RESEARCH ARTICLE

Multi-subtype gp160 DNA immunization induces broadly neutralizing anti-HIV antibodies

E Rollman¹, J Hinkula¹, J Arteaga², B Zuber¹, A Kjerrström¹, M Liu^{1,3}, B Wahren¹
and K Ljungberg¹

¹Department of Virology, Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden; ²Department of Medicine, Clinical Research Center, Huddinge Hospital, Karolinska Institutet, Stockholm, Sweden; and ³Transgene, Strasbourg, France

A highly desirable feature for an human immunodeficiency virus type 1 (HIV-1) vaccine is the ability to induce broadly reactive anti-envelope antibodies that can neutralize primary HIV-1 isolates. Two immunizations with an HIV-1 envelope-encoding plasmid together with recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) resulted in high antibody titers in mice. The antibody induction was further enhanced after immunization with genes encoding HIV-1 envelopes originating from subtypes A, B and C. The sera from these animals were able to neutralize A, B and C viral isolates, whereas the sera from animals immunized solely with subtype B DNA neutralized only subtype B virus. The combined DNA vaccine gave serum antibodies with

broad recognition of HIV-1 envelope epitopes as determined by peptide mapping. Cell-mediated immunity was not compromised by the increased humoral immunity. This demonstrates the ability of multiple envelope genes to induce the desired antibody response against several subtypes. Moreover, it documents the ability of rGM-CSF to enhance the potency of such a vaccine when given simultaneously. The strategy may be useful for making an HIV vaccine more potent and broadly effective against strains of different clades.

Gene Therapy (2004) 11, 1146–1154. doi:10.1038/sj.gt.3302275; Published online 22 April 2004

Keywords: HIV-1; DNA immunization; gp160; antibody neutralization; GM-CSF

Introduction

Currently, more than 60 million people have been infected with human immunodeficiency virus type 1 (HIV-1).¹ Over 5 million new HIV-1 infections occurred in 2002, and acquired immunodeficiency syndrome (AIDS) is spreading rapidly.² These facts highlight the need for a prophylactic vaccine against HIV-1. The various HIV-1 subtypes have biological and antigenic differences.³ Whether an HIV-1 vaccine needs to comprise different subtype antigens is a matter for debate, but most investigators agree that broadly crossreactive antibodies and cellular immune responses are desirable.^{4,5}

A recombinant HIV-1 envelope protein (rgp120) was used in the world's first phase III HIV vaccine trial resulting in the induction of immunity, but unfortunately without conferring substantial protection.⁶ New strategies for HIV-1 vaccination include attenuated vaccinia virus carrying HIV gene segments,⁷ recombinant adenovirus vectors⁸ and plasmids encoding HIV-1 proteins or peptides.^{9,10} DNA vaccination is especially suitable since it preferably induces cellular immunity, an important host response against intracellular microbes. DNA immunogens can easily be constructed to express antigens from multiple HIV-1 antigens and/or sub-

types.^{11–13} Since immune responses to plasmid-encoded antigens tend to be weak in primates, numerous strategies for enhancing DNA immunization have been evaluated. Cytokines or cytokine genes have been shown to be potent immune enhancers in genetic immunization. For example, seven out of eight macaques immunized with a gene encoding HIV-1 *gag* together with a fusion protein of interleukin-2 and immunoglobulin (IL-2/Ig) survived challenge with the pathogenic chimeric simian-human immunodeficiency virus (SHIV 89.6P).^{14,15} Immunization with a bicistronic plasmid that coexpresses HIV-1 gp120 and granulocyte-macrophage colony-stimulating factor (GM-CSF) under the control of a single promoter led to a dramatic augmentation of vaccine-elicited CD4+ T-cell responses, but did not affect either antibody or CD8+ T-cell responses in mice.¹⁶ Coinjection of the IL-12, IL-7 and GM-CSF genes with the antigen gene has been used to enhance genetic immunization in mice.^{17–21} Furthermore, the HIV coreceptor gene CCR5 has been used together with recombinant GM-CSF (rGM-CSF) to induce immune responses against CCR5 in macaques.²² Protein subunit vaccines, enhanced by naturally hypomethylated bacterial DNA in a specific nucleotide context (5'-Pu-Pu-CpG-Pyr-Pyr-3') can stimulate both arms of the immune system.²³ Protein together with synthetic immunostimulatory CpG oligonucleotides (CpG-ODN) has been used to activate responses to, for example, the HIV-1 envelope and reverse transcriptase proteins in mice^{24,25} and the hepatitis B surface antigen in mice and man.^{26,27}

Here, we describe immunization with HIV-1 gp160 DNA alone, typically resulting in poor humoral immunity. A single injection of gp160 DNA was given with recombinant GM-CSF, resulting in potent antibody induction. Finally, we performed a prime-boost immunization protocol by priming with gp160 DNA together with rGM-CSF and then boosting with rgp160 protein. Particularly, the priming with HIV-1 gp160 DNA together with recombinant GM-CSF induces high titers of broadly neutralizing antibodies. The HIV neutralization was even broader if the envelope DNA immunogen derived from multiple viral subtypes.

Results

Antibody induction

Immunizations with DNA plasmids expressing the HIV-1 gp160 gene of subtypes A, B or C without any adjuvant did not result in detectable antibodies (0/40 animals), but did give HIV-specific induction of cellular immunity (Table 1, part I). Envelope DNA immunizations together with rGM-CSF resulted in anti-gp160 antibody titers of 1.5×10^4 , at 30 days after a single immunization (Table 1, part II). In a third set of immunizations, priming twice with gp160 genes together with rGM-CSF induced strong antibody responses in all multi-subtype-immunized mice, with a group geometric mean anti-gp160 IgG titer of 2×10^5 (Table 1, part III and Figure 1). After booster immunization with rgp160 and CpG-ODN, animals in the multi-subtype DNA-immunized group retained high antibody responses, with a geometric mean group titer of 4×10^5 , at 14 days after immunization (Figure 1a). Subtype B DNA-primed animals showed lower antibody response frequencies than the multi-subtype DNA-immunized mice, with altogether eight out of 12 mice responding with ELISA antibodies 14 days after the second DNA priming (Figure 1a). This was also reflected in the two groups' mean anti-gp160 IgG titers of approximately 3×10^3 and 2×10^2 , respectively. Antibody titers were augmented approximately one logarithm after protein boosting. The mice receiving rgp160 protein with CpG-ODN (the B1 group) reached a mean titer of

Figure 1 Antibody production. (a) HIV-1 gp160-specific antibodies were analyzed by ELISA before (white boxes) and after (black boxes) the booster immunization. Horizontal lines indicate the group geometric mean antibody titers. (b) The group mean IgG1/IgG2a isotype ratios were determined in the sera from responding animals after priming (white bars) and boosting immunization (black bars). Error bars indicate one standard deviation.

2×10^4 ; animals given rgp160 protein alone (the B2 group) showed a mean titer of 3×10^3 .

The IgG1/IgG2a isotype ratios for responding animals were determined (Figure 1b). All groups had a T-helper 2 (Th2) type of response with predominant IgG1 anti-

Table 1 HIV-1 gp160 DNA immunization with multi-subtype envelope genes and rGM-CSF

	Part I						Part II			Part III			
Immunization schedule													
Subtype gp160 DNA immunogen	AC	A	B	C	mock	—	B	B	ABC	B1	B2	—	
Number of C57Bl/6 mice	10	10	10	10	5	5	6	6	6	6	6	6	
Number of i.m. inoculations	2	2	2	2	2	2	1	1	2	2	2	2	
Adjuvant rGM-CSF	—	—	—	—	—	—	+	—	+	+	+	—	
Immune response													
Anti-rgp160 B IgG geometric mean titer	<100	<100	<100	<100	<100	<100	1×10^4	<100	2×10^5	3×10^3	2×10^2	<100	
T-cell proliferation to rgp160 B	++*	+	+	+	0	0	++	+	++*	++*	+	0	
IFN- γ to rgp160 B	ND	ND	ND	ND	ND	ND	70	20	50	100	110	<40	

– = no antigen, no adjuvant.

++ = SI 3–10.

++ = SI > 10.

* = cross-subtype reactivity.

0 = no proliferation above cutoff.

ND = not done;

Grey area = post-boost data.

IFN- γ , measured by ELISA (pg/ml, part II) or by PBMC ELISpot (SFCs per million cells, part III).

bodies before boost (mean group ratios ranging from 9 to 19). In animals that received CpG-ODN in the booster immunization (the ABC and B1 groups), this profile changed in favor of a more balanced Th2/Th1-like profile, with ratios of 4 and 5, respectively. Animals boosted with rgp160 without CpG-ODN (the B2 group) kept their prominent Th2-like profile, with a mean IgG1/IgG2a ratio of 13. IgG1 levels were augmented two- to three-fold and IgG2a levels increased two- to 15-fold postboost in the two groups that received CpG-ODN.

Figure 2 Anti-envelope antibody epitope mapping. The sera from each group of animals were screened against individual 15-mer peptides covering the gp120 C2–C5 region, before and after protein boost. (a) Antibody epitope mapping with pooled sera from the ABC DNA immunized group, before (filled circles, solid line) and after protein boost (open circles, dotted line). (b) Antibody epitope mapping with pooled sera from the B1 group, before (filled squares, solid line) and after protein boost (open squares, dotted line). (c) Antibody epitope mapping with pooled sera from the B2 group, before (filled triangles, solid line) and after protein boost (open triangles, dotted line). The start position of every fourth peptide is indicated on the x-axis. The assay was run at dilution 1:100 with a naïve serum cutoff of 0.3.

IgG2a responses remained low in the group boosted with rgp160 only (data not shown).

Overlapping peptides covering the subtype B gp120 antigen were used to screen for vaccine-induced humoral responses to antigenic determinants (Figure 2). The peptides corresponded to the gp120 constant regions 2 to 5 (C2–5) and variable regions 3 to 5 (V3–5).²⁸ We show that multi-subtype immunization with HIV-1 gp160 genes of subtypes A, B and C together with rGM-CSF results in the serum IgG recognition of four major gp120 sites (V3, C3, V4 and V5). The serum IgG from ABC DNA primed animals recognized 65% of all peptides in the constant and variable regions (Figure 2a). Boosting the same animals with rgp160 together with CpG-ODN increased the antibody epitope recognition of C2, C3, C4 and C5, with an overall peptide recognition frequency of 73%. Immunization with subtype B DNA resulted in the recognition of epitope regions in C2, C3, C4 and V5 with peptide recognition frequencies of 29% (group B1) and 23% (group B2). Boosting the subtype B DNA-immunized animals with rgp160, with or without CpG-ODN, increased the response to epitopes in the V3 and C3 region (Figure 2b and c). The sera from DNA-primed animals recognized the gp41 peptides NEQLLELDK-WASLWN (optical density (OD) 0.8 ± 0.1) and KDQQLGIWGCSGKLICT (OD 1.7 ± 0.3) to a similar extent (data not shown). The protein boost increased the IgG reactivity towards the two gp41 epitopes in all groups (OD 2.7 ± 0.2 and 3.0 ± 0.2 , respectively).

Multi-subtype HIV-1 neutralization

The sera collected after one immunization with gp160 DNA and rGM-CSF (Table 1, part II) had 90% neutralization capacity at serum dilutions of 1:10 against HIV-1 isolates IIB and SF2 (data not shown). This is to be compared with the sera from the animals that received gp160 DNA without adjuvant, which resulted in the neutralization of approximately 30%. The sera from mice primed with DNA and rGM-CSF (part III) responded with anti-gp160 antibodies with high potency to neutralize the subtype B HIV-1 strains LAI, SF-2 and 6920 already after DNA priming (Table 2). The B1 group responded with similar neutralization titers to the subtype B viruses, whereas the B2 group had lower neutralization titers. The multi-subtype-immunized mice also neutralized the primary vaccine-matched subtypes A (92UG031) and C (92BR025) isolates (Figure 3). A 90% reduction of virus production was seen at 40- and 80-fold serum dilutions, respectively (Table 2). The sera from animals immunized with subtype B DNA showed neutralization below 90% of subtypes A and C at the lowest (1:20) serum dilutions. Instead of raising the neutralizing capacity, the boost with recombinant protein reduced this, despite higher binding antibody titers (Figure 3). The background neutralization was assessed in the sera from the untreated naïve mice. The background neutralization was generally very low (Figure 3, panels d, h and l).

Cellular immunity to multiple epitopes

Spleen T-cell proliferative responses (SI of 3–20) were seen in all (40/40) animals immunized with gp160 DNA without adjuvant (Table 1, part I). Splenocytes from

Table 2 Neutralization titers induced by HIV-1 gp160 DNA

Virus	Subtype	TCID ₅₀	Sera from group						Polyclonal human IgG	
			ABC		B1		B2		HIVIG	
			IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀
92UG031	A	125	90	40	<20	<20	<20	<20	> 20 000	> 20 000
LAI	B	400	>2000	>2000	>2000	>2000	550	50	5000	350
SF-2	B	470	>2000	>2000	>2000	>2000	>2000	580	> 20 000	> 20 000
6920	B	120	>2000	730	>2000	1150	1520	460	15 000	4600
92BR025	C	25	>2000	80	200	<20	80	<20	20 000	5600

TCID₅₀ = tissue culture infectious dose 50%; IC₅₀ = inhibitory concentration 50%; IC₉₀ = inhibitory concentration 90%.

animals given a mixture of plasmids pKCMVgp160 A and C responded better (mean SI >20) to rgp160 restimulation than splenocytes from animals that received one of the envelope genes (mean SI of 3–5). The same splenocytes were also stimulated with overlapping 15-mer peptides designed to match the gp120 V1–V5 region of subtypes A- and C-encoding plasmids. Again, animals immunized with plasmids pKCMVgp160 A and C together showed a higher degree of reactivity to A and C peptides than animals immunized with any of the single envelope genes (Table 1, part I). Immunization with rGM-CSF resulted in significantly increased T-cell proliferation against rgp160 in animals receiving the cytokine in comparison to animals that did not ($P < 0.05$) (Table 1, part II). A distinct enhancement of IL-4 production (32 ± 6 pg/ml of IL-4) was also observed in the T-cell supernatants when splenocytes from animals given rGM-CSF were compared to those from animals given the DNA immunogen alone (4 ± 1 pg/ml of IL-4).

Figure 4 Lymphocyte proliferation. (a) Fresh splenocytes from individual mice were restimulated with rgp160 subtype B antigen followed by the detection of incorporated radiolabeled thymidine. The cutoff for response was determined to 27 000 cpm. Error bars indicate one standard deviation. (b) Frozen splenocytes were pooled and restimulated with subtype A- and C-specific overlapping peptides covering the gp120 V1–V5 region. The cutoff for response was determined to 1500 cpm. Error bars indicate the intra-assay standard deviation.

Figure 5 Interferon- γ ELISpot. Envelope-specific IFN- γ -secreting cells were detected in pooled PBMC from each group isolated 6 weeks after DNA immunization (white bars) and in splenocytes 2 weeks after protein boost (gray boxes). The cutoff for a positive response was 50 SFCs per million cells (line).

All groups in part III showed T-cell proliferative responses after stimulation with rgp160 (Figure 4a and summarized in Table 1). Restimulation with peptide pools matching subtypes A and C resulted in broadly reactive responses in animals primed with the multi-subtype or subtype B DNA and later boosted by rgp160 with CpG-ODN (the ABC and B1 groups, Figure 4b). Animals that received subtype B DNA followed by rgp160 without CpG-ODN were unable to recognize peptides representing subtypes A or C.

Pooled murine PBMC from each group were collected and analyzed for IFN- γ production after stimulation with rgp160, at 6 weeks after the second prime with DNA and rGM-CSF (Figure 5). Subtype B-immunized animals developed intermediate responses while ABC-immunized animals had weak IFN- γ production. Splenocytes isolated 10 days after the booster immunization also produced IFN- γ . The B1 group appeared to have the strongest CD8+ reactivity as assessed by stimulation with rgp160. IFN- γ responses were also detected in multi-subtype-immunized mice after the boost with rgp160 plus CpG-ODN. Only background levels were, however, seen in mice primed with subtype B DNA boosted with rgp160 without immunostimulatory ODNs (group B2).

Discussion

The induction of potent neutralizing anti-HIV antibody responses by any type of vaccination has proved difficult.^{29,30} We show that recombinant GM-CSF together with HIV-1 envelope genes from multiple subtypes (A, B and C) can change the envelope DNA immunogen into a potent entity that induces high titers of broadly reactive antibodies as well as cellular responses.

Plasmid-encoded GM-CSF has previously been used as a genetic adjuvant to DNA vaccination and has proved efficient in inducing both Th1- and Th2-type responses.^{16,20,21,31} The timing of the administration of the GM-CSF gene has been shown to be relevant for the type of immune response induced. When the GM-CSF gene was inoculated 3 days prior to genetic immunization, the immune response was mainly of the Th2 type, whereas simultaneous inoculation gave a balanced Th1/Th2 profile. Moreover, inoculation with the GM-CSF gene 3 days postimmunization gave a Th1 response.³² As rGM-CSF protein was administered in our study at the time of immunization, we are likely to imitate the first of these scenarios, since the GM-CSF is present before antigen expression. Indeed, the addition of rGM-CSF to our DNA immunogen strongly favors antibody production with a Th2 tilted IgG subclass profile³³ and enhanced IL-4 production. The plasmids were able to induce general lymphocyte proliferation, as seen in the first two immunization protocols, with significantly increased proliferation when rGM-CSF was delivered together with the gp160 DNA.

The humoral immunity was enhanced by the combination of envelope genes from viruses of subtypes A, B and C. Multi-subtype DNA immunization contributed to a larger number of responders with higher antibody titers than immunization with subtype B DNA alone. Antibody epitope mapping of the gp120 revealed

broad antigen recognition in the sera obtained from multi-subtype-immunized animals in comparison to the sera from subtype B-immunized mice. The gp41 region in all DNA constructs was identical. By analyzing the IgG serum response towards two highly conserved gp41 regions, we show that the sera from the subtype B and the multi-subtype-immunized animals recognized the gp41 epitopes equally well. In addition, the multi-subtype-immunized mice were able to neutralize primary isolates of subtypes A, B and C, whereas mice immunized with the gp160 gene derived from subtype B were not.

We believe that the enhancement of antibody production, as well as the broader antibody specificity in the multi-subtype-immunized animals, are due to the presentation of multiple epitopes and thereby the stimulation of a broad clonal expansion of B cells. This is consistent with our previously reported finding that multi-subtype-immunized mice were more prone to develop antibody responses after experimental HIV challenge when compared to mice receiving the envelope gene from a single HIV-1 subtype.²⁹ Similarly, broad antibody responses have been reported in the clinic with multi-subtype infected individuals being capable of producing IgG that recognizes HIV from several subtypes.³⁴

Enhanced crossreactive T-cell proliferative responses were detected after immunization with a mixture of plasmids encoding subtype A and C proteins in comparison to inoculation with the individual plasmids. Combining plasmids encoding A, B and C antigens resulted in cellular immunity detected as gp160-specific IFN- γ production of PBMCs.

The rgp160 protein boost resulted in a reduced neutralizing ability despite the induction of higher binding antibody titers and broader antigenic recognition. This may be explained by the fact that the rgp160 is most likely not present in its native trimeric form. Rather, it would consist of monomeric structures displaying non-neutralizing epitopes not shown on the native protein (reviewed in Poignard *et al*³⁵). Investigators have reported that gene-based immunogens may be more effective than protein antigens in inducing antibodies to conformational epitopes,³⁶ and that boosting with synthetic peptides may refocus the antibody response and can sometimes abrogate neutralizing capacity.²² Similar mechanisms are most likely responsible for the postboost reduction of the neutralizing capacity in our study. After DNA priming, the subtype B-immunized animals (Figure 2, group B1) show an antibody response directed to the C3 region. This response was refocused towards the V3 region after the protein boost. In theory, these postboost V3-directed antibodies should be very potent in neutralizing the homologous LAI strain, but this was not the case in our study. The boost of V3 titers may not be sufficient to replace the neutralizing responses to conformational epitopes that were lost after the protein boost. Antibodies to conformational epitopes may be more relevant, but are difficult to detect using a standard peptide ELISA. We believe that the DNA immunogens are more efficient at presenting correctly folded epitopes. It is worth noting, however, that the neutralization of the LAI strain after the protein boost was still high.

The lymphocyte proliferation results, as measured by rgp160 B restimulation, were similar in all groups after

protein boost. Priming with multi-subtype DNA followed by protein boosting together with CpG-ODN resulted in broad T cellular responses. In addition, the group primed with gp160 B DNA that received a protein boost with CpG-ODN (the B1 group) showed a higher degree of crossreactivity against subtypes A and C peptide pools compared to the group primed with gp160 B DNA followed by protein boost without CpG-ODN (the B2 group). Therefore, it could be argued that the CpG-ODN component helps in creating a Th milieu, likely to affect the overall cellular as well as humoral responses.

Cellular recall responses are known to be sensitive to antigen differences. The relatively low level of IFN- γ detected in the ABC DNA primed and subtype B protein boosted animals could therefore be explained by the phenomenon of original antigenic sin at the cellular level.³⁷ More specifically, in HIV-1 infected patients therapeutically immunized with rgp160, it has been shown that boosting an already existing cellular immunity to the HIV-1 envelope must be carried out with a highly similar immunogen to evoke functional CTL.³⁸ Therefore, it is likely that only the subfraction of T cells responding to the subtype B antigen in the ABC group were able to produce IFN- γ upon restimulation with the subtype B recombinant protein. Recombinant subtype A and C envelope proteins were unfortunately not commercially available at the time of this study.

The B2 group, boosted with rgp160 only, did not respond with IFN- γ production above background levels, results that are in concordance with previous findings.^{24,39} We therefore argue that the CpG component was essential for the induction of the IFN- γ response, something that is strengthened by the switch in antibody isotype from a Th2-type response towards a more balanced Th1/Th2-like response.^{33,40–42}

In conclusion, we had developed a gene-based immunization strategy capable of strongly enhancing humoral immune responses to the HIV-1 envelope through rGM-CSF mediated immune enhancement in mice. Moreover, multi-subtype immunization increased the response frequency and broadened antibody neutralization and peptide reactivity. In clinical protocols, genetic immunization has been moderately successful.^{43–45} Therefore, optimization of preclinical protocols is crucial for the future success of the DNA vaccine field. A similar DNA priming regimen may increase the potency and breadth of anti-HIV responses needed for an HIV vaccine to be effective in humans. Ultimately, phase I clinical trials with the HIV-1 envelope gene and rGM-CSF as a molecular adjuvant in healthy volunteers will show if the working hypothesis that the envelope DNA immunogen can be adjuvanted by GM-CSF also holds true in man. Such trials are in progress.

Materials and methods

Plasmids and immunizations

The gene-based immunogens pKCMVgp160 A, pKCMVgp160 B, pKCMVgp160 C and pKCMVrev have been described elsewhere.^{29,46} The gp160 B plasmid encodes the entire gp160 molecule, in which the furin cleavage site responsible for post-translational modification into gp120 and gp41 was deliberately modified by

site-directed mutagenesis, resulting in amino-acid changes R511S and A512M. The gp160 gene encoded by pKCMVgp160 B originates from a subtype B (LAI) virus, whereas the A and C plasmids encode chimeric molecules in which the V1–V5 region of the subtype B backbone has been replaced with corresponding fragments from WHO strains A92UG031 (HIV-1 subtype A) and 92BR025 (HIV-1 subtype C), respectively. All plasmids were grown in *Escherichia coli* and bacterial endotoxins were removed (QIAGEN Endofree mega plasmid kit, QIAGEN, Germany). Three immunization protocols (I–III) were used (Table 1). *Part I*: Four groups of 10 C57Bl/6 mice (6- to 8-week-old female) were given 50 µg by the intramuscular (i.m.) route of either pKCMVgp160 A, pKCMVgp160 B or pKCMVgp160 C or a 25 + 25 µg mixture of plasmids pKCMVgp160 A and C (Table 1, part I). Each envelope DNA dose was mixed with 50 µg pKCMVrev and dissolved in 50 µl H₂O. An untreated group and a mock vector backbone (pKCMV)-immunized group were also included. Animals were killed 14 days after the last immunization. *Part II*: Two groups of C57Bl/6 mice were immunized i.m. by a single dose of 50 µg pKCMVgp160 B + 50 µg pKCMVrev with or without 1 µg of murine rGM-CSF (R&D Systems, UK) (Table 1, part II). The rGM-CSF was mixed with the DNA in a total volume of 100 µl PBS. Animals were killed 30 days after the immunization. *Part III*: Two groups of C57Bl/6 mice were immunized twice i.m. with a total of 2 × 100 µg of DNA (75 µg pKCMVgp160 B + 25 µg pKCMVrev) (Table 1, part III). One group was given a mixture of 25 µg of plasmids pKCMVgp160 A, B and C together with 25 µg pKCMVrev. The total gp160 DNA dose in the multi-subtype group (75 µg) was the same as that of the subtype B-immunized group. The DNA was delivered in 50 µl H₂O. rGM-CSF (1 µg) was administered to the site of immunization 15 min prior to vaccination, dissolved in 50 µl H₂O. Booster immunizations were delivered at week 11 at the site of DNA immunization and consisted of 20 µg of rgp160 formulated in aluminum hydroxide (MicroGeneSys (now Protein Sciences), CT, USA) with or without 50 µg of the 1826 CpG-ODN motif (TCCATGACGTTCTT GACGTT, Cybergene, Sweden).²³ The B DNA-primed animals given a protein boost of rgp160 together with CpG-ODN is referred to as B1 group and the group given rgp160 only as B2. Blood was obtained 14 days after the second DNA immunization, followed by protein boosting and killing 14 days later.

Antibody IgG ELISA

ELISA was carried out essentially as described previously.²⁹ Briefly, ELISA plates (Nunc Maxisorp, Denmark) were coated with rgp160. The serum was diluted in 10-fold serial dilutions and reactive antibodies were detected with goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP) (Bio-Rad, CA, USA). Subclass IgG antibodies were detected using rabbit anti-mouse IgG1 and IgG2a antibodies (Sigma-Aldrich, Sweden) followed by a goat anti-rabbit HRP antibody (DAKO PO449, Denmark). Plates were developed with O-phenylene diamine and the OD was read at 490 nm. Two times the mean OD of the naïve sera was used as cutoff for a positive response. The anti-envelope serum IgG response was mapped by pooled sera using 48

individual peptides (15-mers with 5-mer overlap) representing amino acids 249–514 of gp120 HIV-1 subtype B.^{47,48} Two well-characterized gp41 antibody epitopes (NEQLLELDKWASLWN, amino acid 657–672; and KDQQLGIWGCSGKLICT, amino acid 599–618) were also included.^{49,50} The cutoff for antibody mapping was calculated as the mean OD of negative sera plus three standard deviations.

HIV-1 neutralization assay

The HIV-1 neutralization assay was performed as described previously.⁵¹ The viral isolates used for the neutralization were the WHO reference strains 92UG031 (subtype A) and 92BR025 (subtype C), the subtype B laboratory strains IIB LAI (viral isolates from which the HIV-1 envelope gene fragments encoded by our DNA immunogens originate), the subtype B laboratory strain SF-2 and the primary subtype B isolate 6920. Briefly, the sera from mice responding with binding anti-envelope antibodies were pooled group wise, inactivated at 56°C for 1 h to prevent complement-mediated neutralization. The sera were diluted in RPMI 1640 medium (Invitrogen, Scotland) in 96-well tissue culture plates (Nunc micro-well plates, Nunc, Denmark). The sera and virus were mixed appropriately and incubated at 37°C for 1 h followed by the addition of 100 000 human PBMCs activated by phytohemagglutinin. The cultures were incubated at 37°C in 5% CO₂ over night, after which the cells were washed twice with RPMI 1640. After 6 days of culture, the presence of HIV-1 p24 antigen in the culture medium was measured.⁵² The background in the p24 ELISA was determined for each plate and subtracted from all wells before the percentage neutralization was determined as [1 – (mean p24 OD in the presence of test serum/mean p24 OD in the absence of test serum)] × 100.

Lymphocyte proliferation and cytokine production

The lymphocyte proliferation was performed essentially as described earlier.²⁹ Splenocytes (400 000 per well) were cocultured with antigen (0.1 µg rgp160 per well) for 3 days before H³+-labeled thymidine was added to each well and incubated for 16 h. The cells were harvested and counted in a β-counter (Wallac 1450, Finland). After 72 h, interferon-γ and IL-4 were determined in the culture supernatants by ELISA in accordance with the manufacturer's instructions (R&D Systems, UK). Cryo-preserved splenocytes were thawed, pooled group wise and cultured over night in 10% FCS-RPMI. These cells were used for the analysis of subtype crossreactivity: Two different peptide pools (50 peptides per pool, 15-mers with 8-mer overlap, at 1 µg/peptide/well), corresponding to the subtype variable regions (V1–V5, aa 120–477) of the subtypes A and C viruses, were used to determine proliferation in response to matched A and C antigen. In the prime-boost schedule (part III), murine PBMCs were analyzed 6 weeks after the second immunization with DNA and rGM-CSF by IFN-γ ELI-Spot. Spleen-derived lymphocytes were also analyzed 10 days after the boost with rgp160. Briefly, blood was pooled from each group in EDTA-containing tubes. The blood was centrifuged and the peripheral lymphocytes purified by Ficoll-Paque (Amersham Pharmacia Biotech, Sweden). Alternatively, splenocytes were isolated from mashed spleens by centrifugation at 1000 rpm for

8 min. The cells were resuspended in PBS, purified by Ficoll–Paque as above. The lymphocytes were washed twice and seeded (200 000/well) onto an ELISpot plate (Millipore Multiscreen plates #MAIPN4510, Bedford, MA, USA). Antigens were rgp160 or a baculovirus-derived control protein (1 µg per well respectively). The ELISpot procedure was performed essentially according to the manufacturer's instructions (DIACLONE, France). Spot-forming cells (SFCs) were detected in an AID ELISpot reader (Autoimmun Diagnostika GmbH, Germany). SFC per million cells were calculated as spots in the envelope protein-stimulated wells minus spots in the control protein-stimulated wells. The criterion for a positive response was more than 50 SFC per million cells. ConA was used as a positive control in T-cell proliferation as well as in the ELISpot assay. A stimulation index (SI) for T-cell proliferation was calculated as the ratio of *in vitro* antigen stimulation divided by medium stimulation. Student's *t*-test (Statistica 6.0, Uppsala, Sweden) was used for the analysis of the cellular data. The criterion for statistical significance was $P < 0.05$.

Acknowledgements

We thank Reinhold Benthin and Gunnel Engström for outstanding technical assistance and Roya Hakimnia, Sayi Kimwaga-Stika, Sandra Melki and Marwa Osman for the gp41 ELISA. This work was supported by the Foundation for Strategic Research: the Infection and Vaccinology programme and the Inflammation programme; and by the Swedish Research Council and EU Grant ICA4-CT-2002-10036.

References

- UNAIDS/WHO. AIDS epidemic update: December 2003. UNAIDS/WHO, (www.unaids.org) 2003.
- Wahren B. HIV in Europe – a growing threat. *J Clin Virol* 2001; **21**: 171–173.
- Kanki PJ et al. Human immunodeficiency virus type 1 subtypes differ in disease progression. *J Infect Dis* 1999; **179**: 68–73.
- McMichael AJ, Hanke T. HIV vaccines 1983–2003. *Nat Med* 2003; **9**: 874–880.
- Nabel GJ. HIV vaccine strategies. *Vaccine* 2002; **20**: 1945–1947.
- Berman PW VaxGen Announces Results of its Phase III HIV Vaccine Trial in Thailand: Vaccine Fails to Meet Endpoints, <http://www.vaxgen.com/pressroom/index.html> VaxGen Announces Results of its Phase III HIV Vaccine Trial in Thailand: Vaccine Fails to Meet Endpoints, <http://www.vaxgen.com/pressroom/index.html> > 2003.
- Hanke T, McMichael AJ. Design and construction of an experimental HIV-1 vaccine for a year – 2000 clinical trial in Kenya. *Nat Med* 2000; **6**: 951–955.
- Shiver JW et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002; **415**: 331–335.
- Calarota SA et al. Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *J Immunol* 1999; **163**: 2330–2338.
- Boyer JD et al. Therapeutic immunization of HIV-infected chimpanzees using HIV-1 plasmid antigens and interleukin-12 expressing plasmids. *AIDS* 2000; **14**: 1515–1522.
- Gaschen B. Diversity considerations in HIV-1 vaccine selection. *Science* 2003; **299**: 1515–1518.
- Moore JP, Parren PW, Burton DR. Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. *J Virol* 2001; **75**: 5721–5729.
- Muthumani K ZD et al. Novel engineered HIV-1 East African Clade-A gp160 plasmid construct induces strong humoral and cell-mediated immune responses *in vivo*. *Virology* 2003; **15**: 134–146.
- Barouch DH et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. *Nature* 2002; **415**: 335–339.
- Barouch DH et al. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 2000; **290**: 486–492.
- Barouch DH et al. Potent CD4+ T cell responses elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF. *J Immunol* 2002; **168**: 562–568.
- Sin JI et al. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell-mediated protective immunity against herpes simplex virus-2 challenge. *J Immunol* 1999; **162**: 2912–2921.
- Sin JI et al. Interleukin 7 can enhance antigen-specific cytotoxic-T-lymphocyte and/or Th2-type immune responses *in vivo*. *Clin Diagn Lab Immunol* 2000; **7**: 751–758.
- Kato H et al. Rectal and vaginal immunization with a macromolecular multicomponent peptide vaccine candidate for HIV-1 infection induces HIV-specific protective immune responses. *Vaccine* 2000; **18**: 1151–1160.
- Kim JJ et al. Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J Interferon Cytokine Res* 1999; **19**: 77–84.
- Moore AC, Kong WP, Chakrabarti BK, Nabel GJ. Effects of antigen and genetic adjuvants on immune responses to human immunodeficiency virus DNA vaccines in mice. *J Virol* 2002; **76**: 243–250.
- Zuber B et al. Induction of immune responses and break of tolerance by DNA against the HIV-1 coreceptor CCR5 but no protection from SIVsm challenge. *Virology* 2000; **278**: 400–411.
- Davis HL et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J Immunol* 1998; **160**: 870–876.
- Zuber B, Mäkitalo B, Zuber AK, Wahren B. A novel potent strategy for induction of immunity to HIV-1 reverse transcriptase in primates. *AIDS* 2002; **16**: 1839–1840.
- Deml L et al. Immunostimulatory CpG motifs trigger a T helper-1 immune response to human immunodeficiency virus type-1 (HIV-1) gp 160 envelope proteins. *Clin Chem Lab Med* 1999; **37**: 199–204.
- Halperin SAVNG et al. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine* 2003; **21**: 2461–2467.
- McCluskey MJ, Weeratna RD, Payette PJ, Davis HL. Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA. *FEMS Immunol Med Microbiol* 2002; **32**: 179–185.
- Gorny MK et al. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. *J Virol* 2002; **76**: 9035–9045.
- Ljungberg K et al. Enhanced immune responses after DNA vaccination with combined envelope genes from different HIV-1 subtypes. *Virology* 2002; **302**: 44–57.
- Chakrabarti BK et al. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. *J Virol* 2002; **76**: 5357–5368.

- 31 Iwasaki A *et al.* Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J Immunol* 1997; **158**: 4591–4601.
- 32 Kusakabe K *et al.* The timing of GM-CSF expression plasmid administration influences the Th1/Th2 response induced by an HIV-1-specific DNA vaccine. *J Immunol* 2000; **164**: 3102–3111.
- 33 Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 1997; **158**: 2278–2284.
- 34 Guay LA *et al.* Phase I/II trial of HIV-1 hyperimmune globulin for the prevention of HIV-1 vertical transmission in Uganda. *AIDS* 2002; **16**: 1391–1400.
- 35 Poignard P, Saphire EO, Parren PW, Burton DR. gp120: Biologic aspects of structural features. *Annu Rev Immunol* 2001; **19**: 253–274.
- 36 Wang B *et al.* Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1993; **90**: 4156–4160.
- 37 Klenerman P, Zinkernagel RM. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* 1998; **394**: 482–485.
- 38 Kundu SK *et al.* Role of preimmunization virus sequences in cellular immunity in HIV-infected patients during HIV type 1 MN recombinant gp160 immunization. *AIDS Res Hum Retroviruses* 1998; **14**: 1669–1678.
- 39 Maurer T *et al.* CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *Eur J Immunol* 2002; **32**: 2356–2364.
- 40 Liu HM *et al.* Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor. *Blood* 1998; **92**: 3730–3736.
- 41 Ayash-Rashkovsky M *et al.* Induction of antigen-specific Th1-biased immune responses by plasmid DNA in schistosoma-infected mice with a preexistent dominant Th2 immune profile. *Biochem Biophys Res Commun* 2001; **282**: 1169–1176.
- 42 Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol Med Microbiol* 2001; **32**: 65–71.
- 43 Wang R *et al.* Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998; **282**: 476–480.
- 44 Calarota S *et al.* Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* 1998; **351**: 1320–1325.
- 45 MacGregor RR *et al.* First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis* 1998; **178**: 92–100.
- 46 Kjerrström A *et al.* Interactions of single and combined human immunodeficiency virus type 1 (HIV-1) DNA vaccines. *Virology* 2001; **284**: 46–61.
- 47 Akerblom L *et al.* Neutralizing cross-reactive and non-neutralizing monoclonal antibodies to HIV-1 gp120. *AIDS* 1990; **4**: 953–960.
- 48 Broliden PA *et al.* Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. *Proc Natl Acad Sci USA* 1992; **89**: 461–465.
- 49 Calarota S *et al.* Immunodominant glycoprotein 41 epitope identified by seroreactivity in HIV type 1-infected individuals. *AIDS Res Hum Retroviruses* 1996; **12**: 705–713.
- 50 Wang JJ, Steel S, Wisniewolski R, Wang CY. Detection of antibodies to human T-lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment of gp41 envelope protein. *Proc Natl Acad Sci USA* 1986; **83**: 6159–6163.
- 51 Hinkula J *et al.* Immunological and virological interactions in patients receiving passive immunotherapy with HIV-1 neutralizing monoclonal antibodies. *J Acquir Immune Defic Syndr* 1994; **7**: 940–951.
- 52 Devito C, Levi M, Broliden K, Hinkula J. Mapping of B-cell epitopes in rabbits immunised with various gag antigens for the production of HIV-1 gag capture ELISA reagents. *J Immunol Methods* 2000; **238**: 69–80.