Immunology and Cell Biology (2004) **82**, 539–546 doi:10.1111/j.1440-1711.2004.01278.x

Special Feature

Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen

JC AGUILAR,[1] Y LOBAINA,[1] V MUZIO,[1] D GARCÍA,[1] E PENTÓN,[1] E IGLESIAS,[1] D PICHARDO,[1] D URQUIZA,[1] D RODRÍGUEZ,[1] D SILVA,[2] N PETROVSKY[2] and G GUILLÉN[1]

[1]*Biomedical Branch, Center for Genetic Engineering and Biotechnology, Havana, Cuba and* [2]*Autoimmunity Research Unit, ANU Medical School, The Canberra Hospital, Canberra, ACT 2606, Australia*

Summary There are estimated to be 350 million chronic carriers of hepatitis B infection worldwide. Patients with chronic hepatitis B are at risk of liver cirrhosis with associated mortality because of hepatocellular carcinoma and other complications. An important goal, therefore, is the development of an effective therapeutic vaccine against chronic hepatitis B virus (HBV). A major barrier to the development of such a vaccine is the impaired immune response to HBV antigens observed in the T cells of affected patients. One strategy to overcome these barriers is to activate mucosal T cells through the use of nasal vaccination because this may overcome the systemic immune downregulation that results from HBV infection. In addition, it may be beneficial to present additional HBV epitopes beyond those contained in the traditional hepatitis B surface antigen (HbsAg) vaccine, for example, by using the hepatitis B core antigen (HBcAg). This is advantageous because HBcAg has a unique ability to act as a potent Th1 adjuvant to HbsAg, while also serving as an immunogenic target. In this study we describe the effect of coadministration of HBsAg and HBcAg as part of a strategy to develop a more potent and effective HBV therapeutic vaccine.

Key words: cellular, hepatitis B, immune, nasal, Th1, Th2, vaccine.

Introduction

Hepatitis B core (HBcAg) and surface (HBsAg) antigens are the main structural antigens of hepatitis B virus (HBV). Both antigens constitute potent immunogens for experimental animals as well as in humans acutely infected with HBV.[1] In vaccine studies, HBcAg is a potent immunogen even in the absence of adjuvant, and can be used as a carrier molecule for homologueous and heterologous epitopes.[2–6] HBsAg is the antigen used in the commercial recombinant HBV vaccine, which has been proven over the last 20 years to be a very safe and effective prophylactic vaccine against HBV infection.[7] However, there remains a need for development of more potent hepatitis B vaccines, both for use as prophylactic vaccines in poor responders to the current HBV vaccines and for use in therapeutic HBV vaccination. Recent developments include combined prophylactic vaccines,[8] plant-derived vaccines,[9] vaccines administered through novel routes[9–12] and therapeutic vaccination.[13–17]

There are estimated to be approximately 350 million chronic carriers of HBV worldwide.[7,18] Patients with chronic HBV are at risk of developing liver cirrhosis, which is associated with a high rate of mortality because of complications of portal hypertension and hepatocellular carcinoma.[19] Simple immunization protocols with the existing HBV vaccine have been ineffective in solving the problem imposed by HBV chronic infection.[14] This emphasizes the need for more potent HBV antigens and adjuvant strategies. These new strategies take into account the effect of HBV on the immune system plus virological and immunological aspects of patients who recover from infection compared to those who are unable to clear the virus.[17] A combination of antiviral treatments with therapeutic vaccination is a promising new strategy.[17] Impaired T-cell immune responses to HBV antigens are observed in patients with chronic HBV infection.[20–22] Chronic infection is also associated with functional defects in the dendritic cells (DC).[23–25] Thus, in order for therapeutic vaccination strategies to be effective in eradicating the virus they must restore the immune system and overcome the well-documented state of unresponsiveness.

Our recent work on a therapeutic vaccine has been directed at the delivery of HBV antigens through the mucosal route. Nasal administration of HBV antigens targets the nasal-associated lymphoid tissue (NALT). Nasal administration enables antigens to access very specialized mechanisms for antigen sampling,[26] including antigen uptake by M cells. M cells transport antigens from the luminal surface through a thin cytoplasm to a pocket at the basal surface. These pockets, considered cellular modifications, are M-cell functional adaptations reflecting their role in mucosal uptake of luminal antigens. M-cell pockets enable the interaction of the antigen

Correspondence: Julio Aguilar, Biomedical Branch, Center for Genetic Engineering and Biotechnology, Apdo 6162, Havana 10600, Cuba. Email: Julio.aguilar@cigb.edu.cu

Received 18 May 2004; accepted 19 May 2004.

with the cells of the immune system in a compartment protected from the modulatory effect of systemic immunity.[26] Another potentially important mechanism of mucosal immunization is the interaction of the antigen with DC in the tonsils. These professional APC are organized in a surface network of approximately 500 DC/mm^2.[26]

Previous studies have demonstrated that the strong immunogenicity of HBcAg is explained by its dual behaviour as a T-cell dependent and independent antigen.[27] This is related to the ability of HBcAg to act as a potent B-cell activator, enabling activated B cells to work efficiently as primary APC.[4,5] Another explanation for the strong immunogenicity of HBcAg is the presence of nucleic acids bound to the C-terminal region of the HBcAg molecule. These nucleic acids are copurified with HBcAg as a nucleoprotein from *Escherichia coli*. Toll-like receptor 3 (TLR3) binds double-stranded RNA and activates MyD88, MAP kinases and NF-κβ.[28,29] A similar interaction of capsid-derived RNA with TLR3 in endosomes suggests their potential use as adjuvants or immunomodulators. Furthermore, particle-incorporated RNA has 1000-fold higher potency as a Th1-inducing adjuvant than free RNA mixed to a protein antigen.[30]

The HBV has been detected in many different tissues apart from hepatocytes.[1] Therefore, we considered that the nasal route would be a very efficient route to induce immunity against the 183 amino acid HBcAg nucleoprotein.[31] In this work we describe the effect of coadministration of HBsAg and HBcAg on the immunogenicity of both antigens and advance a novel strategy for the development of more potent HBV therapeutic vaccine candidates.

Materials and Methods

Antigens

HBsAg was produced to more than 95% purity at the Center for Genetic Engineering and Biotechnology production facilities (CIGB, Havana, Cuba) as a component of the commercial HBV prophylactic vaccine, Heberbiovac-HB. HBsAg for this vaccine is expressed and purified from the yeast *Pichia pastoris.* HBcAg was expressed in *E. coli* strain W3110, which had previously been transformed with a plasmid containing the entire core antigen gene under the control of a tryptophan promoter (A Musacchio *et al*., unpubl. data). Luria Bertani (LB) medium supplemented with 50 μg/mL kanamycin was used to culture the *E. coli* strain. After centrifugation the supernatant was discarded and the pellet was resuspended. Three passes in a French press (Typ KDL, Basel, Switzerland) were used for rupture and the supernatant was collected and precipitated with ammonium sulfate. The resulting pellet was resuspended and applied to a Q-sepharose fast flow column (Pharmacia, Uppsala, Sweden). The peak containing HBcAg was concentrated and applied to a Sepharose CL-4B column (Pharmacia). The resulting HBcAg had a purity of >95% and measured 28 nm as characterized by electron microscopy. The formulation containing both antigens was obtained by simple mixture in PBS (0.1 mol/L NaCl, 2 mmol/L KCl, 10 mmol/L Na2HPO4, 1 mmol/L KH2PO4, pH 7.2).

Electron microscopy

For electron microscopy (EM) analysis we prepared the combined formulation as a simple mix of 5 μg of each antigen (HBsAg and HBcAg) in PBS at 0.1 mg/mL. Samples of each individual antigen were also prepared at the same concentration to be used as controls.

Direct EM was carried out using negative stain with 2% uranil acetate. We followed the procedure described by Roth *et al*.[32] Briefly, two drops of the sample were deposited for 5 min on a copper grill containing 400 holes covering a previously de-ionized membrane (JEOL, Peabody, MA, USA). Three random fields were observed using a transmission electron microscope (JEOL) with an acceleration voltage of 80 Kv and 40 000× magnification.

Immunization schedules

The immunization schedules were carried out using 8–12-week-old female Balb/c mice in groups of 6–10 mice. Intramuscular (i.m.) or intranasal (i.n.) immunization routes were used. For the i.n. route, a volume of 50 μL per mouse was used (25 μL per nostril). Mice were anaesthetized using an i.p. injection of 30 μL of ketamin 10 mg/mL. For the systemic route we used a volume of 100 μL administered i.m. in the back.

The first immunization schedule employed four groups of eight mice each. The antigen administrations were carried out on days 0 and 14, and the sera were collected on day 21. Groups 1 and 4 received 5 μg of HBsAg or HBcAg, respectively, in PBS using the i.n. route. Group 2 was immunized with 5 μg HBsAg and 5 μg HBcAg i.n. As a control we immunized mice i.m. with 5 μg HBsAg in alum (0.5 mg/mL) (Group 3).

To examine the behaviour of the antibody subclass response in a different strain of mice, a similar protocol was followed in C57/Bl6 mice using only the groups immunized i.n. with the combination of antigens and a control group immunized i.m. with HBsAg in alum.

A second immunization schedule was carried out using four groups of 10 mice each to assess the cellular immune response against HBsAg using the ELISPOT technique. In this schedule we administered four doses on days 0, 15, 30 and 90. The first group was immunized i.n. with 5 μg of HBsAg in PBS. The second group received the combined formulation: 5 μg HbsAg + 5 μg HBcAg using the i.n route. As controls we employed two groups, the first group received i.m. HBsAg 5 μg in alum (0.5 mg/mL) and the other was a placebo group immunized i.n. with PBS.

Several experiments were carried out with the aim of evaluating the cellular proliferative response induced by the i.n. coadministration of HBsAg and HBcAg. For that purpose five groups of 10 mice were employed. All groups were immunized using the i.n route, except group 1, which was immunized i.m. Antigen administrations were carried out on days 0, 14, 28 and 90, and the sera were collected 10 days after each dose. The first group was a control and received 5 μg of HBsAg in alum (0.5 mg/mL) (Group 1). Group 2 received 5 μg of HBsAg and 5 μg of HBcAg. The following groups were immunized with 5 μg of HBsAg or HBcAg, respectively, in PBS (Groups 3 and 4). The final group (Group 5) was used as a placebo and received PBS. On day 42, four animals per group were killed to evaluate the lymphoproliferative response.

Biological fluids

Sera were collected via the retrorbital plexus. The blood was centrifuged at 6000 *g* for 10 min in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany) and the serum was conserved at –20°C until evaluation.

ELISA

Isotype-specific IgG was analysed using ELISA. In brief, high binding plates (Costar, Corning, NY, USA) were coated with 100 μL of HBsAg or HBcAg 10 μg/mL in coating buffer (11 mmol/L

Na_2CO_3, 35 mmol/L $NaHCO_3$, pH 9.6) and incubated over night at 4°C. Plates were blocked with 2% skim milk in PBS for 1 h at 37°C. The plates were incubated with the serum samples diluted with 1% skim milk and 1% Tween 20 in PBS for 2 h at 37°C. The antimouse IgG peroxidase conjugate (Sigma, St Louis, MO, USA) was incubated for 1 h at 37°C. Subsequently the plates were incubated with the substrate solution (52 mmol/L Na_2HPO_4, 25 mmol/L citrate, 1 mg/mL o-phenylene-diamine (OPD), 0.1% H_2O_2) for 15 min at room temperature. The plates were washed with 0.05% Tween 20 in PBS solution three to five times between each step. The reaction was stopped with 3 mol H_2SO_4 solution. Finally, the plates were read at 492 nm in a microplate reader (Sensident Scan; Labsystem, Helsinki, Finland). Subclass analysis was carried out with the kit ISO-2 Mouse Monoclonal Antibody Isotyping Reagents (Sigma).

Statistics

Cut-off values were considered to be twice the optical density (OD) values of the negative control means at 492 nm (serum coming from a mixture of pre-immune serums). The OD values from the samples were analysed using an Excel program to determine a value of titre plotting the OD values on a standard curve. The statistical treatment of titres was carried out using *F*- and *T*-tests or by using one-way ANOVA and Kruskal–Wallis or Newman–Keuls tests depending on the specific case.

Lymphoproliferation assays

Suspensions of unfractionated splenocytes were prepared from groups of four mice and incubated (0.1×10^6 cells/well) for 4 days at 37°C in the presence of HBV antigens (2.5 μg/mL and 5 μg/mL). Assays were conducted 3–5 times per antigen and per antigen concentration during the stimulation. The results are representative of at least four repetitions. 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 the stimulation index (SI), which represents the ratio between the mean c.p.m. obtained in the presence and absence of antigen. Stimulation index values three standard deviations above the mean of all negative SI values were regarded as positive.

Enzyme-linked immunospot assay for IFN-γ

Preparation of target and effector cells

Ten days after the last immunization, spleens were aseptically removed and individual cell suspensions were prepared. Erythrocytes were lysed by 5 min of 0.83% NH_4Cl incubation. The cells were extensively washed with medium, resuspended in RPMI-1640 (Gibco, Scotland, UK) supplemented with 10% FCS, 2 mmol/L glutamine, 2 mmol/L piruvate, 50 mmol/L 2-mercaptoethanol and antibiotics (complete medium) and counted. Meanwhile, H-2^d mastocytome cells p815 were pulsed for 1 h at 37°C, 5% CO_2 in complete medium with 10 mmol/L $S_{(28–39)}$ peptide IPQSLDSWWTSL from HBsAg.[33] After incubation p815 cells were further incubated for another 15 min with Mitomycin C (Sigma). The cells were extensively washed to avoid any trace of Mitomycin C and resuspended in complete medium for counting. The p815 cells without peptide were also treated as controls.

In vitro *re-stimulation of primed CTL*

After washing, the cells were counted and distributed at 2×10^6 cells/mL in 10 mL RPMI FCS in 25 cm^2 flasks (Nalge-Nunc, Rochester, NY, USA) and stimulated with 10 mg/mL of peptide $S_{(28–39)}$. After culturing for 4 days in 5% CO_2, half of the total medium was substituted and new medium containing 2×10^4 U/mL of IL-2 was added. On day 7, cells were collected and counted. Subsequently, 10^4 and 5×10^4 cells/well were added to 10^5 p815 cells, previously pulsed with the peptide for 1 h. As a positive control for the assay we used stimulation with concanavalin A (2 μg/well). Every group was controlled using the same number of wells incubated with non-pulsed p815 cells as a negative control and the experimental controls of non-immunized mice.

ELISPOT assay

Nitrocellulose-backed 96-well plates (MAHA S45; Millipore, Bedford, MA, USA) were coated with 100 mL of 5 mg/mL murine IFN-γ-specific mAb R4-6A2 (Pharmingen, San Diego, CA, USA) overnight at 4°C, washed three times with PBS and blocked using complete medium at 37°C for 1 h. Two dilutions (2×10^5 and 1×10^5) of freshly isolated or restimulated splenocytes and 1×10^5 p815-pulsed cells were incubated for 20 h at 37°C in 5% CO_2. Unpulsed p815 cells were incubated as negative controls and splenocytes stimulated with 2.5 μg/mL Concanavalin A were used as positive controls.

The plates were washed three times with PBS and five times with 0.05% Tween 20 in PBS, and then 0.5 μg/mL of secondary biotin-conjugated antibody XMG1.2 (Pharmingen) was added and reacted at room temperature for 2 h. The wells were washed five times with 0.05% Tween 20 in PBS and peroxidase-labelled streptavidine (Sigma) was added at a dilution of 1:1000 for 1 h. The wells were washed again with 0.05% Tween 20 in PBS and PBS and the spot were developed by adding 3,3′-diaminobenzidine (3,3′,4,4′-tetra-aminobiphenyl) tetrahydrochloride (Sigma) in 50 mmol/L Tris-HCl, pH 7.4, with 0.3% H_2O_2. After 15 min the wells were washed with tap water, dried and spot counted using a dissection microscope. The ELISPOT was assayed under restimulation with the described peptide.

Results

The combined formulation of HBsAg and HBcAg (HBs & HBc) was initially characterized using EM. In line with previous literature, HBsAg and HBcAg individually had the physical appearance of virus-like particles using EM. Both particles were obtained and purified to a minimum of 95% purity and a size analysis revealed that both antigens were homogeneous in size and in the range of 20–30 nm. HBcAg was ~30 nm (Fig. 1b) and HBsAg ~20 nm (Fig. 1a). It was possible to discriminate between HBcAg and HBsAg based on the well-recognized high electron density nucleus of the HBcAg particle (Fig. 1b). It has been reported previously that the cause of this high electron density is the presence of nucleic acids encapsulated in the particle, which is associated with the arginine-rich C-terminal region of the HBcAg protein 36.

Based on the characteristic nucleus of HBcAg we could easily observe the aggregation of HBsAg and HBcAg particles when mixed in the resulting HBs and HBc formulation (Fig. 1c). This aggregation could result from a natural tendency of both particles to aggregate, which was evident from the tendency of both antigens alone to form aggregates (Fig. 1a,b). Aggregates of various sizes, up to 200 nm, were observed.

Serum IgG antibody response

There was a significant increase in specific IgG response against HBsAg obtained in sera of mice nasally immunized

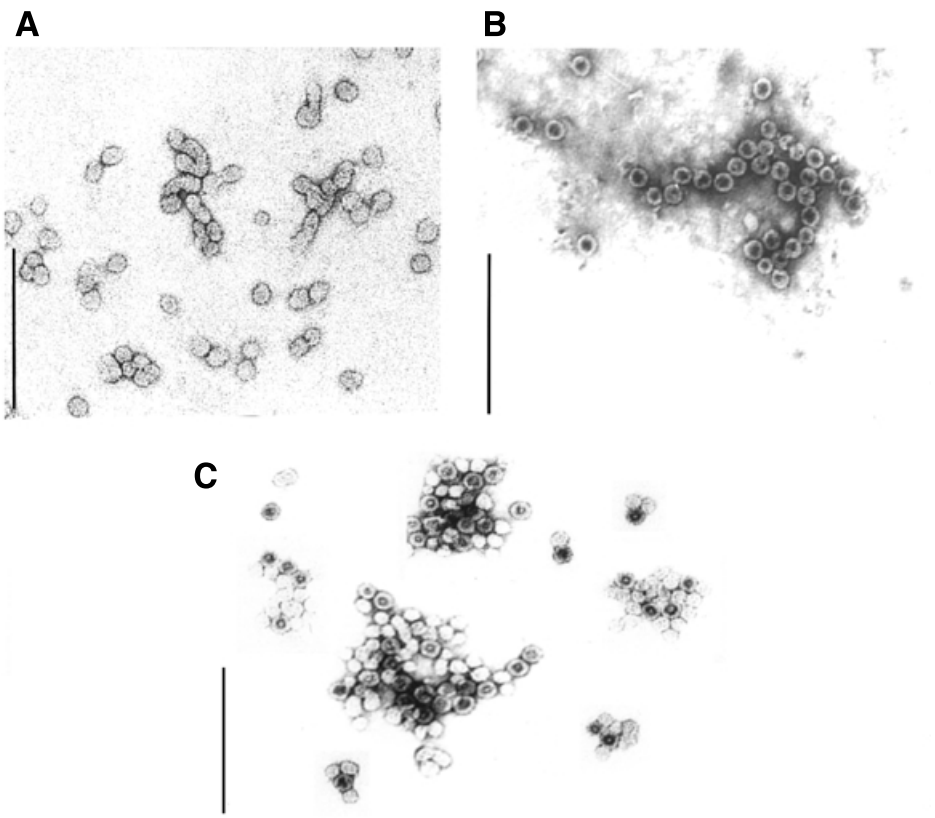

Figure 1 Transmission electron microscopy of (a) particles of HBsAg, (b) particles of HbcAg and (c) formulation of HBsAg and HBcAg in PSB. Bar equals 200 nm.

with the HBs and HBc formulation compared with the group nasally immunized with the HBsAg in PBS ($P < 0.001$). A comparison with control mice administered HBsAg i.m. confirmed the superiority of the response induced by nasal immunization of the combined HBs and HBc formulation ($P < 0.001$) (Fig. 2a). In the case of the HbcAg-specific IgG response, the combined HBs and HBc formulation gave significantly higher responses than HBcAg alone ($P < 0.001$), indicating that HBcAg increases the immune response to HbsAg and conversely HBsAg increases the response to HBcAg (Fig. 2b).

IgG subclass pattern analysis

Serum IgG subclass patterns were compared in the groups of mice immunized nasally with the combined HBs and HBc formulation and the corresponding parenteral control immunized with HBsAg in alum. Both C57/Bl6 mice and Balb/c mice revealed a strong IgG2a immune response to HBsAg after nasal immunization with the combined HBs and HBc formulation. As a result, the ratio of HbsAg-specific IgG1/IgG2a (Fig. 3a,b) and IgG1/IgG2b (data not shown) were significantly decreased ($P < 0.05$) in both mice strains, indicating a Th1 switch induced by HBcAg. In the case of HbcAg-specific IgG subclass response, a similar pattern of a switch to an IgG2a immune response was observed with the combined HBs and HBc formulation. For HBcAg alone there was a very potent IgG2a and IgG2b antibody response (data not shown), which is consistent with nasally administered HBcAg predominantly inducing a Th1 antibody response to itself.

Lymphoproliferative and IFN-γ responses after nasal or i.m. immunization with HBsAg and HBcAg

The capacity of the nasal route to induce cellular responses in systemic compartments was evaluated by studying lymphoproliferative and IFN-γ responses of unfractionated spleen cells to HBsAg and HBcAg after nasal immunization with HBsAg or HBcAg alone or the combined HBs and HBc formulation compared to i.m. immunization with HBsAg in alum. The IFN-γ ELISPOT assay was conducted using the immunodominant $HBs_{(28–39)}$ CTL peptide from HBsAg as previously described. Measurement of lymphoproliferative activity revealed the induction of strong proliferative responses in systemic compartments against both HBsAg and HBcAg after nasal-combined immunization. The antibody responses obtained with the combined HBs and HBc formulation were consistently higher than the responses induced by HBsAg or HBcAg alone, whether administered i.n. or i.m. (Fig. 4).

It was possible to detect IFN-γ secretion to HBsAg 1 month after a booster dose on day 90 using an ELISPOT assay incorporating a restimulation protocol. A significant increase in the secretion of IFN-γ was obtained for the group immunized with the nasal-combined formulation compared with the HBsAg nasally administered in PBS ($P < 0.05$) and the parenteral control of HBsAg in alum ($P < 0.05$) (Fig. 5).

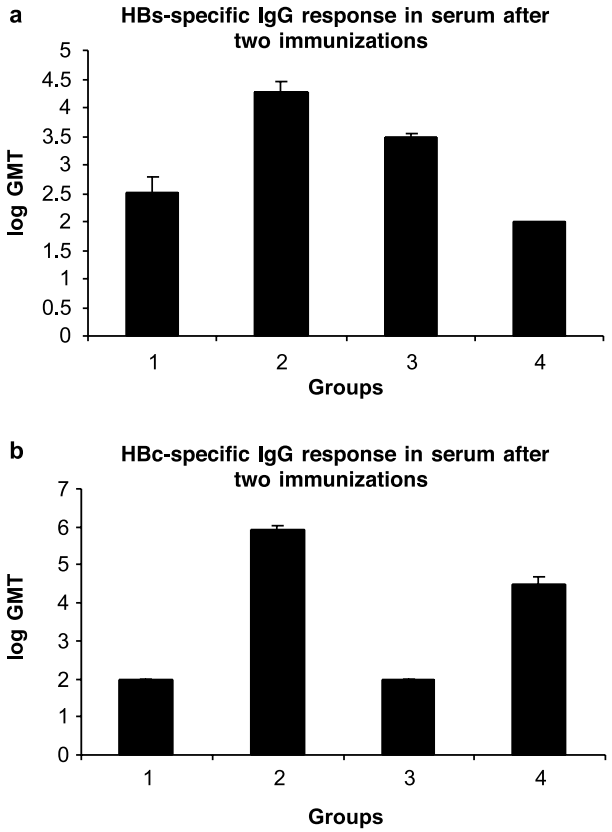

Figure 2 Levels of IgG antibody in sera 10 days after Balb/c mice received a second immunization dose. Immunization groups: (1) intranasal HBsAg in PBS, (2) intranasal HbsAg + HbcAg, (3) i.m. HBsAg in alum and (4) i.m. HBcAg in PBS. (a) The HBsAg-specific IgG response and (b) the HBcAg-specific IgG response. Group 4 in (a) and groups 1 and 3 in (b) represent the minimum dilution assayed in which no positive signal was detected.

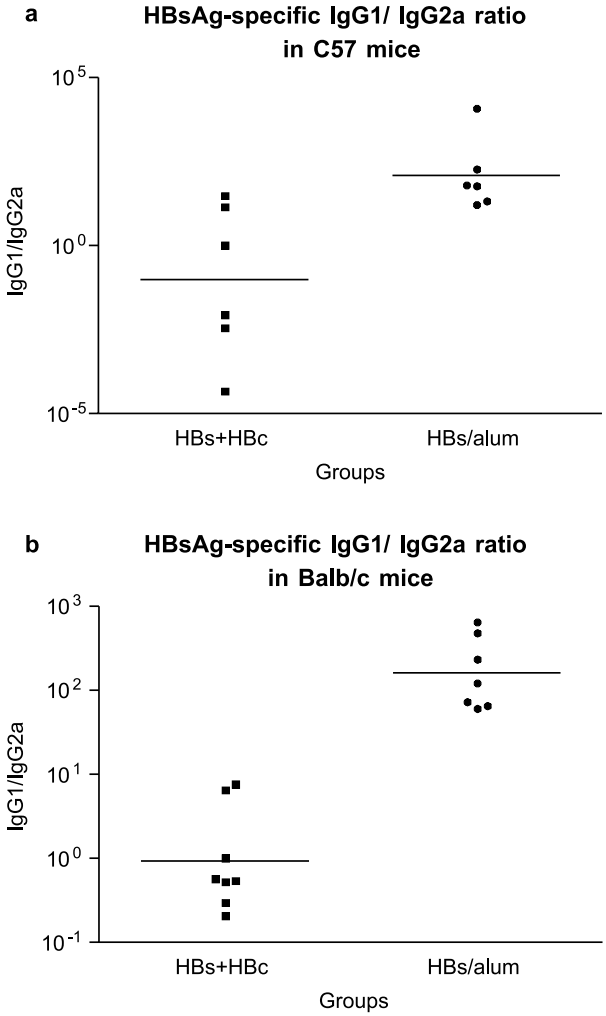

Figure 3 HBsAg-specific IgG subclass patterns in (a) C57/Bl6 mice and (b) Balb/c mice after i.n. immunization with HBs and HBc or i.m. immunization with HBsAg in alum. Each dot represents the ratio of IgG1 titre to IgG2a titre for an individual mouse.

Discussion

A marked enhancement in antibody and cellular responses towards both HBsAg and HBcAg is observed after the nasal coadministration of a formulation containing a mixture of HBsAg and HBcAg. Although this synergism is also observed when HBsAg and HBcAg are coadministered i.m., the total antibody response was 1–2 logs higher after nasal compared to parenteral administration. Furthermore, the use of the nasal route enhanced the production of IgG2a, suggesting the ability of nasally administered HBsAg and HBcAg to favour a Th1 response. This feature held true when using either Balb/c or C57/Bl6 mice, despite the fact that the former have an overall Th2 bias.

The apparent adjuvant effect of HBcAg for HBsAg could be explained in several ways. New properties may arise in formulations containing both HBsAg and HBcAg as a result of the ability of such mixtures to form self-organizing aggregated structures. Both HBsAg and HBcAg naturally aggregate into particles with HBsAg forming particles measuring 22 nm and HBcAg forming particles measuring 28 nm. Both antigens interact to generate aggregated structures ranging in size from 22 nm to 200 nm (Fig. 1). The particulate nature of antigens is an important feature for the immunogenicity of nasally administered antigens.[33–35]

Another mechanism that may explain how HBcAg can act as an adjuvant relates to its unique 3-D folding, which generates a regular spacing between repetitive spikes on its surface. This feature enables HBcAg to bind and activate a high number of naive B cells. Following binding of HBcAg, the cross-linking of immunoglobulin membrane receptors on B cells results in intracellular signalling, secretion of IgM and IgG and upregulation of costimulatory molecules.[36] Taking into consideration the very fast and specific uptake of HBcAg by B cells,[36] aggregated particles may be taken up and processed by B cells preferentially, driving the HBsAg through a very effective route for antigen processing and presentation.

The above results are highly relevant to therapeutic vaccination for chronic hepatitis B infection. It has been suggested that the effect of serum anti-HBcAg antibodies in blocking the interaction between B cells and HBcAg may be an important factor in viral immunopathogenesis.[36] Mucosal

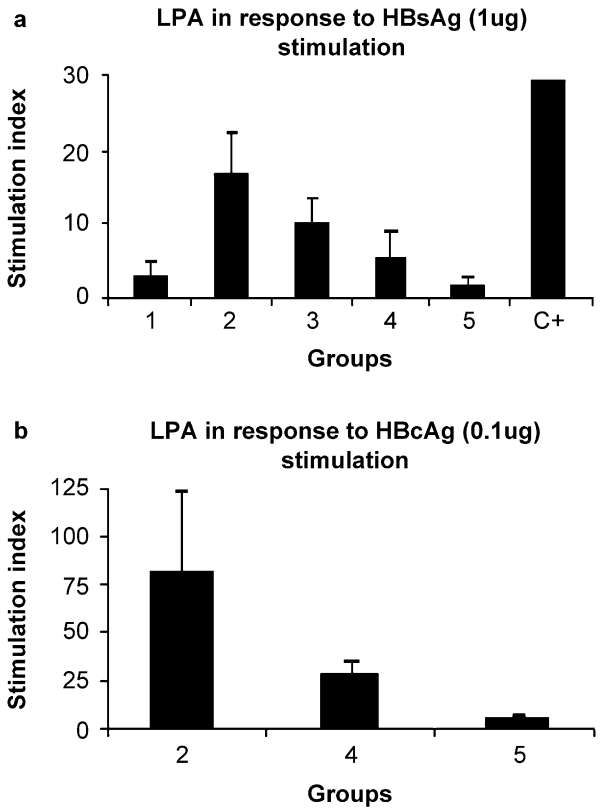

Figure 4 Lymphoproliferation assays using spleen cells 2 weeks after the third immunization dose. Assays were performed in pools using three mice per group. (a) HBs-specific proliferative response and (b) HBc-specific proliferative response. Immunization groups: (1) i.m. HBsAg in alum, (2) i.n. HbsAg + HbcAg, (3) i.n. HBsAg in PBS, (4) i.n. HBcAg in PBS and (5) placebo and control (C+).

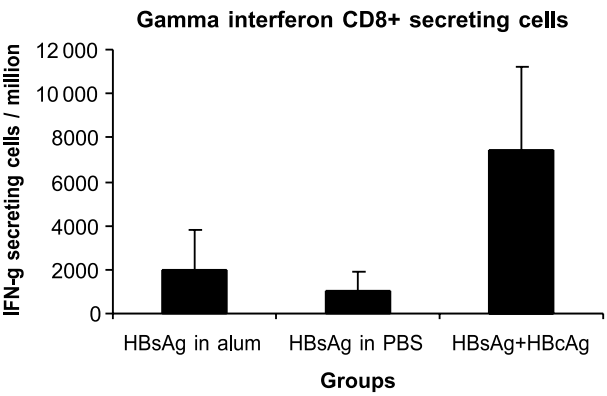

Figure 5 Restimulated ELISPOT assays. Splenocytes were cultured *in vitro* with peptide 28–39 of HBsAg and then IFN-γ-producing cells were detected using an ELISPOT assay. n = five mice. There was a fourfold higher number of peptide 28–39-specific IFN-γ-producing cells in animals immunized with the combination of HBsAg plus HBcAg compared with either antigen alone.

administration of HBsAg and HBcAg is directed at avoiding this negative effect of systemic anti-HBcAg antibodies that are present as a general feature of chronic HBV infection. The protected compartment offered by M cells may enable the administered antigen to avoid the modulatory effect of high serum anti-HBcAg titres.

Another point of interest regarding mucosal administration concerns the recent discovery of HBV replication in salivary glands and the secretion of HBeAg.[37] These results suggest that oral tolerance may develop towards HBeAg or against HBcAg (which shares around 80% of the HBeAg primary sequence) by virtue of the HBeAg being secreted by the salivary glands, swallowed and treated by the immune system as a dietary antigen. It is hypothesized that mucosal administration of antigens in a Th1-immunomodulatory environment may help in the subversion of such oral tolerance.[38]

Recent reports from Bertoletti *et al*. reveal an important degree of T-cell functional recovery after antiviral treatment with Lamivudine.[39,40] This suggests that it is possible to rescue T cells from non-responsive states. Other reports demonstrate that in patients naturally recovering from HBV chronic infection, there is a rebound in cellular immunity that correlates with viral clearance.[41] This raises the possibility of combining nasal vaccination with antiviral treatment as a means of overcoming non-responsiveness and tolerance to hepatitis antigens in chronically infected individuals. The interaction between HBcAg-activated B cells and T cells results in the development of a Th1 phenotype, followed by antibody isotype switching and the increased production of IgG anti HBcAg antibodies.[36,42] The same effect was obtained in the HBsAg immune response in the combined HBs and HBc formulation studied in two different mice strains (Fig. 3).

There was a marked increase in the number of IFN-γ-producing spleen cells in mice immunized with the combined HBs and HBc formulation. The ELISPOT assay using the P815 mastocytome cell line pulsed with the $CD8^+$-restricted $HBs_{(28–39)}$ peptide indicated that nasal immunization with the combined HBs and HBc formulation activates antigen specific $CD8^+$ cells, consistent with a strong Th1 response. This result is in line with the finding that B cells that bind viral capsids can prime cytotoxic T cells.[43] We were also able to show that proliferative T-cell responses were enhanced using nasal administration of the combined HBs and HBc formulation. This was evident from the strong adjuvant effect of HBcAg on the cellular immune response against HBsAg and vice versa.

These results demonstrate the benefits of mucosal vaccination as a strategy to induce strong systemic immunity and overcome immune non-responsiveness. To our knowledge this concept has never before been applied to therapeutic immunization. In our opinion, mucosal therapeutic vaccination should not be seen as a replacement for parenteral vaccination. In the specific case of chronic HBV therapeutic vaccination, the nasal administration of a combination of HBsAg and HBcAg should help in the generation of more potent and generalized anti-HBV responses in infected patients and can be used as an adjunct to parenteral therapeutic vaccination with standard or enhanced HBV vaccines. As revealed by this study, new properties arise from the mixture of HBsAg and HBcAg as a consequence of the adjuvant and immunomodulatory actions of both antigens. Nasal administration of both antigens in a combined formulation induces a

heightened humoral and cellular immune response against both HBsAg and HBcAg. We hope that these results will contribute to the design of an effective HBV therapeutic vaccine candidate that can be used alone or together with existing HBV parenteral vaccines.

Acknowledgements

The Center for Genetic Engineering and Biotechnology (CIGB) has proprietary interests in the hepatitis B prophylactic and therapeutic vaccines described in this paper. The authors recognize the support received for this work from the CIGB and from the National Health Sciences Centre, Canberra, Australia.

References

1 Mandell GL, Bennet JE, Dolin R. *Principles and Practice of Infectious Diseases* 5th edn [CD Rom]. New York: Churchill Livingstone, 2000.
2 Stahl SJ, Murray K. Immunogenicity of peptide fusions to hepatitis B virus core antigen. *Proc. Natl Acad. Sci. USA* 1989; **86**: 6283–7.
3 Schodel F, Moriarty AM, Peterson DL *et al.* The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. *J. Virol.* 1992; **66**: 106–14.
4 Yoshikawa A, Tanaka T, Hoshi Y *et al.* Chimeric hepatitis B virus core particles with parts or copies of the hepatitis C virus core protein. *J. Virol.* 1993; **67**: 6064–70.
5 Borisova GP, Berzins I, Pushko PM *et al.* Recombinant core particles of hepatitis B virus exposing foreing antigenic determinants on their surface. *FEBS Lett.* 1989; **259**: 121–4.
6 Brown AL, Francis MJ, Hastings GZ *et al.* Foreing epitopes in immunodominant regions of hepatitis B core particles are highly immunogenic and conformationally restricted. *Vaccine* 1991; **9**: 595–601.
7 Hilleman MR. Overview of the pathogenesis, prophylaxis and therapeusis of viral hepatitis B, with focus on reduction to practical applications. *Vaccine* 2001; **19**: 1837–48.
8 Pichichero ME, Blatter MM, Reisinger KS *et al.* Impact of a birth dose of hepatitis B vaccine on the reactogenicity and immunogenicity of diphtheria-tetanus-acellular pertussis-hepatitis B-inactivated poliovirus-Haemophilus influenzae type b combination vaccination. *Pediatr. Infect. Dis. J.* 2002; **21**: 854–9.
9 Smith ML, Richter L, Arntzen CJ, Shuler ML, Mason HS. Structural characterization of plant-derived hepatitis B surface antigen employed in oral immunization studies. *Vaccine* 2003; **21**: 4011–21.
10 Aguilar JC, Acosta-Rivero N, Duenas-Carrera S *et al.* HCV core protein modulates the immune response against the HBV surface antigen in mice. *Biochem. Biophys. Res. Commun.* 2003; **310**: 59–63.
11 Debin A, Kravtzoff R, Santiago JV *et al.* Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. *Vaccine* 2002; **20**(21–22): 2752–63.
12 Isaka M, Yasuda Y, Mizokami M *et al.* Mucosal immunization against hepatitis B virus by intranasal co-administration of recombinant hepatitis B surface antigen and recombinant cholera toxin B subunit as an adjuvant. *Vaccine* 2001; **19**: 1460–6.
13 Chen X, Li M, Le X, Ma W, Zhou B. Recombinant hepatitis B core antigen carrying preS1 epitopes induce immune response against chronic HBV infection. *Vaccine* 2004; **22**: 439–46.
14 Yalcin K, Acar M, Degertekin H. Specific hepatitis B vaccine therapy in inactive HBsAg carriers: a randomized controlled trial. *Infection* 2003; **31**: 221–5.
15 Yalcin K, Danis R, Degertekin H, Alp MN, Tekes S, Budak T. The lack of effect of therapeutic vaccination with a pre-S2/S HBV vaccine in the immune tolerant phase of chronic HBV infection. *J. Clin. Gastroenterol.* 2003; **37**: 330–5.
16 Ren F, Hino K, Yamaguchi Y *et al.* Cytokine-dependent antiviral role of CD4-positive T cells in therapeutic vaccination against chronic hepatitis B viral infection. *J. Med. Virol.* 2003; **71**: 376–84.
17 Michel ML. Towards immunotherapy for chronic hepatitis B virus infections. *Vaccine* 2002; **20**(suppl 4): A83–8.
18 Lee WM. Hepatitis B virus infection. *New Engl. J. Med.* 1997; **337**: 1733–45.
19 Hoofnagle JH, di Bisceglie AM. The treatment of chronic viral hepatitis. *New Engl. J. Med.* 1997; **336**: 347–56.
20 Kakimi K, Isogawa M, Chung J, Sette A, Chisari FV. Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: implications for immunotherapy of persistent viral infections. *J. Virol.* 2002; **76**: 8609–20.
21 Chisari FV. Hepatitis B virus immunopathogenesis. *Annu. Rev. Immunol.* 1995; **13**: 29–60.
22 Reignat S, Webster GJ, Brown D *et al.* Escaping high viral load exhaustion: CD8 cells with altered tetramer binding in chronic hepatitis B virus infection. *J. Exp. Med.* 2002; **195**: 1089–101.
23 Zheng BJ, Zhou J, Qu D *et al.* Selective functional deficit in dendritic cell – T cell interaction is a crucial mechanism in chronic hepatitis B virus infection. *J. Viral Hepat.* 2004; **11**: 217–24.
24 Beckebaum S, Cicinnati VR, Zhang X *et al.* Hepatitis B virus-induced defect of monocyte-derived dendritic cells leads to impaired T helper type 1 response in vitro: mechanisms for viral immune escape. *Immunology* 2003; **109**: 487–95.
25 Li YG, Chen M, Zhang DZ *et al.* Clinical research on the treatment effect of autologous dendritic cell vaccine on the patients with chronic hepatitis B. *Zhonghua Gan Zang Bing Za Zhi.* 2003; **11**: 206–8.
26 Neutra RM, Pringault E, Kraehenbuhl J-P. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 1996; **14**: 275.
27 Milich DR, McLachlan A. The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. *Science* 1986; **234**: 1398–401.
28 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001; **413**: 732–8.
29 Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem. Biophys. Res. Commun.* 2002; **293**: 1364–9.
30 Riedl P, Stober D, Oehninger C, Melber K, Reimann J, Schirmbeck R. Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. *J. Immunol.* 2002; **168**: 4951–9.
31 Lobaina Y, García D, Abreu N, Muzio V, Aguilar JC. Mucosal immunogenicity of hepatitis B core antigen. *Biochem. Biophys. Res. Commun*. 2003; **300**: 745–50.
32 Bullock GR, Petrusz P. *The Colloidal Gold Marker System for Light and Electron Microscopic Cytochemistry*. London: Academic Press, 1983.
33 Schirmbeck R, Wild J, Reimann J. Similar as well as distinct MHC class I-binding peptides are generated by exogenous and endogenous processing of hepatitis B virus surface antigen. *Eur. J. Immunol.* 1998; 0**28**: 4149–61.

34 Tan WS, Dyson MR, Murray K. Hepatitis B virus core antigen: enhancement of its production in Escherichia coli, and interaction of the core particles with the viral surface antigen. *Biol. Chem.* 2003; **384**: 363–71.

35 Jiang HL, Park IK, Shin NR, Yoo HS, Akaike T, Cho CS. Controlled release of Bordetella Bronchiseptica Dermonecrotoxin (BBD) vaccine from BBD-loaded chitosan microspheres *in vitro*. *Arch. Pharm. Res.* 2004; **27**: 346–50.

36 Chattaraj SC, Das SK. Physicochemical characterization of influenza viral vaccine loaded surfactant vesicles. *Drug Deliv.* 2003; **10**: 73–7.

37 He Q, Mitchell A, Morcol T, Bell SJ. Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2. *Clin. Diagn. Lab. Immunol.* 2002; **9**: 1021–4.

38 Milich DR, Chen M, Schodel F, Peterson DL, Jones JE, Hughes JL. Role of B cells in antigen presentation of the hepatitis B core. *Proc. Natl Acad. Sci. USA* 1997; **94**: 14 648–53.

39 Zhevachevsky NG, Nomokonova NY, Beklemishev AB, Belov GF. Dynamic study of HBsAg and HBeAg in saliva samples from patients with hepatitis B infection: diagnostic and epidemiological significance. *J. Med. Virol.* 2000; **61**: 433–8.

40 Weiner HL. Oral Tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol. Today* 1997; **18**: 335–41.

41 Boni C, Bertoletti A, Penna A *et al.* Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J. Clin. Invest.* 1998; **102**: 968–75.

42 Boni C, Penna A, Ogg GS *et al.* Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. *Hepatology* 2001; **33**: 963–71.

43 Lazdina U, Alheim M, Nystrom J *et al.* Priming of cytotoxic T cell responses to exogenous hepatitis B virus core antigen is B cell dependent. *J. General Virol.* 2003; **84**: 139–46.

44 Walker RI. New strategies for using mucosal vaccination to achieve more effective immunization. *Vaccine* 1994; **12**: 387–400.