RESEARCH

Comparison of the immune response induced in mice by five commercial vaccines based on recombinant HBsAg from different sources, implications on their therapeutic use

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ABSTRACT

Several Hepatitis B surface antigen (HBsAg)-based formulations are used in therapeutic immunization studies, but further studies are needed on the immune response elicited by different HBsAg-based formulations to optimize future immunotherapeutic approaches. Here we compare the immunological properties of five HBsAg based commercial vaccines. The formulations are based on recombinant HBsAg produced in three different yeasts. Mice were immunized with these vaccine formulations and the immune response was evaluated by ELISA, enzyme-linked immunospot and lymphoproliferation assays to compare total IgG and the main IgG subclasses in the sera, as well as the frequency of IFN-γ secreting CD8+ T cells and the lymphoproliferation activity of spleen cells. Our results indicate that the Heberbiovac-HB vaccine based on a *Picchia pastoris* produced antigen, elicited a more complete response showing the most potent humoral immune responses while having a remarkable capacity to induce a high frequency of IFN-γ secreting CD8+ T cells and a superior lymphoproliferation response. A potential relationship between antigen aggregation and lipid composition with immunogenicity results is suggested. In conclusion, our results demonstrate that similar formulations based on recombinant HBsAg obtained in different hosts differ in their capacity to induce cellular immune responses and, in some cases, in the intensity of the resulting humoral responses. This would indicate that these formulations would not have a similar effect when treating different chronically infected Hepatitis B patients. Future immunotherapeutic studies using recombinant HBsAg-based vaccines should take into account these differential properties.

Keywords: HBV, HBsAg, vaccine

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RESUMEN

Comparación de la respuesta immune inducida en ratones por cinco vacunas comerciales basadas en el HBsAg recombinante obtenido de diferentes fuentes, implicaciones en su uso terapéutico. Varias formulaciones basadas en el antigeno de superficie de la hepatitis B (HBsAg) han sido usadas con fines terapéuticos, sin embargo, con el objetivo de optimizar futuros desarrollos se requieren estudios más amplios de la respuesta inmune generada por las diferentes formulaciones. El presente trabajo compara las propiedades inmunológicas de cinco vacunas comerciales basadas en el HBsAg obtenido de forma recombinante en tres diferentes cepas de levaduras. Con este objetivo se inmunizaron ratones y la respuesta inmune generada fue evaluada por ensayos de ELISA, ELISPOT y linfoproliferación permitiendo estudiar comparativamente las respuestas de IgG total y subclases de IgG en suero, así como la frecuencia de células T CD8+ secretoras de IFN-γ y la actividad linfoproliferativa en células del bazo. Los resultados obtenidos indican que la vacuna Heberbiovac-HB, basada en el HBsAg producido en Pichia pastoris, genera la respuesta inmune mas completa, induciendo una potente respuesta humoral, una alta frecuencia de células T CD8+ secretoras de IFN-γ y la mayor respuesta linfoproliferativa. Como potenciales causas de estos resultados se sugieren en la discusión la relación con el estado de agregación y la composición lipídica del antigeno. Concluyendo, los resultados obtenidos demuestran que formulaciones similares, basadas en el HBsAg recombinante obtenido de diferentes hospederos, difieren en su capacidad de inducir una respuesta inmune celular, y en algunos casos en la intensidad de la respuesta humoral generada. Esto sugiere que dichas formulaciones pudieran tener un comportamiento diferente en su uso terapéutico en pacientes enfermos por hepatitis B crónica. Las propiedades diferenciales entre HBsAg recombinantes reportadas por este trabajo son de interés en el desarrollo de futuros estudios que empleen este antigeno en la inmunoterapia.

Palabras clave: HBV, HBsAg, vacunas

*I*ntroduction

The infection by the Hepatitis B Virus (HBV) is still an important health problem at the global scale in spite of the very effective vaccines existing since the 1980's. Two billion people alive today show evidence of a past or current infection and more than 350 million people are persistently infected. The state of chronicity correlates with an increased risk of developing liver cirrhosis, hepatocellular carcinoma and other complications such as portal hypertension and liver failure. As a consequence one million people die each year worldwide [1].

The hepatitis B surface antigen (HBsAg) is the main protective antigen of the HBV and the basis of all

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available prophylactic vaccines. Natural HBsAg can be found as spherical or tubular particles in the blood of HBV-infected patients. These 22 nm virus-like particles contain viral-encoded membrane proteins (S, M and L) and ~ 30% (per weight) of host-cell-derived lipids [2]. Since the early 1980´s it was possible to obtain recombinant HBsAg (rHBsAg) purified from yeast, essentially indistinguishable from plasma-derived antigen [1]. Different yeast strains have been used for this purpose, which include *Saccharomyces cerevisiae*, *Picchia pastoris* and *Hansenula polymorpha*, among others [1, 2]. HBsAg has also been expressed and purified from Chinese hamster ovary (CHO) and plant cells with similar immunological and physical characteristics [3, 4].

Recently, yeast-expressed rHBsAg was shown to behave as an apoptotic-like particle, suppressing lipopolysaccharide (LPS) -induced secretion of pro-inflammatory cytokines but increasing the secretion of IL-10 by monocytes. Additionally, rHBsAg binds to monocytes through the interaction with the LPS – binding protein and the CD14 receptor suppressing their activation. Remarkably, plasma derived HBsAg does not have these characteristics. It is suggested that the differences are due to the different lipid content between both antigens. Considering these and other observations the authors proposed that the anti-inflammatory and immunosuppressive potential of yeast-expressed HBsAg is another factor that might affect the immunogenicity of rHBsAg compared to the natural antigen. They also speculated that a similar mechanism could be use by the HBV to interfere with the normal function of antigen-presenting cells and induce T cell anergy preventing the antibody-mediated neutralization of the virus. The latter effects are typical of chronic HBV infected patients [2, 5, 6].

In the field of therapeutic vaccination against chronic hepatitis B, the use of the current preventive vaccines has been previously reported [7-9]. The general conclusion of these trials evidenced that in this complex immunological scenery more powerful antigen formulations and novel adjuvant strategies are required to overcome the state of unresponsiveness of chronic patients [10, 11]. A combination of antiviral treatments with therapeutic vaccination is a promising new strategy [12]. The impaired immune response to HBV-encoded antigens at T cell subsets level [13-15] is well-documented in these patients. Chronic infection by HBV is also associated with functional defects in dendritic cells [16- 18].

The role of potent cellular immune responses in HBV clearance of chronically infected patients has now been consistently demonstrated [19, 20]. The ability to affect clearance by the passive transfer of bone marrow from a naturally immune HBV donor, and the fact that chronic patients recovering from infection develop cytotoxic T lymphocyte (CTL) responses, similar to acute patients [21, 22], further support the previous statement. It is essential for a therapeutic vaccine candidate to elicit an effective and potent cellular immune response in order to subvert the state of immune tolerance against HBV antigens [20].

In the present study we compared the immunological properties of five commercial vaccines based on recombinant hepatitis B surface antigens produced in three different hosts. Specifically, we explored the humoral and cellular immune responses elicited by each formulation in Balb/c mice.

Materials and methods

Vaccine formulations

We used five commercial vaccine formulations based on rHBsAg produced on three different yeasts, adsorbed in alum. All antigens had over 95% purity and have been extensively used in vaccination programs around the world. Two of the vaccines used in this study, Heberbiovac-HB (HeberBiotec, Cuba) and Shanvac™-B (Shantha Biotechnics, India) (A and B respectively), contained rHBsAg purified from *P. pastoris*. Euvax-B (LG PhD, Korea) and Engerix™-B (GlaxoSmithKline, Belgium) vaccines (C and E respectively) employed rHBsAg purified from *Saccharomyces cerevisiae*. The Hepavax-Gene® vaccine formulation (GreenCross, Korea) used an antigen produced in *H. polymorpha* (D). All vaccines have the same presentation consisting of 20 mg of rHBsAg adsorbed in alum per milliliter.

Immunization schedules

Three immunization schedules were carried out using groups of 10 Balb/c female mice of 8 to 12 weeks old. The intramuscular (i.m.) immunization route was used, administering a volume of 100 µL per animal of each vaccine (without dilution) corresponding to a dose of 2 µg of each rHBsAg per mouse. A placebo group with 0.5 mg/mL of alum was always included. The doses were administered on days 0, 15, 30 and 90 and blood was collected ten days after each dose through the retrorbital plexus. All experiments were conducted in accordance to institutional guidelines [23].

ELISA for determining IgG total and subclass response

Specific IgG against HBsAg was evaluated by ELISA. Briefly, high binding plates (Costar, USA) were coated with 100 µL of rHBsAg expressed in *Picchia pastoris* (provided by HeberBiotec) 5 µg/mL in coating buffer (11 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Plates were blocked with 2% (w/v) skim milk in phosphate saline buffer (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) (PBS) for 1 h at 37 °C. Subsequently, the plates were incubated with the serum samples diluted with 1 % (w/v) skim milk, 1% (v/v) Tween 20 in PBS, for 2 h at 37 °C. The anti-mouse IgG peroxidase conjugate (Sigma, USA) was incubated for 1 h at 37 °C. Subsequently the plates were incubated with the substrate solution (52 mM Na_2HPO_4, 25 mM citrate, 1 mg/mL OPD, 0.1% (v/v) H_2O_2) for 15 min at room temperature. Washes with 0.05% (v/v) Tween 20 in the PBS solution were carried out between each step three to five times, and a volume of 100 µL was employed for each incubated solution. The reaction was stopped by adding 50 µL/well of the 3 M H_2SO_4 solution. Finally the plates were read to 492 nm in a microtiter plate reader (Sensident Scan, Merck).

The IgG subclass evaluations were done by a similar ELISA assay, using the ISO-2 Mouse Monoclonal Antibody Isotyping Reagents kit and

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following the manufacturer's recommendations (SIGMA, USA).

Positive samples for antibody titers were detected using cut-off values of twice the optical density (OD) of negative controls (preimmune serum). Each sample was analyzed using an Excel program that could interpolate the OD values on the standard curve consisting of a pool of hyperimmune sera of known titers. This standard curve was included in each individual plate. Finally, the results of total IgG and subclasses obtained were represented as logarithms of the geometric mean of the titer (GMT) for each treatment group (with a confidence interval of 95%).

Enzyme-linked immunospot (ELISPOT) assay for determining interferon gamma (INF-γ) response

Preparation of target and effector cells

Ten days after the last immunization, the spleens were aseptically removed and individual-cell suspensions were prepared. Erythrocytes were lysed after 5 min of incubation with 1 mL per spleen of 0.83% (w/v) NH_4Cl. The cells were extensively washed with the medium, resuspended in RPMI 1640 (Gibco, USA), supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco, USA), 2 mM glutamine, 2 mM piruvate, 50 mM 2-mercaptoethanol and antibiotics (complete medium) and counted. Meanwhile, H-2^d mastocytome cells p815 were pulsed for 1 h at 37 °C, 5% CO_2 in complete medium with 10 mM of the HBsAg S_{28-39} peptide IPQSLDSWWTSL (Center for Genetic Engineering and Biotechnology, CIGB, Cuba) [24]. After incubation, p815 cells were further incubated for another 15 min with mitomycin C (Sigma, USA). They were extensively washed to avoid any trace of mitomycin C, and resuspended in a complete medium for counting. The p815 cells without peptide were also treated as controls.

In vitro re-stimulation of primed CTL

After the washing steps, the cells were counted and distributed in 25 cm^2 flasks (Becton Dickinson, England) at $2x10^6$ cells per milliliter in 10 mL of a complete medium, and stimulated with 5 µg/mL of the S_{28-39} peptide. After growing for four days at 37 °C and 5% CO_2, half of the total medium was substituted and a new medium containing 20 IU/mL of IL-2 (CIGB, Cuba) was added. On day 7 the cells were collected and counted.

ELISPOT assay

Nitrocellulose bottom 96-well, MAHA S45 plates (Millipore, France) were coated with 100 µl of 5 µg/mL murine IFN-γ specific mAb R4-6A2 (Pharmingen, Becton Dickinson, England) overnight at 4 °C, washed three times with PBS and blocked using a complete medium at 37 °C for 1h. Two dilutions of freshly isolated ($2x10^5$ and $1x10^5$) or re-stimulated splenocytes (10^4 and $5x10^4$) and $1x10^5$ p815-pulsed with the peptide S_{28-39} were incubated 20 h at 37 °C in 5% CO_2. Splenocytes incubated with 2.5 µg/mL of concanavalin A (Sigma, USA) were used as positive controls. Every group was controlled by the same number of wells incubated with un-pulsed p815 cells as a negative control and the experimental controls of placebo mice.

After 20 h of incubation the plates were washed three times with PBS and five times with PBS-0.05% (v/v) Tween 20, then 0.5 µg/mL of anti-IFN-γ biotin conjugated (antibody XMG1.2, Pharmingen, Becton Dickinson, England) was added and reacted at room temperature for 2 h. Then the plates were washed five times with PBS- 0.05% (v/v) Tween 20, and peroxidase-labeled streptavidine (SIGMA, USA) was added at a 1:1000 dilution for 1 h. The wells were washed again with PBS-0.05% (v/v) Tween 20 and PBS and the spots were developed by adding 3,3´-diaminobenzidine (3, 3´, 4, 4´-tetraaminobiphenyl) tetrahydrochloride (Sigma, USA) in 50 mM Tris-HCl, pH 7.4 with 0.3% (v/v) H_2O_2. After 15 min, the wells were washed with tap water, dried and the spots counted under a dissection microscope (Zeiss, Germany). In our case the ELISPOTs were assayed under re-stimulation with the S_{28-39} peptide and using five individual samples per group.

Lymphoproliferation assays

Individual non-fractionated splenocyte suspensions were prepared for each mouse and incubated (10^5 cells/well) for 4 days at 5% CO_2 and 37 °C in the presence of rHBsAg expressed in *Picchia pastoris* (provided by HeberBiotec) (2.5 and 5 µg/mL). Cells incubated with concanavalin A (SIGMA, USA) were used as the positive controls and cells incubated with complete RPMI medium were employed as negative controls. All proliferation assays were performed in triplicate in 96-well plates and [^{3}H] thymidine (^{3}H-TdR; 0.5 µCi/well; specific activity, 2.0 Ci/mM; Amersham International, Buckinghamshire, UK) was added 12 h before harvesting. Results are expressed as the stimulation index (SI), which represents the ratio between the mean number of scintillations per minute (cpm) obtained in the presence and absence of the antigen. SI values ≥ 3 were regarded as positive.

Statistical procedures

The statistical treatment of titers was carried out using the F test to evaluate variance homogeneity followed by the Student test (t test) in the case of two group comparisons. For multiple group comparisons the results were analyzed using the GraphPad Prism version 4.00 program (GraphPad Software, USA), selecting One-way Anova and Newman Keuls test as parametric tests, or Kruskal Wallis and Dunns tests in non-parametric cases. The same procedure was used to analyze the humoral and cellular responses.

Results

The present study describes the evaluation of five commercial vaccines containing HBsAg produced in three different yeasts. It was designed as a comparative study of the humoral and cellular immune responses. The humoral response was evaluated measuring total IgG titers as well as the main subclasses. In the case of the cell-based immune response, the capacity of the different formulations to induce IFN-γ secreting CD8+ T cells was tested as well as lymphoproliferative responses, both in spleen cells.

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HBsAg-specific humoral immune response in sera

HBsAg-specific total IgG response was evaluated in sera after each dose. As described above, we used the rHBsAg expressed in *P. pastoris* (provided by HeberBiotec) for coating the plates. Ten days after the first dose we did not observe seroconversion in any tested serum; this behavior is typical of the HBsAg that need the T-cell cooperation for the production of a specific response in sera. The IgG response obtained after the third dose was high for all groups (geometric mean of the titer equal to or higher than 10^4) (Figure 1).

The anti-HBsAg IgG response elicited by both vaccines containing antigens expressed in *P. pastoris* (A and B) was similar in all evaluated points ($p>0.05$) and statistically superior to the rest of the vaccines assayed after the third dose -except between groups B and E, where the *P. pastoris* produced HBsAg formulation (Shanvac™-B) induced higher titers compared to that of *S. cerevisiae* (Engerix™-B), but this was not significant ($p>0.05$).

In general, the most marked differences in total IgG were obtained between *P. pastoris* rHBsAg-based formulations (A and B) and the formulation comprising the antigen produced in *H. polymorpha* (Hepavax-Gene) (D) ($p<0.001$). Euvax-B (C) and Engerix™-B (E) vaccines, containing *Saccharomyces*-derived antigens, showed an intermediate antibody level. However, Engerix™-B (E) induced a higher response compared to the Euvax-B vaccine (C) ($p < 0.01$).

Response of IgG subclasses

The specific IgG subclass response was evaluated after the third dose (Figure 2). The results of a within -group analysis showed that the serum IgG1 responses generated by all tested vaccines were higher than their respective IgG2a and IgG2b ($p \leq 0.001$). Between groups, both Heberbiovac-HB and Shanvac™-B vaccines (A and B, respectively) induced a higher IgG1 antibody response compared to the rest of the formulations, containing *S. cerevisiae* and *H. polymorpha* derived antigens ($p \leq 0.001$). A similar behavior was shown by the IgG2a and 2b subclasses, where groups C and D also induced lower responses ($p \leq 0.001$) compared to the rest of the vaccine formulations (Figure 2).

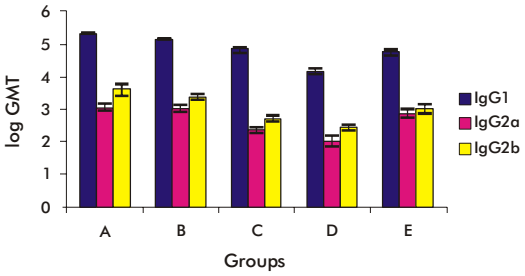

Figure 2. HBsAg-specific IgG subclass responses in sera after the third dose. Groups A to E were immunized with different vaccine formulations. Antigen produced in *Picchia pastoris:* Heberbiovac-HB (A) and Shanvac™-B (B), antigen produced in *Saccharomices cerevisiae:* Euvax-B (C) and Engerix™-B (E), and *Hansenula polimorpha* derived antigen: Hepavax-Gene® (D). (The error bars represent the interval of confidence in each case (95%). Log GMT: logarithm of the geometric mean of titer.

An intermediate level of titers was also obtained for the subclasses induced by *Saccharomyces* derived formulations, groups C and E, (Figura 2). However, similarly to the total IgG assay, Engerix™-B (E) induced a higher response compared to Euvax-B (C), the IgG2a levels were not statistically different compared to both *Picchia*-derived formulations but in the case of the IgG2b Heberbiovac-HB (A) it induced a greater response compared to Engerix™-B (E) ($p < 0.001$).

HBsAg-specific secretion of INF-γ measured by ELISPOT assays

The frequency of IFN-γ secreting cells was evaluated after the third dose by ELISPOT. The assay was carried out under re-stimulation conditions with the Balb/c immunodominant CTL peptide S_{28-39}. The

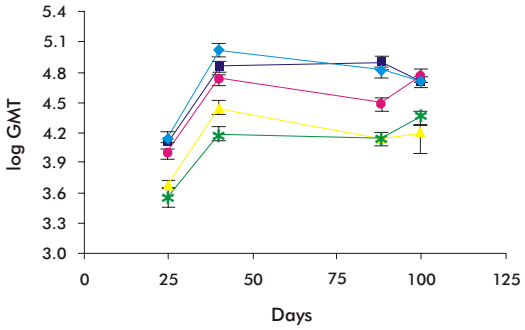

Figure 1. Kinetics of HBsAg-specific IgG response in sera. Groups A to E were immunized with different vaccine formulations. The antigen produced in *Picchia pastoris:* Heberbiovac-HB (A) and Shanvac™-B (B), the antigen produced in *S. cerevisiae:* Euvax-B (C) and Engerix™-B (E), and the antigen derived from *Hansenula polimorpha:* Hepavax-Gene® (D). (The error bars represent the confidence interval in each case (95%)). Log GMT: logarithm of the geometric mean of the titer.

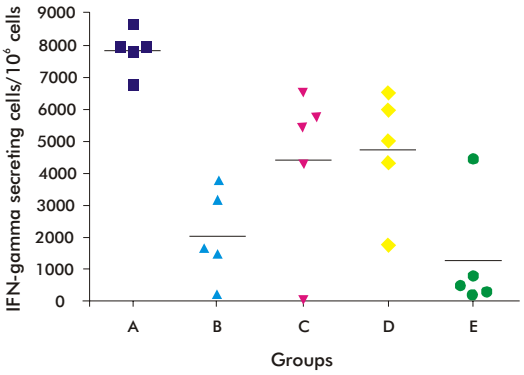

Figure 3. Frequency of IFN-g secreting cells measured by ELISPOT assay after the third dose. Groups A to E were immunized with different vaccine formulations. The antigen produced in *Picchia pastoris:* Heberbiovac-HB (A) and Shanvac™-B (B); the antigen produced in *Saccharomices cerevisiae:* Euvax-B (C) and Engerix™-B (E), and the *Hansenula polimorpha* derived antigen: Hepavax-Gene® (D). In this assay we used cells of each individual animal incubated with concanavalin A (ConA) as the positive control and cells incubated with an RPMI culture medium as the negative control.

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formulations studied elicited responses with different intensities (Figure 3). The Heberbiovac-HB vaccine (A) induced the highest frequency of IFN-g secreting cells. The response generated by Shanvac™-B (B) was significantly reduced ($p \leq 0.05$), as well as the response induced by Engerix™-B (E) ($p \leq 0.001$). Euvax-B and Hepavax-Gene vaccines (C and D, respectively) developed an intermediate level of response but did not significantly differ from the rest.

HBsAg-specific lymphoproliferative response in spleen cells

We measured the lymphoproliferative response after the third dose using five individual samples per group and employing for the immune stimulations 2.5 µg/mL of the rHBsAg expressed in *P. pastoris*. As shown in figure 4, under our conditions, positive proliferative responses were detected only for the formulations containing antigens expressed in *S. cerevisiae* (E) (mean SI = 3.46) and *P. pastoris* (A) with a mean of SI = 7.36. We were not able to find statistical differences between them.

Discussion

Several therapeutic clinical trials using HBsAg-based vaccines have been carried out with modest results [7, 9, 11], suggesting that more potent formulations and different therapeutic strategies should be developed.

In this study we evaluated the immunogenicity of five commercial vaccines based on rHBsAg produced in three different hosts. In line with the role of cellular immune responses on HBV clearance, the present study examines cell-based immune responses in addition to the evaluation of serum IgG levels and their respective subclasses.

All vaccines evaluated in this study developed strong antibody responses, which are expected as they have proven their efficacy in prophylactic campaigns around the world [25-28]. However, we have detected differences in humoral responses among the vaccine formulations tested. The Heberbiovac-HB and Shanvac™-B vaccines, containing *P. pastoris* derived antigens, induced similar levels of humoral responses both at total IgG as well as in their respective subclasses. Engerix™-B and Euvax-B, both *Saccharomyces*-derived formulations, developed intermediate levels in titers and the Hepavax-GeneÒ, *Hansenula*-derived formulation induced the lowest responses. This pattern of anti-HBsAg IgG response was consistent when we used an rHBsAg produced in hamster ovary cells for coating the ELISA plates.

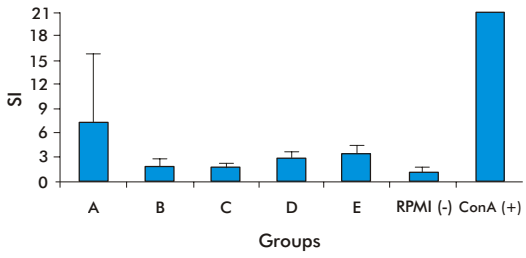

Figure 4. Lymphoproliferation assay after the third dose using five individual animals per group and 2.5 µg/ml of the rHBsAg expressed in *Picchia pastoris* (HeberBiotec) for the immune stimulation. Groups A to E were immunized with different vaccine formulations. The antigen produced in *P. pastoris:* Heberbiovac-HB (A) and Shanvac™-B (B); the antigen produced in *S. cerevisiae:* Euvax-B (C) and Engerix™-B (E), and the *Hansenula polimorpha* derived antigen: Hepavax-Gene® (D). (The error bars represent the standard deviation). In this assay we used cells of each individual animal incubated with concanavalin A (ConA) as the positive control and cells incubated with the RPMI culture medium as the negative control.

From this result it is interesting to note that in the present study that the humoral immunogenicity depended on the yeast specie expressing the antigen. This could be related to the potential differences in the glycolipid pattern produced by the host. It is reported that the rHBsAg expressed in yeast contains only the non-glycosylated S protein [29], although differences in lipid composition have been previously described [30].

It is known that various physical and/or nutritional factors, such as growth rate, culture medium, respiratory quotient, pH, temperature, aeration, minerals and nitrogen source, have been shown to greatly influence recombinant protein expression [31] and lipid composition [32]. Differences in the lipid content between *Saccharomyces* and *Hansenula*-expressed antigens have been reported [30].

At present we know that the rHBsAg from the Heberbiovac-HB vaccine (*P. pastoris-expressed*) has phosphatidylserine as one of its structural lipids, thus differing from the rHBsAg of Engerix™-B (*Saccharomyces*-expressed) [33]. Further ongoing experiments explore the influence of the difference in lipid composition on the immune response to the rHBsAg.

Our results are in line with previous reports where *P. pastoris* derived vaccine was assayed in two comparative clinical trials using a *Saccharomyces* expressed antigen formulation as a control [27, 34]. Additionally, it has been shown that a *P. pastoris* derived antigen is immunogenic through the nasal route when administered in PBS. Conversely, in previous reports, mice given a nasal administration of rHBsAg from other sources under similar conditions failed to induce a detectable antibody response, showing a very low immunogenicity under such conditions [35, 36].

Although the concept of cytokine mediated HBV clearance has been widely accepted [37, 38], the majority of the reports using commercial hepatitis B vaccines in a prophylactic scenario have focused on the antibody response and not the induced cellular immune response. In this study we explored the cellular immune response using the ELISPOT assay which is able to measure the frequency of IFN-γ secreting CD8+ T cells specific for the immunodominant epitope S_{28-39}. ELISPOT assays showing that the formulations assessed elicited different frequencies of IFN-γ secreting CD8+ T cells. In this case, the most marked differences obtained were between the Heberbiovac-HB and Engerix™-B vaccines. In line with the results obtained from antibody evaluations, not all vaccine formulations induced similar immune responses at the cellular compartment. It was surprising to note that the other antigen produced in *Picchia* (Shanvac™-B vaccine) was not able to induce a similarly potent response, suggesting that specific conditions in the production and purification of the antigen could also

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36. Debin A, Kravtzoff R, Santiago JV, Cazales L, Sperandio S, Melber K, *et al*. Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. Vaccine 2002;20(21-22):2752-63.

37. Bertoletti A and Gehring AJ. The immune response during hepatitis B virus infection. J Gen Virol 2006;87(Pt 6):1439-49.

38. Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci USA 2004;101(17):6669-74.

influence the resulting CD8+ T cell immunogenicity. Also interesting was the result of the low response of the Engerix™-B vaccine, compared to the Euvax-B (both based on *Saccharomyces*-expressed rHBsAg) and the Hepavax-Gene® vaccines, showing the lowest immunogenicity in terms of humoral response.

Another group of results come from experiments comparing the frequency of IFN-γ secreting CD8+ T cells elicited by different batches of Heberbiovac-HB and Engerix™-B vaccines after a single dose of the vaccine and employing the same ELISPOT assay (data no shown). This set of results correlates with those reported here; we observed a statistically superior response for certain Heberbiovac-HB batches compared to some specific Engerix™-B batches, mainly based on the reduced immunogenicity of certain Engerix-B batches. A series of ten consecutive batches of Heberbiovac-HB did not differ from a control batch used for all ELISPOT assays.

For a more complete characterization of cellular immune responses, we studied the proliferative capacity of spleen cells from immunized mice. The [^{3}H] thymidine lymphoproliferation technique evidenced positive results only in the case of two vaccines, Heberbiovac-HB and Engerix™-B. The SI of Heberbiovac-HB was twice that of Engerix™-B and the rest of the evaluated vaccines did not respond to the stimulus in our experimental conditions. The poor results could be a consequence of the low immunization doses used to compare the formulations, selected to avoid immune response saturation and to optimize CTL responses. More sensitive proliferation techniques could also detect differences among the rest of the group. However, this assay is further proof of the differential behavior of vaccine formulations in their ability to elicit cellular immune responses.

The differences in the immune response between Heberbiovac-HB and Engerix™-B vaccines may be explained on the basis of the differential presence of PS in the rHBsAg from Heberbiovac-HB, as mentioned above. It has been extensively reported that PS plays a key role in the recognition and engulfment of apoptotic cells by phagocytes [39, 40]. Considering together the presence of PS and the evidence of the aggregation obtained for this rHBsAg, we suggest that this antigen could mimic the interactions between macrophages and apoptotic cells through the PS and PS-receptors, followed by a more efficient way of processing and presentation. Further data that reinforce this hypothesis is the demonstration, by Hoffmann PR *et al.* [40], that the ligation of many receptors, including CD14, CD68, CD36, and a_vb_3 integrin, resulted in particles binding to macrophages. However, bound particles were not ingested unless PS was present [40].

In line with our suggestion Vanlandschoot *et al.* [29, 41] demonstrated that the rHBsAg expressed in yeast bind to the cell surface of monocytes through an interaction with the lipopolysaccharide binding protein and the lipopolysaccharide receptor, CD14. This attachment is suggested to depend on the presence of charged phospholipids in the particles. This study was limited to the use of three different lots of rHBsAg produced in *S. cerevisiae*, obtained from Glaxo SmithKline. Nevertheless, clear differences in binding to CD14, immune suppression, and T-cell immunogenicity were observed among the evaluated preparations. Curiously, phosphatidylinositol is the only charged phospholipid found in the HBsAg expressed in *Saccharomyces*-expressed HBsAg differing from that expressed in *P. pastoris* and *H. polymorpha*-expressed which also contains phosphatidylserine [30, 42]. We have preliminary data showing that the rHBsAg from *P. pastoris* (present in the Heberbiovac-HB vaccine) has a reduced binding capacity to CD14 compared with the rHBsAg from *S. cerevisiae* (present in the Engerix-B vaccine, provided by Glaxo SmithKline) [33]. The observation that reduced binding could be correlated to a reduced inhibition of the LPS-induced activation of monocytes raised the idea that the immune-suppressive attachment to monocytes might influence HBsAg-specific T-cell proliferation. If this assumption is correct, an enhanced proliferation may be predicted when using the low binding rHBsAg preparations. This suggestion is in line with the results published by Vanlandschoot and colleagues [29] and also with our results.

Another possible explanation for the differential immunogenicity found in the rHBsAg expressed in *P. pastoris* used in the Heberbiovac-HB vaccine could be related to the presence of a significant amount of an aggregated fraction in the final preparations [43]. This idea agrees with reports suggesting that the full immunogenicity of HBsAg is highly dependent on its tertiary and possibly quaternary structure [42]. We hypothesized that the differences in size among the 22 nm native antigens and the aggregates would probably involve a more efficient uptake and antigen processing, resulting in a better immunogenicity. We have previously reported that preparations containing the aggregated fraction obtained by chemical treatment induce more potent immune responses in mice [33].

An electron microscopy characterization of rHBsAg from different sources is required to validate this hypothesis. However, some reports in this field offer interesting data. Diminsky D *et al.* [44] showed by electron microscopy that HBsAg expressed in a *Hansenula*-expressed HBsAg presented the following distribution in particle size, 89% of the particles were 30 ± 8 nm, while 11% of the particles were aggregated to 208 ± 79 nm, differing from HBsAg expressed in CHO cells-expressed HBsAg, whose particle population has a homogeneous size of 32.1 ± 10 nm. Both rHBsAg in this study are very similar in their size, shape, gross lipid composition, biodistribution in lymph nodes and their humoral response. The main difference found between these rHBsAg was in their ability to stimulate CTL response. They suggest that the difference found in the uptake of the particles by the target cells, which was 4.5-fold higher for the *Hansenula*-derived antigen may explain the difference in the CTL response. Although Diminsky D *et al.* did not explain the differences in the cellular immune response by the aggregation of rHBsAg, their results are in line with our hypothesis.

We ignore if the rHBsAg derived from *H. polymorpha* derived rHBsAg employed in our study has a similar particle size distribution to that referred above, considering that both come from different providers. However, if we analyze the IFN-γ secretion response elicited by this antigen in the present study,

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43. Tleugabulova D, Falcon V, Sewer M, Penton E. Aggregation of recombinant hepatitis B surface antigen in *Picchia pastoris*. J Chromatogr B 1998;716:209-219.

44. Diminsky D, Schirmbeck R, Reimann J, Barenholz Y. Comparison between hepatitis B surface antigen (HBsAg) particles derived from mammalian cells (CHO) and yeast cells (*Hansenula polymorpha*): composition, structure and immunogenicity. Vaccine 1997;15(6-7):637-647.

is interesting that, in contrast to the pattern observed for the antibody response, the cellular immune response for this antigen tended to be better compared to the Engerix^{TM}-B vaccine.

It is now known that the inclusion of new antigens such as hepatitis B nucleocapsid or the use of more potent adjuvants are crucial in the success of HBV therapeutic candidates, however, the inclusion of the surface antigen in the formulations is still considered to be important. Therefore, the selection of the most immunogenic rHBsAg has implications in the development of new therapeutic vaccine candidates.

Conclusions

Our results indicate that all formulations evaluated induce strong humoral responses. However, significant differences can be found in their capacity to induce humoral and cellular responses, which is a key issue in the use of these rHBsAg in a therapeutic setting.

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Statement of Interests

All the authors are employees of the CIGB who own a patent (European Patent Publication No. WO 2005/037311) that claimed a group of results included in this paper.

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