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Comparison of Serum Humoral Responses Induced by Oral Immunization with the Hepatitis B Virus Core Antigen and the Cholera Toxin B Subunit▽

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The hepatitis B virus core (HBc) virus-like particle (VLP) is known as one of the most immunogenic antigens and carrier vehicles in different immunization strategies. Recent findings are suggesting the potential of the HBc VLPs as an oral immunogen. Here, we focus on the induction of serum humoral responses by oral administration of HBc VLPs in preparations substantially free of lipopolysaccharide and immunomodulating encapsidated RNA. The full-length HBc antigen was used, because the C-terminal arginine-rich tail may contribute to the immunogenicity of the antigen as the region is involved in cell surface heparan sulfate binding and internalization of the protein. Serum antibody levels and isotypes were determined following oral administration of the HBc VLPs with the perspective of using the HBc VLP as an immunostimulatory and carrier molecule for epitopes of blood-borne diseases in oral immunization vaccination strategies. Following oral administration of the HBc VLP preparations to mice, a strong serum humoral response was induced with mainly immunoglobulin G2a (IgG2a) antibodies, pointing toward a Th1 response which is essential in the control of intracellular pathogens. Intraperitoneal immunization with the HBc VLP induced a stronger, mixed Th1/Th2 response. Finally, a comparison was made with the induced serum humoral response following oral administration of the recombinant cholera toxin B pentamer, a commonly used oral immunogen. These immunizations, in contrast, induced predominantly antibodies of the IgG1 isotype, indicative of a Th2 response. These data suggest that the HBc VLP can be an interesting carrier molecule in oral vaccine development.

The hepatitis B virus core (HBc) virus-like particle (VLP) is a strong immunogen that is remarkably tolerant to substitutions, insertions, and deletions in its two immunodominant loop regions and its C-terminal tail (28, 30, 31, 45, 53, 54). The VLP is also capable of transferring its immunogenic capacities to these additions and is therefore often used as a vehicle for foreign B- and T-cell epitopes. With the HBc carrier platform, protective immunity could be induced against a range of pathogens, including several *Plasmodium* spp. (43, 56, 57), the influenza A virus (48), and the Puumala hantavirus (64).

Fifis et al. (15) suggested that the strong immunogenicity of VLPs is based on their size, which is suitable for uptake by dendritic cells, thereby directly promoting dendritic cell maturation and migration, a process essential for the stimulation of an immune response (15, 17). In addition, some VLPs that resemble infectious viruses retain their receptor binding regions and are able to target and enter cells by using their normal receptor (21). For HBc VLPs, a specific role for the arginine-rich C-terminal tail in cell surface heparan sulfate binding and uptake has been demonstrated (8, 9, 66). Furthermore, HBc VLPs are generally known to be extremely stable as they are resistant to denaturing agents and variations in pH (between 2 and 13) and temperature (1 h at 70°C) (3, 49), which makes them suitable for use as oral immunogens. It has been shown that recombinant Norwalk VLPs (2) and hepatitis E VLPs (36) are at least partially resistant to degradation in the gastrointestinal tract and thus able to induce mucosal and systemic immune responses following oral delivery. In such a case, the mucosal response can provide a first barrier in preventing viral infection. After oral vaccination of mice with human papillomavirus-like particles, it was demonstrated, using an in vitro assay, that systemic virus-neutralizing antibodies could be induced, though the systemic (immunoglobulin G [IgG]) immune response against these orally delivered particles was rather weak (36, 55). Moreover, it is generally known that when VLPs are used as a carrier platform, the response against the integrated epitopes is weaker than the VLP-induced response. Therefore, there is a need to screen for VLPs that induce a high systemic humoral response after oral vaccination. These VLPs, used as a carrier platform, would still be able to induce a strong systemic response against integrated epitopes. As the HBc VLP is known as one of the most immunogenic and stable VLPs (46) and as recent findings suggest the potential of the HBc VLP as an oral immunogen (37), we investigated whether this particle, following oral administra-

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tion, can induce a serum humoral response of a potentially protective level. Because the C-terminal arginine-rich region might contribute to the immunogenicity of the VLP, the full-length protein was used. Immunostimulating contaminants, such as LPS and incorporated RNA, were extracted from the preparations during purification. The strength and nature of the serum humoral response in mice were determined following four gavages with 50 μg recombinant HBc (rHBc) and compared to those observed after intraperitoneal (i.p.) immunization with the VLPs. In addition, the induced antibody levels and isotypes were compared to those observed following oral immunization with the recombinant cholera toxin B subunit (rCTxB), a frequently used oral immunogen (for reviews, see references 11 and 68).

MATERIALS AND METHODS

Expression and purification of the rHBc VLPs. The molecular cloning of the HBc gene (no. LMBP 2470; collection, University of Ghent, Belgium), the expression of full-length HBc particles in *Escherichia coli* and the purification of the rHBc VLPs were described previously by Broos et al. (6). The purity and identity of the samples were evaluated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and on Western blots. The correct particle assembly was ascertained by electron microscopy after negative staining using 1% phosphotungstic acid and ultracentrifugation on a linear sucrose gradient. Purified protein samples were dialyzed overnight against phosphate-buffered saline (PBS) before administration to mice.

Plasmid construction, expression, and purification of the CTxB. To express CTxB in *E. coli*, we used the *ctxB* gene, which was designed according to the codon usage by *E. coli* (1). The gene was kindly provided by Paulo Lee Ho (Centro de Biotecnologia, Instituto Butantan, São Paulo, Brazil). CTxB was secreted in the periplasm of *E. coli* cells, allowing intramolecular disulfide bridge formation, essential for efficient folding (and pentamerization) and, consequently, efficient binding of the toxin to the GM1 receptor (38). To achieve secretion, the *ctxB* gene was fused to the secretion signal coding sequence of *E. coli* outer membrane protein A (sOmpA), as described by Guisez et al. (23). Briefly, bacteria were allowed to grow at 37°C until an optical density at 600 nm of 0.5 was reached and induction of the CTxB occurred in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside) at 28°C. Periplasmatic proteins were extracted by osmotic shock. The periplasmatic protein mixture was extracted in 20 mM Tris (pH 7.4) and 20 mM NaCl and loaded on a DEAE-Sephacel column (Amersham Biosciences). Retained proteins were eluted from the column by using a gradient of increasing NaCl: most of the CTxB was recovered at 125 mM NaCl. This fraction was diluted 1/5 in 20 mM Tris (pH 7.4) and loaded on a HiTrap SP FF column (1 ml; GE Healthcare) by using an Äkta chromatographic purification platform. After elution using a linear NaCl gradient from 20 mM to 1 M NaCl in 20 mM Tris (pH 7.4), CTxB was found as a single peak at 400 mM NaCl. The purity of the recombinant protein was analyzed on a 15% SDS-PAGE gel with a Bio-Rad Protean II system (Bio-Rad) according to Laemmli (32). Gels were stained with Coomassie brilliant blue G-250 or silver stained (SilverQuest silver stain kit; Invitrogen). For CTxB, a monomeric protein band of 13 kDa was expected. The identity of the protein was determined by Western blot analysis, using goat anti-CTxB (1/2,000; List Biological Laboratories, Inc.) and horseradish peroxidase (HRP)-labeled human anti-goat as the secondary antibody (1/2,000; immunodetection kit; Invitrogen). Pentamerization of the CTxB monomers was shown with SDS-PAGE by loading nonboiled protein samples in 2-mercaptoethanol free loading dye (35). Protein concentrations were determined using the Bradford assay (5).

Administration of the rHBc VLP and rCTxB pentamers to mice. For oral immunization with HBc, two groups of six 8-week-old BALB/c BY Jico mice (Charles River Laboratories, France) were adapted to conditions in a dedicated animal house. To one group of mice, 50 μg of purified rHBc in PBS was administered intragastrically by gavage. This was repeated four times: on days 0, 13, 24, and 48. An administered volume of 300 μl liquid was not exceeded. The control group received an equal volume of PBS. For oral vaccination with CTxB, two groups of six 8-week-old mice were orally immunized with 50 μg purified recombinant CTxB at the same time points. For i.p. injection of HBc, one group of six mice received 10 μg HBc antigen (HBcAg) i.p. at days 0, 13, and 24, while another group was injected with PBS as a negative control.

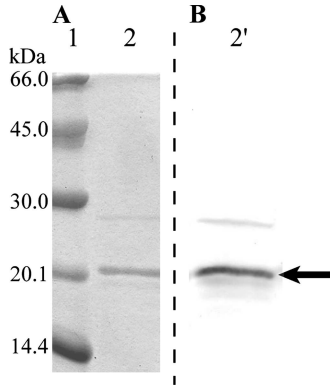

FIG. 1. Analysis of the purified HBc protein samples on a Coomassie brilliant blue-stained 12.5% SDS-PAGE gel (A) and a Western blot (B). (A) Lane 1, LMW protein mixture (Amersham Biosciences); lane 2, purified HBcAg. The HBc monomer at 21 kDa is indicated by an arrow. Multimeric structures appear as higher protein bands. (B) Lane 2′, Western blot analysis of the purified HBcAg.

Sample collection. Blood samples were obtained from mice orally immunized with HBc by tail bleeding on days 0, 16, 42, 62, and 72 and by heart puncture on day 106. Similarly, blood samples were taken from i.p.-injected mice on days 0 and 36 and from mice orally immunized with CTxB on days 0 and 62. Samples were kept on 37°C for 30 min and centrifuged twice at 9,000 × *g* for 15 min to collect the serum. Samples were stored at −20°C until testing.

Antibody ELISA. Serum HBc-specific antibody levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as described by Broos et al. (6), making a serial twofold dilution of the serum samples. For characterization and isotyping of the humoral response, HRP-conjugated goat anti-mouse IgG, IgA, IgG1, IgG2a, and IgG2b were obtained from Zymed Laboratories (San Francisco, CA) (1/2,000), and HRP-conjugated goat anti-mouse IgG3 was obtained from Centaur BVBA (Brussels, Belgium). After addition of the enzyme substrate, the absorbance was measured at 492 nm and 630 nm (background) (UltraMicroplate Reader EL_x808; Bio-Tek Instruments, Inc.). The data were analyzed by Student's *t* test ($P < 0.05$). When pooled serum fractions were analyzed, the samples were considered to be reactive when the measured absorbance at 492 nm was two and a half times higher than the negative control absorbance at the same dilution.

Serum CTxB-specific antibody levels were determined in an ELISA, based on the method described by Fingerut et al. (16). Briefly, ELISA plates were incubated overnight at 4°C with 100 μl of 15 μg/ml GM1 (monosialoganglioside GM1; Sigma) in bicarbonate buffer (pH 9.5). Blocking was performed with 1% bovine serum albumin in PBS-0.2% Tween 20. Purified CTxB at a concentration of 20 μg/ml in 1% bovine serum albumin in PBS-0.2% Tween 20 was allowed to bind to the receptor for 1 h at 37°C. Twofold serum dilutions were added and incubated for 2 h at 37°C. The presence of serum CTxB-specific antibodies was shown using HRP-conjugated goat anti-mouse IgG following oxidation of the added *o*-phenylenediamine substrate.

RESULTS

Characterization of rHBc VLPs and rCTxB. rHBc VLPs from *E. coli* were purified, and protein samples were analyzed on a 12.5% SDS-PAGE gel and a Western blot (Fig. 1A and B). The HBc monomer was observed as a 21-kDa band; multimeric complexes appeared as higher-molecular-mass bands. Examination of these preparations by electron microscopy revealed the presence of a large number of well-assembled particles (data not shown).

Quantification of periplasmatically expressed and purified CTxB revealed that our two-step purification protocol yields 2 mg CTxB from 1 liter culture. Periplasmatic secretion is appropriate since periplasmatic chaperones (58) and thiol-disulfide oxidoreductase (disulfide bridge formation) carry out ef-

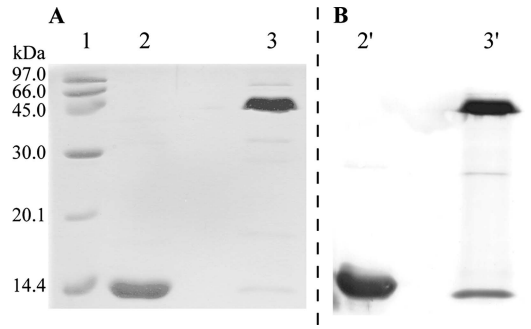

FIG. 2. (A) Purified rCTxB analyzed on a 15% SDS-PAGE gel stained with CBB. Lane 1, LMW protein mixture (Amersham Biosciences); lane 2, purified CTxB samples boiled in loading dye with 2-mercopto-ethanol. A monomeric protein band appears at 13 kDa. Lane 3, nonboiled, purified CTxB samples loaded in 2-mercoptoethanol free loading dye. A pentameric protein band appears at 50 kDa. (B) Western blot analysis of the purified CTxB samples. Lane 2′, CTxB monomer at 13 kDa in a loading dye with β-mercapto; lane 3′, CTxB pentamer at 50 kDa in a loading dye without β-mercapto (sample not boiled).

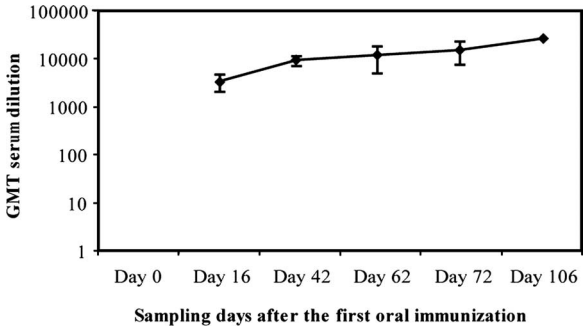

FIG. 4. Kinetics of serum IgA antibody response of BALB/c mice after oral immunization with 50 μg rHBc VLPs in PBS without any adjuvant. The *y* axis shows the geometric mean titer (GMT) of the antibody dilution at which an antigen-specific response was detected. The *x* axis shows the days on which samples were taken after oral immunization. The error bars show the standard deviations of the mean. On day 106, in the sera of all orally immunized mice HBc-specific IgA antibodies could still be detected at the highest dilution tested (1/25,000).

ficient folding and assembly of the different subunits (47, 71), leading to the formation of a completely functional CTxB pentamer complex. The purity of the recombinant proteins and their pentameric nature were analyzed on an SDS-PAGE gel (Fig. 2A) and a Western blot: the goat anti-CTxB antibody recognized the protein bands at 13 and 50 kDa (Fig. 2B), corresponding to the CTxB monomeric and pentameric structures, respectively.

Kinetics of serum IgG and IgA after oral immunization with rHBc VLPs. To evaluate the potential of the full-length rHBc as an oral immunogen, 50 μg of the protein in PBS (without adjuvant) was orally administered to six BALB/c mice on days 0, 13, 24, and 48. Blood samples were taken, and serum IgG and IgA antibody responses were monitored by ELISA on days 0, 16, 42, 62, 72, and 106. An HBc-specific serum IgG response could be detected 2 weeks after the first immunization and continuously increased until day 62 (Fig. 3). At this time point, IgG antibodies could be detected in sera of all immunized mice at dilutions between 1/50,000 and 1/250,000. The IgG titers remained at the same level for at least another 6 weeks after the last immunization. HBc-specific serum IgA responses could be detected from day 16 after the first gavage, and these responses increased until day 106 (Fig. 4). At the end of the experiment, all mice showed HBc-specific IgA reactivity at a dilution of 1/50,000, the highest dilution tested.

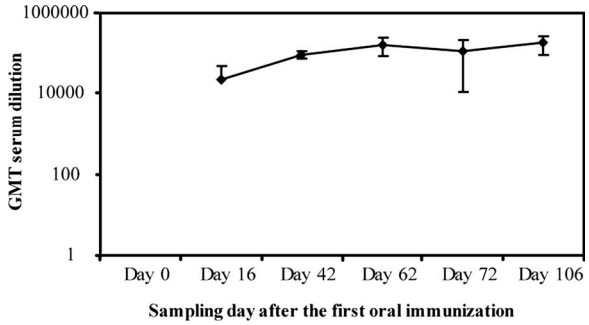

FIG. 3. Kinetics of serum IgG antibody response of BALB/c mice after oral immunization with 50 μg rHBc VLPs in PBS without any adjuvant. The *y* axis shows the geometric mean titer (GMT) of the antibody dilution at which an antigen-specific response was detected. The *x* axis shows the days on which samples were taken after oral immunization. The VLPs were orally given on days 0, 13, 24, and 48. The error bars show the standard deviations of the mean. On day 16, the sera of two out of the six orally immunized mice were not HBc reactive on the lowest tested dilution (1/5,000); therefore, no negative standard deviation could be shown on this logarithmic scale.

Comparison of serum IgG levels and IgG subclasses after oral and parenteral immunization with HBc VLPs. To determine the nature of the induced immune responses, HBc-specific IgG subclasses of the pooled serum samples were characterized by ELISA 2 weeks after the last oral or i.p. administration of the HBc VLPs. Pooled serum samples were considered to be reactive when the measured absorbance at 492 nm is two and a half times higher than the negative-control absorbance at the same dilution. The predominant subclass induced following oral delivery of the HBc VLP was the IgG2a subtype (Fig. 5A). HBc-reactive serum IgG2a antibodies could still be detected at dilutions up to 1/100,000. Serum HBc-specific IgG1 and IgG2b responses were weaker (detectable up to dilutions of 1/5,000), and no IgG3 antibodies were seen.

Following i.p. injection with the HBc VLP, all IgG subclasses were present (Fig. 5B) in the sera of immunized mice. High levels of HBc-specific IgG2a and IgG1 antibodies could be demonstrated (detectable up to dilutions of 1/1,000,000), while levels of IgG3 and IgG2b were lower. Although high levels of HBc-specific antibodies can be detected after oral vaccination, the HBc-specific humoral response induced after i.p. immunization is at least 10 times stronger (Fig. 6).

Comparison of serum IgG levels and subclasses after oral immunization with HBc VLP and CTxB. Two weeks after the last oral administration of rCTxB, pooled serum fractions were analyzed for CTxB-specific IgG subclasses. The humoral response induced by orally delivered CTxB appeared to be mainly composed of the production of antibodies of the IgG1 isotype (Fig. 5C). Antibodies of the IgG1 isotype could be detected in dilutions of up to 1/500,000. No antigen-specific

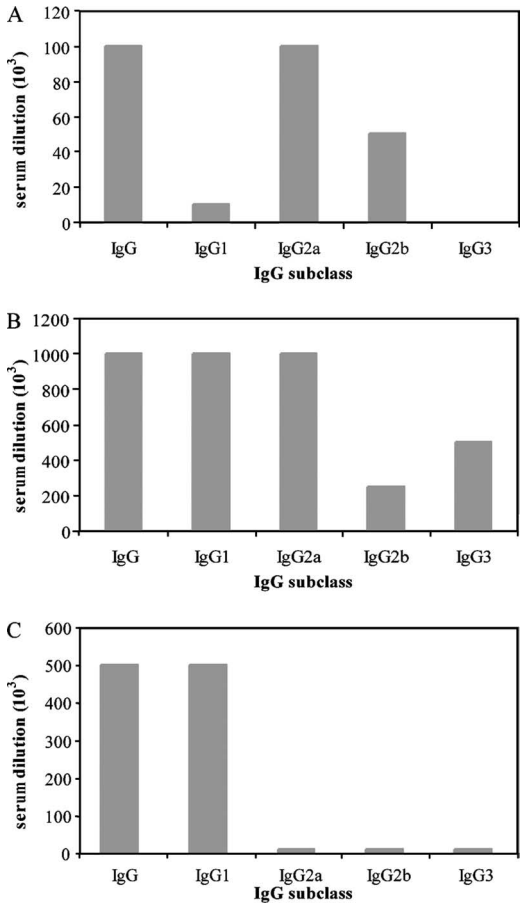

FIG. 5. IgG subclasses (2 weeks after the last immunization) of pooled sera from BALB/c mice immunized orally with 50 μg rHBc particles (A), i.p. with 10 μg rHBc particles (B), and orally with 50 μg rCTxB (C). The *y* axis shows the antibody dilution at which an antigen-specific response was detected. The *x* axis shows the analyzed IgG subtypes.

IgA antibodies could be detected after oral vaccination with rCTxB.

DISCUSSION

When administered parenterally or nasally, VLPs have proven to be highly immunogenic and capable of inducing a protective immune response, even against integrated epitopes. Lobaina et al. (37) showed that orally administered HBc VLPs can induce a systemic humoral response following sublingual and oral immunization of 5 μg proteins. From our data, it can be concluded that four doses of 50 μg orally administered HBc VLPs induce a strong humoral response of a potentially protective level as HBcAg-specific antibodies could still be detected at a dilution of 1/100,000. Moreover, this induction occurs even in the absence of any oral delivery system (as liposomes) or oral adjuvant (such as CTxB). Also, immunomodulating lipopolysaccharide and encapsidated RNA were restrained in the administered preparations. The induced systemic humoral response is at least 10 times higher than that induced by other orally administered VLPs (Fig. 6). Considering that immune responses against integrated epitopes are

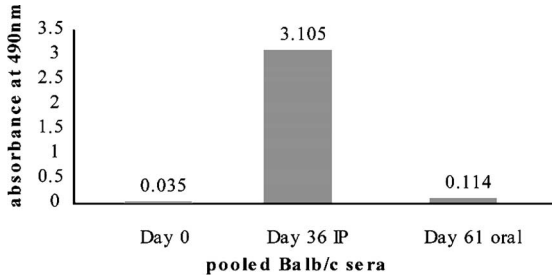

FIG. 6. Absorbance values of pooled BALB/c sera analyzed for their HBc-specific IgG levels at a dilution of 1/40,000. Pooled sera were examined 2 weeks after the last immunization with either 50 μg orally administered rHBc particles or 10 μg i.p. injected rHBc VLPs. The *y* axis shows the absorbance at 490 nm, while the analyzed mouse groups are represented on the *x* axis.

generally weaker, the HBc particle vaccine might be the most suitable VLP for use as carrier molecule in oral vaccination strategies, capable of inducing sufficiently high antibody titers against integrated epitopes. The detection of serum HBc structure-specific antibodies following oral administration proved that the capsid structure and antigenicity of HBc were, at least partially, conserved during passage through the gastrointestinal tract. This is most likely due to the extreme pH resistance of the VLPs and their capacity to reassemble after denaturation (3, 49).

Furthermore, de Aizpurua and Russell-Jones (12) showed that several types of antigens, especially lectins, could induce systemic responses after oral delivery using normal dosage and not inducing systemic tolerance. The involvement of specific glycoconjugates in the interaction and uptake of oral pathogens, antibodies, and vaccine antigens by M cells in mice has already been shown (19).

Recently, it was also proposed that C-terminal protamine-like domains of the HBc have a specific role in guiding the binding of capsids to host cells through interaction with membrane heparan sulfate proteoglycans (8, 66). These proteoglycans are present on the surfaces of mucosal cells, B cells, macrophages, and dendritic cells. Therefore, we suggest that following oral vaccination heparan sulfates might be of particular importance, functioning as membrane receptors to facilitate transcytosis by M cells and uptake by other antigen-presenting cells associated with the gut-associated lymphoid tissue. In that respect, Milich and coworkers (44) reported on the importance of B cells in antigen presentation and concomitant induction of an immune response following HBc administration in an in vivo mouse model. Nevertheless, the importance of specific glycoconjugates in the uptake of oral pathogen antigens should not be underestimated, and there is, in addition, no evidence for such a T-cell-independent B-cell response in humans (65).

Furthermore, the protamine-like C-terminal domain of the HBc protein appears of particular interest in conferring full protection after immunization. De Filette et al. (13) showed that after i.p. administration of the M2e epitope, integrated in the N-terminal loop of a C-terminal-truncated HBc VLP, only partial protection against influenza was induced. In contrast, immunization with the epitope integrated in particles induced complete protection, and this was correlated with a high IgG2a

level (14). Although it is assumed that the protamine domain is easily truncated following proteolytic treatment, it might be possible that some intact particles reach the mucosal surfaces and establish a binding interaction with the mucosal surfaces that facilitates internalization.

The nature of the induced immune response was determined because it has been stated that there is a correlation between the antigen-induced isotypes and their protective efficiency (50). Antibodies of the IgG2a subtype are often induced following administration of viral antigens (10, 70) and are believed to be most effective in antibody-dependent protective immunity (14, 40). Therefore, the isotypes of the induced IgG response in all immunized mouse groups were determined. The predominant IgG subtype after oral administration of the HBc VLP was the IgG2a isotype, indicating a Th1 response. Th1 lymphocytes are specialized in the production of gamma interferon and are essential in the control of intracellular pathogens, such as respiratory syncytial virus (52), measles virus (29), agents of malaria (7), mycobacteria that cause tuberculosis (60), and human immunodeficiency virus (41). After i.p. immunization with rHBc VLPs, we could detect high levels of both IgG1 and IgG2a antibodies, pointing to a mixed Th1/Th2 response. Mixed Th1/Th2 responses are often induced after immunization with antigens of a polymeric nature (e.g., that of a repetitive and spatially ordered array of the same epitope) (67). Mazumdar et al. (42) showed that following vaccination against *Leishmania*, a mixed Th1/Th2 response is induced, which instructs a protective Th1 response after challenge.

The HBc-specific antibody levels produced after oral immunization are 10-fold lower than those obtained following i.p. injection of the VLP, even though the orally delivered antigen dose is 5 times higher than that which we administered i.p. These lower antibody titers are probably due to proteolytic degradation of the orally administered antigen. Oral immunization with higher doses of the antigen does not induce higher serum antibody titers but might even induce oral tolerance (18, 61). Reduction in the dose by protection against proteolytic degradation can be obtained by antigen encapsulation through layering in polymethacrylates and skim milk (26), by the use of oral adjuvants, or by administration of recombinant protein expressed in edible plant parts (surrounded by the cellulose cell wall) (for reviews, see references 20, 33, 59, and 62).

Oral immunization is enjoying great public interest in developing and Western countries, because it offers many advantages over parenteral immunization. Oral immunogens are easy to deliver, avoid nosocomial infections, reduce the need for specialized medical assistance, and are thus potentially useful for mass immunization (22). Expression of VLPs in edible plant parts can reduce antigen production costs and avoid the need for expensive and time-consuming purification protocols. It has been shown that after freeze or air drying of Norwalk VLPs expressed in tomato fruits, specific IgG and IgA responses could still be induced in mice fed with these preparations (25, 72). Moreover, the corn-expressed B subunit of heat-labile toxin, the S glycoprotein of transmissible gastroenteritis, and the lettuce-expressed measles virus hemagglutinin protein have all been shown to be stable for at least 1 year, even when stored at ambient temperatures (34, 69). This suggests the potential for accessibility and use of these vaccines in developing countries, as cold chains are not required for antigen preservation.

We compared the HBc-induced antibody levels and isotypes with those induced after oral immunization with CTxB. Together with the similar *E. coli* heat-labile enterotoxin, CTx is one of the most commonly used carrier and adjuvant molecules in mucosal vaccination strategies, capable of inducing serum IgG antibody responses (11). Its excellent carrier capacities result from facilitating protein uptake upon binding on the glycosphingolipid GM1 ganglioside (51), present on virtually all cells, including immune cells. Our results (Fig. 5C) are in agreement with those of Marinaro et al. (39), who showed that cholera toxin especially induces a Th2 immune response associated with high titers of IgG1 isotype antibodies. This confirms the modulating nature of the CTxB molecule in T-helper-cell differentiation and activity, resulting in Th2 stimulation. For infections with *Tricuris muris* and *Ascaris*, triggering such a Th2 response is most beneficial (27). The shift toward a Th2 response is also important when gene fusions with the CTxB subunit are used to induce peripheral tolerance to the coupled antigen, an application needed in order to prevent autoimmune diseases (24, 33). After oral vaccination with rCTxB, no IgA levels could be measured in the serum. Similarly, Blanchard et al. (4) could not detect antigen-specific IgA following immunization with an antigen that was genetically coupled to the CTxB toxin. These authors suggested that CTxB is acting solely as a carrier molecule and not as an adjuvant in this system.

In summary, our observation that the HBc VLP induces a strong serum humoral response following oral immunization suggests the high potential of the HBc VLP for use as a carrier in oral vaccination strategies. The Th1-biased response can be of particular interest where the HBc is used as a carrier vehicle for vaccination strategies against viral and parasitic infections, such as, e.g., malaria, tuberculosis, and leishmaniasis. These are all infections thriving in tropical, developing regions where needle-free, oral mass immunization can be of particular importance.

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