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Research Article

Coinoculation with hepatitis B surface and core antigen promotes a Th1 immune response to a multiepitopic protein of HIV-1

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Summary It has been defined that strong and multispecific cellular immune responses correlate with a better prognosis during the course of chronic diseases. A cross-enhancing effect on the resulting immune response obtained by the coadministration of recombinant hepatitis B virus (HBV) surface and core Ag was recently observed. With the objective of studying the effect of such Ag on the immune response to coinoculated heterologous Ag and vice versa, several formulations containing the recombinant HBV Ag and a multiepitopic protein (CR3) composed by CTL and Th epitopes from HIV-1 were evaluated by s.c. and mucosal administration. Combinations of two and three Ag were evaluated for cellular and humoral immune responses. The results showed that the best Ag combination for nasal immunization was the mixture comprising the CR3 recombinant HIV protein and both HBV Ag. Similarly, it was also the best formulation for s.c. immunization in aluminium phosphate adjuvant. In conclusion, it is possible to induce a Th1 stimulation of the cellular immune response specific for a HIV-based recombinant protein by formulating this Ag with the recombinant HBV Ag.

Key words: adjuvant, AIDS vaccine, hepatitis B vaccine, Th1 cell.

Introduction

There is a need for new adjuvants and strategies to promote immune responses in systemic and mucosal compartments to vaccine candidates for chronic infections. Developing a vaccine to control the AIDS pandemic is an area of intensive work. Although important discoveries have been made in the pathogenesis and molecular biology of HIV-1, the task remains elusive. Unfortunately, the results of the only phase III trial using the AIDSVAX vaccine candidate (produced by VaxGen, Brisbane, CA, USA) were disappointing.[1] There is a call to study novel strategie and to accelerate research and testing of novel candidate vaccines. However, the success of therapies might change the pandemic to a new scenario with increasing prevalence of new recombinant isolates,[2–4] resistance mutations[5,6] and many more patients coinfected with other viruses. Coinfection with HIV–hepatitis C virus (HCV) is estimated to be higher than 50%,[7] and it has been shown that 70–90% of HIV-infected individuals have evidence of past or active infection with hepatitis B virus (HBV).[8–10] These coinfections modify the outcome of therapies, having worse prognosis as have been shown for chronic HBV and HCV.[11,12] So, although we are still waiting for a vaccine against HIV-1, we already need combined prophylactic or therapeutic vaccines (e.g. for HIV–HBV and HIV–HCV).

The hepatitis B core Ag (HBcAg) is highly immunogenic during HBV infection,[13] and the hepatitis B surface Ag (HBsAg) has been shown to be very immunogenic and protective as the main vaccine Ag for seronegative persons.[14,15] Additionally, both structural Ag from HBV are potent immunogens when tested in mice, resembling events in humans.[16] They elicited good immune responses even without adjuvants.[17,18] Hence, they have been used as carriers for heterologous Ag.[19–21] Most importantly, it was recently shown that the transfer of HBcAg-reactive T cells is associated with the resolution of chronic HBV infection.[22] Then, the HBsAg has proved to be good for prophylaxis,[23] and the use of the HBcAg Ag has been suggested for developing therapeutic candidates. Our group has previously shown a marked enhancement in antibody and cellular responses towards both HBsAg and HBcAg after nasal immunization (i.n.).[24,25] Additionally, Riedl *et al.* reported the same adjuvant effect of HBcAg particles on i.m. or s.c. codelivered HBsAg.[26]

In the case of HIV-1, accumulating evidence emphasizes the importance of cellular immune responses in controlling HIV replication in humans.[27] Several proteins or multiepitopic polypeptides from HIV-1 are currently studied under different approaches aimed to elicit such an immune response. The leading approaches are based on recombinant viral vectors such as canarypox or adenovirus,[28,29] but they have several drawbacks. Among them is the impossibility of using these viral vectors in schedules with several administrations because of the immunity

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elicited against the vector itself. The immunity against the viral vectors is 20–30 fold higher than the immune response elicited to the foreign recombinant protein.[30] This is the principal reason why the prime-boosting approach is essential when working with these viruses. Additionally, viral vectors are not efficient in eliciting specific humoral immune responses against recombinant Ag. Finally, for some such viral vectors, there is a strong pre-existing immunity in populations with a high incidence of infection with the wild-type virus.[31]

Soluble polypeptides are very bad immunogens for eliciting cellular responses. In general, subunit Ag need potent adjuvants to improve their immunogenicity, even when results show the delivery of exogenous proteins into cells using membrane permeable carrier peptides.[32,33] Nonetheless, the conjugation step is an important drawback in this approach.

The CR3 recombinant protein comprises B-cell, Th and CTL epitopic rich regions of HIV-1 from several subtype B isolates.[34] It was conceived to elicit a broad cellular response to different virus isolates. Here, we studied the potential of HBcAg and/or HBsAg as adjuvants to enhance the cellular and humoral immune responses to the CR3 protein without interfering with the immune response against them.

Materials and methods

Antigens

The entire rHBcAg particle of 183 amino acids was expressed in *Escherichia coli* and purified for immunization experiments as described.[35] Recombinant HBsAg was taken from the production process of the Cuban Hepatitis B vaccine HeberBioVac HB (CIGB, La Habana, Cuba). This protein was expressed in *Pichia pastoris* yeast, and its purification procedure was published elsewhere.[36]

CR3 is a multiepitopic protein composed of CTL and Th epitope rich regions comprising T1, T2 from gp120, an epitope from gp41, another from vpr, a fragment of p66/p51 (HIV retrotranscriptase [RT]) protein (position 2663–3109 HIV-1 SF2 provirus), a part of Nef (position 8516–8818 HIV-1 LAI isolate) and Gag (position 1451–1696 HIV-1 SF2).[34] It was purified from *E. coli* BL21 (DE 3 codon Plus RLI) strain after transforming the bacteria with plasmid pCR3T7. The protein was expressed as inclusion bodies after induction with isopropylthiogalactoside (IPTG) at 200 mmol/L for 2–3 h. Bacteria were collected by centrifugation, suspended in 10 mmol/L Tris (pH 8.0) with 1 mmol/L EDTA (TE), 25 μg/mL lysosyme, 2 mmol/L PMSF, 1% Triton X-100 at 0.08 g/mL, and disrupted using a French press. Cells were centrifuged at 18 000 *g* for 10 min at 4ºC, and the pellet was washed twice with 10 mL of 4 mol/L urea and 2 mmol/L PMSF in TE buffer. The suspension was blended each time at 8000 r.p.m. using a tissue homogenizer (IKA Labortechnik, Staufen, Germany) and pelleted at 18 000 *g* for 10 min. The proteins were extracted with 8 mol/L urea, 2 mmol/L PMSF, and 5 mmol/L dithiothreitol (DTT) in TE. The CR3 protein was further purified using anionic exchange chromatography with Q-Sepharose FF (Amersham Biosciences, Piscataway, NJ, USA) in buffer containing 20 mmol/L Tris-HCl, 8 mol/L urea, 1 mmol/L DTT, pH 8.0–8.5 and cationic exchange with SP-Sepharose FF (Amersham Biosciences) using the same buffer and a linear gradient of 0–1.5 mol/L NaCl. Then, the protein was dialyzed against 8% acetic acid and lyophilized. A pyrogen-free product with more than 95% purity was achieved. The solubilization of the lyophilized material was in 0.1% acetic acid.

Interaction between CR3 protein and HBV Ag

A solid phase assay similar to the indirect ELISA described in serology was developed. Briefly, high-binding 96-well plates (Costar, Austin, TX, USA) were coated for 3 h at 37ºC with 100 μL of the CR3 protein in the concentration range of 0.05–12.5 μg/mL in coating buffer. After extensive washing with 0.05% Tween-20, the wells were blocked with milk as described in the Serology section. Then, plates were washed again, and 5 μg/mL HBcAg or HBsAg was added to wells in PBS for 2 h at 37ºC. Following extensive washing, to detect any possible interaction, polyclonal sera anti-HBcAg at 1:1000 and anti-HBsAg at 1:3000 dilutions in buffer were incubated in the wells for 1 h at 37ºC. Subsequently, rabbit-antimouse IgG peroxidase at the proper dilution was incubated in the dilution buffer for 1 h at 37ºC. The reactions were developed as described in the Serology section.

Several controls were included to verify the specificity of the interaction: (i) noncoated wells receiving all treatments including the interaction with HBV Ag (to rule out any interactions with the milk); (ii) noncoated wells, not incubated with HBV Ag receiving the rest of the treatments (to rule out any interaction of antisera against HBV Ag with the milk); (iii) coated wells with CR3 at 20 μg/mL that were not incubated with HBV Ag but treated with antisera against HBV Ag (to rule out any cross-reaction of the antisera with CR3); (iv) coated wells, as before, receiving all treatments except the antisera against HBV Ag (to rule out any cross-reaction between the antimouse IgG with CR3 alone or joined HBV Ag); (v) coated wells, as before, not incubated with HBV Ag or with antisera against them but with the mAb 6.2 specific for CR3[34] (control of coating quality); and (vi) wells coated with 5 μg/mL of HBV Ag and incubated with antisera against them (positive controls for the antisera against the HBV Ag).

Immunization

Female BALB/c mice, 6–8 weeks old, were purchased from CENPALAB (Habana, Cuba). Groups of nine mice were immunized in two schedules, through the nasal route or s.c., with PBS (placebo), the combined formulation HBsAg + HBcAg, CR3, HBcAg + CR3, HBsAg + CR3 and HBcAg + HBsAg + CR3. For the nasal route, proteins were dissolved in sterile PBS and dispensed in 50 μL (25 μL/nostril). For s.c. inoculations, they were given in 100 μL adjuvated in 1 mg/mL aluminium phosphate (Superfos Biosector, Vedbaek, Denmark). Immunizations were carried out at 0, 7, 14, 35 and 63 days for the mucosal route and at 0, 14, 35 days for s.c. inoculations. In all cases, mixtures of Ag were prepared the day before and stored at 4°C until inoculation. Animals were anaesthetized by i.p. administration of 30 μL ketamine 10 mg/mL. The doses of HBsAg, HBcAg and CR3 were 5, 5 and 10 μg per mice in i.n. and 2, 4 and 10 μg in s.c. inoculations, respectively. All experiments were conducted in accordance to institutional guidelines.

Biological fluids

Blood was collected through the retrorbital plexus at day 44. It was centrifuged at 7830 *g* for 10 min (Eppendorf centrifuge; Eppendorf, Hamburg, Germany) and the serum stored at −20°C until evaluation.

Vaginal washes were collected on the same day by a skilled technician to avoid the incidence of trauma and blood contamination. They were obtained by reflushing 100 μL of the sterile PBS solution with a micropipette and then centrifuged as above, and the supernatant was stored at −20°C.

Serology

The specific IgG and IgA antibody responses in serum and vaginal fluid were measured by ELISA. High-binding 96-well plates (Costar) were coated with the Ag at 5 μg/mL in 100 μL of coating buffer (11 mmol/L

Na_2CO_3 and 35 mmol/L $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Plates were blocked with 2% skim milk in PBS for 1 h at 37°C. Subsequently, they were incubated with serum samples diluted in 1% skim milk, 5% calf serum and 0.5% Tween-20 in PBS, for 2 h at 37°C. The rabbit-antimouse total IgG, IgG_1, IgG_{2a} and IgA peroxidase conjugates (ICN Biomedicals, Aurora, OH, USA) were incubated for 1 h at 37°C at appropiate dilutions. The reactions were then developed with substrate solution (52 mmol/L Na_2HPO_4, 25 mmol/L sodium citrate, 1 mg/mL *o*-phenylenediamine (Sigma, Milwaukee, WI, USA) and 0.1% H_2O_2) for 10 min at room temperature. The reaction was stopped with 50 μL 3mol/L H_2SO_4. Finally, the plates were read at 492 nm in a microplate reader (Sensident Scan, Merck, Germany). Washes with 0.05% Tween-20 in distilled water were carried out between each step at least five times. Results were expressed as $\log_{10}$ titre calculated by interpolation of absorbance values at a fixed serum dilution into a linear regression analysis plotting the dilution versus A_{492} of the standard curve of a known titre serum. The titre was defined as the $\log_{10}$ highest dilution that gave twice the absorbance of the negative control sera diluted 1:100. Titres are given as the geometric mean ± SD from the individual sera. In the case of the IgA response, data from mucosal washes corresponded to 1:10 dilution of the samples.

Quantification of anti-CR3 IFN-γ-secreting cells

The number of IFN-γ-secreting cells was detected by an ELISPOT assay. Ten days after the last immunization, the spleens of three mice per group, drawn randomly, were aseptically removed and single-cell suspensions prepared. Erythrocytes were lysed after 5 min incubation in 0.83% NH_4Cl. The cells were extensively washed with the medium and diluted in the complete medium (RPMI 1640 (Gibco, Paisley, Scotland, UK) supplemented with 10% FCS, 2 mmol/L glutamine, 2 mmol/L sodium pyruvate, 50 μmol/L 2-ME and antibiotics) and counted. Nitrocellulose-backed 96-well plates (MAIPN4550; Millipore, Bedford, MA, USA) were coated with 100 μL of 5 μg/mL murine IFN-γ-specific mAb R4-6A2 (Pharmingen, San Diego, CA, USA) overnight at 4°C, washed three times with PBS and blocked using the complete medium at 37°C for 1 h. Three dilutions (2×10^5, 1×10^5 and 0.5×10^5) of freshly isolated splenocytes were incubated for 50 h at 37°C, 5% CO_2 in the complete medium with sterile and non-pyrogenic CR3 protein at 2.5 μg/mL. Wells with splenocytes without protein were incubated as negative controls and with 2.5 μg/mL concanavalin A as positive controls. The plates were washed three times with PBS and five times with 0.05% Tween-20 in PBS. Then, 0.5 μg/mL of secondary biotin-conjugated antibody XMG1.2 (Pharmingen) was added and incubated for 2 h at room temperature. The wells were washed five times with 0.05% Tween-20 in PBS, and peroxidase-labelled streptavidin (Sigma) was added at 1:1000 dilution for 1 h. Then, the plates were washed again with 0.05% Tween-20 in PBS, and after with PBS and the spots were developed by adding 100 μL 3-amino-9-ethylcarbazole (AEC; Sigma) solution.[37] After 15 min, the reaction was stopped with tap water. Plates were dried, and spots were counted under a dissection microscope. The results were expressed as the number of spot-forming cells per 10^6 splenocytes after subtracting the spots of negative wells. Values above the mean number of spots in the placebo or negative groups plus three standard deviations were considered positives.

Western blot anti-HIV-1

Mice sera were tested for anti-HIV protein recognition using the DAVI-BLOT kit (LISIDA, La Habana, Cuba) according to the manufacturer's directions. Single serum suspensions were prepared per group and tested at 1:10 dilution for the i.n. groups and 1:100 for the systemic counterpart. The reactions were developed with the antimouse IgG–peroxidase conjugate.

Statistical procedures

GraphPad Prism version 4.00 statistical software (GraphPad Software, San Diego, CA, USA) was used to carry out ANOVA and *t*-tests to determine differences between the means of groups, and Tukey's test for post-ANOVA comparisons. All titres were transformed to $\log_{10}$ for a normal distribution. For the nonsero-converting sera, an arbitrary titre of 1:50 was assigned for statistical processing. A P value of <0.05 was considered statistically significant.

Results

Interaction between the multiepitopic protein CR3 and the HBV Ag

The HBV surface and core Ag, obtained as recombinant particles, were shown to elicit CTL responses against themselves by cross-primming,[38,39] and the potent Th1 adjuvant activity of HBcAg formulated with HBsAg[24] was shown. The formulation containing both particles generates supra-aggregated structures that may be mediating this adjuvant effect.[24] A solid phase assay, aimed at evaluating the potential interaction of hepatitis B Ag and CR3, was developed based on the immobilization of CR3 and the addition of the HBV Ag in solution. The results showed the interaction of CR3 with both HBV Ag. Moreover, the binding of CR3–HBcAg was stronger than that of CR3–HBsAg interaction (Fig. 1). These results were further corroborated using a liquid phase assay where the interactions were verified in saline buffer (data not shown).

Th1 immune response in spleen following i.n. and s.c. immunization of CR3 coadministered with HBV Ag

Considering the advantage of the interaction between CR3 and HBV Ag in the potential enhancing of the immune response against CR3, we decided to immunize animals to evaluate the immune response against all the Ag assayed in different formulations. The capacity of the i.n. and s.c. routes to elicit cellular responses in the spleen of immunized mice was evaluated using unfractionated splenocytes. Cells of immunized mice were tested *ex vivo* for IFN-γ secretion in ELISPOT assays.

To measure anti-CR3 cellular responses after i.n. immunization, we gave a final fifth dose to increase the level of effector cells through memory cell reactivation. It was based on the fact that the *ex vivo* ELISPOT assay will detect the IFN-γ secretion mainly by effector cells in relation to the contribution made by resting memory cells.[40] Several days after the immunization, we studied the anti-CR3-specific cellular response. Only mice inoculated with HBsAg + CR3 and HBsAg + HBcAg + CR3 showed values above the threshold (Fig. 2A). The evaluation of the immune response after s.c. immunization was also carried out. In this case, mice inoculated with HBcAg + CR3, HBsAg + CR3 and HBsAg + HBcAg + CR3 showed values above the threshold in the ELISPOT (Fig. 2B). However, the first two groups were only slightly positive.

All Ag elicited specific IgG antibodies in serum following i.n. and s.c. immunization

To address whether mucosal and s.c. immunization of the CR3 protein with hepatitis B Ag result in enhanced humoral immunity to any of the Ag in the serum, we evaluated the IgG antibody

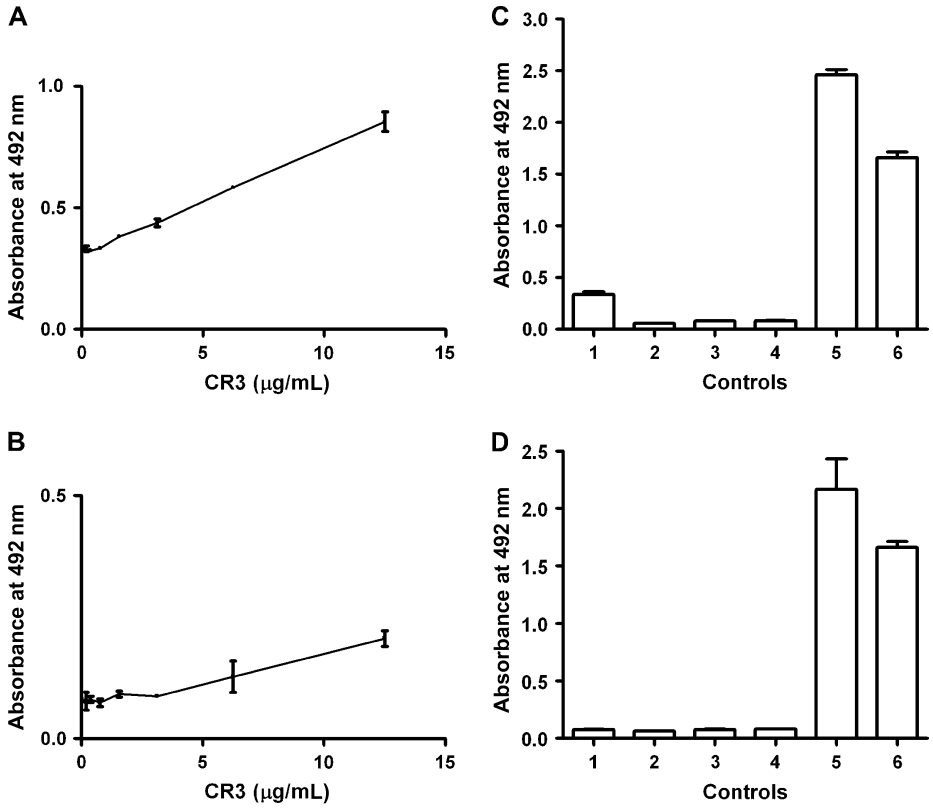

Figure 1 Non-covalent interactions between the recombinant multi-epitopic protein CR3 of HIV-1 and the HBsAg and HBcAg antigens of HBV. The wells from ELISA plates were coated with the CR3 protein at a concentration of 0.05–12.5 μg/mL. After blocking the plates to avoid nonspecific binding, the HBcAg (A) and HBsAg (B) were added to wells at 5 μg/mL in PBS to promote the interaction with the CR3 protein. Anti-sera against HBV antigens were added to reveal any interaction following extensive washes. Absorbance values were obtained after the addition of antimouse whole IgG coupled to peroxidase and the chromogenic substrate. Controls included in this assay to verify the specificity of the interactions with HBcAg (C) and HBsAg (D) were: 1, non-coated wells that received all treatments including the interaction with HBV antigens; 2, non-coated wells, not incubated with HBV antigens that received the rest of the treatments; 3, wells coated with CR3 that were not incubated with HBV antigens but treated with anti-sera against HBV antigens; 4, wells coated as before that received all treatment except the anti-sera against HBV antigens; 5, wells coated as before, that were neither incubated with HBV antigens nor with the anti-sera against them but with the mAb 6.2 specific for CR3; 6, wells coated with HBV antigens and incubated with the anti-sera against them.

response of mice inoculated with different formulations as described above.

After four i.n. inoculations with the CR3 protein, we detected similar low IgG titres against it in the sera of all groups (Fig. 3A). The IgG subclass pattern was predominantly IgG_1 for all groups, without statistical differences among the IgG_1/IgG_{2a} ratios (Fig. 4A). However, in the group of mice where the HBsAg + HBcAg + CR3 mixture was given, approximately 55% of them had an IgG_1/IgG_{2a} ratio close to one or below that value showing a notable increase in the level of IgG_{2a} antibodies compared with other groups.

The recognition pattern to HIV-1 proteins was evaluated by western blot, using sera pools from every group. All groups with anti-CR3 IgG specifically reacted with p55. In addition, the group immunized with HBsAg + CR3 detected p24, gp160 and gp120 (Fig. 5A).

As expected, only mice immunized with mixtures of HBcAg + HBsAg, HBsAg + CR3 and HBcAg + HBsAg + CR3 seroconverted to HBsAg in sera (Fig. 3A). The group immunized with the HBcAg + HBsAg combination reached the highest IgG titre and that immunized with the HBsAg + CR3 combination reached the lowest ($P < 0.01$). Because of the importance of the antibody response to HBsAg, we also evaluated the composition of IgG subclasses in the sera (Fig. 4B). As reported previously, the group given HBcAg + HBsAg elicited a balanced IgG_1/IgG_{2a} ratio,[24] which was similar to HBcAg + HBsAg + CR3 ($P > 0.05$). In contrast, mice immunized with HBsAg + CR3 had a predominantly IgG_1 pattern ($P < 0.01$).

The whole group of mice inoculated with HBcAg + HBsAg developed strong IgG titres in the sera against HBcAg. In contrast, only one mouse in the HBcAg + CR3 group and three mice in the CR3 + HBsAg + HBcAg group elicited titres against the core Ag (Fig. 3A).

In the case of s.c.-immunized mice, the IgG response in the sera was assayed 10 days after the last immunization. Animals immunized with CR3 in mixtures and as a single protein adjuvated in aluminium phosphate elicited a potent specific IgG antibody response to the multiepitopic protein (Fig. 3B). The statistical analysis showed no differences

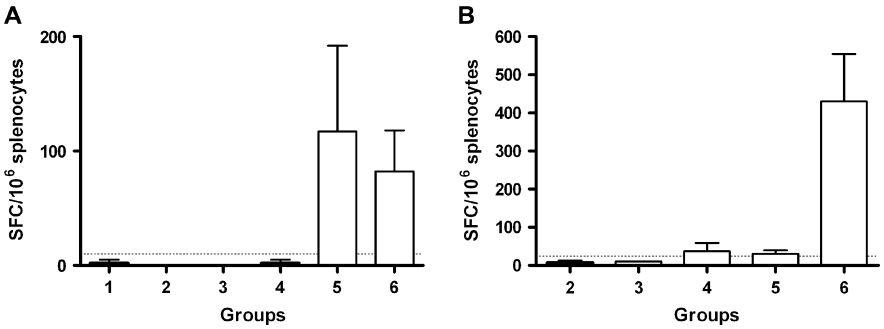

Figure 2 Quantitation of the CR3-specific response using an ELISPOT-IFN-γ assay. BALB/c (H-2^d) mice were immunized i.n. (A) and s.c. (B) with: 1, PBS (placebo); 2, the combined formulation HBsAg + HBcAg; 3, CR3; 4, HBcAg + CR3; 5, HBsAg + CR3; 6, HBcAg + HBsAg + CR3. Splenocytes were removed 10 days after the last immunization and the number of IFN-γ-secreting cells, in response to the stimulation with CR3 protein and the medium alone, were measured. The results shown are the number of IFN-γ-secreting cells per million splenocytes in pools of cells from three mice per group. Standard deviations from three individual wells are represented. Threshold values are shown by dotted lines in the graphs.

between the mean titres of the groups. Additionally, all groups showed a preferential IgG_1 antibody response (Fig. 4C). Pools of sera from all groups of animals immunized with CR3 protein elicited specific antibodies for p24, p53, p55 and p68 proteins of HIV-1 (Fig. 5B). Additionally, mice in the HBcAg + CR3 group recognized the gp120 protein.

Mice in the groups immunized with HBsAg + HBcAg, CR3 + HBsAg and CR3 + HBsAg + HBcAg (aluminium phosphate) seroconverted to HBsAg (Fig. 3B). Titres did not differ between the groups. All groups showed an IgG_1 antibody response higher than the IgG_{2a} (Fig. 4D).

All groups of mice immunized with HBcAg seroconverted against the core protein. There were no differences among groups immunized with HBcAg, HBsAg + HBcAg and HBcAg + CR3. However, mean titres of these groups were slightly higher than those of HBsAg + HBcAg + CR3 in the aluminium phosphate group ($P < 0.05$) (Fig. 3B).

HBcAg increased the level of IgG_{2a} antibodies to coadministered CR3 multiepitopic protein of HIV-1 after i.n. inoculation

We have previously shown the capacity of HBcAg to induce strong IgG_{2a} immune responses against itself and against coadministered HBsAg after i.n..[24] An overall analysis was carried out to study if a bias similar to the IgG_{2a} pattern was induced in the IgG response to the CR3 protein. Mice were then arranged in two groups, those immunized with core and CR3 and those immunized with CR3 but without core. Afterwards, the IgG_1/IgG_{2a} ratios from the groups of mice were compared.

Our results showed a significant increase in anti-CR3 IgG_{2a} antibodies induced after i.n. inoculation in groups of mice having HBcAg included in combined formulations with CR3 ($P = 0.0357$). In contrast, the same effect was not detected after s.c. immunization. To exclude the possible contribution of HBsAg to this effect, we carried out a similar analysis. The IgG_1/IgG_{2a} ratios from the groups of mice immunized in combined formulations with the HBsAg were compared with those where this Ag was not added. It was observed that the HBsAg did not promote any change in the IgG subclass pattern ($P > 0.05$).

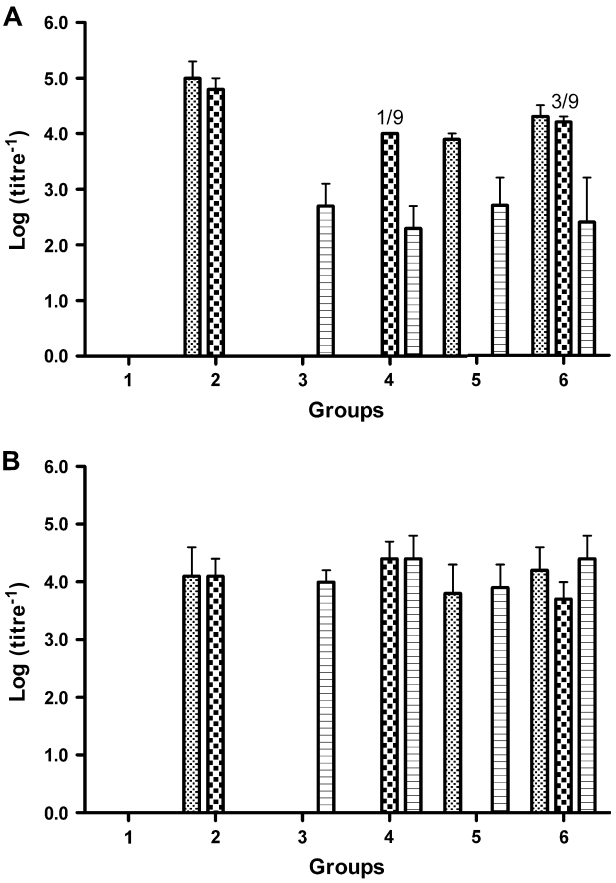

Figure 3 IgG antibody response in the sera against CR3, HBsAg and HBcAg antigens. Mice were immunized i.n. (A) and s.c. (B). Sera samples were obtained 10 days after the last immunization. Groups: 1, PBS (placebo); 2, the combined formulation HBsAg + HBcAg; 3, CR3; 4, HBcAg + CR3; 5, HBsAg + CR3; 6, HBcAg + HBsAg + CR3. The frequency of mice responding against each antigen was 100% in most cases; otherwise the plotted titre comprised only positive sera and the real frequency is shown above the particular column. ▨, HbsAg; ▩, HbcAg; ▭, CR3.

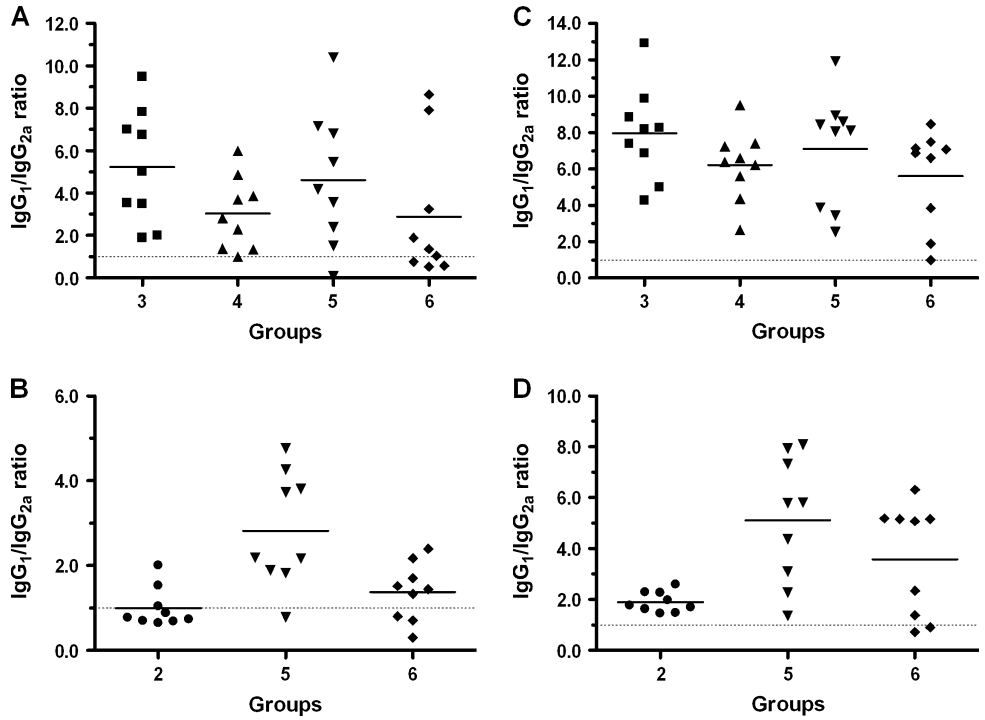

Figure 4 CR3 and HBsAg-specific IgG subclass patterns in BALB/c mice. Animals were immunized i.n. (A and B) and s.c. (C and D). Results are shown for humoral responses against CR3 (A and C) and HBsAg (B and D) antigens. Groups: 2, the combined formulation HBsAg + HBcAg; 3, CR3; 4, HBcAg + CR3; 5, HBscAg + CR3; 6, HBcAg + HBsAg + CR3. Each dot represents the IgG1 to IgG2a titres ratio for an individual mouse. The dotted line represents the IgG1/IgG2a = 1 ratio.

Intranasal coadministration with the CR3 multiepitopic protein of HIV-1 elicited IgA antibodies to HBV Ag in the vagina

In contrast to s.c. immunization, which did not elicit any response in the vagina, we found specific IgA antibodies against HBV Ag after i.n. inoculation. For CR3 protein, however, no specific IgA antibodies were detected. Regarding HBV Ag, we detected IgA antibodies in vaginal washes against HBsAg in the HBsAg + HBcAg group, which elicited the highest humoral response, followed by the mixture of the three Ag. They were the sole immunogens eliciting a humoral response to HBsAg in vaginal washes (Fig. 6A). Nevertheless, in all groups where the HBcAg Ag was added, (HBsAg + HBcAg, HBcAg + CR3 and HBsAg + HBcAg + CR3) most of the animals elicited an IgA response in the vagina against the core Ag (Fig. 6B). There were statistical differences between these groups. The level of antibodies in the HBsAg + HBcAg were the highest followed by HBcAg + HBsAg + CR3 and HBcAg + CR3 ($P < 0.05$).

Discussion

The use of genetic engineering techniques to obtain recombinant vaccine Ag has become a potent tool to obtain pure soluble subunit Ag. An important problem of these soluble, nonparticulated recombinant Ag is their poor immunogenicity. The main goal in current adjuvant development is to improve the immunogenicity of subunit Ag, avoiding the risks and toxicity associated with the administration of the Ag in the original microorganism. However, the intrinsic toxicity of the adjuvants has become an important drawback in the development of new vaccine adjuvants and their formulations.

The CR3 protein (CIGB) was obtained as a soluble recombinant protein containing several CTL, Th and B-cell epitopes from different HIV-1 strains. Previous studies showed that epitopes included in CR3 are recognized by CTL from chronic HIV-1-infected patients.[41] This study takes into consideration the ability of the important Ag from HBV, the HBsAg and the HBcAg to elicit potent CTL $CD8^+$ and Th responses against themselves[38,39] as well as their immunoenhancing effect when combined in formulations given mucosally and s.c.[24,26] Here, we evaluate the capacity of those Ag from HBV to stimulate the immunogenicity of the multiepitopic protein CR3.

Our results showed that it is possible to elicit anti-CR3-specific IFN-γ-secreting cells in the spleens of mice after i.n. and s.c. immunization if the CR3 protein is combined, as a simple mixture, with HBsAg and HBcAg Ag from HBV. This Th1 immune response seems to depend on the presence of HBV Ag because the CR3 protein alone was not able to elicit any IFN-γ secretion after i.n. or s.c. immunization. Perhaps, the stimulus for CR3-specific Th1 cells to become activated is caused by immune deviation as a result of a bystander stimulation due to the immune response elicited by HBcAg Ag in the vicinity.[42] Further work will address the cellular immune response elicited in lymphoid tissues associated with mucosa as we consider this to be a main target for

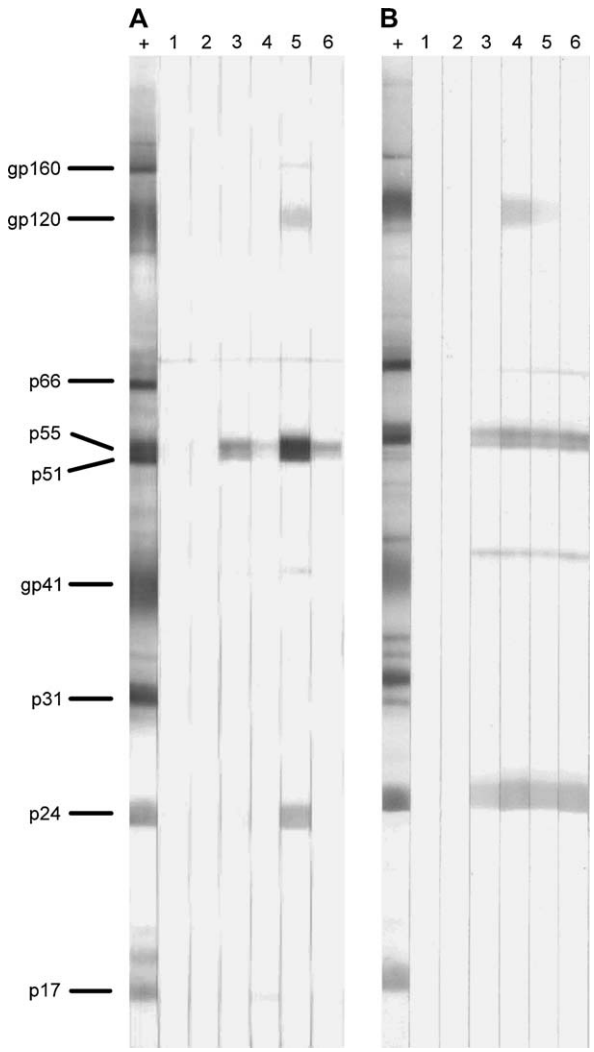

Figure 5 Western blot anti-HIV-1 from mice sera. Animals were immunized i.n. (A) and s.c. (B). Immunization groups: +, sero-positive patient; 1, PBS (placebo); 2, HBsAg + HBcAg; 3, CR3; 4, HBcAg + CR3; 5, HBsAg + CR3; 6, HBcAg + HBsAg + CR3.

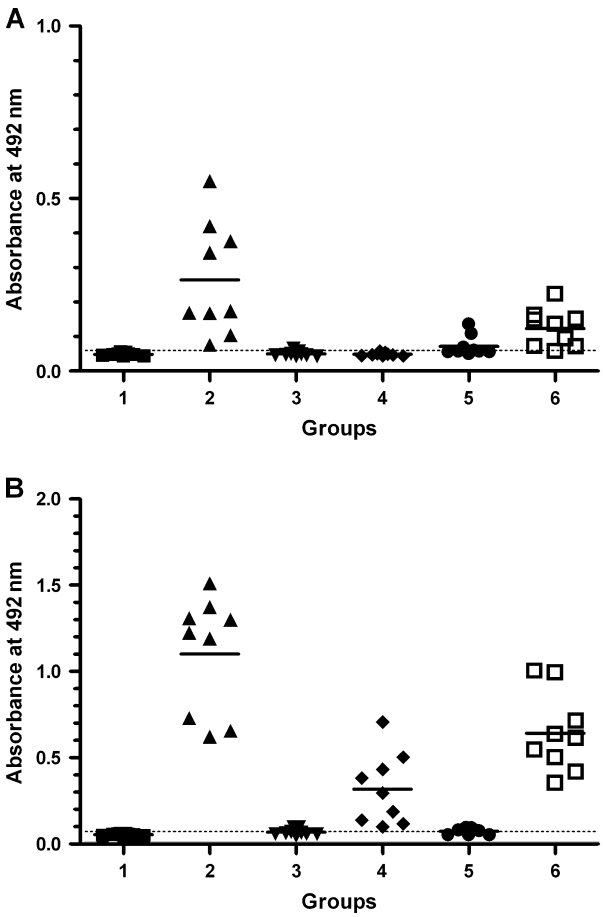

Figure 6 IgA response in the vagina after i.n. immunization. (A) Anti-HBsAg response and (B) anti-HBcAg. Each dot represents an individual mouse. The dotted line represents the cut off value. Immunization groups: 1, PBS (placebo); 2, the combined formulation HBsAg + HBcAg; 3, CR3; 4, HBcAg + CR3; 5, HBsAg + CR3; 6, HBcAg + HBsAg + CR3.

infection and reservoirs for both viruses. The notion that only after mucosal stimulation is it possible to get mucosal immune responses is well supported.[43]

Previous work has used the HBcAg as a carrier protein. The general approaches have been limited to include epitopic sequences in the permissive regions of the Ag by means of molecular biology techniques[18] or by the chemical conjugation of peptides and proteins.[44] The CR3 protein, however, has 139 amino acids, several proline residues dispersed in its primary structure and a nonhomogenous distribution of charges.[34] These features make it unsuitable for HBcAg structure preservation. Additionally, it has been impossible for us to couple both Ag using conventional chemistry. Therefore, our results are of importance because similar problems have been reported by others.[45]

However, humoral immune responses against all Ag in mice sera were measured after immunization. Although the CR3 protein was designed to induce cellular immune responses, the analysis of its humoral response is not devoid of importance. First, in a prophylactic setting, it is important to discriminate an infected person from a vaccinated one. Second, the protein also includes B-cell epitopes associated with several immune-effector mechanisms.[46–53] Our results show that CR3 is able to induce an IgG recognition pattern in sera to HIV-1 proteins, after i.n. and s.c. immunization, similar to seropositive persons. This result stresses the need to carefully differentiate seronegative immunized persons from those truly infected for a planned clinical trial.

It was also shown that in groups where HBcAg was coimmunized with CR3 through the nasal route, a biased IgG-subtype pattern mirroring an immune deviation to a Th1-like immune response was obtained. This may be relevant in a therapeutic setting because there is evidence that a change to a Th2-like immune response is induced in chronic HIV-1-infected patients.[54,55] The same effect was not as strong in s.c.-immunized groups and showed no statistical significance, but the aluminium phosphate Th2 inducer was also contained in the immunized formulations and was probably playing a role in this regard.[56] It is noteworthy that anti-CR3 IgG levels in the sera were similar in all groups of mice in both immunization

schedules, showing that the HBV Ag did not enhance the CR3 humoral response. However, further work will address the anti-CR3 IgA antibodies in mucosal secretions and the cellular responses in mucosal compartments, which are current limitations here.

The humoral response to HBsAg in the serum protects against HBV infection, and it has proven to be of value in large vaccination programmes all over the world. Consequently, it is imperative not to inhibit this response. We showed that it is possible to immune modulate and enhance that response in combined HBsAg–HBcAg formulations.[24,25] We then measured the IgG to HBsAg in the sera of immunized mice and carried out comparative analyses. The CR3 added to the HBsAg + HBcAg mixture slightly decreased the IgG levels to HBsAg in the sera after i.n. compared with the HBsAg + HBcAg group. However, the geometric mean of titres was still high and the IgG_1/IgG_{2a} ratio did not differ, preserving the balanced response. Therefore, the immune modulation provided by the HBcAg Ag was not affected.

In contrast, after s.c. immunization the IgG-subtype pattern was biased to IgG_1 in all groups. Again, the effect of the alum-based adjuvant could mediate this result. In vaginal washes, IgA antibodies were only found after i.n. in the HBsAg + HBcAg and CR3 + HBsAg + HBcAg groups. Nevertheless, including CR3 in the mixture of HBV Ag reduced the level of IgA antibodies to HBsAg. Perhaps the CR3–HBsAg interaction obstructed the recognition of some B-cell epitopes of the surface Ag by the immune system, which may also explain the slight reduction in IgG titres in the sera. The same phenomenon is probably occurring after s.c. immunization and might be overcome by the alum-based adjuvant. Consequently, differences in titres were not found between the groups.

We do not envisage the use of anti-HBcAg antibodies for vaccination purposes. This response was only measured to get some insight into the Ag interactions in combined formulations. Our results showed that the CR3 protein deeply affected the humoral response in sera to HBcAg after mucosal immunization as evidenced in groups CR3 + HBcAg and CR3 + HBsAg + HBcAg. It is not an unexpected result considering the strength of the CR3–HBcAg interaction. Regarding s.c. immunizations, as explained for the HBsAg, the alum adjuvant could influence the results, although it was not enough to overcome the reduction in IgG titres in the CR3 + HBsAg + HBcAg group compared with the HBcAg + HBsAg group. Taken together, the strong interaction between CR3 and HBcAg was of benefit to the anti-HBsAg humoral response compared with the anti-HBcAg. It is important to point out that the aluminium phosphate adjuvant was needed to elicit a good immune response after s.c. inoculation. This was verified in preliminary experiments where the Ag were immunized in phosphate saline buffer as well (data not shown).

This study as a whole showed that the best Ag combination with the CR3 protein for i.n. is the mixture comprising both HBV Ag in phosphate saline buffer. It was based on the anti-CR3 IFN-γ secretion in the spleen, the balanced anti-HBsAg IG_1/IgG_{2a} ratio in the sera and the presence of IgA antibodies in the vagina. Similarly, the best formulation for s.c. immunization is the same mixture in aluminium phosphate. In this case, the former formulation elicited the highest anti-CR3 IFN-γ secretion and the anti-HBsAg IgG titres in the sera were still high. In our opinion, the effect described in the mixture of CR3, HBsAg and HBcAg rests on the physicochemical nature of HBV recombinant Ag. The HBsAg is a proteoliposome and its adjuvant activity might be related to this feature. Also, the enhancing effect of HBcAg, based on its nucleic acid content,[26] further increases CR3 immunogenicity.

Our results stress the fact that the formulation with both HBV Ag further contributes to the immunogenicity of the heterologous Ag compared with the mixtures containing each HBV Ag alone. Hepatitis B Ag are very important per se, increasing the valence of the resulting formulations. Additionally, more immunogenic HIV vaccine candidates may result from the enhancement and/or immune modulation of the HIV-specific immune response. In this regard our approach, comprising the surface and core Ag of HBV and a recombinant protein from HIV-1, has several advantages compared with other strategies: first, the immune system is not stressed with a huge nonspecific and irrelevant response; second, in a therapeutic setting, immune-deficient patients will not confront a potential hazard of an uncontrolled infection with live vectors, which might spread in turn to healthy people; and third, pre-existing immunity to virus-like particles (VLP) does not impair the immune response to other Ag after coinoculation.[57]

In conclusion, it is possible to induce a better quality Th1 stimulation of the immune response specific for the HIV-based recombinant protein by formulating this Ag with the surface and core recombinant HBV Ag. This stimulation is effective after i.n. and s.c. immunization. Our data suggest a novel approach to develop vaccine formulations.

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