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Influence of Aluminum-Based Adjuvant on the Immune Response to Multiantigenic Formulation

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ABSTRACT

Several adjuvants have been described and tested in humans. However, the aluminum-based adjuvants remain the most widely used component in vaccines today. Emerging data suggest that aluminum phosphate and aluminum hydroxide adjuvants do not promote a strong commitment to the helper T cell type 2 (Th2) pathway when they are coadministered with some Th1 adjuvants. In this regard, subtle differences between both aluminum-based adjuvants have been demonstrated. We have previously shown that subcutaneous immunization, in aluminum phosphate, of a mixture comprising the surface and core antigens of hepatitis B virus (HBV) and the multiepitopic protein CR3 of human immunodeficiency virus type 1 elicits a CR3-specific Th1 immune response. In these experiments, the antigens were adjuvated at the same time. As the final selection of the best adjuvant should be based on experimental evidence, we asked whether aluminum hydroxide allows a better Th1 immune deviation than aluminum phosphate. We also studied several ways to mix the antigens and the impact on CR3-specific interferon (IFN)-γ secretion. Our findings indicate that aluminum hydroxide allows better Th1 immunodeviation than aluminum phosphate adjuvant for the mixture of HBV antigens and CR3. In addition, CR3-specific IFN-γ secretion of the various formulations tested was the same irrespective of the order in which the antigens were combined.

INTRODUCTION

SELECTION OF THE RIGHT ADJUVANT is a key element in any vaccine formulation. Aluminum-containing adjuvants were first described by Glenny and coworkers in the early years of the last century (13). Further improvements of the former work arrived soon with the substitution of the so-called protein aluminates by the use of preformed aluminum hydroxide (AlOOH) (28) and aluminum phosphate ($AlPO_4$) hydrated gels (11). Although many experimental adjuvants have been described and tested in animal models and humans, the aluminum-based adjuvants remain the most widely used component of prophylactic vaccines in use today (6).

It is known that antigen adsorption efficiency on aluminum adjuvants is dependent on ligand exchange and electrostatic interactions (24,25). Because of the chemical nature of the adjuvant, it is known that proteins with an acid isoelectric point adsorb better to aluminum hydroxide than those with a basic isoelectric point; in the case of aluminum phosphate the opposite is true (36). Thus, only with these two aluminum-based adjuvants is the adsorption of most proteins possible.

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The work of researchers has long focused on the action mechanisms of adjuvants. In this regard, current thinking indicates that adjuvants may exert their immune-enhancing effect according to five immune-functional activities (34): (1) enhanced translocation of antigens to the lymph nodes; (2) physical protection and prolonged delivery of antigens; (3) interaction with PRRs (pathogen recognition receptors) present on accessory cells; (4) capacity to cause local reactions at the injection site, inducing danger signals; and (5) induction of inflammatory cytokines at the injection site. For aluminum adjuvants, their depot effect and immunostimulatory capacity are the mechanisms generally cited to explain their adjuvant effect. Although the depot effect has been a matter of debate (17) it has been demonstrated that adsorption allows efficient antigen uptake by dendritic cells (29). The resulting immunomodulation induced by aluminum adjuvants is characterized by interleukin (IL)-4 production, leading to a helper T cell type 2 (Th2) antibody response dominated by murine IgG1 antibodies. Because of their preferential Th2 immune response, aluminum adjuvants are appropriate for vaccines against exotoxins and extracellular infectious agents. On the other hand, they are considered ineffective in the case of vaccines for intracellular microorganism such as viruses, for which a Th1 immune response is desirable. The Th2 immunomodulatory effect of aluminum-based adjuvants is a direct consequence of immune mechanisms elicited by themselves (17,25). Nevertheless, experimental evidence suggests that they do not promote a strong commitment of the immune system to the Th2 pathway. For instance, adjuvant formulations including CpG and cytokines are able to induce a shift to a Th1 response (8,42). Moreover, it is known that Algammulin, prepared by mixing aluminum hydroxide with γ-inulin, promotes an immune deviation to a Th1 immune response (7). Consequently, there are no reasons, in principle, to exclude aluminum-based adjuvants in vaccine formulations for eliciting Th1 immune responses against intracellular pathogens such as human immunodeficiency virus (HIV)-1 and hepatitis B virus (HBV).

The core antigen (HBcAg) of HBV promotes Th1 immunomodulation of the immune response to coadministered antigens, including the surface antigen (HBsAg) of HBV (27,31). Riedl and coworkers have related this adjuvant activity to the nucleic acid content in the particle, which interacts with Toll-like receptor-3 (TLR3) (31,32). However, there is evidence of a synergistic effect in the enhancement of the immunogenicity for both antigens of HBV, the surface and core antigens, in the combined formulation after nasal administration, indicating that HBcAg increases the anti-HBsAg response and, conversely, HBsAg increases the specific HBcAg response (1,27). On the basis of that evidence we hypothesized that a mixture of HBsAg and HBcAg would act as a Th1 adjuvant formulation. We have shown that a simple mixture containing the recombinant protein from HIV-1, CR3, and the surface and core antigens from hepatitis B virus adjuvated in aluminum phosphate elicited a Th1 response against the recombinant antigen after subcutaneous immunization far better than the same formulation without adjuvant and the combination of CR3 with each HBV antigen separately (19). In this sense, the recombinant antigens derived from HBV had a significant role in the resulting Th1 immune modulation. In addition, further immuno-enhancing activity provided by the aluminum phosphate adjuvant was evident when the same multiantigenic formulation was inoculated in saline solution by the subcutaneous route.

In the literature, it is generally assumed that all aluminum-based adjuvants behave the same way in every experimental situation. However, unexpected differences between the aluminum hydroxide and the aluminum phosphate might arise in particular experimental settings as demonstrated by Wang and coworkers, who used IL-12 with the surface antigen of HBV (42). In addition, in our previous work (19), the antigens CR3, HBsAg, and HBcAg were mixed and adjuvated at the same time; however, a comparison of different ways to formulate the three antigens in the mixture is an important issue. In some cases, the antigens compete for adsorption to the adjuvant (39); also, when working with several antigens it is possible that interactions among them influence the adsorption of the resulting aggregates to the adjuvant and/or their presentation to the immune system.

In the present work, we studied the influence of aluminum-based adjuvants on the resulting immune response to a mixture of CR3, HBsAg, and HBcAg proteins as well as the effect of various ways of mixing the antigens on the cellular anti-CR3 immune response. Although our investigation was focused mainly on the development of a vaccine candidate against HIV-1, we also measured specific HBcAg and HBsAg responses to gain some insight concerning antigen interactions in the multiantigenic formulation. In particular, the anti-HBsAg response elicited by the former formulation was further evaluated and is discussed because of its protective effect against hepatitis B.

MATERIALS AND METHODS

Antigens

The entire recombinant (r)HBcAg particle of 183 amino acids was expressed in *Escherichia coli* and purified for immunization experiments as already described (26). Recombinant HBsAg was taken from the produc-

tion process of the Cuban hepatitis B vaccine Heberbiovac HB (Centro de Ingeniería Genética y Biotecnología [CIGB], Havana, Cuba). This protein was expressed in the yeast *Pichia pastoris* and its purification procedure has been published elsewhere (14).

The HIV-1 antigen CR3 is a multiepitopic protein composed of cytotoxic T lymphocyte (CTL) and helper T cell (Th) epitope-rich regions comprising T1 and T2 from gp120, an epitope from gp41, another from Vpr, a fragment of the p66/p51 (reverse transcriptase [RT]) protein (positions 2663–3109, HIV-1 SF2 provirus), a part of Nef (positions 8516–8818, HIV-1 LAI isolate), and a part of Gag (positions 1451–1696, HIV-1 SF2) (18). It was purified from *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA [Cat. No. 230245]) and the purification process was as described previously (19). A pyrogen-free product with more than 95% purity was achieved.

Determination of antigen binding to aluminum adjuvants

To estimate the degree of adsorption the protocol reported by Berthold and coworkers was followed with few variations (3). Several aliquots were tested: (1) antigens without adjuvants (positive control), (2) $AlPO_4$ without antigens (negative control), (3) AlOOH without antigens (negative control), (4) antigens adjuvated with $AlPO_4$, and (5) antigens adjuvated with AlOOH. After overnight incubation at 4°C under agitation, aliquots were centrifuged (centrifuge 5415C; Eppendorf, Hamburg, Germany) at 1500 rpm ($200 \times g$) for 3 min to pellet the aluminum gel. The supernatants were carefully removed and concentrated at least twice by centrifugation, using Centricon-10 concentrators (Amicon, Beverly, MA). Protein in the supernatants was measured with the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). The low-protein protocol (5–250 μg) for the microtiter plate assay was followed. The degree of adsorption was estimated on the basis of the calculated difference between the amount of protein in aliquot 1 and the fraction recovered in the supernatants of aliquots 4 and 5. All aliquots were treated as for the immunizations.

Immunizations

Female BALB/c mice, 6–8 wk old, were purchased from CENPALAB (Havana, Cuba). Antigens were administered subcutaneously in a 100-μL volume, adjuvated in $AlPO_4$ or AlOOH (1 mg/mL) (Brenntag Biosector, Frederikssund, Denmark). In all cases mixtures of antigens were prepared 1 d ahead and stored at 4°C until inoculation. Animals were anesthetized by intraperitoneal administration of 30 μL of ketamine (10 mg/mL).

In the first immunization schedule three groups of nine mice were inoculated with the following: (1) HBsAg (AlOOH) (current hepatitis B vaccine), (2) HBcAg + HBsAg + CR3 ($AlPO_4$), and (3) HBcAg + HBsAg + CR3 (AlOOH). Antigen administrations were carried out on days 0, 14, and 35, and sera were collected 10 d after the last immunization. Doses of HBsAg, HBcAg, and CR3 were 7, 9, and 20 μg/mouse, respectively.

A second immunization schedule was carried out with 6 groups of 12 mice each. They were inoculated with the following: (1) phosphate-buffered saline (PBS; placebo), (2) HBsAg + HBcAg, (3) HBcAg + HBsAg + CR3, (4) (HBcAg + HBsAg) + CR3, (5) HBcAg + (HBsAg + CR3), and (6) HBsAg + (HBcAg + CR3). As highlighted by parentheses for groups 4–6, two antigens were mixed and incubated for 16 h, in PBS at 4°C, before addition of the last antigen and the adjuvant. In this schedule, we administered three doses on days 0, 14, and 42 and sera were collected 10 d after the last immunization. The dose for all antigens was 5 μg/mouse in AlOOH adjuvant. All experiments were conducted in accordance with institutional guidelines.

Biological fluids

Blood was collected through the retrorbital plexus 10 d after the last immunization. It was centrifuged at 10,000 rpm for 10 min (centrifuge 5415C; Eppendorf) and the serum was stored at –20°C until evaluation.

Serology

The specific IgG antibody responses in serum were measured by enzyme-linked immunosorbent assay (ELISA). High binding capacity 96-well plates (Corning Life Sciences, Acton, MA) were coated with the antigen at 5 μg/mL in 100 μL of coating buffer (11 mM Na_2CO_3 and 35 mM $NaHCO_3$, 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. Rabbit anti-mouse total IgG, IgG1, and IgG2a peroxidase conjugates (MP Biomedicals, Aurora, OH) were incubated for 1 h at 37°C at appropriate dilutions. The reactions were then developed with substrate solution (52 mM Na_2HPO_4, 25 mM sodium citrate, *o*-phenylenediamine [OPD, 1 mg/mL] [Sigma-Aldrich, Milwaukee, WI], and 0.1% H_2O_2) for 10 min at room temperature. The reaction was stopped with 50 μL of 3 M H_2SO_4. Last, the plates were read at 492 nm in a microplate reader (Sensident Scan 352, Labsystems, Finland). Washes (at least five) with 0.05% Tween 20 in distilled water were carried out between each step. Results were expressed as the $\log_{10}$ titer calculated by interpolation of absorbance values at a fixed serum dilution into a linear regression analysis plotting dilution versus A_{492} of the standard curve of a known titer

serum. The titer was defined as the $\log_{10}$ highest dilution that gave twice the absorbance of negative control sera diluted 1:100. Titers are given as the geometric mean ± SD from the individual sera.

Measurement of interferon-γ and IL-4 cytokines in culture supernatants

Mice were immunized as described above. Ten days later, four animals per group were randomly selected and killed and duplicate aliquots (2×10^5 cells) of splenocytes were placed into 96-well microplates in complete medium and stimulated separately with CR3 and HBsAg (2.5 μg/mL). Cells were also cultured in medium alone and with concanavalin A (5 μg/mL) as negative and positive controls, respectively. Aliquots of supernatant were removed 50 h later and stored at −20°C. Interferon (IFN)-γ (Th1-type) and IL-4 (Th2-type) were assessed by a sandwich ELISA according to the manufacturer's recommendations (Invitrogen Pharmingen, San Diego, CA). Standard curves were obtained with recombinant mouse IFN-γ and IL-4 (Invitrogen Pharmingen).

Quantification of IFN-γ-secreting cells

The number of IFN-γ-secreting cells was detected in an enzyme-linked immunospot (ELISPOT) assay. Ten days after the last immunization, the spleens of four mice per group, drawn randomly, were aseptically removed and cell suspensions were prepared. Erythrocytes were lysed after a 5-min incubation in 0.83% NH_4Cl. The cells were extensively washed with medium, diluted in complete medium (RPMI 1640 [Invitrogen GIBCO, Paisley, UK] supplemented with 10% fetal calf serum [FCS], 2 mM glutamine, 2 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and antibiotics), and counted. Nitrocellulose-backed 96-well plates (MultiScreen-IP filter plates [cat. no. MAIPN4550]; Millipore, Bedford, MA) were coated with 100 μL of murine IFN-γ-specific mAb R4-6A2 (5 μg/mL; Invitrogen Pharmingen) overnight at 4°C, washed three times with PBS, and blocked with 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 in 5% CO_2 in complete medium with sterile and nonpyrogenic CR3 and HBsAg proteins at 2.5 μg/mL. Wells with splenocytes without protein were incubated as negative controls and with concanavalin A (2.5 μg/mL) as positive controls. The plates were washed three times with PBS and five times with 0.05% Tween 20 in PBS. Secondary biotin-conjugated antibody XMG1.2 (0.5 μg/mL; Invitrogen Pharmingen) was then added and incubated for 2 h at room temperature. The wells were washed five times with 0.05% Tween 20 in PBS, and peroxidase-labeled streptavidin (Sigma, St. Louis, MO) was added at a 1:1000 dilution for 1 h. The plates were then washed again with 0.05% Tween 20 in PBS, and afterward with PBS, and the spots were developed by adding 100 μL of 3-amino-9-ethylcarbazole (AEC; Sigma) solution (15). After 15 min, the reaction was stopped with tap water. Plates were dried, and spots were counted under a dissection microscope. Results were expressed as the number of spot-forming-cells (SFC) per 10^6 splenocytes after subtracting the spots of negative wells. Values above the mean number of spots in the placebo or negative group plus 3 standard deviations were considered positive.

Statistical procedures

GraphPad Prism version 4.00 statistical software (GraphPad Software, San Diego, CA) was used to carry out analysis of variance (ANOVA), *t* tests, and Mann–Whitney and Wilcoxon signed rank tests to determine the significance of differences between the means of groups. The Tukey test was chosen for post-ANOVA comparisons. All titers were transformed to $\log_{10}$ to obtain a normal distribution of the values. For the nonseroconverting sera, an arbitrary titer of 1:50 was assigned for statistical processing. $p < 0.05$ was considered statistically significant.

RESULTS

Adjuvant effect of $AlPO_4$ versus AlOOH

First, we studied the adsorption of the mixture HBsAg + HBcAg + CR3 to both aluminum-based adjuvants and no differences were observed. To study *in vivo* the role of the adjuvants, mice were immunized with the mixture HBsAg + HBcAg + CR3 in aluminum phosphate and aluminum hydroxide by the subcutaneous route. In addition, a group of animals was immunized with the current vaccine formulation based on HBsAg absorbed in aluminum hydroxide for comparison. The IgG1:IgG2a ratio, considered a useful parameter to discriminate Th1 and Th2 profiles (30,37,38,41), was determined against CR3 and HBsAg after the third dose in animal sera. In this regard, considering the CR3-specific response, better Th1 immunomodulation was obtained with the aluminum hydroxide adjuvant ($p = 0.0105$; two-tailed unpaired *t* test) (Fig. 1A). However, no difference was observed when the HBsAg-specific response was evaluated. In analyzing further, we noted that HBsAg-specific IgG1:IgG2a ratios induced by the multiantigenic formulation with aluminum hydroxide and aluminum phosphate were significantly lower than with HBsAg alone in aluminum hydroxide (the vaccine formulation) ($p = 0.0147$; one-way ANOVA and Tukey multiple comparison test). Whole IgG responses specific for CR3, HB-

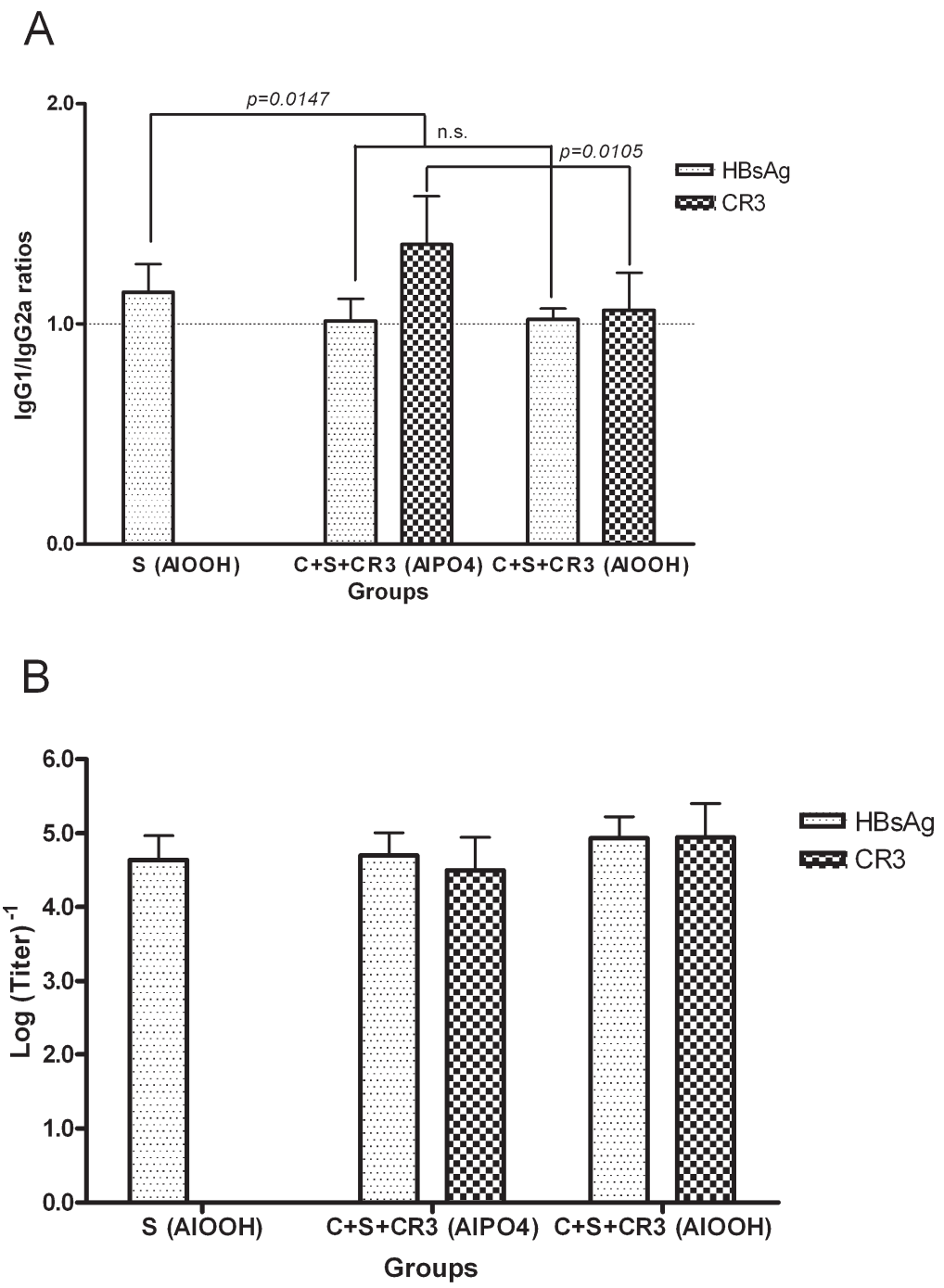

FIG. 1. Humoral immune response anti-HBsAg and anti-CR3. BALB/c mice were immunized three times with the following: (1) HBsAg (S) (subcutaneous, AlOOH), a similar formulation to the current commercial vaccine; (2) HBcAg (C) + HBsAg + CR3 (subcutaneous, $AlPO_4$); (3) HBcAg + HBsAg + CR3 (subcutaneous, AlOOH). Sera were collected 10 d after the last dose and titers were calculated by ELISA. (**A**) IgG1:IgG2a ratios and (**B**) total IgG titers. Data represent means + SD of nine mice per group. The dotted line in (**A**) represents the IgG1:IgG2a = 1 ratio.

sAg, and HBcAg antigens were not different between both adjuvants (Fig. 1B).

On the other hand, four animals per group were randomly selected and killed to evaluate IFN-γ and IL-4 secretion in the supernatant of splenocytes stimulated with CR3 and HBsAg antigens, and the frequency of IFN-γ-secreting cells in an *ex vivo* ELISPOT IFN-γ assay. After an overall analysis of the data, no differences were

found between groups of mice immunized with aluminum phosphate and aluminum hydroxide on considering the level of total IFN-γ and IL-4 secreted in the supernatants. However, intragroup analysis showed that IFN-γ levels were significantly higher than IL-4 levels only in the group inoculated with aluminum hydroxide adjuvant ($p = 0.0391$; Wilcoxon signed rank test) (Fig. 2). The median level of total IFN-γ secretion increased more than 4000 times compared with IL-4 secretion with AlOOH (median, 158,219; [range, 0 to 430,396] vs. 37.73 [range, 0.36–60.57], respectively). In the $AlPO_4$ group the same coefficient increased only about 10 times (273.1 [range, 0–497,258] vs. 27.75 [range, 7.225–138.9], respectively). To characterize further the Th1 response the frequency of IFN-γ-secreting cells was measured. In analyzing the specific anti-HBsAg response, we noted a significant increase in the median frequency of IFN-γ-secreting cells of 313 SFC/10^6 cells (range, 295 to 1085 SFC/10^6 cells) in the aluminum hydroxide group compared with 170 SFC/10^6 cells (range, 140 to 218 SFC/10^6 cells) in the aluminum phosphate group ($p = 0.0286$; two-tailed Mann–Whitney test). A similar evaluation for the anti-CR3-specific response was carried out. Unfortunately, for this antigen an unexpectedly high frequency of IFN-γ-secreting cells was scored in all animals (data not shown). Because of that it was impossible to determine any difference between groups.

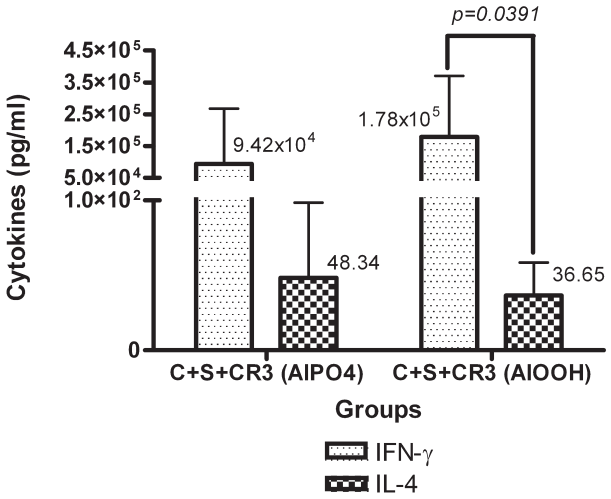

FIG. 2. Splenic IFN-γ and IL-4 responses after immunization with aluminum phosphate and aluminum hydroxide adjuvants. Mice were immunized on days 0, 14, and 35 with the following: HBcAg + HBsAg + CR3 (subcutaneous, AlPO4) or HBcAg + HBsAg + CR3 (subcutaneous, AlOOH). Ten days after the end of the schedule animals were killed and the splenocytes were purified, followed by HBsAg and CR3 stimulation in culture for 50 h. Culture supernatants were then harvested and assayed by ELISA. Results were calculated by subtracting the background of unstimulated control cultures from the net values of antigen-pulsed cultures. Data represent averages + SD of nine mice per group.

Immune responses elicited by several mixtures of HBsAg, HBcAg, and CR3

Considering the preceding results, aluminum hydroxide allowed better Th1 immunomodulation for the mixture of the recombinant protein CR3 from HIV-1 with HBV antigens. Because of that, we decided to continue the experiments with aluminum hydroxide instead of aluminum phosphate as adjuvant.

Three additional formulations, variations of the simple mixture of antigens, were prepared for comparison. First, the recombinant core and surface antigens of HBV were incubated together for several hours before the addition of CR3 and the adjuvant ([HBsAg + HBcAg] + CR3); second and third, the CR3 protein was incubated with HBsAg or HBcAg before addition of the other antigen of HBV ([HBsAg + CR3] + HBcAg and [HBcAg + CR3] + HBsAg, respectively). In addition, groups of mice immunized with placebo and with HBsAg + HBcAg were included for comparison. After three doses the humoral response against the antigens as well as the cellular immune response against CR3 were measured.

Whole IgG anti-HBsAg titers did not differ significantly among the four formulations of CR3, HBsAg, and HBcAg antigens tested (groups 3 to 6) or with the HBsAg + HBcAg group (Fig. 3). Regarding the anti-HBcAg response, mice immunized with the simple mixture HBsAg + HBcAg + CR3 elicited the highest titers compared with those inoculated with other mixtures of the same antigens (groups 4 to 6) ($p < 0.0001$; one-way analysis of variance and Tukey multiple comparison test).

As shown in Fig. 3, the highest anti-CR3 titers were elicited with the formulations HBsAg + HBcAg + CR3 and (HBsAg + HBcAg) + CR3 and the lowest titers with HBsAg + (HBcAg + CR3) ($p = 0.0212$; one-way analysis of variance and Tukey multiple comparison test). In mice immunized with HBcAg + (HBsAg + CR3) the geometric mean of titers was between those of the other groups and no statistically significant differences were observed.

Finally, although the simple mixture of antigens promoted the highest anti-CR3 frequency of IFN-γ-secreting cells in mouse spleen, significant differences from animals immunized with the rest of the formulations under study were not found (Fig. 4).

DISCUSSION

Data emerging from various therapeutic trials in humans suggest that multiple doses are required to increase

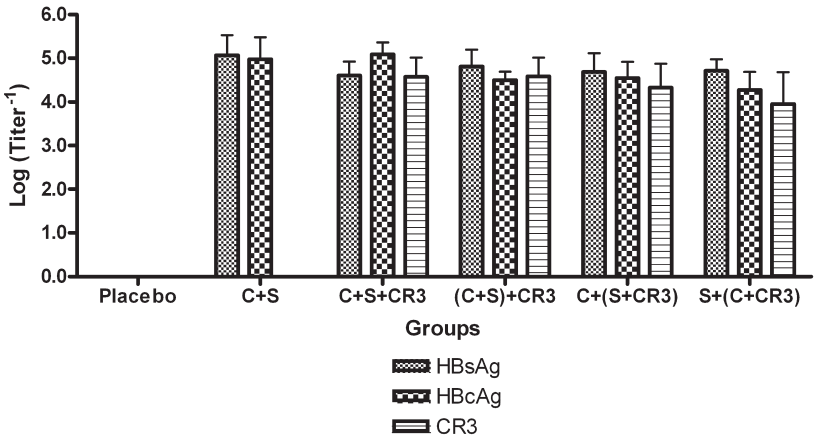

FIG. 3. Total IgG anti-HBsAg, anti-HBcAg, and anti-CR3 immune response for various antigen formulations. Twelve BALB/c mice per group were immunized subcutaneous on days 0, 14, and 42 with the following: (1) PBS (placebo), (2) HBsAg + HBcAg, (3) HBcAg + HBsAg + CR3, (4) (HBcAg + HBsAg) + CR3, (5) HBcAg + (HBsAg + CR3), or (6) HBsAg + (HBcAg + CR3). Ten days after immunization, sera were collected and titers were determined by ELISA. Data represent means + SD.

responses and the number of responding individuals (20). Therefore, vaccine candidates formulated with adjuvants having low toxicity would be advantageous. The only widely use adjuvants for human immunizations are aluminum-based adjuvants. Both aluminum hydroxide and aluminum phosphate are currently used in commercial vaccines (2). They have shown a good safety profile for many years, inducing only mild local reaction at the site of injection in most cases (10). Hence, they should be considered in therapeutic formulations. Many efforts have been made to elucidate the mechanisms of antigen absorption and immune enhancement of aluminum-based adjuvants. However, it is not fully understood how these adjuvants actually work. Consequently, selection of the specific aluminum-containing adjuvant must be based on experimental evidence.

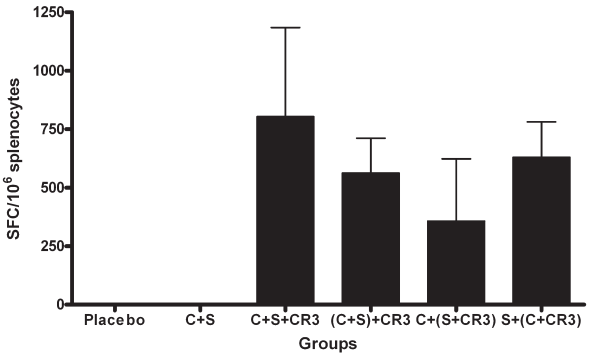

FIG. 4. CR3-specific response in the ELISPOT IFN-γ assay. BALB/c ($H\text{-}2^d$) mice were immunized subcutaneously on days 0, 14, and 42 with the following: (1) PBS (placebo), (2) HBsAg + HBcAg, (3) HBcAg + HBsAg + CR3, (4) (HBcAg + HBsAg) + CR3, (5) HBcAg + (HBsAg + CR3), or (6) HBsAg + (HBcAg + CR3). Splenocytes were removed 10 d after the last immunization and the number of IFN-γ-secreting cells, in response to stimulation with CR3 protein and medium alone, was measured. Results are expressed as the average number + SD of IFN-γ-secreting cells per million splenocytes from four mice per group.

In a series of previous experiments we demonstrated the capacity of the formulation HBsAg + HBcAg + CR3 to promote a Th1 immune response to the CR3 recombinant protein from HIV-1. The mixture was effective through the subcutaneous route of inoculation (19). In that case the aluminum phosphate adjuvant was selected as recommended (24), based on the predicted basic isoelectric point for the CR3 protein. However, emerging data suggest that the immune response is not affected by the degree of adsorption of the antigens to aluminum-containing adjuvants (3,5,22,42,43). Consequently, we decided to test whether aluminum phosphate could be considered the best adjuvant for our formulation before future developments. Therefore, an experimental comparison between both adjuvants was necessary to rule out any possibility to induce a better Th1 immunomodulation after inoculation with the mixture HBsAg + HBcAg + CR3.

In this study, several criteria were used to compare and contrast differences between the aluminum phosphate and hydroxide adjuvants. In mice, the associations Th1/IFN-γ-IgG2a and Th2/IL-4-IgG1 are well supported by experimental evidences (30,37,38,41). However, IgG1 antibodies can be secreted under an ongoing Th1 immune response (44). It is possible because the IgG1-coding sequence is upstream of the IgG2a-coding sequence, and

switching to IgG1 may also be induced by IL-2, which is a Th1 interleukin (9,12). Consequently, *sensu strictisimo,* only the presence or deficiency of IgG2a antibodies may be considered a surrogate marker of helper T cell immune status. In this regard, a high or low IgG1:IgG2a ratio results in a Th2 or Th1 immune response, respectively.

Our results indicate that BALB/c mice immunized with a mixture of HBsAg + HBcAg + CR3 with aluminum hydroxide adjuvant developed a better Th1-biased response than with the aluminum phosphate, as indicated by the increased level of CR3-specific IgG2a antibodies (lower IgG1:IgG2a ratio). However, the HBsAg-specific IgG1:IgG2a ratios were not different between the adjuvants. These observations support the notion that a differential Th1–Th2 commitment of the immune system might arise to antigens in combined formulations (21). It is important to note that whole IgG titers against CR3, HBsAg, and HBcAg were of similar magnitude between both groups of animals and it suggests the absence of a differential immunoenhancing effect on the specific IgG responses. In addition, the lower IgG1:IgG2a ratio observed with both adjuvant formulations compared with the vaccine formulation suggests the possible anti-HBV therapeutic application of the mixture of antigens.

When considering the potential role of the aluminum adjuvants ($AlPO_4$ and AlOOH) on anti-CR3 and anti-HBsAg IFN-γ and IL-4 secretions, as a whole, we did not find significant differences. However, we did find better Th1 polarization (IFN-γ vs. IL-4 secretion) in the CR3 + HBsAg + HBcAg group adjuvated with aluminum hydroxide. Consistent with this finding, a higher frequency of specific anti-HBsAg IFN-γ-secreting cells was also induced. These results provide an explanation and confirm the previous differences in the IgG1:IgG2a ratios.

Previous studies have shown differential induction of helper T cell patterns between the aluminum phosphate and hydroxide adjuvants. Wang *et al*. demonstrated that IL-12 combined with aluminum phosphate induced a far better Th1 response against HBsAg than did aluminum hydroxide (42). Interestingly, the adjuvant effect of IL-12 bound to aluminum phosphate was not related to differential adsorption to either adjuvant. In our investigation, we demonstrated that the simple mixture of the CR3, HBsAg, and HBcAg antigens is equally adsorbed to aluminum phosphate and aluminum hydroxide whereas the last adjuvant allowed a stronger commitment of the immune system to the Th1 response. The fact that the immunoenhancing activity of aluminum-based adjuvants does not involve any TLR pathway in antigen-presenting cells (35) might explain why Th1 immunomodulating agents dominate in combining formulations. Taken together, these observations suggest that there are subtle differences in the effect of both aluminum-based adjuvants that modulate the resulting helper T cell pattern, depending also on the nature and influence of the antigens. Systematic comparisons of the adjuvant effect addressing quantitative and/or quality aspects are needed.

In multiantigenic formulations the resulting immunity will be a consequence of the individual characteristics of the antigens as well as of new features that emerge as a result of reciprocal influences among them. HBcAg induces a strong Th1 response, allowing deviation of the immune response elicited to coadministered antigens (1,27,32). On the other hand, animals immunized with CR3 and HBsAg elicit a Th2 response after inoculation with alum-based adjuvants (19). Thus, we consider the influence of HBcAg as the main cause of the specific anti-CR3 and anti-HBsAg IFN-γ secretion and IgG2a antibody production in the mixture of CR3 + HBsAg + HBcAg. However, it is worth noting the immunoenhancing effect contributed by HBsAg to the ongoing anti-HBcAg Th1 response in combining formulations with HBcAg (1,27) and to the ongoing anti-CR3 Th1 response in combining formulations with the former antigen and CR3 protein (19). In that sense, the proteoliposomal nature of the surface antigen (33) might be related to this effect. In addition, there is a spontaneous tendency of the surface and core antigens to aggregate to form supramolecular structures up to 200 nm in size (1). In our multiantigenic formulation, assuming the association with CR3 protein (19), the size might increase further than 200 nm. Such structures will increase the phagocytic internalization of the antigens (4) and consequently their immunogenicity. Finally, bystander stimulation due to TLR stimulation (23,40) and antigen-specific ongoing immune responses elicited in the vicinity might also contribute to the individual immunogenicity of the antigens.

A preliminary experiment was also carried out with various formulations of the antigens. Previous studies showed that CR3, HBsAg, and HBcAg aggregate in solution (19). Thus, it could be argued that preferential interactions between particular pairs of such antigens might impact on the specific immune response elicited against some of them. This hypothesis corresponds well with our data showing that the antibody levels among groups of animals differed with the anti-HBcAg and anti-CR3 humoral responses. Nevertheless, anti-HBsAg levels did not differ among the groups. These results are in line with previous findings showing a stronger interaction between CR3 and HBcAg than with CR3-HBsAg and it might explain the former results. Because the anti-HBcAg and anti-CR3 antibodies might be of limited practical value, we emphasize the protective value of the anti-HBsAg humoral response (16). In this regard, the strong interaction between CR3 and HBcAg could benefit the anti-HBsAg humoral response. In addition, because we did not find any difference in the frequency of anti-CR3 IFN-γ-se-

creting cells among the antigen formulations it could be argued that no other formulation elicited a better immune response than the simple mixture of HBsAg, HBcAg, and CR3.

In summary, the results thus far revealed evidence that aluminum hydroxide is a better choice than aluminum phosphate for Th1 induction after immunization with the mixture CR3 + HBcAg + HBsAg. In addition, in the absence of evidence against the former formulation of antigens this work suggests the use of aluminum hydroxide as a potential adjuvant in antigen formulations eliciting cellular anti-HBV and anti-HIV-1 immunity in humans.

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REFERENCES

1. Aguilar JC, Lobaina Y, Muzio V, Garcia D, Penton E, Iglesias E, Pichardo D, Urquiza D, Rodriguez D, Silva D, Petrovsky N, and Guillen G: Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. Immunol Cell Biol 2004;82:539–546.
2. Baylor NW, Egan W, and Richman P: Aluminum salts in vaccines: US perspective. Vaccine 2002;20:S18–S23.
3. Berthold I, Pombo ML, Wagner L, and Arciniega JL: Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants. Vaccine 2005;23:1993–1999.
4. Brewer JM, Pollock KG, Tetley L, and Russell DG: Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles. J Immunol 2004;173: 6143–6150.
5. Chang M, Shi Y, Nail SL, HogenEsch H, Adams SB, White JL, and Hem SL: Degree of antigen adsorption in the vaccine or interstitial fluid and its effect on the antibody response in rabbits. Vaccine 2001;19:2884–2889.
6. Clements CJ, and Griffiths E: The global impact of vaccines containing aluminium adjuvants. Vaccine 2002;20: S24–S33.
7. Cooper PD: Vaccine adjuvants based on γ inulin. Pharm Biotechnol 1995;6:559–580.
8. Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, and Krieg AM: CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–876.
9. DeKruyff RH, Mosmann RR, and Umetsu DT: Induction of antibody synthesis by $CD4^+$ T cells: IL-5 is essential for induction of antigen-specific antibody responses by T_H2 but not T_H1 clones. Eur J Immunol 1990;20:2219–2227.
10. Duclos P: Safety of immunisation and adverse events following vaccination against hepatitis B. Expert Opin Drug Saf 2003;2:225–231.
11. Ericsson H: Purification and adsorption of diphtheria toxoid. Nature 1946;158:350–351.
12. Finkelman FD, Katona IM, Urban JF Jr, Snapper CM, Ohara J, and Paul WE: Suppression of *in vivo* polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1. Proc Natl Acad Sci USA 1986;83:9675–9678.
13. Glenny A, Pope C, Waddington H, and Wallace U: The antigenic value of toxoid precipitated by potassium alum. J Pathol Bacteriol 1926;29:38–45.
14. Hardy E, Martinez E, Diago D, Diaz R, Gonzalez D, and Herrera L: Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*. J Biotechnol 2000;77:157–167.
15. Helms T, Boehm BO, Asaad RJ, Trezza RP, Lehmann PV, and Tary-Lehmann M: Direct visualization of cytokine-producing recall antigen-specific CD4 memory T cells in healthy individuals and HIV patients. J Immunol 2000;164:3723–3732.
16. Hilleman MR: Overview of the pathogenesis, prophylaxis and therapeusis of viral hepatitis B, with focus on reduction to practical applications. Vaccine 2001;19:1837–1848.
17. HogenEsch H: Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 2002. 20: S34–S39.
18. Iglesias E, Ruiz M, Carrazana Y, Cruz LJ, Aguilar A, Jimenez V, Carpio E, Martinez M, Perez M, Martinez C, Cruz O, Martin A, and Duarte C: Chimeric proteins containing HIV-1 T cell epitopes: Expression in *E. coli*, purification and induction of antibodies in mice. J Biochem Mol Biol Biophys 2001;5:109–120.
19. Iglesias E, Thompson R, Carrazana Y, Lobaina Y, Garcia D, Sanchez J, Garcia J, Cruz O, Brown E, Martin A, Muzio VL, and Aguilar JC: Coinoculation with hepatitis B surface and core antigen promotes a Th1 immune response to a multiepitopic protein of HIV-1. Immunol Cell Biol 2006;84:174–183.
20. Im EJ, and Hanke T: MVA as a vector for vaccines against HIV-1. Expert Rev Vaccines 2004;3:S89–S97.
21. Ismail N, and Bretscher PA: The Th1/Th2 nature of concurrent immune responses to unrelated antigens can be independent. J Immunol 1999;163:4842–4850.
22. Iyer S, HogenEsch H, and Hem SL: Effect of the degree of phosphate substitution in aluminum hydroxide adjuvant on the adsorption of phosphorylated proteins. Pharm Dev Technol 2003;8:81–86.

23. Kamath AT, Sheasby CE, and Tough DF: Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-$\alpha\beta$ and IFN-γ. J Immunol 2005;174:767–776.

24. Lindblad EB: Aluminium adjuvants: In retrospect and prospect. Vaccine 2004;22:3658–3668.

25. Lindblad EB: Aluminium compounds for use in vaccines. Immunol Cell Biol 2004;82:497–505.

26. Lobaina Y, Garcia D, Abreu N, Muzio V, and Aguilar JC: Mucosal immunogenicity of the hepatitis B core antigen. Biochem Biophys Res Commun 2003;300:745–750.

27. Lobaina Y, Palenzuela D, Pichardo D, Muzio V, Guillen G, and Aguilar JC: Immunological characterization of two hepatitis B core antigen variants and their immunoenhancing effect on co-delivered hepatitis B surface antigen. Mol Immunol 2005;42:289–294.

28. Maschmann E, Küster E, and Fischer W: Über die Fähigkeit des Tonerde-Präparates B, Diphtherie-Toxin zu adsorbieren. Ber Dtsch Chem Ges 1931;64:2174–2178.

29. Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, and Hem SL: Role of aluminum-containing adjuvants in antigen internalization by dendritic cells *in vitro*. Vaccine 2005;23:1588–1595.

30. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, and Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348–2357.

31. Riedl P, Buschle M, Reimann J, and Schirmbeck R: Binding immune-stimulating oligonucleotides to cationic peptides from viral core antigen enhances their potency as adjuvants. Eur J Immunol 2002;32:1709–1716.

32. Riedl P, Stober D, Oehninger C, Melber K, Reimann J, and Schirmbeck R: Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. J Immunol 2002;168:4951–4959.

33. Satoh O, Imai H, Yoneyama T, Miyamura T, Utsumi H, Inoue K, and Umeda M: Membrane structure of the hepatitis B virus surface antigen particle. J Biochem (Tokyo) 2000;127:543–550.

34. Schijns VE: Immunological concepts of vaccine adjuvant activity. Curr Opin Immunol 2000;12:456–463.

35. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, and Medzhitov R: Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2001;2:947–950.

36. Seeber SJ, White JL, and Hem SL: Predicting the adsorption of proteins by aluminium-containing adjuvants. Vaccine 1991;9:201–203.

37. Sideras P, Bergstedt-Lindqvist S, and Severinson E: Partial biochemical characterization of IgG1-inducing factor. Eur J Immunol 1985;15:593–598.

38. Snapper CM, and Paul WE: Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;236:944–947.

39. Timofeev AV: Unexpected problem of the traditional approach to the addition of aluminium hydroxide to vaccine preparations. Vaccine 2005;23:2953.

40. Tough DF, Borrow P, and Sprent J: Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. Science 1996;272:1947–1950.

41. Vitetta ES, Ohara J, Myers CD, Layton JE, Krammer PH, and Paul WE: Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. J Exp Med 1985;162:1726–1731.

42. Wang S, Liu X, and Caulfield MJ: Adjuvant synergy in the response to hepatitis B vaccines. Vaccine 2003;21:4297–4306.

43. Weissburg RP, Berman PW, Cleland JL, Eastman D, Farina F, Frie S, Lim A, Mordenti J, Nguyen TT, and Peterson MR: Characterization of the MN gp120 HIV-1 vaccine: Antigen binding to alum. Pharm. Res. 1995;12:1439–1446.

44. Yip HC, Karulin AY, Tary-Lehmann M, Hesse MD, Radeke H, Heeger PS, Trezza RP, Heinzel FP, Forsthuber T, and Lehmann PV: Adjuvant-guided type-1 and type-2 immunity: Infectious/noninfectious dichotomy defines the class of response. J Immunol 1999;162:3942–3949.

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