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Association between Virus-Specific Cytotoxic T-Lymphocyte and Helper Responses in Human Immunodeficiency Virus Type 1 Infection

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Cellular immune responses are thought to be an important antiviral host defense, but the relationship between virus-specific T-helper and cytotoxic-T-lymphocyte (CTL) responses has not been defined. To investigate a potential link between these responses, we examined functional human immunodeficiency virus type 1 (HIV-1)-specific memory CTL precursor frequencies and p24-specific proliferative responses in a cohort of infected untreated persons with a wide range of viral loads and CD4 cell counts. Levels of p24-specific proliferative responses positively correlated with levels of Gag-specific CTL precursors and negatively correlated with levels of plasma HIV-1 RNA. These data linking the levels of HIV-specific CTL with virus-specific helper cell function during chronic viral infection provide cellular immunologic parameters to guide therapeutic and prophylactic vaccine development.

Induction and maintenance of cytotoxic-T-lymphocyte(s) (CTL) responses has been considered a key element for the development of an effective human immunodeficiency virus (HIV) vaccine, but the factors that regulate the magnitude and breadth of these responses have not been defined. A dependence of CTL on CD4 helper function during chronic viral infections has been suggested by murine studies (reviewed in reference 12). Depletion of CD4 cells by monoclonal antibodies (MAbs) (20), as well as genetic knockout of CD4 (29), result in progressive loss of initial virus-specific CTL responses and an inability to control viremia in lymphocytic choriomeningitis virus (LCMV) infection. A recent study by Zajac et al. has furthered these observations by demonstrating that under conditions of CD4 T-cell deficiency, CTL can persist in a nonfunctional state (31). The potential contribution of help to CTL persistence in humans has been suggested from studies of adoptively transfused cytomegalovirus (CMV)-specific CTL, which persist only in the presence of preexisting CMV-specific Th cells (24).

A recent study in HIV-infected persons demonstrated a negative correlation between Gag-specific T-helper (Th) responses and viral load (26). In addition, CTL studies, including tetramer analysis, show a negative correlation between virus-specific CTL frequency and viral load in human leukocyte lymphocyte antigen (HLA) A*0201-positive persons (21, 22). However, a direct link between virus-specific Th cell function and CTL in a human chronic viral infection and in HIV-1 infection in particular has not been demonstrated. In this study we performed a detailed analysis of functional memory CTL and Th cell responses in persons with a wide range of viral loads to determine the relationship between these two immune effector mechanisms.

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MATERIALS AND METHODS

Subjects. Subjects with a wide range of plasma HIV-1 RNA levels were enrolled from the San Francisco city clinic cohort (9, 10, 19) and from the Boston area. CD4 cell counts and viral loads in the subjects studied ranged from 166 to 1,099 (mean, 685) and from <400 to 264,000 (mean, 37,237) respectively. Blood was drawn in ACD tubes and shipped to Massachusetts General Hospital overnight. Investigators were blinded with respect to subject's CD4 cell counts and viral loads. All patients were antiretroviral-therapy naive when these studies were performed and gave written informed consent. Four subjects previously included in an earlier cohort where T-helper-cell responses were examined (161j, CTS-01, PG-9011, and MK-089 [26]) are included in this study for an extended analysis of the association of CTL with T-helper-cell responses. The subject numbers of these individuals in the present study are as follows: 161j, subject 20; CTS-01, subject 21; PG-9011, subject 9; and MK-089, subject 12.

CTL precursor frequency analysis. Precursor frequencies of HIV-1-specific CTL were estimated by performing limiting dilution on freshly isolated peripheral blood mononuclear cells (PBMC) followed by in vitro stimulation with the anti-CD3 MAb 12F6 as previously described (11). PBMC were cultured at 250 to 16,000 lymphocytes per well in 24 replicate wells of 96-well microtiter plates. To each well of the precursor assay plate was added 5×10^4 gamma-irradiated PBMC from an HIV-1-seronegative donor and 0.1 μg of 12F6 per ml. After 10 to 14 days, the wells were split and assayed for cytotoxicity against ^{51}Cr-labeled autologous B-LCL infected with vaccinia virus expressing Gag, RT, Env, or Nef proteins. The fraction of nonresponding wells was the number of wells in which ^{51}Cr release did not exceed the mean plus three standard deviations of the average spontaneous release of the 24 control wells (16, 18). The activated cell frequency was estimated by the maximum-likelihood method (4, 5, 7). The sensitivity of this assay is 50 CTL precursors/10^6 PBMC.

Lymphoproliferative responses. Cells (2×10^5/well) were cultured in six replicate wells of 96-well U-bottom plates in the presence of HIV-1 recombinant p24 antigen, gp160 antigen, control proteins, tetanus toxoid (final antigen concentration, 0.5 μg/ml), or medium alone. Six days later the cells were pulsed with [^{3}H]thymidine at 1.0 μCi/well, and the uptake was measured 6 h later with a scintillation counter (Topcount; Packard Instrument, Meriden, Conn.) as previously described (26). Based on experiments performed with seronegative controls, a stimulation index (SI) of >3 was considered significant.

RESULTS

Chronically infected subjects differ in the magnitude of their virus-specific immune responses. Initial simultaneous evaluations of helper and CTL responses were performed on two subjects with a duration of infection of >10 years and stable CD4 counts of >500/mm^3 but with markedly different viral loads. In our prior study of HIV-1-specific helper responses,

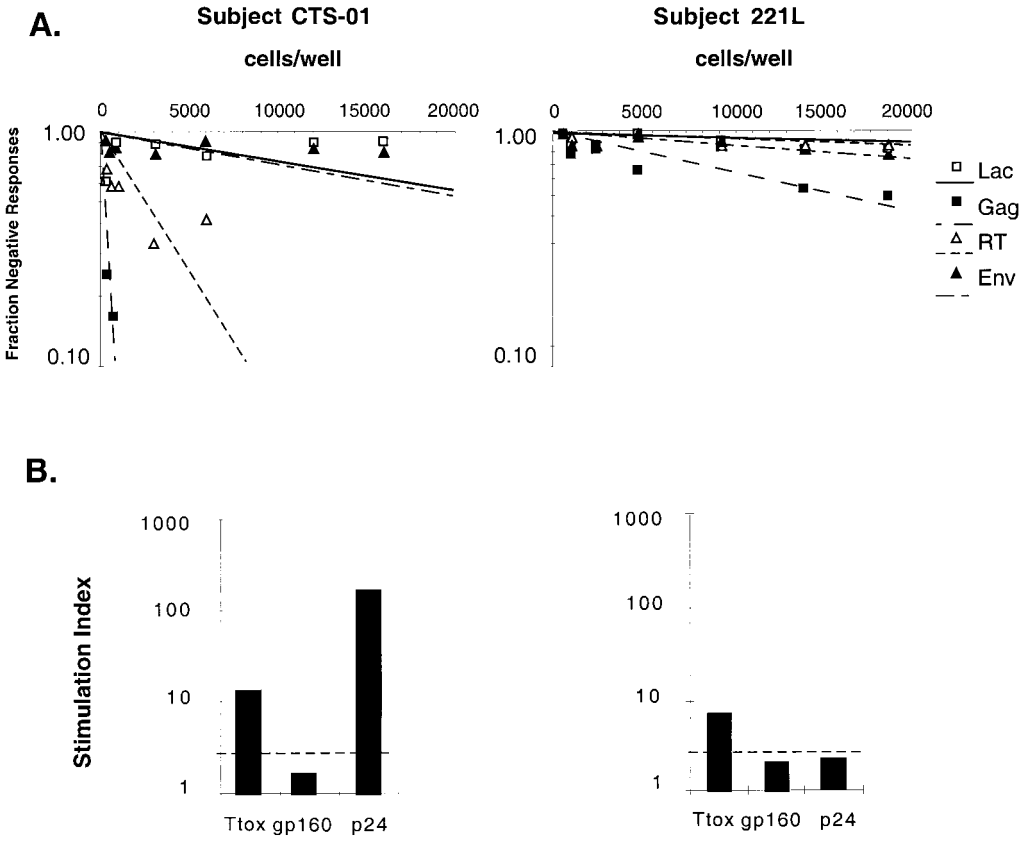

FIG. 1. HIV-1-specific CTL precursor frequencies in two subjects with high and low viral loads. For CTLp analysis, serial dilutions of freshly isolated PBMC were placed in 24 replicate wells and stimulated with a CD3-specific MAb (11). For the assessment of proliferative responses, freshly isolated PBMC were placed in six replicate wells in the presence of tetanus toxoid, p24 antigen, or gp160. Viral load measurements were performed on cryopreserved plasma (Amplicor HIV Monitor Test; Roche Molecular Systems, Branchberg, N.J.) according to the manufacturer's specifications. (A) CTLp in subject CTS-01 (subject 21) (viral load, <400 copies/ml; CD4 count, 900 cells/mm^3; duration of infection, 15 years at time of assay) and CTLp in subject 221L (subject 8) (viral load, 200,000 RNA copies/mm^3; CD4 count, 800 cells/mm^3; duration of infection, 11 years at time of assay). (B) Proliferative responses in subjects CTS-01 (subject 21) and 221L (subject 8). The dotted line represents an SI value of 3.

we described a subject with robust p24-specific helper responses and CTL precursor frequencies of >1/200 PBMC (subject 161j). Another subject (CTS-01) in that study, with documented asymptomatic HIV infection for 15 years, a persistently low viral load (≤700 copies/ml), and a stable CD4 cell number of 900 cells/mm^3 was demonstrated to have strong proliferative responses to HIV-1 p24 antigen (26). At a subsequent time point we performed simultaneous limiting-dilution CTL precursor and lymphoproliferative assays on this subject in order to test the hypothesis that strong HIV-specific proliferative responses were associated with high levels of virus-specific CTL precursors. As shown in Fig. 1, this subject had high levels of CTL precursors directed at the Gag and RT proteins. A second individual (221L) with a CD4 count of 800 cells/mm^3 10 years after his documented seroconversion and a viral load of 200,000 copies/ml also had demonstrable CTL precursors, but they were barely within the detectable range. In addition, this subject's CTLp were directed solely against Gag, with no responses to Env, RT, or Nef (Fig. 1A). These responses were mediated by CD8$^+$, class I-restricted lymphocytes as defined by limiting-dilution cloning (data not shown). The magnitudes of T-helper activity to p24 antigen (performed concurrently with CTLp assays on freshly isolated PBMC) revealed similar differences between the responses of these two individuals: the SI of PBMC from subject CTS-01 to p24 was >100, whereas subject 221L had no significant proliferative response despite having a detectable response to tetanus toxoid (Fig. 1B). Over the next 6 months the HIV-1 RNA level rose in subject 221L to 641,000 copies/ml and was accompanied by a decline in CD4 cell number to 457 CD4 cells/mm^3, prompting institution of antiretroviral therapy. In contrast, subject CTS-01 maintained a low viral load in the absence of antiretroviral therapy.

HIV-1 p24-specific helper responses correlate with control of viremia. These data suggested a possible association between CTL, CD4 proliferation, and viral load. We next evaluated these parameters in 19 additional, antiretroviral-naive, HIV-1-infected individuals. Two of these additional subjects were described in our previous work (subjects PG-9011 and MK-089), but only had lymphoproliferative assays performed at that time. In order to extend our studies we used freshly isolated PBMC from this cohort to simultaneously evaluate p24-specific proliferative responses and a broad range of CTL responses directed against vaccinia virus vectors expressing HIV-1 Gag, Env, and Nef antigens.

Although there was only a weak negative trend between levels of Gag-specific CTL precursors and viral burden (R = 0.38, P = 0.09) (Fig. 2A), there was a highly significant negative correlation between the ability of PBMC to proliferate in response to soluble p24 antigen and viral burden (R = −0.64,

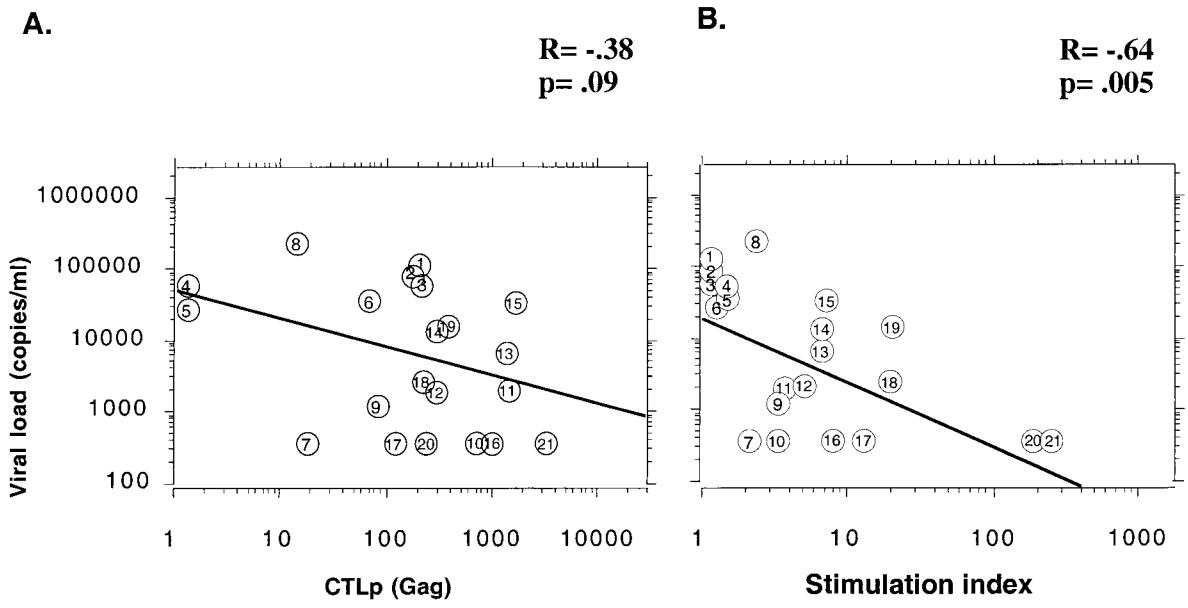

FIG. 2. Relationship between Gag-specific CTLp and proliferative responses and plasma HIV-1 RNA level. (A) Levels of Gag-specific CTLp do not significantly correlate with low levels of plasma viremia. (B) Increasing p24-specific helper responses are negatively correlated with viral load. Assays were performed on 21 antiretroviral-naive individuals with a wide range of CD4 counts and viral loads. HIV RNA was measured with the Amplicor HIV monitor test according to the manufacturer's specifications. Individual subjects are numbered.

$P = 0.005$; Fig. 2), which was similar to our findings in the previously described smaller cohort of antiretroviral-naive subjects (26). If the four previously reported patients are removed from this analysis, the R value is -0.61 with a P value of 0.01. These responses were of the Th-1 type, associated with gamma interferon (IFN-γ) production and a lack of interleukin-4 (IL-4) or IL-10 production (reference 26 and data not shown). A multiple regression analysis demonstrated that both p24-specific proliferative responses and CD4 count were independently associated with a low viral load ($P = 0.02$ and $P = 0.04$, respectively). Although there was a highly significant correlation between p24-specific and tetanus toxoid-specific proliferation ($R = 0.76$, $P = 0.0001$), tetanus toxoid recall responses were not associated with lower plasma HIV-1 RNA levels ($R = 0.36$, $P = 0.14$), and HIV-1 p24-specific proliferation was again independently associated with lower viral burden (multiple regression, $P = 0.017$). Proliferative responses to gp160 were detected infrequently. Only four subjects had an SI of >3, and these subjects all had significant p24-specific proliferative responses (data not shown).

HIV-1 p24-specific proliferative responses correlate with the magnitude of Gag-specific CTL responses. Since CD4 cell proliferative responses have been shown to be required for the maintenance of effective CTL responses during chronic murine viral infections (20, 29), we directly examined the relationship between CTLs directed against HIV-1 Gag, Env, RT, and Nef and CD4 cell helper responses. Only CTL responses directed against Gag were tightly linked with the level of HIV-1-specific help ($R = 0.6$, $P = 0.007$, Fig. 3A), and the data suggested a threshold at a p24 SI value of 3, in that all subjects with an SI of >3 had levels of Gag CTLp of >100/10^6 PBMC. There were no statistically significant correlations between the levels of p24-specific helper responses and the levels of CTL precursors directed against Env, RT, or Nef (Fig. 3B to D). When cell numbers were sufficient (14 subjects), direct ex vivo CTL lysis was measured against target B-LCL infected with vaccinia virus vectors expressing Gag, RT, Env, and Nef at an effector/target ratio of 100:1. There was no relation between the levels of p24-specific help and HIV-specific CTL lysis, nor between HIV-specific CTL lysis and HIV-1 plasma viremia (data not shown).

Although in the cohort as a whole there was no significant relation between higher CTL precursor frequencies and lower viral loads ($R = -0.38$, $P = 0.09$) (Fig. 2A), these functional CTLp assays involve the addition of exogenous help in vitro in the form of IL-2 and irradiated feeder cells. Thus, CTL may be detectable in the in vitro assay even if they have impaired ability to proliferate and kill in vivo. We therefore assessed the relationship between viral load and CTL precursor frequencies, controlling for the presence or absence of a significant helper cell response. The choice of an SI of 3 as the definition of a significant helper response was based on studies of p24-specific Th cell responses of 17 unexposed HIV-1 seronegative individuals (26) (mean ± 1 standard deviation, 1.3 ± 0.6; range, 0.5 to 3). This choice was further supported by the threshold effect observed in Fig. 3. In the chronically infected persons, the presence of a significant p24-specific proliferative response was associated with a 1.2-$\log_{10}$-lower viral burden (mean ± standard deviation, 3.3 ± 0.71 versus 4.6 ± 0.86 $\log_{10}$ copies/ml; analysis of variance [ANOVA], $P = 0.001$) (Fig. 4B), and an SI of 3 also correlated with a >10-fold increase in the number of Gag-specific CTL precursors (2.66 ± 0.48 $\log_{10}$ CTL precursors/10^6 PBMC versus 1.4 ± 0.95 $\log_{10}$ CTL precursors/10^6 PBMC; ANOVA, $P = 0.0005$) (Fig. 4A). If one uses a more conservative definition of an SI of 5 as the cutoff for a significant helper response, Gag CTLp were still significantly associated with the presence of Th-cell responses ($P = 0.018$). Also, in the presence of help the mean level of Gag-specific CTL precursors was higher than the level of CTLp directed against Env ($P = 0.04$) or Nef ($P = 0.004$) but not against RT ($P = 0.09$).

In the absence of T-helper-cell responses, the presence of HIV-1-specific CTLp was not associated with decreased viral replication. Four of eight subjects lacking p24-specific helper

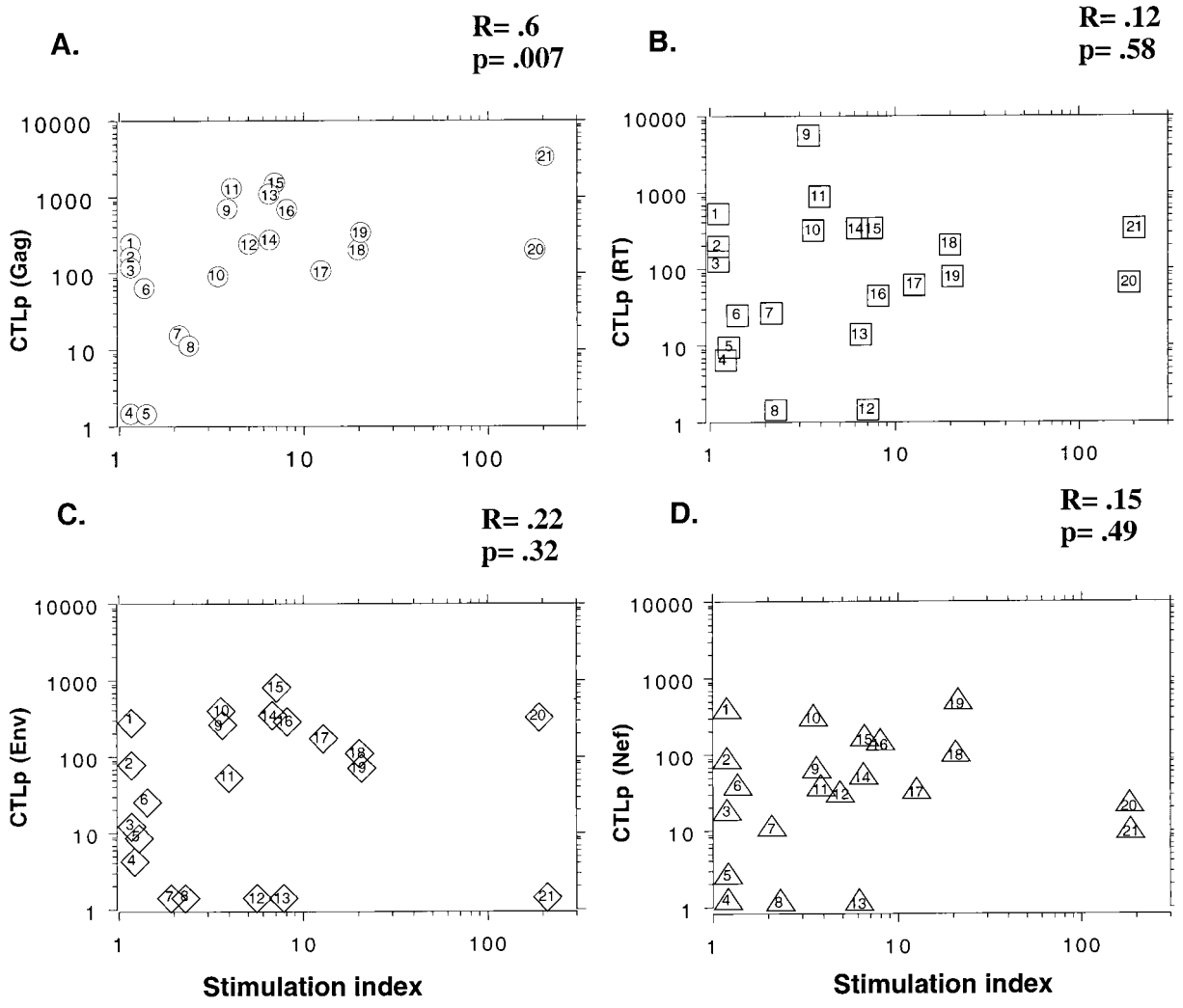

FIG. 3. Relationship between HIV-1-specific proliferative responses and CTL responses. CTL precursor frequencies directed against Gag, Env, RT, and Nef were plotted against p24 SI values. (A) The presence of a p24-specific proliferative response is strongly associated with increasing levels of Gag-specific CTL precursors. (B to D) Levels of Env, RT, or Nef-specific CTL are not strongly associated with the level of p24-specific help. Individual subjects are numbered, as in Fig. 2.

responses had no detectable CTLp to any of the HIV-1 antigens tested (Fig. 3). There was no difference in the mean level of HIV-1 plasma viremia when subjects with CTLp were compared to those without measureable CTLp (4.8 ± 0.2 versus 4.3 ± 1.2 $\log_{10}$ copies/ml; $P = 0.4$). An independent effect of CTL precursor frequency on viral load in the presence of HIV-1-specific helper activity could not be assessed, since all subjects with significant proliferative responses had high levels of Gag-specific CTL precursor frequencies. In other words, in persons with virus-specific Th cell function, CTL were always present.

DISCUSSION

These data demonstrate a strong association between virus-specific T-helper responses and CTL responses and provide evidence that the levels of HIV-1-specific CTL precursors are dependent on the presence of virus-specific helper cell function. In the absence of such help, CTL can occasionally be present in high numbers, yet they are not associated with control of viral replication. Our ability to detect CTL precursors in some subjects in the absence of detectable T-helper-cell function may be related to the assay conditions of the CTL precursor frequency assay. The addition of irradiated allogeneic feeder cells and IL-2 may provide in vitro replacement of help that is lacking in vivo. This provides an explanation for the observation that functional CTLp assays can indicate that HIV-1-specific CTL are present without containment of viremia (6). HIV-1-specific CTL can clearly mediate potent inhibition of HIV-1-replication in vitro (30), and a number of studies suggest that CTL responses may contribute to the control of the virus in vivo (2, 13, 17, 25). However, strong in vitro CTL responses can also be seen in persons with high viral loads and progressive illness (3, 14), suggesting that these responses may be suboptimal in vivo. This is consistent with observations in murine models of chronic viral infection, in which depletion of CD4 cells leads to progressive loss of CTL activity and an inability to control viremia (20, 29).

Only p24-specific T-helper-cell responses were detected in a substantial number of subjects, and gp160 rarely elicited lymphoproliferative responses (data not shown). This may be due to the higher conservancy of Gag compared to Env. We also tested for the ability of RT and Nef proteins to induce prolif-

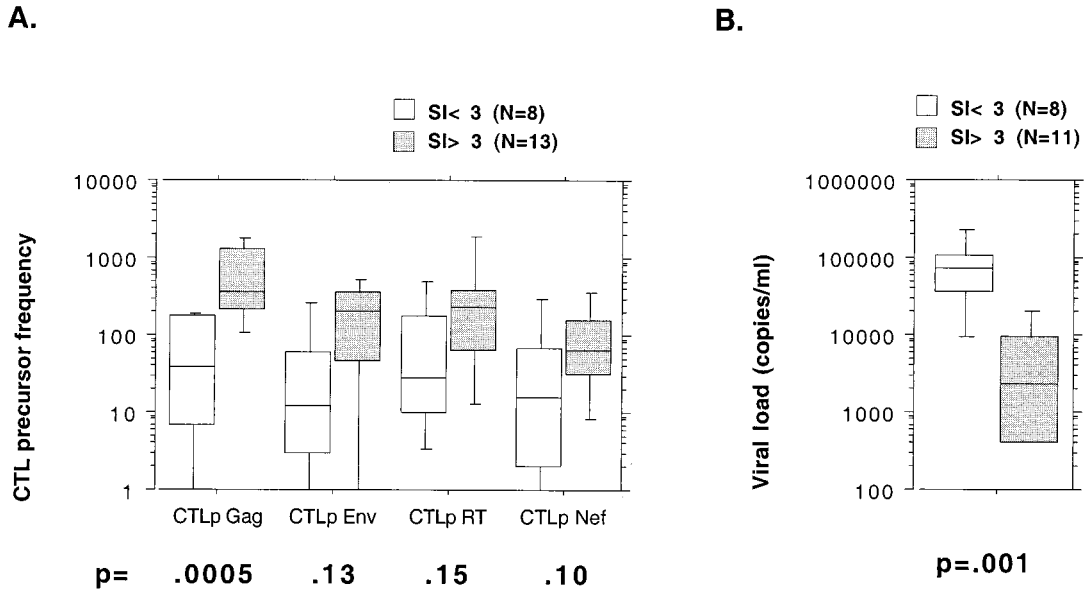

FIG. 4. CTL precursor frequencies in the presence or absence of HIV-1-specific T-helper responses. (A) Subjects with a p24 SI value of >3 have higher numbers of circulating HIV-1-specific Gag CTL precursors. (B) Subjects with significant helper responses have lower viral RNA levels. Box plots show the mean, upper bounds of the 75th and 90th percentiles and the lower bounds of the 10th and 25th percentiles.

erative responses in a small number of subjects and have not detected such responses, even in persons with strong Gag-specific T-helper-cell responses (data not shown). There may be several reasons for this lack of detection. Although RT is relatively conserved, it may not be expressed at high enough levels to stimulate robust immune responses (28). The lack of response to the Nef protein may be due to the fact that Nef, like Env, is less well conserved (15, 27). The finding that p24-specific helper responses only correlated with Gag-specific CTL responses likely reflects the immunodominance of Gag for inducing CTL responses (3, 8, 23). That is, for a given level of help, one is more likely to find higher frequencies of Gag-specific CTL compared to CTL specific for other HIV proteins. Although we found trends for increased numbers of CTL precursors directed against Env, RT, and Nef in the presence of helper responses, these results were not statistically significant with this sample size (Fig. 4).

The high frequency of Gag-specific T-helper and CTL responses in subjects with control of viremia may also reflect the effectiveness of functional Gag-specific CTL in controlling viremia. In our previous work we have shown that HLA-A2-restricted CTL specific for an immunodominant epitope in p17 were more effective at suppressing viral replication than CTL specific for an HLA-A2-restricted RT epitope (30). One explanation for this enhanced CTL effectiveness may be the higher level of p17 epitope expressed on the cell surface, due to the greater expression level of the Gag compared to the RT protein (28). The high level of Gag expression in HIV-1-infected cells may therefore be responsible both for the degree of immunogenicity of the protein, leading to vigorous T-helper and CTL responses, and for the control of viremia in subjects with CTL able to target epitopes in Gag. Our data indicate that some persons had high levels of HIV-1-specific CTLp, yet in the absence of helper cell function these cells were less effective at controlling viremia.

This inability of CTL to control viremia despite high frequencies of virus-specific CTL has been ascribed to a unique phenotype of CTL lacking effector function, which is more pronounced in situations where CD4 help is limiting (31). Zajac et al. found that CD4 +/+ mice infected with a weakly virulent strain of LCMV (Armstrong) were able to clear the infection. Analysis of virus-specific CTL with major histocompatibility complex (MHC) class I tetramers revealed that after viral clearance, high levels of virus-specific CTL persisted, and 100% of these cells maintained effector function as measured by the ability to produce IFN-γ after antigenic stimulation. Infection of mice with a rapidly replicating and widely disseminating LCMV strain led to the development of a subset of CTL which stained positive with MHC class I tetramers but which were not able to produce IFN-γ or to lyse infected cells. These "silenced" effector cells, while able to undergo large expansions and to persist in high numbers, are nevertheless unable to clear the chronic infection. The proportion of these nonfunctional CTL was even higher when CD4 helper cells were limiting (12, 31). Our data support a direct functional interaction between virus-specific Th cell function and effective CTL control in vivo in human viral infections. Although we found that subjects with high levels of HIV-specific T-helper cell responses generally had higher numbers of virus-specific CTL precursors, we also identified a subset of individuals with detectable CTL precursors despite an absence of virus-specific helper function. Future studies of this subset of individuals with more sensitive techniques may similarly demonstrate a population of circulating, yet nonfunctional CTL in subjects with high levels of HIV-1 plasma viremia.

Identification of correlates of immune protection is critical for the rational design of therapeutic and prophylactic vaccines. The data presented here are consistent with coordinate control of viral replication by virus-specific T-helper cells exerting their effect at least in part through CTL. It is likely that other effector mechanisms, such as humoral immune responses, are also influenced by these helper cells (1). However, assessment of neutralizing antibody activity in this same cohort has shown that persons with the lowest viral loads have the weakest neutralizing responses (10). It will be important to determine the precise mechanisms by which CD4 cells contrib-

ute to CTL persistence and whether CD8 cells with a silenced phenotype, as described by Zajac et al., are present with high frequency in HIV-infected subjects with high levels of viremia. If so, then immunotherapeutic strategies designed to augment T-helper-cell responses and restore CTL to functional competence may be a valuable adjunct to current antiretroviral drug regimens. Our findings that significant control of viremia is associated with a significant virus-specific helper response to p24 and strong virus-specific CTL responses suggest that the simultaneous induction of these immune responses will likely be a prerequisite for the development of a successful prophylactic HIV-1 vaccine.

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