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Posintro™-HBsAg, a modified ISCOM including HBsAg, induces strong cellular and humoral responses

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ABSTRACT

To improve the hepatitis B vaccines on the market new adjuvant systems have to substitute aluminium. In this study the hepatitis B surface antigen (HBsAg) was incorporated into a novel adjuvant system, the Posintro™, a modification of the traditional immune stimulatory complexes (ISCOMs). This new HBsAg vaccine formulation, Posintro™-HBsAg, was compared to two commercial hepatitis B vaccines including aluminium or monophosphoryl lipid A (MPL) and the two adjuvant systems MF59 and QS21 in their efficiency to prime both cellular and humoral immune responses. The Posintro™-HBsAg induced the strongest humoral response with high titers of HBsAg specific antibody, high number of antigen specific B-cells and a strong T helper 1 (Th1) antibody profile when compared to the other adjuvant formulations. The Posintro™-HBsAg was also a strong inducer of cellular immune responses with induction of delayed type hypersensitivity (DTH) reaction and $CD4^+$ T-cell proliferation. In addition, Posintro™-HBsAg was the only vaccine tested that also induced a strong cytotoxic T lymphocyte (CTL) response, with high levels of antigen specific CD8 T-cells secreting IFN-gamma mediating cytolytic activity. The results demonstrate that this novel experimental vaccine formulation, the Posintro™-HBsAg, is strongly immunogenic and can induce both class I and class II responses in experimental animals. This shows promise both for the protection against hepatitis B virus infection and as a potential therapeutic vaccine.

1. Introduction

Hepatitis B is one of the major diseases of mankind with approximately 2 billion people being infected with hepatitis B virus and more than 350 million people live with chronic infections (http://www.who.int/mediacentre/factsheets/fs204/en/). People with chronic infection are at high risk of developing cirrhosis of the liver or both cirrhosis and liver cancer, two diseases with a death rate of 1 million people per year. The hepatitis B virus is a double shelled DNA virus with an outer lipoprotein envelope containing the hepatitis B surface antigen (HBsAg) (Seeger and Mason, 2000). Commercial vaccines on the market consist of HBsAg formulated with aluminium phosphate, used as adjuvant (e.g. Twinrix®) (Van der Wielen et al., 2006). Aluminium based hepatitis B vaccines only induce protection in approximately 70–90% of the vaccinated population (Van Damme and Van der Wielen, 2001; Bryan et al., 1995). In, for example, dialysis patients who are known to have increased risk to be infected with hepatitis B, the aluminium induced humoral immune response is not enough, and thus a stronger adjuvant is needed, like the TLR4 agonist monophosphoplipid A (MPL), found in the commercial hepatitis B vaccine Fendrix® (Beran, 2008). It is therefore very important to increase the potency of the hepatitis B vaccines by using new adjuvant systems.

The ISCOMs are cage like particles with a mean diameter of 40 nm, when observed in transmission electron microscope (TEM), consisting of cholesterol, phospholipids and saponin. The saponin is the adjuvant part of the ISCOM and it is composed of Quil A, from the bark of *Quillaja saponaria* Molina (Morein et al., 1984). The immune stimulatory complexes (ISCOMs), can induce both a humoral and a cellular immune response with induction of both the MHC class II and I pathways (Takahashi et al., 1990; Mowat et al., 1991; Heeg et al., 1991; van Binnendijk et al., 1992). ISCOMs have been

Abbreviations: HBsAg, hepatitis B surface antigen; CTL, cytotoxic T-lymphocyte.

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used with success in parenteral, oral and intranasal immunization routes (Aguila et al., 2006), but only few papers have evaluated the efficacy of the combination of hepatitis B proteins and ISCOMs (Howard et al., 1987; Guan et al., 2002; Pandey and Dixit, 2009). In this paper we have modified the charge of the traditional ISCOMs by embedment of positively charged cholesterol derivate, the DC-cholesterol, and giving rise to a new generation of ISCOMs denoted Posintro™. The HBsAg is a complex antigen with a number of membrane embedded domains (Gavilanes et al., 1982). These domains make it difficult to separate the antigen into monomers as it has a tendency to form large Dane particles spontaneously, which makes it hard to incorporate HBsAg into the Posintro™. We have mildly denaturated the HBsAg prior to incorporation into the Posintro™, giving rise to the vaccine formulation Posintro™-HBsAg.

In this paper, we describe the new experimental hepatitis B vaccine, the Posintro™-HBsAg, inducing both a stronger humoral and CTL response to hepatitis B in comparison to two commercial hepatitis B vaccines, Twinrix® (aluminium adjuvant) and Fendrix® (aluminium and MPL adjuvant). Posintro™-HBsAg is also more potent than the adjuvant systems MF59 (an oil-in-water emulsion) and QS21 (a Saponin fraction, included in ISCOMs). The induced humoral response makes the Posintro™-HBsAg experimental vaccine suitable as a high-potency prophylactic vaccine. And the strong induction of CTLs by the Posintro™-HBsAg experimental vaccine, which is required for clearance of a hepatitis B infection in chronically infected patients, makes it a good candidate as a therapeutic vaccine.

2. Materials and methods

2.1. Animals and cell lines

Female NMRI mice were used for DTH reaction, antibody titer, B-cell activation and T-cell proliferation analysis. BALB/cJ (H-2^d) mice were used for all CTL response analysis.

All mice were purchased from Taconic (Copenhagen, Denmark) and were used at an age of 6–8 weeks. Donkin–Hartley guinea pigs were purchased from HB Lidköpings Kaninfarm (Lidköping, Sweden). All guinea pigs were used at an age of 5–6 weeks.

The number of animals used for each graph is shown in the legend and no animals have been excluded.

Target cells for the CTL analysis the DBA/2 mastocytoma cell line P815 (H-2^d) was obtained from European Collection of Cell Cultures, ECACC, (Salisbury, UK) and maintained in RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 10% (v/v) FBS (Gibco, Grand Island) 50 μg/mL gentamycin (Life Technologies, Rockville, MD) and 50 μM mercaptoethanol (Sigma–Aldrich) and is referred to below as R10 (10% FBS) culture medium. The cells were kept at 37 °C, 5% CO_2 in a humidified incubator.

2.2. Antigen and vaccines

HBsAg was a generous gift from Serum Institute of India (SII). Twinrix® and Fendrix® were bought from GSK, Middlesex, UK. Twinrix® is constituted of inactivated hepatitis A virus adsorbed to $Al(OH)_3$ and HBsAg adsorbed to $ALPO_4$. Fendrix® is constituted of HBsAg and $ALPO_4$ and 3-*O*-desacyl-4′-monophosphoryl lipid A (MLP) called AS04C. Incomplete Freund's adjuvant (FIA) was diluted to 1:1 in PBS and HBsAg, vortexed and passed through a small syringe before use.

The amount of the HBsAg given to the different species, mixed or incorporated in the formulations or included in the commercial vaccines, were 5 μg HBsAg/guinea pig and 4 μg HBsAg/mouse in all animal experiments except in the cytolytic assays where 2 μg HBsAg/mouse was used.

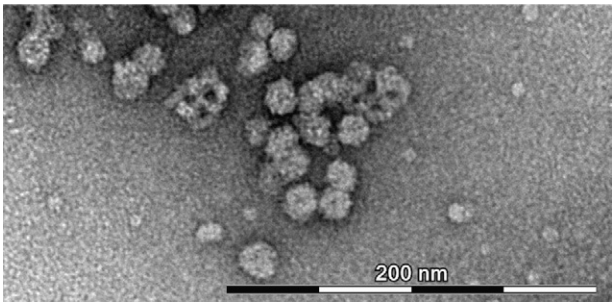

Fig. 1. A transmission electron microscopy (TEM) picture of Posintro™-HBsAg.

2.3. Preparation of Posintro™-HBsAg

HBsAg was mildly denaturated and reduced in 20 mg N-decanoyl-N-methylglucamide (Mega-10) (Bachem, Bubendorf, Switzerland) using 28.5 nmol DL-Dithiothreitol (DTT) (Sigma–Aldrich, St. Louis, MO) or DTT and 2.4 mg Sodium Dodecyl Sulfate (SDS) (Sigma–Aldrich, St. Louis, MO) per mg of HBsAg to facilitate exposure of hydrophobic domains. The addition of only DTT or both DTT and SDS induced the same antibody-titer in guinea pig or mice (data not shown), but the addition of SDS increased the sustained solubilisation of HBsAg. Everything was dissolved in Milli-Q water before mixing, and the denaturation was performed at 37 °C with continuous stirring for 2 h. Posintro™ nano-particles containing HBsAg (Posintro™-HBsAg) were prepared by the standard dialysis method as first described by Hoglund et al. (1989). Briefly cholesterol, 3ß-[N-(N′,N′-dimethylaminoethane)-carbamoyl] Cholesterol Hydrochloride (DC-cholesterol) and 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) (all from Avanti Polar Lipids, Alabaster, AL) were dissolved in 20% (w/v) mega-10 in Milli-Q water. Then, mildly denatured HBsAg, cholesterol, DC-cholesterol, POPC and Quil A (dissolved in Milli-Q water) were mixed in the weight-ratio of 5:3:1:4:20. Final concentration of detergent was 74 mg/mL. The mixture was stirred for 2 h before extensive dialysis (Slide-A-Lyzer® casette, 10,000 MW cut-off, Thermo Scientific, Denmark) against 0.1X PBS pH 7.4 (24 h at 37 °C, 24 h at RT and 48 h at 4 °C with buffer changes every 24 h) in order to remove Mega-10, unincorporated Quil A and unincorporated lipids. Particle size was determined by Dynamic Light Scattering using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK). Transmission electron microscopy (TEM) pictures were obtained of Posintro™-HBsAg on carbon-coated glow-discharged mesh 400 grids after negative staining with 2% Uranyl acetate (Vironova, Sweden) (Fig. 1). Posintro™-HBsAg was diluted in PBS and the final saponin amount was 6–10 μg/mice and 13 μg/guinea pig.

2.4. Preparation of QS21

QS21 was purified from the saponin mixture Quil-A (Brenntag Biosector) by semi-preparative HPLC using a Vydac C_4 column (5 μm particle size, 10 mm × 250 mm) as in Kensil et al. (1991), separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex (Kensil et al., 1991). The collected QS-21 fraction was freeze-dried before analytical HPLC analysis (Kensil et al., 1991). The HPLC chromatogram of the purified QS21 was compared to that of Quadri 2 (a pure standard of QS-21 (Dalsgaard et al., 1995) and QS21 had a purity of about 90% compared to Quadri 2. QS21 was stored at −80 °C until use. One day prior to the immunization, QS21 was mixed with HBsAg in PBS at a ratio of 4 μg QS-21 per μg HBsAg. The QS21 dose in mice was 16 μg and in guinea pigs 20 μg.

2.5. Preparation of MF59

MF59 is an oil-in-water emulsion consisting of 4.3% squalene, 0.5% Tween 80, 0.5% Span 85 (all from Sigma–Aldrich) in a 10 nM isotonic citrate buffer. MF59 was prepared, as previously described (Ott et al., 1995), by high pressure homogenization at 12,000 psi with a Microfluidizer (model 110Y; Microfluidics, Newton, MA) cooled with ice water (5–7 cycles). Before high pressure homogenization, the mixture was pre-mixed with Ultra Turrax at 15,000 rpm for 5 min. The MF59 emulsion was then sterilized by passage through a 0.22 μm pore size filter (Sterilflip) (Millipore) and stored at 4 °C until used. The average particle size of the emulsion droplets, determined by Dynamic Light Scattering using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK), was 173 ± 11 nm and the polydispersity index was 0.12 ± 0.04. MF59 was used within a month from the day of preparation, and showed no increase in particle size during that period. The MF59 was mixed 1:1 with HBsAg in 0.9% NaCl 1–2 h before use to prepare the MF59-HBsAg formulation.

2.6. Immunization of mice and guinea pig

NMRI mice were immunized intradermally (i.d.) in the base of the tail with the vaccines at a HBsAg dose of 4 μg/mice (PBS was used as diluents for QS-21 and Posintro™-HBsAg). The mice were immunized with undiluted Twinrix® and Fendrix® corresponding to 1/5 of a human dose and guinea pigs were immunized with 1/4 of a human dose. The mice were boosted with the identical formulations 4 weeks later. Blood were drawn before priming, just before boosting and 12 days after boosting, draining lymph nodes and spleen were taken simultaneously. Axillary and inguinal lymph nodes were identified as draining lymph nodes for i.d. immunization after injection with methylene blue. The animals were sacrificed 12 days after boosting and axillary and inguinal lymph nodes were isolated and pooled. Simultaneously, the spleen was also isolated.

For cytolytic assays, BALB/cJ mice were immunized i.d. as described above. In distinction, a dose of 2 μg HBsAg was used and the animals were boosted twice with 2 weeks interval. Guinea pigs were primed intra muscularly (i.m.) and boosted four weeks later with the equivalent of 5 μg HBsAg in a volume of 200–500 μl, in accordance with the mice study. Blood was drawn pre-immunization, prior to boosting and 4 weeks after boosting.

2.7. Delayed type hypersensitivity test

NMRI mice were immunized intradermally in the base of the tail, as described in the immunization section followed by a boost dose with the same amount 3 weeks later. One week after the boost all mice were injected with only PBS in one of the hind footpad and PBS mixed with 5 μg HBsAg in the other footpad. Before treatment the footpad thickness was measured with a vernier calliper (with a ability to measure 0.01 mm) 24 h later the footpad thickness was measured. The footpad thickness increment due to antigen addition was recorded for each adjuvant system.

2.8. Preparation of cells from the lymph nodes and the spleen

The lymph nodes and spleens were isolated and minced separately through a 70 μM cell strainer (Becton Dickinson, San Jose, CA). The erythrocytes in the spleen were lysed in 5 mL of 0.2% NaCl for 30 seconds. All cells were counted using Trucount tubes (Becton Dickinson) and flow cytometry analysis were performed on a FACScaliber (Becton Dickinson). All cells were cultured *in vitro* in R10-medium.

2.9. Flow cytometry analysis of proliferating CD4+ T-cells

Lymph node cells from immunized NMRI mice were washed twice in PBS and incubated together with 0.5 μM CFSE (Invitrogen), diluted in PBS, for 5 min at RT. The reaction was stopped with 5 mL of 0.5% BSA (Sigma–Aldrich) in PBS. The cells were diluted in R10 culture medium. Lymph node cells (10^5 cells/well) were plated in flat bottom 96-well plates and cultured for 5 days with a final concentration of 10 μg/mL HBsAg or only medium. Five replicate cultures were performed for each assay. After the incubation time, the five replicate wells were pooled and the cell number counted using the Trucount tubes (Becton Dickinson). The rest of the cells were incubated with phycoerythrin conjugated anti-CD4 and 2 μg/mL 7AAD (Sigma–Aldrich) (detecting dead cells). PBS containing 0.5% BSA (Sigma–Aldrich) was used in all cell labelling and washing steps. Cells were stained for 20 min at 4 °C, washed and re-suspended in PBS/BSA. Flow cytometry analysis was performed on a FACScaliber (Becton Dickinson) using the FCS Express V3 software (De Novo Software, Ontario, Canada) for data analysis. Approximately 5000 $CD4^+$ were analysed in each FACS analysis. The percentage proliferating $CD4^+$T-cells were detected as $CFSE^{low}$ CD^{high} cells and the percentage was multiplied with the total cell number, counted from the five replicate wells, both for cells incubated with HBsAg and cells incubated with medium only. The HBsAg induced number of proliferating cells or $CD4^+$ T-cells was obtained by subtracting the number of positive cells incubated with medium only from the number of positive cells incubated with HBsAg.

2.10. Cytokine secretion of antigen specific T-cells in the draining lymph nodes

The supernatant from draining lymph nodes from immunized NMRI mice cultured 5 days *in vitro* with HBsAg or medium only was analysed using a Luminex based cytokine assay, described by the manufacturer (Panomics, Fremont, CA or Biosource, Camarillo, CA) and analysed on a xMAP Luminex machine (Ramcon A/S, West Sacramento, CA).

2.11. ELISPOT for anti-hepatitis B secreting B-cells

The ELISPOT technique was used to measure the number of hepatitis B specific antibody secreting B-cells. Briefly a PVDF membrane micro 96-well-plate (Pierce, Rockford, IL) was coated with 1 μg/well of HBsAg diluted in a carbonate coating buffer (0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$, pH 9.6) over night at 4 °C. Tetanus Toxoid, 1 μg/well (SSI, Copenhagen, Denmark), was used as a negative control and medium only was used as background control. The wells were washed in washing buffer (Pierce) and a blocking buffer (Pierce) was added for 1 h at 37 °C. The blocking buffer was replaced with a single cell suspension from the spleen from each individual animal, from immunized NMRI mice, was added in an amount of 10^6 cells/well diluted in R10 culture medium, with duplicates for each animal. The plate was wrapped in aluminium foil and incubated in an incubator at 37 °C for 3 h. The plate was washed 6 times in washing buffer and was shaken between each washing step. Biotinylated anti-mouse IgG (DakoCytomation, Glostrup, Denmark) was diluted 4000 times in dilution buffer containing 1% BSA and incubated for 1 h at 37 °C. The wells were washed with washing buffer and Streptavidine-alkaline phosphate 100 μl (Pierce) was added for 30 min at room temperature. The wells were washed with washing buffer and NBT/BCIP (nitroblue tetrazolium/bromochlorindophenol) substrate solution (Pierce) was added 100 μl to each well for 15 min at room temperature. The colorimetric reaction was stopped by the addition of ultra pure water. The plates were dried over night at room temperature

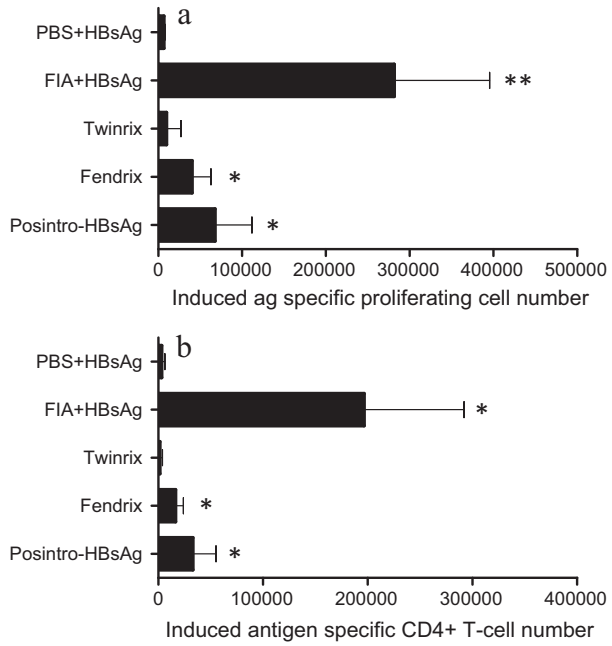

Fig. 2. (a) HBsAg induced proliferation of total cell population or (b) of $CD4^+$T-cells. Cells from the draining lymph nodes were cultured *in vitro* with HBsAg or medium only as control for five days. The cells were counted and analysed on FACS. The antigen induced number of cells was obtained by counting the number of CFSE positive cells incubated with HBsAg and subtracting the background, CFSE positive cells incubated in medium only. The graph is showing mean values + SD ($n = 3$). Statistical analysis, shown in the graph, was performed by comparing groups to the group receiving PBS + HBsAg, using unpaired *t*-test with one-tailed distribution, shown in the graph as $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. The experiment was performed twice, yielding the same conclusions. Data shown are from the second experiment.

dark and evaluated in high resolution (pixel size <5 μm) using an automated ELISPOT reader system (Carl Zeiss) with KS Elispot Software 4.8. (Zellnet Consulting Inc., Fort Lee, NJ).Anti-HBsAg specific B-cell spots were calculated by counting the spots in each well subtracting spots from wells coated with irrelevant antigen (Tetanus toxoid) and calculating an average from the duplicate.

2.12. Determination of anti-HBsAg antibody responses

Antibody levels, both total anti-HBsAg guinea pig IgG antibody levels and total anti-HBsAg mouse IgG and mouse isotype IgG1 and IgG2a antibody titer, were assessed in serum and measured by ELISA. Serum was obtained from the blood that was allowed to clot and afterwards centrifugated at 2160 × *g* for 10 min at RT. Microtiter plates (96 well, MaxiSorb, Nunc, Roskilde, DK) were

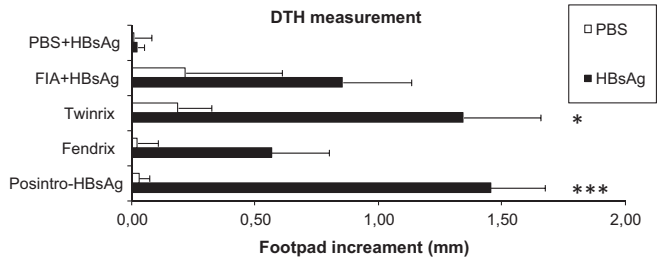

Fig. 3. HBsAg induced cellular responses measured by DTH-assay. The increments in the footpad size, due to a DTH reaction, were measured. Mice were immunized twice. After boost either PBS or HBsAg was injected into the footpads. The mean increase, of the size of the footpads in mm between before and after footpad injection + SD ($n = 4$), was recorded for each adjuvant system. Statistical analysis, using parametric ANOVA test, shown in the graph, was performed by comparing groups with the group receiving PBS + HBsAg.

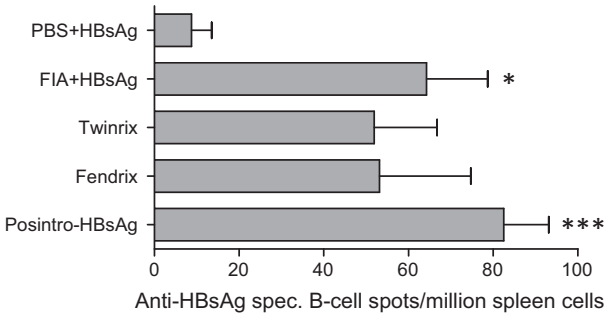

Fig. 4. ELISPOT analyses for anti-HBsAg IgG secreting B-cells in the spleen. Spleen cells from immunized animals were incubated on an HBsAg coated ELISPOT plate, HBsAg specific B-cells were detected with anti-mouse-IgG specific antibodies. The graph shows the mean values ± SEM of number of HBsAg specific antibody secreting B-cells/million spleen cells. Statistical analysis, using parametric ANOVA test, was performed by comparing groups with the group receiving PBS + HBsAg. The plots are from two individual experiments for all vaccines except Fendrix®, where the plots are from one experiment ($n = 3$–7).

coated with 50 μl of 0.4 μg/mL HBsAg (Serum Institute of India) in carbonate buffer (50 mM, pH 9.6). Prior to use, the plates were washed three times with PBS containing 1% (w/v) Triton X-100 (washing buffer) and blocked with 1% (w/v) bovine serum albumin (BSA) (Sigma–Aldrich) in PBS. Serum was added in a series of five ranging from fivefold dilutions to 390,625-fold dilutions and incubated for 1 h at 37 °C. The plate was washed and alkaline phosphatase labelled goat-anti-mouse IgG, IgG1 or IgG2a (Southern Biotech, Birmingham, AL) or alkaline phosphates labelled goat-anti-guinea pig IgG (Southern Biotech) diluted 1:4000 in PBS containing 1% (w/v) BSA was added and incubated for one hour at 37 °C. The plate was washed with washing buffer and p-NPP (Kem-En-Tec Diagnostics, Copenhagen, Denmark) was added as substrate for 30 min at RT and the reaction was stopped by 1 M NaOH. The absorbance was determined at 405 nm using a Vmax kinetic Microplate Reader (Molecular Devices Corporation, CA). Hepatitis B specific antibody titers were calculated from a plot where the OD values were put against the correlated dilution factor, on a log scale. A 4-parameter curve is set up and to determine the titer an s-shaped curve has to be obtained and at the linear part of the curve at 50% of binding, the dilution figure is equal to the titer. All values were correlated to an internal standard, a pool of serum from the same species immunized with HBsAg.

2.13. Cytolytic assays

Spleens were removed from immunized BALB/cJ mice seven days after the last immunization. The spleen cell population were re-stimulated *in vitro* over night for IFN-γ ELISPOT and intracellular staining for IFN-γ. For Pentamer and CD107α mobilizing assay the cell population were re-stimulated for five days and human IL-2 (20 U/mL) (with cross-reactivity to mouse) was added for another 2 days.

The spleen cell population was restimulated with the cell line P815 ($H\text{-}2^d$) pre-incubated with HBsAg (1 μg/mL) and a synthetic peptide derived from HBsAg, the L^d MHC class I-restricted peptide IPQSLDSWWTSL (Ulrich et al., 2000), (Proimmune, Oxford, UK) (30 μg/mL) for 90 min at 37 °C. The p815 cells were then irradiated (10,000 rad) and used as responder cells (1×10^6 cells/well) and put in culture with spleen cells (the effector cells) (4×10^6 cells/well) in a 24 well plates (Nunc) and cultured at 37 °C, 5% CO_2 in a humidified incubator. The p815 cells pre-incubated with medium only served as negative responder cells in the assay for intracellular production of IFN-γ.

IFN-γ assays: The ELISPOT technique was used for detection of IFN-γ producing cells according to the manufacture's protocol

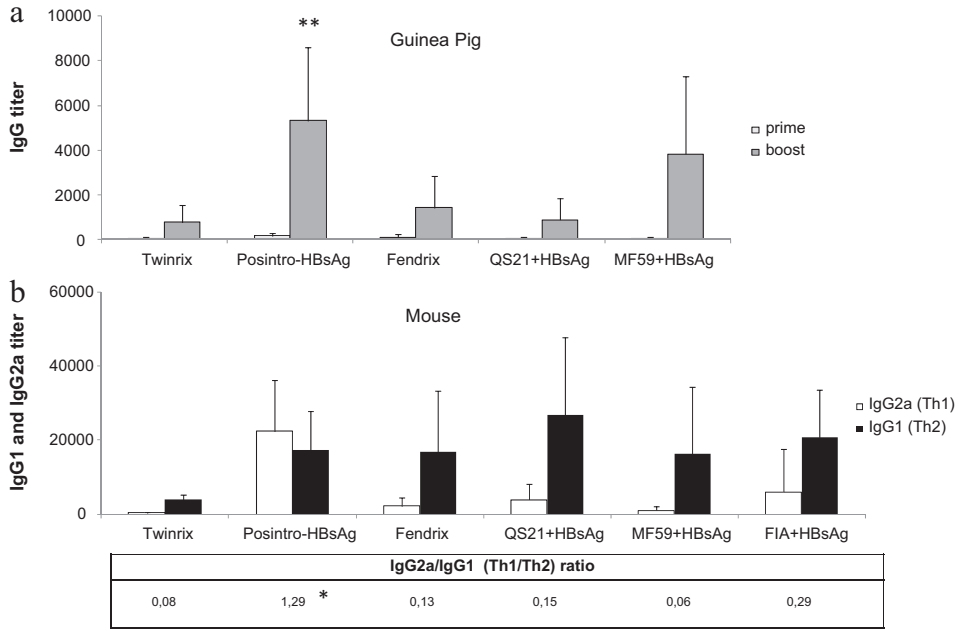

Fig. 5. (a) HBsAg-specific total IgG titers induced in guinea pigs and (b) HBsAg-specific IgG1 and IgG2a titers induced in mice. TheIgG2a/IgG1 ration is shown in the table under the graphs. The total IgG, IgG1 and IgG2a titers were measured by ELISA and bars represents mean values + SD ($n = 10$ for GP and $n = 6$ for mice). Statistical analysis was performed by a parametric ANOVA test comparing groups with the group receiving Twinrix® reported in graph 4a and in the table (showing IgG2a/IgG1 ratio).

(Becton Dickinson). Briefly, effector cells (0.2×10^6 cells/well) were cultured with irradiated responder cells (P815, 5×10^4 cells/well) in 96-well ELISPOT plates pre-coated with anti-mouse IFN-γ antibody. R10-medium alone and non-peptide loaded irradiated responder cells served as negative controls. Plates were incubated at 37 °C, 5% CO_2 for 20 h. Wells were then washed with washing buffer provided from the kit and spots detected after the addition with anti-mouse IFN-γ biotinylated detection antibody (Becton Dickinson) for 2 h, 37 °C, followed by streptavidin-HRP antibody and AEC substrate solution (AEC Chromogen mixed with AEC Substrate buffer from the kit). Plates were incubated at RT until spots appeared and the colorimetric reaction was stopped by the addition of ultra pure water. The plates were dried over night at RT and Ag-specific spot-forming units (SFU) were analysed as described above.

In addition, intracellular detection of IFN-γ was performed by tracking the phenotype of the IFN-γ producing cells. Intracellular cytokine staining was performed as described in the Cytofix/Cytoperm™ kit instruction manual (BD Biosciences,Mississauga, ON). In brief, the cells were *in vitro* stimulated with antigen as for ELISPOT assay and after 16 h of stimulation GolgiPlug™ was added and the cells were incubated for another 4 h. Afterwards the cells were stained with anti-CD8-APC for 5 min at RT and washed and fixated in Fixation/Permeabilization solution from the kit. The cells were then washed and stained intracellular with anti-IFN-γ-FITC for 30 min at 4 °C, washed and analysed.The pentamer staining was performed according to the manufacture's protocol (Proimmune), where one specific and one control phycoerythrin florochrome labelled Pro5® MHC class I pentamers were used. One is being the MHC class I allele H-$2L^d$ and loaded with the HBsAg derived peptide IPQSLDSWWTSL and a control pentamer being the MHC class I allele H-$2L^d$ loaded with the Beta-galactosidase derived peptide, TPHPARIGL (Proimmune). Briefly, re-stimulated spleen cells from immunized BALB/cJ mice were incubated for 10 min at RT with either pentamer. After washing, the cells were incubated with anti-CD8-APC for 10 min at 4 °C, washed and re-suspended in PBS and then fixated in 4% paraformaldehyd. The control pentamer was negative for all animals.

The CD107α mobilizing assay described by Betts et al. (2003), was performed in order to detect functional effector cells (Betts et al., 2003). After *in vitro* activation the spleen cells were harvested and seeded ($1{,}33 \times 10^6$ cells, cell amount from start of the 7 days *in vitro* activation) in a 96-well plate with fresh responder cells P815 cells (1×10^4 cells/well), either peptide loaded or non-loaded irradiated p815 cells, incubated in a flat bottom 96-well plate (Nunc). Anti-CD107α (4 μl/mL) was added to the effector cells before addition of responder cells, followed by incubation for 1 h at 37 °C. Subsequently, GolgiPlug™ (BD Biosciences) (1 μg/mL Brefeldin A) was added and the cells were further incubated for 3 h. The cells were harvested and stained with anti-CD8-APC (Becton Dickinson).

All FACS tubes were analysed on a FACScaliber (Becton Dickinson) using the FCS Express V3 software (De Novo Software, Ontario, Canada) for data analysis.

3. Results

3.1. Induction of cellular responses

Different vaccine formulations were tested in combination with HBsAg in their ability to induce an antigen specific cellular response. Both total numbers of proliferating cells and specifically proliferating $CD4^+$T-cells from immunized mice were analysed with flow cytometry analysis where proliferating cells were separated by CFSE staining (Fig. 2a and b). The adjuvant systems tested were Twinrix® and Fendrix® two commercial hepatitis B vaccines including aluminium and aluminium and MPL as adjuvant, Freund's incomplete adjuvant (FIA) mixed with HBsAg and the Posintro™-HBsAg. FIA induced the strongest antigen specific proliferation, whereas Posintro™-HBsAg and the commercial vaccine Fendrix® induced comparable levels of proliferating cells, with significantly higher number of both proliferating and $CD4^+$T-cells than in the control group receiving HBsAg without adjuvant (HBsAg in PBS). To determine the cellular response induced by the adjuvant systems, the well-known DTH assay was performed (Fig. 3). The Posintro™-HBsAg induced significantly stronger DTH than the commercial

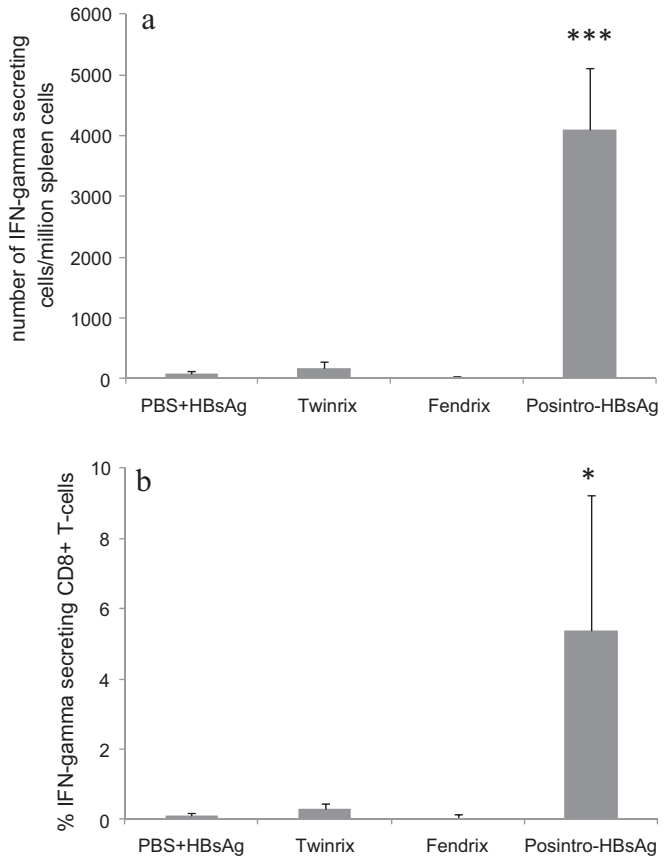

Fig. 6. IFN-gamma secretions measured by two methods. Spleen cells from immunized mice were cultured *in vitro* in the presence of HBsAg and a peptide derived from HBsAg and analysed for: (a) IFN-gamma secreting cells by ELISPOT or (b) IFN-gamma secreting $CD8^+$ T-cells by intracellular staining. The results are expressed as mean values + SD ($n = 4$). Statistical analysis, shown in the graph, was performed by comparing groups with the group receiving PBS + HBsAg using a parametric ANOVA test ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

vaccine Fendrix® ($p < 0.05$) and comparable DTH reaction as the commercial vaccine Twinrix®.

3.2. Induction of antigen specific B-cells

The induction of antigen specific B-cells was detected using ELISPOT. Mice were immunized twice intradermally and the spleen cells were incubated on HBsAg coated membrane plate. The spots detected were specific B-cells producing antibodies specific for the HBsAg. The numbers of positive spots were subtracted from the spots induced by an irrelevant antigen, tetanus toxoid (ranging 0–3 spots in each well) (Fig. 4). Posintro™ was the adjuvant system that induced the highest number of antigen specific B-cells. The number of B-cells was significantly ($p < 0.001$) higher than in the control group receiving HBsAg without adjuvant (HBsAg in PBS). The two commercial vaccines including aluminium or aluminium and MPL, as adjuvants, did not induce a number of specific B-cells significantly higher than HBsAg in PBS.

3.3. Induction of systemic antibody response

Different adjuvant systems in combination with HBsAg were tested in their ability to induce antigen-specific IgG antibodies and the antigen-specific isotypes, IgG1 and IgG2a, the latter two in order to analyse the Th2 and Th1 antibody response, respectively. In both guinea pigs (Fig. 5a) and mice (Fig. 5b) a large panel of different adjuvant systems in combination with HBsAg were compared to Posintro™-HBsAg. In guinea pigs the Posintro™-HBsAg induced the highest average IgG antibody titer compared to the other formulations, and the IgG titer was significantly higher than Twinrix® ($p < 0.01$), Fendrix® ($p < 0.05$) and QS21 + HBsAg ($p < 0.01$) (Fig. 5a). Posintro™-HBsAg induces also a strong boost effect. In mice, two IgG isotypes were analysed, IgG1 (a Th2 induced isotype) and IgG2a (a Th1 induced isotype) (Fig. 5b). Posintro™-HBsAg induced the highest Th1 antibody response with high IgG2a titers compared to all the other vaccine formulations, with significantly higher IgG2a level than Twinrix®, Fendrix® (both $p < 0.001$), QS21and MF59 (both $p < 0.01$) mixed with HBsAg. The aluminium based commercial vaccines Twinrix® is known to induce a strong Th2 response, but even so, Posintro™-HBsAg induced also significant higher IgG1 titer than Twinrix® ($p < 0.01$). Posintro™-HBsAg was the only vaccine formulation tested that induced a ratio of IgG2a/IgG1 above 1, correlated to a stronger Th1 than Th2 induced immune response and significantly higher ratio than PBS + HBsAg, Twinrix®, Fendrix® and MF59 ($p < 0.05$).

3.4. Antigen specific cytokine production

Draining lymph node cells from mice immunized with the different formulations of HBsAg were cultured *in vitro*. The cytokine production was measured in the supernatant after 5 days of culture using the Luminex analysis system (Table 1). The strong induction of Th1 and Th2 antibody titer by Posintro™-HBsAg is also seen on the Th1 and Th2 cytokine levels. Posintro™-HBsAg induces IFN-γ (Th1 induced cytokine), IL-5 and IL-13 (both Th2 induced cytokines) IL-10 (suppressive cytokine), and TNF-α (pro-inflammatory cytokine). Posintro™-HBsAg induced significantly higher titers of IL-5 ($p < 0.01$) and IL-13 ($p < 0.01$) than the commercial vaccine Twinrix®. Posintro™-HBsAg and Fendrix® induce approximately the same amount of IFN-γ but Posintro™-HBsAg induced significantly higher levels of both IL-5 ($p < 0.05$) and IL-13 ($p < 0.01$) than Fendrix®. Posintro™-HBsAg induced also significantly higher amount of IL-10 compared to Twinrix® ($p < 0.001$) and Fendrix® ($p < 0.05$).

3.5. Induction of antigen specific cytotoxic T-cells

To verify the amount of induced cytotoxic T-cells, reactive to a L^d restricted S38-39 epitope of the S protein (HBsAg) (Schirmbeck et al., 1998), four analysis methods were used. The classical analysis of the antigen-induced IFN-γ producing cells using both the ELISPOT technique and determination of intracellular cytokine production by flow cytometry was applied. The latter was used to analyse the IFN-γ secretion by $CD8^+$ T-cells, in particular. Posintro™-HBsAg induced significantly higher level of IFN-γ than the two commercial vaccines in both methods, with a p-value of <0.001 for the ELISPOT method (Fig. 6a) and $p < 0.05$ for intracellular staining of IFN-γ (Fig. 6b). To analyse the amount of HBsAg specific $CD8^+$ T-cells induced by the different adjuvant systems, hepatitis B-positive pentamer staining were used. Again, Posintro™-HBsAg induced significantly stronger induction of $CD8^+$ T-cells binding the HBsAg derived pentamer in comparison to both Twinrix® and Fendrix® ($p < 0.01$) (Fig. 7). To verify the amount of $CD8^+$ T cells mediating cytolytic activity in an antigen-specific manner, the CD107α analysis method was used. In this fourth analysis method Posintro™-HBsAg demonstrated again to be the best inducer of activated cytotoxic T-cells with highly significantly induction of CD107α positive $CD8^+$ T-cells ($p < 0.001$) (Fig. 8).

Table 1
Cytokine secretion by *in vitro* HBsAg stimulated draining lymph node cells from animals immunized with different adjuvants.

Adjuvant system	IFN-g (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IL-10 (pg/ml)	TNF-a (pg/ml)
PBS + HBsAg	0	0	50(±50)	0	0
Twinrix®	500(±300)*	150(±50)**	2600(±1300)*	150(±100)**	35(±15)*
Fendrix®	750(±550)*	50(±100)	2300(±1200)*	200(±150)*	120(±55)*
Posintro™-HBsAg	1050(±1000)	400(±200)**	8100 (±2400)**	600(±150)**	125(±45)**

Results are expressed as mean value ± SD ($n = 3$). Statistical analysis was performed by comparing groups with the group receiving PBS + HBsAg, using unpaired *t*-test with one-tailed distribution. The experiment was performed twice, yielding the same conclusions. Data shown are from the second experiment.

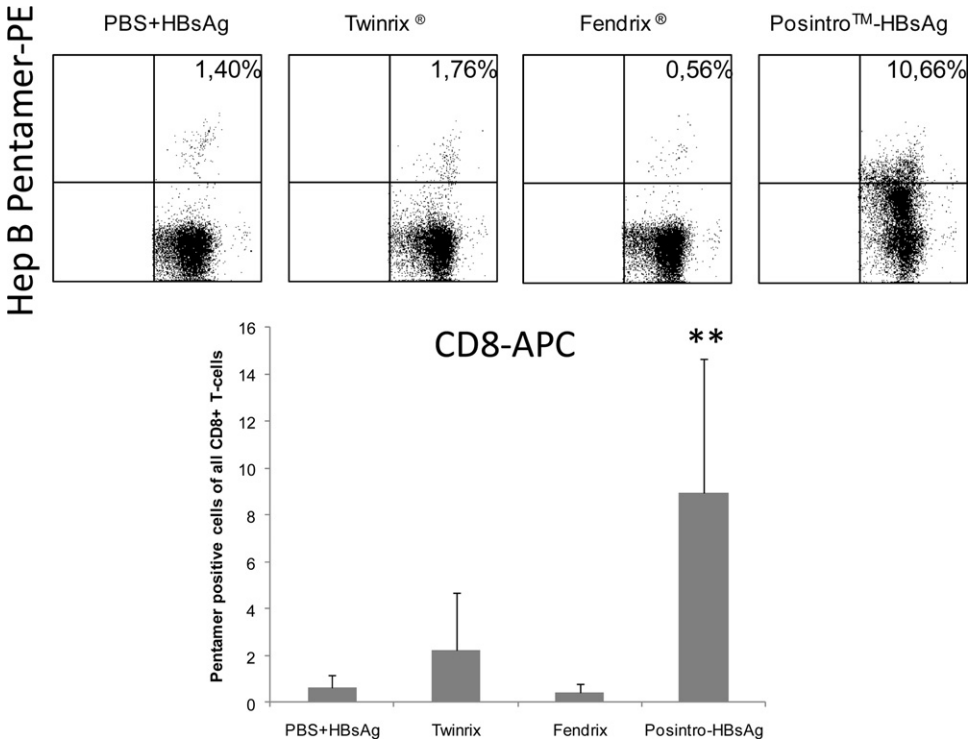

Fig. 7. Pentamer staining of $CD8^+$ lymphocytes. Spleen cells from immunized mice were cultured *in vitro* in the presence of HBsAg and a peptide derived from HBsAg and analysed for HBsAg-peptide loaded pentamer positive cells in the $CD8^+$ T-cell population. The results are expressed as mean values + SD ($n = 4$). Statistical analysis, shown in the graph, was performed by comparing groups with the group receiving PBS + HBsAg using a parametric ANOVA test ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

4. Discussion

To combat hepatitis B infections in chronic hepatitis B carriers many scientists have marked out that the activation of the CTLs is crucial as demonstrated in both chimpanzee and mice (Guidotti et al., 1994, 1999). The CTL status in acute infected hepatitis B carriers (subjects that successfully clear the virus infection) and chronic infected carriers (subjects that do not clear the virus infection) are totally different. The acute hepatitis B carriers have much higher frequency of antigen specific CTLs than chronic hepatitis B carriers (Penna et al., 1991; Missale et al., 1993). To activate the CTLs, new adjuvant system has to be developed. The prophylactic vaccines, on the market, induce a strong humoral response and low or no CTL response against hepatitis B infections. To inhibit the HBV replication, antiviral drugs like Interferons or Lamivudine are used (Lai et al., 1998; Lok et al., 1993). In the present study a new hepatitis B vaccine formulation, the Posintro™, with incorporated HBsAg, was compared to five well known adjuvant systems in their ability to induce both a strong humoral and a cellular immune responses to HBsAg. The adjuvant systems tested in this study were the aluminium phosphate found in the commercial vaccines Twinrix® (GSK) and Fendrix® (GSK), the latter combined with MPL. MPL is a potent vaccine adjuvant that activates antigen presenting cells through the Toll-like receptor 4 (TLR4)/MD-2 complex (Alderson et al., 2006). Furthermore the oil-in-water emulsion MF59, the purified saponin fraction QS21 were compared (see the review (Peek et al., 2008)) and the strong adjuvant Freunds incomplete, FIA, was also used (Bandholtz et al., 2002). All these vaccine formulations include the antigen, HBsAg. In the literature, only few examples of the combination of ISCOMs and hepatitis B are found and to our knowledge no comparative study with commercial hepatitis B vaccines, has been reported. Pandey et al. have also incorporated HBsAg into ISCOMs but they have not denaturated HBsAg prior to incorporation (Pandey and Dixit, 2009). In our hands, HBsAg could not be incorporated into the Posintro™ without a denaturation step otherwise HBsAg will after a while reconstitute into Dane particles. An additional difference between their and our formulation is the ratio of the antibody titers IgG2a/IgG1. The Posintro™-HBsAg formulation induced a IgG2a/IgG1 ratio of above 1.0 compare to Pandeys formulation that induced a ration clearly bellow 1.0 and with approximately the same IgG2a titer as for the adjuvant aluminium, known to induce a Th2 shifted immune response. In this paper, a full range of both humoral and cellular assays are performed to evaluate the potency of the new vaccine formulation Posintro™-HBsAg. To verify the potency of this vaccine, in comparison to other vaccine candidate, it was tested in two species, mice and guinea pigs.

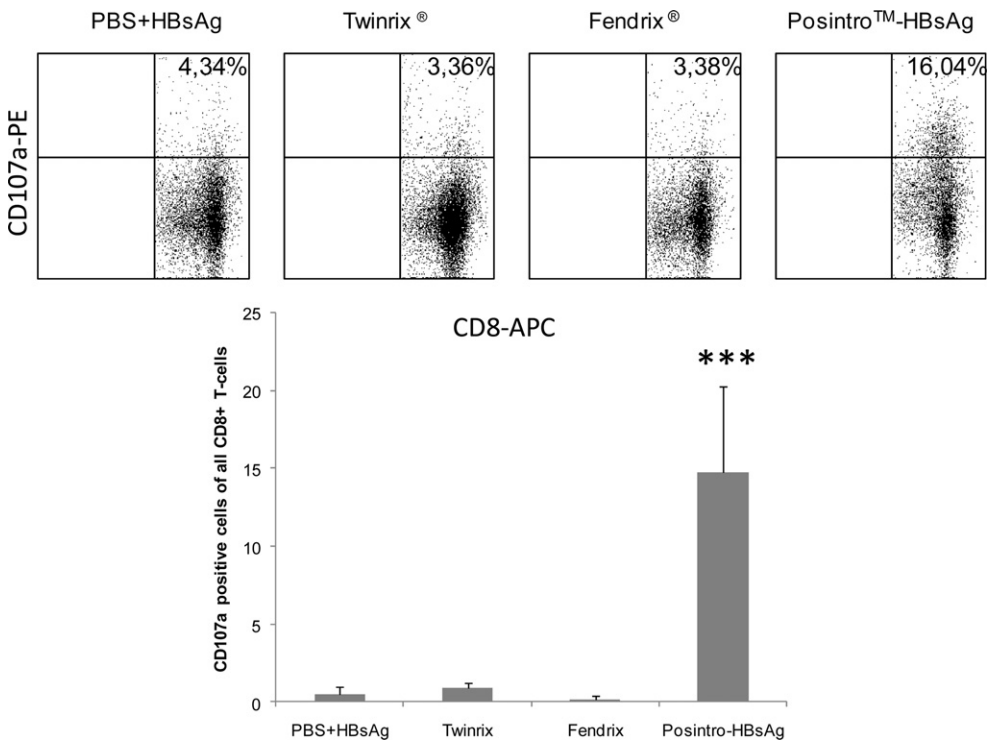

Fig. 8. CD107α staining. Spleen cells from immunized mice were cultured *in vitro* in the presence of HBsAg and a peptide derived from HBsAg and analysed for CD107α positive cells in the $CD8^+$ T-cell population. The results are expressed as mean values + SD ($n = 4$). Statistical analysis, shown in the graph, was performed by comparing with the group receiving PBS + HBsAg using a parametric ANOVA test (*$p < 0.05$).

To suppress a hepatitis B virus infection the cytotoxic T-cells have been demonstrated to be important (Guidotti et al., 1994; Shimada et al., 2003). This CTL response is diminished in chronic hepatitis B virus carriers and only detected in acute viral hepatitis B (Penna et al., 1991; Missale et al., 1993; Maini et al., 2000; Rehermann et al., 1995). It has been shown that hepatocytes express hepatitis antigen determinates in complex with MHC class I in hepatitis B virus transgenic mice (Moriyama et al., 1990), thus allowing susceptibility to a CTL response. Vaccination with HBsAg in complete Freund's adjuvant (CFA), known to induce a strong CTL response, decreased both the HBsAg and HBeAg levels in HBV transgenic mice (Akbar et al., 1997). In comparison, vaccination of hepatitis B chronic carriers with aluminium based hepatitis B vaccines, resulted in no or only minor decrease in HBV DNA content in serum between vaccinated and unvaccinated subjects (Pol et al., 2001; Couillin et al., 1999). In chronic hepatitis B carrier, not only the induction of a CTL response is of importance for viral clearance, but also the induction of $CD4^+$ T-cells of Th1 subtype, correlating to reduced HBV DNA content, since these cells secrete large amount of IFN-γ (Couillin et al., 1999) and the HBV replication is inhibited by IFN-γ through IFN-γ mediated nitric oxide production (Guidotti et al., 1996; Guidotti, 2002). The Posintro™-HBsAg formulation is the only HBsAg vaccine formulation tested here that induced antigen specific cytotoxic T-cells. Posintro™-HBsAg also induced high amount of IFN-γ producing $CD8^+$ T-cells and these cells expressed the marker CD107α upon antigen activation, which is correlating to cytolytic activity (Betts et al., 2003). These $CD8^+$ T-cells also bound HBsAg-specific pentamers upon antigen activation. In acute HBV infection IFN-γ but also TNF-α is the superior cytokines at mediating viral clearance (McClary et al., 2000). We demonstrate that Posintro™-HBsAg induces both IFN-γ and TNF-α producing cells in the draining lymph nodes. Our results are in agreement with other data demonstrating the induction of IFN-γ secreting T-cells by ISCOMs in combination with HBsAg (Guan et al., 2002). Others have demonstrated that the ISCOMs induce a stronger antigen specific CTL response than even Freunds complete adjuvant (Morein, 1988). In contradiction to published data, we could not induce IFN-γ producing $CD8^+$T-cell population by the addition of the TLR-agonist MPL included in the commercial vaccine Fendrix® (Alderson et al., 2006). IL-10 is another cytokine induced by Posintro™-HBsAg immunization and according to Wu et al., high serum levels of IL-10 and IL-12 is associated with protection against HBV infection by the induction of high levels of HBV specific antibody (Wu et al., 2010). Others have demonstrated that HBsAg incorporated into ISCOMs induce a strong IgG antibody response (Howard et al., 1987). In this paper, specific B-cell response induced by four HBsAg formulations were compared, and Posintro™-HBsAg was superior to FIA, Twinrix® and Fendrix® at inducing the largest pool of antigen specific B-cells with plasma and memory B-cells. Posintro™-HBsAg induces also a strong boost response, which is crucial for a strong vaccine formulation. Like others, we observed that Twinrix® induced a strong specific antibody response to hepatitis B (Van der Wielen et al., 2006). However, even though Twinrix®, with its aluminium phosphate adjuvant system induced a strong Th2 antibody profile, Posintro™-HBsAg induced an even higher titer of Th2 antibodies in addition of the strong induction of Th1 antibody titer. The Posintro™-HBsAg induced both a strong Th1 and Th2 immune response demonstrated in both the antibody profile and the cytokine profile. Although Fendrix®, including MPL, induced a higher level of Th1 antibody profile than Twinrix®, the Posintro™-HBsAg induced significantly higher levels of IgG2a than Fendrix®. The water-in-oil emulsion MF59 has been demonstrated previously to be superior to aluminium at inducing a high IgG titer in response to HBsAg (Singh et al., 2006); these data are confirmed in this paper. In this study the novel Posintro™-HBsAg vaccine formulation is compared with MF59 mixed with HBsAg and Posintro™-HBsAg induced an even stronger immune response than MF59, based on total IgG titer and also induced a significantly stronger Th1 response.

In this paper the effect of the experimental vaccine Posintro™-HBsAg has been evaluated and compared to other adjuvant systems. The results show that Posintro™ is a efficient adjuvant candidate for vaccines against intracellular pathogens, since Posintro™-HBsAg ensures a strong induction of both humoral and cellular immune responses and drives a strong Th1 profile with the induction of an activated anti-hepatitis B cytotoxic $CD8^+$ T-cell pool.

5. Conclusion

Our experimental results demonstrated that the novel Posintro™-HBsAg vaccine formulation induced both a robust humoral and cellular immune responses including a strong CTL response. In comparison to both commercial hepatitis B vaccines and experimental vaccines, the Posintro™-HBsAg vaccine formulation induced a higher IgG titer with a shift towards Th1. The Posintro™-HBsAg vaccine formulation was also the only vaccine tested that induced a CTL response, which is needed to combat intracellular pathogens.

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