Cross-Clade-Specific Cytotoxic T Lymphocytes in HIV-1-Infected Children

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We studied cytotoxic T lymphocyte (CTL) cross-reactivity between human immunodeficiency virus type 1 (HIV-1) subtypes within a group of infants infected with either HIV-1 B or non-B clade. Fifteen children were infected with a clade B virus. Nine were infected with non-B virus, including two clade A, four clade D, two clade F, and one clade G. CTL activities from in vitro activated peripheral blood mononuclear cells were tested against autologous cell line infected with recombinant vaccinia viruses encoding for Env, Gag, Pol, or Nef proteins from a clade A or B isolate. HIV-1-specific CTL elicited from infection with clade B virus could lyse targets expressing clade A proteins, and vice versa. In infants with positive CTL responses, cross-clade recognition was predominant and was detected within 88% of the Pol, 83% of the Nef, 67% of the Gag, and 55% of the Env responders. Longitudinal studies showed that CTL cross-reactivity to both B and A targets was stable for several years. Elicitation of CTL reactivities capable of elimination of virus-infected cells is an important goal for the development of an efficient AIDS vaccine. The significant cross-reactivity of CTL shown in this study supports the concept that vaccines developed using a single-clade immunogen may be applicable to induce broadly reactive T cell responses.

INTRODUCTION

The functions of antigen-specific T lymphocytes are required for recovery from viral infections, clearance of virus, control of persistent infections, or a combination. Cytotoxic T lymphocyte (CTL) activities specific to most viral proteins have been detected in the peripheral blood mononuclear cells (PBMCs) of human immunodeficiency virus (HIV)-infected individuals (Johnson and Walker, 1994; Rivière et al., 1994, for review). Although HIV-specific CTL cannot fully clear the virus from infected hosts, they appear to play a role in controlling disease progression (Greenough et al., 1997; Klein et al., 1995; Ogg et al., 1998; Rivière et al., 1995). Virus-specific CTL are the first virus-specific immune response detected, and they are temporally associated with the reduction of viral load in HIV-infected adults (Borrow et al., 1994; Koup et al., 1994; Lamhamedi-Cherradi et al., 1995). In experimental models of viral infections, it has been demonstrated that protective immunity after a viral challenge requires the preexistence of memory virus-specific CTL (Fu et al., 1997; Martins et al., 1995; Yokoyama et al., 1995). Thus elicitation of CTL capable of control of viral replication and elimination of virus-infected cells is an important goal for the development of an efficient AIDS vaccine. In view of the great sequence variability of HIV-1, a study of the intensity of the cross-reactive immune responses able to recognize the various HIV-1 strains is of particular importance.

The different HIV-1 strains are classified into two groups based on phylogenetic analysis with the use of DNA sequences (Myers et al., 1996). The majority of the sequences constitute the major (M) group of HIV-1 viruses, and a smaller number of sequences are classified as O (outlier). The M group has been subdivided into 10 subtypes or clades (A–J) that differ from each another by an average nucleotide percentage distance of 30% based on env sequence. Subtype B is the most prevalent subtype in the Caribbean, Europe, and North America. The main geographic area is West, Central, and East Africa for subtype A; India and East and South Africa for subtype C; Central and East Africa for subtype D; East Asia and Central Africa for subtype E; and South America, East Europe, and Central Africa for subtype F (Jansens et al., 1997; Myers et al., 1996; Workshop Report from the European Commission and UNAIDS, 1997).

The ability of infants, including newborns, to mount a virus-specific cytotoxic response has been reported by different investigators (Buseyne et al., 1993; Luzuriaga et al., 1991, 1995; McFarland et al., 1994). We studied the HIV-specific CTL activities in a cohort of children (Buseyne et al., 1993). These children are part of the French pediatric cohort of whom approximately one third of infants are of African origin and thus may be infected by a subtype different from clade B. These patients present two important characteristics regarding their immune responses: they were infected when their immune
system was not fully mature, and the diversity of HIV-1 strains is much broader than that in most other risk groups in Western countries. In this study, we typed the virus strains infecting these infants and compared their CTL responses against HIV proteins from two clades (B and A). This is the first description of significant CTL cross-reactivity between clade B and non-B viruses in HIV-1-infected infants. These results support the concept that vaccines developed using a single-clade immunogen may be applicable to induce broadly reactive T cell responses.

RESULTS

Genetic subtyping of HIV-1 isolates

The genetic classification of HIV-1 isolates from these infected infants was determined by HMA in 20 cases and by phylogenetic analysis in four cases (EM50, EM66, EM95, and EM97) as described under Materials and Methods. Fifteen children were infected with a clade B virus and nine with a non-B virus (Table 1). The geographic origins of the 15 infants infected with a clade B virus were Europe (nine), North Africa (two), sub-Saharan Africa (one), Haiti (one), and Asia (two). Eight of the nine infants infected with a non-B clade virus were from sub-Saharan Africa, and one was from Europe (Romania). As shown in Table 1, two clade A (EM7, EM59), four clade D (EM17, EM79, EM95, EM97), two clade F (EM50, EM66), and one clade G (EM103) viruses were characterized. For two D strains (EM95, EM97) and the two F strains (EM50, EM66), the phylogenetic tree based on the C2-V3 nucleotide alignment and including reference strains was constructed with the Neighbor–Joining method. Trees constructed with the Fitch–Margoliash method and maximum parsimony gave similar branching patterns.

CTL cross-reactivity among different HIV-1 clades

T cell lines were derived after in vitro activation of peripheral blood mononuclear cells (PBMCs) as described under Materials and Methods. We previously shown that HIV-specific effectors induced by this stimulation protocol were MHC class I restricted CD3+ CD8+ T-lymphocytes (Buseyne et al., 1993). The 24 children were tested for their CTL activities. HLA typing, clinical stage, and age of the children are summarized in Table 1. The percentage of infants with a positive CTL
targets expressing clade A protein, and HIV-1-specific EM64, EM68, EM76, and EM84) could cross-react with HIV-1 clade B (EM42, EM45, EM47, EM48, EM54, EM60, EM64, EM68, EM76, and EM84) could cross-react with targets expressing clade A protein, and HIV-1-specific CTL responses elicited from infection with HIV-1 clade A, D, or F (EM7, EM17, EM50, EM66, EM95, and EM97) could cross-react with targets expressing clade B proteins.

Longitudinal studies of CTL responses

For two infants infected with a non-B virus, a longitudinal study was possible. As shown in Fig. 1, the Gag-specific activity of infant EM7, infected with a subtype A virus, was measured on five occasions from 2.3 to 9.3 years of age. The Gag-specific cytotoxic response was found to be positive with a similar intensity to both A and B targets, showing that cross-reactivity was stable over a long period of time. The Nef-specific response was tested on two different occasions (2.3 and 31 years of age) showing cross-reactivity but with a consistently higher recognition of the A targets compared with the B targets (Table 2 and data not shown).

Infant EM50 was infected with a subtype F virus. His Env-, Gag-, Pol-, and Nef-specific CTL responses were measured on four occasions from age 10.4 to 14.8 years. Despite variations in the intensity of the specific lysis, the Pol-specific activity was found to be consistently positive to the B- but negative to the A-expressing targets (Fig. 2A). In contrast, the Nef-specific activities were detected consistently to the A but only occasionally to the B targets (Fig. 2B). The Gag-specific activities were detected over time with varying intensity but with a consistent cross-reactivity to both A and B targets (Fig. 2C). Env-specific responses were not detected (not shown).

Thus in these two examples of longitudinal follow-up of CTL activities, cross-reactivity to both B and A targets was stable for several years. Furthermore, for one of these individuals, the CTL response was multispecific and the pattern of clade recognition differed between different proteins.

**DISCUSSION**

We studied the extent of cross-reacting CTL activities from 24 infants infected with HIV-1. Fifteen infants were infected with a subtype B virus, and nine were infected with a non-B-subtype virus. For most infants with a positive CTL response, there was a significant cross-reactivity of CTL between clade B and non-B subtypes. The cross-clade CTL responses were found in 88%, 83%, 67%, and 55% of infants with a positive CTL responses to the Pol, Nef, Gag, and Env proteins, respectively. Cross-reactive CTLs were found in both HIV-1 clade B- and non-B-infected children.

The typing of HIV-1 strain was performed with the heteroduplex mobility assay or phylogenetic analysis. Most children (nine) infected with a clade B virus were from Europe, where the clade B subtype is prevalent (Workshop Report from the European Commission and UNAIDS, 1997). The other six infants infected with a clade B virus were from North Africa, Africa, Asia, and...
Haiti, where this viral subtype has been described. A total of nine infants were infected with non-B virus. Two (EM50 and EM66) were infected by blood transfusion with an F-subtype virus. Child EM50, who was born to an African mother, was infected in Congo, where this subtype is present (Janssens et al., 1997), and child EM66, who was born to a European mother, was infected in Romania. Infection of children by clade F virus after blood transfusion in Romania has been previously reported (Dumitrescu et al., 1994). The seven remaining children, who were infected with a clade A, D, or G virus, were born to mothers from sub-Saharan Africa, where these clades have been frequently described (Janssens et al., 1997).

Six of the nine infants infected with a non-B virus developed CTL activities to one or more of the four proteins tested from a clade A virus. In all infants in whom there was a positive CTL response, cross-recognition of at least one of the viral proteins tested from a clade B virus was also observed (EM7, EM17, EM50, EM66, EM95, and EM97). Infant EM7 was infected with a clade A virus by perinatal transmission. His Gag-specific CTL lines were repeatedly cross-reactive to both B and A targets over time (7 years), and the Nef-specific CTL

![Figure 1: Longitudinal analysis of clade A- and B-specific CTL activities from infant EM7.](image)

FIG. 1. Longitudinal analysis of clade A- and B-specific CTL activities from infant EM7. Gag-specific CTL activities of T cell lines derived from in vitro activated PBMCs measured at different effector-to-target (E/T) ratios, from age 2.3 to 9.3 years. Targets were autologous B-EBV targets infected with recombinant vaccinia virus encoding for the Gag gene of clade A or B isolate or with a control vaccinia virus (+).
activity was predominantly directed to clade A protein. In a few cases, such as EM7 and EM95 for Nef, EM17 for Gag, and EM42 for Pol, CTL activities were directed to both A and B subtypes but with a consistently higher lysis to one subtype (Table 2). This suggests the presence of cross-clade and type-specific CTL responses at the same time. Infant EM50 was infected by transfusion with an F-subtype isolate. His pattern of CTL recognition was remarkably stable over time: the Gag proteins from the A and B subtypes were recognized equally well, but the Nef- and Pol-specific CTL activities were directed to the A and B subtypes, respectively. These data from child EM50 provide an example, in the same individual, of different patterns of reactivities: (1) absence of envelope-specific activities, (2) cross-reactive Gag-specific activity, and (3) B or A type-specific activities to the Pol or Nef protein, respectively.

Fourteen of the 15 infants infected with a clade B virus had CTL activities directed to at least one of the four proteins tested. As in the case of non-B-infected infants, most cytotoxic activities were cross-reactive to both clade A and B proteins: one of one Nef, 10 of 10 Pol-, four of eight Env-, and three of the six Gag-specific responses were directed to both clade A and B proteins.

A number of T cell epitopes have been reported in the highly conserved Pol and Gag proteins. In view of the great homology of the Pol and Gag amino acid sequences expressed in the recombinant vaccinia vectors (91.9% and 94% amino acid identity for Pol and Gag, respectively, between clade ACAR3253 and clade B LAI virus), the presence of cross-reactive CTL is not surprising. The comparison of the Nef amino acid sequences from these two viral isolates (clade ACAR3253 and clade B LAI virus) reveals 79.5% amino acid identity. However, most CTL epitopes have been described in the highly conserved central region (amino acids 63–146, with 86% amino acid identity) of the Nef protein (Rivière et al., 1994 for review), and it is likely that this central region is the

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**FIG. 2.** Longitudinal analysis of clade A- and B-specific CTL activities from infant EM50. Percentage-specific lysis of T cell lines derived from in vitro activated PBMCs at 10.4, 11.6, 13.2, and 14.8 years of age. CTL activities were measured at different effector-to-target (E/T) ratios against autologous B-EBV targets infected with recombinant vaccinia virus encoding for the Pol (triangles, Fig. 2A), Nef (circles, Fig. 2B), or Gag (squares, Fig. 2C) genes of clade A (○, ■) or B (△, □) isolate or with a control vaccinia virus (+).
The target of the cross-reactive CTL responses detected in the majority of the children with a positive Nef-specific CTL response.

The type-specific CTL responses (Gag specific for infants EM45, EM84, and EM96; Pol specific for infants EM50 and EM75; Nef specific for EM50; and Env specific for EM48, EM65, EM76, and EM78) are probably directed to epitopes located in nonconserved regions of the proteins.

Within the 15 clade B virus-infected infants, eight had detectable envelope-specific CTL activities: four cross-reactive and four type specific. Of the eight infants infected with non-B-subtype virus (A, D, F, or G) and tested for envelope-specific activities, only one (EM17) had detectable CTL activities, cross-reactive to both A and B targets. CTL responses directed to the envelope glycoprotein were more frequently detected in infants infected with B-subtype virus compared with infants infected with non-B-subtype virus, probably reflecting the viral diversity between each subtype (30% nucleotide divergence in the $env$ gene).

Differences in allele and haplotype frequencies for HLA has been reported between Caucasians and Africans. For example, allelic frequency for HLA-A2 is 21.3% and 13.8% in the populations of France and Zaïre, respectively (Imanishi et al., 1991). HLA molecules determine the viral peptides presented to CTL and may affect the pattern of CTL recognition. In this study, both sub-Saharan African and European infants present a high degree of CTL cross-clade reactivity for Pol, Nef, and Gag. Thus differences in HLA do not influence HIV-specific CTL cross-reactivity at the level of protein recognition, at least in this group of patients.

CTL cross-reactivity among HIV-1 clades was initially reported in adult volunteers immunized with an HIV-1 clade B-based canarypox vector vaccine (Ferrari et al., 1997). CTL clones derived from clade B-infected adults in the United States were shown to be cross-reactive to a B virus and at least to one non-B clade virus (Cao et al., 1997). In the same study, CTL lines from 14 Senegalese adults infected with a non-B clade virus were able to recognize at least one of the clade B proteins tested. Similar results were reported in a study of Zambians individuals: six of the eight subjects infected with a clade C virus elicited CTL activities specific for the clade B proteins (Betts et al., 1997). Frequent cross-clade reactivities in the CTL responses were also reported from six Africans from the Central African Republic infected with clade A virus and from eight Caucasians infected with a clade B virus (Durali et al., 1998). In Ugandan patients
infected with non-B clade virus, the cross-clade recognition of the Gag protein by CTL was shown against four different subtype viruses (McAdams et al., 1998). Most of the children studied in this work were born to infected mothers, and thus the HIV-1 subtype diversity reflects the migration of the parents from Africa (Simon et al., 1996). This study provides the first example of cross-clade CTL activity in a sample of infants living in France and infected with B- and non-B-subtype virus, and as in previous studies, we found a predominance of the CTL cross-clade reactivity.

Elicitation of CTL reactivities capable of elimination of virus-infected cells is an important goal for the development of an efficient AIDS vaccine. In HIV-1-infected infants, the significant cross-reactivity of CTL between clade B and non-B viral proteins supports the concept that vaccines developed based on a single-clade immunogen may be applicable to induce broadly reactive T cell responses, but such a vaccine should include large sequences containing several epitopes to improve the probability of cross-reactivity between the vaccine strain and the field isolate.

MATERIALS AND METHODS

Patients

The children studied were followed at the Département de Pédiatrie, Hôpital Necker (Paris, France). The legal guardians gave informed consent before their infants entered the study. Most children were born to HIV-1-infected mothers. Three children, one from Romania (EM66), one from Africa (EM50), and one from Asia (EM76), were infected by blood transfusion before 5 years of age. The diagnosis of HIV infection was based on HIV isolation from PBMCs in the first months of life or on the presence of anti-HIV antibodies in the serum at 18 months of age. The clinical status of the children was recorded according to the 1994 revised pediatric classification system (Centers for Disease Control and Prevention, 1994). HLA genotyping was performed in the Department of Immunology at the Chelsea and Westminster Hospital (London, UK) using amplification-refractory mutation system PCR (Krausa et al., 1995). These infants were included in the longitudinal follow-up of HIV-spe-
specific cytotoxic activities at Institut Pasteur (Buseyne et al., 1993).

Cytotoxic assay

Target cells. For each patient, an autologous Epstein–Barr Virus (EBV)-transformed B cell line was obtained by infection of PBMCs with supernatant from the EBV-producing cell line B95-8 (American Type Culture Collection CRL 1612). EBV-B cells (3 × 10^6 cells) were infected with the wildtype vaccinia virus or with the various recombinant vaccinia viruses (rvv), at a multiplicity of infection of 10 PFU/cell, for 1 h at 37°C and then washed and cultured for 16 h at 37°C in 5% CO_2 before use in the cytotoxic assays.

Viruses. All the vaccinia viruses used in this study were provided by Transgène (Strasbourg, France). The parental virus was the Copenhagen strain of wildtype vaccinia virus (vv-WT). Foreign genes were inserted under the control of the 7.5-kb vaccinia virus promoter, inside the thymidine-kinase gene. Recombinant vaccinia viruses vvTG1139, vvTG1144, vvTG1147, and vvTG3167 encode the Env, Gag, Nef, or Pol protein of HIV-1LAI, respectively (Buseyne et al., 1993; Rivière et al., 1989), vvTG6059 encodes the Env protein of a clade A isolate HIV-192CAR3253, and vvTG6026, vvTG6015, and vvTG6052 encode the Gag, Nef, or Pol protein of a clade A isolate HIV-192UG037, respectively (Durali et al., 1998).

Effector cells. PBMCs were isolated from heparinized blood by density gradient centrifugation on Ficoll–Hamegar (Ficoll-Paque, Pharmacia, Les Ulis, France). The generation of CTL lines was performed after in vitro activation of PBMCs with phytohemagglutinin A (PHA-p, 5 μg/ml) in RPMI 1640 medium (Bioproducts, reference M12167, F, Gagny, France) supplemented with 5% human AB serum (ETS, Bobigny, France), L-glutamine (2 mM, Gibco reference 043±05030H, Cergy Pontoise, France), sodium pyruvate (1 mM, Gibco reference 043±01360 H), and nonessential amino acids (1×; Gibco reference 043±01140H), and recombinant human interleukin-2 (100 IU/ml, a generous gift from D. Lando, Roussel Uclaf, Cergy Pontoise, France). The cultures were fed every 3–5 days for 3–5 weeks. The cytolytic activity of the T cell lines was evaluated from day 20 to day 35 after in vitro stimulation.

Chromium release assays. Cytotoxic assays were conventional 4-h ^51Cr release assays as previously described (Rivière et al., 1989). Briefly, 1 × 10^5 target cells were labeled for 1 h with 3.7 MBq of Na_2^51CrO_4 (reference NEZ030, NEN, Le Blanc Mesnil, France), washed three times, and distributed at 5 × 10^3 cells/well in 0.1 ml of medium in round-bottomed 96-well microtiter plates (Costar, OSI, Maurepas, France). Various concentrations of effector cells in 0.1 ml (in triplicate or quadruplicate) were added. The plates were incubated at 37°C in 5% CO_2 for 4 h and then centrifuged at 150 × g for 5 min. Supernatant (50 μl) from each well was transferred with 100 μl of scintillation liquid (Optiphase Hisafe 3, EGG, Evry, France) into a 96-well harvesting plate (reference 81150401; EGG, Evry, France), and the radioactivity was measured in a beta counter (Microbeta 1450, EGG). Percent specific lysis was calculated as 100 × (experimental release — spontaneous release)/(maximal release — spontaneous release). Maximal release was obtained from targets lysed by 5% Triton X-100/1% SDS. Spontaneous release was obtained from targets incubated 4 h in medium alone. Assays in which spontaneous ^51Cr release exceeded 35% were discarded. The CTL responses against HIV antigens were considered positive if they exceeded the mean of vv-WT-specific lysis by 3 SDs and by 10% for ≥1 effector-to-target ratio. Similarly, clade A-specific CTL response was considered higher than the clade B-specific CTL response if it exceeded the mean of vv-B-specific lysis by 3 SDs and by 10% for ≥1 effector-to-target ratio, and vice versa.

PCR, heteroduplex mobility assay (HMA), and sequence determination

DNA was extracted from PHA-activated PBMCs. Env fragments were amplified by nested PCR using ED3/ED12 as outer primers and ES7/ES8 as inner primers (Delwart et al., 1993). For the HMA, reference plasmids for each subtype (A–H) were also amplified with inner primers. Heteroduplexes were analyzed as previously described (Delwart et al., 1993). For sequence determination, amplified PCR fragments were purified with a PCR product purification kit and sequenced by using dye terminator chemistry (Perkin-Elmer) on an automated DNA sequencer 373A.

Sequence analysis

Sequences were aligned with the multiple sequence analysis program CLUSTAL W with minor adjustments. Reference strains for each subtype for the region analyzed were included in this study. For construction of the trees, the pairwise evolutionary distances were estimated by using Kimura’s two-parameter method. These methods were implemented using the Phylip package (Felsenstein, 1993).

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