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Immunological characterization of two hepatitis B core antigen variants and their immunoenhancing effect on co-delivered hepatitis B surface antigen

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Abstract

The hepatitis B virus (HBV) core and surface antigens are potent immunogens in animal models and humans. They have been used in vaccine studies for prevention or therapy of HBV diseases and also as carrier molecules in new developments. In this study we explored the nasal immunogenicity of two different variants of the recombinant complete nucleocapsid (HBcAg) as well as their adjuvant effect on hepatitis B surface antigen (HBsAg). To characterize the immune response, the serum IgG antibody response was tested during one year against both antigens, and the serum and vaginal secretions were tested for recognition of linear epitopes of HBcAg for both HBcAg variants. The results obtained evidenced that the intranasal immunogenicity of both HBcAg variants was similar and high, developing early and long lasting IgG responses. A similar recognition pattern to all sera and vaginal washes samples was generated by the two variants of HBcAg, also similar to a pool of human anti-HBcAg positive sera. A synergistic effect in the enhancement of the immunogenicity for both antigens was evidenced in the combined formulation after nasal administration. Taken together, these results would be of interest in the design of more potent therapeutic and preventive vaccines complementing systemic and mucosal responses.

Keywords: HBcAg; HBsAg; Hepatitis

1. Introduction

The hepatitis B surface antigen (HBsAg) is one of the major antigens of the hepatitis B virus, and the mean antigen of the commercial vaccine accepted worldwide as a very efficient tool to prevent the disease by said virus.

The hepatitis B core antigen (HBcAg), is also an immunodominant component in hepatitis B virus (HBV) infection (Hoofnagle et al., 1981; Milich et al., 1987). This antigen constitutes a potent immunogen for experimental animals as well as for humans acutely infected with the HBV. The strong immunogenicity of the HBcAg has been explained for its dual behavior as a T-cell dependent and independent antigen (Milich and McLachlan, 1986), which is related to a potent B-cell activation to work efficiently as primary antigen-presenting cells (Milich et al., 1997a; Lazdina et al., 2001). Naturally, the antibodies directed against the HBcAg appear early during the course of the HBV infection and they often persist per year after the recovery of the disease (Tordjeman et al., 1993). In vaccine studies, the HBcAg has shown to be a potent immunogen even without adjuvants as well as an attractive carrier molecule for homologous and heterologous epitopes (Stahl and Murray, 1989; Schodel et al., 1992; Yoshikawa et al., 1993; Borisova et al., 1989; Brown et al., 1991; Jegerlehner et al., 2002).

Abbreviations: HBV, hepatitis B virus; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen

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Immunogenicity studies administering the 183-amino acid 21 kDa core protein by parenteral route, indicated that the HBcAg preferentially but not exclusively elicits Th1-like cells, conversely to HBeAg that elicits Th0 or Th2-like cells (Milich et al., 1997b) although both proteins share >70% of their amino acid sequence and most T and B cell epitopes. Priming specific Th1 immunity by recombinant HBcAg depends on its arginine (Arg)-rich, 34–36-residue-long C terminus and nucleotides bound to it (Riedl et al., 2002a).

The mucosal immunogenicity of HBV antigens has been poorly studied. Recent works evidenced the high immunogenicity of complete core antigen nasally administered (Lobaina et al., 2003), conversely, truncated core variants have shown poor immunogenicity (Francis et al., 1990). In the case of surface antigen, it was able to induce a significant immune response only after mixing with cholera toxin or CpG motifs (McCluskie and Davis, 1998). However, both antigens have never been co-administered before through the nasal route. Recently, Riedl and co-workers reported the adjuvant effect of complete HBcAg particles on intramuscular or subcutaneous co-delivered protein antigens. They observed that HBcAg induced a Th1-biased pattern on unrelated co-administered antigens such as HBsAg and OVA. This behavior of the HBcAg was explained by the presence of RNA bound to the arginine-rich domain of the HBcAg particles. RNA-containing HBcAg particles, but not truncated HBcAg-149, stimulated IL-12 release from dendritic cells and INF-γ release from non-immune spleen cells. This can support its role as a potent enhancer of specific priming of Th1 immunity (Riedl et al., 2002a; Riedl et al., 2002b). Also the binding of nucleic acids to cationic peptides may protect them from degradation by nucleases during the extracellular phase, facilitating their uptake by cells, and/or directing their delivery to an endolysosomal signaling compartment (Gursel et al., 2001).

The present work explores the intranasal immunogenicity of two variants of the complete recombinant hepatitis B nucleoprotein antigen. We also study the recognition pattern of linear peptides from HBcAg in serum and vaginal lavages using two administration routes and different formulations. Furthermore, we present the evidences on the crossenhancing effect of HBsAg and HBcAg antigens after their nasal co-administration in mice.

2. Materials and methods

2.1. Preparation of recombinant antigens

The first rHBcAg variant, called HBcRIV-2 was obtained by Palenzuela et al. (2002). Shortly, the HBcAg gene was amplified by polymerase chain reaction (PCR) from pR2M6-HBcAg construction (Domínguez et al., 1999). The amplified gene was cloned into pRIV-2 vector (Pharmacia, Sweden), under the control of pL promoter, using the restriction sites *Nco*I/*Bam*HI (Biolab, USA). Expression was assayed in *E. coli* W3110 cells previously transformed with the pGP1-2 plasmid, which confers kanamycin resistance and codes for the protein cI857, the pL promoter repressor. The HBcRIV-2 has a carboxyl-terminal eight amino acids (aa) insertion. The sequence of the inserted peptide was KL-GSVDLQ. The expression and purification process of this variant was carried out as described by Palenzuela et al. (2002).

The second rHBcAg variant, called HBcM492, was expressed in *E. coli* strain W3110, previously transformed with a plasmid containing the entire core antigen gene under the control of the tryptophan promoter (Musacchio et al., publication in course). The LB medium supplemented with 50 μg/mL kanamycin was used to culture the *E. coli* strain. After centrifugation, supernatant was discarded and the pellet was resuspended. Three passes in French press were used for the rupture and the supernatant was collected and precipitated with ammonium sulfate. The resulting pellet was resuspended and applied to a Q-sepharose fast flow (Pharmacia, Sweeden) column. The peak containing HBcAg was concentrated and applied to a sepharose CL-4B column. The resulting rHBcAg was obtained with purity over 95% and size of 28 nm as characterized by electron microscopy (data not shown).

2.2. Immunization schedules

Three immunization schedules were carried out using groups of 8–10 Balb/c mice (females) of 8–12 weeks old each. The immunization routes tested were subcutaneous (s.c.) and intranasal (i.n.). The i.n. administration was conducted in two parts of 25 μL per nostril, in mice anesthetized using i.p. administration of 30 μL of ketamine 10 mg/mL. Parenteral injections were conducted in a volume of 100 μL.

Seven groups of eight mice each were employed in the first immunization schedule. The inoculations were conducted on days 0 and 14, and blood samples were collected on days 12 and 24 by retrorbital puncture. The groups 1, 2 and 5 received 10 μg of HBcRIV-2, 10 μg of HBcM492 and 5 μg of HBsAg, respectively, in saline-phosphate buffer (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) (PBS) by the i.n. route. Group 3 and 4 were immunized nasally with 5 μg HBsAg and 10 μg of each core variant (HBcRIV-2 or HBcM492), respectively. As a parenteral route control, we immunized mice subcutaneously with 5 μg HBsAg in alum (group 6). A placebo group was immunized with PBS by s.c. route (group 7).

2.3. Biological fluids

Sera were collected via retrorbital plexus. The blood was centrifuged to 3000 rpm for 10 min (Eppendorf centrifuge, 5415C), and the serum was conserved at −20 °C until evaluation.

2.4. ELISAs

Specific IgG against HBsAg and both HBcAg variants were evaluated by ELISA. Briefly, high binding plates (Costar, USA) were coated with 100 μL of HBsAg or HBcAg (5 μg/mL) in coating buffer (11 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Plates were blocked with 2% skim milk in PBS for 1 h at 37 °C. Subsequently, the plates were incubated with the serum samples diluted with 1% skim milk, 1% Tween 20 in PBS, for 2 h at 37 °C. The anti-mouse IgG peroxidase conjugate (Amershan, U.K.) was incubated for 1 h at 37 °C. Subsequently the plates were incubated with the substrate solution (52 mM Na_2HPO_4, 25 mM citrate, 1 mg/mL OPD, 0.1% H_2O_2) for 15 min at room temperature. Washes with 0.05% Tween 20 in PBS solution were carried out between each step three- to five-times. The reaction was stopped with 3 M H_2SO_4 solution. Finally the plates were read to 492 nm in a microtiter plate reader (Sensident Scan, Merck).

2.5. Statistical procedures

Cut-off values were considered twice the optical density (OD) values generated by negative controls (pre-immune serums). OD values from samples were processed using an Excel program able to interpolate the OD values on the standard curve of known titers.

The statistical treatment of titers was carried out using the *F*-test to evaluate variance homogeneity followed by the *t*-test for two group's comparisons. Alternatively for multiple comparisons, using one-way ANOVA and Kruskal–Wallis or Newman–Keuls test according to the specific case.

2.6. Mapping of peptide epitopes on cellulose membrane

2.6.1. Synthesis of overlapping 12-mer peptides corresponds to the amino acid sequence of the HBV nucleocapsid protein on cellulose support

To identify the regions of the HBV core protein recognized by immunization with different formulation containing the HBcAg, the peptide spot synthesis approach was used, as previously described by Frank (1992). The derivation of Whatman 540 paper was carried out sterifying the first anchor component, Fmoc-β-Ala-OH, using *N*,*N*′-diisopropylcarbodiimide (DIC) and *N*-methylimidazole (NMI) in dry *N*,*N*-dimethylformamide (DMF). The spot array on the cellulose membrane was defined anchoring Fmoc-β-Ala-OH on the previously marked positions, according to the required number of 12-mer peptides. For the assembly of the overlapping HBc sequence peptides, the standard Fmoc-/*t*-Bu chemistry was used. After the final cycle of synthesis, all peptides were N-terminally acetylated.

2.6.2. Binding of anti-HBc Abs to overlapping 12-mer peptides corresponding to the amino acid sequence of HBc protein

Cellulose sheet exhibiting the overlapping 12-mer peptides corresponding to HBcRIV-2 protein was soaked in ethanol to prevent possible hydrophobic interactions between the peptides. Ethanol was exchanged against Tris-buffered saline (TBS) (10 mM Tris, pH 7.6, 150 mM NaCl) by sequential washing, and non-specific binding was blocked by incubating overnight in 10 mL of T-TBS blocking buffer (5% Powder milk, 0.05% Tween-20 in TBS). The sheet was subsequently incubated for 3 h with the serum samples diluted in 10 mL of T-TBS blocking buffer. Serum samples were diluted according to the predetermined anti-HBcAg Ab titers (we used, 1:100 dilution for murine serum, 1:10 for murine vaginal lavages and 1:50 for a pool of human positive serum). Cellulose sheet was washed three-times with T-TBS buffer. Then an alkaline phosphatase-conjugated anti-mouse IgG (Amershan, U.K.) at 1:3000 dilution, or a biotin-conjugated anti-mouse IgA at 1:500 dilution, or an alkaline phosphatase-conjugated anti-human IgG at 1:3000 dilution, respectively, was added in T-TBS blocking buffer for 1 h. The cellulose sheet was then washed again for three-times with T-TBS. Detection of peptide bound was achieved by incubating the sheet with 0.3 mg/mL of 5-bromo 4-chloro 3-indolyl phosphate (BCIP) (Sigma), 4.5 mg/mL 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma), in substrate buffer (100 mM Tris, pH 8.9, 100 mM NaCl, 2 mM $MgCl_2$). Positive spots developed a blue/violet color. Washing with PBS stopped staining. Cellulose sheet was finally regenerated for others assays as described (Frank, 1992).

3. Results

3.1. High immunogenicity and immunoenhancing effect of two HBcAg variants

The HBcAg-specific IgG response was clearly detected in the sera of all nasally immunized mice as soon as day 10 after the first dose (data not shown). The response after a second nasal administration resulted in high titers for both HBcAg variants. After the first and second immunizations, the IgG response against both antigens resulted in non-significant differences (Fig. 1a).

The HBc-specific antibody response was higher in the groups receiving the formulation containing the mixture of HBcAg and HBsAg (groups 3 and 4) compared to the groups receiving the HBcAg alone (groups 1 and 2) (Fig. 1a). The same comparison in the case of anti-HBsAg IgG titers evidenced a superior response in the groups immunized with the combined formulation, generating a significantly higher response compared with the control group immunized nasally with the HBsAg in PBS (Fig. 1b). The specific antibody response generated by the groups containing the combination of antigens was also higher than the titers generated by

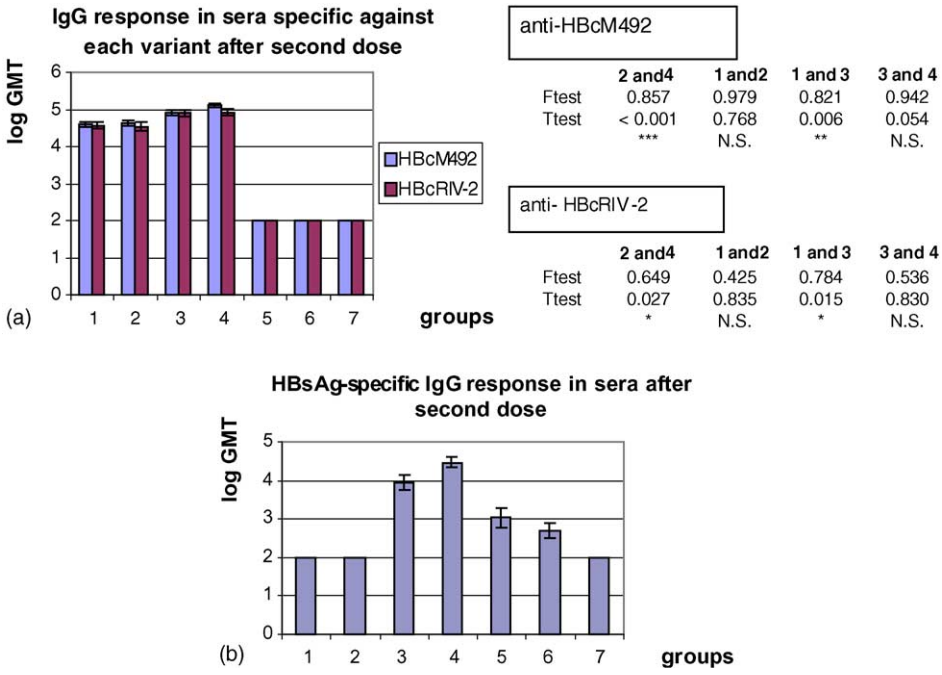

Fig. 1. Levels of IgG antibody in sera 10 days after the second dose. (a) HBcM492- and HBcRIV-2-specific IgG response. (b) HBsAg-specific IgG response.

the group immunized with the HBsAg in alum parenterally (group 6).

3.2. Linear peptide mapping on cellulose membrane

To evaluate if the inclusion of eight aa at the carboxyl-terminal region induced differences in antigenicity, the antibody response was mapped using a cellulose membrane containing 31 overlapping 12-mer peptides covering the entire sequence of the HBcRIV-2 variant. The membrane was incubated with different sera samples coming from nasally immunized mice with HBcRIV-2 (S1) and HBcM492 (S2), respectively. The recognition of the peptides 7, 8, 10, 11, 17, 18, 30 and 31 with similar intensity was observed for both variants (Table 1).

When we incubated the membrane with a pool of sera coming from mice immunized nasally with HBcRIV-2 and HBsAg (S5) we observed a decreased intensity in the recognition of peptides 7 and 8, and a slightly increase in the recognition of the carboxyl-terminal region (Table 1, S1 and S5 samples). These results were in line with the recognition obtained assaying a pool of vaginal lavages coming from the same group of mice (VL1). These results evidenced that the immunologic behaviors of the HBcAg antigen vary when we co-administered the core and surface antigens in the same formulation.

The recognition induced by a pool of anti-HBc-positive human sera coming from blood donors (S4) was evaluated comparatively. Interestingly, the results obtained showed a similar recognition pattern for the murine sample immunized with the combined formulation and the human sample. Similar to the pool of sera coming from mice immunized with HBcRIV-2 and HBsAg (S5), the human pool recognized with higher intensity the peptide 17 instead of the 18, presents a slight response against peptides 7 and 8, and a detectable recognition of the peptides 25–30 (Table 1, samples S4 and S5 respectively). In the case of the human sample, in contrast to the murine, the intensity of the recognition of peptide 30 was lower, which is in line with a previous report that refers that this specific region of the HBcAg is poorly immunogenic in humans (Tordjeman et al., 1993).

4. Discussion

4.1. High immunogenicity and immunoenhancing effect of two HBcAg variants

The HBcAg-specific IgG responses were clearly detected in the sera of all nasally immunized mice as soon as day 10 after the first dose (data not shown). These observations are in line with the reported T-cell independent behavior of the core antigen (Milich et al., 1986a; Milich et al., 1986b). The obtained results of HBcAg-specific IgG response in sera using the two variants of HBcAg demonstrate that the HBcAg was highly immunogenic by the intranasal route. Moreover, the eight aminoacids residues inserted by the carboxyl-terminal region of the HBcRIV-2 variant do not seems to affect the intensity of total IgG titers compared to the response induced by the HBcM492 (183 aa) variant.

In summary, the obtained results suggest that HBcAg is a highly potent antigen through the nasal route and a synergistic effect was obtained in formulations containing the HBcAg and the HBsAg antigens. The improvement of the

Table 1
Visual analysis of the positive recognition on cellulose membrane

	S1	S2	S3	S4	S5	VL1	S6	S7	VL2	Electric charge
1-MDIDPYKEFGAT	–	–	–	–	–	–	–	–	–	2–
2-KEFGATVELLSF	–	–	–	–	–	–	–	–	–	1–
3-VELLSFLPSDFF	–	–	–	–	–	–	–	–	–	2–
4-LPSDFFPSVRDL	–	–	–	–	+	+	–	–	–	1–
5-PSVRDLLDTASA	–	–	–	–	–	–	–	–	–	1–
6-LDTASALYREAL	–	–	–	–	–	–	–	–	–	1–
7-LYREALESPEHC	++++	+++	+++	+	+	++	–	–	–	1–
8-ESPEHCSPHHTA	++	+++	++	+	+	++	–	–	–	1+
9-SPHHTALRQAIL	–	–	–	–	–	–	–	–	–	3+
10-LRQAILCWGELM	++++	+++	+++	+++	+++	+++	*	*	*	0
11-CWGELMTLATWV	+	+	+	+	++	++	–	–	–	1–
12-TLATWVGNNLED	–	+	+	–	+	–	–	–	–	2–
13-GNNLEDPASRDL	–	–	++	–	–	–	–	–	–	2–
14-PASRDLVVNYVN	–	–	–	–	–	–	–	–	–	0
15-VVNYVNTNVGLK	–	–	–	–	–	–	–	–	–	1+
16-TNVGLKIRQLLW	–	–	–	+	+	+	–	–	–	2+
17-IRQLLWFHISCL	++	++	++	+++	+++	+++	*	*	*	2+
18-FHISCLTFGRET	+++	+++	+++	++	++	++	–	–	–	1+
19-TFGRETVLEYLV	–	–	–	–	–	–	–	–	–	1–
20-VLEYLVSFGVWI	–	–	–	–	–	–	–	–	–	1–
21-SFGVWIRTPPGY	–	–	–	–	–	–	–	–	–	1+
22-RTPPGYRPPNAP	–	–	–	+	+	+	–	–	–	2+
23-RPPNAPILSTLP	–	–	–	–	–	–	–	–	–	1+
24-ILSTLPETTVVR	–	–	–	–	–	–	–	–	–	0
25-ETTVVRRRGRSP	–	–	–	+	+	–	–	–	–	3+
26-RRGRSPRRRTPS	–	–	–	+	+	+	–	–	–	6+
27-RRRTPSPRRRRS	–	–	–	+	+	+	–	–	–	7+
28-PRRRRSQSPRRR	–	–	–	+	+	+	–	–	–	7+
29-QSPRRRRSQSRE	–	–	–	+	+	+	–	–	–	4+
30-RSQSRESQCKLG	+++	++++	++++	++	++++	++++	–	–	–	2+
31-SQCKLGSVDLN	++	++++	+++	+	++++	++	–	–	–	0

S1: sera pool of mice immunized i.n. with HBcRIV-2, S2: sera pool of mice immunized i.n. with HBcM492, S3: sera pool of mice immunized subcutaneously with HBcRIV-2 in alum, S4: HBc-positive human sera pool, S5: sera pool of mice immunized i.n. with HBcRIV-2 and HBsAg, S6: HBc-negative murine sera pool, S7: human HBc-negative sera pool, VL1: pool of vaginal lavages of mice i.n. immunized with HBcRIV-2 and HBsAg and VL2: HBc-negative vaginal lavages pool. (*) Negative controls that generate a yellow coloration, different to the positive blue, in the peptides 10–17.

immune response against HBsAg at IgG level after nasal administration is a valuable result as the antibody levels in blood mediate the protection against HBV. As it is known, mucosal immunization needs potent adjuvant strategies to compete in efficacy and costs with the parenteral route of immunization. The properties found in the combined formulations are useful in the development of a new strategy to improve the HBsAg titers up to protection levels and HBcAg antigenic determinants can be introduced with the addition of HBcAg. The HBcAg has been regarded also as a protective antigen although HBsAg has shown superior results at this regard, however a formulation containing the mixture of both antigens is a more potent and cost-effective candidate as the core antigen behaves at the same time as the vaccine adjuvant.

The adjuvant activity of HBcAg may result from their chemical composition as a nucleoprotein, containing around 1% of nucleic acids inside of the particle. These molecules have important stimulatory effects. However, we do not discard the potential involvement of HBcAg-specific B cells as professional antigen presenting cells in the presentation of HBsAg epitopes as HBsAg physically associates to HBcAg (data not shown). Peptides inserted in the HBcAg sequence could follow the same pathway.

4.2. Linear peptide mapping on cellulose membrane

The results obtained indicated that the antibody elicited by both HBcAg variants have a similar recognition pattern, evidencing that the eight additional aa inserted at HBcRIV-2 variant does not generated variations in the linear recognition pattern, suggesting that the conformation was not affected by this kind of insertions. Interestingly the recognition observed for the two last peptides by murine sera samples was particularly intense, which does not correspond with the consulted literature that refers a poor immunogenicity for the carboxyl-terminal region of the HBcAg (Ulrich et al., 1992; Schodel et al., 1990).

In addition to the above explained immunoenhancing effect of HBsAg on HBcAg, we also observed the influence of this co-administration in the recognition of linear sequences on the HBcAg.

The similar results obtained from the comparative study with a pool of anti-HBc-positive human sera were interesting

if we have into account that the HBc-specific antibody response was generated in humans where this antigen co-exist with the HBsAg after the HBV infection.

5. Conclusions

Although more experimentation should be done in terms of cellular immune response, the available results indicated that the HBcAg has an immunoenhancing effect on nasally co-delivered HBsAg. The insertion of peptides at the carboxyl-terminal region of the HBcAg doesn't affect their intrinsic adjuvant property or the antigenicity of HBcAg. The formulation containing the surface and core antigens for intranasal administration elicited a potent immune response to HBsAg, a promising result in the design of new candidates for therapeutic or preventive approaches. The linear epitopes recognized on the HBcAg sequence after vaccination with the combined formulation reproduced the natural behavior of anti-HBcAg positive blood donors.

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