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Biochemical and Biophysical Research Communications 300 (2003) 745–750
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Mucosal immunogenicity of the hepatitis B core antigen☆

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Received 26 November 2002

Abstract

The hepatitis B virus (HBV) core antigen (HBcAg) is a potent immunogen in animal models and humans and has been used as a carrier for several antigens, however, the mucosal immunogenicity of HBcAg or chimeric HBcAg proteins has been poorly studied and only using the truncated variant of the HBcAg. In this study we explored the mucosal immunogenicity in mice of the recombinant complete nucleocapside of HBcAg. The antigen was administered by different mucosal and parenteral routes. The antibody response in sera was evaluated after each immunization and mucosal lavages were tested with the final extraction. To characterize the immune response, the serum IgG antibody response was tested during six months and also the ratio IgG2a to IgG1 was determined. The results obtained evidenced that the mucosal immunogenicity of HBcAg depended on the administration route, being the intranasal (i.n.) route the one that generated the higher IgG responses in sera, similar in intensity and duration to parenteral administrations. The IgA response in mucosal washes was superior for nasally immunized mice compared to the rest of mucosal and parenteral groups. The nasal route also induced the higher IgG2a to IgG1 ratio, evidencing a Th1-like Ab subclass pattern. In addition to the high Ab responses, preliminary results of the cellular response induced by nasal administration evidenced the induction of strong lymphoproliferative responses in spleen cells.

Keywords: HBsAg; Mucosal; Core; Hepatitis

The hepatitis B core antigen (HBcAg) is one of the immunodominant components in hepatitis B virus (HBV) infection [1,2]. 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 [3]. Also by several properties derived from their potent activation of B cells which functions as primary APC, explaining the enhanced immunogenicity of HBcAg [4,5]. The antibodies directed against the HBcAg appear early during the course of the HBV infection and they often persist for years after the recovery of the disease [6].

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 [7–12]. The hybrid core proteins, achieved by the insertion of different sequences within the immunodominant region around the amino acid 80, can still be folded in the HBcAg chimeric particles, inclusive in the case of large insertions as the green fluorescent protein [13].

Immunogenicity studies using parenteral routes, comparing the 183-amino acid 21 kDa core protein and the HBeAg, indicate that the HBcAg preferentially but not exclusively elicits Th1-like cells conversely to HBeAg that elicits Th0 or Th2-like cells [14] although both proteins share >70% of their amino acid sequence and most T and B cell epitopes. HBcAg contains 5–20 ng RNA/μg protein while nucleotide binding to HBeAg is not detectable. Priming specific Th1 immunity by recombinant HBcAg depends on its arginine (Arg)-rich, 34–36-residue-long C terminus, and nucleotides bound to it. Particle-incorporated RNA has a 1000-fold potency as a Th1-inducing adjuvant than free RNA mixed to a protein antigen [15].

☆ *Abbreviations:* HBV, hepatitis B virus; HBcAg, hepatitis B virus core antigen; HbsAg, hepatitis B virus surface antigen.

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doi:10.1016/S0006-291X(02)02897-8

There are few studies of mucosal immunization of HBcAg. The oral administration of recombinant *Salmonella* as an antigen delivery system of HBcAg particles with homologous and heterologous insertions evidenced the effectivity of this system to induce strong responses against both antigens, however, the particle in this study was carried by the attenuated *Salmonella* as an antigen delivery system [16].

To our knowledge, only one report uses the HBcAg particle as a carrier through the nasal route in mice. This study evidenced that it is possible to induce an Ab response after nasal administration of truncated HBcAg chimeric protein. Interestingly, the antibody response against the carrier and the inserted antigen demonstrated poor immunogenicity [17].

The present work explores the mucosal immunogenicity of the recombinant hepatitis B nucleoprotein antigen, expressed in *Escherichia coli* as the entire particle of 183 amino acids, after intranasal, oral, sublingual, intrarectal or intravaginal administration, using HBcAg in buffer solution as an immunogen and comparing the response obtained by mucosal routes with the parenteral controls immunized with the same formulation without adjuvants.

Materials and methods

Antigen. The rHBcAg protein was expressed in *E. coli*, strain W3110, previously transformed with a plasmid that contained the entire core antigen gene under the control of the tryptophan promoter [18]. 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 (pressure 1500 kgf/cm^2) were used for the rupture and the supernatant was collected and precipitated twice at 10% and at 30% of saturation with ammonium sulfate. The resulting pellet was resuspended and applied to a Q-Sepharose fast flow (Pharmacia, Sweden) column. The peak containing HBcAg was concentrated and applied to a column XK 50/100 (Pharmacia) packed with Sepharose CL-4B gel. The resulting rHBcAg was obtained with a purity over 95% and size of 28 nm as characterized by electron microscopy (data no shown). From the same electronic microscopy analysis a central zone of strong electronic density was clearly detected. A posterior spectroscopic analysis at 260 and 280 nm evidenced a higher optical density ratio 260–280 nm compared to non-nucleoprotein antigens like HBsAg and BSA (Table 1). Both characteristics, capture ELISA test and Western blotting using specific McAb, were used to follow and identify the complete HBcAg particles during and at the end of the purification process.

Immunization schedules. The first immunization schedule employed seven groups of eight female Balb/c mice, of 8–12 weeks old each. Five of these groups were immunized for several mucosal routes intranasal (i.n.), oral, sublingual (s.l.), intravaginal (i.v.), and intrarectal (i.r.) using 5 μg HBcAg in phosphate-buffered saline (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.2) (PBS). The administrations were on days 0, 15, 30, and 180. Two groups systemically immunized were used as controls, intraperitoneal (i.p.) and subcutaneous (s.c.). For the i.n., oral, and s.l. immunizations, mice were anesthetized by i.p. administration of 30 μL ketamine 10 mg/mL. For systemic immunizations and the rest of the mucosal routes, mice were not anesthetized. A volume of 100 μL per mouse for systemic routes and 50 μL for mucosal routes was used. In the i.n. group, each mouse received 25 μL per nostril administered with a micropipette. Sera were collected on days −2, 10, 25, 40, and 190. On day 190 pulmonary and vaginal washes were carried out.

A second experiment was carried out in 8–12 week old female Balb/c mice, to assess the immunogenicity of HBcAg in terms of lymphoproliferative response in spleen cells. The schedule was the same until the third dose. Two control groups of non-immunized and placebo mice were included in the experiment. Groups of three mice were sacrificed 30 and 60 days after the third immunization to carry out the lymphoproliferative assay (LPA).

Biological fluids. Serum was collected without anesthesia, via retrorbital plexus. The blood was centrifuged to 12,000 rpm for 10 min (Eppendorf centrifuge, 5415C) and the serum was conserved at −20 °C until evaluation.

The pulmonary lavages were obtained by homogenization of lungs in 1 mL PBS and centrifuged to 12,000 rpm for 10 min (Eppendorf centrifuge 5415C) in order to separate cellular debris. The obtained supernatant was stored at −20 °C until analysis.

Vaginal washes were carried out reflushing 100 μL of sterile PBS solution with a micropipette. Finally collected volumes were centrifuged to 12,000 rpm for 10 min (Eppendorf centrifuge 5415C) and supernatants were stored at −20 °C until analysis.

ELISAs. The specific IgG and IgA responses in serum and mucosal washes were analyzed by ELISA. Briefly, high binding plates (Costar, USA) were coated with 100 μL HBcAg 10 μg/mL in coating buffer (11 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Plates were blocked with 1% skim milk in PBS during 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 goat-anti-mouse IgG, IgG1, and IgG2a peroxidase conjugate (Amersham, UK) or goat-anti-mouse IgA peroxidase conjugate (SIGMA, USA) was incubated 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, and 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 microplate reader (Sensident Scan, Merck). In the case of the IgA response, data from mucosal lavages corresponded to 1:10 dilution of the samples.

Lymphoproliferation assays. Unfractionated PBMC (0.1×10^6/well) were incubated for 4 days at 37 °C in the presence of HBV nucleocapside antigen (0.1 and 1 μg/ml). All proliferation assays were performed in triplicate in 96-well plates and [^{3}H]thymidine (^{3}H-TdR; 0.5 μCi/well; specific activity, 2.0 Ci/mmol/L; Amersham International, Buckinghamshire, UK) was added 12 h before harvesting. Results are expressed as stimulation index (SI) which represents the ratio between the mean cpm obtained in the presence and absence of antigen. SI values above three standard deviations above the mean of all SI values were regarded as positive.

Statistical procedures. Cut–off values were considered twice the OD values of negative controls mean at 492 nm (serum coming from a mixture of preimmune serums). OD values from samples were processed using an Excel program that is able to determine a value of titer plotting the OD values on the standard curve of known titers.

The treatment of titers was carried out using the program PRISM. For the statistical analysis we initially used a one-way ANOVA for all

Table 1
Absorbance ratios 260/280 nm for several proteins

Protein	A_{260} nm/A_{280} nm
BSA	0.71
HBsAg	0.958
HBcAg	1.617

the groups involved. In the case of $p < 0.05$, we used Kruskal Wallis test. In the other case, if $p > 0.05$ then we used the Newman Keuls parametric test.

Results

IgG antibody response in serum

Specific IgG responses were clearly detected as soon as day 10 in the sera of all mucosally immunized mice. The immunogenicity of HBcAg was variable, depending on the immunizing route (Fig. 1). The response after nasal administration resulted in titers significantly higher than those reached through the other mucosal routes. This nasal group reached levels of serum IgG titers very close to parenterally immunized mice during all the schedule. The kinetics of the IgG antibody response in serum after each nasal administration also resembled the one obtained by the i.p. and s.c. controls. Conversely, groups immunized by i.r., i.v., and oral routes evidenced a rapid seroconversion at day 10 but did not increase after the second dose, generating a variable increase after the third dose. Reaching a medium level in the case of the i.r. route and a low level for mice immunized by i.v. and oral routes. Interestingly, the group immunized sublingually developed a rapid seroconversion and also a second increase after second dose, resembling the subcutaneous control. This group maintained this intermediate level after the third administration, reaching similar levels as by i.r. administration. A booster administered with the same formulation on day 180 increased significantly the total IgG response for all groups except for i.r. and s.l. groups. Using as a reference the titers generated in the i.n. group, the immune response was sustained, with a similar slope compared to the parenteral controls during 180 days.

The i.n. route induced a significant increase in the IgG2a titers (Fig. 2), conversely the i.v. route induced significantly more IgG1 compared to the IgG2a level, the rest of the groups induced statistically similar titers of both IgG subclasses. In general, with independence of the route, there was a very balanced IgG subclass response for the major IgG subclasses.

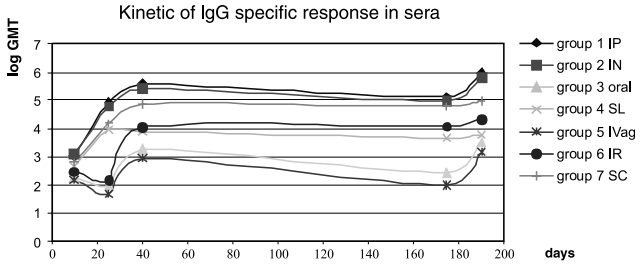
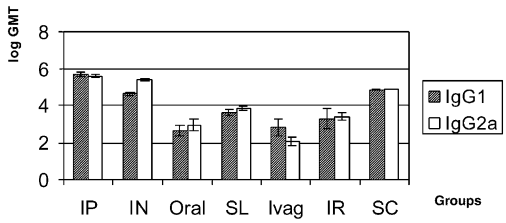


Fig. 1. Kinetics of serum IgG antibody response in mice immunized through intraperitoneal (IP), intranasal (IN), oral, sublingual (SL), intravaginal (Ivag), intrarectal (IR), and subcutaneous (SC) routes.



Relation	IP	IN	Oral	SL	IVag	IR	SC
IgG2a/IgG1	0.9847	1.1651	1.1229	1.0599	0.7004	1.0322	1.0117

Fig. 2. Serum IgG antibody subclass response after fourth dose in mice immunized through intraperitoneal (IP), intranasal (IN), oral, sublingual (SL), intravaginal (Ivag), intrarectal (IR), and subcutaneous (SC) routes.

IgA antibody response

The IgA Ab response was measured at the end of the immunization schedule. The geometric mean of titer (GMT) of IgA antibody responses in serum was superior to 1:100 and statistically similar for all the mucosally immunized groups. The s.l. and i.v. groups induced significantly higher IgA titers in sera compared to the intraperitoneal group (Fig. 3). There was no difference between the i.n. immunized group and the rest of mucosal and parenteral groups in terms of serum IgA titers.

The mucosally secreted IgA response was tested in vaginal and pulmonary lavages of all immunized groups. In lung washes, we observed statistical differences in IgA titers favoring the group immunized by the i.n. route compared to control groups immunized by parenteral routes and also to groups immunized by the rest of the mucosal routes (Fig. 4).

In the case of the vaginal response, titers generated against the HBcAg were also greater after nasal administration compared to i.v. immunization ($p < 0.001$) (Fig. 5). The same result was obtained for the rest of the mucosal and parenteral groups.

Lymphoproliferative response in spleen cells

With the aim of evaluating in a preliminary study the capacity of the nasal route to induce cellular responses in spleen cells, we tested the specific lymphoproliferative response after nasal administration of HBcAg. We used

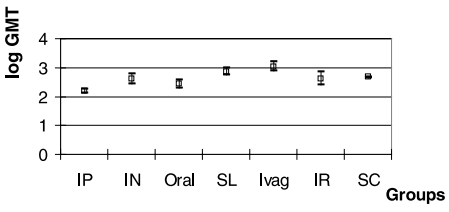


Fig. 3. Serum IgA antibody response after fourth dose through intraperitoneal (IP), intranasal (IN), oral, sublingual (SL), intravaginal (Ivag), intrarectal (IR), and subcutaneous (SC) route.

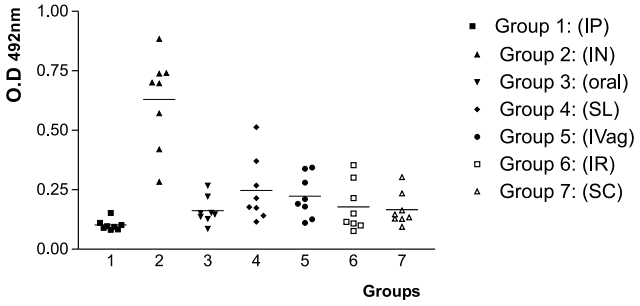


Fig. 4. IgA antibody response in pulmonary lavages after fourth dose. The OD 492 nm shown results correspond to a 1:10 dilution of the samples. The obtained OD value for the negative control (pool of lavages coming from non-immunized mice) was 0.088.

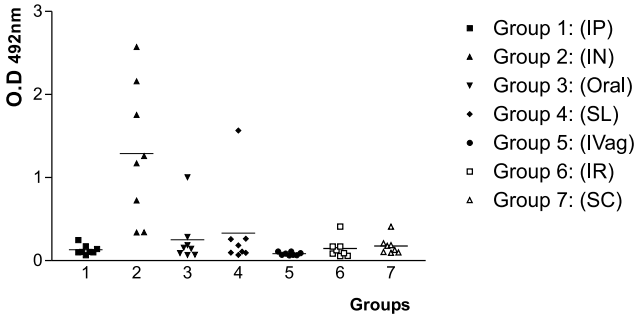


Fig. 5. IgA antibody response in vaginal lavages after fourth dose. The shown OD 492 nm results correspond to a 1:10 dilution of the samples. The obtained OD value for the negative control (pool of lavages coming from non-immunized mice) was 0.091.

non-immunized and placebo mice as control groups. The specific LP responses against HBcAg were measured at two different times, 1 and 2 months after the third dose, to explore the duration of this cellular response. The results evidenced that the administration of HBcAg was able to induce a strong LP response in spleen cells 1 month after the last immunization and was maintained at a lower but still high level after the second month. Results are presented in Fig. 6.

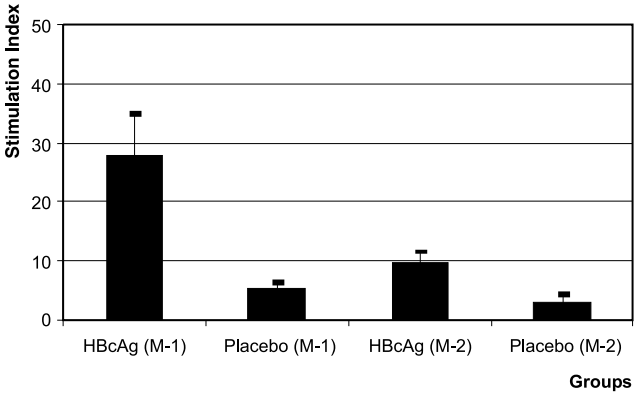


Fig. 6. Lymphoproliferative response of nasally administered HBcAg 1 and 2 months after the last immunization. Placebo corresponding to BS immunized mice.

Discussion

The mucosal immunogenicity of HBcAg has been poorly studied, however, this antigen is regarded as an excellent carrier molecule [7–12] and also as a candidate for preventive or therapeutic strategies in their natural form or using their several T-cell epitopes [19–22]. In the present work we have studied the immunogenicity of the HBcAg after mucosal administration. The experiments assessed the intensity and duration of humoral immune responses in serum and mucosal secretions of mice administered by several mucosal and parenteral routes. Also, a preliminary study to evaluate the capacity of the nasal route to induce cellular responses in spleen cells.

Our results evidenced that the mucosal immunogenicity of HBcAg depended on the administration route. All mucosal routes induced early IgG responses in sera. After the first nasal and sublingual administrations, the intensity of serum IgG responses was similar in intensity to parenterally immunized control groups. This rapid development of the IgG immune response has been reported for parenteral routes, and has been explained by the strong capacity of HBcAg to induce both T-cell independent and T-cell dependent B-cell responses [3].

Nasal administration of HBcAg finally resulted in a higher response and s.l. and i.r. groups induce medium levels of IgG titers in sera, respectively. The rest of the routes tested evidenced a low IgG response (Fig. 1). The behavior after second and third i.n. administrations of HBcAg followed the same kinetics and also induced similar IgG titers compared to i.p. and s.c. groups (Fig. 1). The behavior after second and third doses was different in kinetics and intensity for the rest of the mucosal routes. Nevertheless, there are similarities in the relative intensity of IgG subclasses (IgG2a to IgG1 ratio), evidencing a balanced pattern for both subclasses in most of the groups (Fig. 2). With the exception of i.n. group, where the IgG2a was significantly higher compared to IgG1, and the i.v. route with the contrary effect, the rest of the groups—including parenteral controls—did not present statistical differences (Fig. 2).

Although the role of the humoral immune response against HBcAg does not seem to be important for protection against the HBV [23,24], we used this response as an indicative of immunogenicity in our studies, also, to explore the IgG subclass pattern after mucosal administration that will be an important indicative of Th1–Th2 balance in the development of the immune response [14]. Our result evidenced a Th1-like pattern.

To our knowledge, this is the first study using the complete HBcAg particle in PBS by mucosal routes. This result contrasts with previous studies using chi-

meric proteins evidencing poor immunogenicity against both the HBcAg and the heterologous insert [17].

The strongest IgA specific response found in pulmonary and vaginal washes after i.n. administration of HBcAg (Figs. 4 and 5) indicates that the i.n. route is the most efficient route to induce specific IgA antibody responses in airways and vaginal secretions. This result has obvious interest for the possible nasal use of HBcAg as a carrier of heterologous epitopes from pathogens colonizing nasopharynx, vagina, or those having in airways or vagina their portals of entry.

The analysis of the electronic microscopy of the HBcAg particle obtained in our laboratory evidenced that the HBcAg contains a central zone clearly visualized by their high electronic density, this zone not being presented by the HBsAg particle. Also, the measure of the optical density at 260 and 280 nm showed a clear superiority for HBcAg for the ratio of optical density values at 260 and 280 nm compared to the HBsAg and BSA as control proteins (Table 1). These results are physical evidences of *E. coli* nucleic acids [25]. The nucleic acid component is bound to the arginine-rich domains inside the particles in a proportion of 5–20 ng/μg of protein resulting from a purification process characterized by the isolation of the HBcAg produced in the cytoplasm of *E. coli* without disrupting the particle. It is known that priming specific Th1 immunity by recombinant HBcAg depends on its arginine (Arg)-rich, 34–36-residue-long C terminus, and nucleotides bound to it [15]. The presence of this component in our antigen contributes to the strong nasal immunogenicity and to the resulting balanced response found in most of the groups. Also, these responses correlate with strong cellular immune responses found in our preliminary studies of specific lymphoproliferative activity.

This result in terms of cellular immunity has obvious implications in the development of therapeutic strategies of immunization. A recent report has demonstrated that the presence of HBV HBcAg-specific but not HBsAg specific CD4+ T-cells was detected in seronegative partners, virus-exposed individuals, indicating that the antiviral protective immune response had been established upon subclinical infection. These HBV-specific T-cells may contribute to protection from HBV-infection in the absence of an anti-HBV humoral immune response [26].

Usually, HBV chronic infection is accompanied by a state of hyporesponsiveness or tolerance to the major antigens including the HBcAg. This fact has been correlated with the induction of chronicity [24]. We hypothesize that a mucosal therapeutic administration of the HBcAg could offer a potential way to scape from this state of tolerance. The assumption is based mainly on the characteristic compartmentalization of the mucosal immune system and their capacity to overcome concomitant systemic immune depressions [27,28].

Conclusions

Based on our experience administering the complete HBcAg nucleoprotein particle without adjuvants we concluded that the intranasal immunization of HBcAg is the most immunogenic mucosal route, able to promote a Th1-like antibody pattern and the strongest mucosal responses. Although more experimentations should be done in terms of cellular immune response, preliminary results in LP activity evidenced the capacity of the route to induce strong and long lasting humoral and cellular responses in sera and spleen cells, respectively. These results could have implications in the design of mucosal preventive and therapeutic vaccine candidates.

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