Recognition of BCR-ABL Positive Leukemic Blasts by Human CD4+ T Cells Elicited by Primary In Vitro Immunization With a BCR-ABL Breakpoint Peptide

By George J.A. ten Bosch, Antonia M. Joosten, Jan H. Kessler, Cornelis J.M. Melief, and Onno C. Leeksma

In chronic myeloid leukemia (CML) the classical 9;22 translocation results in a BCR-ABL fusion gene, which encodes a chimeric BCR-ABL fusion 210 kD oncoproteins (p210BCR-ABL). The two main p210BCR-ABL fusion variants in CML, b2a2 and b3a2, are examples of well characterized antigens expressed by malignant cells. The possibility of an immunotherapeutic approach involving the fusion part of p210BCR-ABL in CML has previously been illustrated by observed peptide binding to major histocompatibility complex (MHC) class I alleles and by demonstrating the immunogenicity of p210BCR-ABL breakpoint peptides. In this report we show that in vitro immunization of human T cells with a 17 amino acid (aa) peptide representing the p210BCR-ABL fusion region resulted in peptide specific CD4+ T-cell lines designated P4, P6, and P7. HLA DR4 (DRB1*0401) restricted T-cell line P4 and several subsequently derived clones recognized HLA-DRB1*0401 and p210BCR-ABL mRNA expressing blasts from an allogeneic patient with CML in blast crisis. Recognition appeared DR expression-dependent. No responses were observed with DR4 positive p210BCR-ABL negative cells or with p210BCR-ABL leukemic cells with absent or insufficient expression of DR4. These observations indicate that oncoprotein p210BCR-ABL can be degraded and processed for presentation by MHC class II molecules at the surface of leukemic cells. The BCR-ABL fusion region is in all likelihood presented as peptides by HLA DR and thus capable to act as a distinctive tumor antigen to peptide specific CD4+ T cells.

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This study was approved by a local ethical committee. Blood samples were collected from patients and healthy donors after informed consent only.

Preparation of BCR-ABL Specific T Cells and Clones

Blood was obtained at regular intervals from a healthy male blood group A Rh-D negative donor P with the following HLA type: A2, B7, B44; Cw5, Cw7; Dr4(DRB1*0401), DR5(DRB4*0101), DR2(DRB1*1501, DR5(DRB5*0101), DR7(DRB7*0101), DQ6, DQ8, DPB*0201, DPB*0401. Peripheral blood mononuclear cells (PBMC), 20 to 30 × 10⁶, were isolated from whole blood using Ficoll-amidotrizoate centrifugation and cultured at 37°C in 5% CO₂. Tissue culture medium (Iscove's Modified DMEM, Gibco-Life Technologies, Paisley, Scotland) was supplemented with antibiotics (100 IU/mL penicillin) and 10% to 15% autologous heat inactivated serum. Final 17mer b2a2 or b2a2 peptide concentration was 10 µg/mL (approximately 6 nmol/mL). Responding cells were restimulated with 10⁷ irradiated autologous PBMC and peptide every 10 days. From day 40 an additional 2 × 10⁷ allogeneic pooled irradiated PBMC from six random healthy donors and 10⁷ autologous irradiated PBMC were used as antigen presenting cells (APC) for restimulation. From this moment on, 10% T-cell growth factor (TCGF, Lymphocult, Biotest) was added to the tissue culture medium, after which T-cell cloning was performed by limiting dilution.

Peptides

Amino acid sequences of the p210BCR-ABL and p210BCR-ABL fusion region and of its physiological counterparts 1A-a2 and b3b4 were used to design the breakpoint representing 17 aa length peptides (b2a2-
The peptides were synthesized using solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422, Langenfeld, Germany) with the Fmoc procedure, purified by C18 reversed phase high performance liquid chromatography (HPLC), and characterized by amino acid sequence analysis and mass spectrometry. Peptides were stored at -70°C in aliquots of 1 mL in a concentration of 10 mg/mL in phosphate-buffered saline (154 mmol/L NaCl, 1.4 mmol/L NaHPO₄, pH 7.5).

**Patient Material**

Cells designated HB were from a male, 44 years old, blood group A Rh-D positive patient with CML in myeloid blast crisis. HLA type: A1, A2; B37, B40; Cw3, Cw6; DR1(DRB1*0101); DR4(DRB1*0401), DR53(DRB4*0101); DQ8. A stable high leukocyte count (20 to 35 × 10⁹/L, >90% blast cells) was observed under maintenance chemotherapy (6-mercaptopurine, hydroxyurea) and prednisone. Cytogenetics showed the 9;22 translocation, as well as translocation (12;14)(p12;q13) and deletion 15q22q24. HB cells were found b3a2 positive/b2a2 negative by reverse transcriptase-polymerase chain reaction (RT-PCR) at two separate occasions. Blast surface marker expression was analyzed by fluorescence-activated cell sorter (FACS) using monoclonal antibodies (MoAb) [anti-CD15 B4.3: Central Laboratory of the Netherlands Bloodtransfusion Service, Amsterdam, the Netherlands; anti-CD33 My9: Coulter Clone, Hialeah, FL; anti-CD34 My10: Becton Dickinson, San Jose, CA; anti-CDw65 VIM2: Behring, Marburg, Germany; anti-HLA DR: Becton Dickinson, San Jose, CA]. B-LCL HB were obtained by Epstein-Barr virus (EBV) transformation of B cells from peripheral blood.

Blasts from CML patient FM (male, 44 years), with an accelerated phase of the disease, were b2a2 positive/b3a2 negative. HLA type: A2, A3; B12, B44, B47; Cw5, Cw6; DR7, DR11, DR52, DR53; DQ2, DQ7.

Leukemic cells from b2a2 positive/b3a2 positive CML patient IL (female, 43 years) were collected during blast crisis and kindly provided by Dr H.J. Kolb, Munich, Germany, HLA type: A2; B7; DR15, DR51; DQ6, DQ7.

Cells ES were obtained from a 34-year-old man with CML in chronic phase, HLA type: A1, A2; B8, B12; Cw5, Cw7; DR4(DRB1*0404), DR17, DR52, DR53; DQ2, DQ7. Blast showed the 9;22 translocation and were b2a2 negative/b3a2 positive by RT-PCR.
PCR. Blasts were isolated by ficoll density gradient centrifugation and cultured in tissue culture medium in 75 cm² flasks (Falcon, Becton Dickinson Co, Franklin Lakes, NJ) with 10% heat inactivated autologous serum without further supplements. Cell viability was assessed by trypan blue exclusion. In case cell viability did not exceed 75%, dead cells were removed by ficoll gradient centrifugation. Enrichment for DR positive cells was achieved by means of FACs sorting (Becton Dickinson Immunocytometry Systems, Mountain View, CA) using preservative free fluorescein isothiocyanate (FITC)-labeled anti-HLA DR (Becton Dickinson, San Jose, CA). Sorted cells were cultured overnight before being used.

**Proliferation Assay**

Proliferation tests were performed using a standard ³H-thymidine incorporation assay. Briefly, 10⁰ irradiated APC, were incubated with 2.5 to 5·10⁴ responder cells (in round-bottomed microtiter-wells, Costar Co. Cambridge, MA) in 150 μL culture medium with 15% heat inactivated serum autologous to the responder cell donor. After 48 hours, 1 μCi ³H-thymidine was added for another 15 hours before harvesting (Microcell harvester; Skatron Co, Traraby, Norway) and counting (1205 Betaplate counter; Wallac Oy, Turku, Finland). All tests were repeated at least two times on separate occasions. Results are expressed as the mean plus the standard error of the mean (SEM) of counts per minute (cpm) from triplicate experiments, unless stated otherwise.

**MHC Blocking**

HLA-DR blocking experiments were performed as described using murine MoAb B8.11.2.2. The DQ restricted anti-Mycobacterium leprae CD4⁺ clone R3F10 was used to exclude aspecific blocking by anti-DR.

**RESULTS**

**BCR-ABL Breakpoint Peptide-Specific T-Cell Lines**

Primary in vitro immunization of peripheral blood lymphocytes (PBL) from a healthy donor with 17mer b3a2 peptide resulted in three CD4⁺ b3a2 peptide-specific T-cell lines (P4, P6, and P7). There was no reactivity of any of these cell lines to the physiological 17mer counterparts b3b4 and IA-a2 or to b2a2 (Fig 1). Presentation by APC was essential because no proliferation to peptide was seen in the absence of APC. Minimal peptide concentration required for inducing a response was approximately 50 ng/mL (30 nmoL L) for all T-cell lines.

**MHC Restriction of BCR-ABL b3a2 Peptide-Specific T-Cell Lines**

MHC blocking experiments indicated that the responses of P4, P6, and P7 to the b3a2 peptide were HLA-DR restricted. Table 2 shows the specificity of the response of BCR-ABL b3a2 peptide-specific T-cell line P4. The results indicate that the T-cell lines were specific for the b3a2 peptide and that the response was HLA-DR restricted.

**Table 2. Specificity of the Response of BCR-ABL b3a2 Peptide-Specific T-Cell Line P4**

<table>
<thead>
<tr>
<th>Stimulator Cells</th>
<th>CML Stage of Disease</th>
<th>b3α2/b2α2 mRNA</th>
<th>HLA DR</th>
<th>DR Positive Population (%)</th>
<th>Background cpm ± SEM</th>
<th>P4 Response cpm ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>F T CML HB</td>
<td>BP</td>
<td>+/-</td>
<td>1, 41, 53</td>
<td>76</td>
<td>6,726 ± 573</td>
<td>294 ± 220</td>
</tr>
<tr>
<td>F B LCL HB</td>
<td>-</td>
<td>-/-</td>
<td>1, 41, 53</td>
<td>92</td>
<td>833 ± 73</td>
<td>330 ± 20</td>
</tr>
<tr>
<td>F CML IL</td>
<td>BP</td>
<td>+/-</td>
<td>15, 51</td>
<td>NT</td>
<td>677 ± 58</td>
<td>266 ± 134</td>
</tr>
<tr>
<td>T T CML ES</td>
<td>CP</td>
<td>+/-</td>
<td>45, 17, 52, 53</td>
<td>19j</td>
<td>601 ± 113</td>
<td>144 ± 23</td>
</tr>
<tr>
<td>F CML FM</td>
<td>AP</td>
<td>-/+</td>
<td>7, 11, 52, 53</td>
<td>7</td>
<td>826 ± 201</td>
<td>440 ± 87</td>
</tr>
</tbody>
</table>

Abbreviations: BP, blast phase; CP, chronic phase; AP, accelerated phase.

* ³H thymidine incorporated by stimulator cells in the absence of responder cells.
† The prefix F or T denotes the status when used: fresh or subsequently thawed.
‡ DRB1*0401.
§ DRB1*0404.
∥ Six % of the cells showed a DR fluorescence comparable to HB cells. DR fluorescence intensity of the other 13% was low.
BCR-ABL* BLAST RECOGNITION BY HUMAN T CELLS

Fig 3. Effect of anti HLA-DR antibodies B8.11.2 on the response of T-cell line P4 to CML blasts HB. Final concentration anti-DR IgG in the test approximately 12.5 μg/mL (1/200 dilution). The background 3H thymidine incorporation of HB blasts (without T cells) was 675 ± 53 cpm.

Fig 2. Recognition of CML Blasts by T Cells P4

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>DR Positive Population (%)</th>
<th>P4 Reactivity</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB-blasts 21 days in culture</td>
<td>50</td>
<td>3,613 ± 71</td>
<td>249 ± 75</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5,295 ± 427</td>
<td>165 ± 19</td>
</tr>
<tr>
<td>HB-blasts 5 days in culture</td>
<td>91</td>
<td>8,610 ± 100</td>
<td>280 ± 55</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>12,304 ± 1,025</td>
<td>175 ± 78</td>
</tr>
<tr>
<td>Control APC</td>
<td>NT</td>
<td>608 ± 2</td>
<td>568 ± 399</td>
</tr>
<tr>
<td>Control APC plus b3a2 peptide (5 μg/mL)</td>
<td></td>
<td>10,980 ± 612</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The fusion regions in BCR-ABL oncoproteins have been acknowledged as potential tumor-specific targets for an immunotherapeutic approach of associated malignancies.27 HLA class I and class II molecules capable of presenting BCR-ABL breakpoint peptides have recently been identified.20,28 Intermediate to high-affinity MHC binding of BCR-ABL peptides has been observed.26 However, evidence for intracellular processing of endogenous BCR-ABL proteins leading to the presentation of MHC-bound native BCR-ABL peptides at the cell surface is lacking thus far. The present study suggests that such processing of p210BCR-ABL proteins may, in fact, occur by showing recognition of BCR-ABLb3a2 expressing CML blasts by a BCR-ABLb3a2 breakpoint representing peptide-specific CD4+ T-cell line and several of its clones in an HLA DR4-restricted fashion. This phenomenon cannot be explained as recognition of exogenous blast cell-derived BCR-ABL peptide presented by APC (from patient or T-cell donor origin). FACS sorted CML blasts from patient HB, 96% double positive for DR and progenitor marker CD34, were effective in stimulating P4 cells to proliferate making a role of patient-derived residual CD34 negative professional APC unlikely. Furthermore, incubation of the T-cell line P4 with BCR-ABL peptide in the absence of any added APC did not result in proliferative activity above the background level (data not shown).

Although we cannot fully exclude the possibility that this apparent specific T-cell response is based on cross reactivity with a DR bound peptide unrelated to p210BCR-ABL peptide presented by these particular allogeneic CML blasts, several indications argue against such an alternative explanation. First, p210BCR-ABL negative B-LCL from the same patient were not recognized, making cross reactivity with, for instance, an HLA-derived peptide unlikely. Second, several other allogeneic CML cells without expression of either the appropriate HLA molecules and/or BCR-ABLb3a2 mRNA were unable to stimulate T cells to proliferate. Nonspecific, eg, leukemic blast-derived, cytokine-mediated T-cell proliferation is virtually excluded by the fact that two other BCR-
ABL peptide-specific non-DR4 restricted CD4+ T-cell lines did not respond to the same CML blasts. Such a nonspecific stimulatory effect is also difficult to reconcile with the HLA-DR dependency of the T-cell responses. One would have to assume a unique sensitivity of these DR-restricted and peptide-specific T cells P4 for an immature myeloid differentiation stage-specific cytokine produced by certain CML blasts only. The production of which is furthermore inhibited by anti HLA-DR antibodies. Experiments are underway in our laboratory to solve this issue by searching for the BCR-ABL L\(\beta\) junction region aa sequence among the many different peptides eluted from immunopurified HLA-DR molecules of CML blasts of patient HB.

Intriguing recent findings of very low numbers of BCR-ABL transcripts in some healthy individuals\(^3\) are in order to establish if the presence or absence of BCR-ABL mRNA in healthy individuals is correlated with their ability to mount T-cell responses to BCR-ABL peptides. The present observations, albeit confined to a single donor-patient combination, provide a first argument for the feasibility of peptide-specific adoptive immunotherapy or possibly BCR-ABL peptide vaccination in CML.

NOTE ADDED IN PROOF
During the review process of this manuscript Bocchia et al\(^3\) reported in this journal that cytolytic HLA class I-restricted human T cells directed against BCR-ABL fusion sequence encoded synthetic peptides could be established. However, recognition of native BCR-ABL peptides was not yet demonstrated.

REFERENCES

Table 4. Effect of Phenotypic Changes of CML Blasts HB During Culture on Recognition by T-Cell Line P4

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Days in Culture</th>
<th>CD15 (%)</th>
<th>CD33 (%)</th>
<th>CD34 (%)</th>
<th>CDw65 (%)</th>
<th>HLA-DR (%)</th>
<th>P4 Reactivity (% of response to b(\beta)a2 peptide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>15</td>
<td>76</td>
<td>70</td>
<td>72</td>
<td>71</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>15</td>
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<td>77</td>
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</tr>
<tr>
<td>4</td>
<td>21</td>
<td>80</td>
<td>98</td>
<td>1</td>
<td>91</td>
<td>19</td>
<td>8</td>
</tr>
</tbody>
</table>

Four fresh HB blast samples obtained at different occasions were cultured, tested, and phenotyped for myeloid and progenitor cell markers. The response of P4 is given as the percentage of the maximal response to autologous APC with 10 μg/mL (6 nmol/mL) 17mer b\(\beta\)a2 peptide.


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