

Use of DNA priming and vaccinia virus boosting to trigger an efficient immune response to HIV-1 gp120

Research Article

Dolores Rodríguez¹, Juan Ramón Rodríguez¹, Mercedes Llorente², Pilar Lucas², Mariano Esteban^{1,3}, Carlos Martínez-A.² and Gustavo del Real²

Departments of Molecular & Cellular Biology¹ and Immunology & Oncology², Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, E-28049 Madrid, Spain

³Corresponding author: Tel: (+34) 91-585-4503; Fax: (+34) 91-585-4506; E-mail: mesteban@cnb.uam.es

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Summary

To enhance the efficiency of DNA vaccines to HIV-1, we immunized BALB/c mice sequentially with a gp120 DNA vector and a recombinant vaccinia virus (VV). We have also evaluated the effect of granulocyte macrophage colony stimulation factor (GM-CSF) expression by a DNA vector on both cellular and humoral immune responses when coadministered with the gp120-encoding DNA at priming. Our results show a significant enhancement of both arms of the immune system when the DNA prime/VV boost regime is used, as compared with the immunization protocol based on priming and boosting with vector DNA. A 100-fold increase in the number of antigen-specific IFN- γ -secreting CD8⁺ T cells was observed in splenocyte cultures from mice immunized with the combined vector DNA/VV protocol. The humoral immune response is also improved in animals receiving the vector DNA/VV combined vaccine, as shown by the increase in both env-specific antibody titers and HIV-1 neutralizing activity in sera. IgG1 was the predominant isotype detected in sera from the immunized animals. This, together with the IL-4 and IFN- γ production in splenocyte cultures from these animals, indicated that both Th1 and Th2 responses are induced by the combined immunization approach. Coadministration of a GM-CSF-expressing DNA vector in the priming step resulted in enhanced T cell proliferation rates, irrespective of whether the booster was given with vector DNA or recombinant VV. In addition, a slight increase in the humoral immune response was also observed in animals primed with gp120 and GM-CSF-expressing plasmid and boosted with recombinant VV. These findings describe a combinatorial priming/booster immunization approach that may be effective in the control of HIV-1 infection and of other pathogens.

I. Introduction

Since the mid-1980's, when HIV was first isolated from patients, a variety of vaccine constructs and vaccination strategies have been explored to combat this virus, although none has yet been demonstrated to be effective in preventing *in vivo* HIV infection.

Effective vaccines must stimulate the correct balance between cellular (Th1-driven) and humoral (Th2-driven) immune responses according to the particular infectious agent; strategies aimed toward triggering a both humoral and cellular responses are thus receiving considerable attention. Although the HIV-1 envelope (env) glycoprotein

contains epitopes that activate T cell memory, and against which neutralizing antibodies as well as anti-HIV-1 cytotoxic T cells (CTL) are directed, vaccination with recombinant env glycoprotein induces only modest anti-HIV-1 CTL activity.

DNA immunization represents a novel approach to vaccine development and immunotherapy aimed toward immune response control. DNA vaccination induces antigen-specific cellular and humoral immune responses through the delivery of non-replicating transcription units, which drive the synthesis of specific foreign proteins within the inoculated host. Inoculation with plasmid DNA has been shown to be protective in several viral disease

models (Tighe *et al.* 1998). DNA vaccines are simple to manufacture, provide prolonged antigen expression, and allow manipulation of protein antigenicity at the cDNA level with no need for protein production and purification. Unlike conventional protein vaccines, plasmid DNA vaccination leads to antigen processing and presentation by MHC class I and class II molecules and thus more closely resembles a viral infection. DNA vectors are particularly effective in inducing a strong MHC class I-restricted CTL response. Virus-specific CTL have been induced by gene vaccination with plasmid DNA encoding influenza (Raz *et al.*, 1994) and HIV-1 viral proteins (Fuller *et al.*, 1994); high CTL activity levels have been detected more than one year after intradermal gene vaccination with plasmid DNA encoding influenza virus nucleoprotein (Raz *et al.*, 1994). Intradermal gene vaccination in mice induces antigen-specific Th1 cells secreting high IFN- γ levels, and stimulates IgG2a isotype antibody production (Raz *et al.*, 1996; Sato *et al.*, 1996). The safety, feasibility and immunogenicity of DNA vaccines are currently under evaluation in clinical trials. Immunization with DNA coding for HIV-1 gp160 elicits neutralizing antibodies and CTL responses in mice and in primates (Wang *et al.*, 1995), and HIV-1-infected chimpanzees immunized with DNA expressing env substantially decreased the HIV-1 viral load (Ugen *et al.*, 1997).

In general, the antibody response induced by gene vaccination is lower than that induced by protein vaccination, possibly due to the minute amount of gene product produced *in vivo*. The overall potency of naked DNA vaccines is less than that of recombinant vaccines; since DNA does not undergo replicative amplification as occurs with live recombinant viral and bacterial vaccines, the amount of antigen ultimately presented to the immune system may be limited. Although DNA vaccination appears to be a promising strategy, it may nonetheless require the use of immunomodulators, adjuvants such as cytokines, or specific immunization regimes to enhance immune responses.

Several groups have recently shown enhancement of the response triggered by DNA vaccination by coinjection of cytokine-encoding plasmids (Xiang & Ertl, 1995). Codelivery of vectors encoding a cytokine such as GM-CSF can augment antigen-specific responses following either intramuscular or epidermal plasmid DNA delivery (Xiang & Ertl, 1995). We have recently demonstrated that a recombinant vaccinia virus (VV) expressing a chimeric GM-CSF/gp120 fusion protein induces a significant increase in cellular immune responses against the HIV-1 env antigen (Rodríguez *et al.*, 1998). It has also been shown that GM-CSF, given at priming in the form of a DNA/GM-CSF chimeric vaccine, increases the magnitude of the anamnestic response, irrespective of the antigen form used in the subsequent booster immunization (Gerloni *et al.*, 1998). GM-CSF enhances viability and function of dendritic cells (DC) (Heufler *et al.*, 1998; Witmer *et al.*, 1987), activates cells of the dendritic lineage *in vitro* (Inaba *et al.*, 1992), and potentiates antigen

presentation *in vivo* (Disis *et al.*, 1996). GM-CSF is neither a Th1 nor a Th2 cytokine and can exert its adjuvant effect without skewing the Th1/Th2 balance.

We previously described a vaccination protocol based on priming with influenza virus expressing a CD8⁺ T cell epitope of circumsporozoite (CS) protein from *Plasmodium yoelii*, followed by boosting with a VV recombinant expressing the entire CS protein which induces high protection levels against malaria in the murine model system (Li *et al.*, 1993; Rodrigues *et al.*, 1994). Other recent experiments in mice have shown that priming with DNA and boosting with recombinant vaccinia virus (VV) expressing a circumsporozoite protein is associated with high immunogenicity and protective efficacy against *P. berghei* (Sedegah *et al.*, 1998). Delivering recombinant VV in the boost rather than in the priming dose may elude development of host immunity to the viral portion of the immunogen, which would reduce the response to the booster. The critical event in priming may depend on the processing properties of the immunogen expressed (influenza or DNA better than VV), whereas the secondary response is dependent on the quantity of immunogen expressed (VV better than influenza or DNA).

To improve the efficiency of a HIV-1 vaccine, we have implemented a vaccination regime consisting of priming with DNA vectors expressing gp120 and GM-CSF, followed by boosting with a recombinant VV expressing env. We also present compelling data that support the enhancement of both humoral as well as a cytotoxic T cell response by priming with DNA plasmids containing the appropriate antigen plus GM-CSF and boosting with a recombinant vaccinia virus containing the same antigen as that used for the initial immunization.

II. Results

A. Enhanced humoral immune response to HIV-1 gp120 by the priming/booster approach: vector DNA/VV recombinant

We have attempted to develop new strategies for triggering efficient immune responses by integrating current procedures with new techniques, using combinations of plasmids and live viruses. More specifically, we focused on the immune response to the HIV-1 env protein, using a DNA plasmid containing the env gp120 gene (pCdm7) to immunize mice intradermally; we then analyzed the antibody response to HIV-1 gp120. In a parallel set of experiments, we compared this response to that of mice injected intraperitoneally with a recombinant vaccinia virus encoding HIV-1 gp120. Finally, given the increase in immunogenicity of GM-CSF-linked antigens, we coimmunized mice with a plasmid DNA vector encoding murine GM-CSF.

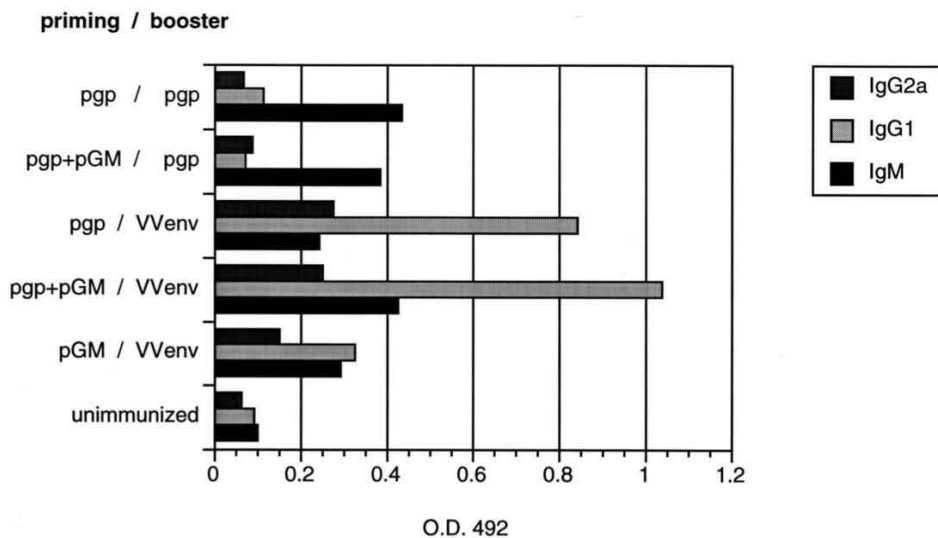
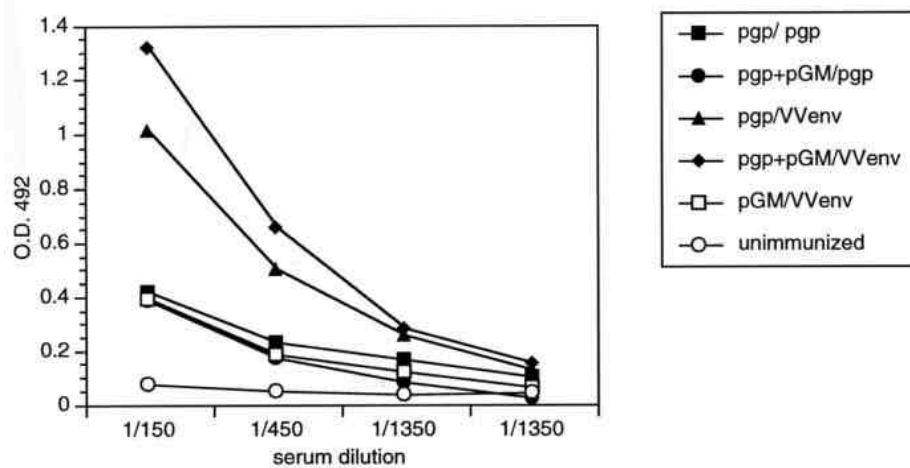
Balb/c mice were primed and boosted with combinations of DNA vectors expressing HIV-1 gp120 or

Table 1. Immunization regimes of mice

prime	pgp (50 µg) i.d.	pgp (50 µg) pGM (50 µg) i.d.	pgp (50 µg) i.d.	pgp (50 µg) pGM (50 µg) i.d.	pGM (50 µg) i.d.
boost	pgp (50µg) i.d.	pgp (50 µg) i.d.	VVenv (5 x 10 ⁷ pfu) i.p.	VVenv (5 x 10 ⁷ pfu) i.p.	VVenv (5 x 10 ⁷ pfu) i.p.

Injections were intradermic (i.d.) for plasmid DNA or intraperitoneal (i.p.) for vaccinia virus. Booster injections were given two weeks after priming.

Figure 1. Humoral anti-HIV-1 gp120 immune response elicited in mice primed with vector DNA and boosted with DNA or recombinant VV. BALB/c mice (four per group) were primed by intradermal injection with the plasmid expressing gp120 (pgp), with the plasmid expressing GM-CSF (pGM) or with a combination of both (pgp+pGM). After 21 days, mice were boosted with pgp or with the recombinant VV-env as indicated in **Table 1**, and serum samples collected two weeks after the boost. **(A)** ELISA analysis of anti-gp120 reactivity in pooled serum samples. **(B)** ELISA determination of anti-gp120 antibody isotypes in pooled serum samples.



GM-CSF and VV recombinants expressing env (**Table 1**). Serum samples were collected from mice two weeks after booster for quantitation of gp120-specific antibodies by titration of sera in gp120-coated ELISA plates (**Fig. 1A**). Sera from mice of the groups primed with plasmid DNA and boosted with VV-env showed higher antibody titers than those from animals primed and boosted with plasmid DNA alone, indicating that boosting with VV-env is an efficient system for triggering antibody responses. In addition, coinjection of the GM-CSF-DNA for priming resulted in a further increase in antibody titer in mice boosted with VV-env. Priming with gp120-DNA was essential for eliciting high specific antibody levels, since control animals primed with GM-CSF-DNA alone and boosted with VV-env showed low antibody levels.

ELISA analysis of antibody isotypes showed production of both IgM and IgG following immunization. There is a predominance of IgG1, with lower IgG2a levels, in sera from mice receiving a VV boost (**Fig. 1B**). Concurring with the data presented in **Fig. 1A**, the IgG1 titer increased when GM-CSF was used for priming. The gp120-specific antibody response in DNA/DNA-immunized mice was only of the IgM isotype, whereas control animals immunized with GM-CSF-DNA/VV-env showed reduced levels of all three isotypes, indicating that the presence of the appropriate antigen at priming is required to trigger antigen-specific responses.

To determine the antiviral activity of sera from immunized mice, we performed neutralization assays in which activated human PBMC were challenged with cell-free HIV-1/NL4-3 virus preincubated with sera from mice immunized using the protocols described above. The amount of p24 present in culture supernatants was measured at ten days post-infection (**Fig. 2**). As expected,

unimmunized mice or mice immunized in conditions producing low antibody titers display no neutralizing capacity. In contrast, we found potent neutralizing activity in sera from mice primed with gp120-DNA+GM-CSF-DNA and boosted with VV-env. Less potent antiviral activity was observed in sera from gp120-DNA/VV-env-immunized mice, and activity was insignificant in DNA/DNA-immunized and control mice.

B. Analysis of T cell proliferation and cytokine production triggered by DNA vaccination

Activation and proliferation of T helper lymphocytes is critical in inducing humoral immune responses, *via* expansion of antigen-activated B cells, as well as cellular immune responses, *via* CD8⁺ cytotoxic T lymphocyte expansion. Exposure to antigen can stimulate at least two patterns of cytokine production by CD4⁺ T cells (Mosmann *et al.*, 1986). Responses that result in secretion of interferon- (IFN-) and interleukin 2 are classified as T helper 1 (Th1), whereas CD4⁺ T cells producing IL-4, IL-5 and IL-10 are classified as T helper 2 (Th2). Differentiation of CD4⁺ T cells into either Th1 or Th2 is influenced by the milieu in which antigen priming takes place. The identification of the conditions leading to a Th1 or Th2 response is critical, as under some circumstances the successful elimination of infectious agents depends on expansion of the appropriate CD4⁺ T cell subset. In general, Th1 cells are responsible for generating cellular immunity against intracellular pathogens and Th2 cells promote the development of humoral responses (Clerici *et al.*, 1992).

Figure 2. *In vitro* anti-HIV-1 neutralization activity of sera from immunized mice. Purified cell-free HIV-1 (IIIB) virus was incubated with serial dilutions of sera from control and immunized mice. After 1 h at 37°C, the serum-virus mixture was used to infect PHA-activated human PBMC. At 10 days post-infection, the amount of p24 antigen in the culture medium was measured by ELISA. The mean of triplicate samples is represented.

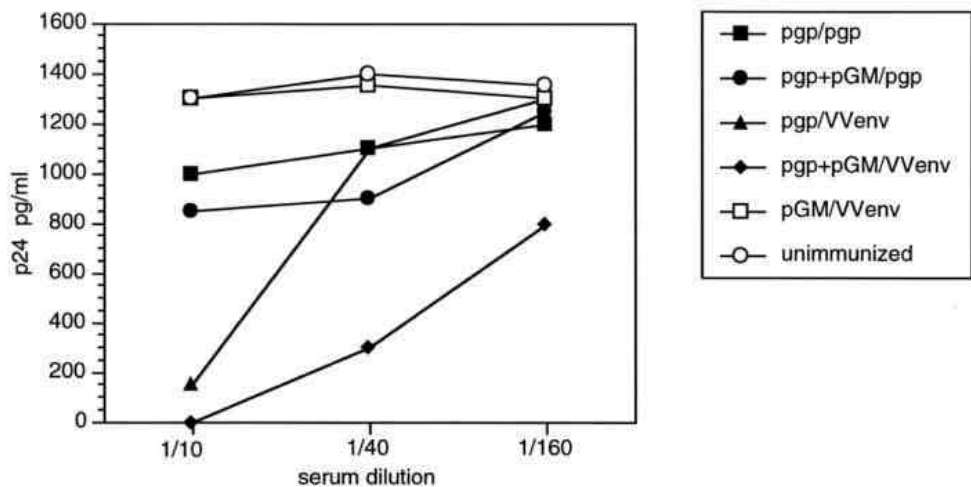


Figure 3. Cellular anti-HIV-1 gp120 immune response induced after vaccination of mice with vector DNA followed by boosting with DNA or recombinant VV. Proliferative response of spleen cells from mice immunized as described for Fig. 1. Spleens were removed 14 days after boosting and spleen cells incubated with purified gp120 (1 µg/ml). [³H]-thymidine incorporation was measured as described in Methods. The Stimulation Index (SI) was determined as the ratio of ³H cpm in gp120-stimulated cultures/³H cpm in unstimulated cultures.

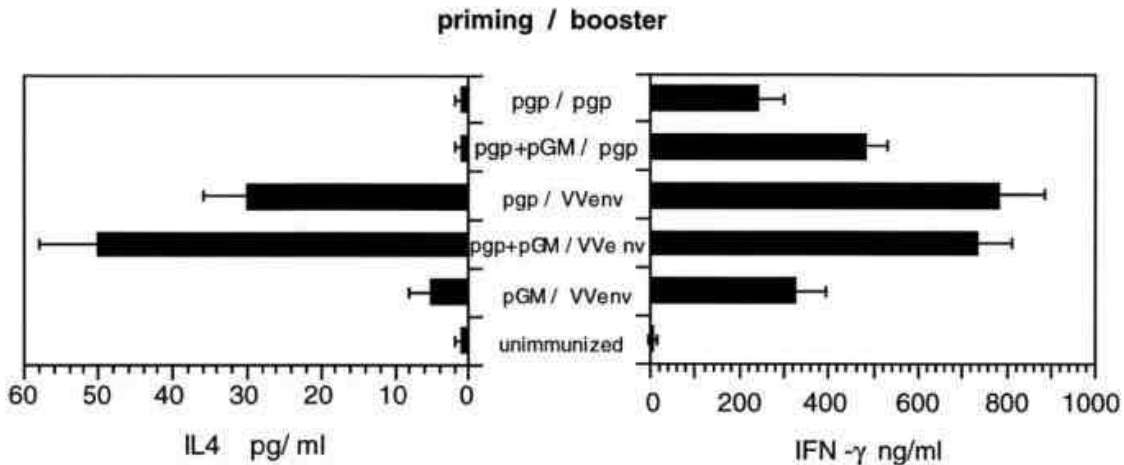
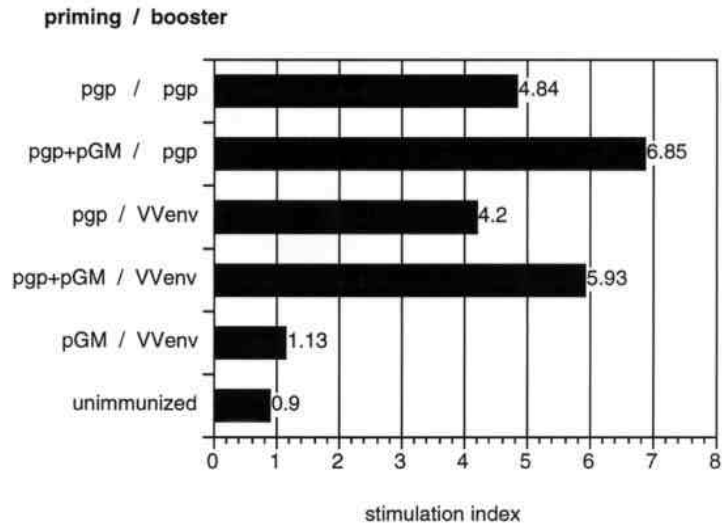


Figure 4. Cytokine response in spleen cells from immunized mice. The gp120-stimulated splenocyte culture supernatants described above were collected after 48 h, and IFN- and IL-4 levels determined as described in Methods. The mean of triplicate samples is represented.

Here we have analyzed the effect of different immunization protocols on triggering T cell activation. Two weeks after the last injection, splenocytes from immunized mice were tested for T cell proliferation. Antigen-specific T cell proliferation was determined after incubation of the splenocyte cultures with purified gp120. Cell proliferation was measured by addition of [³H]-thymidine to cultures and determination of the [³H]-thymidine incorporation ratio in gp120-stimulated cultures vs. unstimulated cultures, to derive the stimulation index (SI). The highest SI was obtained when the GM-CSF-DNA vector was coinjected with the gp120 DNA vector in the priming; this enhancement was observed regardless of

whether the booster was given with vector DNA or VV-env (**Fig. 3**).

To determine the type of immune response, we analyzed the pattern of cytokine responsiveness. IL-4 and IFN- levels were measured in *in vitro* gp120-stimulated splenocytes from immunized animals (**Fig. 4**). IFN- levels in DNA/VV-immunized mice were significantly higher than in DNA/DNA-immunized mice. There was a significant increase in IFN- production when GM-CSF-DNA was incorporated in the priming of mice immunized with DNA/DNA, but not when the priming/booster regime consisted of vector DNA/VV-env. In addition, IL-4 was only detectable in splenocyte cultures from DNA/VV-

env-immunized mice; coinjection of GM-CSF-DNA at priming induced increased production of this cytokine.

C. DNA vaccination activates efficient CD8⁺ T cells

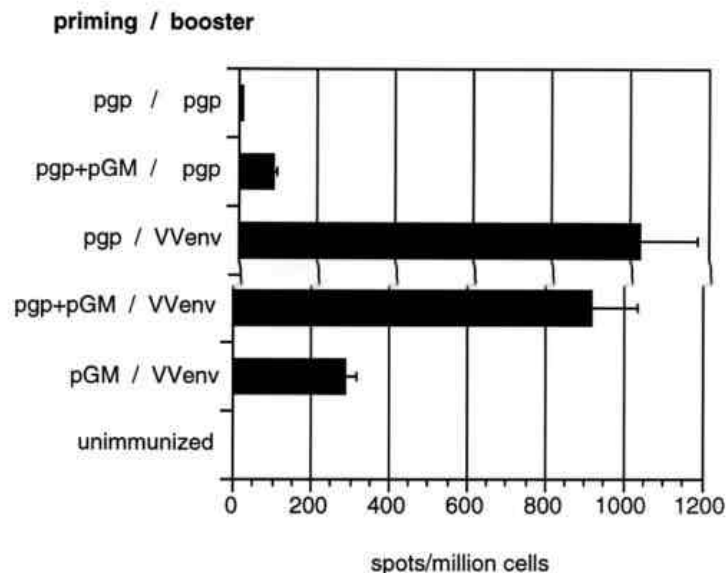
The ELISPOT assay uses peptides of defined MHC class I-restricted CTL epitopes to quantitate epitope-specific IFN- γ release by individual CD8⁺ T cells in unstimulated splenocyte cultures, correlating with levels of cytotoxic activity (Hanke *et al.*, 1998, Rodrigues *et al.*, 1994). As detection of IFN- γ -producing cells is more sensitive and quantitative than a ⁵¹Cr-release cytotoxicity assay, the ELISPOT assay is a useful method for evaluating the success of vaccination. The priming/boosting vaccination regime, using the DNA/VV-env, induces a dramatic stimulation of IFN- γ -producing CD8⁺ cells, as compared to vaccination with the DNA/DNA approach (Fig. 5). A single VV-env inoculation was even more effective in inducing an env-specific CD8⁺ T cell response than the DNA/DNA double immunization regime, as seen by the larger number of IFN- γ -secreting cells among splenocytes from mice primed with GM-CSF-DNA and boosted with recombinant VV-env. In accordance with the observed increase in IFN- γ production, there was a 10-fold increase in the CD8⁺ T cells number in mice primed with GM-CSF-DNA and the gp120-expressing DNA vector, and boosted with gp120-DNA. This enhancing effect of GM-CSF was not, however, observed in animals treated with the DNA/VV-env approach.

III. Discussion

The ideal HIV-1 vaccine would probably elicit both humoral and cellular immune responses. Such a dual response would aid in clearing virus before persistent infection is established, as well as in eliminating infected cells by recognizing processed viral fragments associated with host-specific MHC class I antigens and presented on the infected cell surface. The induction of antibodies against the gp120 surface protein of HIV-1 would be important, as this protein is the principal viral determinant interacting with host receptors and the major antigenic determinant to which neutralizing antibodies are directed. Moreover, HIV-specific antibodies that mediate ADCC are found very early in acute infection and correlate well with a decline in plasma virus load (D'Souza *et al.*, 1996). On the other hand, the dramatic decrease in HIV-1 viral load following the initial appearance of CTL after primary infection, and the temporal association between HIV-specific CTL activity and stable viral load or CD4⁺ cell counts during asymptomatic stages are the best indicators of CTL efficiency.

Immunization with DNA vaccines induces protective CTL responses in many experimental models, and can prevent HIV-1 infection or trigger a neutralizing HIV-1 response in certain non-human systems (Wang *et al.*, 1995; Ugen *et al.*, 1997); the safety, feasibility and immunogenicity of DNA vaccines are currently under evaluation in clinical trials (unpublished data).

Figure 5. HIV-1 specific IFN- γ -secreting CD8⁺ T cells in splenocyte cultures from mice primed with vector DNA and boosted with DNA or recombinant VV-env. Splens from mice, immunized as in Fig. 1, were removed 14 days after boosting, and spleen cells incubated with P815 cells coated with a synthetic peptide corresponding to the gp120 V3 loop. After stimulation of the splenocytes with V3 loop peptide-presenting APC, the number of IFN- γ -secreting CD8⁺ T lymphocytes was quantitated by the ELISPOT assay following the protocol described in Methods. Each bar represents the mean of triplicate samples.



Although DNA vaccines generally elicit strong cytotoxic responses, the antibody response generated by gene vaccination is lower than that induced by protein vaccination. An additional boost may be required for the generation of a protective humoral immune response, and combined prime-boost immunization approaches using different vector combinations are under evaluation. Priming with a DNA vector followed by a boost with an attenuated VV (MVA) recombinant has recently been shown to result in unexpectedly high levels of protection against *P. berghei* in mice (Schneider *et al.*, 1998). The rationale for using two distinct vaccine vehicles for the same antigen in combined prime/boost regimes lies in the observation that sequential vaccination with the same vehicle complex decreases vaccine immunogenicity; for example, prior host exposure to VV has been observed to reduce the immunogenicity of VV-based vaccines. VV recombinants expressing HIV-1 envelope antigens were among the first prototype vaccine constructs generated against AIDS (Chakrabarty *et al.*, 1986; Hu *et al.*, 1986). In animal models, these constructs elicit both humoral and cellular immune responses, which are nonetheless unable to control viral infection after HIV challenge (Earl *et al.*, 1989).

A DNA prime-MVA boost regime has also been evaluated as a candidate HIV vaccine, given its ability to potentiate the induction of specific CTL (Hank *et al.*, 1998); this study focuses, however, on cellular and not on antibody responses. Here we report the effect of the combined vaccine on both humoral and cellular responses, and take advantage of the immunostimulant properties of GM-CSF in the priming step. We and others have demonstrated that this cytokine increases the potency of immunization against tumor cells and protein antigens (Tao & Levy, 1993; Disis *et al.*, 1996). Using a VV expressing the GM-CSF/gp120 fusion protein, we observed significant enhancement of the cellular immune response against the env antigen (Rodriguez *et al.*, 1998).

Here we show an increase in both humoral and cellular immune responses using the DNA-prime/VV-boost regime, as compared to vaccination with DNA alone. This enhancement is especially relevant in the induction of IFN- γ -producing CD8⁺ cells, for which a 100-fold increase was observed. The antibody response was also significantly augmented both in the titer of specific antibodies and in the viral neutralizing activity of the serum. Whereas sera from mice immunized with the DNA/DNA regime contained only IgM antibodies, IgG1 was the predominant isotype in sera from DNA/VV-immunized animals, although IgM and IgG2a were also detectable. This response appears to be paradoxical, since the induction of high CTL levels corresponds to a typical Th1-type response, whereas IgG1 antibody production is characteristic of a Th2-type response. In fact, we observed cytokines representative of both types of response (IFN-

and IL-4) in culture supernatants of splenocytes from mice receiving the combined immunization, whereas IFN- γ but not IL-4 was present in DNA-immunized mice. It has been claimed that the effect of DNA is dominant, as preimmunization with plasmid DNA followed by boosting with protein in alum prevents the induction of the IgE antibody response or the activation of Th2 cells that would be expected in an alum-based vaccine (Raz *et al.*, 1996). Our results do not confirm this observation, since boosting with gp120-expressing vaccinia virus induces a combined immune response characterized by IL-4 and IgG1 production, as well as by a large number of IFN- γ -producing CD8⁺ cells. The only reports of significant antigen-specific IL-4 and IgG1 production following DNA immunization are associated with the gene gun route, since direct intramuscular or intradermal inoculation of naked DNA results specifically in Th1 responses (Feltquate *et al.*, 1997). Immunization with the chicken ovalbumin (OVA) gene induces primarily an IgG1 rather than an IgG2a response, although the OVA-specific T cell response includes IFN- γ -secreting Th1 cells (Tighe *et al.*, 1998). An unbiased increase in all immune responses was also observed by increasing the period between immunizations, with significant enhancement of IFN- γ , IL-4, IgG1 and IgG2a production (Prayaga *et al.*, 1997). Immune responses of this nature may be more desirable for protection against mucosally-transmitted viral diseases and certain inflammatory autoimmune disorders such as rheumatoid arthritis.

Finally, we have observed that coimmunization of gp120-DNA with GM-CSF-DNA results in the enhancement of antigen-specific T cell proliferation rates in both DNA/DNA- and DNA/VV-immunized mice. The GM-CSF stimulation effect on the number of IFN- γ -producing CD8⁺ cells can clearly be seen in DNA/DNA-immunized mice, but not in DNA/VV-vaccinated mice, probably due to a saturation effect of the VV boost. A modest but significant increase was seen, however, in the humoral response when GM-CSF-DNA is included in the DNA/VV immunization protocol.

Given the acceptability and potential safety of both DNA and VV vaccine vehicles in humans, it is reasonable to believe that this priming/booster regime based on these vectors may be effective not only for HIV, but also for other infectious agents and some forms of cancer.

IV. Materials and methods

A. Plasmids and recombinant virus

Plasmid DNA expressing gp120 was kindly provided by Dr. Andreas Bültmann (Max von Pettenkofer-Institut, Genzentrum, Munich, Germany) (André *et al.*, 1998). The plasmid expressing GM-CSF was previously described (Rodriguez *et al.*, 1998). Plasmids for immunization were purified using Qiagen Maxiprep Columns (Hilden, Germany).

The VV-env recombinant virus has been previously described (Rodríguez *et al.*, 1989).

B. Immunization of mice

Six-week-old female BALB/c mice received 50 µg of DNA in PBS intradermally. Fifteen days later, they received an intraperitoneal injection of 5×10^7 plaque-forming units (pfu) of purified VV env.

C. Anti-gp120 ELISA

Anti-gp120 levels in immunized mouse serum were quantitated by titrating on ELISA plates (Maxi-sorb, Nunc) coated with gp120 (IIIB; Intracel) at 1 µg/ml in PBS (100 µl/well) overnight at 4°C. Remaining protein binding sites were blocked with 0.5% BSA in PBS (200 µl/well, 60 min, 37°C). After washing plates with distilled water, the diluted sera were added to the wells and incubated for 60 min at 37°C, followed by a peroxidase (PO)-labeled goat anti-mouse immunoglobulin antibody (GAM-PO, Tago Inc., Burlingame, CA) and OPD (Sigma Chemical Co., St. Louis, MO). The reaction was terminated with 3N sulphuric acid and optical density determined at 492 nm. The titer is expressed as the highest serum dilution giving an absorbance value three times higher than that of the preimmune serum.

D. Viral neutralization assays

HIV-1 strain IIIB cell-free virus (NL4-3 strain; 2 ng p24/10⁶ cells) was incubated with serial dilutions of mouse antisera for 1 h at 37°C. PHA-activated PBMC were incubated with the virus-antiserum mix for 2 h, then washed three times. Triplicate 0.5 ml cultures were tested for p24 production at 5, 7 and 10 days post-infection. p24 antigen levels were measured by ELISA (Coulter, Miami, FL).

E. T cell proliferation assay

Spleens were removed from infected mice, single-cell suspensions prepared in complete medium (RPMI-1640 with 10% FCS, 2 mM L-glutamine and 10 µM 2-mercaptoethanol), and splenocytes (10⁶/well) dispensed into 96-well microtiter plates. Culture triplicates were challenged with 2 µg/ml of gp120 or concanavalin A (Con A, Sigma) and incubated for three days at 37°C in 5% CO₂, after which 1 µCi of [³H]-thymidine (5 Ci/mmol; Amersham) was added to each well. Cells were harvested after 16 h and [³H]-thymidine incorporation into DNA measured by liquid scintillation counting. Stimulation index (SI) was determined as the ratio: experimental count (mean of [³H]-thymidine incorporation in triplicate wells incubated with antigen) divided by the spontaneous count (mean of [³H]-thymidine incorporation in triplicate wells incubated with medium alone).

E. Cytokine assays

In vitro gp120-specific IFN- and IL-4 production was measured using gp120-stimulated splenocytes as previously described (Rodríguez *et al.*, 1998). Conditioned medium containing the secreted cytokines were collected from all cultures after 48 h.

F. ELISPOT assay

The ELISPOT assay was used to detect epitope-specific IFN- secreting cells (Miyahira *et al.*, 1995). Briefly, nitrocellulose-bottomed 96-well plates were coated with anti-mouse IFN- mAb R4-6A2 (8 µg/ml, Pharmingen, San Diego, CA). After overnight incubation at room temperature, wells were washed three times with RPMI 1640, then 100 µl of medium supplemented with 10% FCS were added to each well, and plates incubated at 37°C for 1 h. Duplicate cultures were prepared with serial doubling dilutions of immunized splenocytes, beginning with 10⁶ cells/well. P815 cells (H-2^d), used as antigen-presenting cells (APC), were pulsed with 10⁻⁶ M of the synthetic peptide GPGRAFVTI, corresponding to the V3 loop of gp120, and treated with mitomycin C (30 µg/ml, Sigma). After several washes with culture medium, 10⁵ P815 cells were added to each well. Control P815 cells were not pulsed with the peptide. Plates were incubated for 26-28 h at 37°C, washed with PBS containing 0.05% Tween-20 (PBS-T) and incubated overnight at 4°C with biotinylated anti-mouse IFN- mAb XMG1.2 (2 µg/ml, Pharmingen) in PBS-T. Plates were washed with PBS-T and PO-labeled avidin (Sigma; 100 µl, 1/800 dilution in PBS-T) was added to each well. One hour later, wells were washed with PBS/T and PBS. Spots were developed by adding 50 mM Tris-HCl, pH 7.5 containing 1 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.015% H₂O₂. When the plates were completely dry, the number of spots was determined with the aid of a stereomicroscope.

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