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Study of Different Coupling Agents in the Conjugation of a V3-Based Synthetic MAP to Carrier Proteins

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Abstract: The conjugation of synthetic peptides to carrier proteins is a widely used method for immunological studies. Different coupling agents have been described to form the conjugate with carrier proteins. In this paper, we demonstrate that the antibody response toward V3-based synthetic MAPs derived from HIV-1, JY1 isolate, conjugated to two different carrier proteins using either *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) or *β*-maleimidopropionic acid *N*-hydroxysuccinimide ester (MPS), or succinic anhydride (SA) show different behaviors. An excellent anti-JY1 response without a strong response to the coupling agent is observed in the case of succinic anhydride spacer. In contrast, MBS produces total abrogation of the antibody response with a high response toward the coupling agent.

Keywords: anti-peptide antibody; *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; *β*-maleimidopropionic acid *N*-hydroxysuccinimide ester; peptide conjugate; succinic anhydride

INTRODUCTION

Synthetic peptides have been widely used for generation of anti-peptide antibodies (Abs) with predetermined specificity. Because of their size, peptides may not be immunogenic on their own. They must be coupled to carrier proteins that provide the required T-cell help to elicit specific Abs. Another strategy to enhance the immunogenicity of peptides is the use of a multiple antigen peptide (MAP). It is possible to generate higher epitopic antigenic densities coupling MAPs to carrier proteins. This approach, at the same time, could be the base of combined vaccines. Several cross-linking agents are currently used to conjugate peptides to proteins: glutaraldehyde [1,2], *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) [3], carbodiimides [4,5], *β*-maleimidopropionic acid *N*-hydroxysuccinimide ester (MPS) [6], succinic anhydride (SA) [7] and others. During immunization, antibodies against the carrier proteins and/or the coupling agent might be elicited. Ideally, a good coupling agent should not generate any Ab response against itself.

Both MBS and MPS are heterobifunctional agents that allow the linkage of a cysteine thiol group of the peptide with an amino group of the carrier protein. Different strategies have been used for MBS- and MPS-mediated conjugation. Most employ a two-step procedure: peptide or carrier activation, followed by elimination of non-reacted MBS [8]. Occasionally, owing to unclear immunological reasons, a given conjugation scheme may be successful at producing a peptide-carrier conjugate, but unsuccessful at generating antibodies [9]. Also, the conjugate may

Abbreviations: Boc, *t*-butyloxycarbonyl; BSA, bovine serum albuminin; DIPC, 1,3-diisopropylcarbodiimide; DMF, *N,N,*-dimethylformamide; DCM, dichloromethane; DIPEA, diisopropylethylamine; EDAC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole; HF, hydrogen fluoride; MAP, multiple antigen peptide; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; MPS, *β*-maleimidopropionic acid *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate.

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produce reactive Abs against the peptide, but that are not cross-reactive with the parent protein from which the peptide sequence was derived.

The V3 loop is the primary target for neutralizing antibodies in HIV-1 infection [10,11], and is involved in many aspects of virus infectivity. Sequence changes in V3 can affect chemokine receptor usage and, therefore, modulate which types of cells are infected [12]. This region comprises helper T-cell and CTL epitopes [13]. Accordingly, the V3 loop has been included in many HIV-1 vaccine candidates [14]. To develop a peptide-based vaccine for HIV infection, a V3-peptide from HIV-1 MN isolate has been conjugated to proteins using glutaraldehyde [15].

Different attempts have successfully demonstrated the feasibility of using recombinant cDNA clones expressing V3-specific sequences [16,17]. In comparison, the conjugation approach permits the coupling of different V3 sequences to a single carrier protein at higher epitopic density. Furthermore, relevant carrier epitopes may be preserved, thus providing a strategy to obtain combined vaccines. A further consideration that V3-based vaccines must contain a cocktail of sequences in order to overcome virus variability [18]. A vaccine that comprises a set of specific V3 peptides could be used in therapy, with the hope of slowing or preventing the appearance of SI variants. Based on the fact that distinct V3 sequence subtypes have been reported for different geographic areas, conjugated vaccines offer a very dynamic approach. From this point of view, it is very important to study the quality of the immunological response using different coupling agents.

If a V3 synthetic peptide is coupled to a carrier protein, one should expect an Ab response against it to appear. In this study, the Ab response against a V3 epitope from a HIV-1, JY1 isolate, presented both as a single peptide and as a MAP, showed different behavior, depending on the coupling agent. Antibody response was abrogated when MBS was employed as a coupling reagent on two carrier proteins: P64k, a recombinant protein derived from *Neisseria meningitidis* [19] and HBsAg, the recombinant surface antigen from the hepatitis B virus. In contrast, a strong humoral response was obtained when either MPS or succinic anhydride were used as coupling agents.

MATERIALS AND METHODS

Materials

P64k and HBsAg were obtained from Vaccine Division, CIGB (Havana, Cuba). BSA, MBS, MPS and succinic anhydride were obtained from Sigma Chemical (St Louis, MO, USA). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

Synthesis of Peptide and MAP

The peptide sequence (RQSTPIGLGQALYTT) belongs to the central part of the V3 loop (residues 317-331) from the gp120 envelope protein of the HIV-1, JY1 isolate [20]. The JY1-based peptide and tetrameric MAP (JY1-MAP4) were synthesized with an additional cysteine residue at the *C*-terminus of the sequence, to allow coupling to carrier proteins. The syntheses were accomplished manually by stepwise solid-phase procedure using *tert*-butoxycarbonyl/benzyl chemistry [21]. The lysine core was assembled using Boc-Lys(Boc) via 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TBTU) activation. Cleavage of the Boc group was carried out with 37.5% TFA in DCM. The TFA salt was neutralized by 5% DIPEA in DCM. The coupling reactions were mediated with 1,3-diisopropylcarbodiimide (DIPC)/HOBT in DMF, and monitored by the qualitative ninhydrin test. After the last coupling cycle, each MAP and peptide-resin system was cleaved with anhydrous hydrogen fluoride (HF) and the corresponding mixture. The JY1-peptide was extracted with 30% HAc in water and lyophilized. The JY1-MAP4 was extracted into 8 M urea and 100 mM Tris–HCl buffer (pH 8.0). Next, it was dialyzed in 2 M urea and 100 mM Tris–HCl buffer, pH 8.0, for 24 h. Finally, the MAP was dialyzed in 1 M HAc, to remove all the urea, and was then lyophilized.

Chromatography

The JY1-peptide and JY1-MAP4 were analyzed by reversed-phase, high-performance liquid chromatography (RP-HPLC) (Pharmacia-LKB), using an analytical RP-C18 column (4.6 × 100 mm, Vydac). A linear acetonitrile/water gradient from 5 to 60% of acetonitrile over 40 min and a flow rate of 0.8 mL/min were used. The absorbance was monitored at 226 nm. The main fractions from each profile were characterized by amino acid analysis.

Amino Acid Analysis

Samples of peptide and MAP were hydrolyzed for 24 h in vacuum-sealed ampoules, with 6 mol/L HCl containing 0.1% phenol and 0.1% 2-mercaptoethanol. After evaporation, free amino acids were dissolved in the adequate buffer and loaded onto an

automatic amino-acid analyzer Alpha Plus 4151 (Pharmacia-LKB, Sweden), using a sodium buffer system for amino-acid separation and ortho-phthalaldehyde derivatization for fluorescence detection. All samples were analyzed in three replicates.

Preparation of MPS and MBS Conjugates

The coupling reaction was done according to Van Denderen [22]. Briefly, 5 mg of P64k or HBsAg were dissolved in 1 mL of 0.01 M phosphate buffer (pH 6.0), and then activated with 50 μL of MBS or MPS solution (10 mg/mL in DMF) for 30 min at room temperature. The mixture was dialyzed against phosphate buffer at saline (PBS), pH 7.5. 5 mg of JY1-peptide or JY1-MAP4 were dissolved in 3 M guanidine hydrochloride in PBS at 5 mg/mL. The MPS or MBS-proteins were mixed with the JY1-peptide or JY1-MAP solution for 3 h at room temperature with gentle stirring.

Preparation of Succinic Anhydride Conjugate

The modified method described by Deen [23] was used. Briefly, 5 mg of HBsAg was dissolved in 1 mL of 0.2 M K_2HPO_4, pH 8.0. Solid succinic anhydride (1 mg) was added, and the solution was stirred until all anhydride was dissolved. The pH was kept at 8–8.5 using 3.0 M NaOH. The mixture was dialyzed against water at pH 4–5. Then, 7 mg of 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDAC) was added to the activated protein and stirred for 10 min at 4°C. Further, 5 mg of the JY1-MAP8 was dissolved in 3 M guanidine hydrochloride in PBS at 5 mg/mL. The succinic-protein and JY1-MAP8 solutions were mixed for 3 h at room temperature with gentle stirring.

The conjugates were purified by gel filtration through Sephadex CL-4B (Pharmacia, Sweden) equilibrated in PBS. The concentration of the conjugates was determined using the Coomassie reagent method [24].

ELISA for the Quality Control of Conjugates

Plates were coated with 1.25 μg/mL of the conjugates overnight at 4°C in PBS. They were analyzed as in indirect ELISA, except that, after blocking, the plates were incubated with anti-JY1 mAb at 5.0 μg/mL at 37°C for 3 h. The anti-JY1 monoclonal antibody 332 was kindly provided by Dr E Carpio.

Immunizations

Balb/c mice (10 female, 4–6 weeks old in each group) were immunized with different antigens and several doses. A summary is presented in Table 1. Mice in the first schedule were immunized using complete Freund's adjuvant (1:1) on day 0 and incomplete Freund's adjuvant (Sigma, St Louis, MO, USA) on days 14, 28 and 56. They were bled on day 66. In the case of HBsAg, animals were immunized three times on days 0, 14, 28 and bled on day 38.

A second similar schedule was performed in parallel, with the only difference being that a fifth dose was given 15 days after the fourth, and a last bleeding performed 2 weeks later.

Indirect and Competitions ELISAs

Indirect ELISAs were done by procedures already published [25]. Briefly, plates were coated with either BSA-JY1, BSA-Prt (non-related protease peptide from HIV-1), JY1 peptide, HBsAg, BSA-MPS,

Table 1 Summary of the Immunization Schedules

Schedule	Groups	Coupling agents	Adjuvant	Doses (μg)	Via
1	P64k-JY1	MBS	CFA-IFA	5	s.c.
	P64k-JY1 MAP4	MBS	CFA-IFA	5	s.c.
	P64k	–	CFA-IFA	5	s.c.
	JY1-MAP8	–	CFA-IFA	20	s.c.
	HBsAg-JY1 MAP4	MBS	Alum	10	i.p.
2	JY1-MAP8	–	CFA-IFA	50	i.p.
	HBsAg-JY1 MAP4	MPS	CFA-IFA	10	i.p.
	HBsAg-JY1 MAP8	SA	CFA-IFA	10	i.p.

s.c.—subcutaneous, i.p.—intraperitoneal.

BSA-succinic anhydride or TAB9 protein a recombinant quimeric protein bearing six V3 gp120 loops from HIV-1, peptide JY1 being one of them [26] at 4 μg/mL and MBS at 100 μg/mL over night a 4°C in PBS. After one wash with 0.05% Tween 20 in distilled water, plates were blocked with 0.5% BSA in PBS (blocking solution) for 1 h at 37°C. Mice sera from each group were pooled, and Abs were detected beginning at 1/100 dilution. After 2 h at 37°C, the plates were washed and incubated, or an additional 1 h with anti-mouse IgG conjugated to horseradish peroxidase. A binding reaction was visualized by the addition of *o*-phenylenediamine (0.05%) and H_2O_2 (0.05%). The reaction was stopped using 3 M H_2SO_4 after 10 min, and the plaques were read at 492 nm.

The Prt peptide conjugated to BSA using MBS as coupling agent was provided by Carmen E. Gómez (CIGB, Havana, Cuba).

The competition ELISAs were made as with the indirect ELISA, but sera were used at dilutions that reported an OD value between 0.5 and 1.5. Different concentrations of peptides and MBS were incubated with the sera in solution.

Statistical Analysis

All ELISA OD values were transformed to the natural logarithm to get a normal distribution in the immunized groups. An *F*-test was performed to assess variance homogeneity between groups and a Student's *t*-test was done ($p < 0.05$ was considered statistically different).

RESULTS

The V3 Amino Acid Sequence was Properly Preserved in all the Conjugates and the MAP

All the conjugates were evaluated in an indirect ELISA using the anti-JY1 mAb and their OD values at 1.25 μg/mL of coating were measured. In all cases, the JY1 epitope was properly recognized by the mAb (Figure 1).

The Anti-V3 Antibody Response was Abrogated by MBS

The humoral response against the VP3 epitope in mice immunized with four doses of the different immunogens was evaluated in a direct ELISA using as plate antigen a BSA-JY1 conjugate prepared by the MBS method. Unexpectedly, no seroconversion

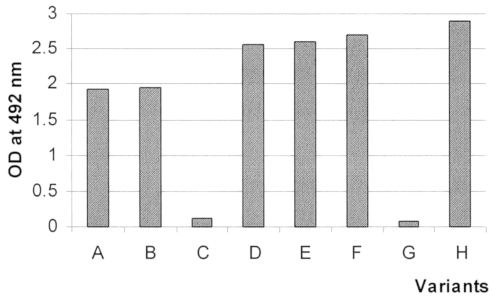

Figure 1 Study for the quality control of conjugates. An indirect ELISA was performed using a mAb. For details, see text. (A) P64k-JY1 peptide, (B) P64k-JY1 MAP4, (C) P64k, (D) HBsAg-JY1 MAP4 (MBS), (E) HBsAg-JY1 MAP4 (MPS), (F) HBsAg-JY1 MAP8 (succinic anhydride), (G) HBsAg, (H) JY1-MAP8.

directed to the V3 epitope was observed for MBS-based conjugates P64k-JY1 and P64k-JY1 MAP4 at serum dilutions 1:100 or lower. Also, antisera from mice immunized with P64k-JY1 (MBS) conjugate showed similar titers for BSA-JY1 than for BSA-Prt, a conjugate of an irrelevant peptide also prepared via MBS (Figure 2). Further, free JY1 peptide did not compete with the antisera on plates coated with BSA-JY1 over a large concentration range (data not shown). These results suggested that the antibodies might be recognizing either the carrier protein or the maleimidobenzoyl spacer. The first possibility was discarded in a control assay using BSA-coated plates. To confirm the presence of an anti-MBS response, we performed a competition experiment which showed that MBS effectively displaced the

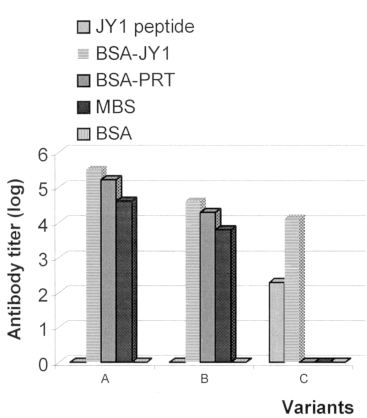

Figure 2 Analysis of the Ab response titers of pooled sera from mice immunized with P64k-JY1 MAP4 or P64k-JY1 peptide conjugates. Plates were coated with JY1 peptide, BSA-JY1, BSA-PRT, MBS and BSA as referred in the text. (A) P64k-JY1 MAP4, (B) P64k-JY1 peptide, (C) JY1-MAP8.

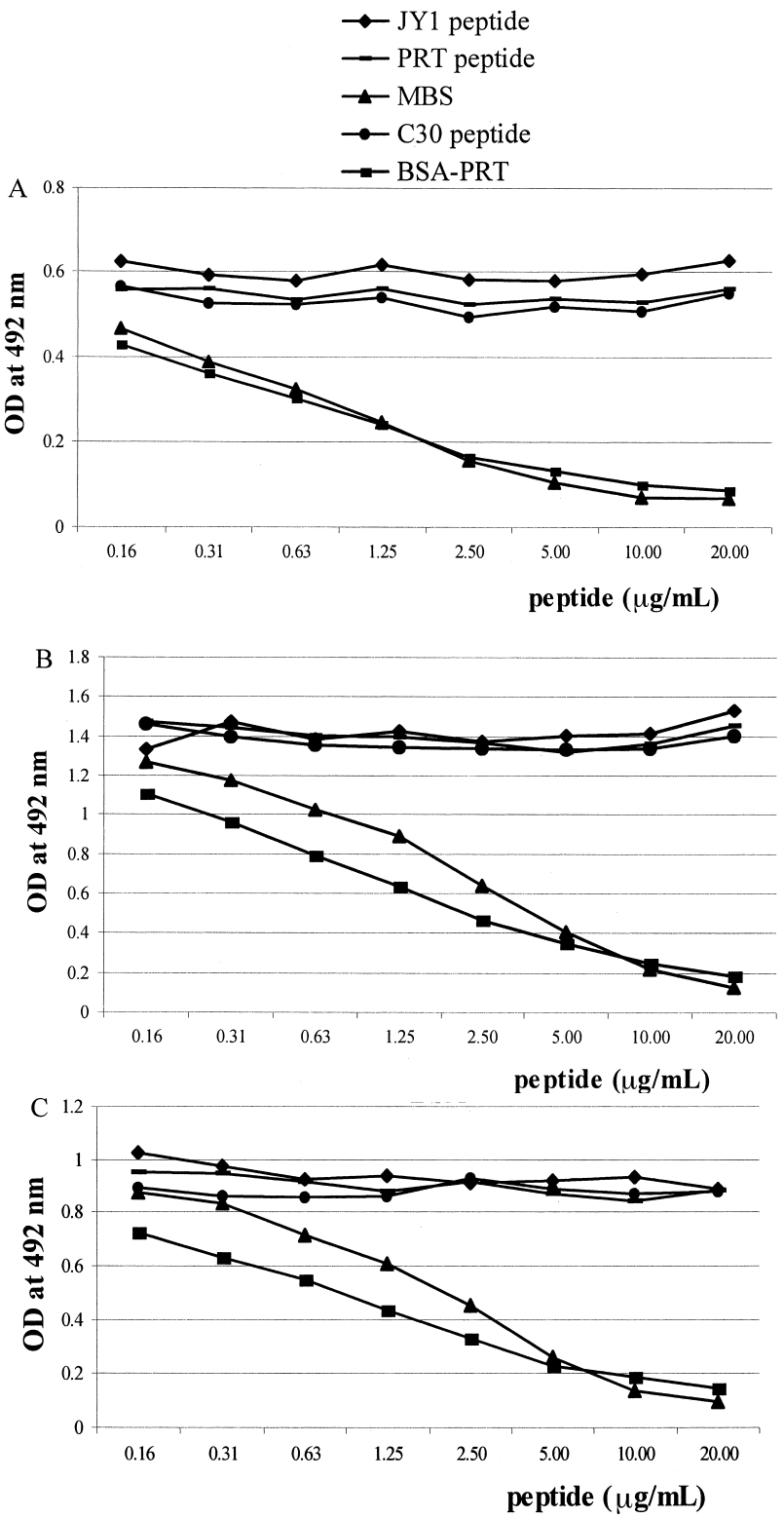

Figure 3 Competition ELISAs. (A) Pooled sera from mice immunized with P64k-JY1 MAP4, (B) from mice immunized with P64k-JY1 peptide, and (C) mice immunized with HBsAg-JY1 MAP4.

antisera from plates coated with MBS-based conjugates such as BSA-Prt or BSA-JY1, with inhibition curves similar to the positive controls (Figure 3(A) and (B)). Similar behavior was observed for antisera from mice immunized with HBsAg-JY1 MAP4 (Figure 3(C)). Taken together, these findings demonstrate that the observed antibody response was directed to MBS, not to the JY1 peptide.

Antibody Responses Elicited using MPS and Succinic Anhydride as Coupling Agents

After three doses, both V3 conjugates generated higher titers against the JY1 epitope in most of the mice, with 100% seroconversion. In particular, for the succinic anhydride conjugates, titers were higher than 10^{-4} in all mice. There were statistically significant differences between the geometric means of both groups ($p < 0.01$). There was no difference in anti-JY1 response between the JY1-MAP8 and the succinic anhydride conjugate groups. However, the humoral response against the JY1-MAP8 compared with the MPS groups was stadistically higher ($p < 0.05$) (Table 2).

The pooled sera from mice immunized with HBsAg-MAP4 (MPS) and HBsAg-MAP8 (SA) were titered in an indirect ELISA using plates coated with BSA-MPS or BSA-SA conjugates. It was found that sera from the group of mice immunized with the succinic anhydride conjugate gave a positive signal even at 1:5000 dilution. On the contrary, for mice immunized with the MPS conjugate, no positive signal was found at 1:20 dilution or below.

After four doses, there was no statistical difference in anti-JY1 response between groups immunized with JY1-MAP8 and MPS-conjugate. In contrast, there were significant differences between the succinic anhydride-conjugate group and the two former groups ($p < 0.05$) (Table 2).

Table 2 ELISA Anti-JY1 IgG Titers

Animals immunized with	GM ± S.D. after doses[a]	
	Three	Four
JY1-MAP8	4.3 ± 0.5	4.7 ± 0.3
HBsAg-JY1 MAP4 (MPS)	3.9 ± 0.5	4.4 ± 0.3
HbsAg-JY1 MAP8 (SA)	4.7 ± 0.4	5.1 ± 0.2

GM ± S.D.: geometric mean ± standard deviation.
[a] GM are expressed as log of the titers.

DISCUSSION

The similar results obtained with P64k-JY1 peptide and P64k-JY1 MAP4 immunization groups lead us to conclude that MBS abrogates the Ab response against the V3 peptide coupled to carrier protein P64k. The MAP molecules were immunogenic on its own, and generated a good anti-V3 JY1 Ab response in Balb/c mice after three doses. However, it seems difficult that the carrier protein mediates the phenomenon, because similar results were obtained when the JY1-MAP4 was coupled via MBS spacer to the HBsAg carrier.

The antibody response generated against MBS is not directed to a new epitope generated after the coupling, because the binding of these antibodies was abrogated by intact MBS. Conflicting results have been published about the use of MBS as a coupling agent for immunizations. It has been reported that high antibody titers are generated against the MBS spacer when coupling a peptide to Tetanus toxoid [27]. Nevertheless, the anti-peptide response was not completely abolished, and the influence of the carrier protein was not assessed. In contrast, good anti-peptide response has been generated to a peptide coupled to KLH by MBS [8]. Likewise, a similar conjugate using a peptide from a human mucin induced a high titer of antibodies and protection in a mouse model for cancer [28].

Our results indicate that MBS abolishes the Ab response against the JY1 V3 peptide, even as a MAP structure, and regardless of the carrier protein. This finding suggests that the MBS coupling agent should be carefully compared with other coupling agents working with V3-conjugated peptides because it might probably abolish the antibody response. This unwanted phenomenon makes the MBS an unsuitable agent for immunizations procedures.

Several aspects might influence the anti-peptide response elicited by the conjugates. The conjugation procedure is one of them. Conjugates made by coupling MBS first to the protein carrier and then to the peptide can leave MBS molecules with no peptide coupled at all. These unreacted MBS molecules can favor an immune response against themselves. Blocking unreacted maleimido sites molecule could be a successful adaptation to decrease the immunodominance of MBS. This immunodominant response against MBS but not V3 peptide might then be explained by the mice's repertoire having been selected for that feature.

As the MPS conjugate generated a good anti-JY1 response, even though the same conjugation procedure as for MBS was used, we can argue that non-reacting MPS molecules did not promote in this case a humoral response against themselves. The MBS aromatic ring, the only differential feature with MPS, could explain this phenomenon.

The best anti-JY1 response was achieved using the HBsAg-JY1 MAP8 conjugate. This can be explained because the MAP is coupled as a dimeric structure that contains twice the number of B-cell epitopes. In comparison, a limited humoral response is targeted to the coupling agent.

Comparison between HBsAg-JY1 MAP4 and HBsAg-JY1 MAP8 (AS) conjugates suggests that the amount of couple epitope influence the titer level. For the last immunogen, the response was highest, in agreement with the fact that it displayed twice the amount of epitopes. However, the anti-HBsAg responses were quite similar between both groups. On the other hand, the humoral responses for conjugate HBsAg-JY1 MAP4 (using MPS as a coupling agent) are similar to those of JY1-MAP8, even though the conjugated MAP has twice less JY1-V3 epitope and the total amount of V3 epitope in the immunized conjugate is 20 times smaller. The result shows the potency of conjugation in enhancing immunogenicity.

In conclusion, these results show that a successful conjugation procedure can improve the humoral response for a MAP. Using five times less the amount of conjugate, it is possible to obtain a high and statistically superior humoral response. The anti-carrier response is also high, providing a strategy to obtain combined vaccines.

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