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HCV core protein modulates the immune response against the HBV surface antigen in mice

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Abstract

Mucosal vaccination is currently arousing a great deal of interest, since mucosally induced immunity is able to protect not only against microorganisms using mucosa as a door of entry, but also against those parenterally transmitted. Hepatitis C virus (HCV) is considered a worldwide health problem and a current vaccine is not available. In the present work, immunogenicity of particulate HCcAg was evaluated, administered alone and also in formulations with the main protective antigen of HBV, the surface antigen (HBsAg), both by mucosal (i.n) and parenteral (i.m) routes. HCcAg was able to induce strong immune responses after nasal as well as parenteral administration, developing a strong Th1-like antibody response in serum. Preliminary data also suggested the ability of HCcAg to efficiently enhance and modulate the host immune response against HBsAg. These results support the use of the particulate HCcAg in the rational design of candidates for HCV therapeutic or preventive vaccine strategies or inclusively in the development of future combined vaccines.

Keywords: Hepatitis C virus; Core antigen; Virus-like particles; Hepatitis B virus

Mucosal vaccination is currently arousing a great deal of interest, since mucosal immunization allows the induction of strong immune responses both in mucosal and systemic compartments. These responses involve the overall secretory as well as the central immune system [1,2]. Thus, mucosally induced immunity is able to protect not only against microorganisms colonizing mucosa or using mucosa as a door of entry, but also against those parenterally transmitted.

Except for a small number of vaccines, with the live polio vaccine being a notable example, most vaccines are administered parenterally [3,4]. The main reason is that the antigens required for inducing the protective immune responses are large molecular weight compounds which penetrate absorption barriers poorly and are generally highly labile to enzymes present at the portals of drugs absorption [4,5].

Numerous approaches have been attempted in order to develop mucosal vaccines. One example is the use of living bacterial or viral vectors as carriers [6]. Mucosal delivery of non-proliferating vaccine antigens usually requires mucosal adjuvants to elicit immune responses [7]. However, several particulate antigens have evidenced a strong mucosal immunogenicity such as the whole-cell pertussis vaccine [1], group B meningococcal outer membrane vesicles [8], and the hepatitis B virus core antigen (HBcAg) [9,10]. This property is related to their particulate state and physico-chemical nature, enabling antigen sampling and transport by M cells from nasopharyngeal epithelium to immune-inductive lymphoid tissues [11]. Interestingly, these antigens also evidenced their capacity to immunoenhance the host immune response against co-administered heterologous antigens [10,12].

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It is known that parenteral administration of vaccines has been associated to insecure practices like repeated use of syringes in developing countries [13]. The prevention of hepatitis B virus (HBV) infection is one of the targets to develop a mucosal vaccine. Although commercially available vaccines against HBV are quite effective, a mucosal vaccine could be effective too, taking into consideration that the sexual transmission is one of the main routes of transmission for HBV [14]. A mucosal vaccine will also avoid the use of syringes, potentially lowering the cost of vaccination, and increasing comfort and security.

One of the challenges facing the development of hepatitis C virus (HCV) vaccine is their extensive genetic variation [15]. The HCV core protein (HCcAg) is among the most conserved proteins in HCV [16] and is known to induce sensitization of both helper (Th) and cytotoxic T lymphocytes (CTL) [17]. Therefore, HCcAg could constitute an important vaccine component.

We have previously described the HCcAg comprising the first 120 aa of the HCV polyprotein (HCcAg.120), as a 30 nm particle [18]. This structure possesses appropriate physico-chemical and geometric attributes for effective presentation to the mucosal lymphoid tissue. Additionally, we had demonstrated that the particulate HBcAg enhanced the immune response against the hepatitis B surface antigen (HBsAg), after nasal co-administration of both antigens [10]. So, we decided to explore the nasal immunogenicity of HCcAg.120 and also its capacity to enhance or modulate the HBsAg-specific immune response to obtain a potential vaccine candidate for hepatitis B and C viruses.

Materials and methods

Antigens. The HBsAg was produced with more than 95% of purity at the CIGB production facilities (CIGB, Cuba). The antigen was expressed and purified from the yeast *Pichia pastoris* and was quantified by producers and accepted by the quality control department.

HCcAg.120 was produced for research use only up to 90% and characterized as a particle of approximately 30 nm as previously described [18,19].

Immunization schedule. HCcAg.120 and HBsAg were used to raise specific antibodies in mice. Six groups of 10 female Balb/C mice (6–8 weeks old) each were immunized either intramuscularly (i.m.) or intranasally (i.n.) as follows: Group 1 was i.n. immunized with 10 μg of HCcAg in phosphate-buffered saline solution (PBS) (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.4), Group 2 was i.n. immunized with 5 μg of HBsAg in PBS, Group 3 was i.n. immunized with 10 μg of HCcAg.120 and 5 μg of HBsAg in PBS, Group 4 was i.m. immunized with 10 μg of HCcAg.120 adsorbed to alum hydroxide, Group 5 was i.m. immunized with 5 μg of HBsAg adsorbed to alum hydroxide, and Group 6 was i.m. immunized with 10 μg of HCcAg.120 and 5 μg of HBsAg adsorbed to alum hydroxide. All groups were boosted at 14 and 28 days after primary immunization. Serum samples were taken before immunization and on day 42 by retro-orbital punction.

Enzyme-linked immunosorbent assay. The 96-well microtiter plates (Costar, Cambridge, MA) were coated either with 100 μL of HCcAg.120 (10 μg/mL) or HBsAg (10 μg/mL) diluted in coating buffer (50 mM carbonate buffer, pH 9.6) at 4 °C overnight. After three washes with PBS containing 0.05% Tween 20 (PBST), the wells were blocked with 100 μL of PBST containing 1% skimmed milk at room temperature for 1 h. Each well received 100 μL of the mice sera at different dilutions in PBST and the plates were incubated at 37 °C for 1 h. The plates were washed five times with PBST and 100 μL of 1:3000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, UK) was added at 37 °C for 1 h. The plates were then washed five times with PBST and incubated at room temperature for 10 min with 100 μL of a solution containing 0.05% (p/v) *o*-phenylenediamine (Sigma, St. Louis, USA) and 0.05% (v/v) H_2O_2. Subsequently, 50 μL of 3 N H_2SO_4 was added to stop the reaction and the absorbance at 492 nm was read in a microplate reader (Sensident Scan, Merck). The cutoff values were established by duplication of the mean absorbance (OD) value of mice pre-immune serum from three determinations.

In the case of IgG1 to IgG2a subclass determination, two specific biotinylated monoclonal antibodies for IgG1 and IgG2a (Amersham, UK) were used at 1:3000 dilution. After 1-h incubation, a second conjugate of streptavidine and peroxidase (Amersham, UK) was used at 1:3000 dilution in 100 μL.

Statistical procedures. Comparisons between groups of mice were analyzed using Student's t test. All tests were 2-tailed and differences were considered significant when $P \leqslant 0.05$.

Results and discussion

Several formulations containing HCcAg.120 and HBsAg either mixed or alone, in solution or adsorbed to alum, respectively, were assayed for i.n. and i.m. administration. In general, HCcAg-specific serum IgG titers obtained after nasal administration of HCcAg.120 without adjuvants were comparable to those obtained after i.m. immunization of HCcAg.120 adjuvated with alum (Fig. 1A). The strong HCcAg-specific antibody (Ab) response obtained is in agreement with the high immunogenicity shown by other particulate nucleocapsid antigens, i.e., HBcAg both parenterally and nasally [9].

In addition, i.n co-immunization of HCcAg and HBsAg induced a strong HCcAg-specific serum IgG antibody response. Although this response was apparently higher in intensity to that obtained by using HCcAg alone, no significant differences were observed (Fig. 1A). However, when both antigens were co-administered by the i.m. route and adjuvated with alum, a statistically significant improvement in the HCcAg-specific serum IgG antibody response was observed. It is interesting to note that the use of nasal administration resulted in a higher variability of titers. Furthermore, the intensity of the resulting HCcAg-specific IgG titers after i.n and i.m inoculation of 10 μg of HCcAg alone was extremely high, decreasing the capacity to evidence differences using a reduced number of animals (Fig. 1A).

On the other hand, high HBsAg-specific serum IgG titers were obtained after HBsAg immunization (Fig. 1B). In addition, no significant differences in the HBsAg-specific IgG antibody titers were observed between nasal and intramuscular routes (Fig. 1B).

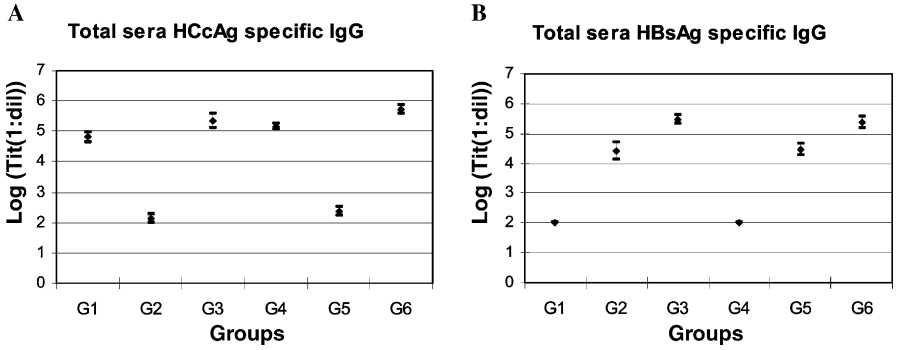

Fig. 1. (A) Titers of the HCcAg specific IgG response in sera of mice after immunization are shown. G6 > G4*, G6 > G1*. (B) Titers of the HBsAg-specific IgG response in sera of mice after immunization are shown. G3 > G2*, G3 > G5*, G6 > G5*, and G6 > G2*. Results are shown as logarithm (log) of the reciprocal of the serum dilution equivalent to twice the mean optical density (OD 492 nm) of negative control. Error bars indicating the standard errors of the means are shown. Stat: *Student's t test ($p < 0.05$).

The effect of HCcAg on HBsAg immunogenicity was also analyzed (Fig. 1B). Interestingly, after addition of HCcAg to the formulation of HBsAg for both nasal and parenteral administration, a statistically significant improvement in the HBsAg-specific serum IgG antibody response was observed as compared to that obtained when HBsAg was administered alone (Fig. 1B). This clearly demonstrated an immunoenhancing effect of HCcAg over HBsAg immunogenicity. Note that the observed adjuvant effect by i.n and i.m routes was similar in intensity.

These results support the strong immunogenicity of HCcAg by nasal and parenteral routes as well as its capacity to act as an adjuvant after inoculation by both nasal and intramuscular routes when co-immunized with HBsAg. These properties are likely to be related with the particulate nature of HCcAg as has been previously reported for other particulate antigens [9,20,21].

Isotype analysis of the specific antibodies induced by HBsAg and HCcAg were also explored by the evaluation of the major IgG subclasses in sera for all the groups involved in the experiment. Interestingly, HCcAg induced a predominant specific IgG2a response independent of the route and formulation used. In addition, this Th1-like IgG subclass pattern was not affected by the co-immunization with HBsAg (Fig. 2, Table 1).

Table 1
HCcAg-specific and HBsAg-specific IgG1/IgG2a ratio

Groups	G1	G2	G3	G4	G5	G6
HCcAg-specific IgG1/IgG2a ratio	0.40	—	0.24	0.25	—	0.33
HBsAg-specific IgG1/IgG2a ratio	—	14.63	0.98	—	66.25	6.43

On the contrary, HBsAg induced a Th2-like IgG subclass pattern independent of the immunization route and formulations used (Fig. 3, Table 1). Remarkably, HCcAg modulated the HBsAg-specific IgG subclass pattern to the Th0-like sense for both alum-based i.m as well as for PBS-based nasal formulations. Note that HBsAg-specific IgG1/IgG2a subclass ratio decreases from 15 and 66 in those mice i.n and i.m immunized with HBsAg alone to 1 and 6, respectively, after the co-administration of both antigens. Thus, HCcAg not only induced a Th1-like pattern against itself, but also demonstrated a strong immunomodulatory activity over the host immune response against HBsAg.

Recently, it has been demonstrated that nucleotides bound to the positive residues of HBcAg are responsible for an intrinsic adjuvant activity of this antigen, which

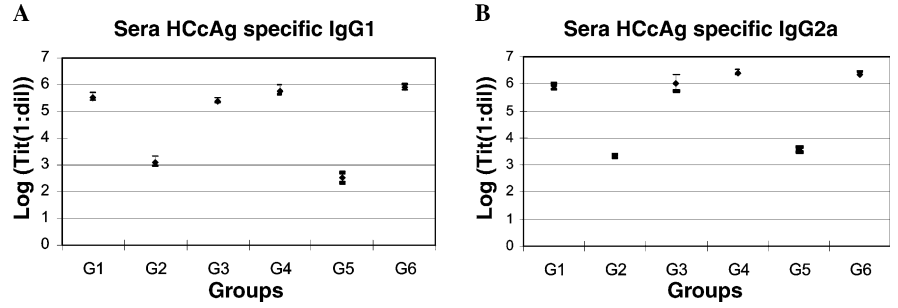

Fig. 2. Titers of the HCcAg-specific IgG1 (A) and HCcAg-specific IgG2a (B) responses in sera of mice after immunization are shown. Results are shown as log of the reciprocal of the serum dilution equivalent to twice the mean optical density (OD 492 nm) of negative control. Error bars indicating the standard errors of the means are shown. Stat: Student's t test ($p < 0.05$).

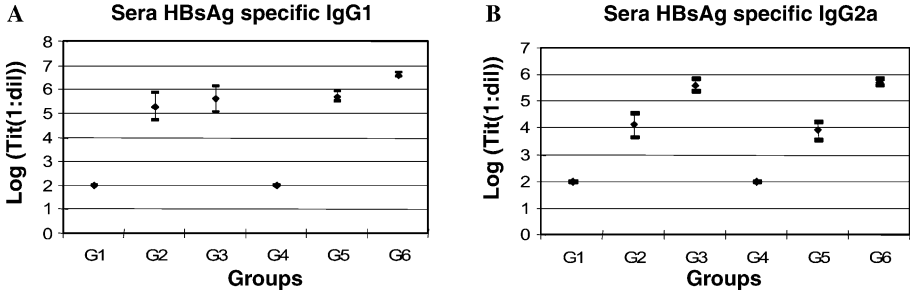

Fig. 3. Titers of the HBsAg-specific IgG1 (A) and HBsAg-specific IgG2a (B) responses in sera of mice after immunization are shown. Results are shown as log of the reciprocal of the serum dilution equivalent to twice the mean optical density (OD 492 nm) of negative control. Error bars indicating the standard errors of the means are shown. G6 > G5* (A); G3 > G2*, G3 > G5*, G6 > G2*, and G6 > G5** (B). Stat: *Student's t test ($p < 0.05$), **($p < 0.01$).

induces Th1 immunity against the homologous antigen [22]. In addition to the HCcAg particulate nature, a similar property of HCcAg to interact with nucleotides could explain its adjuvant activity as well as its capacity to induce a Th1-biased isotype switching. In this regard, previous studies have shown that HCcAg binds to nucleotides [23,24].

The antigens used in the present study induced strong immune responses after mucosal and parenteral immunization. This work also evidenced the synergistic interaction between HBsAg and HCcAg in terms of adjuvanticity. Previously, it had been shown that a strong Th1-like T cell response specific for HCcAg in HCV infected patients is related either with a benign course of the infection or with a successful immune response against HCV [17,25]. So, HCcAg.120 could be considered as an attractive target for HCV vaccines. On the other hand, further studies directed to explore the capacity of HCcAg to improve and modulate the immune response to homologous and heterologous antigens coupled, inserted or added to the particle are in progress.

In conclusion, HCcAg was able to induce strong humoral immune responses after nasal as well as parenteral routes, developing a strong Th1-like antibody response in serum. Preliminary data also suggested the ability of HCcAg to efficiently enhance and modulate the host immune response for co-administered antigens. These results support the use of HCcAg in the rational design of candidates for HCV therapeutic or preventive vaccine strategies or inclusively in the development of future combined vaccines.

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