A new strategy for the induction of specific CD4+ and CD8+ cellular responses against a recombinant multiepitopic protein of HIV-1

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ABSTRACT

Modern vaccine development usually requires highly purified recombinant proteins. However, these antigens in general are poorly immunogenic, and when administered in aluminum-based adjuvants—the only universally accepted formulation—the elicited response has a Th2 bias that is inadequate for immunizing against pathogens such as the Human Immunodeficiency Virus type 1 (HIV-1), whose control requires a vigorous cellular immune response. The purpose of this work was to obtain a strong cellular immune response specifically directed against a recombinant multiepitopic antigen that includes HIV-1 sequences of relevance to vaccine development. This was achieved by taking advantage of the adjuvanticity of the surface (HBsAg and S) and nucleocapsid (HBcAg and C) antigens of the hepatitis B virus (HBV) and using the parenteral and/or mucosal immunization routes. After immunizing mice with formulations containing these antigens, it was evidenced that the mixtures of the HIV-1-derived recombinant multiepitopic antigen (CR3) with the surface and nucleocapsid antigens from HBV generate a strong Th1-type response. The purpose of this work was to obtain a strong cellular immune response specifically directed against a recombinant multiepitopic antigen that includes HIV-1 sequences of relevance to vaccine development. This was achieved by taking advantage of the adjuvanticity of the surface (HBsAg and S) and nucleocapsid (HBcAg and C) antigens of the hepatitis B virus (HBV) and using the parenteral and/or mucosal immunization routes. After immunizing mice with formulations containing these antigens, it was evidenced that the mixtures of the HIV-1-derived recombinant multiepitopic antigen (CR3) with the surface and nucleocapsid antigens from HBV generate a strong Th1-type response—detected by an increased secretion of IFN-γ and the proliferation of CD4+ and CD8+ cells—after parenteral and nasal inoculations. Furthermore, it was shown that co-immunization through the nasal and parenteral routes generates more potent immune responses than parenteral delivery alone; and that a pre-existing specific response against HBV antigens does not inhibit the development of a strong CR3-specific CD4+ and CD8+ cellular responses.

Introduction

The development of vaccine candidates against the Human Immunodeficiency Virus type 1 (HIV-1) currently needs new adjuvants and strategies that promote the generation of immune responses in both systemic and mucosal compartments. Although the scientific community has invested a considerable amount of research on the development of a prophylactic or therapeutic vaccine against this pathogen, the results so far have been disappointing. Meanwhile, the current success of antiretroviral therapies and the extension of their use to a growing number of patients can potentially change the landscape of the current pandemic to a situation characterized by the predominance of recombinant isolates, with mutations conferring resistance to antiretroviral drugs and a significant increase in the number of co-infections with other viruses. For instance, it is known that the rate of co-infections of HIV-1 with the hepatitis C virus (HIV-HCV) is as high as 50% among HIV-seropositive patients in some territories [1], and 70 to 90% of these patients have had an active HBV infection [2-4]. These co-infections decrease the efficacy of antiretroviral therapies while increasing their side effects; furthermore, they accelerate the progression of some diseases, as has been shown for HCV and HBV [5, 6]. In some parts of the world the HIV epidemic continues to grow alarmingly, like in sub-Saharan Africa—where the South African epidemic is one of the worst in the world—and in the Caribbean [7]. Therefore, the development of an effective vaccine against HIV-1 is still a high priority for the international scientific community.

A considerable body of evidence obtained through the study of the HIV-1 infection supports the notion that a cellular immune response is essential for the control of viral replication [8]. Currently, several HIV-1 proteins and multiepitopic polypeptides are studied in different strategies aimed at generating a cellular response. The leading approaches in this sense are based on the use of recombinant live vectors, such as canarypox or adenovirus [9, 10]; however, these approaches have several disadvantages. One of their main drawbacks is that live vectors cannot be used in immunization schemes that include several parenteral inoculations, since anti-vector immunity can reach levels 20- to 30-fold higher than those against the recombinant protein [11]. Additionally, for some of these vectors there is a very strong pre-existing immune response in human populations due to a high incidence of infection by their wild-type counterparts [12]. Because of these, the eventual success of these vectors is limited.

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The genetic engineering technology is a powerful tool for obtaining pure recombinant vaccine antigens. However, it is widely recognized that these antigens are poorly immunogenic. Therefore, one of the main objectives for adjuvants development is the improvement of the immunogenicity of recombinant antigens (Ag), thus eliminating the risk and toxicity associated to the delivery of Ag obtained directly from the microorganism. However, the inherent toxicity of adjuvants is a significant stumbling block for the development of new vaccine adjuvants and formulations.

To obtain a multiepitopic protein for a vaccine, several different epitopic regions from HIV-1 were selected, taking into consideration: 1, the presence, during natural infection, of important cellular responses against the selected fragments; 2, that the variability of the regions should be as low as possible; 3, the design should strive for a wide representation of viral proteins and 4, the protein should comprise epitopes present in the context of the most frequent HLA haplotypes. These efforts resulted in the design of the recombinant protein CR3, which contains several cytotoxic T lymphocyte (CTL), helper T cell (Th) and B cell epitopes from different isolates belonging to subtype B of HIV-1 [13]. In a previous report, it was shown that the epitopes included in CR3 are efficiently recognized by the CTL generated during natural infection in HIV-positive patients [14]. On the other hand, it has also been evidenced that it is possible to induce a Th1 immunodominance in the anti-HBsAg response after mucosal and parenteral inoculations with HBcAg + HBsAg mixture, simultaneously synergizing the immune response to both Ags [15]. This result highlighted the potential of this mixture for adjuvanting purposes.

The aim of this study was the development of a new formulation for parenteral and/or mucosal inoculations to increase the immunogenicity of a multiepitopic Ag derived from HIV-1 (CR3). This paper opens the way to the development and manufacturing of prophylactic and/or therapeutic vaccine candidates to be used in immunization strategies that involve the administration of repeated doses, for obtaining mucosal and systemic immune responses.

**Results and discussion**

**Demonstration of the Th1 adjuvant effect of the mixture of HBsAg plus HBcAg**

In order to study the adjuvanticity of the surface and nucleocapsid Ag of HBV on the anti-CR3 response, mucosal and parenteral inoculations (using the intranasal (in) and subcutaneous (sc) routes, respectively) were performed using separate mixtures of CR3 with each HBV Ag or with both Ags simultaneously. The inoculations also included control groups, one of them comprising the CR3 protein alone. For mucosal inoculations, the immunogen was diluted in phosphate-buffered saline; the parenteral immunizations used aluminum phosphate as the adjuvant, which was chosen after taking into account the proven safety of this adjuvant in humans, the isoelectric point of CR3, and the results from preliminary studies which showed that the mixture of HBcAg with HBsAg and CR3 in saline solution without adjuvants is poorly immunogenic. Therefore, one group was immunized with the combination of the three antigens (HBcAg, HBsAg and CR3) by the sc and in routes, it was possible to detect frequencies of CR3-specific IFN-γ-secreting cells in response to stimulation with the CR3 protein with each HBV Ag or with both Ags which had a similar behavior (Figure 1). In spite of this, the inclusion of HBcAg in the formulation proved to be important for the induction of a Th1 response. Further studies in mice inoculated with the HBcAg + HBsAg + CR3 formulation evidenced that it stimulated the proliferation of CR3 and HBsAg-specific CD4+ and CD8+ cells in mice spleen. Another interesting finding from *in vitro* experiments was the existence of a bystander stimulation effect between the CR3 and HBsAg Ags that potentiates the response against both Ag. This could be the basis of the observed adjuvant effect. Lastly, it was proven that the anti-HBsAg humoral response in mice immunized subcutaneously with CR3 + HBsAg + HBcAg remains at the same levels as in mice inoculated with HBsAg + HBcAg, and although the titers decrease if the mice are inoculated intranasally, they are still significantly lower than those observed in mice immunized with the CR3 + HBsAg + HBcAg mixture.

**Figure 1.** Quantification of the specific anti-CR3 response with a ELISPOT assay. Balb/c mice (H-2d) were immunized in (A) and sc (B) with: 1, Placebo; 2, S+C; 3, CR3; 4, CR3+C; 5, CR3+S; 6, CR3+S+C. The splenocytes were obtained 10 days after the last dose, and the frequency of IFN-γ-secreting cells in response to stimulation with the CR3 protein was quantified. The results are shown as the number of spot-forming cells (SFC) per million splenocytes in mixtures of three spleens per group. The standard deviation is obtained from measures on three wells. The threshold values are represented with dotted lines.

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significantly high. Hence, the mixture of the three Ag was chosen for further work [16].

**Comparison of the adjuvant effect of aluminum phosphate vs. aluminum hydroxide**

The selection of an appropriate adjuvant is a major issue during the design of a vaccine formulation. Aluminum-based adjuvants were first described at the beginning of the past century, and in spite of the many experimental adjuvants described so far, those based on aluminum are still most widely used in vaccines currently available. On the other hand, the data collected from clinical trials with therapeutic vaccine candidates have revealed that multiple doses are required to increase the level of the immune response as well as the frequency of responding patients. Therefore, there is a need for adjuvants with very low toxicity; and in this sense aluminum-based adjuvants represent the ideal choice due to their excellent safety profile, demonstrated with millions of doses administered in human beings. Currently, the aluminum-based adjuvants most widely used in commercial vaccines are aluminum hydroxide (AlOOH) and aluminum phosphate (AlPO4). The AlOOH is currently used in the formulation of the Heberbiovac HB vaccine, manufactured at the Center for Genetic Engineering and Biotechnology (CIGB) for the prevention of HBV infections. Therefore, we decided to compare the immune response induced by both adjuvants in the formulation previously selected as described above [17].

The experiments with mice immunized with the mixture of the three Ag (HBcAg + HBsAg + CR3) showed that AlOOH favored a better Th1 bias of the anti-CR3 response (IgG1/IgG2a ratios) (p < 0.05) and potentiated the secretion of IFN-γ compared with that of IL-4 (p < 0.05) (Figure 2). Ag adsorption to both adjuvants is highly efficient; therefore this factor does not account for the differences found in this experiment. Other authors have also found differences between both adjuvants [18]; however, the causes underlying this behavior are still unknown. Based on these results, we decided to use AlOOH as the adjuvant for all further parenteral immunizations.

**Comparison between methods for preparing the Ag mixture**

In the previous experiments, the Ag were mixed and adjuvanted at the same time with aluminum hydroxide (simple mix); however, the method used to mix the components of the formulation may be relevant, and it is therefore important to compare different methodologies for this operation. When working with several Ag, it is possible for Ag-Ag interactions exert some influence over the adsorption of the resulting aggregate and therefore, its presentation to the immune system, thus generating quantitative and/or qualitative differences in the immune response elicited in experimental animals.

In order to study and compare in vivo the effect of the specific method used to mix the Ag formulation on the frequency of IFN-γ-secreting cells, several mixtures were prepared in which two Ag were first mixed, followed by the inclusion of the third component. These mixtures were compared with mixing the three Ag at the same time. After the immunizations, there were no statistically significant differences between the mixtures as evaluated by the frequency of anti-CR3 IFN-γ-secreting cells, although the simple mixture reached the highest frequency levels (Figure 3). Since the more complex and labor intensive variants with a defined mixing sequence showed no increase in the immune response, the simple simultaneous mixing of the three Ag was chosen to be used in future experiments [17].

**Co-inoculations using the mucosal and parenteral routes**

Several reports in the literature have shown that co-inoculations through the mucosal and parenteral routes generates a better immune response, both

![Figure 2. Evaluation of the IgG1:2a ratio (A) and the secretion of cytokines (IFN-γ and IL-4) after stimulation with CR3 (B). The sera and splenocytes to study the antibodies and cytokines were obtained 10 days after the last inoculation.](image-url)

The study of the pre-existing response to HBV antigens is very important in the context of this investigation, since it could limit the development of antigens.

Influence of a pre-existing anti-HBsAg and anti-HBcAg response on the anti-CR3 response

The study of the pre-existing response to HBV antigens is very important in the context of this investigation, since it could limit the development of antigens. Therefore, we decided to compare the immune responses generated after parenteral immunization with those obtained from nasal and parenteral co-inoculations.

Mice from experimental and control groups were immunized with identical doses for each Ag, using mixtures prepared following the same procedures and adjuvanted identically. The results agreed with earlier reports in the literature in that the animals immunized intranasally first and then subcutaneously developed a higher frequency of anti-CR3 IFN-γ-secreting cells compared to those inoculated solely through the subcutaneous route (Table 1). Furthermore, there was a reduction of the anti-HBsAg IgG1:2a antibody ratio. These studies proved that the combination of inoculation routes improves immunological parameters measured in systemic compartments as compared to parenteral immunization alone.

Table 1. Comparison of groups immunized parenterally or through both routes (parenteral and mucosal). The results are presented as the number of spot-forming cells per 10⁶ spleen cells in an anti-CR3 IFN-γ ELISPOT after in vitro re-stimulation for seven days.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse</th>
<th>CR3</th>
<th>Group</th>
<th>Mouse</th>
<th>CR3</th>
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</thead>
<tbody>
<tr>
<td>CR3 + HBsAg</td>
<td>1</td>
<td>1260</td>
<td>CR3 + HBcAg +</td>
<td>1</td>
<td>3710</td>
</tr>
<tr>
<td>SC route</td>
<td>2</td>
<td>1160</td>
<td>HBsAg 1st and</td>
<td>2</td>
<td>&gt;8000</td>
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<td></td>
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In conclusion, the formulation might provide the added benefit of immunity against hepatitis B. In order to protect this novel vaccination strategy, a patent application was submitted in 2003 [23]. Currently, a number of studies are being conducted to gain insight into the mechanisms underlying these results.

Figure 3. Quantification of the anti-CR3 response using an IFN-γ ELISPOT. Balb/c mice were immunized with different antigen formulations. The couple of antigens mixed before the addition of the last one is shown in parentheses.

Figure 4. Study of the proliferation of anti-CR3 CD4+ and CD8+ cells after staining with CFSE and analysis by flow cytometry. Groups: 1, positive control; 2, placebo; 3, with pre-existing response against HBcAg; 4, with pre-existing response against HBcAg and HBsAg.