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Immune Responses in Asymptomatic HIV-1-Infected Patients After HIV-DNA Immunization Followed by Highly Active Antiretroviral Treatment

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Intensive chemotherapy is capable of reducing the viral load in HIV-1-infected individuals while infected cells are still present. A special property of DNA immunization is to induce both new CTL and Ab responses. We evaluated the possibility of inducing new immune responses in already infected individuals by means of DNA constructs encoding the nef, rev, and tat regulatory HIV-1 genes. Significant changes in viral loads and CD4+ counts were observed in four patients who started highly active antiretroviral treatment (HAART) during the immunization study. The DNA immunization induced Ag-specific T cell proliferation, which persisted up to 9 mo after the last DNA injection, and cytolytic activities but did not, by itself, reduce viral load. Increased levels of CTL precursor cells were induced in all nine DNA-immunized patients. The profile of IFN-γ secretion observed when human PBMC were transfected with the nef, rev, and tat DNA resembled that found in the CTL activity (nef > tat > rev). Ab responses that occurred after immunizations were of a low magnitude. In accordance with the high IL-6 production induced by the nef DNA plasmid, IgG titers were highest in patients immunized with nef DNA. The initiation of HAART appears to contribute to the induction of new HIV-specific CTL responses, but by itself did not cause obvious re-induction of these activities. The Journal of Immunology, 1999, 162: 2330–2338.

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2 Abbreviations used in this paper: HAART, highly active antiretroviral treatment; CTLp, CTL precursor; SI, stimulation index; B-LCL, EBV-transformed B-lymphoblastoid cell line; ELISPOT, enzyme-linked immunospot; MuLV, murine leukemia virus.
SV40 origin of replication (positions 5092–5207). The immunization schedule was approved by the local ethical committee of the South Hospital in Stockholm and the Swedish Medical Products Agency. Informed consent before immunization was given by all patients in accordance with the Swedish ethical regulations.

Vaccination

Nine asymptomatic HIV-1-infected patients were included in the study (Table I). The immunization scheme and the initiation of HAART treatment are shown in the figures. The patients belong to a cohort of 40 originally asymptomatic HIV-1-infected individuals who have been vaccinated with rgp160 and responded to gp160 with B and T cell-specific immunity (11, 14). Sera from these patients were repeatedly tested by ELISA, using peptides and proteins representing the HIV-1 nef, rev, or tat genes. They were selected for having no or low Ab reactivities to the protein coded by the gene used for the vaccination. Thus, three patients were immunized with HIV-1 nef DNA (patients 7, 3, and 39), three with HIV-1 rev DNA (patients 37, 25, and 34), and three with HIV-1 tat DNA (patients 29, 6, and 12). Eight patients were immunized at days 0, 60, and 180; patient 7 was immunized at days 0, 40, and 180. The DNA constructs (100 μg) were administered in distilled water (1 ml) by i.m. injection in the right deltoid muscle. Blood was collected before and on the day of each immunization, 14 days after each immunization, and every 2–3 mo thereafter. Patient 34 moved abroad in 1997 and was lost to follow-up. Ten asymptomatic HIV-1-infected patients, who also belong to the well-controlled cohort of 40 individuals repeatedly vaccinated with rgp160, who had not received HIV-1 DNA immunization as well as 14 HIV-1 seronegative laboratory workers were recruited as controls.

Antiviral chemotherapy

Table I shows the nine patients and the antiretroviral treatment received. Patient 7 received zidovudine (3’-azido-3-deoxythymidine; AZT) and didanosine at entry and started HAART (AZT, lamivudine, and indinavir) after the third DNA immunization. Patient 29 started HAART on the day of the second DNA immunization, and patients 3 and 37 did so 3 mo after the third DNA immunization. Five patients are naive to antiretroviral therapy.

Table I. Patients included in the study

<table>
<thead>
<tr>
<th>Patient</th>
<th>DNA Immunogen</th>
<th>Antiretroviral Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>nef</td>
<td>AZT + didanosine at entry and started HAART* 3 mo after the third immunization</td>
</tr>
<tr>
<td>3</td>
<td>nef</td>
<td>Started HAART 3 mo after the third immunization</td>
</tr>
<tr>
<td>39</td>
<td>nef</td>
<td>Naive</td>
</tr>
<tr>
<td>37</td>
<td>rev</td>
<td>Started HAART 3 mo after the third immunization</td>
</tr>
<tr>
<td>25</td>
<td>rev</td>
<td>Naive</td>
</tr>
<tr>
<td>34</td>
<td>rev</td>
<td>Naive</td>
</tr>
<tr>
<td>29</td>
<td>tat</td>
<td>Started HAART on the day of the second immunization</td>
</tr>
<tr>
<td>6</td>
<td>tat</td>
<td>Naive</td>
</tr>
<tr>
<td>12</td>
<td>tat</td>
<td>Naive</td>
</tr>
</tbody>
</table>

* HAART; combination of AZT, lamivudine, and indinavir.

37°C; and thereafter with the substrate o-phenylene diamine (Sigma, St. Louis, MO). The plates were washed six times after each incubation step. The absorbance was measured at 490 nm. Serum samples from 10 uninfected individuals were used as negative controls. For comparison, sera from non-DNA-immunized HIV-1-infected individuals, who belong to the cohort of 40 individuals (14), were also tested. Values higher than the mean of negative controls plus 3 SD were considered positive.

Lymphocyte proliferation assay

T cell subsets (CD4 and CD8 cell counts) were determined at every sampling collection as mentioned above. The T cell proliferation of fresh PBMC was done as previously described (11, 14). The following purified Ags were used at concentrations of 1 μg/ml and 10 μg/ml: recombinant Nef protein from HIV-1 Bru derived from Escherichia coli (provided by V. Erle, Gesellschaft für Strahlung und Umweltforschung (GSF), Neuenberg, Germany), recombinant Rev protein from HIV-1 LAI derived from a baculovirus-lepidopteran cell system (provided by G. Engström, Swedish Institute for Infectious Disease Control, Stockholm, Sweden), and E. coli-recombinant HIV-1 Bru Tat protein (provided by C. Svanholm, Karolinska Institute, Stockholm, Sweden). These proteins are useful to measure virus-specific responses to the respective DNA plasmids encoding nef, rev, or tat (25). The Ags were not toxic. PHA (HA16; Glaxo Wellcome Orion Diagnostica, Irosa, Sweden) at 1 μg/ml was used as the positive control and complete medium (RPMI 1640) with 1-glutamine supplemented with 10% human serum AB+ from blood donors, 100 IU/ml penicillin, and 100 μg/ml streptomycin; Life Technologies, Taby Sweden) was used to correct for spontaneous proliferation. The mean cpm was calculated for triplicates of Ags, mitogen, or the medium control. The stimulation index (SI) was calculated by dividing the mean cpm for each Ag or mitogen by the mean cpm for a medium control. An SI of ≥3 was considered positive.

Cytotoxicity assay

PBMC were isolated by Ficoll-Hypaque density gradient separation (Pharmacia Biotech, Uppsala, Sweden). PBMC collected during the first 6 mo of the study were stimulated in vitro in culture medium (RPMI 1640) with t-glutamine supplemented with 10% human serum AB+ from blood donors, 100 IU/ml penicillin, and 100 μg/ml streptomycin; Life Technologies) in the presence of recombinant human IL-2 (Amersham Pharmacia Biotech, Uppsala, Sweden) for 14 days as described (23). Briefly, on days 0 and 7, cultures were stimulated by the addition of paraformaldehyde-fixed autologous EBV-transformed B-lymphoblastoid cell lines (B-LCL) infected with recombinant vaccinia vectors expressing either the nef (MVA-nef), the rev (VVTG-rev), or the tat (VVTG-tat) HIV-1 LAI gene (multiplicity of infection 2–5), or with HIV-1 pseudotyped with amphotropic murine leukemia virus (MuLV) 4070A (HIV-1/MuLV) (23, 31). In addition, frozen PBMC obtained after the third DNA immunization were stimulated in vitro with autologous lymphocytes infected with recombinant vaccinia vectors vP1218 (expressing the HIV-1 MN nef), VVTG-rev or VVTG-tat at a multiplicity of infection of 5. Cultures also contained 330 IU/ml of IL-7 (R&D Systems, Minneapolis, MN) (32). Cytolytic activity was measured in detail 4-51Cr release assays at various E:T ratios (23). Targets cells were autologous and heterologous B-LCL infected with MVA-nef (or vP1218), VVTG-rev, VVTG-tat, or HIV-1/MuLV. Targets infected with wild-type vaccinia virus (MVA-WT) or uninfected B-LCL served as controls. HIV-1 expression in infected targets was determined by indirect immunofluorescence assay, using the anti-p24 mAb EF7 (33) and mAbs against Nef, Rev, or Tat (29). Culture supernatants from cells infected with
HIV-1/MuLV were tested for HIV-1 p24 release. CTL assays were considered positive if the lysis of Ag-expressing targets exceeded the lysis of control targets by $>10\%$. The frequency of HIV-1-specific CTLp was estimated by limiting dilution analysis (34).

**Human cytokine enzyme-linked immunospot (ELISPOT) assays**

A detailed description of cytokine ELISPOT assays has been published (35, 36). Briefly, 96-well nitrocellulose-backed microtiter plates (Millipore, Bedford, MA) were coated with 10 $\mu$g/ml of monoclonal anti-cytokine Ab overnight at 4°C. The plates were then blocked with 5% BSA in PBS for 1 h and washed extensively with PBS-Tween 20. Mononuclear cells from peripheral blood of normal volunteers were separated by density gradient centrifugation over Ficoll-Hypaque. Cells were washed twice in RPMI 1640 supplemented with 10% heat-inactivated FCS. Serial dilutions of a single cell suspension, starting with 10$^5$ cells/well, were incubated on the anti-cytokine Ab-coated plates at 37°C for 10 h in humidified 5% CO$_2$ in an air incubator in the presence of 50 $\mu$g/ml of DNA plasmid. E. coli, HIV-1 plasmid p37 (25), or calf thymus DNA (Sigma) were used as positive and negative control DNAs as well as medium. The plates were washed with Tween in PBS and overlaid with biotinylated anti-cytokine Ab for 2 h at room temperature. Plates were washed again, treated with a 1/300 dilution of avidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 1 h, and washed a final time. The cytokine secreted by single cells was visualized by the addition of a solution of 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT; Kirkegaard & Perry Laboratories, Gaithersburg, MD). This solution yields a purple precipitate in the presence of phosphatase. The colorimetric reaction was halted after 20 min by washing with water, and spots were enumerated under $\times 40$ magnification.

**Results**

**Cytokine production**

Recent studies indicate that CpG motifs in the plasmid backbone of a DNA vaccine can influence the nature and magnitude of the immune response. Before administering the nef, rev, and tat encoding DNA plasmids to humans, we examined their immunostimulatory activity on human PBMC in vitro. Cells from normal donors were incubated in vitro for 10 h with immunostimulatory bacterial DNA, control calf thymus DNA, or plasmid. As expected, CpG motifs present in the E. coli DNA but absent from mammalian DNA stimulated human PBMC to produce IFN-$\gamma$ and IL-6 (Fig. 1). Increased IFN-$\gamma$ production was also elicited by the DNA vaccines, with the nef encoding gene being the most active. A similar hierarchy was observed when IL-6 production was monitored. These findings indicate that the DNA plasmids used in this study contained motifs that would be immunostimulatory in man, with the nef encoding plasmid being most active in this regard. Two types of controls were included; the number of cells secreting both cytokines was highest for transfection with E. coli bacterial DNA when compared with the mammalian DNA transfection. For comparison, a plasmid expressing the p24 gene of HIV-1 (p37 DNA) and known to produce a Th1 response in mice, induced a high production of IFN-$\gamma$.

**Lymphocyte proliferative responses**

Fresh PBMC from all patients were assayed for lymphocyte proliferation in response to Nef (patients 7, 3, and 39), Rev (patients 37, 25, and 34), and Tat Ags (patients 29, 6, and 12), respectively. The results are shown in Fig. 2. Before the first DNA immunization, low or no proliferations were seen to these Ags in all nine patients. After the first nef DNA immunization (Fig. 2A), the three patients responded to Nef Ag, with SI $>3$ in repeated assays. Continued high levels were observed in patients 7 and 3 after the initiation of HAART and a further increase in patient 39 (who did not receive antiretroviral therapy) after the third immunization.

Patient 37, who was rev DNA immunized, was consider a non-responder by proliferation. However, a significant response was observed after the initiation of HAART (Fig. 2B). Proliferative response to the Rev Ag was found positive from day 74 (2 wk after the second immunization) in patient 25. Strong proliferation to Rev was detected in patient 34 after the first immunization and on the day of the second injection. Over the course of follow-up, the latter two patients showed an increased or stable T cell response to Rev without HAART.

Tat-specific proliferation was detected at most time points in patient 29, who started HAART on the day of the second immunization (Fig. 2C). In patients 6 and 12, proliferative responses to Tat were detected at least twice after tat DNA immunizations.

In summary, significant increases from baseline were seen in 93/138 (67%) experiments conducted with fresh cells after immunization as compared with 20/45 (44%) experiments conducted with fresh cells before immunization ($p < 0.01$).

Proliferative responses to the two other regulatory HIV Ags unrelated to that used in the immunizations were also evaluated at several occasions (not shown in the figures). Patients 3, 39, and 37 did not respond to both unrelated Ags (SI $\leq 3$) at any time points tested. Four patients (numbers 25, 34, 29, and 12) showed no responses to one of the two unrelated Ags (Tat or Rev), whereas a positive response was observed to Nef Ag (SI range, 3.1–19.2); this response increased in two patients (numbers 34 and 12) and decreased in two patients (numbers 25 and 29). Proliferative responses to both unrelated Ags were detected at some time points in patients 7 and 6; the responses decreased during the follow-up (patient 7) and an increase was observed in patient 6 against Rev (SI from 1.5 to 6).

Stimulation with PHA resulted in high levels of proliferation which did not change significantly over time (data not shown). Fresh PBMC from non-DNA-immunized infected or noninfected individuals were also assayed for T cell proliferation against Nef, Rev, and Tat at several time points. None of the 29 noninfected individuals showed a response; the mean SI values to Nef, Rev, and Tat were 1.4 ($n = 10$), 1.4 ($n = 12$), and 1.2 ($n = 7$), respectively (data not shown). Eight non-DNA-immunized HIV-1-infected individuals (four are naïve to antiretroviral treatment and...
four received HAART) had detectable but low HIV-specific proliferative responses. Results from two representative individuals from each group are shown in Fig. 2D. No responses were detected in two patients without antiretroviral therapy, whereas in two patients a decrease to one of the three Ags was observed. In two of the patients receiving HAART, an increase was observed to one of the three Ags, whereas the other two patients were stable and negative at all time points.

**CD4** lymphocyte counts

The CD4 lymphocyte development is detailed in Fig. 2. In three of four patients (numbers 3, 37, and 29) who received both DNA immunizations and HAART, an increase was observed, whereas in patient 7 the response was transient.

No significant change from base levels was seen in four of five patients following DNA vaccination without HAART, whereas in patient 25 (rev DNA immunized) a moderate decrease was observed. CD4 levels thus appeared to increase considerably in patients receiving HAART.

**HIV-specific CTLp**

The frequencies of HIV Nef-, Rev-, and Tat-specific CTLp were estimated by limiting dilution assays before and during DNA immunizations. All nine patients developed detectable HIV-specific CTLp after DNA vaccination. Fig. 3 presents the CTLp frequencies estimated longitudinally in five patients. The CTLp frequencies correlated well with the CTL assays and ranged from 1 to 166/10^6 PBMC in nef DNA immunized patients (Fig. 3, A–C). The frequency of CTLp against Rev-expressing targets ranged from 16 to 31/10^6 PBMC (data not shown); the highest level was detected in patient 25 (Fig. 3D). A high level of CTLp to Tat-infected targets was detected in patient 6 (Fig. 3E), whereas in the other two patients immunized with tat DNA the level of CTLp ranged from 2 to 46/10^6 PBMC (data not shown). CTLp frequencies to Nef, Rev, and Tat transfected target cells were also estimated at some occasions in five HIV-infected individuals non-DNA immunized. CTLp frequencies ranged from 1 to 26/10^6 PBMC, from 1 to 13/10^6 PBMC, and from 1 to 12/10^6 PBMC against Nef-, Rev-, and Tat-expressing targets, respectively.
Tat-infected targets, respectively. Fig. 3F shows repeated data from three of the control non-DNA immunized HIV-infected patients.

**Cytotoxic T lymphocyte responses during DNA immunizations**

CTL results from these patients as well as from control individuals were reported in Calarota et al. 23. Briefly, CTL assays were negative before DNA vaccination in all nine patients. After DNA immunization, cytotoxic activity was quite marked in patients immunized with nef DNA. Overall, HIV-1-specific CTL increased and remained in five immunized patients; in three patients the response was transient and one patient did not react (patient 37). Cytolysis was mediated by CD8+ MHC class I-restricted CTL as determined by depletion of CD8+ cells (23). CTL activity against MHC class I mismatched targets was also tested. As shown in Fig. 4, mismatched target cells were not recognized by the CTL.

**Correlation between CTL activity and lysis of HIV-1 Ag expressing targets**

The target cells used for demonstration of CTL activity were analyzed with respect to Ag occurrence and content. HIV-1 p24 Ag was detected in all culture supernatants from targets infected with HIV-1/MuLV. The expression of HIV-1 proteins in targets was detected by indirect immunofluorescence assay and the detectable percent of infected cells varied among the autologous B cell lines.

For example, 50% of B-LCL from patient 7 were infected with HIV-1/MuLV (Fig. 5A) and at an E:T ratio of 25:1, CTL from this patient lysed half the fraction of visibly infected targets. The percent of B-LCL from patient 34 infected with HIV-1/MuLV was 30% (Fig. 5B) and, at an E:T ratio of 25:1, CTL lysed a fraction of targets equal to all detectable HIV-1/MuLV-infected targets. A similar pattern was observed between CTL from patient 3 to autologous targets infected with MVA-nef (Fig. 5C). As shown in Fig. 5D, 25% of B-LCL from patient 12 expressed HIV-1 Tat and, at an E:T ratio of 50:1, the percent of specific lysis was 14%, which is equivalent to ~60% of the Ag-stained cells. We conclude that the lysis

![FIGURE 3](image-url) Frequencies of HIV-specific CTLp from five asymptomatic HIV-infected and DNA immunized patients. A–C, nef DNA immunized patients; D, rev DNA immunized patient; and E, tat DNA immunized patient. F, Consecutive samples from HIV-infected patients 16, 2, and 13, who were non-DNA immunized. Frequencies were normalized to number of CTLp/10⁶ PBMC. Error bars indicate 95% confidence intervals. *, Not tested. DNA immunizations are indicated (†). Absence of columns indicates undetectable.

![FIGURE 4](image-url) CTL activity of stimulated PBMC against MHC class-I mismatched targets. A, Patients 39 and 7, nef DNA immunized, at day 180. B, Patient 34, tat DNA immunized, at day 74. C, Patient 6, tat DNA immunized, at day 180. Results are expressed as percent specific lysis, determined by subtracting the percent lysis of control targets from that of Ag-expressing targets. The number of the patient from whom the B-LCL were derived is indicated. HLA A phenotypes of the patients are: HLA A2 (patients 39 and 6); HLA A9 (patient 7); HLA A11,19 (patient 34); and HLA A3,10 (patient 29).
is effective against autologous HIV-infected cells that express the largest amount of Ag, although this point was not directly proven.

**Correlation between HIV-specific CTL responses and viral load in patients with both DNA vaccination and antiretroviral treatment**

Results from the four patients who started HAART during the present study are shown in Fig. 6. Significant decreases ($\geq 1 \log_{10}$ copies/ml change) in viral RNA were noted in all of them. Patient 7 (Fig. 6A) had detectable CTL activity 16 mo after the last nef immunization and 13 mo after HAART started. In patient 29 (Fig. 6D), after 2 wk of the last tat immunization, CTL responses were weak and later disappeared. In these two patients the viral level has again increased. However, when viral load at baseline is compared with the latest time point, the HIV-1 RNA level is still considerably decreased. Patient 7 showed a positive CTL response when viral load started to increase. No CTL responses were detected after 8–9 mo of HAART treatment in patients 3 and 37 (Fig. 6, E), when a significant reduction of plasma RNA viral load was still noted. Patient 37 was continuously a CTL nonresponder.

The initiation of HAART treatment appears not to contribute to the induction of new HIV-specific CTL responses, CTL responses to Nef, Rev, and Tat were detected in three HIV-infected non-DNA-immunized patients, who also started HAART treatment. A representative patient is shown in Fig. 6E. Positive CTL assays from these patients ranged from 13 to 16% specific lysis.

**Longitudinal analysis of HIV-specific CTL responses and viral load in patients receiving DNA immunization only**

The five patients who received DNA immunizations without antiretroviral treatment showed no significant changes in HIV-1 RNA levels (Fig. 7). Remarkably, CTL responses to Nef-infected targets were detected in patient 39 up to 17 mo after the third nef DNA immunization (Fig. 7A). After the last rev DNA immunization, patient 25 showed a positive cytotoxic response (Fig. 7B), whereas in patient 34 weak CTL responses were seen only during immunization (Fig. 7C). In patient 6 (immunized with tat DNA), CTL activity to Tat-infected targets was marginal (11%) or more pronounced (19%) after the third immunization (Fig. 7D). No cytolytic activity against Tat was detected in the follow-up of patient 12 (Fig. 7E). Thus, the DNA-induced CTL responses appear to wane when immunizations were stopped.

CTL responses in two HIV-infected individuals who did not receive either DNA immunization or HAART treatment were also evaluated. Results are shown in Fig. 7F. CTL responses detected in both patients against targets expressing Nef, Rev, or Tat were of a low magnitude.

In addition, CTL responses to the two other unrelated Ags to that used in the immunization were evaluated during the follow-up of the eight patients and three of them (patients 25, 3, and 12) showed negative CTL responses at the time points tested. In three patients (numbers 39, 29, and 7) CTL responses against Rev were detected at one occasion, whereas in patients 6 and 37 against Nef, these responses ranged from 11 to 20% specific lysis to the corresponding Ag (not shown in figures).

**Ab responses**

The patients were selected for having no or low initial Ab reactivities to Nef (three patients), Rev (three patients), and Tat (three patients) HIV-1 Ags. In general, the IgG titer increases which occurred after HIV-DNA immunization were of a low magnitude and are summarized in Table II.

Abs to Nef peptides were detected from 14 days after the first immunization in patient 39 and at day 180 (third immunization) in the other two patients immunized with nef DNA (patients 7 and 3).

Abs to Rev peptides were detectable from 14 days after the first immunization in patient 25 and at day 180 in patient 37. After the
third immunization, Abs in the serum samples from patient 34 reacted with almost all Rev peptides tested. Very low IgG reactivity (titers of 24–54) was found before immunization to HIV-1 Tat peptides in patients immunized with tat DNA (Table II). No increase in Ab titers was observed in the three patients after immunizations (titers of 23–56). Thus six of the nine individuals (nef and rev but not tat DNA immunized) developed IgG serum Abs to the respective peptides and of a low and variable magnitude.

Sera from the DNA immunized patients were tested after the third immunization to peptides representing the two other unrelated Ags to that used in the immunization. None of the nine patients reacted to the unrelated Ags (Table II).

For comparison, sera from 31 non-DNA-immunized HIV-1-infected individuals were also tested. Sera from seven individuals reacted with the peptides covering the C-terminal region of HIV-1 nef, whereas sera from five and three individuals reacted to Rev and Tat peptides, respectively (Table II).

Clinical follow-up
After the last plasmid injection, eight patients have been monitored for 11–14 mo. None has developed an AIDS complication, and one patient (number 34) moved abroad in a healthy state. The immunizations were well tolerated, except for one patient who once developed local erythema at the site of injection. He continued immunizations with no further complications.

Discussion
DNA immunization in asymptomatic HIV-1-infected patients was attempted to evaluate the feasibility of inducing immune reactivities in humans. We have demonstrated that immunization with plasmid DNA expressing the HIV-1 nef, rev, or tat regulatory genes induces new HIV-specific T cell proliferative and cytolytic responses in asymptomatic HIV-1-infected patients.

Antiretroviral combination therapy with at least two nucleoside analogues and at least one protease inhibitor (HAART) has been shown to decrease viral load, increase CD4+ lymphocyte counts, delay disease progression and prolong survival of HIV-infected individuals (37–39). In our study, the four patients who started HAART showed increased CD4+ counts and significantly decreased viral loads. Probably, these changes were influenced by HAART. We found no evidence that HIV-DNA immunization alone caused any significant decrease in viral load and/or increase in the CD4+ lymphocyte counts. However, these parameters remained stable during the study period; a moderate decrease in CD4+ counts was observed only in patient 25 (rev DNA immunized).

The most relevant immune response induced by DNA vaccination is perhaps the Ag-specific T cell proliferative responses, because they are lost early in infection. It is clear that these responses were induced in the majority of the DNA immunized patients, can be seen in a higher frequency than in non-DNA immunized patients, and may persist for many mo after the last plasmid immunization. A correlation between Ag-specific proliferative responses and cytolytic activities was observed at several but not all time points. HIV-specific proliferative T cell responses remarkably remained or improved in the four patients treated by HAART.

It has been shown that bacterial DNA induces B cell proliferation and cytokine secretion (40). A growing body of evidence indicates that the unmethylated CpG dinucleotides in the carrier bacterial DNA can enhance the Th1 immune response (41–43). In our study, the profile of IFN-γ secretion observed when treating human PBMC with plasmids encoding the HIV-1 nef, tat, or rev genes in similar plasmid backbones resembles that found in the CTL activity. The CTL response was marked in patients immunized with nef DNA, whereas a moderate cytotoxic activity was observed in patients immunized with tat or rev DNA. Subsequent studies performed in our group, after Ag-specific in vitro stimulation of PBMC from patients immunized with a combination of plasmids encoding the HIV-1 regulatory genes, have shown that lymphocytes stimulated with Nef Ag effectively released IFN-γ (our unpublished data). We must therefore consider that the regulatory genes themselves contribute to the cytokine profile.

HIV-specific Ab, T cell proliferation, and CTL responses were absent before immunization. The results presented here, although based on a small number of patients, demonstrate that CTL against Nef, Rev, and Tat may be induced by DNA vaccination. We showed previously that the cytolytic activity was mediated by CD8+ MHC class I-restricted CTL (23). It has been shown that CTL responses against Rev and Tat are inversely correlated with rapid disease progression to AIDS (7), and our intention now is to magnify these responses by increased doses of HIV-DNA.

The appearance and persistence of CTL activity varied from individual to individual following DNA immunization. Detectable CTLp cells were induced in all nine patients. Our results indicate that nef DNA immunization induced the highest CTL response. This activity remained up to 17 mo after the last immunization in one patient who is still naive to antiretroviral treatment (patient 39). CTL responses appeared to decrease or disappear after the initiation of HAART. It has been suggested that these responses...
were observed in sera from patients immunized with HIV-DNA immunization induced humoral immune responses, although of a very low magnitude. The highest peptide Ab titers observed in sera from patients immunized with DNA to unrelated antigens.

The nine patients were selected for having no or low Ab reactivities to the HIV-1 regulatory proteins. These variables were initially chosen to provide measuring points. Our data demonstrate that HIV-DNA immunization induced humoral immune responses, although of a very low magnitude. The highest peptide Ab titers were observed in sera from patients immunized with nef DNA to peptides covering the C-terminal region of HIV-1 Nef. These results confirm that Nef is the most immunogenic of the HIV-1 regulatory genes and are also in accordance with the IL-6-inducing capacity of the nef plasmid. Rev Ab titers were low, which may be due to the low antigenicity of the HIV-1 Rev protein (46) and the poor Th2 inducing capacity of the plasmid.

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The induction of immunity to HIV-1 by vaccination with regulatory genes has been described in animal models (25, 26, 47). The regulatory proteins are expressed earlier than the structural proteins in the replicative cycle of the virus; thus, the induction of immune responses against them may allow the elimination of infected cells before progeny virus is released. Nef is expressed during the early phase of virus replication (48) and plays an important role in HIV-1 replication and pathogenesis (49). Both Rev and Tat proteins are absolutely essential for virus replication (50). CTL against these essential HIV-1 regulatory proteins may prevent further viral replication and may contribute to the duration of the asymptomatic period.

depend on continued viral replication (44). A correlation has also been shown between the decrease in plasma HIV RNA to undetectable levels and the disappearance of CTL activity in individuals with acute HIV primary infection treated with HAART (45). However, our non-HAART treated and DNA immunized individuals also appeared to lose their CTL reactivity, indicating that the DNA-induced CTL activity is short lived in the immunodeficient individuals.

The nine patients were selected for having no or low Ab reactivities to the HIV-1 regulatory proteins. These variables were initially chosen to provide measuring points. Our data demonstrate that HIV-DNA immunization induced humoral immune responses, although of a very low magnitude. The highest peptide Ab titers were observed in sera from patients immunized with nef DNA to peptides covering the C-terminal region of HIV-1 Nef. These results confirm that Nef is the most immunogenic of the HIV-1 regulatory genes and are also in accordance with the IL-6-inducing capacity of the nef plasmid. Rev Ab titers were low, which may be due to the low antigenicity of the HIV-1 Rev protein (46) and the poor Th2 inducing capacity of the plasmid.

The induction of immunity to HIV-1 by vaccination with regulatory genes has been described in animal models (25, 26, 47). The regulatory proteins are expressed earlier than the structural proteins in the replicative cycle of the virus; thus, the induction of immune responses against them may allow the elimination of infected cells before progeny virus is released. Nef is expressed during the early phase of virus replication (48) and plays an important role in HIV-1 replication and pathogenesis (49). Both Rev and Tat proteins are absolutely essential for virus replication (50). CTL against these essential HIV-1 regulatory proteins may prevent further viral replication and may contribute to the duration of the asymptomatic period.

Our results clearly show that DNA immunization with HIV-1 regulatory genes is capable of inducing new HIV-1-specific T cell immune responses, even in immunocompromised individuals. The DNA immunization was well tolerated and no adverse effect was observed. A recent study demonstrates the safety of a DNA-based vaccine in infected individuals (21), supporting our findings. Few HAART-treated infected individuals spontaneously recover their HIV-specific Th activity (14) unless they are treated very early during primary disease (51). The combination of DNA vaccination with HAART might thus result both in the induction of immune responses and in viral load reduction.

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References


Table II. IgG Ab responses to HIV-1 Nef, Rev, or Tat Ags in sera from nine asymptomatic HIV-1-infected patients following DNA vaccination with regulatory HIV-1 genes

<table>
<thead>
<tr>
<th>Patients</th>
<th>Antigen</th>
<th>Before</th>
<th>After</th>
<th>Non-DNA (n = 31) + DNA (n = 6) Immunized HIV-Infected Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>nef (n = 3)</td>
<td>Ne peptides</td>
<td>aa 151–170</td>
<td>0/3</td>
<td>2/3 (110–180)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 166–185</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 181–200</td>
<td>0/3</td>
<td>2/3 (100–140)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 196–206</td>
<td>0/3</td>
<td>1/3 (130–150)</td>
</tr>
<tr>
<td>rev (n = 3)</td>
<td>Rev peptides</td>
<td>aa 1–20</td>
<td>0/3</td>
<td>1/3 (35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 16–35</td>
<td>0/3</td>
<td>1/3 (29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 31–50</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 46–65</td>
<td>0/3</td>
<td>1/3 (20–36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 61–80</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 76–95</td>
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<tr>
<td></td>
<td></td>
<td>aa 91–110</td>
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<td>2/3 (22–29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 106–116</td>
<td>0/3</td>
<td>2/3 (20–24)</td>
</tr>
<tr>
<td>tat (n = 3)</td>
<td>Tat peptides</td>
<td>aa 1–20</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 16–35</td>
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<td>3/3 (23–34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aa 31–50</td>
<td>1/3 (24)</td>
<td>3/3 (23–46)</td>
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<td></td>
<td></td>
<td>aa 76–86</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Results are presented as number of patients with measurable titers (≥20)/number of patients analyzed, describing two to four events before and five events after DNA immunization. Titer range and/or titer of a single sample is shown in parentheses.

a Sera from 31 non-DNA-immunized HIV-infected individuals were tested together with the serum obtained after the third immunization from the DNA immunized patients. These tests included six sera from rev and tat immunized patients tested with Nef peptides, six sera from nef and tat immunized patients tested with Rev peptides, and six sera from nef and rev immunized patients tested with Tat peptides. None of the DNA-immunized patients reacted to the unrelated antigens.


