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Temporal Association of Cellular Immune Responses with the Initial Control of Viremia in Primary Human Immunodeficiency Virus Type 1 Syndrome

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Virologic and immunologic studies were performed on five patients presenting with primary human immunodeficiency virus type 1 (HIV-1) infection. $CD8^+$ cytotoxic T lymphocyte (CTL) precursors specific for cells expressing antigens of HIV-1 Gag, Pol, and Env were detected at or within 3 weeks of presentation in four of the five patients and were detected in all five patients by 3 to 6 months after presentation. The one patient with an absent initial CTL response had prolonged symptoms, persistent viremia, and low $CD4^+$ T-cell count. Neutralizing antibody activity was absent at the time of presentation in all five patients. These findings suggest that cellular immunity is involved in the initial control of virus replication in primary HIV-1 infection and indicate a role for CTL in protective immunity to HIV-1 in vivo.

The rapid and spontaneous decline in viremia during primary human immunodeficiency virus type 1 (HIV-1) seroconversion syndrome suggests a potent antiviral role for host immunity (8, 11, 33). It remains uncertain, however, which components of the immune system control acute viremia. In other virus infections, both virus-specific and nonspecific immune responses have been implicated in protection from, or the clearance of, acute infection; these include natural killer cells (3, 4, 49), cytotoxic T lymphocytes (CTL) (5, 26, 34, 36, 37), and antibodies (14, 35, 41). A better characterization of the initial host immune response to HIV-1 infection may help to define protective immunity to HIV-1.

We therefore characterized the temporal relationship between changes in virus load and the development of immune responses in five patients with primary HIV-1 infection syndrome. All five patients had acute, self-limited symptomatic illnesses with measurable viremia followed by seroconversion. Four of the five patients have been described previously as V (AD6), F (AD8), L (AD10), and R (AD11) (40, 51). Patient AD13 has not previously been reported. For the analysis of viral load and immunologic function, blood was obtained from AD6 and AD11 before, during, and after evident seroconversion. For AD8, AD10, and AD13, the first available blood sample was obtained 13, 19, and 15 days after presentation with symptoms, respectively, and after evident seroconversion. The day on which the patient presented to a physician with symptoms of primary infection syndrome is taken as day zero.

To measure the frequencies of precursor CTL (CTLp) specific for cells expressing the protein products of HIV-1 *gag*, *pol*, and *env*, limiting-dilution analysis was performed (20). Frozen peripheral blood mononuclear cells (PBMC) were thawed and seeded at 250 to 16,000 cells per well in 200 μl of medium supplemented with 100 U of interleukin-2 per ml in 24-replicate wells of 96-well tissue culture plates. To each well was added 2.5×10^4 gamma-irradiated PBMC from a seronegative donor and 0.1 μg of anti-CD3 antibody per ml. The plates were incubated at 37°C for 14 days with twice weekly medium changes. The contents of each well were then split into four separate wells (50 μl each). Cells in these wells were then assayed for ability to lyse an autologous B-lymphoblastoid cell line infected with the control vaccinia virus or with vaccinia virus carrying HIV-1 *env*, *gag*, or *pol*. The number of wells for each dilution of effector cells which did not exhibit significant cytotoxicity (<3 standard deviations above the mean cytotoxicity for 24 control wells without effectors, or 10% specific lysis) was determined. The precursor frequency of effector cells with a given specificity was determined by plotting the log of the fraction of negative wells (F_n) versus the number of input cells and solving for $F_n = 0.37$ by the maximum-likelihood method (12).

For all five patients, CTLp were measured on the earliest available blood sample (before seroconversion in AD6 and AD11; after seroconversion but within 19 days of presentation in AD8, AD10, and AD13) and a second sample taken 3 to 6 months after presentation. HIV-1-specific CTLp were detected in the initial blood sample from four of the five patients (Fig. 1). In the four patients with detectable CTLp, the frequencies ranged from 0.3 to 38 per 10^4 PBMC, which is consistent with the range of HIV-1-specific CTLp previously reported in infected patients (7, 15, 20). Despite a previous report of high HIV-1-specific CTLp in uninfected individuals (18), we have been unable to detect (<0.1/10^4 PBMC) HIV-1-specific CTLp in three low-risk seronegative laboratory workers (data not shown). The measured responses were therefore unique to individuals infected with HIV-1.

Three to six months into HIV-1 infection, all five patients had detectable HIV-1-specific CTLp (Fig. 1). With the exception of patient AD11, who had no CTLp in the initial sample, the CTLp at the second time point were comparable to, or slightly lower than, those measured at the initial time point. This result suggests that the initial cellular immune response

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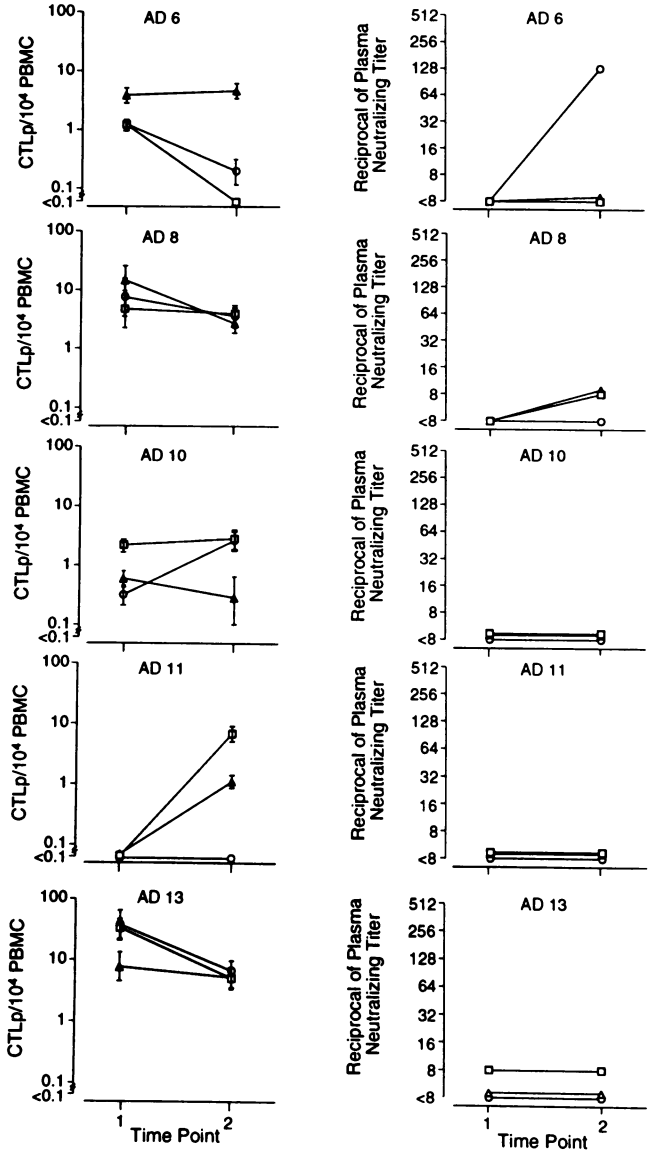

FIG. 1. CTLp (left) and neutralizing antibody titers (right) in five acute seroconvertors (AD6, AD8, AD10, AD11, and AD13), measured at two different time points. Time point 1 was the earliest time point available (preseroconversion in patients AD6 and AD11; days 13, 19, and 15 for AD8, AD10, and AD13, respectively). Time point 2 was 3 to 6 months after presentation. CTLp were determined against autologous targets infected with vaccinia virus vectors expressing HIV-1 *gag* (○), *pol* (△), and *env* (□) genes. Vaccinia vectors used in these assays were as follows: Gag, vAbT141 (23); Pol, vAbT204 (23); and Env, vAbT299, which is similar to vAbT271 (21) except that a short deletion has been inserted beyond the signal peptide which inhibits processing and surface expression of the HIV-1 envelope glycoprotein. Vaccinia virus strain NYCBH (23) was used as a control in all assays. Error bars represent 95% confidence limits of the calculated frequencies. CTLp could not be detected in this assay if present at <0.1/10,000 PBMC. Neutralizing antibody titers were determined against laboratory strains of HIV-1, IIIB (□) and JR-CSF (△), or a first-passage virus isolated from the patient (○).

remains elevated at least 3 to 6 months after the acute infection.

Both $CD4^+$ and $CD8^+$ CTL specific for HIV-1 have been found in infected individuals (22, 24, 39, 44, 46). In addition, a vigorous alloreactive CTL response often accompanies acute viral infections (31, 42). We therefore sought to determine the surface phenotype and HLA restriction of the lytic cells measured in the precursor frequency assays. Using an early blood sample from patient AD6, depletion of $CD8^+$ cells with immunomagnetic beads (Dynal Inc., Great Neck, N.Y.) prior to frequency determination resulted in a loss of HIV-1-specific CTLp, while depletion of $CD4^+$ cells resulted in no significant change in the measured CTLp (Table 1). A high frequency of alloreactive killer cells was found when HLA-mismatched targets were used in the assays ($3.6/10^4$ PBMC; data not shown), but HIV-1-specific precursors were much more frequent when tested against HLA-matched targets than when tested against HLA-mismatched targets ($6.02/10^4$ versus $1.04/10^4$ PBMC for Gag, $37.0/10^4$ versus $3.54/10^4$ PBMC for Pol, and $1.48/10^4$ versus $<0.1/10^4$ PBMC for Env) (Table 1). For all five patients, PBMC from within 3 weeks of presentation were also subjected to limiting dilution cloning for HIV-1-specific CTL (45). In four of the five patients (AD6, AD8, AD10, and AD13), HLA class I-restricted, HIV-1-specific CTL clones were generated and characterized (40, 41a). No HLA class II-restricted clones were recovered from these patients (data not shown). The one patient from whom no HIV-1-specific CTL clones could be generated was the same patient from whom CTLp were absent at the time of presentation (AD11). These results collectively indicate that the CTL response during primary HIV-1 infection is largely mediated by $CD8^+$, HLA-restricted CTL.

It is notable that no single antigen was dominant in the initial cellular immune response to HIV-1; in patients AD6 and AD8, the highest CTLp response was directed against Pol, in AD13, the highest CTLp response was against Gag, and in AD10, the highest CTLp response was against Env (Fig. 1). This finding is to be expected in studies of an outbred population, in which heterogeneity of the major histocompatibility antigens (HLA in humans), rather than the viral antigens, may have the dominant effect on the specificity of the cellular immune response.

The capacity of plasma samples from the five patients to neutralize infectivity of two standard strains of HIV-1 (JR-CSF and IIIB) and autologous primary isolates was tested (Fig. 1). Aliquots of singly passaged (P1) viral stock generated in short-term cultures on phytohemagglutinin-stimulated PBMC

TABLE 1. CTLp in acute HIV-1 infection are $CD8^+$ and HLA restricted[a]

HIV-1 antigen	No. of precursors/10,000 cells			
	Target cells		Effector cells	
	HLA matched	HLA mismatched	$CD8^+$	$CD4^+$
Gag	6.02	1.04	5.58	0.21
Pol	37.00	3.54	29.60	0.56
Env	1.48	<0.10	1.82	<0.10

[a] CTLp frequencies were determined in PBMC taken from patient AD6 32 days after presentation. HLA restriction was determined by assaying on HLA-matched or HLA-mismatched target cells. Surface phenotype of the CTL was determined by separating PBMC into $CD8^+$ and $CD4^+$ fractions by immunomagnetic selection prior to limiting dilution and stimulation. The $CD8^+$ fraction contained <6% $CD4^+$ cells and the $CD4^+$ fraction contained <1% $CD8^+$ cells by flow cytometric analysis. All frequencies as listed have had vaccinia virus-specific frequencies subtracted. For example, Gag (HLA matched) = precursor frequency of unseparated PBMC tested on autologous targets infected with vAbT141 minus the precursor frequency of unseparated PBMC tested on autologous targets infected with NYCBH.

were used in neutralization assays. Fifty to one hundred 50% tissue culture infective doses ($TCID_{50}$) of each P1 virus or laboratory strain of HIV-1 was incubated for 60 min with serially diluted, heat-inactivated plasma from the corresponding patient before inoculation onto 2×10^6 phytohemagglutinin-stimulated PBMC. Cells were incubated at 37°C for 24 h and then washed three times to remove the virus-plasma mixture. After culture for 5 days, supernatants were assayed for p24 antigen by enzyme immunoassay. Neutralization results were expressed as the greatest plasma dilution which caused ≥90% inhibition of p24 production. All assays were performed in duplicate.

In contrast to the observation that CTLp were present in the initial blood sample from four of the five patients, antibodies capable of neutralizing autologous virus were not detected in the initial plasma sample from any of the five patients. Plasma from one patient (AD13) was, however, able to neutralize $HIV\text{-}1_{IIIB}$ infectivity, though at a very low titer (1:8). Even 3 to 6 months into HIV-1 infection, only one of the five patients (AD6) had developed detectable neutralizing activity against the autologous virus, and one other patient (AD8) had developed neutralizing activity against standard strains of HIV-1. These results indicate that in comparison with the CTL response, a detectable neutralizing antibody response is delayed in acute HIV-1 infection.

To more closely assess the timing of the initial immune response in acute HIV-1 infection, two patients (AD6 and AD11) were studied in detail at multiple time points. These two were chosen from the five original patients because both had multiple blood samples available from before, during, and after seroconversion. In addition, while they had similar symptoms of primary HIV-1 syndrome, they experienced profoundly different clinical courses. Patient AD6 maintained a stable $CD4^+$ lymphocyte count, and his symptoms quickly abated. Patient AD11, however, was found to be severely CD4 lymphocytopenic at presentation, and he continued to experience malaise for several weeks after seroconversion. His $CD4^+$ T-cell count never rose substantially, and as a result, zidovudine therapy was initiated on day 178 (Fig. 2A).

At the time of presentation, no antibodies to HIV-1 were detectable in the plasma of patient AD6 or AD11. Seroconversion to HIV-1 was confirmed by enzyme-linked immunosorbent assay and Western blotting (immunoblotting) on day 8 for AD6 and day 10 for AD11. The levels of HIV-1 in the blood of both patients were determined sequentially by endpoint dilution coculture of plasma and PBMC (11, 17, 19) and by branched DNA assay for particulate HIV-1 RNA in plasma (Chiron Corp.) (6). The results are summarized in Fig. 2B. The initial high levels of HIV-1 in the blood of both patients were rapidly and substantially controlled within 4 to 6 weeks of presentation. Aside from minor differences in the levels of plasma- versus PBMC-associated virus, the major apparent difference between these two patients was the level of viremia at which they stabilized. Patient AD6 was able to clear culturable virus from blood, while patient AD11 maintained a level of culturable HIV-1 in plasma and PBMC through day 178. It was only after initiation of zidovudine on day 178 that this level of viremia decreased. Consistent with previous reports, the ability to detect HIV-1 RNA in plasma by a signal amplification method was more sensitive than the ability to detect HIV-1 in plasma by culture (6, 33). Both patients therefore maintained a detectable level of HIV-1 particles in their blood despite clearance of culturable virus.

A major difference in the initial HIV-1-specific CTL response was noted between these two patients (Fig. 2C). HIV-1-specific CTLp to all three major gene products were detected in the day 4 blood sample of AD6. This sample was from 4 days before seroconversion. The levels of the CTLp in AD6 were highest from the time of presentation through day 32, corresponding to the concurrent high levels of viremia. After partial clearance of the viremia, the levels of CTLp appeared to decrease and stabilize. It has previously been reported that HIV-1-infected patients maintain these levels of CTLp in the circulation for many years (7, 15, 20, 22, 39, 44, 46). Patient AD11, however, had no detectable HIV-1-specific CTLp in the initial blood sample. Indeed, aside from a single finding of *env*-specific CTLp on day 10, no CTLp specific for any of the three major HIV-1 gene products were detected on three separate determinations from blood samples spanning days 0 to 23. This is the time frame in which AD6 exhibited the highest levels of HIV-1-specific CTLp and the other three patients (AD8, AD10, and AD13) all had easily detectable CTLp (Fig. 1). The lack of detectable CTLp early in the course of infection in AD11 was not due to our inability to measure cross-reactive CTL, since *env*- and *pol*-specific CTLp were easily detected at day 178 (Fig. 2C). These results suggest that the delayed HIV-1-specific CTL response in patient AD11, as compared to the other four patients, may have been one factor contributing to his persistent viremia, symptoms, and low $CD4^+$ cell counts.

Neutralizing antibodies were found to appear relatively late in infection in both patients (Fig. 2D). The ability of plasma to neutralize HIV-1 infectivity was tested against three separate virus isolates: $HIV\text{-}1_{IIIB}$, $HIV\text{-}1_{JR\text{-}CSF}$, and the HIV-1 isolate recovered from the patients' own blood and passaged once in PBMC (P1 isolate). Neither patient developed neutralizing antibodies against the IIIB or JR-CSF isolate during the almost 300 days of follow-up. In contrast, both patients developed neutralizing activity against their P1 isolates. This activity was first detected in the plasma of AD6 at day 77, and over the ensuing months, the neutralizing antibody titer increased from 1:32 to ≥1:512. The neutralizing activity in the plasma of AD11 was detected later (day 233), and samples beyond day 261 will be collected and tested in the future.

We were thus unable to detect neutralizing antibodies to HIV-1 until several weeks after viremia had decreased in either patient. This finding needs to be interpreted within the constraints of the sensitivity of the assay. The neutralizing antibody assay may not be sufficiently sensitive to detect biologically relevant amounts of neutralizing antibody at early time points. Indeed, using a different neutralization assay, Albert et al. (1) found type-specific neutralizing antibodies within 2 to 3 weeks of infection in four acute seroconvertors. This early a neutralizing response, however, has not been reported as a common finding (2, 16, 28, 48). One way to address this discrepancy would be to use a more sensitive assay which would detect antibodies to known neutralization sites on the virus envelope. These studies are summarized elsewhere (29). It should be noted that a neutralizing antibody response was detected very early after acute infection of rhesus monkeys with the simian immunodeficiency virus SIVmac251 (38). SIVmac251 is able to grow in T-cell lines and was known to be sensitive to neutralization by serum antibodies before being inoculated into the monkeys. Most primary HIV-1 isolates, however, are inherently resistant to neutralization by soluble CD4 (10) and antibodies (30), a fact that will always hinder our ability to measure neutralizing antibodies to HIV-1 with similar sensitivity to that described in acute SIV infection.

It has also been suggested that the loss of infectious virus from plasma during acute seroconversion may represent in vivo neutralization of virus due to formation of antibody-virus complexes (25). If so, no free neutralizing antibodies would be

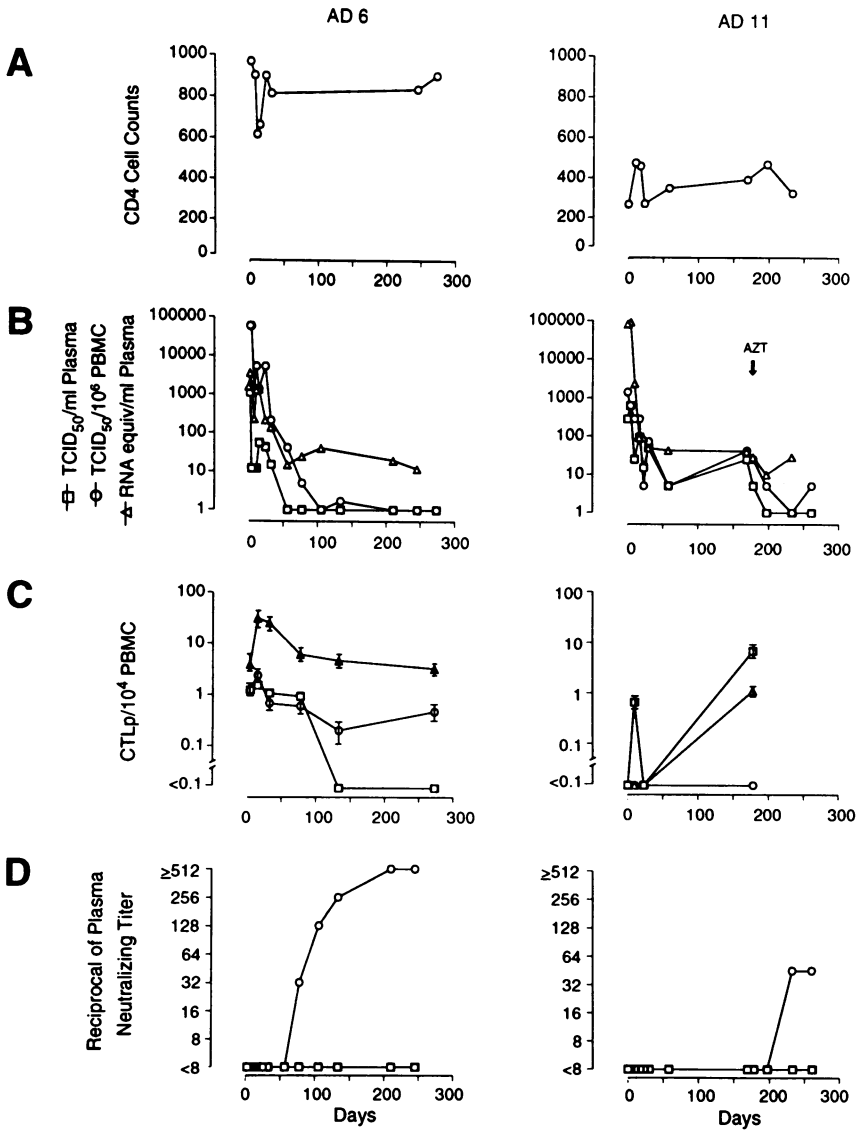

FIG. 2. Day zero is taken as the day the patient presented with symptoms. HIV-1 seroconversion occurred on day 8 for AD6 and day 10 for AD11. (A) Absolute number of $CD4^+$ T cells per microliter of blood as determined by flow cytometry. (B) Amount of HIV-1 in blood as determined by endpoint dilution coculture of plasma (□) and PBMC (○) or branched DNA amplification of particulate HIV-1 RNA in plasma (△). Results are expressed as the number of $TCID_{50}$ per milliliter for plasma, the number of $TCID_{50}$ per 10^6 cells for PBMC, and the number of RNA equivalents (10^3) per milliliter of plasma for the branched DNA assay. Initiation of zidovudine (AZT) therapy on patient AD11 is indicated with an arrow. (C) Precursor frequencies of CTL directed at antigens derived from HIV-1 *gag* (○), *pol* (△), and *env* (□) genes. The same methods and vectors were used in these assays as were used in the assays depicted in Fig. 1. (D) Reciprocal of the plasma neutralizing titer against laboratory strains of HIV-1, IIIB (□) and JR-CSF (△), or a first-passage virus isolated from the patient (○).

detectable in plasma until sufficient neutralizing antibodies were produced to result in neutralizing antibody excess. If such a scenario were operative during acute HIV-1 infection, one would predict that the number of HIV-1 RNA copies in plasma would remain relatively high while the titer of infectious HIV-1 was decreasing. In patients AD6 and AD11, however, we found that the loss of plasma HIV-1 RNA copies paralleled the loss of infectious virus (Fig. 2B), suggesting the absence of increasing amounts of virus-antibody complexes in the circulation during the period of declining plasma virus load. We have, however, estimated that there is a significant excess of serum gp120-reactive antibody very early in HIV-1 infection (29), and these antibodies could theoretically facilitate clearance of HIV-1 in lymphoid tissue (13, 32).

In these studies, the CTL response was the first virus-specific immune response that we were able to detect in acute HIV-1 infection and the one which predicted the consequences of the acute disease course. If CTL were solely responsible for viral clearance, however, one could not explain the initial clearance of 2 logs of HIV-1 from the blood of AD11. This observation clearly indicates that viral clearance mechanisms are multifactorial. Natural killer cells are involved in the initial defense against many virus infections (31, 42), and we have noted transient elevations in natural killer cell activity during acute

seroconversion (AD6) (data not shown). In addition, antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity, and opsonization may all contribute in some way to clearance of HIV-1. Our studies do not, however, address these other potential virus clearance mechanisms.

What, then, could be the role of CTL in clearance of HIV-1 from both PBMC and plasma when CTL are active against virus-infected cells and not cell-free virus? CTL could effect the loss in plasma virus by eliminating virus-producing cells or by blocking virus production through nonlytic mechanisms (27). It has been shown that cells with a CTL phenotype are able to limit HIV-1 antigen release into the supernatant of HIV-1-infected PBMC cultures (43, 47). It should also be noted that the presence of virus-specific CTL is temporally associated with clearance of SIV antigenemia in acutely infected rhesus monkeys (50), and adoptive transfer experiments in mice have demonstrated the ability of CTL to block the early spread of other virus infections in the absence of any humoral immunity (5, 26, 34, 36, 37). Since a single infected cell produces many progeny virions, elimination of such cells is an efficient method for limiting the cell-free virus load.

This is the first report of the timing of the CTL response in acute HIV-1 infection. The presence of CTL early in HIV-1 infection, the temporal association between the high numbers of circulating CTL in the blood and the fall in viremia, and the relatively poor clearance of virus which occurred when the CTL response was delayed (as in AD11) combine to strengthen the argument for the role of CTL in protective immunity to HIV-1 in vivo. This is consistent with what has been observed in other viral infections. The increase in viral load (9) and decrease in measurable CTL activity (7, 18) during the chronic course of HIV-1 infection suggest that CTL continue to be involved in protection from HIV-1-related CD4 depletion long into infection. These observations raise the possibility that stimulation of a CTL response might be an important goal in the development of an effective prophylactic vaccine against AIDS.

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