Cytotoxic T Cell Response against the Chimeric ETV6-AML1 Protein in Childhood Acute Lymphoblastic Leukemia

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Abstract

Cytotoxic T lymphocytes (CTL) are potent effector cells that could provide long term antitumor immunity if induced by appropriate vaccines. CTL recognize 8–14 amino acid–long peptides processed intracellularly and presented by MHC class I molecules. A well-characterized example of a potential tumor antigen in childhood pre-B Acute Lymphoblastic Leukemia (ALL) results from the chromosomal translocation 12;21 leading to the fusion of the ETV6 and AML1 genes. This translocation is observed in $>$ 25% of ALL-patients. In this study, we have examined whether the chimeric ETV6-AML1 protein could serve as a tumor specific antigen for CTL in HLA-A2.1 individuals. We have identified a nonapeptide (RIAECILGM), encoded by the fusion region of the ETV6-AML1 protein, that binds to HLA-A2.1 molecules and induces specific primary CTL in peripheral blood lymphocytes from healthy donors. These CTL specifically lysed HLA-A2.1 tumor cells endogeneously expressing the ETV6-AML fusion protein. CTL with similar functional capacities were found with high frequencies and cloned from one patient's bone marrow indicating that ETV6-AML1–specific anti-ALL CTL are, at least in some patients, spontaneously stimulated and might participate to host antileukemia defense. (*J. Clin. Invest.* 1998. 102:455–462.) Key words: ETV6-AML1 • cytotoxic T lymphocytes • chromosomal translocation • leukemia

Introduction

The cell-mediated immune response against tumors is becoming a focus of cancer immunotherapy. Successes have already been achieved in the absence of a precise knowledge of which tumor antigens are recognized, by infusing in vitro lymphokine-activated and polyclonally expanded killer cells derived from either PBL or tumor-infiltrating lymphocytes. More efficient immunotherapy should be expected using tumor antigen specific immune cells. This depends on the identification of the target antigens, which has been achieved in melanomas (1–3) and renal carcinomas, testing tumor specific cytotoxic T lymphocyte (CTL)[1] clones against transfected cells expressing tumor derived cDNA. An alternative approach is to identify a mutated gene whose product might serve as a specific antigenic marker for malignant cells. The products of translocations, recurrently associated with certain malignancies, particularly in human leukemias, are promising candidates.

We and others have shown that translocation 12;21 [t(12;21)] is the most frequently observed recurrent genetic abnormality in childhood pre-B acute lymphoblastic leukemia (ALL), occurring in 25% of cases (4–6) exclusively in B-lineage leukemia. Contrary to leukemia reactive allogeneic CTL, a cytolytic T cell response to autologous leukemic blasts has been documented rarely. Others have described a correlation between the presence of the ETV6-AML1 fusion gene and a generally more favorable prognosis for patients (6). We, therefore, decided to evaluate whether the fusion protein product of t(12;21) could act as a specific target for a T cell–mediated immune response.

In most cases of t(12;21), the breakpoint is in the frame between ETV6 exon 5 and AML1 exon 2. The chimeric ETV6-AML1 protein of 336 amino acids comprises the helix-loop-helix domain of ETV6 (7), a member of the ETS-like family of transcription factors, fused to AML1 (8), the DNA binding subunit of the AML1/CBFb (9) transcription factor complex. In the majority of cases, patients with t(12;21), as well as the patients included in this study, have exactly the same breakpoint (8) (see Fig. 1).

CTL recognize fragments of endogeneously synthesized cellular proteins of 8–14 amino acid residues bound to MHC class I molecules. We have examined whether the chimeric ETV6-AML1 protein can serve as a tumor-specific antigen in HLA-A2.1 individuals. We have identified a peptide, encoded by the fusion region, that binds efficiently to the HLA-A2.1 molecules. We demonstrate here that in vitro induction of a primary CTL response is feasible against this MHC-binding peptide. Furthermore, we show that patients harbor CTL that recognize autologous tumors, indicating that effective immunization occurs in vivo at least in some cases. This is the first report showing that a product of chromosomal translocation in human acts as a specific tumor antigen being recognized by both in vitro and in vivo generated CTL and can consequently be proposed for immunotherapy.

Methods

ALL patients

The bone marrow (BM) samples used in this study represent a small fraction of those, withdrawn under general anesthesia, for diagnosis.

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1. *Abbreviations used in this paper:* ALL, Acute Lymphoblastic Leukemia; APC, antigen-presenting cells; BM, bone marrow; CTL, cytotoxic T lymphocytes; R9M, RIAECILGM; RT, reverse transcription; t(12;21), translocation 12;21.

In all cases, informed consent was obtained from parents. Patient 1 was a 2-yr-old girl with hepatosplenomegaly and an initial white blood cell count of 30,000/μl. Patient 2 was a 9-yr-old boy studied when a relapse off therapy was diagnosed 6 yr after the onset of the disease. This patient had a pancytopenia with 2% circulating blasts. Patient 2 has a healthy syngeneic twin whose BM was harvested for transplantation, a fraction being used for critical control experiments. In both patients the BM was infiltrated with > 90% leukemic cells with early pre-B stage ($CD19^+$ $CD10^+$ $cIgM^-$) immunophenotype. In both cases, t(12;21) was detected using a combination of fluorescence in situ hybridization and reverse transcription (RT)–PCR analysis of the ETV6-AML1 fusion transcripts, as previously described (4). In both patients, the RT-PCR transcripts correspond to in frame fusion transcripts. Western blot analysis was performed on blast cells using polyclonal antibodies raised against a recombinant peptide of the junctional region of ETV6-AML1 (10). Two bands corresponding to ETV6-AML1 protein products were detected in each case. For both patients and healthy controls, the HLA-A2 subtype was A2.1.

Histopathological studies

Tissues. BM specimens were obtained before treatment from seven patients with t(12;21) translocation. All BM biopsies were decalcified, formalin fixed, and paraffin embedded. Serial 3–4 μm sections were made and stained with hematoxylin eosin, giemsa, or reticulin. Histological analysis of these BM biopsies, in all cases, revealed a diffuse blastic infiltration without mutilant fibrosis.

Immunohistological techniques. Indirect three-stage immunoperoxidase techniques were performed on serial sections of the seven fixed BM biopsies. mAb specific for CD45RO (UCHL-1; DAKO A/S, Glostrup, Denmark), a pan-T antigen (UCHT-1; DAKO A/S), or TIA-1, a cytolytic granule-associated protein (11) (TIA-1; Coultronics, Miami, FL), were used. The numbers of CD45RO and TIA-1–expressing cells were evaluated by randomly counting 10 fields at G $\times$ 400 (each field = 0.5 mm^2) in each BM biopsy. Omission of the primary mAb and use of an irrelevant isotype-matched mAb served as negative control.

Detection of apoptotic cells. Analysis of DNA fragmentation, characteristic of apoptotic cells, was performed by DNA nick end labeling of the fixed BM sections using the TdT-mediated dUTP-biotin nick end labeling method (12). As a negative control, the terminal deoxytransferase reaction step was omitted.

Colocalization of TIA-1–expressing cells and apoptotic cells. In all cases, immunohistological techniques, using anti–TIA-1 mAb and DNA nick end labeling, were performed on the same tissue section for the simultaneous detection of TIA-1–expressing cells and apoptotic cells. Diaminobensidine and fast-red were used as chromogens for visualization of TIA-1 and DNA nick-end labeling, respectively.

Synthetic peptides and binding assays

Peptides were purchased from Eurogentec (Seraing, Belgium; see Fig. 1). Peptide purity was checked by reverse-phase HPLC analysis. Peptide Pol (ILKEPVHGV) from HIV-1 was used in cytolytic assays as negative control. The relative binding characteristics of this peptide to the HLA-A2.1 molecules have been described previously (13, 14). Peptide binding to MHC class I molecules was measured (as described previously in reference 15) in competition assays using a ^{125}I-radiolabeled standard hepatitis B virus core 18–27 (HBc18–27) (FLPSDYFPSV) peptide and soluble purified HLA-A2.1 molecules (16).

Primary CTL induction using synthetic ETV6-AML1 junction peptides

CTL were elicited from PBL of healthy donors using synthetic peptides (17, 18). PBMC from healthy HLA-A2.1 donors were purified by centrifugation on Ficoll-Hypaque. Antigen-presenting cells (APC) were prepared by incubating PBMC with Concanavalin A (5 μg/ml) in the presence of human rIL2 (100 U/ml; Boehringer Mannheim, Mannheim, Germany). The culture medium was RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1mM), penicillin (100 u/ml), streptomycin (100 μg/ml; RPMI complete medium), and FCS (10%). After 6 d, APC were pelleted and resuspended in a 0.5-ml stripping buffer (0.13 M citric acid, 66 mM Na_2HPO_4, 150 mM NaCl, 17 μg/ml phenol red). After 30 s, the cell suspension was neutralized by adding 300 μl of a saturated solution of Na_2HPO_4 containing the peptide to be cell loaded. After incubation with peptide, 15 min at RT, APC were washed, then irradiated at 3,000 rads and cultured for 7 d at a 3:1 ratio with autologous PBMC responder cells. Secondary or tertiary stimulations were similarly weakly performed, the medium being further supplemented with rIL7 (50 ng/ml PharMingen, San Diego, CA) rIL2 (100 U/ml).

Generation of ALL-specific CTL lines and clones

Mononuclear cells were isolated on a Ficoll-Hypaque gradient from ALL BM cells harvested at diagnosis. A T cell was obtained from patient 1 after culture of these cells (a majority of leukemic cells and few T lymphocytes) in the presence of the soluble trimeric form of CD 40 Ligand (CD 40L, 40 μg/ml, kindly provided by Dr. J. Bonnefoy [Glaxo] and by Immunex). After 10 d of culture the T cell line was restimulated with allogeneic feeder cells (in RPMI 1640 complete medium with 10% decomplemented human serum AB, phytohemagglutinin [PHA 1 μg/ml], and human rIL2 [100U/ml]) and tested for CTL activity. T cell lines from patient 2 were isolated before any in vivo or in vitro treatment using immunomagnetic beads coated with anti-CD3 mAb. These T cells were then restimulated weekly with feeder cells from pooled healthy donor PBMC irradiated at 2,500 rads, culture medium being supplemented with PHA and rIL-2. Clones were obtained by limiting dilution and expanded using the same nonspecific stimulation.

Cytotoxicity assays

CTL were tested for cytotoxicity in a standard 4 h ^{51}Cr-release assay (17). In all experiments, T cell lines or clones were used 8 or 15 d after the last stimulation with feeders. Peptide-pulsed targets were prepared by incubating CEM $\times$ 721 (T2) line (HLA-A2.1,TAP1−/TAP2−) overnight at 26°C with synthetic peptide (5 μg/ml) in RPMI 1640 medium without FCS. For cytolysis inhibition assays with anti–HLA-A2.1 [BB7.2, F(ab)′2 IgG] mAb, target cells were first incubated for 30 min with mAb at a final concentration of 200 μg/ml, then the cells were washed before incubation with effector cells. Cold-target inhibition of cytolysis were performed using effector to hot target ratio of 100:1 and cold to hot target ratios ranging from 7:1 to 210:1.

FACS® analysis

Analysis was performed by either direct or indirect immunofluorescence using the following purified specific mAb: PE- or FITC-conjugated anti-CD3 (UCTH-1), PE–anti-TCRαβ (BMA031), PE–anti-CD4 (13B8.2), FITC–anti-CD8 (B8.11), anti-CD10 (ALB12), FITC–anti-B7.1 (MAB104), FITC–anti-CD25 (B1.49.9), FITC–anti-CD45RO (UCHL-1). All of these mAb were purchased from Immunotech (Marseille, France). FITC–anti-CD3/PE-HLA-DR (SK7/L243) and PE– or FITC–anti-CD19 (SJ25C1) were from Becton Dickinson (San Jose, CA). Anti-HLA–ABC (IgG2a, W6/32), PE–anti-B7.2, (FUN-1; PharMingen). FITC-conjugated anti-Vb17 (E17.5F3.15.13), anti-Vb8 (56C5.2), anti-Vb21 (IG.125), anti-Vb3 (LE-89), anti-Vb2 (E22E7.2), anti-Vb13.6 (JU74.3), and unlabeled anti-Vb14 (CAS1.1.3), anti-Vb9 (FIN9), anti-Vb6.1 (CRI304.3), anti-Vb5.2 (36213), anti-Vb5.3 (3D11), anti-Vb13.1 (IMMU222), and anti-Vb12 (VER2.32.3) were purchased from Immunotech. Cells were labeled with mAb on ice for 30 min, then washed four times. For analysis with unlabeled mAb, cells were incubated for 30 min on ice with FITC-F(ab)′2 goat anti–mouse Ig. All cells were analyzed on a FACScan® flow cytometer (Becton Dickinson). The Lysis II program was used for data analysis.

Limiting dilution assays

Relative frequencies of ALL-specific CTL were determined by limiting dilution assays. Increasing numbers of lymphocytes (from 50 to

10,000) were seeded per well, and tested 10 d later for cytolytic activity by adding 5,000 ^{51}Cr-labeled target cells per well, in a final volume of 200 μl. Each dilution was tested in 24 replicated wells. Supernatants were collected after 9 h incubation at 37°C. A well was considered positive if ^{51}Cr-release exceeded by 3 SD the mean spontaneous release evaluated from control-wells containing target cells alone. Effector cell frequencies were estimated by Poisson distribution analysis. Minimal estimates were verified by using the statistical method of $\chi 2$ minimization, applying Poisson probability theory to the single hit model.

Cell lines

ALL pre-B cell line REH (HLA-A2.1-[ATCC]) that express ETV6-ALM1 protein10; CEM × 721 (T2) line (HLA-A2.1,TAP1−/TAP2−; and patient 2 EBV-transformed lymphoblastoid B cell line were used in this study. EBV cell line was established from PBL by standard method using supernatant from the EBV-producing marmoset cell line B95.8. REH cells were cotransfected with HLA-A2.1 plasmid and hygromycin resistant plasmid by electroporation (900 μF and 1050 μF, 320 V, Tms = 28) as described previously (19).

Results

One peptide from the junction region stably binds to the HLA-A2.1 molecules. Nine nonapeptides (listed in Fig. 1) derived from the fusion region of ETV6-AML1 were synthesized and tested for their ability to bind to purified soluble HLA-A2.1 molecules. One of them, R9M (RIAECILGM), exhibited high binding activity in a competitive assay with a standard iodinated peptide. Kinetic binding studies using the transporter associated with antigen presentation–deficient T2 line indicated that the MHC-peptide interaction had a moderate dissociation rate (half-life of the complexes of 3 h, data not shown). Therefore, one might expect such complexes, formed in the endoplasmic reticulum, to reach the surface of ALL-cells and be available for CTL recognition.

A ETV6 nts : **ATGCCCATTGGGAGAATAGCAG**ACTGTAGA............

AML1 nts : **AATGCATACTTGGAATGAATCCTTCTAGAGACGTCCAC**

B nts : TCC CCG CCT GAA GAG CAC GCC ATG CCC ATT GGG AGA ATA GCA GAA
aa : S P P E E H A M P I G R I A E

nts : TGC ATA CTT GGA ATG AAT CCT TCT AGA GAC GTC CAC
aa : C I L G M N P S R D V H

C SPPEEH**AMPIGRIAECILGMNPS**RDVH

D

Peptide sequences	Inhibition (%) of ^{125}I labelled F10V fixation
AMPIGRIAE	28
MPIGRIAEC	8
PIGRIAECI	18
IGRIAECIL	0
GRIAECILG	0
RIAECILGM (R9M)	91
IAECILGMN	0
AECILGMNP	0
ECILGMNPS	0
FLPSDYFPSV (F10V)	94

Figure 1. Binding of junctional nonapeptides to HLA-A2.1 molecules. (*A*) Nucleotide (*nts*) sequence of ETV6 and AML1 involved in the ETV6-AML1 fusion. (*B*) Nucleotide sequence and deduced amino acid sequence of the fragment representative of in-frame–fused ETV6-AML1 transcripts. Nucleotide 1033 of ETV6 is underlined. (*C*) Amino acid sequence of the ETV6-AML fusion region. (*D*) The ability of ETV6-AML1 peptides to bind soluble HLA-A2.1 was evaluated in a competitive test by the percent inhibition of binding of iodinated hepatitis B virus peptide (*F10V*).

Induction of primary CTL responses from healthy donors by ETV6-AML1 peptide. Next, we studied whether the MHC-binding ETV6-AML1 (R9M) peptide would be effective in raising HLA-A2.1–restricted CTL. Using stripped autologous T blast cells loaded with R9M peptide for CTL induction, we have induced specific primary CTL in three healthy donors. These CTL specifically lysed the HLA-A2.1$^+$ T2 cell line when incubated with peptide R9M, compared with lysis of cells pulsed with the irrelevant HIV-1 peptide (Table I). Specificity of lysis was further warranted using HLA-mismatched R9M peptide-pulsed target cells that were not lysed (data not shown). However, it cannot be assumed that CTL generated under such conditions will kill targets expressing the protein from which the peptide was derived. For this to occur, the endogeneous protein must be processed in such a way that this particular antigenic peptide is produced, efficiently transported into the endoplasmic reticulum, and associated with the relevant MHC class I molecules. Therefore, we asked whether CTL-lines, generated from HLA-A2.1 healthy donors and ALL patients, would be able to kill HLA-matched ETV6-AML1$^+$ tumor cell.

Specific HLA-A2.1–restricted CTL from a ALL-patient and a healthy donor can lyse tumor cells expressing endogeneously the ETV6-AML1 fusion protein. Having established that primary CTL from healthy donors induced in vitro by synthetic exogeneous peptides exhibited peptide-dependent specific lysis of HLA-matched target cells, we next tested whether such CTL could recognize tumor target cells expressing endogeneously the ETV6-AML1 fusion protein. Two CTL-lines were generated as described in Methods. CTL-line from ALL-patient 1 BM not only lysed R9M-pulsed HLA-matched cells, but, more importantly, HLA-matched ALL-tumor cells and the HLA-A2.1$^+$-transfected REH cells expressing endogeneously the ETV6-AML1 protein. Neither autologous EBV-transformed cells in the absence of R9M peptide (Fig. 2 *A*) nor HLA-A2.1- (nontransfected) REH cells (Fig. 2 *B*) were lysed indicating CTL specificity. The second T cell line derived from healthy donor was similarly able to recognize ETV6-AML1

Table I. Primary Induction of CTL from Healthy Donors

	DON 590		DON 888		DON 801	
E/T ratio	10:1	3:1	10:1	3:1	10:1	3:1
Specific lysis (percent)	30	21	34	27	24	17
Nonspecific lysis (percent)	18	7	8	5	1	0

Primary CTL were generated from PBL in mixed lymphocyte with autologous concanavalin A–activated lymphocytes that had been acid-treated in the presence of peptide R9M. After two rounds of stimulation, these lymphocytes were tested for specific lytic activity at different E/T ratios. Specific lysis was measured in a standard ^{51}Cr-release assay using the T2 cells loaded with peptide R9M as targets. Nonspecific lysis was measured on T2 cells loaded with an irrelevent HIV-1 Pol peptide.

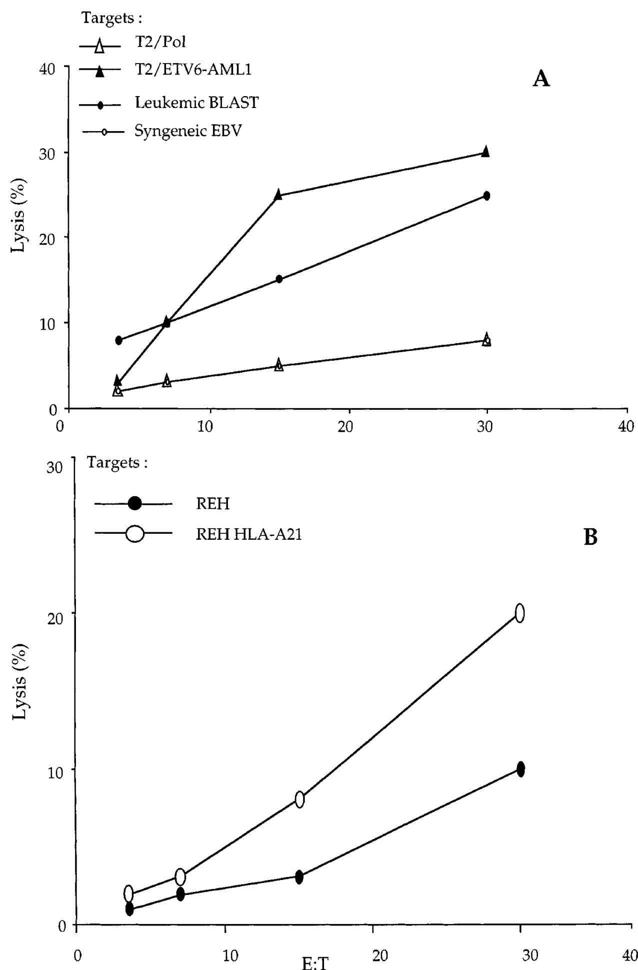


Figure 2. The T cell line derived from patient 1 BM recognized peptide R9M and autologous leukemic cells. Total BM, including leukemic blasts and T cells, were cultured 10 days with CD40L and then restimulated with allogeneic feeder cells. Cytotoxic activity was assessed 9 d after restimulation at E/T ratios of 30:1, 15:1, 7.5:1, 3.75:1, and 1.8:1. Lytic activity of the T cell line is shown against syngeneic EBV (*open circles*) and autologous leukemic blasts (*closed circles*). R9M specific lysis is also shown using the T2 line pulsed with peptide R9M (*closed triangles*) and the irrelevant peptide Pol (*open triangles*). HLA-A2.1 restricted lysis is shown using the REH line (*closed circles*) and HLA-A2.1–transfected REH line (*open circles*).

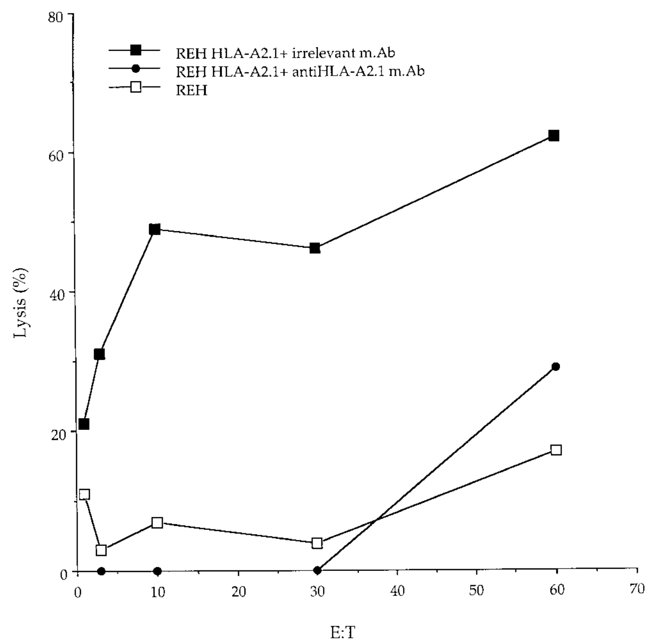


Figure 3. HLA-A2.1 restriction of ETV6-AML1 junction peptide-specific T cell response. HLA-A2.1$^-$, ETV6-AML1$^+$ REH cells (*open squares*, HLA-A2.1$^+$-transfected, ETV6-AML1$^+$ REH cells preincubated with irrelevant mAb [F(ab)′2, 200 μg/ml, *closed squares*], and HLA-A2.1$^+$–transfected, ETV6-AML1$^+$ REH cells preincubated with anti–HLA-A2.1 BB7.2 mAb [F(ab)′2, 200 μg/ml, *closed circles*]) were used as targets in a 4-h ^{51}Cr-release assay using healthy donors, R9M peptide–induced, primary CTL line.

peptide-pulsed HLA-A2.1$^+$ target cells and HLA-A2.1-transfected REH cells (data not shown). These T cell lines generated in vitro were not alloreactive since HLA-A2.1–nontransfected REH cells were not killed. To confirm the HLA-A2.1 restriction, blocking experiments were performed. Lysis of HLA-A2.1–transfected REH cells was completely inhibited by anti–HLA-A2.1 (BB7.2) mAb (Fig. 3) as well as by cold target cells expressing both the HLA-A2.1 and ETV6-AML1 fusion molecules. Irrelevant antibodies or HLA-A2.1–nontransfected REH cold targets did not inhibit lysis (Fig. 4). These results showed that the two CTL cell lines recognized a R9M neoantigenic determinant restricted by the HLA-A2.1 molecules. We concluded that the ETV6-AML1 protein is efficiently endogeneously processed and presented by HLA-A2.1 molecules, and therefore represents a potential target for specific cell-mediated immunity against tumors bearing the t(12;21) translocation. Since the ETV6-AML1 (R9M) peptide is presented at the surface of the ALL leukemia-cells, we next wondered whether these tumor cells could be immunogenic in vivo.

Presence of T lymphocytes expressing lytic markers and elevated frequencies of leukemia-specific CTL in ALL patient BM at the time of diagnosis. We reasoned that the minor subset of infiltrating T lymphocytes before treatment (< 10% of BM cells at diagnosis) could be enriched in leukemia-specific T lymphocytes similarly to tumor-infiltrating lymphocytes in solid tumors. This hypothesis was supported by the identification of activated T cells in the BM of ALL patients, in contrast to normal donors. The expression of CD45RO and TIA-1 was studied in BM biopsies from seven patients with t(12;21) (Fig. 5 is representative of the data obtained from biopsies of seven patients). These markers were expressed with a mean density of 6 cells per 0.5 mm^2 for CD45RO and 5.8 cells per 0.5 mm^2 for TIA-1. In each case, a higher TIA-1/CD45RO ratio (96%) was found compared with a normal T cell population (49–64%), arguing for the recruitment and activation of potential cytotoxic cells in these leukemic BM. Apoptotic cells with a large nuclear size, typical of leukemic cells, were present in each specimen studied (Fig. 5) and double labeling experiments showed that TIA-1–expressing cells were in close contact with apoptotic cells, suggesting a specific cytopathic effect of these T cells on the tumor cells. Control biopsies from the bone marrow of several children suffering from other malignancies as Hodgkin lymphoma or Neuroblastoma were used.

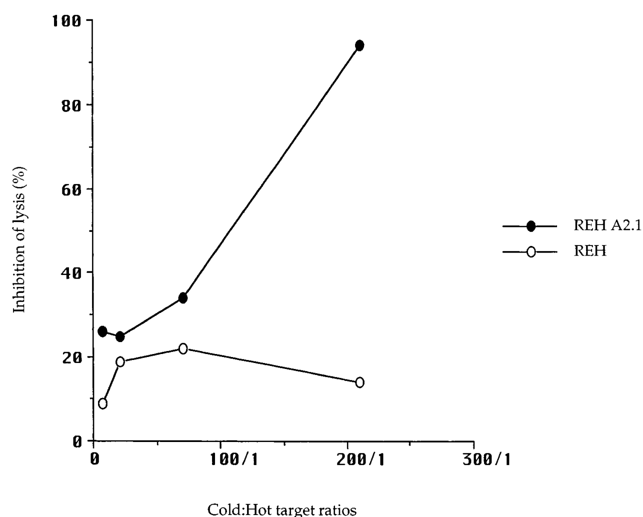


Figure 4. Cold-target inhibition of the lysis by R9M-specific CTL of HLA-A2.1$^+$, ETV6-AML1$^+$ ALL cells. Freshly isolated HLA-A2.1$^+$, ETV6-AML1$^+$ ^{51}Cr-labeled ALL cells were tested as hot targets in a 4-h assay using as effector (E/T ratio 100:1) primary CTL induced in vitro with the R9M junction peptide. HLA-A2.1$^-$, ETV6-AML1$^+$ REH cell (*open circle*) and HLA-A2.1$^+$-transfected, ETV6-AML1$^+$ REH cell (*closed circle*) were used as cold targets at 210, 70, 21, and 7:1 cold/hot target ratios. The results are expressed in percentages of inhibition of the lysis of ALL cells in the absence of cold target.

Very few infiltrated T cells can be detected in these biopsies, < 10 cells for 15 mm^2 (representing around 30 fields), meaning 10-fold less cells than what is observed in acute lymphoblastic leukemia. Furthermore, in these two pathologies no T cells expressing the activation marker TiA1 can be detected in BM biopsies. The rate of apoptosis BM cell of normal donors was compared with that observed in ALL donors. The biopsies show a higher percentage of apoptotic cells in normal samples; these results were expected because of the normal high turnover of the cells in the hematopoietic tissues. However, no colocalization of apoptotic cells and activated T cells was found on those biopsies.

The frequency of leukemia-specific CTL in BM from patient 2 was determined and compared with the frequency of such CTL in the BM of his syngeneic healthy twin. CTL specific for the tumor in BM of patient 2 were found with a 1:100 frequency. In contrast, no leukemia-specific CTL were detected in the BM of the syngeneic twin demonstrating natural specific priming in the ALL-patient (Fig. 6). However, these experiments were performed with uncloned, in vitro–activated T lymphocytes. To more precisely characterize the effector cells contained in patient 2 BM, those effectors were cloned.

Cytotoxic T lymphocyte clones isolated from the BM of ALL patients are specific for the ETV6 AML1 fusion protein. To confirm that ETV6-AML1–specific CTL exist in vivo in ALL-patients, cloned T lymphocytes from patient 2 BM were tested for their specific antitumor activity. Lymphocytes were sorted out from BM using anti-CD3 mAb linked to magnetic beads and cloned by limiting dilution using allogeneic feeder cells (see Methods). Clones were screened for CD4 and CD8 markers. 30% of clones were found to be CD8$^+$ and were analyzed for their cytolytic specificity and Vβ T cell receptor chain

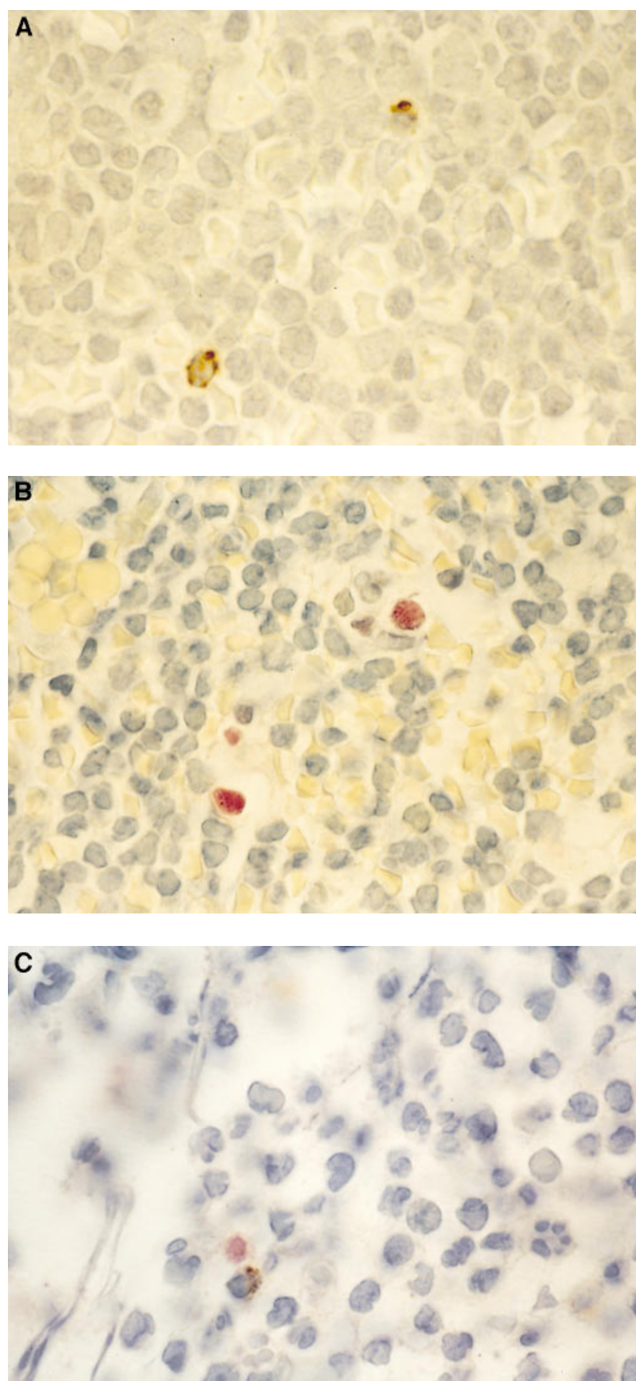
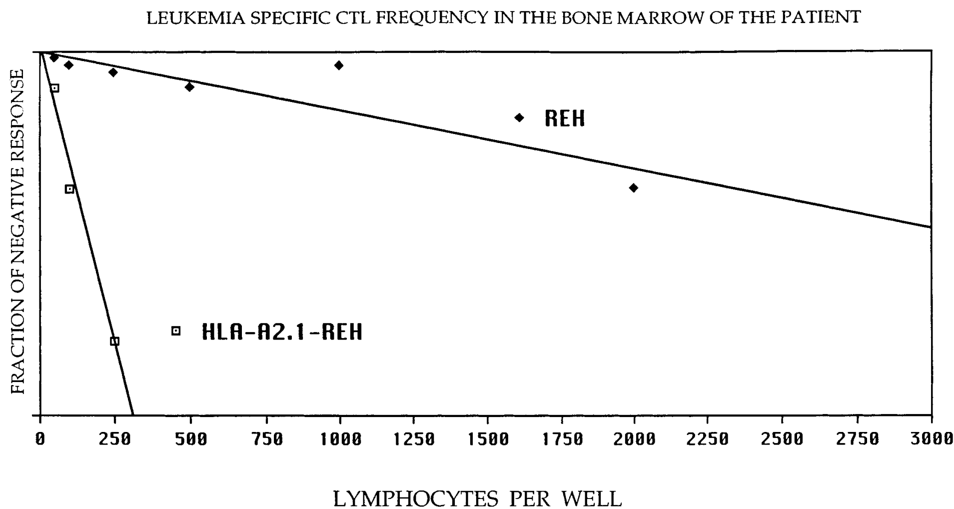


Figure 5. Histological analysis of serial sections of ALL BM biopsies at time of diagnosis. Notice the diffuse infiltration by lymphoblasts with large nuclei, readily identified by hematoxylin counterstaining (×2,000). (*A*) Immunohistochemical detection of TIA-1 expressing cells with intracytoplasmic brown granules, the small size of these cells and the expression of TIA-1 molecules differentiate them from pre-B lymphoblasts. (*B*) DNA nick end labeling using the TdT-mediated dUTP-biotin nick end labeling method of the adjacent section identifies large apoptotic lymphoblasts in the vicinity of the TIA-1 positive cells. (*C*) Close contact of TIA-1$^+$ and apoptotic cells illustrated by immunohistochemical identification of TIA-1 molecules and DNA nick end labeling on the same BM section.



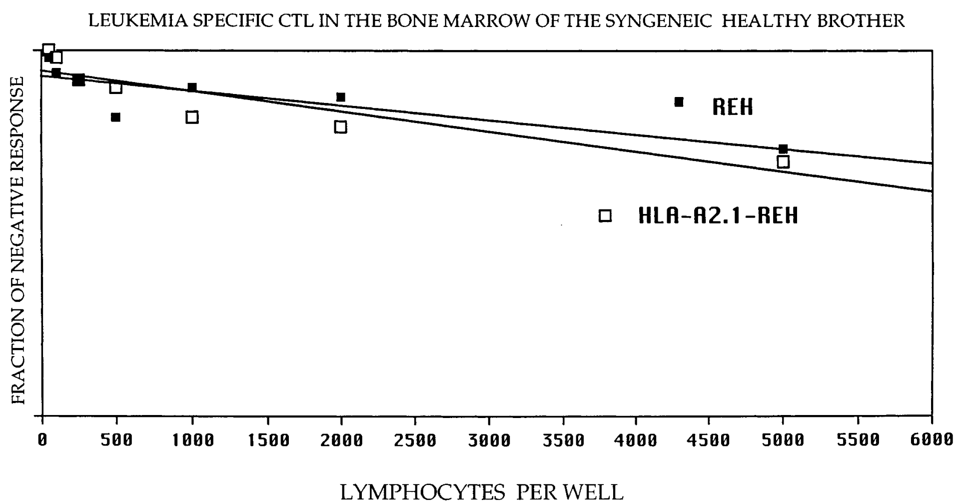


Figure 6. Leukemia-specific CTL frequencies. Leukemia-specific CTL frequencies were estimated in parallel in the BM of patient 2 and his healthy twin. After 10 d of culture, the microculture was split and tested on both HLA-A2.1–transfected REH and on nontransfected REH target cells.

usage. Specificity was determined on HLA-matched target cells pulsed with the ETV6-AML1 R9M peptide and on autologous tumor cells (Fig. 7). These clones recognized autologous tumor cells and syngeneic EBV-blasts pulsed with the R9M peptide and not the irrelevant peptide. Interestingly, these clones expressed three different Vβ (02, 03, 13.6) chains although they all recognized the same MHC–peptide complex. These results show that, in vivo some patients generate a specific immune response against the ETV6-AML1 translocation product.

Discussion

This study demonstrates that in ALL, a fusion ETV6-AML1 molecule, resulting from a t(12;21) chromosomal translocation, acts as a tumor specific antigen for $CD8^+$ CTL. A nine amino acid–long, nonself, junction peptide comprising four ETV6 and five AML1 residues binds to HLA-A2.1 molecules and induces in vitro CTL responses from both HLA-A2.1$^+$ healthy donors and ALL-patients. Specific recognition by these CTL of tumor cells expressing endogeneously the ETV6-ALM1 fusion protein indicates efficient spontaneous processing and presentation of the junction peptide. Furthermore, CTL specific for the junction peptide were identified with high frequency among the T lymphocytes infiltrating the leukemic BM (patient 2), prior treatment. Finally, the $CD8^+$ phenotype, Vβ usage and junction peptide-specificity of the effector cells of this patient could be determined at the clonal level, leading to us to think that reinforcement of host antileukemia CTL responses might be feasible and of therapeutic value.

The nine amino acid junction epitope does not exhibit the HLA-A2.1 canonical binding motif at position 2, where a leucine residue is usually found. There are already many such examples in the literature, on this subject, and, in some cases, these unusual peptides are essential for host defense (20). Therefore, a search for class I–restricted antigenic peptides should not be limited to peptides displaying canonical binding motifs, especially in the case of tumor antigens (21). The identified R9M junction peptide being nonself, induction or reinforcement of R9M-specific CTL responses should not be harmful for the host. In fact, polyclonal stimulation of patient CTL only resulted in the expansion of ETV6-AML1 junction-

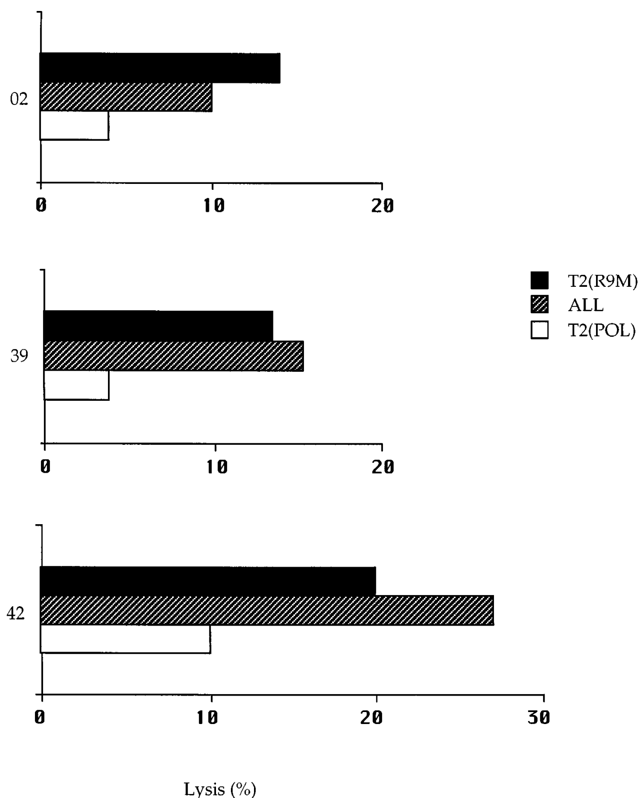


Figure 7. Cytolytic activity of CD8+ clones derived from patient 2's BM. Immunopurified CD3+ T lymphocytes, nonspecifically restimulated in vitro with allogeneic irradiated feeder cells, were subsequently cloned by limiting dilution and expanded as indicated in Methods. CD8+ T cells clones 02, 39, and 42 were tested at a 1:1 E/T ratio in a standard 4-h ^{51}Cr-release assay using autologous ALL (*hatched bars*), R9M peptide–pulsed (*closed bars*), and I9V control Pol peptide-pulsed (*open bars*) HLA-A2.1+, ETV6-AML1-T2 target cells.

specific effector cells with no detectable lysis of cells not expressing the fusion protein. Thus, the ETV6-AML1 fusion protein is a more attractive immunotherapeutic target than other (p53, erb/neu, etc.) proteins, overexpressed and not mutated in certain leukemia, but also expressed in most of the normal tissues.

In spite of efficient R9M peptide-presentation by ALL-cells, the elicited CTL response documented in patient 2 does not suffice for leukemic cell eradication. This may simply reflect the fact that the proliferative potential of the tumor cells overwhelms the lytic capacity of the patient CTL. There is also evidence that tumor cells can interfere negatively with cytolytic T lymphocytes by releasing inhibitory cytokines (22) and can induce CTL anergy or apoptose. In addition, tumor cells often lack some accessory costimulatory molecules required for optimal CTL induction (as the B7.1 in ALL). Detailed analysis of these possibilities is underway in our laboratory for more adapted immunotherapeutic actions. In parallel, having identified a tumor-specific immunogenic peptide, the vaccine potential of this peptide is currently evaluated in a HLA-A2.1–transgenic, H-2 class I–negative animal model. Preliminary results indicate that different vaccine regiments (intramuscular injection of naked DNA, infusion of in vitro–activated, peptide-pulsed professional APC), which can be proposed in human trials, effectively induce R9M-specific CTL responses. Once optimized, these vaccine strategies could be proposed as a complementary therapeutic action, in those ALL cases that resist chemotherapy.

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