
Gisella Volpe, Alessandro Cignetti, Cristina Panuzzo, Mirela Kuka, Katiuscia Vitaggio, Mara Branaccio, Giuseppe Perrone, Monica Rinaldi, Giuseppina Prato, Milena Fava, Massimo Geuna, Marisa Pautasso, Claudia Casnici, Emanuela Signori, Giancarlo Tonon, Guido Tarone, Ornella Marelli, Vito M. Fazio, and Giuseppe Saglio

Introduction

Imatinib is more effective than any other previous pharmacologic approach for chronic myelogenous leukemia (CML) and has become the golden standard for its front-line treatment (1, 2). In spite of the very high percentage of patients obtaining a complete cytogenetic response and of those obtaining a major molecular response (3), which corresponds to a decrease of >3 logs in the BCR/ABL amount compared with the mean amount normally observed at diagnosis (3, 4), patients achieving a complete molecular remission [undetectable BCR/ABL transcript at the nested reverse transcriptase PCR (RT-PCR) analysis] are ranging between 5% and 25% of the reported series (3, 5–7). Studies on bone marrow samples have shown that residual cells are part of the leukemic stem cell compartment (8) and consequently most of those who discontinue imatinib treatment relapse in a very short period (9). The mechanisms underlying the resistance of these cells are unknown, but their final eradication is believed to be needed to obtain a definitive cure for the disease.

CML is considered one of the potential responsive malignancies to immunotherapy (10, 11). The evidence that leukemic cells present human leukocyte antigen (HLA)–associated immunogenic peptides derived from Bcr/Abl fusion protein has provided the basis for tumor vaccine development (12–14). Furthermore, the reinduction of complete molecular remission by means of donor lymphocyte infusions into patients relapsed after allogeneic bone marrow transplantation further confirms the role of T cells in the control of the disease (15, 16).

Up to now, vaccination approaches have been based essentially on the use of Bcr/Abl junctional peptides, derived from amino acidic sequences spanning the e14a2 fusion junction between Bcr and Abl (17, 18). Other approaches tried to exploit the presence of proteins overexpressed by CML cells, such as proteinase-3 and prame (19, 20).

In the present article, new potential tumor-specific antigens in BCR/ABL–positive leukemias are described. We report the presence of BCR/ABL transcripts arising from alternative splicing and of their corresponding proteins. In particular, those with junctions involving BCR exons 1, 13, or 14 on one side and ABL exon 4 or 5 on the other side result in the production of fusion proteins characterized by an initial and correct Bcr portion attached to a
sequence of amino acids of variable length arising from the out of frame (OOF) reading of the ABL exons 4 and 5 gene sequence. We have found that HLA-binding peptides derived from Ab1 OOF are immunogenic and able to elicit an antigen-specific T-cell response in vitro. OOF-specific T cells were found in the peripheral blood of CML patients, and they could lyse primary autologous CML cells after in vitro sensitization. These observations highlight the importance of OOF-derived epitopes as new leukemia-specific antigens suitable for vaccination approaches.

Materials and Methods

Patients and cell lines. Sixty-three primary samples were collected at diagnosis from 58 patients with BCR/ABL-positive CML and from 5 patients with BCR/ABL–positive acute lymphoblastic leukemia (ALL) after informed consent. Of 63 patients analyzed, 24 of 63 expressed the e1a2, 33 of 64 expressed the e1a4, and 6 of 64 expressed the e1a2 transcript. Furthermore, we analyzed 6 BCR/ABL–positive cell lines: 4 were e1a2 positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1). Peripheral blood of healthy subjects and HL60 cells, a positive (CMLT-1, JK, KCL22, and BV173), 1 was e14a2 (K562), and 1 was e1a2 (TOM-1).

RNA extraction, nested RT-PCR, and direct sequence. One microgram of total cellular RNAs, extracted from mononuclear cells using the guanidine/thiocyanate/phenol/chloroform method (21), was reverse transcribed in cDNA using random exomers. To amplify splice variant e1a4 transcript, we used a specific set of primers and a Taqman TAMRA probe designed on the E13a5 and E14a5 splice junctions. I step: E13EST, 5′-gaagtgtttcagattgagag-3′ (forward primer) and ABLex 5′-aggccgagttgatga-3′ (reverse primer); II step: E13INT, 5′-cagatgctgaccaactcgtgt-3′ (forward primer) and ABLa5 5′-tgccacgacttgatga-3′ (reverse primer).

(b) e1a4 splice junction. I step: E1a4EST, 5′-ggagtgctca-gaagccctcc-3′ (forward primer) and ABLa5 5′-tcgctgcttcatggtgatgtcc-3′ (reverse primer); II step: E1a4INT, 5′-ctagtgtgggcaccaagctgtg-3′ (forward primer) and ABLa5 5′-tcgctgcttcatggtgatgtcc-3′ (reverse primer).

(c) e1a5 splice junction. I step: E1a5EST, 5′-ggagtgcggcttcatggtgatgtcc-3′ (forward primer) and ABLa5 5′-gcgttcccgtaggtcatgaact-3′ (reverse primer).

(d) e1a3 splice junction. I step: E2EST, 5′-aggtgcgctctctctctcag-3′ (forward primer) and ABLa6 5′-gtggccagttgatga-3′ (reverse primer); II step: E2INT, 5′-cagatgctgaccaactcgtgt-3′ (forward primer) and ABLe6x 5′-tgaggtgtttcatctctctcag-3′ (reverse primer).

The retrotranscription and amplification conditions were those defined by the BIOMED1 concerted action (22). The amplification products were sequenced using the sequencing system dye terminator cycle sequencing kit, as described previously, to verify the correct reading frame of the cloned insert. E. coli TOP10 cells were transformed with the above-mentioned plasmids and fusion proteins, which were induced by isopropyl-ß-D-thiogalactopyranoside (IPTG) according to the manufacturer's instructions. Lastly, bacteria were pelleted, resuspended in lysis buffer, and sonicated. After centrifugation at 20,000 x g for 30 min at 4°C, the fusion proteins were purified by affinity chromatography.

Antiserum reacted against OOF protein portion. Polyclonal rabbit antiserum against OOF protein portion was obtained by immunizing a rabbit with repeated i.m. injections of 300 μg Mbp-oof fusion protein, emulsified in complete Freund adjuvant. Antibody specificity was shown by immunoprecipitation of the total protein extracts of bacterial colonies transformed with GST-OOF gene fusion vector after IPTG induction and in Western blots on total protein extracts.

Identification of the Bcr/Abl-oof fusion protein. Protein extracts from K562 and HL60 cell lines were obtained using a lysis solution (radioimmunoprecipitation assay buffer) able to break the nuclear membrane and to recover whole-cell proteins. Briefly, cell lines were washed twice with cold PBS and lysed for 15 min on ice with a solution containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and the following protease inhibitors: 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 trypsin inhibitor units/ml apropin. Afterwards, protein extracts were clarified by centrifugation and protein concentration was quantified using Bio-Rad protein assay (Bio-Rad Laboratories). Immunoprecipitation was done overnight at 4°C by adding 5 μl polyclonal anti-OOF antiserum along with protein A-agarose beads to 6 mg protein extracts. The protein/antibody complex was recovered by short centrifugation at 14,000 rpm and boiled in Laemmli buffer. Proteins were loaded and resolved by SDS-PAGE before transfer to nitrocellulose membranes (Hybond C, Amersham International PLC). Membranes were saturated with 5% bovine serum albumin (BSA) in TBS-Tween and incubated overnight at 4°C with a monoclonal antibody (mAb) against the NH2 terminus of Bcr (Santa Cruz Biotechnology, Inc.) in TBS-1% BSA. After washing, the filters were incubated with peroxidase-conjugated antimouse antibody for 2 h at room temperature and detection was done with Lite-Abbot chemiluminescent substrate (Cellbio).
polyclonal antibody (Santa Cruz Biotechnology) diluted 1:200 and anti-Ki-67 mouse mAb, clone MIB-1 (DAKO), diluted 1:50. Slides were then incubated with ABC Staining System (Santa Cruz Biotechnology) biotin-conjugated secondary antibodies according to the manufacturer’s instructions. Antibody binding was visualized by incubating with 3,3′-diaminobenzidine solution and counterstained with Mayer hematoxylin and evaluated with an Olympus BX51TF microscope (Olympus Corp.) equipped with Plan-Neofluar objective lens 4×/0.10 NA, 10×/0.25 NA, 20×/0.40 NA, and 40×/0.65 objective lenses. A Digital Sight DS-5M-L1 Nikon camera (Nikon Corp.) was used and the acquisition software was Nikon’s ACT-2U Multifunctional imaging software (Nikon).

**T-cell epitope prediction of OOF protein portion for human HLA class-I.** The amino acid motifs responsible for specific peptide binding to two of the most common types of HLA class-I molecules (HLA-A2 and HLA-A3) were determined using the algorithm developed by Rammensee’s laboratory (SYFPEITHI; ref. 24). Based on the scores obtained with this algorithm, an arbitrary value of 15 was set to define nonamers with high or intermediate binding to HLA-A2 and HLA-A3 (<15, intermediate; >15, high). The following peptides were purchased from PRIMM Ltd.: QQAHCLWCV (OOF#1, score for HLA-A2 16, score for HLA-A3 3), GVRGRVEEI (OOF#2, score 21 and 17), LLREPLQHP (OOF#3, score 19 and 14), CILWCVPQLR (OOF#4, score 11 and 16), RLLEPLQHP (OOF#5, score 13 and 30), and RVLRSCHSH (OOF#6, score 10 and 25). Peptides were grouped into three different mixtures (mixture A, B, or C) according to their high or intermediate score for HLA-A2 and HLA-A3: mixture C was composed of peptides with intermediate score for A3 and high for A*0201 (OOF#1 and OOF#3), mixture B was composed of peptides with high score for A3 and intermediate for A*0201 for (OOF#4, OOF#5, and OOF#6), whereas mixture A was composed only of one peptide with high score for both alleles (OOF#2). All peptides were purified by high-performance liquid chromatography at a minimum purity of 95%. Peptide binding to HLA-A2 was confirmed by doing a MHC stabilization assay using the TAP-deficient T2 cell line (25). Results obtained confirmed the score obtained for the HLA-A2 molecule with the SYFPEITHI algorithm (data not shown).

**Cytokine flow cytometry assay.** For cytokine flow cytometry assay (26–28), peripheral blood samples from HLA-A2 or HLA-A3 healthy donors and BCR/ABL-positive CML patients in complete cytogenetic remission after imatinib treatment were collected and peripheral blood mononuclear cells (PBMCs) were obtained by gradient centrifugation on Lymphoprep (Fresenius Kabi Norge AS). All selected patients showed the presence of BCR/ABL alternative splicing. PBMCs were resuspended in serum-free medium at 2×10⁶ cells/mL (X-vivo 15, BioWhittaker) and incubated for

---

**Figure 1.** A and B, nested RT-PCR analysis (II step) of BCR/ABL alternative splicing in Ph-positive leukemic samples. Next to the main transcripts e1a2, e13a2, and e14a2, the following alternative transcripts can be seen: e14a, e13a4, and e14a4 (A) and e1a5 (B). p190-positive samples (lanes 1–4); p190-negative samples (lanes 5 and 6 and lanes 8–13); negative controls (lanes 7 and 14). M1 size: 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 384, 298, 234, and 154 (top to bottom); M2 size: 1,114, 900, 692, 501/489, 404, 320, 242, 190, 147, 124, 110, and 67 (top to bottom).

**Figure 2.** Alternative reading frame (ARF) of ABL gene into the splice variants involving BCR exon 1, 13, or 14 and ABL exon 4 or 5. A, normal reading frame of ABL exons 4 and 5 into e1a2, e13a2, and e14a2 fusion transcripts. B and C, ARF of ABL exons 4 and 5 into the OOF splice variants. All three kinds of alternative splice junctions with abl exon 4 display the same ARF, as well as all those with abl exon 5.
14 to 16 h at 37°C in a humidified 5% CO₂ atmosphere with 10 ng/mL of the three different peptide mixtures (mixture A, B, or C; see above paragraph). One hour after the beginning of the culture, 10 ng/mL brefeldin A (Sigma) was added. Unstimulated PBMCs were used as negative control, whereas PBMCs stimulated with a superantigen [staphylococcal enterotoxin B (SEB), at 5 μg/mL; Sigma] and with anti-CD14 mAb (1 μg/mL; Becton Dickinson) were used as positive control (29). After 24 h, cells were washed in PBS, stained for 30 min at 4°C in the dark with antihuman CD8 APC (Caltag Laboratories), fixed, and permeabilized using Fix and Perm kit (Caltag Laboratories) according to the manufacturer’s instructions. Cells were then stained with mAb anti-interleukin (IL)-2 FITC and anti-IFNγ phycoerythrin (all from Caltag Laboratories) for 30 min at 4°C in the dark. For all samples, at least 10,000 CD8+ T cells (60,000–200,000 total events) were acquired on a Becton Dickinson FACSCalibur flow cytometer.

In vitro generation of OOF-specific CTLs. PBMCs were obtained from heparinized blood of CML patients and healthy donors. CD8+ cells were positively purified from PBMCs using magnetically labeled mAb to CD8 (Miltenyi). Dendritic cells were generated from PBMCs as already described, positively purified from PBMCs using magnetically labeled mAb to CD8 and cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF; 80 ng/mL) and IL-4 (40 ng/mL) for 40 h. A cocktail of proinflammatory cytokines (PharMingen and Beckman Coulter) was then added. Unstimulated PBMCs were used as negative control, whereas PBMCs stimulated with a superantigen [staphylococcal enterotoxin B (SEB), at 5 μg/mL; Sigma] and with anti-CD14 mAb (1 μg/mL; Becton Dickinson) were used as positive control (29). After 24 h, cells were washed in PBS, stained for 30 min at 4°C in the dark with antihuman CD8 APC (Caltag Laboratories), fixed, and permeabilized using Fix and Perm kit (Caltag Laboratories) according to the manufacturer’s instructions. Cells were then stained with mAb anti-interleukin (IL)-2 FITC and anti-IFNγ phycoerythrin (all from Caltag Laboratories) for 30 min at 4°C in the dark. For all samples, at least 10,000 CD8+ T cells (60,000–200,000 total events) were acquired on a Becton Dickinson FACSCalibur flow cytometer.

Cytotoxicity assay. Cytotoxic activity of cultured CD8+ cells was assessed against peptide-pulsed phytohemagglutinin (PHA) blasts or primary CML cells collected and frozen at diagnosis. To obtain PHA blasts, normal PBMCs were activated by PHA (2% v/v; Invitrogen) in the presence of 100 units/mL IL-2 and 10 ng/mL IL-7 (Chiron Corp. and Peprotech). CML cells were thawed and cultured in the presence of Flt3 ligand and stem cell factor (100 ng/mL; Peprotech) for 6 days and then used as targets. All target cells were labeled with 50 μCi [35S]methionine (GE Healthcare) for 2 h at 37°C, washed thrice, and then incubated 4 h (5 × 10⁵ cells per well) with effector cells at various E:T ratios in a total volume of 150 μL. After incubation, the supernatants were collected and radioactivity was measured on a gamma counter. Spontaneous release was determined by harvesting the supernatant of target cells incubated in the absence of effector cells and maximum release was determined by resuspending target cells incubated in the absence of effector cells. The percentage of specific lysis was calculated using the following formula: experimental release = spontaneous release / maximum release – spontaneous release × 100.

Results

BCR/ABL transcripts with alternative splicing. In addition to the main BCR/ABL fusion transcripts, 50 of 63 (79%) BCR/ABL–positive CML and ALL samples and 5 of 6 (83%) BCR/ABL–positive cell lines showed the presence of transcripts with junctions

Figure 3. Western blot detection of 116-kDa Bcr/Abl-OOF fusion protein. Immunoprecipitation of K562 (K) and HL60 (H) radioimmunoprecipitation assay buffer extracts with rabbit polyclonal anti-OOF serum followed by immunoblotting with anti-Bcr mAb.

Figure 4. K562 (A–D) and HL60 (E–H) immunohistochemical staining. K562 (A) and HL60 (E) cell lines incubated with OOF antisera (dilution 1:50); K562 (B) and HL60 (F) cell lines incubated with preimmune antiserum (dilution 1:50); K562 (C) and HL60 (G) cell lines incubated with anti-Ki-67 antibody (dilution 1:50), as control of nuclear positivity; K562 (D) and HL60 (H) cell lines incubated with anti-BCr antibody (dilution 1:200), as control of cytoplasmic positivity. A strong and homogeneous nuclear positivity in K562 can be seen with OOF antiserum staining (A), whereas no positive evidence is seen in HL60 (E). Original magnification, ×400.
respectively between BCR exons 1, 13, and 14 and ABL exon 4 (e1a4, e13a4, and e14a4). Transcripts arising from an alternative splicing of the main BCR/ABL transcripts were only visible after the second step of the nested RT-PCR (Fig. 1). In addition, a consistent proportion of patients and cell lines positive for abl exon 4 splice variants was also expressing junctions between BCR exon 1, 13, or 14 and ABL exon 5 (Fig. 1).

Direct sequencing of amplification products confirmed in all cases the presence of junctions between BCR exon 1, 13, or 14 on one side and ABL exon 4 or ABL exon 5 on the other side. The sequences of alternative junctions have been deposited in the National Center for Biotechnology Information Database (Genbank accession nos. DQ898313–DQ898315 and DQ912588–DQ912590).

BCR/ABL alternative transcripts containing BCR exon 1, 13, or 14 joined to ABL exon 4 are predicted to code for a type of protein, in which a correct NH2-terminal Bcr portion of different length is attached to a sequence of 112 amino acids arising from the OOF reading of the entire ABL exon 4 and of 68 nt of the ABL exon 5, in correspondence of which a stop codon is generated (Fig. 2B). Interestingly, these three kinds of alternative junctions (e1a4, e13a4, or e14a4) could all code for Bcr/Abl-OOF fusion proteins with the same shift in the reading frame of the ABL gene. Splice variant junctions of BCR 1, 13, or 14 with ABL exon 5 (e13a5, e14a5, or e1a5) are predicted to code for fusion proteins with the same OOF reading of ABL exon 5, although only the last 22 amino acids out of the above-described 112 are retained (Fig. 2C). These findings suggest that, beside the classic p210 and p190 Bcr/Abl fusion proteins, in BCR/ABL–positive leukemia, other Bcr/Abl fusion proteins could be present, characterized at the COOH terminus by unique amino acid sequences resulting from the OOF reading of the ABL gene exon 4 and 5, or only exon 5.

**Quantitative RT-PCR analysis of e14a4 alternative splice junction.** The amount of the BCR/ABL transcript with the e14a4 junction was tested by quantitative real time PCR (Q-RT-PCR) analysis in the peripheral blood and bone marrow samples collected from 11 BCR/ABL patients at diagnosis and, for one of these patients, also during the disease evolution. We detected that the expression of the alternative hybrid transcript is ~3 log lower than that of the main BCR/ABL fusion transcript, with a mean value at diagnosis of 0.15% for bone marrow samples (range, 0.02–0.33%) and 0.065% for peripheral blood samples (range, 0.02–0.11%), whereas all healthy volunteers tested as control were scored completely negative. Analysis of alternative transcript in samples collected at different disease stages revealed that Q-RT-PCR method was able to detect the e14a4 alternative splice junction in a minor cytogenetic response bone marrow sample, but not in a major cytogenetic response bone marrow sample (data not shown). These data suggest that the expression of alternative splice transcript might persist in residual Ph-positive cells during major or complete cytogenetic remission, but not at detectable levels.

**Identification of Bcr/Abl-OOF fusion proteins in K562.** To identify the postulated Bcr/Abl OOF fusion proteins, total protein extracts from K562 were immunoprecipitated with a polyclonal rabbit antiserum generated against the OOF protein portion.

![Figure 5](https://cancerres.aacrjournals.org/article-figures/CancerRes2007;67;11 фиг.5.png)
(see Materials and Methods for details). After immunoblotting with a commercial mouse mAb against the NH2-terminal Bcr portion, we detected a band (Fig. 3), which is not present in the total protein extracts from the HL60 cells, used as negative controls. The size (116 kDa) is compatible with the hypothetical translation product of the above-mentioned e14a4 splice variant transcript. Furthermore, the presence of OOF fusion proteins was shown by immunohistochemical staining. All Bcr/Abl-positive human hematopoietic cell lines incubated with OOF antiserum showed a strong and homogeneous nuclear positivity, whereas neither the same cell lines treated with preimmune antiserum nor the Bcr/Abl-negative cell lines used as controls were positive (Fig. 4). These experiments suggest that the alternative transcripts are normally translated.

**OOF-specific T cells are present in CML CD8+ T-cell fraction.**

To determine whether a T-cell response against the OOF Abl portion occurs in vivo during the course of the disease in CML patients, we analyzed by flow cytometry the intracellular accumulation of IFNγ on patients’ T cells following stimulation with OOF-derived peptides. We used PBMCs collected from four CML patients (two HLA-A2 and two HLA-A3 positive) in complete cytogenetic remission but not in molecular remission after imatinib treatment, as well as PBMCs from HLA-A2 and HLA-A3 healthy donors. When enough PBMCs were collected, the experiment was repeated in a different day.

Following PBMC stimulation with different mixtures (mixtures A, B, and C; see Materials and Methods) of OOF-derived peptides, we detected a specific IFNy production by CD8+ T cells in response to one or more OOF peptide mixtures in all CML patients but not in the four healthy donors used as controls (Fig. 5). In particular, the percentage of CD8+ T cells that were specifically producing IFNγ in response to OOF peptides was 4.5 to 62 times higher in CML patients than in healthy donors (see Fig. 5A and B for patient data and Fig. 5C for plots from one representative sample). IFNy production observed in CD8+ donor T cells following SEB + anti-CD28 stimulation confirmed results validity, showing that also T cells from donors can respond to polyclonal antigenic stimuli similarly to patients. No significant production of IL-2 by CD8+ T cells was detected in CML patients after stimulation with the same mixtures (Fig. 5C; data not shown).

Among the different OOF peptide mixtures, mixture A (i.e., OOF#2 peptide) was the most effective because it could induce IFNy production in three of four cases; two of these positive cases were HLA-A2+ and one of three was HLA-A3+ (see Fig. 5A).

**Induction of a CTL response against OOF-derived peptides in a HLA-A2 healthy donor.** To confirm the immunogenicity of OOF-derived peptides, we tested their capacity to induce a peptide-specific CTL response using PBMCs from HL60--A2+ healthy donors. Purified CD8+ cells were primed in vitro with autologous mature dendritic cells pulsed with the three different OOF mixtures (A, B, and C) and restimulated twice at weekly intervals with irradiated peptide-pulsed autologous dendritic cells. CTL activity was evaluated 1 week after the last stimulation with a cytotoxicity assay using PHA blasts pulsed with the relevant mixture (A, B, or C) or with an irrelevant tumor antigen epitope (survivin 2M peptide) as targets. As shown in Fig. 6A, CTLs generated against mixture A showed high specific lysis against mixture A-loaded target cells, whereas no significant cytotoxicity was observed when the same CTLs were tested against survivin-loaded target cells. In addition, CTLs generated against mixtures B and C were able to kill their respective target cells, but with lower efficiency. These data provide further evidence that OOF-derived peptides, and in particular OOF#2 (i.e., mixture A), are immunogenic and escape central tolerance.

**Stimulation of HLA-A2+ CML patient OOF-specific CTLs and lysis of autologous CML cells.** The presence of OOF-specific CD8+ T cells in CML patients suggests that OOF-derived epitopes (and in particular the OOF#2 peptide) are processed and presented in vivo by antigen-presenting cells. To gain further evidence that the OOF#2 peptide is naturally processed by CML cells, we examined the ability of OOF#2-specific CTLs from one HLA-A2+ CML patient to lyse autologous CML cells endogenously expressing the alternative splice variant. To induce OOF#2-specific CTL, a peripheral blood sample was collected from a HLA-A2+ CML patient in cytogenetic complete remission (patient #2) and purified CD8+ cells were repeatedly stimulated in vitro with peptide-loaded antigen presenting cells as described in the above paragraph.

CTL activity was analyzed in a cytotoxicity assay against autologous PHA blasts collected in cytogenetic complete remission (and therefore not expressing endogenous OOF at detectable levels; data not shown) pulsed with the relevant (OOF#2) or an irrelevant (survivin 2M) peptide and against autologous CML cells collected at diagnosis. As shown in Fig. 6B, OOF#2-specific CTLs specifically lysed PHA blasts loaded with the OOF#2 peptide, whereas no
activity was recovered against survivin-loaded targets. OOF#2-specific CTLs could also kill autologous CML cells, suggesting that the OOF#2 peptide is endogenously processed and presented by CML cells.

Discussion

In Ph-positive leukemias, the t(9;22) translocation results in transcripts where BCR exon 1(e1), 13 (e13), or 14(e14) is "normally" joined to ABL exon 2 (a2) and translated into the canonical p190 or p210 Bcr/Abl fusion proteins, whose constitutive tyrosine kinase activation is responsible for the appearance of the leukemia phenotype (31).

In this study, we show that, beside the main BCR/ABL fusion transcripts, small but detectable amounts of different fusion transcripts are also produced in a high percentage of CML and ALL patients as a consequence of alternative splicing. In particular, BCR/ABL alternative transcripts involving ABL exon 4 prevail in most samples. From an immunologic point of view, their corresponding translational proteins are very intriguing because they contain an initial regular Bcr portion of variable length attached to a sequence of 112 amino acids arising from the OOF reading of ABL exons 4 and 5. This OOF portion is present in Ph-positive CML and ALL patients, independently on the type of existing fusion transcript, and is clearly detectable in ~80% of the cases. Because OOF proteins are expressed only by Ph-positive cells and do not have homology with other known human proteins, they could represent a new group of tumor-specific antigens, suitable as immunologic targets. Besides, the amount of potential T-cell epitopes deriving from OOF protein could be higher than that derived from junctional amino acidic sequence of Bcr/Abl classical fusion proteins. Based on these findings, vaccination might be extended to a greater number of leukemic patients, independently on their HLA genotype and BCR/ABL fusion transcript type (17, 18, 32).

Recently, frameshift sequences have been found also in other tumors. In fact, six sequence variants have been observed in a large subgroup of patients with acute myelogenous leukemia, all leading to a frame shift in the COOH terminus of the nucleophosmin protein, resulting in the replacement of the last amino acids with different residues (33). Also in inherited and spontaneous colorectal cancer, T-cell reactivity against several peptides derived from a frameshift mutation in TGF-beta1 gene was detected. These findings suggest that these newly OOF epitopes represent an attractive target for cancer vaccines (34).

Our data also show that OOF-derived nonpeptides are immunogenic and able to elicit a peptide-specific CTL response. In particular, we report a CTL epitope (OOF#2 peptide) that (a) binds to both HLA-A2 and HLA-A3 molecules, (b) stimulates a specific CTL response in vitro both in HLA-A2+ healthy donors and in a CML patient, and (c) is processed and presented at the surface of HLA-A2+ CML cells. In particular, OOF#2-specific CTLs from a CML patient were able to specifically recognize and kill not only peptide-pulsed target cells but also autologous CML cells endogenously expressing OOF. Although a larger number of patients need to be evaluated at different time points during the course of the disease, this study suggests that a T-cell response against the Bcr/Abl-OOF fusion proteins ensues in vivo and can be reactivated in vitro. Thus, one might hope that the stimulation or reactivation of such responses, following peptide-based vaccination of leukemic patients in cytogenetic remission, might be helpful to eradicating Bcr/Abl-positive cells or at least delaying disease progression.

In summary, Bcr/Abl alternative splice variants could be novel leukemia-specific antigens and their potential use in immunotherapeutic approaches is worth of being further investigated.

Acknowledgments

Received 10/11/2006; revised 3/12/2007; accepted 4/6/2007.

Grant support: Ministero Istruzione Universita e Ricerca scientifica Project FBB 2002-2005 (BBA/01HLRNB; V.M. Fazio), Ministero della Salute Strategic Project 2002-2004 (BF2002240; V.M. Fazio and G. Saglio), Ricerca sanitaria finalizzata 2006 Regione Piemonte (1347; G. Vole), and Ricerca scientifica applicata 2004 Regione Piemonte (A99; A. Cignetti).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. M. Iorio (Transfusional Medical Centre of OIRM Sant’Anna Hospital, Turin, Italy) for his support in HLA patients and donor typing.

References


Gisella Volpe, Alessandro Cignetti, Cristina Panuzzo, et al.

*Cancer Res* 2007;67:5300-5307.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/11/5300

Cited articles
This article cites 34 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/11/5300.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/11/5300.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/67/11/5300.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.