Research paper

Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles

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Abstract

Alginate coated chitosan nanoparticles were previously developed with the aim of protecting the antigen, adsorbed on the surface of those chitosan nanoparticles, from enzymatic degradation at mucosal surfaces. In this work, this new delivery system was loaded with the recombinant hepatitis B surface antigen (HBsAg) and applied to mice by the intranasal route. Adjuvant effect of the delivery system was studied by measuring anti-HBsAg IgG in serum, anti-HBsAg sIgA in faeces extracts or nasal and vaginal secretions and interferon-γ production in supernatants of the spleen cells. The mice were primed with 10 μg of the vaccine associated or not with nanoparticles and associated or not with 10 μg CpG oligodeoxynucleotide (ODN) followed by two sequential boosts at three week intervals. The association of HBsAg with the alginate coated chitosan nanoparticles, administered intranasally to the mice, gave rise to the humoral mucosal immune response. Humoral systemic immune response was not induced by the HBsAg loaded nanoparticles alone. The generation of Th1-biased antigen-specific systemic antibodies, however, was observed when HBsAg loaded nanoparticles were applied together with a second adjuvant, the immunopotentiator, CpG ODN. Moreover, all intranasally vaccinated groups showed higher interferon-γ production when compared to naïve mice.
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1. Introduction

A safe and effective vaccine to prevent infection with hepatitis B virus (HBV) has been available since 1982, and is recommended by the World Health Organization (WHO) for administration to all infants [1]. Therefore, it is not understandable that the number of chronically infected people continues to be a problem in many parts of the World, including Asia, Africa and the western Pacific region [2]. Some progress in the control of the disease in the western Pacific area was recently reported [1] after the inclusion of hepatitis B vaccine in every national immunization programme. Even though the price of the vaccine had dropped significantly in the last years, there were still some countries that could not afford these immunization programmes. Furthermore, the control of the spread of the disease through vaccination of infants would undoubtedly prevent a large number of infections among adults, but it will take several decades to be achieved. So, the implementation of vaccination programmes, at least for

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the vaccination of high-risk adults, like drug users, sex workers and health professionals, would certainly prevent a large number of infections [3].

The intramuscular route of administration of the vaccine currently in the market, is not efficient to induce mucosal antibodies. The presence of antigen-specific sIgA at mucosal surfaces has been shown to help, when the transmission of the disease is through one of the mucosal routes, which is the case of the sexual transmission of HBV. To note that sexual transmission (either homosexual or heterosexual) in high-risk adults is the main mode of transmission in Europe and North America (regions of low HBV prevalence) [2]. On the other side, the induction of mucosal antibodies seemed to be facilitated if the antigens were administered by a mucosal route together with an appropriate adjuvant. So, the development of efficient mucosal adjuvants, which could allow an easy and inexpensive mass vaccination and the production of more stable vaccines is urgently required.

Among the mucosal administration routes, the nasal mucosa is the most attractive site for the delivery of vaccines. Compared with the more challenging oral vaccination, the nasal route is more efficient at inducing the secretory IgA, in particular on the cervicovaginal mucosa [4], simultaneously with the induction of a systemic antibody response (reviewed in [5]). For this reason, several attempts have been made regarding the intranasal vaccination with hepatitis B, in order to identify an effective mucosal adjuvant that elicits specific mucosal and systemic hepatitis B antibodies. Adjuvants, such as recombinant cholera toxin B subunit [6], genetically modified adenovirus [7] expressing the hepatitis B surface antigen, attenuated *Salmonella typhimurium* [8] expressing the hepatitis B nucleocapsid, or *Escherichia coli* expressing the hepatitis B nucleoprotein antigen [9], have all been shown to be efficient as intranasal adjuvants. However, safety concerns have predominantly been the cause for restricting the development and the use of these adjuvants in clinical studies. Synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN) are considered to be potent immunopotentiator adjuvants. It was demonstrated in mice that the intranasal delivery of HBsAg, which alone has no effect, elicits good immune responses when given in combination with CpG ODN [10–12]. Moreover, CpG is superior to cholera toxin (CT) for the induction of humoral and cell-mediated systemic immunity as well as mucosal immune responses (IgA) at local (lung) and distant (intestine) sites [10].

Particulate vaccine delivery systems loaded with HBsAg have also been evaluated by the intranasal route. Some examples already published include polymeric biodegradable poly(lactide-co-glycolide) (PLGA) microparticles [13], cationic particles (SMBV™) [14], cationic lipid emulsion carrying a DNA vaccine [15] and lipid microparticles [16].

Great attention has been devoted to the cationic biodegradable polysaccharide, chitosan. It is an inexpensive raw material, derived from chitin the second largest abundant natural polymer. Additionally, it has important qualities like, low toxicity, mucoadhesive [17] and immunostimulating properties [18], hence showing enormous potential to be used on mucosal vaccination. In fact, chitosan has been used in nanoparticulate formulations for loading and delivering several vaccines (see Section 4). However, as far as we know, the evaluation of chitosan based delivery systems, as a mucosal adjuvant for hepatitis B vaccine, was never reported. So, it is the main purpose of the present work, to evaluate the immune response produced after i.n. vaccination with HBsAg associated with the new recently characterized [19,20] alginate coated chitosan nanoparticles that was specifically designed by us for mucosal vaccination. The delivery system is composed of a chitosan core to which the hepatitis B surface antigen (HBsAg) was adsorbed and then coated with sodium alginate. Alginate coating proved to modify the antigen release profile from the chitosan nanoparticles and at the same time may protect the antigen from the enzymatic degradation during their passage throughout mucosal surfaces. Additionally, the HBsAg adjuvant effect of CpG ODN associated or not with the same coated nanoparticles was investigated as well.

2. Materials and methods

2.1. Materials

2.1.1. Polymers

Chitosan were purchased from Primex BioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation was 95% (titration method) and the viscosity was 8 cP (measured in 1% solution in 1% acetic acid). A low molecular weight, pharmaceutical grade, sodium alginate (MANUCOL LB®) was kindly donated by ISP Technologies Inc. (Surrey, UK). According to the provider's specifications, the typical values for the percentage of mannuronic and guluronic acid for Manucol LB are 61% and 39%, respectively, with an estimated molecular weight of 18 kDa. Both polymers meet the requisites described in the European Pharmacopoeia for use in pharmaceutical formulations.

2.1.2. Antigen, adjuvant and reagents

The hepatitis B surface antigen (HBsAg), (subtype ADW2) was kindly provided by GSK Biologicals (Rixensart, Belgium), Engerix B™ was from GlaxoSmithKline Biologicals (Rixensart, Belgium), Class B CpG ODN (1826) (5′-TCC ATG ACG TTC CTG ACG TT-3′) was purchased from Coley Pharmaceutical Group (Ottawa, Canada). Concanavalin A (Con A), phenylmethanesulfonyl fluoride (PMSF) and avidin peroxidase conjugate were from Sigma Chemicals (St. Louis, USA). Certified foetal bovine serum (FBS) and L-glutamine (200 mM) were from Gibco (Invitrogen Co, Paisley, Scotland, UK), 1 M Hepes buffer (0.85% NaCl), RPMI 1640 without L-glutamine and Pen-Strep

(10,000 U penicillin/ml; 10,000 μg streptomycin/ml) were from Biowhitaker (Cambrex Bio Science, Verviers, Belgium). [Methyl-^{3}H] thymidine (1.0 mCi/ml) was obtained from Amersham Biosciences (UK) and R-phycoerythrin (PE)-conjugated hamster anti-mouse CD69, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 were obtained from BD Biosciences (Madrid, Spain). The FITC-conjugated goat anti-mouse IgM (anti-μ), the anti-mouse IFN-γ and biotin rat anti-mouse IFN-γ was purchased from PharMingen (San Diego, CA, USA). The mouse IgA ELISA quantification kit was obtained from Bethyl Laboratories (Montgomery, USA). All reagents used were of analytic grade. All solutions were prepared in ultrapure water.

2.2. Methods

2.2.1. Preparation of the coated nanoparticles

The preparation of the alginate coated chitosan nanoparticles was performed according to the method previously described [20]. Briefly, chitosan was dissolved at a concentration of 0.25% (w/v) in diluted acetic acid solution. The formation of the particles was achieved after the addition of 3.5 ml of sodium sulphate solution (10% w/v) to 200 ml of the chitosan solution. The resulting suspension was centrifuged for 30 min at 3500 rpm (2800g) and the supernatant was discarded. The particles were re-suspended in ultrapure water and centrifuged twice. They were finally frozen in liquid nitrogen and freeze-dried overnight using a Labconco freeze dry system (Labconco Corporation, Kansas, USA). The dry powder was kept frozen until further use.

The loading of the nanoparticles with HBsAg or with CpG ODN was performed by incubating a solution of HBsAg or the solution of CpG with a suspension of chitosan particles in phosphate buffer at pH 7.4, under mild agitation at room temperature for 120 min. In order to find the system (nanoparticles: hepatitis B antigen) which results in a high loading efficacy, different concentrations of the antigen were experimented (0.5% nanoparticles: 0.1–0.0148% HBsAg). In the subsequent coating step, the suspensions composed of 0.05% (w/v) HBsAg and 0.5% (w/v) nanoparticles and the second with 0.05% (w/v) CpG and 0.5% (w/v) nanoparticles were used.

Alginate coated nanoparticles were obtained by mixing equal volumes of the loaded nanoparticles suspension and a buffer phosphate solution of sodium alginate (1% w/v) under magnetic stirring. The agitation was maintained for 20 min. The suspension was then centrifuged for 10 min at 1600 rpm and the supernatant was discarded. The particles were re-suspended in 0.262 mM CaCl$_2$ in 50 mM Hepes buffer solution, kept under agitation for another 10 min and immediately administered to the mice.

2.2.2. Evaluation of the loading efficacy of HBsAg and CpG ODN in coated nanoparticles

The loading efficacy of the coated particles was calculated in an indirect way, quantifying the antigen that remained in solution as described before [19]. After the coating with alginate, an aliquot of the particle suspension was centrifuged at 14,000 rpm for 15 min and the protein concentration in the supernatant was quantified by micro-BCA-protein assay (PIERCE, Rockford, USA) using a microplate reader with a 570 nm filter.

For the CpG ODN the same procedure was followed and the oligodeoxynucleotide concentration quantified by measuring the OD of the supernatants at 260 nm. To eliminate background interference, the supernatant of unloaded particles was processed by the same way. The results refer to the nanoparticle batches used in the vaccination studies.

The loading efficacy (LE) and the loading capacity (LC) were calculated from the following equations:

$$LE\ (\%) = (\text{Total amount of HBsAg or CpG} - \text{free HBsAg or CpG})/\text{Total amount of HBsAg or CpG} * 100 \tag{1}$$

$$LC\ (\text{μg of HBsAg or CpG/mg chitosan nanoparticles dry weight}) = (\text{Total amount of HBsAg or CpG} - \text{free HBsAg or CpG})/\text{mg chitosan nanoparticles dry weight}. \tag{2}$$

2.2.3. Immunization studies
2.2.3.1. Animals.
Seven-week-old female BALB/cAnNHsd mice were used (Harlan Iberia, Barcelona, Spain) with four or six mice per group. Animals were housed for acclimatization one week before the experiments at the animal resource facilities of the Faculty of Pharmacy at the University of Porto. Animal care, handling and immunization protocols were performed and approved in accordance with institutional ethical guidelines. Animals had free access to food and water and were kept under a 12 h light/dark cycle during all the experiment.

2.2.3.2. Treatment groups.

Group I	suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg
Group II	suspension of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg plus 10 μg of the adjuvant (CpG ODN) in solution
Group III	suspension of the mixture of alginate coated chitosan nanoparticles loaded with 10 μg HBsAg and nanoparticles loaded with 10 μg CpG ODN
Group IV	phosphate buffer saline (PBS) solution with 10 μg HBsAg
Group V	PBS solution with 10 μg HBsAg and 10 μg CpG ODN
Group VI	untreated or negative control
Group VII	subcutaneous administration of Engerix B (1 μg HBsAg) (positive control)

2.2.3.3. Immunization schedule. The primary immunization was followed by two boosts with three weeks between each immunization and the mice were sacrificed four weeks after the last boost. To evoke an immune response a total volume of 15 μl (7.5 μl in each nostril) of the formulations was administered. The non-anesthetized mice were maintained in supine position and the formulation was deposited in the nasal cavity with the aid of a micropipette with a 10 μl tip. The mice were kept in this position for approximately another 2 min to allow optimal spreading of the formulations on the nasal mucosa.

2.2.3.4. Sample collection. Blood samples were taken from the orbital sinus before each boost and by cardiac puncture at the end of the experiment. The sera were prepared by centrifugation and stored at −20 °C until analysis.

Vaginal secretions were collected at the end and four days before the end of the experiment, by rinsing with 150 μl of a cold PBS (containing 0.1% bovine serum albumin (BSA)) through the vagina. The 1% BSA–PBS solution was introduced into the vaginal tract of non-anesthetized mice using a Gilson pipette. These 150 μl aliquots were withdrawn and reintroduced six times.

Nasal secretions were collected just at the end of the experiment, by washing the nasal cavity with 200 μl of cold PBS (containing 1% BSA). The washing solution was forced to pass throughout the trachea of the sacrificed mice and collected from the nostrils side.

The extracts were vortexed and treated with sodium azide and phenylmethylsulfonyl fluoride (PMSF), allowed to stay at room temperature for at least 15 min and then centrifuged (6000 rpm/15 min/4 °C). The clear supernatants (containing 0.1% sodium azide, 0.1% BSA and 1 mM PMSF) were then stored at −80 °C until tested by ELISA for secretory antibody (sIgA) levels.

Faecal pellets (4–8) were collected four days before the end of the experiment into Eppendorf tubes. The pellets (0.2 g/ml) were suspended in PBS (containing 0.1% sodium azide, 0.1% BSA and 1 mM PMSF), vortexed and allowed to rest at room temperature for 1 h. Solid matter was separated by centrifugation at 14,000 rpm for 15 min. The clear supernatants were frozen at −80 °C before being used in ELISA to determine antigen-specific and total IgA.

2.2.3.5. Enzyme-linked immunosorbent assays (ELISA) for HBsAg specific immunoglobulins. Ninety-six-well flat-bottomed microtiter plates (Nunc immunoplate maxisorb) were previously coated with the recombinant HBsAg (1 μg/well) in coating buffer (50 mM sodium carbonate, pH 9.6) by overnight incubation at 4 °C. The plates were washed five times with PBS-T (PBS containing 0.05% Tween 20) and blocked with 3% BSA in PBS-T (200 μl/well) for 1 h at 37 °C. The plates were then washed five times with PBS-T and the serial dilutions of each serum (100 μl/well) from the individual mice were tested in triplicate, starting from a 1:100 dilution in PBS-T. The serum was incubated for 2 h at 37 °C and after washing the plates

with PBS-T, they were incubated for an additional 30 min at 37 °C with peroxidase-labelled goat anti-mouse immunoglobulin G and isotypes (anti-IgG1, anti-IgG2a). The bound antibodies were revealed by adding 100 μl/well of 0.5 mg/ml of *o*-phenylenediamine dihydrochloride (OPD) (Sigma, Spain) in 10 ml of citrate buffer with 10 μl of 30% H_2O_2. The reaction was stopped after 10 min with 50 μl of 3 M HCl to each well. The absorbance was read out at 492 nm in an automatic ELISA reader (Easy Reader 400, SLT-LABINSTRUMENTS). Elisa titers were expressed as mIU/ml and 1 mIU is the OD mean of the pre-immune serum plus two times the standard deviation.

The measurement of IgA was carried out using a mouse IgA ELISA quantification kit (Bethyl Laboratories, Montgomery, Texas, USA) as described by the manufacturer. In order to measure the sIgA levels in the gut, nasal and vaginal secretions, total sIgA and the specific anti-HBs sIgA were determined in the extracts. The results are presented as the anti-HBsAg IgA/total IgA. By this way variations between samples related with the extraction process or stability of the sIgA were minimized.

The standard IgA was diluted to appropriate concentrations in PBS with 1% BSA to create a calibration curve. The extracts were diluted in PBS-T with 1% BSA and added to the plates in series of 2-fold dilutions. The concentrations of the total and specific IgA were determined from the calibration curve generated for each set of samples using a four parameter logistic curve-fit generated by SigmaPlot software (version 8.0, SPSS Inc.).

2.2.3.6. Preparation of spleen cell suspensions. The mice were euthanized by cervical dislocation and their spleens were aseptically removed. Individual spleen cell suspensions were prepared in a Petri dish using curved needles and washed twice with RPMI 1640. The final suspension was adjusted to a final concentration of 1×10^7 cells/ml in a complete RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) glutamine, 1% (v/v) Pen-Strep and 2% (v/v) 1 M Hepes buffer.

2.2.3.7. Cell population of the spleen. The cells (10^6) were washed twice with cold PBS supplemented with 2% FBS and then incubated in the dark for 30 min at 4 °C with saturated concentrations of FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse IgM (anti-μ). After incubation, cells were washed three times with PBS-2% FBS and then re-suspended in 500 μl of PBS-2% FBS. To exclude dead cells, 2.5 μl of propidium iodide (50 μg/ml) was added just before data acquisition. At least 10,000 events were analyzed by flow cytometric acquisition, performed in a fluorescent activated cell sorter (FACS Calibur) (BD, Biosciences, Madrid, Spain). Data were analyzed by CellQuest software (BD, Biosciences, Madrid, Spain).

2.2.3.8. Splenocyte cell culture in the presence of the mitogens. Using sterile 96-well flat-bottomed tissue culture

plates, 25 µl of splenocyte suspension (1×10^7 cells/ml) from each mouse was plated in triplicate along with 25 µl of a complete RPMI solution of the mitogen [Con A (50 µg/ml), CpG ODN (50 µg/ml) plus HBsAg (16 µg/ml), HBsAg alone (16 µg/ml) or without mitogen (control)]. Finally the volume of the well was completed to 200 µl with complete RPMI and incubated at 37 °C with 95% relative humidity and in the presence of 5% CO_2.

2.2.3.9. Interferon-γ (IFN-γ) production by splenocytes.

Spleen cell suspensions were plated with the mitogens (see Section 2.2.3.8.) and incubated in a humidified 5% CO_2 incubator for 96 h at 37 °C. The plates were centrifuged and the clear supernatants stored at −80 °C until analysis of the IFN-γ by ELISA technique, following a procedure described elsewhere [21].

2.2.3.10. Lymphoproliferation assay.

Splenocytes were obtained and cultured together with the mitogens in a flat-bottomed 96-well plate as described before (see Section 2.2.3.8.). The cells were cultured for 96 h at 37 °C and on the last 8 h of incubation each well was pulsed with 1 µCi of [methyl-^{3}H] thymidine. These 96-well plates with the cells were stored at −20 °C until further analysis.

The cells were later thawed and harvested onto a fiberglass filter (filter mats, molecular devices, Skatron, Lier, Norway) using a semiautomatic cell harvester (Scatron Instruments, USA) and DNA thymidine incorporation was determined by standard liquid scintillation techniques with a Beckman LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton, USA). Thymidine incorporation was expressed as counts per minute (cpm).

2.3. Statistical analysis

If another method is not explicitly stated, the data are presented as means ± SEM for at least three experiments and statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's post test using the Prism 4 (GraphPad software, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Characterization of the vaccine delivery system

In a recent publication of our group [20], the development of alginate coated chitosan nanoparticles and the characterization of this new delivery system were reported. Briefly, before coating with sodium alginate, chitosan nanoparticles with a mean diameter of 643 nm (measured by a dynamic light scattering technique) were positively charged (+37 mV). On the other hand scanning electron microscopy (SEM) images of the uncoated particles also revealed the presence of small nanoparticles (around 100 nm) and the same technique also demonstrated that the majority of the coated particles were in a range between 300 and 600 nm. Coated nanoparticles are negatively charged (−35 mV) due to the contribution of the alginate. More recently, we showed the results of the release studies, choosing ovalbumin as a model vaccine [19]. Ovalbumin release studies from coated and uncoated chitosan nanoparticles, performed in several buffers at different pH values, allowed us to conclude that the coating with sodium alginate of the ovalbumin loaded chitosan nanoparticles avoided an ovalbumin burst release observed with uncoated chitosan nanoparticles at pH 5.5, 6.8 and 7.4 (phosphate buffer) within the first 30 min of incubation.

3.2. CpG ODN and hepatitis B antigen entrapment in coated nanoparticles

Different ratios of hepatitis B vaccine to chitosan nanoparticles and CpG ODN to chitosan nanoparticles were investigated (data not shown) and the systems with the highest loading efficacy were used for further studies. It was shown that hepatitis B antigen and CpG ODN were efficiently associated with alginate coated chitosan nanoparticles. The loading efficacy of hepatitis B vaccine in the coated nanoparticles was 84.1 ± 3.0% (mean ± STDEV) and the mean of the loading capacity was 83.9 µg of HBsAg/mg of dry chitosan nanoparticles ±2.77 (STDEV). The loading efficacy of CpG was 97.0 ± 1.3% and the loading capacity was 97.0 ± 0.03 (µg of CpG ODN/mg of dry chitosan nanoparticles).

3.3. Cellular immune responses after intranasal administration of HBsAg loaded nanoparticles

The percentage of T- and B-lymphocytes was determined in order to examine the possible influence of the HBsAg formulations on the cell populations of the mouse spleen. Fig. 1A shows the results of the FACS analysis performed with freshly prepared spleen cell suspensions, using specific mAb against cell surface markers. The percentages of $CD8^+$ and $CD4^+$ T-lymphocytes subpopulations in different groups were shown not to have been significantly different from the control group ($p > 0.05$). The group I vaccinated with the HBsAg associated with the coated nanoparticles was an exception and was shown to have a significantly lower percentage ($p < 0.05$) of $CD8^+$ T-cells (0.7-fold decrease compared to the control mice), whereas no significant difference in the ratio of $CD4^+/CD8^+$ T-cells (Fig. 1B) was observed in the same group when compared with the control group. A slight, but statistically significant difference ($p < 0.01$) in the decrease of B cells was observed in the groups vaccinated without nanoparticles (groups IV, V and VII).

We also tested whether the spleen lymphocytes were susceptible to proliferation when cultured in the presence of the antigen or the antigen plus the adjuvant. The comparison between the results obtained with cells cultured without mitogens (negative control of the experiment) (Fig. 2A) and the results of cells incubated only with the

A

** P < 0.01; * P < 0.05

B
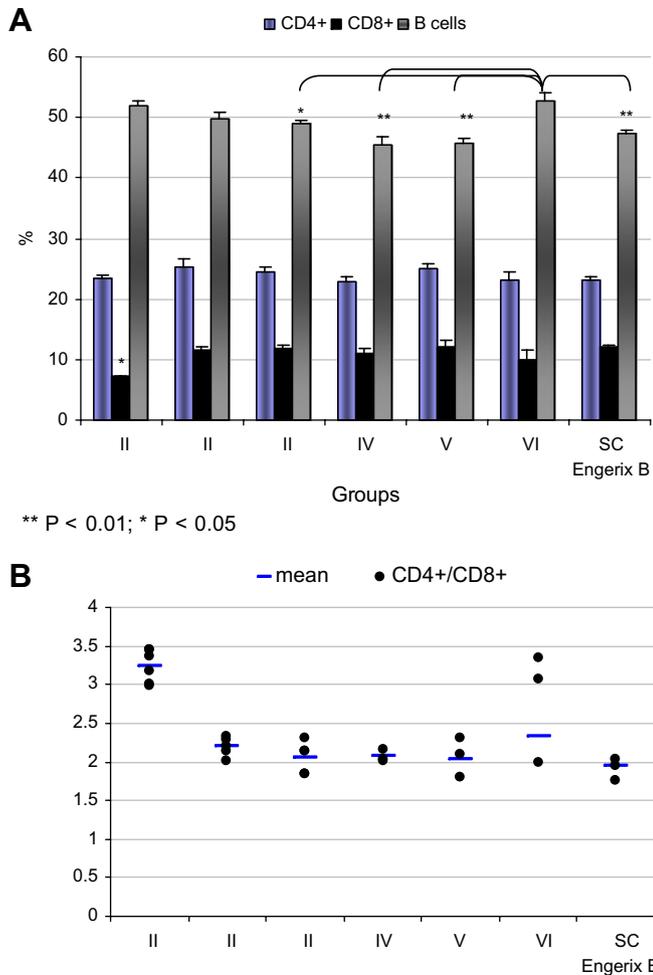
Fig. 1. (A) Percentage of T-lymphocytes CD4^{+}, CD8^{+} and B^{+}-Lymphocytes in the spleens of mice from groups (I, II, III, IV, V, VI and VII) by FACS analysis. Each bar corresponds to the group geometric mean plus the standard error of the mean. The comparison between the control group (VI) and the different treatment groups (I, II, III, IV, V and VII) was made with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test ([**]$p < 0.01$ and [*]$p < 0.05$). (B) T-lymphocytes quota between percentage of CD4^{+} cells and CD8^{+} cells from individual mouse spleen of each group.

HBsAg (Fig. 2B) allowed us to conclude that in general the presence of the antigen in the cell culture proved the ability to induce some division of the cells. However in the concentration used, the stimulation appeared to be non-specific and, in general, the results shown appeared just amplified, when compared with the results without any stimulus. In most cases, the different groups were shown not to be different from the control group. However, two exceptions with a higher mean value ($p < 0.01$) were observed: the group vaccinated with the antigen in solution and the group vaccinated with a solution of the antigen in the presence of the adjuvant (groups IV and V). Nonetheless, due to high variability of the individual values within the groups, these results should not be overestimated. A similar situation was observed for the cells cultured with antigen in the presence of CpG ODN (Fig. 2C).

Finally, Fig. 2D shows the lymphoproliferative results observed in the presence of Con A, a classical mitogen, which served as a positive recall antigen. In this case all the groups vaccinated intranasally showed significantly ($p < 0.01$) lower values. In addition, the group s.c. vaccinated with a commercial formulation showed a higher mean value ($p < 0.01$) when compared to the control (group VI).

The quantification of the interferon-γ (IFN-γ) in the supernatants of the different group splenocytes, cultured under the same conditions is shown in Fig. 3. A direct observation of the results allowed us to conclude that the splenocytes of all the groups vaccinated produced IFN-γ. Moreover, the statistical analysis showed that the amount produced is significantly higher than the amount produced by the cells from the control group in all the groups.

3.4. Humoral immune response to the intranasal administration of HBsAg nanoparticles

3.4.1. Systemic immune response

The positive control group of the experiment received a subcutaneous injection with 1 μg of HBsAg associated with aluminium adjuvant (commercial formulation). The highest concentrations of the anti-HBsAg IgG were observed within this group (Fig. 4A). The presence of HBs specific IgG was also observed in some mice within the groups nasally vaccinated with the antigen and in the presence of CpG ODN. Groups I and IV (absence of CpG ODN) did not induce detectable anti-HBs IgG antibodies in the plasma of any mice. Therefore the presence of a strong mucosal adjuvant seemed to be necessary to induce a systemic humoral immune response in mice vaccinated with 10 μg of the HBsAg. On the other hand, the results also showed that within the nasally vaccinated groups the highest IgG titers were found in the groups where the adjuvant was not associated with the nanoparticles (groups II and V).

The anti-HBsAg IgG subclasses present in the responder mice were also evaluated in order to examine the induced Th profile as shown in Fig. 4B. In the seroconverted nasally vaccinated mice the detected antibodies were predominantly type IgG2 (Th1–like), whereas with the commercial formulation administered subcutaneously, the immune response was predominantly Th2 (IgG1 > IgG2a).

3.4.2. Mucosal immune response

Nasal and vaginal washings and the collection of fresh faeces were performed at the end of the experiment in order to detect the presence of anti-HBsAg sIgA on the mucosal surfaces. The quantification of sIgA in the nasal washings was possible in almost all the groups nasally vaccinated (Fig. 5A). The exception was group IV vaccinated with a solution of the antigen for which no specific sIgA could be detected. In the same way, it was also not possible in the same group (IV) to detect specific antibodies in any fae-
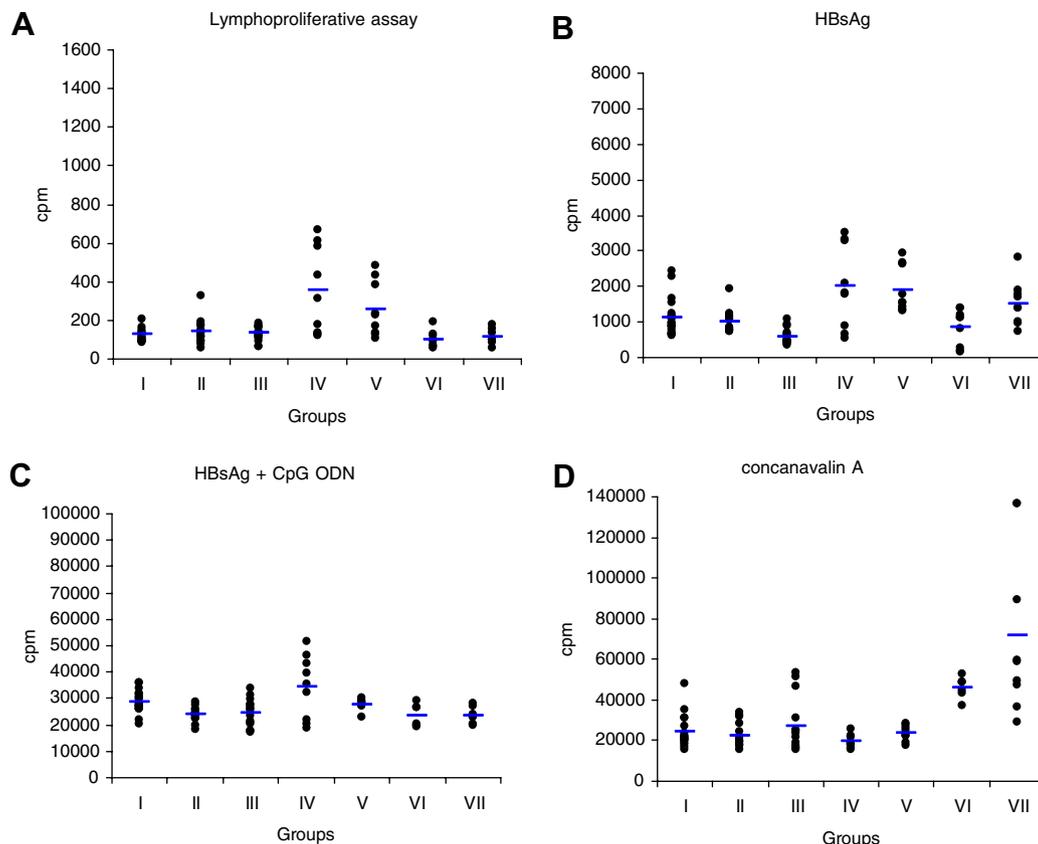
Fig. 2. Lymphoproliferative response after intranasal administration of different hepatitis B vaccine formulations. *In vitro* proliferation of individual mouse splenocytes during a 96 h period stimulated with (A) without stimulus, (B) HBsAg, (C) HBsAg + CpG ODN, (D) Con A. Each circle represents the result of individual samples and the horizontal bar the mean of the group. The results correspond to the thymidine incorporation and are expressed as counts per minute (cpm).
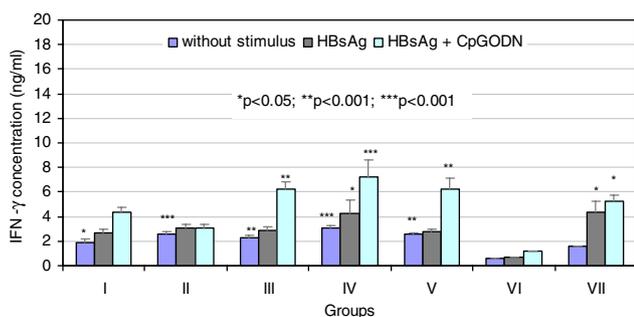
Fig. 3. IFN-γ production by splenocytes of individual mice from each treatment group (Groups I–VII). Each bar corresponds to the group geometric mean plus the standard error of the mean (*n* between 3 and 6). The comparison between the control group (VI) and the different treatment groups (I, II, III, IV, V and VII) was made with a one-way analysis of variance (ANOVA) followed Bonferroni's test.

suspension of the nanoparticles associated with both the antigen and the adjuvant (group III) yielded similar results to group I. Group II differs from group III because in this group the CpG is not associated with the coated nanoparticles. In this case not only the number of responder mice increased (4/5 nasal secretions, 4/5 vaginal secretions) but also the relative amount of the specific antibodies detected was much higher (Fig. 5A and B). A similar and expected result was obtained with the formulation in which the antigen and the adjuvant were not associated with the coated nanoparticles (group V). Therefore, anti-HBsAg sIgA in the lung, gut, saliva, vaginal secretions and in faeces were determined by the researchers.

Finally, the subcutaneous injection of the commercial formulation did not produce any detectable mucosal antibodies in nasal washings extracts.

4. Discussion

In several publications it has been convincingly demonstrated that the subcutaneous (sc) or the intramuscular (im) routes of vaccine application are not effective to induce antigen-specific mucosal antibodies. In the present work, it was possible to confirm once more that the s.c. vaccina-

cal extracts (Fig. 5C) and in the majority of vaginal washings samples (Fig. 5B).

On the other hand, the entrapment of the hepatitis B antigen into the coated nanoparticles (group I) showed to be valuable for the generation of specific mucosal antibodies. This formulation induced the formation of anti-HBsAg sIgA not only at the nasal mucosa (4/6) (Fig. 5A) but also in vaginal secretions (3/6) (Fig. 5B). The application of the

Fig. 4. (A) Serum anti-HBsAg IgG titers of mice nasally immunized with different formulations of hepatitis B vaccine. Values are expressed as antibody titers of individual mice taken at the end of the experiment. The horizontal bar is the mean of the group. (B) Serum anti-HBsAg IgG1 and IgG2a titers of mice nasally immunized with different formulations of hepatitis B vaccine. The bar corresponds to mean titer of responder mice in each group. Titers were defined as the highest plasma dilution resulting in an absorbance value twice that of non-immune plasma (1 mlU/ ml = mean + 2SD of the control group).

tion with a commercial formulation of the hepatitis B (group VII) was unable to induce the generation of detectable sIgA in both, vaginal and nasal secretarial fluids. The induction of mucosal antibodies, with few exceptions described elsewhere [22], seems to be only feasible when a mucosal route of vaccination was chosen and the HBs antigen was associated with an appropriate adjuvant. On the other hand, the importance of inducing mucosal antibodies after an immunization process has been well emphasized in the last few years [4,23,24]. This aspect is even more relevant for those infections that start their deleterious effects on the mucosal surfaces. In these cases, the infection can be resolved before the causative agent enters in the blood. The main protective mechanism is the generation of a local secretory immune response, with secretory IgA (sIgA) antibodies as the primary effectors molecules, but additional humoral and cell-mediated mucosal protective mechanism have also been identified [4,25].
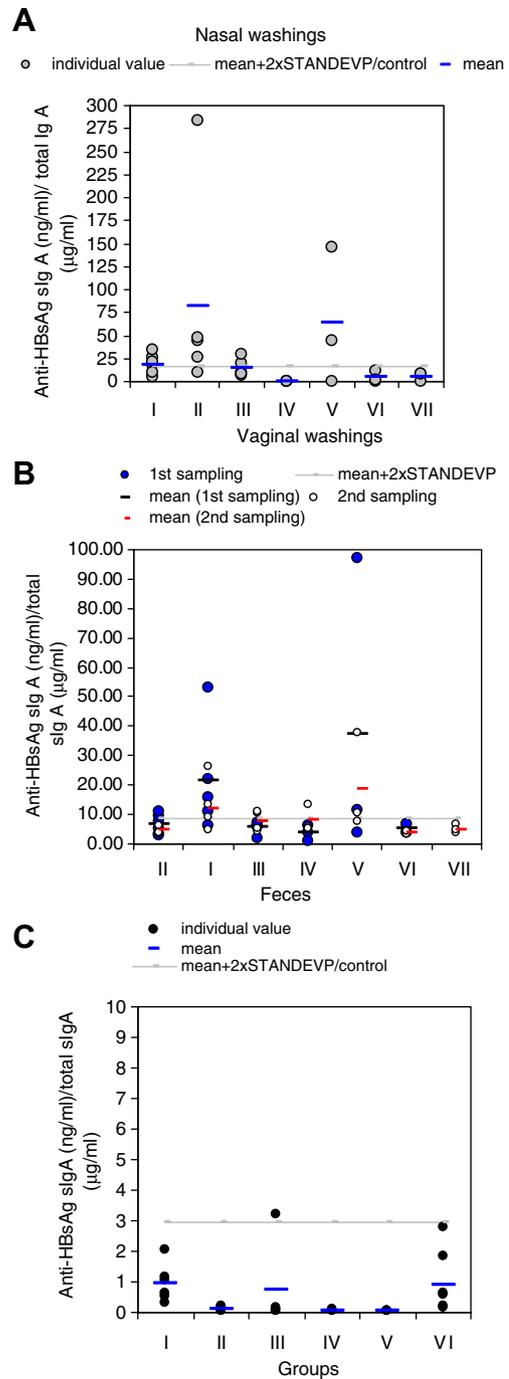
Fig. 5. Secretory anti-HBsAg sIgA profile detected in the individual mice samples of immunized mice with different hepatitis B vaccine oral formulations. Each circle represents the result of individual samples and the horizontal bar the mean of the group. (A) Nasal washings, (B) vaginal washings, (C) faeces. Samples with the value equivalent to mean plus twice the SD of the control group (Group VI) were considered anti-HBsAg sIgA positive. The values were expressed as the ratio between the anti-HBsAg sIgA (ng/ml) and total sIgA (mg/ml).

It is well known that one of the more important modes of hepatitis B virus transmission is the sexual contact with an infected person [26]. Therefore, the induction of the anti-HBsAg sIgA at the urogenital surface would be of great benefit. In the present work, the intranasal adminis-

tration of a saline solution with 10 µg of HBsAg without any adjuvant (group IV) was not able to induce mucosal or systemic antibodies. Similar results were also observed by other research groups [6,10,14]. Therefore, all the evidences show that an adjuvant is required for the intranasal route, in order to obtain a HBV protective immune response. It has been reported that alum (insoluble aluminium salts), one of the few adjuvants approved by the US Food and Drug Administration, is ineffective for the induction of mucosal immunity [27]. Consequently, the investigation of novel, non-toxic adjuvants for the expression of suitable antigens at the mucosal surfaces is necessary. In this work, the evaluation of the immune response followed by the intranasal vaccination with the HBs antigen associated to the alginate coated chitosan nanoparticles is reported for the first time. This delivery system proved to efficiently encapsulate the HBs antigen and in preliminary studies [19] also showed the capability for internalization by intestinal Peyer's patches through specialized M-cells. We did not study the uptake of the alginate coated chitosan nanoparticles into the epithelium of nasopharyngeal lymphoid tissue (NALT). However, it was demonstrated by Fujimura [28,29] in an electron microscopic study that the M-cells of human NALT are ultrastructurally similar to those in Peyer's patches and colonic lymphoid follicles. On the basis of these facts, we assumed that an identical alginate coated chitosan particle uptake in the NALT may well occur. Moreover, to support this hypothesis, the uptake of chitosan nanoparticles into the epithelium of human [30] and trimethyl-chitosan nanoparticles into mice NALT [31] has already been demonstrated. Equally important, in a study performed by Tafaghodi et al. [32], alginate microspheres showed to have a clearance rate in the human nose similar to PLGA microspheres and lower than sephadex microspheres, and were therefore considered as a suitable nasal delivery system. The alginate coating of the chitosan nanoparticles described here may show a similar behaviour as alginate particles on mucosal surfaces, at least during the initial contact with mucosal surfaces.

In the present work, the HBV antigen associated to the alginate coated chitosan nanoparticles was able to induce the generation of mucosal antibodies with strong relevance for nasal secretions. This formulation also induced to some extent a cellular immune response; however, the highly required systemic immune response was not induced. The difference between these results and the ones with the solution of the antigen, proved in an indirect way the superior capacity of the chitosan particles in retaining the antigen in the nose cavity. On the other hand, the systemic nonresponsiveness in this group could be also explained by a mucosally induced tolerance [5]. This is merely a hypothesis since cellular and molecular contributions of the immunocompetent cells present in NALT to the generation of tolerance are unknown [5]. On the other hand, it should be further investigated if this kind of immune response (only mucosal antibodies) would suffice to protect human recipients from a HB infection, especially against transmission by the urogenital tract.

Anti-HBsAg IgG antibodies were detected only in the serum of mice immunized with formulations containing a class B CpG ODN (1826). In a comparable study performed by McCluskie [33], the intranasal immunization of mice with the same doses of the HBsAg plus the CpG ODN in a solution, also induced the generation of both systemic and mucosal antibodies. It has been proven that this adjuvant is a potent immunopotentiator and cannot be compared with an antigen delivery system. The mechanism of the delivery systems seems to be only related with its capacity of slow release the antigen or to facilitate the antigen internalization into antigen-presenting cells (APCs) [27]. Conversely, there are strong indications that responses to CpG are dependent on the presence of TLR9 in the cells [34–36]. After interaction with this receptor, the activation of cell signalling pathways occurs, which results in a cascade of immune events [37,38]. In the present study, the best results were found within the groups vaccinated with the antigen associated with the nanoparticles plus the adjuvant in solution (group II) and in group V, vaccinated with a solution of the antigen plus the adjuvant, being group V with the higher titers. One of the hypotheses to explain this result could be related with the uptake mechanism of the antigen and the adjuvant in the NALT. In group II, the HBsAg associated with nanoparticles may probably have been retained in the NALT during a period that was probably more prolonged than the time period of retention of the adjuvant or the free antigen. This fact may cause a lower amount of the free antigen in close proximity of the adjuvant when compared with the situation observed in group V, vaccinated with a solution of the antigen and the adjuvant not associated with the nanoparticles. The proximity between the HBV antigen and the CpG ODN has been demonstrated to be an important condition for the intensity of the antigen-specific immune response [39–41]. Some examples were reported in the literature, e.g., the adjuvant activity of a non-coding plasmid was demonstrated only when the plasmid and the HBsAg were co-entrapped in the same liposomes, but not in separate vesicles [41]. Interestingly enough, we have observed the same effect: in fact with group III, where both antigen and adjuvant were associated with separate nanoparticles and the result obtained was similar to the result obtained with group I (i.e. the group without CpG ODN). The results indicated that the association of the CpG ODN to separate chitosan nanoparticles was not a good strategy for the intranasal administration. Further studies are needed to evaluate if the co-encapsulation of the antigen and the adjuvant in the same nanoparticle would produce a better immune response. On the other hand, the CpG ODN used in this vaccination study is synthesized with a phosphorothioate (PS) backbone and hence is resistant to nuclease degradation. Consequently, the use of this CpG for intranasal vaccination may not need protection. However, the second

hypothetical mechanism by which the nanoparticles may improve the efficacy of the CpG ODN is by a depot effect, which may result in an extended period during which, both, antigen and CpG ODN are available in the application site, therefore increasing their probability for being internalized by the NALT cells [35]. Therefore in this case the encapsulation may be useful, however the poor immunogenic response observed in group III led us to hypothesise that the free amount of the CpG ODN was not high enough to stimulate the immune system to respond to the HBV antigen. Therefore, the *in vivo* CpG ODN amount released from the coated nanoparticles was most probably, inadequate. This phenomenon could be associated with a strong affinity of the CpG ODN's to the cationic chitosan [42,43]. This hypothesis was addressed in different studies where a poor transfection by chitosan–DNA nanoparticles was observed [44,45] and dependent on several factors, including the degree of deacetylation and molecular weight of the chitosan, and the chitosan/plasmid charge ratio conferred, essentially by amino groups (chitosan) and phosphate groups (DNA) [46,47].

Nevertheless, these results are still not fully conclusive with regard to the advantages of the association of the CpG ODN to nanoparticles. As referred above, theoretically, suitable nanoparticles may increase the residence time in the NALT tissue and facilitate the internalization of the CpG into the mucosal lymphoid tissues. This would decrease the amount of the immunopotentiator needed for eliciting the desirable adjuvant effect. Once inside the target tissue, the ideal delivery system should release the CpG ODN, while most cell types have the capacity to take up CpG ODN via endocytosis [48] or the CpG ODN internalization in cells would be facilitated by the use of CpG associated to suitable nanoparticles.

Several studies have shown that chitosan is a biopolymer with good properties to obtain a high macromolecules (peptides, plasmid vectors, DNA and vaccines) loading efficacy of the corresponding nanoparticles. Promising results of intranasal vaccination using *Bordetella bronchiseptica* [49], meningococcal C conjugate vaccine with mucosal adjuvant LTK63 mutant [50], diphtheria [51,52] and influenza antigens [53] all associated with chitosan particles or just with chitosan powder have been demonstrated in animal models. More recently, the nasal vaccination in human volunteers by simple syringe insufflations of Menjugate-C associated with chitosan produced geometric mean titers of serum bactericidal antibody comparable to parenteral immunization. More importantly, the sIgA antibodies were detected in nasal washings [54].

Compared with the above cited vaccines, recombinant hepatitis B surface antigen is considered to be a weaker antigen. Therefore, chitosan based formulations need to be improved, in terms of a delivery system for the simultaneous intranasal administration of the vaccine and an immunopotentiator yielding synergistic effects.

5. Conclusion

All the intranasal formulations were able to induce the production of the interferon-γ. The values found were comparable with the concentrations obtained in group s.c. vaccinated with the commercial formulation. Again similar to the behaviour found for the commercial formulation was the lower percentage of B-lymphocytes observed in groups i.n. vaccinated with formulations without nanoparticles.

The association of the HBsAg with alginate coated chitosan nanoparticles, administered nasally to mice, gave rise to humoral mucosal immune responses, which were not induced by the HBsAg alone. The generation of systemic antibodies, predominantly Th1-type antibodies, was observed when the HBV antigen entrapped in the particles was administered simultaneously with the adjuvant, CpG ODN, in solution. Future work will focus in the improvement of the chitosan nanoparticles in order to better control the *in vivo* CpG ODN release from the particles and the co-entrapment of the antigen and the adjuvant, in order to elicit not only mucosal antibodies but also, if possible, systemic humoral immune response.

References

[1] C.J. Clements, Y. Baoping, A. Crouch, D. Hipgrave, O. Mansoor, C.B. Nelson, S. Treleaven, R. van Konkelenberg, S. Wiersma, Progress in the control of hepatitis B infection in the Western Pacific region, Vaccine 24 (2006) 1975–1982.

[2] W.C. Maddrey, Hepatitis B: an important public health issue, J. Med. Virol. 61 (2000) 362–366.

[3] G. Quaglio, F. Lugoboni, P. Mezzelani, D.C. Des Jarlais, A. Lechi, Hepatitis vaccination among drug users, Vaccine 24 (2006) 2702–2709.

[4] J. Holmgren, C. Czerkinsky, Mucosal immunity and vaccines, Nat. Med. 11 (2005) S45–S53.

[5] H. Kiyono, S. Fukuyama, NALT- versus Peyer's-patch-mediated mucosal immunity, Nat. Rev. Immunol. 4 (2004) 699–710.

[6] M. Isaka, Y. Yasuda, M. Mizokami, S. Kozuka, T. Taniguchi, K. Matano, J. Maeyama, K. Mizuno, K. Morokuma, K. Ohkuma, N. Goto, K. Tochikubo, 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 19 (2001) 1460–1466.

[7] J.E. Morin, M.D. Lubeck, J.E. Barton, A.J. Conley, A.R. Davis, P.P. Hung, Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters, Proc. Natl. Acad. Sci. USA 84 (1987) 4626–4630.

[8] D. Nardelli-Haefliger, J. Benyacoub, R. Lemoine, S. Hopkins-Donaldson, A. Potts, F. Hartman, J.P. Kraehenbuhl, P. De Grandi, Nasal vaccination with attenuated *Salmonella typhimurium* strains expressing the Hepatitis B nucleocapsid: dose response analysis, Vaccine 19 (2001) 2854–2861.

[9] Y. Lobaina, D. Garcia, N. Abreu, V. Muzio, J.C. Aguilar, Mucosal immunogenicity of the hepatitis B core antigen, Biochem. Biophys. Res. Commun. 300 (2003) 745–750.

[10] M.J. McCluskie, H.L. Davis, CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice, J. Immunol. 161 (1998) 4463–4466.

[11] M.J. McCluskie, R.D. Weeratna, H.L. Davis, Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution, Mol. Med. 6 (2000) 867–877.

[12] M.J. McCluskie, R.D. Weeratna, P.J. Payette, H.L. Davis, Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA, FEMS Immunol. Med. Microbiol. 32 (2002) 179–185.

[13] K.S. Jaganathan, S.P. Vyas, Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally, Vaccine 24 (2006) 4201–4211.

[14] A. Debin, R. Kravtzoff, J.V. Santiago, L. Cazales, S. Sperandio, K. Melber, Z. Janowicz, D. Betbeder, M. Moynier, Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses, Vaccine 20 (2002) 2752–2763.

[15] T.W. Kim, H. Chung, I.C. Kwon, H.C. Sung, T.H. Kang, H.D. Han, S.Y. Jeong, Induction of immunity against hepatitis B virus surface antigen by intranasal DNA vaccination using a cationic emulsion as a mucosal gene carrier, Mol. Cell 22 (2006) 175–181.

[16] S. Saraf, D. Mishra, A. Asthana, R. Jain, S. Singh, N.K. Jain, Lipid microparticles for mucosal immunization against hepatitis B, Vaccine 24 (2006) 45–56.

[17] A. Bernkop-Schnurch, M.E. Krajicek, Mucoadhesive polymers as platforms for peroral peptide delivery and absorption: synthesis and evaluation of different chitosan–EDTA conjugates, J. Control. Release 50 (1998) 215–223.

[18] O. Borges, G. Borchard, A. de Sousa, H.E. Junginger, A. Cordeiro-da-Silva, Induction of lymphocytes activated marker CD69 following exposure to chitosan and alginate biopolymers, Int. J. Pharm. 337 (2007) 254–264.

[19] O. Borges, A. Cordeiro-da-Silva, S.G. Romeijn, M. Amidi, A. de Sousa, G. Borchard, H.E. Junginger, Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination, J. Control. Release 114 (2006) 348–358.

[20] O. Borges, G. Borchard, J.C. Verhoef, A. de Sousa, H.E. Junginger, Preparation of coated nanoparticles for a new mucosal vaccine delivery system, Int. J. Pharm. 299 (2005) 155–166.

[21] A. Cordeiro-da-Silva, J. Tavares, N. Araujo, F. Cerqueira, A. Tomas, P. Kong Thoo Lin, A. Ouaissi, Immunological alterations induced by polyamine derivatives on murine splenocytes and human mononuclear cells, Int. Immunopharmacol. 4 (2004) 547–556.

[22] R.A. Daynes, E.Y. Enioutina, S. Butler, H.H. Mu, Z.A. McGee, B.A. Araneo, Induction of common mucosal immunity by hormonally immunomodulated peripheral immunization, Infect. Immun. 64 (1996) 1100–1109.

[23] C. Nagler-Anderson, Man the barrier! Strategic defences in the intestinal mucosa, Nat. Rev. Immunol. 1 (2001) 59–67.

[24] H. Iijima, I. Takahashi, H. Kiyono, Mucosal immune network in the gut for the control of infectious diseases, Rev. Med. Virol. 11 (2001) 117–133.

[25] J. Holmgren, A. Harandi, M. Lebens, J.-B. Sun, F. Anjuère, C. Czerkinsky, Mucosal adjuvants based on cholera toxin and *E. coli* heat-labile enterotoxin, in: V. Schijns, D. O'Hagan (Eds.), Immunopotentiators in Modern Vaccines, Academic Press, 2006, pp. 235–252.

[26] F. Mahoney, M. Kane, Hepatitis B vaccine, in: S.A. Plotkin, W.A. Orenstein (Eds.), Vaccines, W.B. Saunders Co., Philadelphia, 1999, pp. 158–182.

[27] D. ÓHagan, Microparticles as vaccine delivery systems, in: V. Schijns, D. O'Hagan (Eds.), Immunopotentiators in Modern Vaccines, Academic Press, 2006, pp. 123–147.

[28] Y. Fujimura, M. Hosobe, T. Kihara, Ultrastructural study of M cells from colonic lymphoid nodules obtained by colonoscopic biopsy, Dig. Dis. Sci. 37 (1992) 1089–1098.

[29] Y. Fujimura, Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans, Virchows Arch. 436 (2000) 560–566.

[30] Y. Fujimura, T. Akisada, T. Harada, K. Haruma, Uptake of microparticles into the epithelium of human nasopharyngeal lymphoid tissue, Med. Mol. Morphol. 39 (2006) 181–186.

[31] M. Amidi, S.G. Romeijn, G. Borchard, H.E. Junginger, W.E. Hennink, W. Jiskoot, Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system, J. Control. Release 111 (2006) 107–116.

[32] M. Tafaghodi, S. Abolghasem Sajadi Tabassi, M.R. Jaafari, S.R. Zakavi, M. Momen-Nejad, Evaluation of the clearance characteristics of various microspheres in the human nose by gamma-scintigraphy, Int. J. Pharm. 280 (2004) 125–135.

[33] M.J. McCluskie, R.D. Weeratna, A.M. Krieg, H.L. Davis, CpG DNA is an effective oral adjuvant to protein antigens in mice, Vaccine 19 (2000) 950–957.

[34] H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira, A Toll-like receptor recognizes bacterial DNA, Nature 408 (2000) 740–745.

[35] M.J. McCluskie, R.D. Weeratna, CpG oligodeoxynucleotides as vaccine adjuvants, in: V. Schijns, D. O'Hagan (Eds.), Immunopotentiators in Modern Vaccines, Academic Press, 2006, pp. 73–92.

[36] F. Takeshita, C.A. Leifer, I. Gursel, K.J. Ishii, S. Takeshita, M. Gursel, D.M. Klinman, Cutting edge: role of Toll-like receptor 9 in CpG DNA-induced activation of human cells, J. Immunol. 167 (2001) 3555–3558.

[37] A.M. Krieg, CpG motifs: the active ingredient in bacterial extracts? Nat. Med. 9 (2003) 831–835.

[38] A.M. Krieg, CpG motifs in bacterial DNA and their immune effects, Annu. Rev. Immunol. 20 (2002) 709–760.

[39] W.M. Li, M.B. Bally, M.P. Schutze-Redelmeier, Enhanced immune response to T-independent antigen by using CpG oligodeoxynucleotides encapsulated in liposomes, Vaccine 20 (2001) 148–157.

[40] I. Gursel, M. Gursel, K.J. Ishii, D.M. Klinman, Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides, J. Immunol. 167 (2001) 3324–3328.

[41] M. Gursel, S. Tunca, M. Ozkan, G. Ozcengiz, G. Alaeddinoglu, Immunoadjuvant action of plasmid DNA in liposomes, Vaccine 17 (1999) 1376–1383.

[42] S. Danielsen, K.M. Varum, B.T. Stokke, Structural analysis of chitosan mediated DNA condensation by AFM: influence of chitosan molecular parameters, Biomacromolecules 5 (2004) 928–936.

[43] J.M. Dang, K.W. Leong, Natural polymers for gene delivery and tissue engineering, Adv. Drug Deliv. Rev. 58 (2006) 487–499.

[44] F.C. MacLaughlin, R.J. Mumper, J. Wang, J.M. Tagliaferri, I. Gill, M. Hinchcliffe, A.P. Rolland, Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery, J. Control. Release 56 (1998) 259–272.

[45] K.L. Douglas, C.A. Piccirillo, M. Tabrizian, Effects of alginate inclusion on the vector properties of chitosan-based nanoparticles, J. Control. Release 115 (2006) 354–361.

[46] T. Sato, T. Ishii, Y. Okahata, In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency, Biomaterials 22 (2001) 2075–2080.

[47] T. Ishii, Y. Okahata, T. Sato, Mechanism of cell transfection with plasmid/chitosan complexes, Biochim. Biophys. Acta 1514 (2001) 51–64.

[48] A.M. Krieg, A.K. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky, D.M. Klinman, CpG motifs in bacterial DNA trigger direct B-cell activation, Nature 374 (1995) 546–549.

[49] M.L. Kang, S.G. Kang, H.L. Jiang, S.W. Shin, D.Y. Lee, J.M. Ahn, N. Rayamahji, I.K. Park, S.J. Shin, C.S. Cho, H.S. Yoo, In vivo induction of mucosal immune responses by intranasal administration of chitosan microspheres containing *Bordetella bronchiseptica* DNT, Eur. J. Pharm. Biopharm. 63 (2006) 215–220.

[50] B.C. Baudner, J.C. Verhoef, M.M. Giuliani, S. Peppoloni, R. Rappuoli, G. Del Giudice, H.E. Junginger, Protective immune responses to meningococcal C conjugate vaccine after intranasal immunization of mice with the LTK63 mutant plus chitosan or trimethyl chitosan chloride as novel delivery platform, J. Drug Target 13 (2005) 489–498.

[51] I.M. van der Lubben, G. Kersten, M.M. Fretz, C. Beuvery, J. Coos Verhoef, H.E. Junginger, Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice, Vaccine 21 (2003) 1400–1408.

[52] E.A. McNeela, D. O'Connor, I. Jabbal-Gill, L. Illum, S.S. Davis, M. Pizza, S. Peppoloni, R. Rappuoli, K.H. Mills, A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM(197)) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery, Vaccine 19 (2000) 1188–1198.

[53] A. Bacon, J. Makin, P.J. Sizer, I. Jabbal-Gill, M. Hinchcliffe, L. Illum, S. Chatfield, M. Roberts, Carbohydrate biopolymers enhance antibody responses to mucosally delivered vaccine antigens, Infect. Immun. 68 (2000) 5764–5770.

[54] Z. Huo, R. Sinha, E.A. McNeela, R. Borrow, R. Giemza, C. Cosgrove, P.T. Heath, K.H. Mills, R. Rappuoli, G.E. Griffin, D.J. Lewis, Induction of protective serum meningococcal bactericidal and diphtheria-neutralizing antibodies and mucosal immunoglobulin A in volunteers by nasal insufflations of the Neisseria meningitides serogroup C polysaccharide-CRM197 conjugate vaccine mixed with chitosan, Infect. Immun. 73 (2005) 8256–8265.