

Long-Term Evaluation of Cellular Immunity during Antiretroviral Therapy and Immunization with Human Immunodeficiency Virus Type 1 (HIV-1) Env Glycoprotein in HIV-1–Infected Persons

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Cellular immune responses to human immunodeficiency virus type 1 (HIV-1) antigens, microbial recall antigens, and CD3 monoclonal antibody were studied in HIV-1–infected asymptomatic patients in a phase II, double-blind trial of immunization with recombinant HIV-1 gp160 in or not in association with zidovudine. A vigorous and persistent lymphoproliferative response (LPR) to HIV-1 Env antigens was observed in vaccinated patients. Neither Env-specific lymphocyte cytotoxicity nor LPR to recall antigens was significantly influenced by gp160 administration. The induction of LPRs to HIV-1 envelope proteins did not show positive effects on the course of HIV-1 infection. Patients treated with zidovudine alone or in combination with the immunogen showed improvement of T lymphocyte responses and transient reduction of viremia. These results suggest that antiretroviral therapy is more beneficial than immunization with gp160 and should always be considered in association with future vaccination and immunotherapeutic interventions in HIV-1–infected subjects.

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by an initial acute symptomatic phase followed by a period of asymptomatic chronic infection. This period, which may last several years, was initially interpreted as true viral latency. In view of this interpretation, an intervention aimed at inducing or boosting the HIV-1–specific immune response was proposed as a possible means for preventing a supposed virus reactivation leading to AIDS [1]. Current views on the pathogenesis of AIDS take into account that virus replication is continuous during infection, with a peak during the acute phase and a progressive increase during the late stages [2, 3]. Rather than being latent, HIV-1 is continuously challenging the immune system, which potently reacts against it, possibly generating a balance that underlies the asymptomatic phase. However, a progressive impairment of immune responses is

observed [4], which may also affect HIV-1–specific immunity. Potentiation of the antiviral immune response may therefore lead to a more prolonged containment of the virus.

During the last few years, the course of infection has been modified by the use of antiretroviral drugs. Controlled experimental studies using monotherapy or combined therapies with reverse transcriptase inhibitors demonstrated a decrease of plasma virus load, an increase of CD4 T cell numbers, and a reduction of observed clinical events [5, 6]. In addition, preliminary results of anti-HIV-1 vaccination using proteins of viral envelope to elicit a specific immune response were promising [7, 8] and encouraged the setting-up of phase II controlled studies to evaluate the clinical efficacy of this approach.

To explore both the relevance of postexposure immunotherapy with an HIV-1 subunit and the possible synergism with conventional antiretroviral therapy, we performed a placebo-controlled, double-blind study comparing the use of recombinant (r) gp160 alone, rgp160 in association with zidovudine, and zidovudine alone in 99 HIV-1–infected asymptomatic patients.

Here we report immunologic measures of HIV-1–specific immune response and T cell function for a subset of 59 of the 99 patients randomized in the trial.

Materials and Methods

Patients. Fifty-nine asymptomatic HIV-1–infected subjects (18–50 years old; 23 women [39%], 36 men [61%]) who had never received antiretroviral drugs were randomized in a phase II, double-blind, three-arm, placebo-controlled trial of immunization with rgp160 in or not in association with antiretroviral monotherapy. According to the Centers for Disease Control and Prevention's

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Informed consent was obtained from all patients enrolled in the study.

Human experimentation guidelines of the Italian Department of Health and of the US Department of Health and Human Services were followed.

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HIV classification system [9], the subjects were classified as groups A1 and A2, having mean CD4 T cell counts between 400 and 600 cells/ μ L. The group of subjects was followed at the University of Rome "La Sapienza" and was part of the two-center protocol 809A-205-IT, which included a total of 99 patients. A detailed description of the clinical results and the main immunologic and virologic results of the entire trial is forthcoming (Pontesilli O, et al., unpublished data).

According to risk factors for HIV-1 infection, the subjects were grouped as follows: 16 male homosexuals, 16 intravenous drug abusers, 2 patients who had received a transfusion with HIV-1-infected blood, and 25 patients who had been infected with HIV-1 through heterosexual contact. Candidate patients were screened 1 month before the study to ensure that they met entry criteria. Inclusion criteria and end points were described in detail elsewhere [10]. In brief, randomized patients had to be asymptomatic (group A) and present a median CD4 T cell count between 400 and 600 cells/ μ L at screening. Main end points were progression to symptomatic disease (groups B or C) or CD4 T cell count <200 cells/ μ L. Serodiagnosis of HIV-1 infection was made by standard ELISA and confirmed by Western blot (Cambridge Biotech, Rockville, MD).

Vaccine product and immunization schedule. The test vaccine, a noninfectious subunit derived from HIV-1 gp160, was produced in a baculovirus expression system in cells of lepidopteran insects, biochemically purified, and adsorbed to aluminum phosphate for final formulation (rgp160, VaxSyn; Protein Sciences, formerly MicroGeneSys, Meriden, CT). The patients were randomized into 3 drug-treatment groups: 1 group received 320 μ g of rgp160 intramuscularly (im) and oral placebo (rgp160 group), another group received 320 μ g of rgp160 im and 250 mg of zidovudine orally two times a day (zidovudine plus rgp160 group), and the last group received alum im and 250 mg of zidovudine orally two times a day (zidovudine group). During the first 2 years of the study, the patients received 12 injections: The first was on day one, and the rest were at months 1–4 and 6 and every 3 months thereafter, up to month 24. The study was for 31 months, including the 1-month screening period and the 6-month postimmunization period. In the group treated with zidovudine, 5 patients did not complete the study (3 had reached one of the established end points, and 2 dropped out due to causes unrelated to the study). In the group treated with rgp160, 8 patients did not complete the study (5 had reached one of the established end points, and 3 dropped out due to causes unrelated to the study). In the group treated with zidovudine plus rgp160, 7 patients did not complete the study (4 had reached one of the established end points, and 3 dropped out due to causes unrelated to the study).

Immunologic and virologic tests schedule. Lymphoproliferation tests, cytotoxic T lymphocyte (CTL) activity, and cytofluorimetric analysis of lymphocyte subpopulations were done at baseline and at months 1, 3, 6, 12, 18, 24, and 30. Plasma HIV-1 RNA was quantitated before the vaccination, after 1 month, and at 4 and 24 months.

HIV-1 antigens. Four recombinant HIV-1 antigens derived from the HIV-1_{IIIb} sequence were selected for lymphocyte stimulation assays (rgp160, rgp120, rp24, and rRev protein) as representative of three structural proteins (core and envelope) and one regulatory protein. Baculovirus-expressed rgp160 was obtained from Protein Sciences; baculovirus-expressed rgp120 and *Escherichia*

coli-expressed rp24 and rRev protein were purchased from American Bio-Technologies (Cambridge, MA).

Antigen-specific lymphoproliferative responses (LPRs). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Lymphoprep (Nycomed, Oslo) at 800 g for 30 min. Separated PBMC (10^6 cells/mL) were cultured in 200 μ L of RPMI 1640 with 10% AB human serum that was negative for anti-HIV-1 antibodies (NABI, Miami) in 96-well flat-bottom cell culture plates (Falcon, Lincoln Park, NY) in the presence of 2 μ g/mL of rgp160, rgp120, rp24, or rRev protein. To test the proliferative response to recall antigen, we added 50 μ g/mL mannoprotein (MP) of *Candida albicans* [11] and 1 Lf/mL tetanus toxoid (TT; Wyeth, Marietta, PA) to the cultures, which were run in triplicate.

CD3 monoclonal antibody (MAB)-induced lymphoproliferation. Cultures were set up essentially as described above for antigen cultures, with the following differences: 10^5 PBMC were seeded in each well in 100 μ L of RPMI 1640 with 10% fetal calf serum added. CD3 MAb OKT3 (Ortho Diagnostic Systems, Raritan, NJ) at 25 ng/mL was added.

Calculation of specific stimulation index (SI). After 6 days of culture at 37°C in a humidified 5% CO₂ atmosphere (3 days for cultures with mitogens), 0.5 μ Ci of tritiated thymidine (specific activity of 25 Ci/mmol; Amersham, UK) were added to each well. After 24 h (4 h for cultures with mitogens), cells were harvested onto glass fiber filters, and incorporated radioactivity was measured in a scintillation counter and expressed as counts per minute (cpm). The SI was calculated by dividing cpm of stimulated cultures by cpm of the unstimulated cultures. LPRs to viral antigen were considered significant for SI >2. This cutoff value was chosen because the mean + 2 SD of the SI obtained in cultures of PBMC from HIV-1-seronegative controls for each HIV-1 antigen tested was always lower than 2 [12]. The same cutoff has been used by other researchers in similar assays with HIV-1 antigen [13].

CTL assay. Preparation of recombinant vaccinia viruses, target cells and effector cells is described elsewhere [14]. The assay was done essentially as previously described [15]. Variable numbers of effector cells were mixed with 10^4 ⁵¹Cr-labeled target cells in V-bottomed microtiter wells in order to obtain effector-to-target ratios of 50:1; 25:1; and 12.5:1 (triplicate cultures). Replicates with target cells in RPMI 1640 plus 10% fetal calf serum only and 0.1% Triton X-100, respectively, were also set up to determine spontaneous and total release. The cells were then incubated for 6 h at 37°C and centrifuged at 140 g for 5 min. Supernatant (100 μ L) was collected from each well, and radioactivity was determined. Results are expressed as follows: % specific ⁵¹Cr release = (experimental release – spontaneous release) $\times$ 100/(total release – spontaneous release). Percent Env-specific cytotoxicity = Env-expressing target release – control target release.

Lymphocyte phenotyping. Lymphocyte phenotype was determined by dual-color direct immunofluorescence with MAb combinations OKT3-fluorescein isothiocyanate (FITC)/OKT4-phosphatidylethanolamine (PE) and OKT3-FITC/OKT8-PE (Ortho Diagnostic) followed by flow cytometry.

Plasma HIV-1 RNA determination. Plasma HIV-1 RNA was quantified by a nucleic acid sequence-based assay (NASBA HIV-1 RNA QT; Organon Teknica, Boxtel, Netherlands).

Statistics. Group responder fractions of LPRs to rgp160 were compared by the χ^2 test. Significance of LPR modifications from baseline

values was assessed by the Wilcoxon test, and LPR values between groups at the same time point were compared by use of the Mann-Whitney U test. Correlation of LPR to rgp160 and rgp120 was studied by linear regression. Variations of CD4 T cell numbers and plasma HIV-1 RNA levels from baseline values and between groups were analyzed by Student's *t* test for paired and unpaired data, respectively.

Results

Response to HIV-1 rgp160. To evaluate the immunization to the injected recombinant antigen, we quantified the fraction

of subjects in each study group who showed a specific SI >2 at each time point. Both groups receiving rgp160 showed an increase of the responder fraction: from 10% to 93% after 18 months in the rgp160 group and from 20% to 93% in the zidovudine plus rgp160 group after 24 months. As shown in figure 1, the increase of the responder fraction in the rgp160 group was significant from the third month (*P* < .001). The induction of the response to rgp160 in the zidovudine plus rgp160 group was apparently faster, since a significant increase

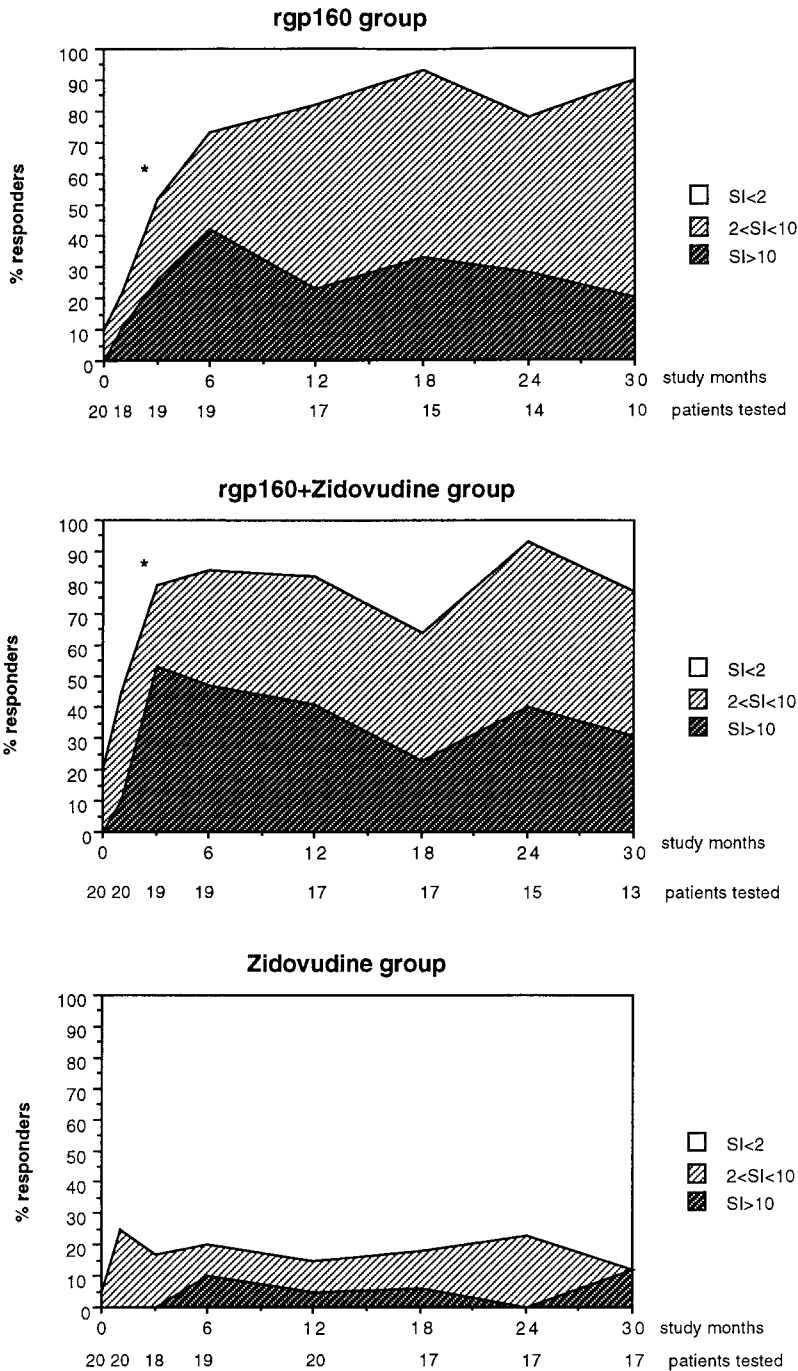


Figure 1. Fraction of patients in each treatment group showing significant recombinant gp160 (rgp160)-specific lymphoproliferative responses, subdivided according to stimulation index (SI) >10 and SI between 2 and 10. Nos. of patients tested at each time point are indicated underneath each graph. **P* < .0001 vs. baseline responder fraction (χ^2 test).

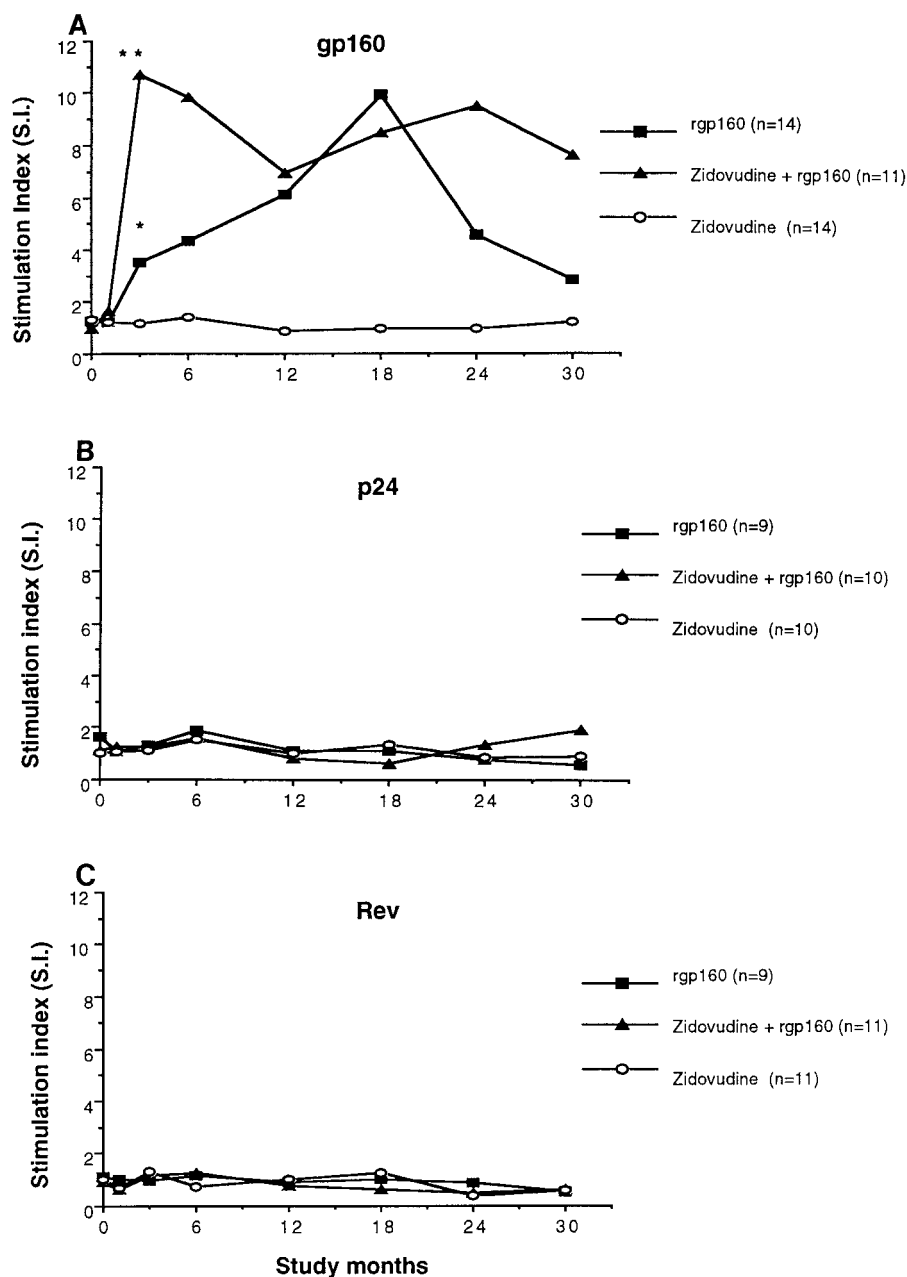


Figure 2. Median SIs of lymphoproliferative responses to HIV-1 gp160 (A), p24 (B), and Rev protein (C) in patients who had complete immunovirologic follow-up. * $P = .034$. ** $P < .005$ vs. baseline values (Wilcoxon test); $P < .01$ vs. zidovudine group (Mann-Whitney test). Nos. of patients studied are shown for each group.

of the responder fraction was seen also after 1 month ($P = .025$). When compared with the increase in the responder fraction in the zidovudine group, the responder fraction at each time point was significantly higher from the third month in the 2 groups receiving rgp160 ($P < .001$). Confirming the faster kinetics of response, the responder fraction at 1 month in the zidovudine plus rgp160 group was significantly higher than that of the zidovudine group at the same time ($P = .039$). A transient moderate increase of the responder fraction was seen also in the zidovudine group only after the first month ($P = .039$).

Longitudinal evaluation of LPRs to HIV-1 antigen. To compare the magnitude of the LPRs to rgp160, rgp120, rp24, and rRev protein, only the patients who completed the immunization protocol and subsequent follow-up were considered. As shown in figure 2A and table 1, no significant variation of the LPR to rgp160 was detected in the zidovudine group. In the rgp160 group, a significant increase of the LPR to rgp160, compared with the baseline SI, was observed after 3 months and in all other subsequent tests ($P = .034$ at month 3 and $P < .01$ subsequently). A significant increase of LPR to rgp160 in the zidovudine plus rgp160 group was also observed starting

Table 1. Median (range) number of lymphoproliferative responses to gp160 in HIV-1–infected persons before and during antiretroviral therapy and immunization with HIV-1 Env glycoprotein.

Treatment group	Time after beginning treatment			
	Baseline	Month 3	Month 12	Month 30
Recombinant gp160	1.16 (0.51–9.04)	2.7 (0.63–31.55)	3.97 (1.52–145.89)	3.5 (1.81–89.9)
Zidovudine + recombinant gp160	1.17 (0.42–5.48)	10.68 (1.01–63.39)	9.53 (1.37–231.38)	7.63 (0.58–75.38)
Zidovudine	1.07 (0.59–9.21)	1.06 (0.17–21.5)	1.02 (0.36–14.13)	1.27 (0.53–20.76)

from the third month ($P < .005$ at all time points). As shown by the increase of the responder fraction, the zidovudine plus rgp160 group had a faster response to rgp160, as reflected by an SI that was significantly higher than that of the zidovudine group at 3 and 6 months ($P < .01$), when the responder fraction for the rgp160 group SI was not significantly different from that of the zidovudine group. After the first year of treatment, both groups receiving rgp160 had significantly higher LPRs to rgp160 compared with that for the zidovudine group. The LPRs to rgp160 were persistently elevated in both groups, even though a trend toward reduction was seen 6 months after the last injection.

A significant correlation was observed between LPR to rgp160 and to rgp120 in all studied groups ($r = .684$ in the zidovudine group, $.962$ in the rgp160 group, and $.862$ in the zidovudine plus rgp160 group; $P < .001$ for all groups), confirming the specificity of the response.

LPRs to HIV-1 antigen other than gp160 induced by natural infection were weak and sporadic, which was consistent with previous observations [12]. At baseline, the median LPRs to rp24 and rRev protein in the study population, regardless of treatment randomization were 1.25 (range: 0.44–5.47) and 1.01 (range: 0.41–6.54), respectively. No significant modification of the LPR to rp24 or rRev protein was observed in any of the 3 groups (figure 2B, C).

LPRs to recall antigen and CD3 MAb. We studied the effect of treatment on T cell function measures, namely responses to recall antigen and to CD3 MAb. A subgroup of 32 patients equally distributed in the 3 groups was evaluated for LPRs to MP and TT, and median SIs are shown in figure 3. No significant modification of the LPR to either microbial stimulant was seen in the rgp160 group. An increase of the LPR to both antigens was seen in the zidovudine group; however, only for MP was the increase significant at months 6 and 18 ($P < .05$) compared with baseline values. Even though not statistically significant, an early transient increase of LPR to MP was seen also in the zidovudine plus rgp160 group.

LPR to CD3 MAb is shown in figure 3C and was studied only in the patients who completed the immunization protocol and subsequent follow-up. As for recall antigen, no modifications of LPR to CD3 MAb were seen in the rgp160 group. A significant increase of LPR to CD3 MAb was observed after 1 year in the other 2 groups receiving zidovudine ($P = .004$

for the zidovudine plus rgp160 group and $P = .027$ for the zidovudine group, compared with baseline values).

Env-specific cytotoxicity. HIV-1 Env-specific CTL activity was measured in a total of 18 patients from the 3 groups (table 2): 6 (33%) had an initial specific release of $>10\%$ and, therefore, were considered as Env-CTL positive at the start of the study. Modifications of the Env-specific CTL activity were observed during the trial in an equivalent fraction of patients in each group. In detail, 3 patients in each group presented increases of variable duration of Env-specific CTL activity, which was defined as the appearance of activity in patients initially considered negative or the doubling of pretreatment activity in patients initially considered positive for Env-specific CTL activity. Variations in Env-specific activity were not associated with immunization with rgp160.

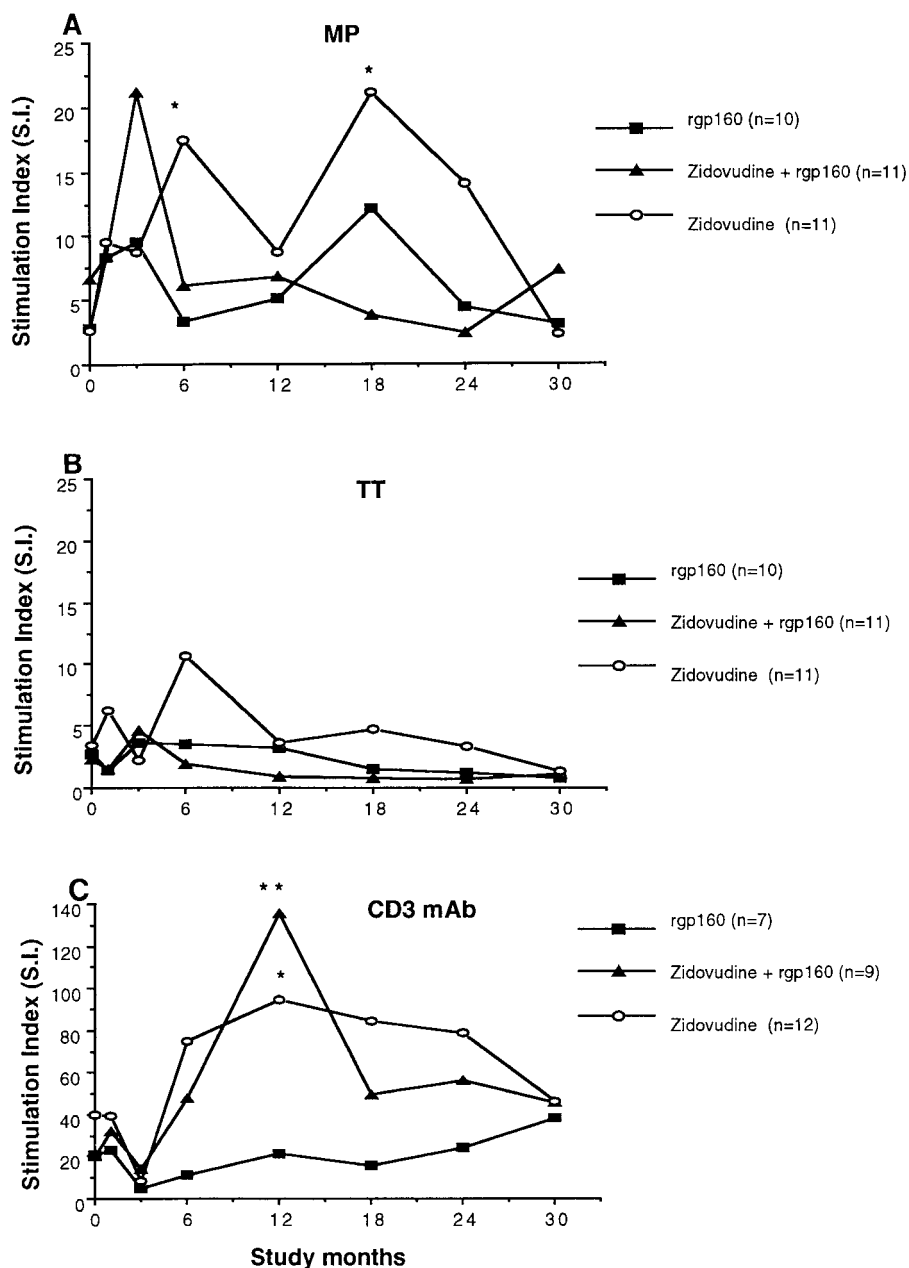
CD4 T cell numbers. To monitor the progression of infection in the groups, we evaluated CD4 T cell counts in patients during 30 months of follow-up (figure 4A). In the zidovudine plus rgp160 group, a significant increase in CD4 T cell numbers compared with baseline values was observed after 3 months ($P = .024$). At that time, the number of CD4 T cells in this group was significantly higher than that for the rgp160 group ($P = .023$). No other significant modifications of CD4 T cell numbers were observed in the 3 groups; however, both groups receiving zidovudine maintained mean CD4 T cell counts comparable to baseline values, whereas the rgp160 group showed slightly lower CD4 T cell counts starting from the sixth month.

Plasma HIV-1 RNA. To study the effect of therapy on virus replication, we measured plasma HIV-1 RNA levels in a subgroup of 47 patients equally distributed in the 3 groups. In both groups receiving zidovudine, a significant decrease of plasma viremia was observed after 1 month with respect to baseline levels ($P = .001$), and at the same time, plasma virus load was significantly lower in these 2 groups compared with the virus load for the group treated with rgp160 alone ($P < .05$); however, after 2 years of follow-up, plasma viremia was comparable to baseline values in all 3 groups (figure 4B).

Discussion

The correlates of protective immunity during HIV-1 infection are still unclear, but major attention is given to the specific cell-mediated immunity [16]. In analogy to other viral infec-

Figure 3. Median SIs of lymphoproliferative responses to recall antigens manno-protein (MM; **A**) and tetanus toxoid (TT; **B**) and to CD3 MAb (**C**) in patients who had complete immunovirologic follow-up. **A:** $*P < .05$ vs. baseline values (Wilcoxon test). **C:** $*P = .027$ and $**P = .004$ vs. baseline values (Wilcoxon test). Nos. of patients studied are shown for each group.



tions [17] and considering the association with the initial reduction of viremia [18], HIV-1-specific CTLs are considered beneficial in containing virus replication and spread. Conversely, LPRs to HIV-1 antigen, which are generally uncommon and weak during the natural infection [12, 19], do not have a clear association with virus control. A phase I study of postexposure immunization with rgp160 suggested a beneficial effect of an immune response to rgp160, measured both as the LPR and as the production of new antibodies with specificity for gp160 epitopes, as reflected in a slower decline of CD4 T cell numbers with respect to patients who did not respond to the immunization procedure [7]. We have previously observed an association

between the maintenance of CD4 T cell numbers and the presence of an LPR to HIV-1 p24 in untreated patients during a short-term follow-up [20]. However a recent analysis suggested that the presence of an LPR to several HIV-1 peptides during the asymptomatic phase is a negative prognostic factor with respect to progression to AIDS [21].

The present study confirms the induction of a strong cell-mediated immune response to rgp160 in HIV-1-infected patients immunized with rgp160. This response is maintained for at least 6 months after the last injection. Further follow-up of patients will provide clarification about whether vaccination-induced immunization is transient or whether the stimulus

Table 2. Env-specific cytotoxicity in HIV-1-infected persons receiving antiretroviral therapy and immunization with HIV-1 Env glycoprotein.

	rgp160 (n = 6)	Zidovudine + rgp160 (n = 5)	Zidovudine (n = 7)
Patients CTL positive at start of study	2	3	1
Patients with CTL increase*	3	3	3

NOTE. CTL = cytotoxic T lymphocyte.

* No. of patients negative for Env-CTL activity at start of study who became positive at any time during trial and patients with positive Env-specific CTL before treatment and at least 2-fold increase during trial.

caused by the persistence of HIV-1 is sufficient to maintain a strong antiviral response. The LPR to other HIV-1 antigen, encoded for by different genes, is not influenced by the vaccination, as previously reported [7].

In contrast to previous observations [22], the Env-specific CTL response is also not significantly influenced by vaccination. In the current study, modifications of Env-specific CTL activity were observed in all 3 groups, suggesting that the specific immunization is not relevant in this respect. Improvement of T cell function associated with treatment with zidovudine might be responsible for a higher frequency of positive CTL assays. Indeed, both groups receiving zidovudine had a transient improvement of T cell function, measured as response to CD3 MAb, mirrored by the expected virus load reduction. The lack of an antigen-specific modification of CTL activity could be found in the modality of immunization, which preferentially activates CD4 T lymphocytes.

The effect on LPRs to recall antigens, such as TT and MP, is less evident since only the group receiving zidovudine showed some increase of LPR to MP with the same kinetics of the improvement of LPR to CD3 MAb. This functional improvement is probably also responsible for the faster response to rgp160 seen in the group receiving both treatments. It may be explained as the result of virus load reduction and thus decreasing toxic effects on immune functions. Alternatively, at least in the case of anti-MP responses, it might also be due to increased clonal expansion of specific CD4 T cells because of the natural stimulation with *C. albicans* in patients with improved immunologic conditions due to antiretroviral therapy. Treatment with rgp160 did not modify any of the LPRs except those to the immunogen itself. A similar finding was described in a phase I trial in which several doses of rgp160 were compared with the administration of an unrelated vaccine (hepatitis B virus vaccine). Short-term follow-up of those patients revealed that specific responses to gp160 were induced without modification of responses to non-HIV-1 antigen [8].

Minor changes of CD4 T cells numbers were observed during the present study even though a trend toward lower set-point values was associated with the immunization procedure. The forthcoming analysis of the entire trial population, which was randomized with respect to clinical events and CD4 T cell

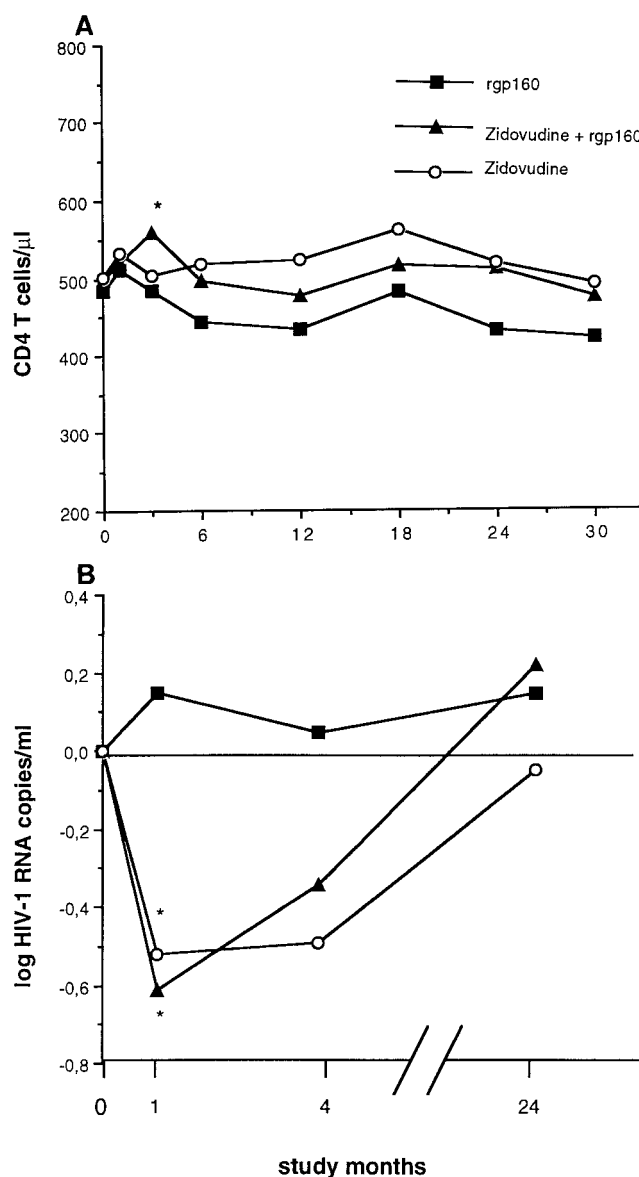


Figure 4. A, Mean CD4 cell counts in all randomized patients; * $P = .024$ vs. baseline values (paired Student's t test), and $P = .023$ vs. rgp160 group (unpaired Student's t test). B, Mean changes from baseline values of plasma HIV-1 RNA levels in subgroup of 47 patients; * $P < .05$ vs. rgp160 group (unpaired Student's t test).

counts (Pontesilli O, et al., unpublished data), will allow a more accurate interpretation.

The discrepancy between the vigorous and persistent response induced by the immunization and the lack of significant effects on established markers of disease progression, as well as on clinical outcome [23, 24], questions the validity of the immunotherapy with viral subunits. A possible explanation of this discrepancy could be found in the induction of immunization to envelope antigen of HIV-1_{III} in subjects infected by different virus strains. The appearance of significant LPRs to gp160 after only two inoculations has been observed also in HIV-1-seronegative volunteers [25]. It is therefore conceivable that the response observed in infected subjects is a primary response rather than an anamnestic one. If this is the case, the question arises of whether such a response has any relevance with respect to natural infection.

Another open question is whether immune activation is advantageous or deleterious in HIV-1 infection. There are some studies describing a positive role of immunization with HIV-1 envelope proteins [7, 26] or inactivated HIV [27]. However, other studies demonstrate that T cell activation is correlated with an increased susceptibility to HIV-1 infection [28], with the induction of virus expression from infected cells [29], and with an increase of virus burden in plasma, PBMC, and lymphoid tissue [30].

Even though advantages have been reported when immunized HIV-1-infected patients are compared with untreated controls [7], we show here that, despite an increase of immune response to rgp160, patients treated with rgp160 alone had, compared to patients treated with rgp160 plus zidovudine or with zidovudine alone, lower CD4 T cell counts, higher levels of plasma HIV-1 RNA, and weaker LPRs to CD3 MAb. These three parameters are considered markers of disease progression [31–33]. Taken together these observations suggest that anti-HIV-1 immunization of HIV-1-infected subjects does not result in an improvement of immune functions or course of infection, while antiretroviral therapy alone or in combination with rgp160 resulted in a transient amelioration of some parameters, which possibly reflect cell-mediated immunity restoration. Whether this conclusion can be extended to other immunizations with different proteins of HIV-1 remains to be elucidated. However, this study does not confirm previous preliminary observations on the positive effect of vaccination with gp160 in HIV-1-infected asymptomatic subjects [7, 8, 26]. The discrepancy with those previous observations can be explained with the more prolonged follow-up of our study, with the more restrictive inclusion criteria with respect to CD4 cell numbers (400–600 CD4 cells/ μ L), and, more important, with the lack of appropriate control groups in those phase I studies. We suggest that antiretroviral therapy should always be used in future therapeutic protocols using anti-HIV-1 immunization, cytokines, or immunostimulant drugs.

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