-E1(214-240)    | GGSGG                  | 1-79        | GSGG        | QSQRWGLGSNPCCHGPWDASPVQHRHSP | PRLRLVDADDPLLLRTAPGPGEVWVTIPvGpQaR | GSGG        | 78-144          | ISLGPGLEKLEFG |

a The RV E1(1-441) sequence is EAAFYTLCTAPGCATQPVPVRAGVFESKIVDGCGFAPWDEATGACIEPITDVSCGELGAWP7PACARIWNGTQACFIVNAYNSGGYQLASYPNGPSYKQYQHTAC0EVFAGEHISA1CWGEPTIDTVSMVFAASLIIIQHPKTVRVKHTETRTVWQLSVAAGSNVTE0EHCNTHPQHGLEQYVPPDGPGLVEINMHTGNNQSQRSWGLGSNPCCHGPWDASPVQHRHSPDCSRLVGATPER | PRLRLVDADDPLLLRTAPGPGEVWVTIPvGpQaR | GSGG        | 78-144          | ISLGPGLEKLEFG |

b Cys residues within the inserted RV E1 sequences are in bold.
collected. The sediment was extracted step by step with 1 M urea and 2 M urea in phosphate-buffered saline (PBS) (with 50 μg/ml PMSF and 0.1% Triton X-100) and centrifuged (30 min at 10,000 × g). The obtained supernatants were collected and loaded on a 20 to 60% (wt/wt) discontinuous sucrose gradient (in PBS with 0.05% Triton X-100). After centrifugation at 25,000 rpm for 20 h at 4°C (Beckman SW 28 rotor), 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 rabbit anti-HBc antibodies. The resulting expression plasmid, pFX7-6His was further eluted in the IMAC elution buffer (8 M urea, 5 mM imidazole and 6 M guanidine-HCl with 8 M urea). The resulting expression plasmid, pFX7-6His, was transformed into the C-terminally truncated RV E1 (1-441) gene lacking hydrophobic domain sequences. The RV E1 protein sequence from aa 1 to 441 was PCR amplified with Pfu DNA polymerase using primers RV E1 5′ (5′-AGTACTAGTACATTGGAGAGCCTTACGATCCTGCAATCAGC-3′) and RV E1 3′ (5′-AGCAGTTTCTTACATCGCCCATC-3′) (MWG-Biotech, Ebersberg, Germany) and plasmid pTopoXL-E1 as a template. The resulting PCR fragment was inserted into the yeast expression plasmid pYEX7-6His (28, 29). The resulting expression plasmid, pEX-RV-E1, was transformed into the Saccharomyces cerevisiae haploid strain AH22 derivative 214 (ura3-1 leu2 his4). Yeast transformation and cultivation were performed essentially as previously described (28, 30, 31). Yeast cells carrying the RV E1 (1-441) gene within the pYEX7-6His 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 of incubation at 30°C in a shaking incubator, yeast cells were harvested by centrifugation at 2500 × g for 5 min, 8 g of wet cells was resuspended in 24 ml of disruption buffer (20 mM PBS [pH 7.5], 2 mM EDTA, 1 mM PMSF), and an equal amount (32 g) of glass beads (Sigma, G9268) was 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 2500 × g for 5 min. The obtained supernatants were centrifuged at 10,000 × 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 × 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 dithiothreitol [DTT]) by shaking at 4°C overnight on a rotary shaker. After extraction, the insoluble material was separated by centrifugation at 10,000 × g for 30 min. Before loading onto immobilized-metal affinity chromatography (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 imidazole.

PAGE and the standard Western blot procedure with monoclonal murine anti-HBc 13C9 antibody (27). To remove sucrose by buffer exchange, continuous sucrose gradient (in PBS with 0.05% Triton X-100) was centrifuged at 25,000 rpm for 20 h at 4°C (Beckman SW 28 rotor), fractions of 2 ml were collected and analyzed for protein content by SDS-PAGE. The resulting fractions 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 endpoint 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.

To assay for protein immunogenicity, full-length HBC (1-183) or RV E1 (1-441) was 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 measured using an automatic reader 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 the negative-control sera derived from preimmunized mice.

**Commercial anti-RV test.** The enzyme immunoassy for the qualitative detection of IgG antibodies to the inactivated RV antigen was performed using a human antibody ELISA kit (Enzymnost anti-rubella virus/IgG/Rub/IgG, OWBF 15; Siemens Healthcare, Marburg, Germany) and 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) was 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 endpoint 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.

**Detection of IgG isotypes.** The IgG1 and IgG2a subsets in the sera of immunized mice were detected using an isotype-specific ELISA with a
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.

RESULTS

Structure and self-assembly of VLPs carrying the RV E1 epitopes. The RV E1 fragment from aa 214 to 285, overlapping the RV-neutralizing epitope, and RV E1 fragment halves from aa 214 to 240 and 245 to 285 were inserted into the MIR of the HBc/H9004 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 HBc/H9004-E1(245-285) variant appeared in the 0.5 M urea extract, whereas the HBc/H9004-E1(214-285) and HBc/H9004-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 was 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(214-285), 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). The presence of the appropriate covalent and noncovalent 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 (SEC) on a Superdex 75 column (not shown). As confirmed by electron microscopy, only HBcΔ-E1(245-285) retained the correct VLPs after purification (Fig. 2F), whereas the HBcΔ derivative dimer-containing fractions contained mostly aggregated nonsymmetric, irregular structures (Fig. 2B, D, and G).

To maintain the consistency of proteins within solutions over time, the samples, which had been stored in 50% glycerol at −20°C after purification, were tested by SEC and dynamic light scattering (DLS) techniques on the 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 to E) and Superdex 75 (data not shown) columns. 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. 4F).

Direct measurement of the particle size in solution using the DLS instrument revealed particles with high mean diameters of 150 to 900 nm for all four preparations (Fig. 4F to I). Two different aggregated non-VLP associations occurred in the protein sample

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 to 18 (B), HBcΔ-E1(214-240) in lysates (C), fractions 16 and 17 (D) and HBcΔ-E1(245-285) (E) in lysates, and fractions 6 to 9 (F) and 16 to 18 (G). (H) HBcAg. Bar, 50 nm.
and 150 nm, respectively. In contrast, the HBc preparation E1(245-285) VLP and dimer preparations in solution obtained from the HBc-E1(214-240) dimer fraction. Both HBc/H9004 120-nm aggregate were observed using the protein sample obtained from the HBc-E1(214-240) dimer fraction. Similarly, a larger 500- to 600-nm aggregate and a smaller 100- to 140-nm aggregate. Two weeks after the third immunization, the anti-HBc antibody response (Fig. 6A) demonstrated a clear dependence (i) on the ability to form VLPs and (ii) on the method of formulation of the chimeric proteins. HBc-E1(245-285) (Fig. 6A, third group of bars) was more immunogenic in correct VLP form than the corresponding capsomeric HBc-E1(245-285) fraction (Fig. 6A, fourth group of bars) or the HBc-E1(214-240) and HBc-E1(245-285) nonstructured aggregates formed by dimers in all three adjuvant formulations. All three proteins induced relatively low anti-HBc antibody responses after immunization in PBS without an adjuvant formulation (Fig. 6A). HBc-E1(245-285) in the VLP form demonstrated a much higher immunogenicity using a CFA/IFA formulation than the other two nonstructured aggregates formed by dimers (Fig. 6, first and second groups of bars). Compared to the CFA/IFA formulation, the Alhydrogel formulation resulted in only a 1.2- to 1.5-times-lower anti-HBc antibody response (Fig. 6A) 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 applied to plates. In both ELISA variants, antigenicity was evident for all of the chimeras, including HBc-E1(214-240), which demonstrated the lowest level of anti-RV recognition.

Humoral response in mice. (i) 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 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). The negative control and the positive control using HBc particles applied to plates. In both ELISA variants, antigenicity was evident for all of the chimeras, including HBc-E1(214-240), which demonstrated the lowest level of anti-RV recognition.
(ii) **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 was developed to test for human antibodies in patients’ sera, we replaced the detecting anti-human IgG antibodies with anti-mouse IgG antibodies in our test. In all cases, the ELISA 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 HBcAg derivatives harboring the RV E1 epitope from aa 214 to 240 (second group of bars). However, the titers of anti-RV antibodies induced by the HBc∆-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, third and fourth groups of bars) in all three formulation variants. Surprisingly, the ELISA 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 HBc∆ derivatives harboring the RV E1 epitope from aa 214 to 240 (second group of bars). However, the titers of anti-RV antibodies induced by the HBc∆-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, third and fourth groups of bars) in all three formulation variants. Surprisingly, the ELISA using the recombinant RV E1(1-441) protein was more sensitive than the commercial one.

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

In general, the anti-RV titers obtained after immunization with HBc∆-E1(214-240) were high compared to the anticarrier titers when proteins were formulated with adjuvants but not diluted in PBS. When immunization was 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 the Alhydrogel and CFA/IFA formulations, the anti-RV E1 titers were very similar to the anti-HBc titers, i.e., 1:31,538 and 1:50,000 for anti-RV compared to 1:41,000 and 1:40,000 for anti-HBc, respectively.

In contrast to HBc∆-E1(214-240), the HBc∆-E1(214-285) chimera carrying the full-length E1 fragment from aa 214 to 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 formulation was in Alhydrogel and CFA/IFA, respectively, whereas the anti-HBc antibody titers were much higher, at 1:28,000 and 1:54,225, respectively.

HBc∆-E1(245-285) displayed a difference in immunogenicity between VLPs and nonstructured 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

**FIG 4** Sizes of particles in the purified samples as measured by size exclusion chromatography (SEC) and DLS analysis. (A to E) SEC Sepharose 4 Fast Flow chromatography. The retention time is indicated on the x axis, and milli-absorbance units (mAU) are indicated on the y axis. (F to J) Results of the DLS size distribution are shown, with the particle diameter in nm on the x axis and the percentage of particles on the y axis. (A and F) HBc∆-E1(214-285); (B and G) HBc∆-E1(214-240); (C and H) HBc∆-E1(245-285), VLPs; (D and I) HBc∆-E1(245-285), purified as dimers; (E and J) HBcAg. Brackets indicate SEC elution times of aggregates, VLPs, and dimers.
showed similar levels of 1:86,667 and 1:95,000, respectively; therefore, the CFA/IFA formulation induced both titers to $10^4$. HBc$_{\Delta}$E1(245-285) VLPs in PBS induced a titer of anti-RV E1 antibodies that was similar to the highest titer of the corresponding nonstructured aggregates formed by dimers and formulated in CFA/IFA, 1:26,000 and 1:31,000, respectively (Fig. 6B, third and fourth groups of bars). 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). HBc$_{\Delta}$E1(214-240) induced more anti-RV E1 antibodies than the other chimeras. The anti-RV E1 antibody titer induced by HBc$_{\Delta}$E1(214-240) in CFA/IFA was 3 times higher than the titer with the Alhydrogel formulation (1:6,000 and 1:1,800, respectively) (Fig. 6C, second group of bars). In contrast, the HBc$_{\Delta}$E1(245-285) protein, which contained the same epitope from aa 214 to 240 within a longer E1 sequence, did not induce a substantial anti-RV antibody response (Fig. 6C, first group of bars). HBc$_{\Delta}$E1(214-240), even in the VLP form, induced a remarkably high level of anti-RV E1 antibodies, although lower than that induced by the HBc$_{\Delta}$E1(214-240) protein (Fig. 6C, third group of bars).

(iii) Immunization with recombinant RV E1(1-441) protein.
To assay the effect of the HBc$_{\Delta}$ 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, fifth group of bars). The observed anti-RV antibody titers were relatively low in all immunized mouse groups, including those given the 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 to 10 times lower than the titers induced by the most active HBc$_{\Delta}$-carried RV E1 fragments injected into mice with PBS or Alhydrogel. Protein HBc$_{\Delta}$E1(214-240) 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), i.e., 1:7,788 and 1:9,025 and 1:10,000 and 1:11,000, respectively.

(iv) 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 dif-
of DNA copies of the epitopes at selected sites in the VLP genes faces of VLPs, two major strategies are currently used: (i) insertion To present foreign epitopes in a native fashion on the outer sur-
face of VLPs, which makes them highly immunogenic, is regarded as a major advantage for the VLP approach to vaccine construction.

**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 reviews, see references 17, 36, 37, and 38).

VLP-based vaccines against hepatitis B virus and human papillomaviruses 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 surfaces 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 surfaces of VLPs, two major strategies are currently used: (i) insertion of DNA copies of the epitopes at selected sites in the VLP genes and (ii) chemical coupling of epitope peptides to the VLP surface (for reviews, see references 17, 36, 37, and 38).

VLPs are likely to provide the inserted foreign protein fragments with a high capacity for induction of 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 nonassembled peptides or the low-molecular-mass carrier proteins (for reviews, see references 16, 36, 37, and 38). However, due to the different stabilities of the chimeric VLPs, the latter may be purified as dimers, which are converted into nonsymmetric high-molecular-mass aggregates. Here, we observed the spontaneous formation of such aggregates from dimers after purification of some of the chimeric HBC-based proteins.

The RV E1 fragment from aa 214 to 285, which is associated with virus-neutralizing activity (7–11), and each half, aa 214 to 240 and 245 to 285, were inserted into the HBC MIR, which is located on the tips of the HBC spikes and represents the most protruding region on the HBC VLP surface (23, 25). HBCΔ-E1(245-285) produced a VLP form, and HBCΔ-E1(214-285) and HBCΔ-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 HBCΔ-E1(245-285) products. Moreover, the HBCΔ-E1(245-285) VLPs aggregated into 280-nm particles in solution, unlike the initial HBC VLPs.

VLP-forming HBCΔ-E1(245-285) products demonstrated a high anti-RV immunogenicity. Surprisingly, the epitope from aa 214 to 240 within the HBCΔ-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 epitope from aa 214 to 240 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 cysteine residues were involved in the anti-RV antibody response.

The remarkably high capacity of protein aggregates to enhance immune responses to the monomeric form of proteins has been described (for a review, see reference 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 the folded epitope with virus-neutralizing activity (7–11), and each half, aa 214 to 240 and 245 to 285, were inserted into the HBC MIR, which is located on the tips of the HBC spikes and represents the most protruding region on the HBC VLP surface (23, 25). HBCΔ-E1(245-285) produced a VLP form, and HBCΔ-E1(214-285) and HBCΔ-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 HBCΔ-E1(245-285) products. Moreover, the HBCΔ-E1(245-285) VLPs aggregated into 280-nm particles in solution, unlike the initial HBC VLPs.

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sponses may be generated against aggregates before T-cell activation, similar to the case for 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 an immunological response. When the epitope from aa 214 to 240 was prolonged with further E1 sequence to position 285 within the full-length fragment from aa 214 to 285, 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 (APCs) 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 a 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 epitope from aa 214 to 240 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 antibody levels were obtained with 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 the 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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