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ARTICLE

Multigene/Multisubtype HIV-1 Vaccine Induces Potent Cellular and Humoral Immune Responses by Needle-Free Intradermal Delivery

Andreas Bråve,[1,2,*] Karl Ljungberg,[3] Andreas Boberg,[1,2] Erik Rollman,[1,2] Maria Isaguliants,[1] Björn Lundgren,[4] Pontus Blomberg,[5] Jorma Hinkula,[1] and Britta Wahren[1,2]

[1]*Swedish Institute for Infectious Disease Control, 171 82 Stockholm, Sweden*
[2]*Microbiology and Tumor Biology Center, Karolinska Institute, 17177 Stockholm, Sweden*
[3]*Carolina Vaccine Institute, University of North Carolina at Chapel Hill, 824 ME Jones Building, CB 7292, Chapel Hill, NC 27599-7292, USA*
[4]*Visionar Biomedical AB, Box 6373, SE-751 36 Uppsala, Sweden*
[5]*Vecura, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden*

**To whom correspondence and reprint requests should be addressed at current address: Avdelningen för Virologi, Smittskyddsinstitutet, 17182 Solna, Sweden. Fax: +46 8 337272. E-mail: andreas.brave@smi.ki.se.*

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Gene vaccination encounters problems different from those of gene therapy since both a short half-life of the gene and a strong immune response to the gene product are desirable. We have evaluated a DNA vaccine consisting of seven plasmids encoding nine HIV-1 proteins. Using a needle-free delivery device, the Biojector, together with recombinant mouse GM-CSF, this vaccine induced strong gp160 Env- and p24 Gag-specific cellular and humoral immune responses in mice. The rGM-CSF was crucial for inducing both antibodies and antigen-specific $CD8^+$ T cell responses against both gp160 and p24. A GMP-produced lot of this vaccine, intended for human use, was delivered intradermally or intramuscularly into BALB/c mice at a GLP-accredited animal facility. This vaccine induced strong cellular responses independent of the route of immunization; moreover, no signs of toxicity were detected after histopathological examination of various tissues. Overall, the results indicate that the intradermal delivery of multigene/multisubtype HIV DNA in combination with recombinant GM-CSF is a safe and efficacious strategy for inducing high levels of specific $CD8^+$ T cells and unusually high titers of antibodies. This vaccine has been approved by the Swedish Medicinal Products Agency and is currently in a Phase I clinical trial.

Key Words: HIV-1, DNA vaccine, GM-CSF, Biojector, multiple genes, multiple subtypes

INTRODUCTION

The search for a vaccine against HIV has occupied the scientific community for more than 20 years. This quest has been hampered by the lack of known correlates of protection, and no protective properties have yet been defined. However, to obtain an efficient vaccination strategy, several criteria are commonly believed to apply [1–3]. First, the vaccine should ideally confer protection against several subtypes and recombinants of the virus circulating in the area where the vaccine is to be used. Thus, a vaccine should represent and protect against the most prevalent subtypes worldwide [4,5]. Second, the vaccine should raise potent cellular immune responses against several virus components as well as neutralizing antibodies to the viral envelope. We have previously shown that a broad neutralizing antibody response can be induced by immunizing mice with combinations of HIV-1 envelope genes of different subtypes [6]. Third, the vaccine has to be readily available in the developing world, where the need for a protective vaccine is greatest. This calls for a vaccine for which a cold chain is not required to maintain its efficiency. For instance, a genetic vaccine may have the suitable characteristics for such a vaccine [7]. However, gene vaccine efficacy needs to be improved. One way to achieve enhanced efficacy and reproducibility of gene-based vaccines would be to increase the effectiveness of delivery. For instance, a reliable needle-free device may greatly enhance DNA delivery to the skin and also improve the safety aspects of genetic immunization. The gene gun's dermal delivery of genes coated on gold beads has been shown to be an efficient way of inducing both potent antibody and T cell responses with a low amount of DNA [8,9]. Devices such as the SyriJet and the Biojector target the epidermis and

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the dermis without the inconvenience of coating the DNA on particles. Intradermal delivery targets the vaccine to an area with a high proportion of antigen-presenting cells (i.e., Langerhans cells in the epidermis and dermal dendritic cells in the dermis), thus facilitating good priming of the immune response [9–12]. For instance, Biojector delivery of vaccine antigens has been shown to induce both antibody responses and cellular responses [13–17].

Anti-envelope antibodies induced by genetic immunization have been shown to differ qualitatively from those induced by protein immunization; the antibodies induced by genetic immunization may be more prone to recognize conformational epitopes and thus may have a higher neutralizing capacity than antibodies induced by protein immunization [6,18]. Inducing high titers of neutralizing antibodies to the HIV-1 envelope by DNA immunization is problematic and needs potent adjuvants. In addition, the expressed envelope proteins must be presented in the correct conformation.

In this study we evaluated an HIV DNA vaccine consisting of a cocktail of seven plasmids encoding 10 HIV-1 proteins delivered with a needle-free system. In addition, two adjuvants were tested: imiquimod and recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF). We, and others, have recently shown immune-enhancing effects on gene-based vaccination using these adjuvants in mice [6,10,19]. Plasmid-encoded GM-CSF has proven to be a potent adjuvant in mouse models [20,21], but has, when tested in nonhuman primates, been shown to be less potent [22]. Recently, plasmid-encoded GM-CSF was shown to have a negative effect on boosting of class I-restricted IFN-γ responses in humans [23]. There are, however, studies showing positive effects in macaques after coadministration of gene-encoded GM-CSF with vaccine antigen genes [24]. We have previously compared GM-CSF, either in the form of recombinant protein or as encoded by plasmid, and found that the adjuvant effect is better after delivery of the recombinant protein [25]. Recombinant GM-CSF has been shown to increase human immune responses when delivered concomitantly with recombinant hepatitis B vaccine [26] as well as with carcinoembryonic antigen [27,28].

In the present study, recombinant (r) GM-CSF and imiquimod were codelivered with vaccine plasmids and GM-CSF was shown to enhance cellular and humoral immunity substantially without inducing any antibody response to GM-CSF itself. These results are encouraging since the success of genetic vaccination in humans appears to depend largely on the mode of delivery and adjuvants used [29]. The vaccine proved safe and immunogenic in mice after intradermal delivery with the Biojector as well as after intramuscular injection and has been approved for a Phase I clinical trial of healthy volunteers in Sweden. The first volunteer was immunized in February 2005.

RESULTS

Antigen Expression and Immunizations

The HIV-1 *gag* gene encodes a polyprotein, p55, which is processed proteolytically to the four independent proteins p17, p24, p6, and p7. The p37 *gag* subtype B construct encodes the antigenically most relevant p17 matrix and p24 capsid proteins. In the HIV viral life cycle, expression of late genes such as *gag* and *env* is tightly regulated and nuclear export of *gag* and *env* mRNA is largely dependent on the expression of the early protein Rev [30,31]. To decrease the Rev dependence, our p37 *gag* subtype B has been modified for enhanced expression by removal of inhibitory sequences mainly in the p17 MA part of the gene, thus rendering the nuclear export of the p37 *gag* Rev-independent [32]. Specifically, codons in AT-rich regions have been altered to have a higher GC content without changing the amino acid composition of the expressed gene. We cloned the p37 *gag* (p17 and p24 polyprotein) gene from the HIV subtype A isolate 92UG031 (GenBank Accession No. AY187680) into the expression vector pKCMV. As expected, the wild-type p37 *gag* A gene was expressed at low levels. To increase the expression of the subtype A construct, we replaced the p17 gene by the optimized subtype B p17 (Fig. 1A). The resulting p37 gene, subsequently referred to as $p37A_{BA}$, produces a 37-kDa protein consisting of p17 Gag of subtype B and p24 Gag of subtype A. There is an amino acid difference of about 15% between the p24 subtype A and subtype B sequences as well as between the p17 subtype A and subtype B sequences. The exchange of p17 from subtype A to expression-optimized B in the $p37A_{BA}$ construct led to 2.5 times higher total expression of the chimeric gene compared to the wild-type subtype A p37 (Fig. 1B). We analyzed protein expression using Image J software and normalized the levels of p37 expression to the expression of the cellular protein tubulin in each sample (Fig. 1C). It has been shown that cellular anti-p24 Gag responses correlate with control of viremia in infected individuals and we therefore reason that p24 is the main vaccine target of the Gag proteins and that the exchange of p17 subtype A for subtype B p17 should not significantly influence the overall breadth of anti-Gag responses elicited by the vaccine [33]. The well-characterized HLA-A2 restricted CTL epitope SLYNTVATL [34,35] found in the p17 Gag is conserved in both the subtype A and the subtype B p17 Gag proteins.

We immunized BALB/c mice three times with the following plasmid constructs: $pKCMVp37A_{BA}$, pKCMVp37B, pKCMVRT, pKCMVgp160B, pKCMVgp160B/A, pKCMV gp160B/C, and pKCMVrev. pKCMVRT encodes an enzymatically inactive variant of

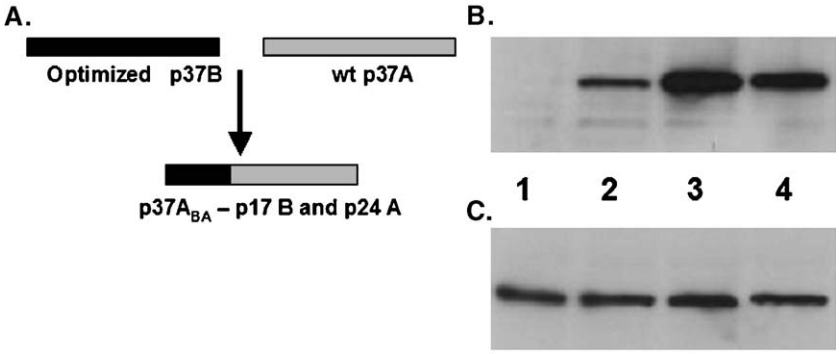

FIG. 1. (A) Optimizing the p37 *gag* subtype A by a combination of an optimized p17 from a subtype B construct and the wild-type p24A gene. (B) Expression of p37 in transfected HeLa cells measured by Western blot. Lane 1, mock-transfected cells. HEK293 cells were transfected with, lane 2, pKCMVp37Awt encoding the wild-type p37A gene; lane 3, pKCMVp37B encoding the optimized p37B gene; and lane 4, pKCMVp37A$_{BA}$, the chimeric p37 construct, consisting of the p17 optimized gene from the subtype B p37 gene and the wild-type p24 subtype A gene. (C) Expression of tubulin by the cells loaded in (B).

reverse transcriptase (RT) of subtype B, and its construction, expression, and immunogenicity have been described elsewhere [36]. The gp160 constructs pKCMVgp160B, pKCMVgp160B/A, and pKCMVgp160B/C have been described previously [37]. Briefly, the pKCMVgp160B encodes gp160 of subtype B, a fusion protein of gp120 and gp41, and pKCMVgp160B/A and pKCMVgp160B/C encode chimeric gp160B proteins with the hypervariable loops (V1–V5) exchanged for subtype A or C sequences, respectively. pKCMVrev encodes the regulatory protein Rev of subtype B, previously described [38]. In the present study the role of Rev was to facilitate the nuclear export of envelope-encoding mRNA and thus to enhance expression of gp160. Therefore, we did not assess immune responses to Rev. The seven plasmids were delivered either as a mixture of all plasmid constructs (DNA$_{Mix}$) or as two entities; one containing the p37 (A$_{BA}$ and B) and *rt* genes and the other the *gp160* (*A*, *B*, and *C*) and *rev* genes (DNA$_{Split}$) (Table 1). The genes were delivered intradermally with Biojector. We used two adjuvants for the immunizations: recombinant mouse GM-CSF, which was delivered subcutaneously at the site of DNA injection, or imiquimod, which was administered topically at the site of DNA injection.

Cellular Responses

We measured cellular responses by IFN-γ ELISpot on splenocytes stimulated with pools of overlapping peptides covering gp120 Env (of subtype A, B, or C), p24 Gag (of subtype A or B), or RT. There were similar responses to gp120 between the animals immunized with the mixture of all genes plus rGM-CSF (group DNA$_{Mix}$ + GM-CSF) and those immunized with the *p37* and *rt* genes separated from the *gp160* and *rev* genes (group DNA$_{Split}$ + GM-CSF) ($P = 0.114$) (Fig. 2A). We did not observe in the BALB/c mice the reduction of responses to envelope that was observed in another mouse strain with the same mixture of genes as used in this experiment [39]. The cellular response to gp120 was dependent on the addition of rGM-CSF since the animals in group DNA$_{Mix}$ + GM-CSF responded significantly more strongly against gp120 than did animals receiving DNA without adjuvant (DNA$_{Mix}$, $P = 0.015$), animals receiving the adjuvant imiquimod (DNA$_{Mix}$ + imiquimod, $P = 0.004$), or untreated animals ($P = 0.004$) (Fig. 2A).

The p24-specific IFN-γ response in all immunized animals was significantly stronger than in the untreated animals when we stimulated splenocytes with peptides covering p24 of both subtypes A and B (Fig. 2B). There were no significant differences in p24 responses between the various groups of immunized animals in terms of magnitude of response or frequency of responders (Fig. 2B).

To determine which population of cells in the spleen was primarily responsible for the production of IFN-γ, we carried out a depletion study of CD8$^+$ T cells from isolated splenocytes. We compared stimulation of pools of splenocytes from all animals in each group to that of pools of splenocytes from the same animals depleted of CD8$^+$ T cells. After CD8$^+$ depletion, the IFN-γ response to gp120 disappeared in the animals that received rGM-CSF in addition to the DNA (Fig. 2C). Thus, rGM-CSF was essential for the induction of gp120-specific CD8$^+$ T cell responses. This was also true for the responses to p24, in that the animals that received rGM-CSF had a noticeable proportion of the IFN-γ produced by CD8$^+$ T cells (Fig.

TABLE 1: Immunization of BALB/c mice

Group	DNA formulation
DNA$_{Split}$ + GM-CSF	Injection 1: pKCMVgp160B/A, pKCMVgp160B, pKCMVgp160B/C, pKCMVrev Injection 2: pKCMVp37A$_{BA}$, pKCMVp37B, pKCMVRT
DNA$_{Mix}$ + GM-CSF	Two injections: all plasmids mixed
DNA$_{Mix}$ + imiquimod	Two injections: all plasmids mixed
DNA$_{Mix}$	Two injections: all plasmids mixed
Untreated	—

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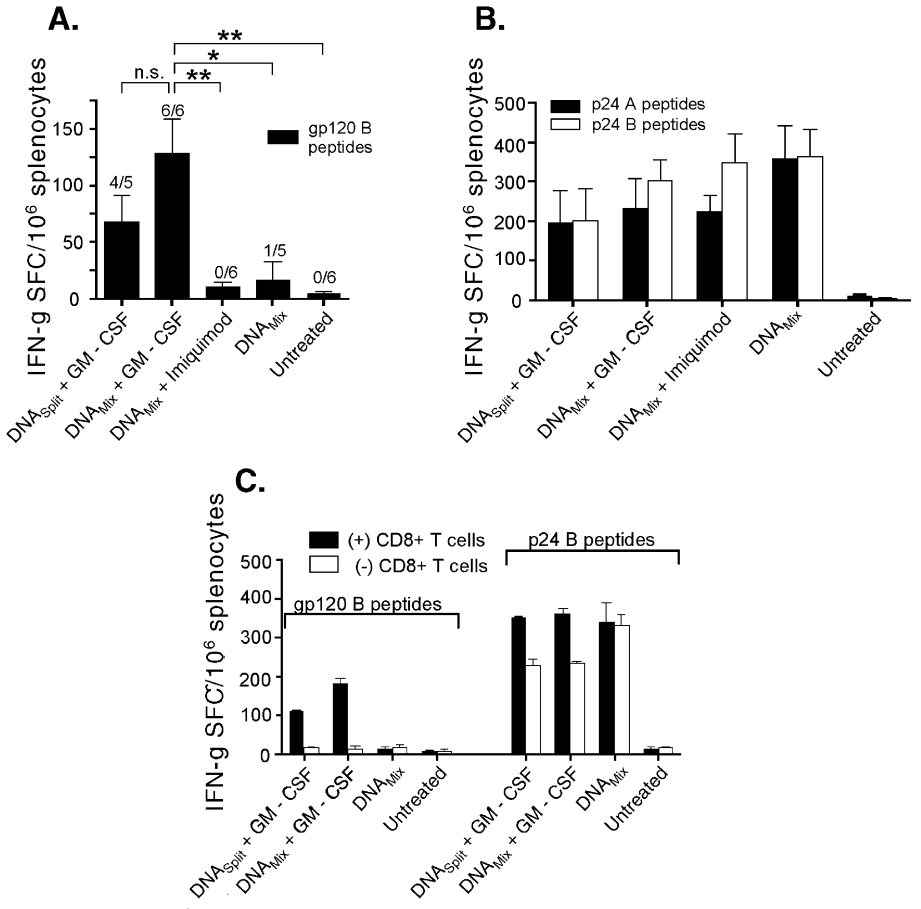

FIG. 2. IFN-γ secretion after three DNA immunizations as measured by IFN-γ ELISpot on splenocytes stimulated with pools of peptides. (A) Stimulation with peptides covering gp120 subtype B. Numbers above bars indicate frequency of responding animals in each group. *Significant difference of $P = 0.015$; **significant difference of $P = 0.004$; n.s., not significant, $P > 0.05$. (B) Stimulation with pools of peptides covering p24 Gag of subtype A or B. All immunized animals responded against both subtypes. (C) $CD8^+$ T cell responses. Splenocytes from each animal in the same group were pooled. The splenocytes, before and after $CD8^+$ T cell depletion, were stimulated with either gp120 B or p24 B peptides.

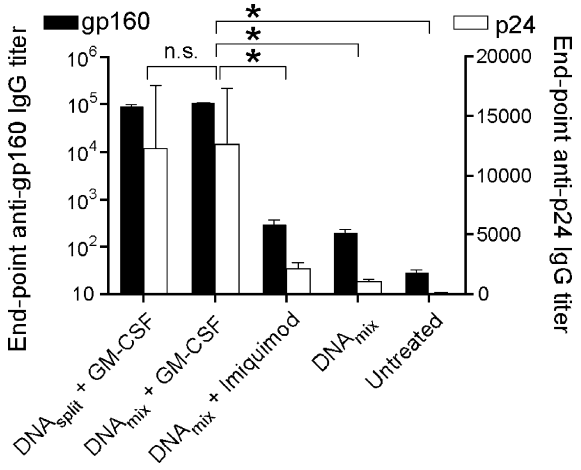

FIG. 3. Humoral immunity induced by the vaccine as measured by gp160 and p24 antibody ELISA 2 weeks after three DNA immunizations. Bars show mean values of end-point titers in each group. *Significant difference, $P = 0.004$; n.s., no significant difference, $P > 0.05$.

2C). Interestingly, the unadjuvanted animals (group DNA_{Mix}) seemed exclusively to elicit IFN-γ production by non-$CD8^+$ T cells. Thus, the p37 *gag* genes induced a non-$CD8^+$ T cell response independent of the adjuvant used. The use of GM-CSF was, however, essential for eliciting $CD8^+$ T cell responses (Fig. 2C).

Humoral Responses

We determined the levels of antibodies binding to gp160 and p24 proteins by enzyme-linked immunosorbent assay (ELISA). The humoral response was strong in all immunized animals adjuvanted by rGM-CSF, with mean end-point IgG titers above 10^5 against gp160 and above 10^4 against p24 (Fig. 3). Recombinant

TABLE 2: IgG1/IgG2a ratio

	gp160 $1/10^4$ serum dilution	p24 $1/10^2$ serum dilution
DNA_{Split} + GM-CSF	2.9 ± 1.3	2.1 ± 0.8
DNA_{Mix} + GM-CSF	2.8 ± 1.4	1.3 ± 0.3
DNA_{Mix} + imiquimod	—	—
DNA_{Mix}	—	—

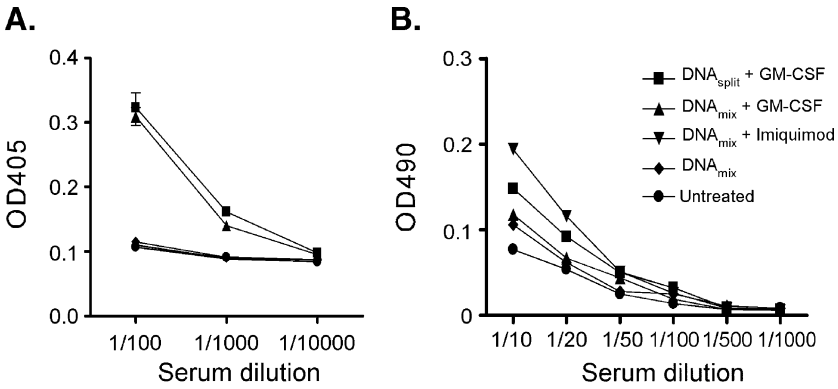

FIG. 4. (A) Mean IgA titers against gp160 in serum 2 weeks after the third and final DNA immunization. (B) Anti-GM-CSF antibodies as measured by ELISA 2 weeks after the third and final immunization.

GM-CSF, but not imiquimod, acted as a potent adjuvant and was crucial for the response to both p24 and gp160, inducing significantly higher antibody titers than in animals receiving the vaccine without rGM-CSF (P = 0.004) (Fig. 3). Similar to what was observed for the cellular responses, the strength of the humoral response in the animals that received the mixture of all vaccine genes together with rGM-CSF, DNA_{Mix} + GM-CSF, was comparable to that in the animals immunized with the two separated entities, DNA_{Split} + GM-CSF (Fig. 3).

We determined the IgG subclass ratio for the immunized animals adjuvanted with rGM-CSF and it indicated a slight skewing toward a Th2 type of response (Table 2). The responses were, however, relatively balanced. We did not determine a subclass ratio for the unadjuvanted animals or the animals adjuvanted by imiquimod, due to the low levels of IgG found in the sera from these animals. In addition to the high titers of IgG antibodies, which are unusually high following a DNA immunization, we noted the induction of IgA antibodies in serum against gp160 and it correlated with the IgG responses, showing high titers of IgA in the animals that received rGM-CSF in addition to the vaccine plasmids (Fig. 4A). To determine if the injection of rGM-CSF induced any specific responses to the adjuvant itself, we analyzed the serum for anti-GM-CSF antibodies. We could not detect any elevated levels of anti-GM-CSF antibodies in the animals that received rGM-CSF (titers below 1/20), compared to the animals in the groups not receiving rGM-CSF (Fig. 4B).

TABLE 3: GLP toxicological study of the vaccine

Group (10 animals/group)	Route of immunization	Adjuvant, route
DNA_{Split} (M)	i.d. with Biojector	—
DNA_{Split} (F)	i.d. with Biojector	—
Saline (M)	i.d. with Biojector	—
Saline (F)	i.d. with Biojector	—
DNA_{Split} (M)	i.m. with needle	rGM-CSF, i.m.
DNA_{Split}(F) +	i.m. with needle	rGM-CSF, i.m.
GM-CSF (M)	—	rGM-CSF, i.m.
GM-CSF (F)	—	rGM-CSF, i.m.

M, males; F, females.

GLP Immunization of BALB/c Mice with GMP-Produced Vaccine

In light of the experimental immunization described above, a GMP-produced vaccine lot intended for a Phase I clinical trial was subjected to a GLP pharmacotoxicity test. The vaccine was formulated and delivered as described above for the DNA_{Split} regimen. We immunized both male and female BALB/c mice (Table 3) intradermally using the Biojector; for comparison, we vaccinated another group with the vaccine plasmids + rGM-CSF intramuscularly with a needle. We included control animals receiving either intradermal injections of saline with Biojector or intramuscular injections of rGM-CSF

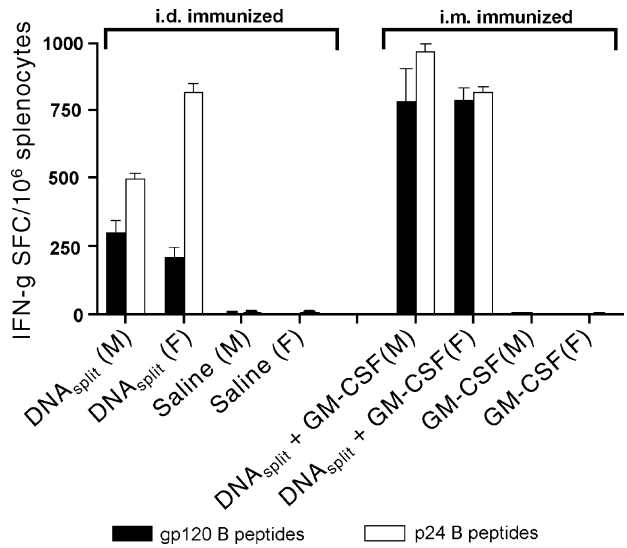

FIG. 5. Cellular responses to envelope and Gag peptides after GLP immunization of BALB/c mice with GMP-produced vaccine batch as measured by IFN-γ ELISpot on splenocytes.

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alone. We immunized the mice three times at 3-week intervals. We sacrificed the mice 6 weeks after the final injection, pooled spleen cells from two animals per group, and measured responses by IFN-γ secretion following stimulation with peptides covering p24 or gp120 (Fig. 5). Splenocytes from animals immunized with the vaccine batch responded strongly when stimulated with Env or Gag peptides (Fig. 5). Intradermal injections of saline with the Biojector or intramuscular injections with rGM-CSF did not render any nonspecific background reactions. We did not analyze any serum samples for humoral responses in these animals because the extensive toxicological analysis required all the available blood. None of the mice showed signs of toxic effects on health status, hematology, clinical chemistry, or histopathology.

DISCUSSION

We evaluated intradermal immunization with an HIV-1 vaccine candidate containing seven plasmids. The plasmids used for immunization encode p37 *gag* of subtypes A and B; gp160 of subtypes A, B, and C; and *rev* and *rt*, both of subtype B. Together with recombinant GM-CSF, the multigene vaccine induced high levels of gp160-, gp120- and p24-specific antibodies when delivered intradermally. It has previously been shown that high titers of anti-HIV-1 envelope antibodies induced after immunization with the gp160 vaccine constructs also induce high titers of broadly neutralizing antibodies [6,37]. Importantly, the induction of antibodies occurred without any significant reduction of the specific $CD8^+$ T cell response. Rather, rGM-CSF appeared to promote antibody as well as $CD8^+$ T cell development. In that the rGM-CSF protein is applied subcutaneously, it may both attract and activate a high number of dendritic cells (e.g., Langerhans cells) and thereby increase both the frequency of antigen-presenting cells and their capacity to present antigens from subsequently expressed plasmid DNA. GM-CSF has been shown to promote proliferation, maturation, and migration of dendritic cells and to induce responses of both the Th1 and the Th2 type [37,40,41]. For responses against gp160 envelope following genetic immunization with the Biojector, the use of rGM-CSF appeared crucial for obtaining strong cellular responses. Moreover, the gp120-specific cellular reactivity was largely dependent on the $CD8^+$ T cell population, since depletion of $CD8^+$ cells resulted in undetectable levels of IFN-γ secretion. In addition, rGM-CSF seemed to increase the number of Gag-specific $CD8^+$ T cells compared to the unadjuvanted animals, in which a lower proportion of Gag-specific $CD8^+$ T cells was seen. Instead, a large population of non-$CD8^+$ T cells contained IFN-γ-secreting cells, most likely $CD4^+$ T cells and/or NK cells. We believe that the differences in the magnitude of the cellular responses to the Env and Gag antigens can be explained by the nature of the different antigens. In immunization studies, as well as during the course of natural infections, *gag* is known to be a strong inducer of $CD8^+$ and $CD4^+$ T immune responses, whereas the *env* genes are weaker immunogens and clearly need a good adjuvant. The rather similar serum levels of IgG1 and IgG2a observed in this study indicate that the combination of Gag-, RT-, and Env-encoding genes together with rGM-CSF induced a relatively balanced Th1/Th2 response. In addition to the high levels of IgG, the vaccine together with rGM-CSF induced high levels of gp160-specific IgA antibodies, something that may correlate with mucosal immunity, which is considered highly important for protection against HIV infection [42–44]. The injection of recombinant GM-CSF did not induce any humoral anti-GM-CSF response in the adjuvanted animals, something that possibly could lead to a reduction of the effects of subsequent GM-CSF injections as well as inducing an unwanted autoreactivity [45,46]. We found no evidence of elevated antibody responses against GM-CSF in the adjuvanted animals. This indicates that also in the clinic it may be possible to use selected GM-CSF preparations to augment immune responses in genetic vaccine trials. Imiquimod, a Toll-like receptor 7 ligand, did not have any significant effects when applied to the skin prior to intradermal immunization with the Biojector. This result correlates with our previous findings showing an augmentation of Th1 responses after gene gun delivery of the antigen, but not after intradermal or intramuscular vaccination together with topical administration of imiquimod [10].

Previously, we have observed competition between the gag and env gene products in another mouse strain [47]. In the present strain of mice there were, however, no significant differences in responses to vaccine antigens between the animals that received all genes as a mixture and those that received the vaccine as two separate, but simultaneous, injections. Also the Gag responses obtained were directed against both subtype A and subtype B. The cellular responses to Env antigens, however, were directed mainly to subtype B, which appears to dominate over subtypes A and C.

The absence of anti-RT responses after immunization with the multiple genes cannot be explained readily. Since RT has been shown to be immunogenic when immunized separately, phenomena such as antigen competition, immunodominance, or tolerance due to endogenous retroviruses could possibly account for the lack of response.

The results from the GLP study of the effects of the GMP-produced vaccine showed the potency of the vaccine in inducing cellular immunity to Gag and Env. At 6 weeks postboost, the cellular responses to envelope were less dependent on rGM-CSF. Furthermore, the vaccine was safe and induced no adverse effects as measured by health status, hematology, clinical blood

chemistry, and histopathology. Our results from the safety study of plasmid DNA vaccines are supported by other studies [48]. The vaccine tested in this study has recently been approved for a clinical Phase I trial by the Swedish Medicinal Products Agency.

In conclusion, we show here that it is possible to raise potent immune responses to several viral antigens following immunization with multiple genes deriving from several subtypes of HIV. High titers of antibodies and strong cellular responses were obtained when the plasmid DNA was delivered intradermally together with recombinant murine GM-CSF. The Biojector is easy to use and gives reproducible results with more consistent results and lower intragroup variations in antibody titers and cellular responses than intramuscular injection with the same antigens.

Materials and Methods

Cloning of the HIV p37 gag subtype A gene. The development and expression of the *envelope* (pKCMVgp160B, pKCMVB/A, and pKCMVB/C), *rev* (pKCMVrev), inactivated *reverse transcriptase* (pKCMVRT), and *gag* B (pKCMVp37B) constructs have been described [32,36–38]. All vaccine immunogens described are encoded in expression vector pKCMV, which contains the promoter sequence from CMV, the poly(A) signal from HPV-16, and the *Escherichia coli* origin of replication and encodes kanamycin resistance. The cloning of the *gag* gene from the subtype A virus originating from Uganda (GenBank Accession No. AY187680) was carried out through standard cloning procedures. The p37 part of the *gag* gene was amplified with PCR primers 5′-TTACATGTCGACCAGCCACCA-TGGGTGCGAGAGC-3′ and 5′-CTCGATGAATTCCATTTACAAAACTC-TTGCTTTATGGC-3′ and subsequently cloned into pKCMV by standard cloning procedures. To increase the expression of the subtype A p24 gene, the first 375 bp of p17 was replaced by a subtype B sequence (GenBank Accession No. L04602) in which inhibitory sequences had been optimized for higher eukaryotic expression [32]. The fusion of the gene fragments resulted in a recombinant p37 gene encoding a 37-kDa protein with the first 119 amino acids of p17 consisting of subtype B and the remainder of p17 and p24 of subtype A. Constructs were propagated in *E. coli*, isolated, and verified by sequencing (ABI 310, Perkin–Elmer). The wild-type subtype A and recombinant construct are herein referred to as pKCMVp37Awt and pKCMVp37A$_{BA}$, respectively, while the subtype B p37 *gag* construct is referred to as pKCMVp37B.

Expression of gag A constructs. pKCMVp37Awt, pKCMVp37A$_{BA}$, and pKCMVp37B were transfected into HEK293 cells grown to 50% confluence. Transfection was performed as has been described previously [37] and cells were incubated at 37°C in 5% CO_2 for 36 h. Cells for immunoblot analysis were counted, lysed, and harvested in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 5% β-mercaptoethanol. The cell lysate was heated for 5 min at 95°C and lysate representing 50×10^3 cells was loaded on a 10% SDS–PAGE gel (Bio-Rad). After electroblotting to a nitrocellulose membrane, p37 *gag* expression was detected using the anti-p24 monoclonal anti-body EF7 [49]. Specific antibody binding was detected with horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies (DAKO A/S, Denmark) with the ECL system (Amersham Pharmacia Biotech, Uppsala, Sweden). Following the detection of p37 expression, antibodies were removed from the membrane by submersion in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.7) at 50°C for 30 min with occasional agitation. After blocking in 5% nonfat dried milk in PBS, tubulin expression was detected using anti-tubulin antibodies (Sigma, Sweden) and antibody binding was detected as described above. For determination of differences in level of expression the films were scanned and analyzed using Image J software (http://rsb.info.nih.gov.ij) and expression of p37 was normalized to the expression of tubulin for the same sample.

Immunizations. Five- to six-week-old BALB/c mice (Charles River, Germany) were immunized with vaccine plasmids (25 μg of pKCMVgp160B, 25 μg of pKCMVgp160B/A, 25 μg of pKCMVgp160B/C, 25 μg of pKCMVrev, 25 μg of pKCMVp37A$_{BA}$, 25 μg of pKCMVp37B, and 50 μg of pKCMVRT) intradermally (i.d.) using the needle-free, CO_2-propelled device Biojector (Bioject Medical Technologies, Inc., OR, USA). Animals were immunized three times at 6-week intervals and sacrificed 12 days after the last immunization. The DNA was diluted in saline and injected at a concentration of 2 mg total DNA/ml. All animals except the untreated naives received two injections (50 μl/injection) during each immunization. The animals were injected at a shaved area on the lower part of the back, just above the base of the tail. The vaccine plasmids were injected either as a mixture of all genes (DNA$_{Mix}$) or as two entities (DNA$_{Split}$), one consisting of the p37- and RT-encoding plasmids and the other of the gp160- and Rev-encoding plasmids. The adjuvants used were murine rGM-CSF (Prospec-Tany Technogene Ltd., Rehovot, Israel) or imiquimod (Aldara, 3M Pharmaceuticals, MN, USA). One microgram of rGM-CSF was diluted in 50 μl of 0.9% saline and administered subcutaneously with a needle at the site of immunization 30 min prior to DNA injection. The imiquimod was administered topically 30 min prior to immunization at the site of vaccination. Due to handling errors, two mice, one in the group DNA$_{Mix}$ + GM-CSF and one in DNA$_{Mix}$, died before the end of the experiment.

GLP toxicity testing of the GMP-produced vaccine. The production of the GMP-produced vaccine lot was carried out by Vecura (Huddinge, Sweden) and was formulated as two entities as described above for the experimental vaccine formulation DNA$_{Split}$. The DNA was formulated at a concentration of 2 mg DNA/ml saline and the rGM-CSF was given as 1 μg in 50 μl saline 30 min prior to DNA. The GLP pharmacotoxicity experiment was carried out by Visionar (Uppsala, Sweden). Eighty BALB/c mice (Charles River), both males and females, were divided into eight groups with 10 animals/group and immunized three times (days 1, 22, and 43) according to Table 3. Each animal receiving the vaccine was injected, either i.d. with Biojector or i.m. using a needle, with both vaccine entities.

The animals were weighed three times a week and checked on a daily basis for changes in food intake, activity, and fur quality as signs of general health status. Mice were sacrificed 6 weeks after the last immunization and organs were subjected to histopathological evaluation. Serum from the animals was subjected to clinical chemistry hematology. Splenocytes from two animals in each group were pooled and antigen-specific secretion of IFN-γ was measured by ELISpot.

ELISA. ELISA was carried out essentially as previously described [12,49]. Briefly, ELISA plates (Nunc Maxisorp; Odense, Denmark) were coated with recombinant subtype B gp160 (1 μg/ml) (Protein Sciences Corp., Meriden, CT, USA), recombinant subtype B p24 (1 μg/ml) (Aalto, Ireland), or rGM-CSF (1 μg/ml) (Prospec-Tany Technogene Ltd.). Briefly, plates were blocked with 5% fat-free milk in PBS and serum was diluted and added to wells. For the GM-CSF ELISA a murine-GM-CSF-specific goat-derived polyclonal antibody (AF-415; R&D Systems, Great Britain) was used as positive control. Reactive antibodies were detected with either goat anti-mouse IgG antibodies conjugated to HRP (DAKO PO449) diluted 1/3500 or, for the positive anti-GM-CSF antibody control, rabbit-derived anti-goat antibodies conjugated to HRP (DAKO) diluted 1:10,000. Plates were then developed for 30 min by adding *O*-phenylene diamine buffer (Sigma). The color reaction was stopped with 2.5 M H_2SO_4 and the optical density (OD) was read at 490 nm. For the IgG subclass and IgA assay, goat anti-mouse IgG1, IgG2a, or IgA (Sigma–Aldrich, Sweden) antibodies were used, followed by incubation with rabbit-derived anti-goat alkaline phosphatase-conjugated antibodies. The plates were then developed for 1 h by adding *p*-nitrophenyl phosphate substrate (Sigma–Aldrich) and the OD was read at 405 nm. Total IgG, subclass IgG, and IgA samples were considered positive if OD exceeded the mean value for

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negative samples (untreated animals and preimmunization bleedings) plus 3 standard deviations.

IFN-γ ELISpot assay. Ficoll–Paque Plus (Amersham Biosciences Europe GmbH, Uppsala, Sweden) was used to purify lymphocytes derived from the spleen (splenocytes). For the depletion of $CD8^+$ T cells, Dynabeads (Dynal Biotech, Oslo, Norway) were used according to the manufacturer's instructions. Splenocytes from individual animals or pooled splenocytes, before and after $CD8^+$ T cell depletion, from all animals in each group were suspended in RPMI 1640 (Sigma) supplemented with penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA, USA) and 10% fetal calf serum (Sigma) and were distributed in anti-interferon-γ (Mabtech, Nacka, Sweden) antibody-coated 96-well polyvinylidene fluoride bottomed plates (MAIPN 4510; Millipore Corp., Bedford, MA, USA). Splenocytes, 2×10^5/well, were stimulated with 1 μg/peptide/well of 15-mers with 10-amino-acid overlaps (Thermo Hybaid, Germany) covering the following HIV-1 proteins: p24 (subtypes A and B), gp120 (subtypes A, B, and C), and RT. All specific peptides were based on the corresponding sequence for the vaccine-encoded proteins. A control peptide library consisting of 18 peptides derived from tick-borne encephalitis virus and medium alone were used as controls. Concanavalin A (1 μg/well) was used as a positive control to test cell viability. The plates were then developed as described by the manufacturer. Results are given as cytokine-producing spot-forming cells (SFC) per million splenocytes and responding animals were defined as having above 50 SFC per million plated cells and twice the number of SFC for unstimulated cells from the same animal.

Statistical analysis. Statistical analyses were performed using the SPSS program, version 10.1.0, for Windows. The criterion for statistical significance was $P = 0.05$. Since most of the data were not normally distributed, the nonparametric Kruskal–Wallis test and the Mann–Whitney U test were used [50]. The former test was used first to identify differences in the comparisons between the intradermally and the intramuscularly immunized animals, the rGM-CSF adjuvanted and the nonadjuvanted animals, and the immunized and the untreated animals. The Mann–Whitney U test was then used for pair-wise post hoc comparison following a statistically significant Kruskal–Wallis test.

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