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

Introduction

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 practical applications. Vaccine 2001;19:1837-48.
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 scenario 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 ~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].

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**Materials and methods**

**Vaccine formulations**

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**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 retroorbital 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 HBsAg expressed in *P. pastoris* (provided by HeberBiotec) 5 μg/mL in coating buffer (11 mM NaCO3, 35 mM NaHCO3, 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 Na2HPO4, 1 mM KH2PO4, 2% (w/v) skim milk in phosphate saline buffer (0.1 M NaCl, 2 mM KCl, 10 mM Na2HPO4, 1 mM KH2PO4, 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 NaHPO4, 25 mM citrate, 1 mg/mL OPD, 0.1% (v/v) H2O2) for 15 min at room temperature. Washes with 0.05% (v/v) Tween 20 in PBS 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 H2SO4 solution. Finally the plates were read to 492 nm in a microtiter plate reader (Sensdient Scan, Merck).

The IgG subclass evaluations were done by a similar ELISA assay, using the ISO-2 Mouse Monoclonal Antibody Isotyping Reagents kit and
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) NHCl. 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 pyruvate, 50 mM 2-mercaptoethanol and antibiotics (complete medium) and counted. Meanwhile, H-2* mastocytome cells p815 were pulsed for 1 h at 37 °C, 5% CO₂ in 5% CO₂ in 95% air (Gibco, USA), 2 mM glutamine, 2 mM piruvate, 50 mM 2-mercaptoethanol and antibiotics (complete medium) with 2.5 μg/mL of concanavalin A (SIGMA, USA), were used as positive controls. 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 cm² flasks (Becton Dickinson, England) at 2 x 10⁶ cells per milliliter in 10 mL of a complete medium, and stimulated with 5 μg/mL of the S28-39 peptide. After growing for four days at 37 °C and 5% CO₂, 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 1 h. Two dilutions of freshly isolated (2 x 10⁶ and 1 x 10⁶) or re-stimulated splenocytes (10⁵ and 5 x 10⁴) and 1 x 10⁴ p815 pulsed with the peptide S28-39 were incubated 20 h at 37 °C in 5% CO₂. Splenocytes incubated with 2.5 μg/mL of concanavalin A (SIGMA, USA) were used as positive controls. Each 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 XM1G1.2, 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 streptavidin (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′, 4,4′-tetramethylbenzidine (SIGMA, USA) in 50 mM Tris-HCl, pH 7.4 with 0.3% (v/v) H₂O₂. 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 ELISPOTrs were assayed under re-stimulation with the S28-39 peptide and using five individual samples per group.

**Lymphoproliferation assays**

Individual non-fractonated splenocyte suspensions were prepared for each mouse and incubated (10⁶ cells/well) for 4 days at 5% CO₂ and 37 °C in the presence of HBsAg expressed in *Pichia 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 [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.

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 different formulations to induce IFN-γ response. The vaccines were compared with the recombinant hepatitis B vaccine (Shanvac-B) in comparison with commercially available vaccine. Indian J Gastroenterol 2000;19(2):71-3. 28. ul-Haq N, Hasnain SS, Umar M, Abbas S, Nagajaran Kumar VR. Safety and immunogenicity of indigenous recombinant hepatitis B vaccine (Shanvac-B) in comparison with commercially available vaccine. Indian J Gastroenterol 2000;19(2):71-3.
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 10^3) (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 Saccharomices-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<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<0.001).

A similar behavior was shown by the IgG2a and 2b subclasses, where groups C and D also induced lower responses (p<0.001) compared to the rest of the vaccine formulations (Figure 2).

An intermediate level of titers was also obtained for the subclasses induced by Saccharomices-derived formulations, groups C and E (Figure 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 S28-39. The anti-HBsAg IgG response elicited by both vaccines containing antigens expressed in *P. pastoris* (A and B) and the antigen produced in *S. cerevisiae*: Euvax-B (C) and Engerix™-B (E), and the antigen derived from Hansenula polymorpha: Hepavax-Gene® (D). (The error bars represent the confidence interval in each case (95%). Log GMT: logarithm of the geometric mean of the titer.)
formulations studied elicited responses with different intensities (Figure 3). The Heberbiovac-HB vaccine (A) induced the highest frequency of IFN-γ secreting cells. The response generated by Shanvac™-B (B) was significantly reduced (p < 0.05), as well as the response induced by Engerix™-B (E) (p < 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 stimuli 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-GeneO, 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.

From this result it is interesting to note that in the present study that the humoral immunogenicity depended on the yeast species 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 S28-39. 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 Pichia (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

![Figure 4. Lymphoproliferation assay after the third dose using five individual animals per group and 2.5 µg/mL of the rHBsAg expressed in Pichia pastoris (HeberBiotec) for the immune stimulation. Groups A to E were immunized with different vaccine formulations. The Heberbiovac-HB (A) and Shanvac™-B (B); the antigen produced in S. cerevisiae: Euvax-B (C) and Engerix™-B (E), and the Hansenula polymorpha 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.](image-url)
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 not 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 [3H] 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 aβ 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 size 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,
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™-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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