[haematologica]
2004;89:1062-1071

Proteosomal degradation of BCR/ABL protein can generate an HLA-A*0301-restricted peptide, but high-avidity T cells recognizing this leukemia-specific antigen were not demonstrated

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A B S T R A C T

Background and Objectives. Cytotoxic T-lymphocytes (CTL) have been generated *in vitro* against chronic myeloid leukemia (CML)-associated BCR/ABL-specific peptides. We analyzed the existence of high-avidity T cells recognizing endogenously processed BCR/ABL-specific proteins.

Design and Methods. We performed binding studies of BCR/ABL-specific peptides, proteosomal digestion of BCR/ABL breakpoint overlapping protein, mass spectrometry of eluates from HLA-*0301-transduced K562 cells, and tried to isolate peptide-specific T-cells using tetramers.

Results. We confirmed the binding of the BCR/ABL-specific peptides KQSSKALQR to HLA-A*0301 and GFKQSSKAL to HLA-B*0801. Proteasomal digestion showed cleavage sites leading to KQSSKALQR but not to GFKQSSKAL. Using mass spectrometry KQSSKALQR could not be detected in the eluates from HLA-A*0301-transduced K562 cells. We attempted to induce BCR/ABL-specific CTL lines from 4 healthy donors using dendritic cells pulsed with KQSSKALQR and performed single cell sorting to isolate tetramer-positive T cells. None of 31 generated clones showed BCR/ABL-specific cytotoxicity. Isolation of tetramer-positive cells from peripheral blood of relapsed CML patients after allogeneic transplantation treated with donor lymphocyte infusion resulted in 38 T-cell clones which did not show peptide-specific cytotoxicity.

Interpretation and Conclusions. We provide evidence that BCR/ABL protein processing can lead to KQSSKALQR peptide binding to HLA-A*0301. However, KQSSKALQR could not be detected in HLA-A*0301-transduced K562 cells, and KQSSKALQR could not be demonstrated to induce high-avidity BCR/ABL-specific CTL.

Key words: BCR/ABL, HLA-A*0301, T-cells, immunotherapy.

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Chronic myeloid leukemia (CML) is a malignant disease caused by dysregulation of a pluripotent hematopoietic precursor cell. CML is characterized by the Philadelphia chromosome, containing the t(9;22) translocation, resulting in the chimeric *BCR/ABL* oncogene.[1,2] This translocation has been found to be essential and sufficient for the development of CML.[3-6] Depending on the exons of the *BCR* and *ABL* genes involved in the translocation, several variants of the fusion gene can be composed of which *b2a2* and *b3a2* are the most frequent.[5,6] These fusion genes encode for a neo-protein, the BCR/ABL protein, which is unique to CML cells.

Intracellular proteins are degraded by the proteasome into small peptides of 8 to 11 amino acids, which are subsequently bound to HLA class I in the endoplasmatic reticulum and transported to the cell membrane for presentation to T cells. Since CML-specific proteins contain amino acid sequences that are novel to the immune system, they might induce tumor-specific cytotoxic T-cell responses when presented by HLA molecules on leukemic cells. A prerequisite for such a response is proteasomal degradation of intracellular BCR/ABL protein resulting in presentation of BCR/ABL-specific oligopeptides by HLA class I or HLA class II molecules on the membrane of leukemic cells and the presence of T cells with a T-cell receptor (TCR) that can recognize these peptide-HLA complexes. Until now there is no definite proof of the existence of such CML-specific responses *in vivo*.

Arguments in favor of the existence of CML-specific T-cell responses are the ability to generate immune responses *in vitro* against BCR/ABL-derived synthetic peptides presented by HLA class I or HLA class II mol-

ecules.[7-17] Also, in a recent study evidence was provided of the presence of the breakpoint-specific peptide KQSSKALQR in HLA class I molecules of HLA A*0301 positive CML cells and K562 cells expressing high levels of BCR/ABL[18,19] transfected with HLA-A*0301, suggesting that proteosomal cleavage of the BCR/ABL protein may result in presentation of this peptide by these HLA class I molecules.[20] In the same study, KQSSKALQR-specific cytotoxic T lymphocyte (CTL) lines were induced from peripheral blood of HLA-A*0301 *b3a2* positive patients with CML using the synthetic peptide and HLA-A*0301/KQSSKALQR tetramer-positive peripheral blood T cells could be detected in the peripheral blood mononuclear cells of the same patients. In addition, we have previously reported a diminished incidence of CML in individuals positive for HLA-A3 or HLA-B8, HLA class I molecules capable of binding BCR/ABL-derived peptides, and also in HLA-DR4 positive individuals.[21,22] These findings may suggest an immune response against BCR/ABL breakpoint peptides *in vivo*, although no formal proof of this hypothesis has been found.

Arguments against the existence of BCR/ABL-specific immune response *in vivo* are that until now no BCR/ABL-specific CTL clones with high-affinity TCR have been isolated and that there is no proof of recognition of endogenously processed antigen. Vaccination of CML patients with breakpoint-specific peptides generated peptide-specific immune responses in a clinical trial, but no cytotoxic BCR/ABL-specific T-cell responses were induced.[23]

Thus, despite evidence suggesting a possible BCR/ABL-specific CTL response there is no formal proof of the existence of BCR/ABL-specific CTL clones recognizing endogenously processed P210. In this study, we attempted to confirm evidence of processing of BCR/ABL protein into BCR/ABL-specific peptides presented by HLA-A*0301 or HLA-B*0801 molecules and tried to induce and/or isolate high-avidity CTL clones specific for BCR/ABL. We show that the BCR/ABL protein was cleaved at several sites in the breakpoint region resulting in the formation of the KQSSKALQR peptide which could bind to HLA-A*0301 on the cell membrane. We performed mass spectrometry of peptide fractions eluted from HLA molecules of cells of the CML-derived K562 cell line retrovirally transduced with HLA-A*0301 but could not detect the BCR/ABL-specific peptide. We explored the immunogenicity of the HLA*A0301 restricted peptide KQSSKALQR using dendritic cells from HLA-A*0301 positive healthy individuals pulsed with this peptide to induce T-cell responses *in vitro*, but failed to induce BCR/ABL-specific CTL responses. In further attempts to isolate and analyze HLA-A*0301/KQSSKALQR positive T-cells, we sorted and cloned HLA-A*0301/KQSSKALQR tetramer positive T-cells from relapsed CML patients responding to donor lymphocyte infusion (DLI), but again failed to show peptide-specific cytotoxicity.

Table 1. Sequencies of peptides used in this study.

Name	*HLA restriction*	*Sequence*
B3A2		VIVHSATGFKQSSKALQRPVASDFEPQ
B3A2	HLA-A3	KQSSKALQR
B3A2	HLA-B8	GFKQSSKAL
HY	HLA-B8	LPHNHTDL
EBV BZLF-1	HLA-B8	RAKFKQLL
EBV EBNA	HLA-B8	FLRGRAYGL
CMV	HLA-B8	DANDIYRIF
HY	HLA-B7	SPSVDKARAEL
FLU	HLA-A3	ILRGSVAHK
MAGE 1	HLA-A3	SLFRAVLITK
CMV pp65	HLA-A3	VLCPKNMIIK
HA-1	HLA-A2	VLHDDLLEA
HA-2	HLA-A2	YIGEVLVSV
HY	HLA-A2	FIDSYICQV
CMV	HLA-A2	NLVPMVATV
A3 consensus	HLA-A3	KVFPCALINK

Design and Methods

Peptides

Peptides were synthesized with an automated multiple peptide synthesizer (Syro II, MultiSynthec, Witten, Germany) using Fmoc chemistry, as described before.[24,25] Fluorescently-labeled reference peptides were synthesized by labeling the corresponding Cys-peptides with 5-(iodoacetamido)fluorescein. Quality was assessed by high-performance liquid chromatography (HPLC) analysis and matrix-assisted laser desorption mass spectrometry. Peptides were dissolved in 50% dimethyl sulfoxide before use in refolding HLA molecules for tetramer production. The sequences of peptides used in this study are shown in Table 1.

HLA/peptide tetramers

Tetramers were synthesized as described before[26,27] with minor modifications. Briefly, recombinant β2-microglobulin and the extracellular portion of MHC class I heavy chain containing the BirA recognition sequence in frame at its C terminus were expressed in *Escheria coli* as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were dissolved in urea, and monomeric HLA class I complexes were refolded around the peptide by dilution in denaturing conditions. After buffer exchange, a specific lysine residue in the heavy chain C-terminal tag was

biotinylated with BirA enzyme. Monomeric complexes were purified by gel filtration and anion exchange chromatography. Tetrameric arrays of biotinylated peptide-MHC class I complexes were formed by the addition of PE- or FITC-labeled avidin. The biochemical quality of tetramer refolding was checked with HLA-specific monoclonal antibodies. The following tetramers were used: the BCR-ABL specific HLA-A3/KQSSKALQR-PE tetramer, the hematopoiesis-restricted mHAg HA-1 HLA-A2/VLHDDLLEA-PE tetramer and HA-2 HLA-A2/YIGEVLVSV-PE,[28] the male-specific HY peptide HLA-A2/FIDSYICQV-PE,[29] the HY/HLA-B7 tetramer[30] and MAGE1 HLA-A3/SLFRAVITK-PE. In addition HLA-A3/KQSSKALQR tetramers kindly provided by Dr. A. Dodi (London) were used.

Binding assays

Peptides were tested for binding to HLA class I alleles using a competition assay as described previously.[31] Briefly, 2 pmol of recombinant denaturated HLA heavy chain was refolded with 15 pmol β2-microglobulin (Sigma), 100 fmol fluorescent standard peptides and a titration of the sample peptide. After 16 hrs of incubation the sample was analyzed on an HPLC column (Synchropak GPC 100; Synchrom, Lafayette, Indiana, USA), and the percentage of bound fluorescent standard peptide was determined. The fluorescent standard peptide used for HLA-A3 (A*0301) binding assays was KVFPC(FI)ALINK, a modified consensus peptide, and for HLA-B8 (B*0801) binding assays FLRGRAC(FI)GL, a modified EBNA-3A 339-347 epitope was used. The binding affinity of the competitor test peptide was expressed as the concentration needed to inhibit 50% binding of the fluorescently-labeled reference peptide (IC_{50}). High affinity binding was defined by an IC_{50} below 100 nM, intermediate affinity binding an IC_{50} between 100 and 1000 nM and low affinity binding an IC_{50} above 1000 nM.

Proteasome-mediated digestion analysis

The b3a2 27-mer peptide VIVHSATGFKQSSKALQRPVASDFEPQ was purified by reversed phase HPLC in an acetonitrile-water gradient and lyophilized. Before use, the peptide was dissolved in proteasome digestion buffer (20 mM hepes, 2mM $MgAc_2$, pH 7.8). 20S proteasomes were isolated from HELA cells (household proteasome) and from an EBV-transformed cell line (immunoproteasome). Three nmol of peptide were digested by 1 μg of proteasome in 300 μL of a proteasome digestion buffer at 37°C. Aliquots (20 μL) were taken after 0, 0.5, 1, 2, 4 and 24 hrs of incubation. The reaction was stopped by addition of 2 μL of 10% trifluoroacetic acid. Aliquots were stored at -20°C until analysis. Digests were diluted in 5% acetonitrile 1% acetic and were analyzed by electrospray ionization mass spectrometry.[32,33] The peaks in the mass spectra were searched for in the precursor peptide sequence using Biolynx software (Micromass, Manchester, U.K.). The intensity of peaks was used to establish the relative amounts of peptides generated after digestion. When the measured amount of fragments at a cleavage site was more than 5% of the total amount of fragments this site was defined a major cleavage site, a cleavage site giving 1-5% of fragments was defined as intermediate and a cleavage site producing < 1% fragments was definied as a minor cleavage site.

The cleavage sites found were compared to the cleavage sites of the same peptide predicted using a computer model available on the Internet (PAProC, prediction algorithm for proteasome cleavages; *http://www.uni-tuebingen.de/uni/kxi*).[34,35]

HLA A*0301 peptide elution and mass spectrometry detection

K562 cells (ATCC CCL 243) were transduced with a retroviral construct encoding HLA-A*0301 allele using the pLZRS system,[36] kindly provided by MHM Heemskerk. Transduced cells were then cloned and cells with the highest expression of HLA A*0301 using flow-activated cells sorter (FACS) analysis were isolated, and expanded. At regular intervals during this expansion the continued expression of HLA A*0301 was assessed with FACS analysis. HLA A*0301 molecules were purified from 30×10^9 transduced K562 cells using methods already described.[37] In short, after lysing cells in NP40 buffer, HLA complexes were isolated with a pan-HLA class I Moab W6/32 bound on CNBr Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted with 10% acetic acid. Peptides were filtered through a 5-kDa cut-off filter, lyophilized and dissolved in 400 μL 10% acetic acid. As an internal control 1 pmol synthetic A*0201 restricted WT1 peptide (RMFPNAPYL) was added. The mixture was loaded on a MiniS PC 3.2/3 cation Exchange Column (Amersham Pharmacia Biotech) using the SMART System (Amersham Pharmacia Biotech), and washed with 0.1 M HCl, and then elution with 0.1 M NaOH was performed. The peptide-containing fractions were acidified with trifluoroacetic acid (TFA), and separated on a μRPC C2/C18 SC 2.1/10 Column (Amersham Pharmacia Biotech). After washing with 10 mL of water with 0.1% TFA the peptides were fractionated using a 0 to 40% gradient of acetonitrile with 0.1% TFA over 60 minutes at a flow rate of 100 μL/min and a fraction size of 100 μL. The same procedure was repeated after addition of 1 pmol of the synthetic A*0301 BCR-ABL b3a2 peptide (KQSSKALQR).

The HPLC fractions were analyzed using electrospray ionization mass spectrometry. This was performed on a hybrid quadrupole time-of-flight mass spectrometer

(Q-TOF1, Micromass, Manchester, UK) as described earlier[37] with modifications: the analytical HPLC pre-column was packed with Hypercarb (20 mm×150 μm ID, 7 μm particle size; Thermo, Breda, The Netherlands). Loading was done in water with 1% acetic acid; trapped peptides were eluted with a steep gradient from 100% of water with 1% acetic acid to 90% acetonitrile and 1% acetic acid in 5 minutes. Mass spectra were recorded from 50-2000 Da every second. In MS/MS mode, ions were selected with a window of 2Da with the first quadrapole. The collision gas applied was argon (4×10^{5} mbar), and the collision voltage was 30 V.

In vitro generation of primary immune response and isolation of T-cell clones

In order to generate peptide-specific immune responses, dendritic cells (DC) were used as stimulator cells. First, immature DC were generated from peripheral blood monocytes or CD34 positive mobilized blood stem cells by stimulating monocytes using granulocyte-monocyte colony-stimulating factor (GM-CSF) (100 ng/mL) and interleukin-4 (IL-4) (500 IU ng/mL) or stimulating CD34 cells using GM-CSF (100 ng/mL), stem cell factor (SCF) (20 ng/mL) and tumor necrosis factor (TNF)-α (2 ng/mL) from day 1 and IL-4 (500 IU/mL) on day 4 and were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 3 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% human serum. Two days before harvesting DC were matured with irradiated (70 Gy) mouse fibroblasts expressing human CD40 ligand or DKTP-vaccine 1:2000 (RIVM, Bilthoven, The Netherlands). These mature DC expressed high levels of HLA-DR, CD80, CD86, CD11c and CD40.

Mature DC were loaded with KQSSKALQR peptide (1 μg peptide/5-10×10^{6} dendritic cells) during two hours of incubation at 37°C. Autologous peripheral blood mononuclear cells (PBMC) were used as responder cells and stimulated with DC at a PBMC:DC ratio of 5:1. Cell mixtures were plated in 24-well plates (Corning Incorporated, Corning, NY, USA) at a concentration of 2.4×10^{6} cell/mL with the addition of IL-2 (concentration 6 IU/mL on day 0 and 60 IU/mL on day 4/5). $CD4^{+}$ cells were depleted prior to the first restimulation. Weekly restimulation was performed with irradiated (15 Gy), peptide-loaded DC at a PBSC:DC ratio of 5:1. The day after restimulation, 120 IU/mL IL-2 were added. Lines were analyzed weekly, by flow cytometry to determine the percentage of $CD3^{+}CD8^{+}$, $CD3^{+}$ $CD4^{+}$ and NK cells, for growth rate and ^{51}Cr-release cytotoxicity assays.

To isolate HLA-A*0301/KQSSKALQR tetramer positive T-cell clones, cells from the cell lines generated with KQSSKALQR-pulsed dendritic cells were stained at 0°C for 30' with the tetramer. CD4-FITC negative, TCRγδ-FITC negative, CD40-FITC negative, KQSSKALQR/HLA-A3-PE positive cells were sorted as single cells per well using a FACSvantage (Beckton Dickinson). Tetramer-positive T-cells were cultured, and stimulated non-specifically on a layer of 50 Gy irradiated allogeneic feeder cells with 0.8 μg/mL PHA and IL-2 (60 IU/mL) in IMDM containing 10% human serum. The phenotypes of the clones were analyzed using FITC- or PE-conjugated monoclonal antibodies against CD3, CD4, CD8, CD56, αβTCR and γδTCR antigens (Beckton Dickinson) and several PE-labeled HLA/peptide tetramers. Cells were stained with these antibodies and tetramers both at 4°C and at 37°C.

*Isolation of HLA-A*0301/KQSSKALQR tetramer positive T cells from patients with relapsed CML treated with DLI after allogeneic stem cell transplantation*

PBMC from HLA-A*0301 of patients with CML characterized by the b3a2 translocation, successfully treated with DLI for a relapse after allogeneic stem cell transplantation, were stained at 0°C for 30' with the tetramer and monoclonal antibodies. CD4-FITC negative, TCRγδ-FITC negative, CD40-FITC negative, KQSSKALQR/HLA-A3-PE positive cells were sorted as single cells per well using a FACSvantage (Beckton and Dickinson). Tetramer-positive T cells were cultured and stimulated non-specifically on a layer of 50 Gy irradiated allogeneic feeder cells with 0.8 μg/mL PHA and IL-2 at a concentration of 60 IU/mL in IMDM with 3 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% human serum.

Cytotoxicity assay

Lytic activity of cell lines and clones was assessed using standard ^{51}Cr-release assays.[38] Briefly, target cells were labeled simultaneously with ^{51}Cr 100 μCi and peptide (1 μg) for 1 hr. Target cells were washed and 5000 cells were aliquoted into wells containing various ratios of T cells. The supernatant was harvested after 4 hr of incubation. The percentage of lysis was determined as follows: (experimental release cpm-spontaneous release cpm)/(maximum cpm-spontaneous cpm) × 100%.

Tetramer analysis

The binding between HLA-A3/KQSSKALQR-PE tetramers and T cells was analyzed by blocking studies using the monoclonal antibodies against CD3 (SK7), CD8 (FK18) and the TCRαβ (WT31-FITC, Beckton and Dickinson; BMA03-PECy5, Immunotech) in FACS analyses and compared to the binding of the HA-1 clone to the HLA-A2/VLHDDLLEA-PE tetramer.[28]

Blocking studies were performed with different concentrations of anti-CD3 and anti-CD8 antibodies and at 2 different temperature conditions (0°C and 37°C). Cells were preincubated for 30' with these antibodies prior to staining with the tetramer.

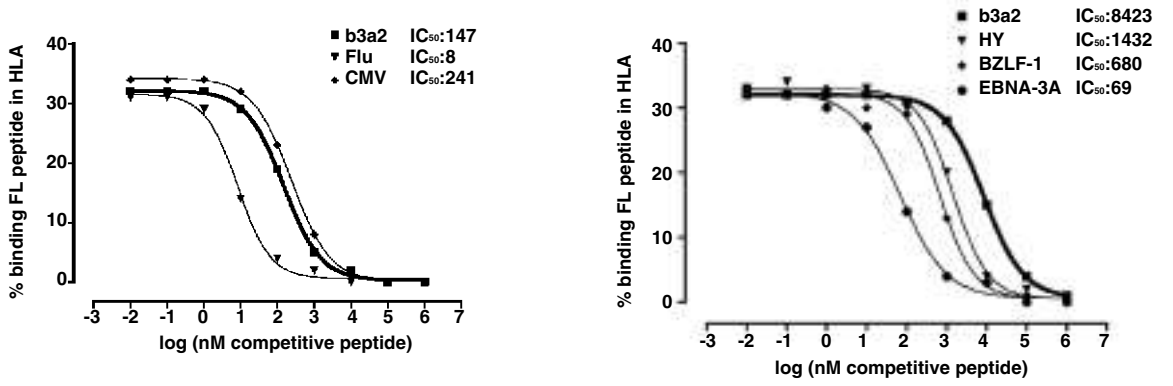

Figure 1. Binding affinity of b3a2-derived peptides, KQSSKALQR to HLA-A3 and GFKQSSKAL to HLA-B8 as assessed with a competition binding assay. Comparisons were made with other known binding proteins for HLA-A3 (FLU A3, CMV A3) and for HLA-B8 (HY B8, BZLF-1 B8, EBNA-3A B8) (Table 1). The binding affinity of the competitor peptide is expressed as the concentration (nM on the X-axis) needed to inhibit 50% binding of the fluorescence(FL)-labeled reference peptide (IC_{50}) and is given in the figure.

Results

Binding of peptides from the BCR/ABL junctional region to HLA-A3 and HLA-B8 molecules

To confirm previous studies showing binding of the junctional peptides KQSSKALQR and GFKQSSKAL to HLA-A3 and HLA-B8,[7,8] respectively, we used a recently developed competition-based HLA class I binding assay.[31] KQSSKALQR showed intermediate to high affinity binding to HLA-A*0301 molecules with an IC_{50} of 147 nM. The binding was in the range of those of reference peptides. GFKQSSKAL displayed a low affinity binding to HLA-B*0801 with an IC_{50} of 8423 nM, lower than those of the reference peptides (Figure 1).

In vitro proteasome-mediated digestions of a 27-mer BCR/ABL junctional peptide

Since a prerequisite for recognition of endogenously processed BCR/ABL junctional peptide by HLA-A*0301 or HLA-B*0801-restricted T-cell responses is cleavage by the proteasome of the BCR/ABL protein into HLA-A*0301 and HLA-B*0801 binding epitopes, we performed *in vitro* proteasome-mediated digestions of a peptide 27 amino acids long (VIVHSATGFKQSSKALQRPVASDFEPQ) spanning the breakpoint region of the b3a2 subtype of BCR/ABL. Both household- and immuno-proteasomes were used for digestions. Products of the digestions were assessed by mass spectrometry at different time intervals. As illustrated in Figure 2, several major, intermediate and minor cleavage sites were characterized. For comparison, the same figure shows the cleavage sites predicted by the

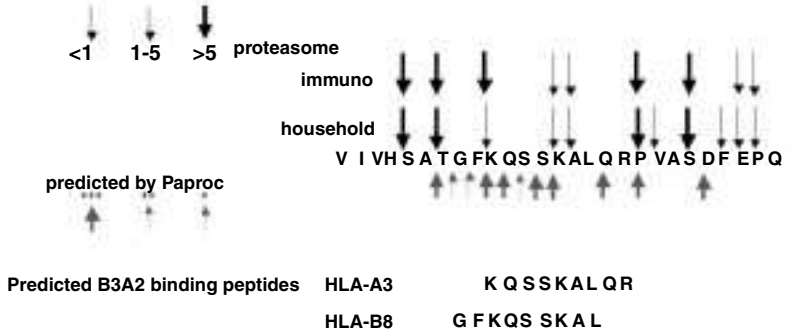

Figure 2. Cleavage sites of a 27-mer peptide spanning the b3a2 BCR/ABL breakpoint region assessed by the percentage of fragments of total summed mass-peak intensities of 27-mer digestion by both the household- and immunoproteasome *in vitro*. Major, intermediate and minor cleavage sites at 4h are defined when the measured mass of fragments at a cleavage site is respectively more than 5%, between 1% and 5% and below 1% of the total mass of fragments. These cleavage sites are depicted as respectively bold, normal and thin black arrows. Cleavage sites predicted by PAProC (*http://www.uni-tuebingen.de/uni/kxi*) are depicted by gray arrows. Predicted BCR-ABL binding peptides to HLA-A3 and HLA-B8 are given at the bottom of the figure.

PAProC algorithm. A major cleavage site was found at the C- and N termini of the HLA-A*0301 binding peptide KQSSKALQR, in line with PAProC predicted cleavage sites, but not of the flanking residues of the HLA-B*0801 binding peptide GFKQSSKAL. Despite the predicted cleavage sites between L and Q, and between T and G cleavage could not be confirmed by mass spectrometry analysis of the fragments.

*HLA-A*0301 peptide elution and mass spectrometry detection*

With a detection limit of 10 fmol, as determined with synthetic KQSSKALQR using the hypercarb setup, no BCR/ABL derived KQSSKALQR was found in one of the eluted fractions from HLA of 30×10^9 HLA-A*0301-transduced K562 cells. Since the amount of the WT1 peptide detected after the HPLC procedure was more than 80% of the amount of input peptide at the start, loss of KQSSKALQR peptide during the procedure was considered unlikely.

*In vitro induction of primary immune response and isolation of T-cell clones directed against the HLA-A*0301 binding peptide KQSSKALQR*

Since we showed that processing of the BCR/ABL protein could lead to the generation of the KQSSKALQR peptide but not to GFKQSSKAL, and since the KQSSKALQR peptide was found to be a good binding epitope to HLA-A*0301, whereas GFKQSSKAL showed only low affinity to HLA-B*0801, only the KQSSKALQR peptide was chosen to induce and/or to isolate a KQSSKALQR-specific T-cell immune response in the context of HLA-A*0301. First, ten cell lines were generated by stimulation of PBMC by KQSSKALQR pulsed DC from four different HLA-A*0301 positive healthy donors. Eight cell lines showed a low percentage of CD8 T-cells positive for HLA-A3/KQSSKALQR-PE tetramers in flow cytrometric analysis. The percentages of tetramer positivity varied from 0.0002% to 0.0033%. Two cell lines did not stain with these tetramers. The two batches of tetramers gave similar results. None of the cell lines showed peptide-specific cytotoxicity in ^{51}Cr-release assays (*data not shown*).

Since the frequencies of specific T-cells measured by tetramer analysis was too low to allow functional characterization of these T-cells, we attempted to obtain peptide-specific clones by sorting the 8 cell lines containing cells staining with HLA-A*0301/KQSSKALQR-PE tetramers using single cell/single well sorting in 11 experiments (3 lines were sorted twice). In total 729 tetramer positive T-cells, with a fluorescence intensity of at least 250 when stained with the HLA-A*0301/KQSSKALQR-PE tetramer, were isolated. After non-specific expansion 31 clones were generated of which 23 clones were evaluable by flowcytometric analysis, and 16 clones could be analyzed in cytotoxicity assays. In control experiments, some HLA-A*0301/KQSSKALQR-PE positive clones also stained positive with other tetramers. A representative example of staining is shown in Figure 3. None of the 16 evaluable clones showed KQSSKALQR-specific cytotoxicity (*data not shown*).

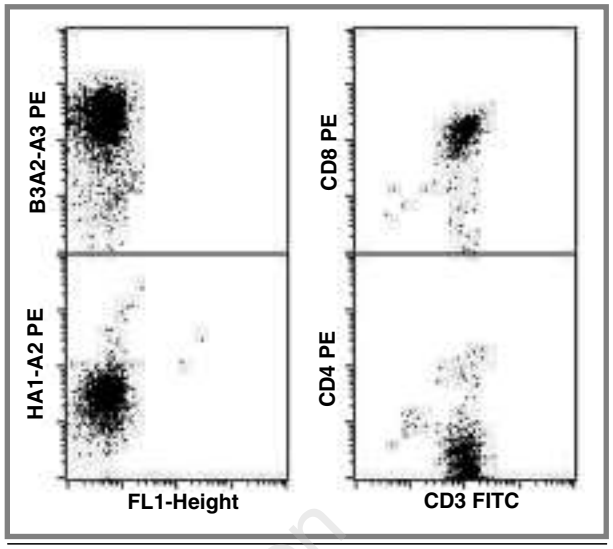

Figure 3. FACS analysis of a T-cell clone isolated by sorting single cell/single well of a cell line, which was generated by stimulation of PBMC with KQSSKALQR pulsed dendritic cells of a HLA-A3 positive healthy donor, using PE-labeled HLA-A3/KQSSKALQR(B3A2) tetramers. The clone stained strongly positive with HLA-A3/KQSS KALQR-PE tetramers but also weakly positive with HLA-2/HA-1-PE tetramer, irrelevant for this clone.

*Isolation and functional studies of HLA-A*0301/KQSSKALQR tetramer-positive T cells from relapsed CML patients treated with DLI after allogeneic SCT*

Since we were unable to induce or isolate a primary CTL response against KQSSKALQR *in vitro* using PBMC from healthy donors we attempted using tetramers to directly isolate KQSSAKALQR-specific T cells present *in vivo* from two HLA-A*0301 positive patients with the b3a2 CML subtype, successfully treated with DLI for a relapse after allogeneic SCT. As described recently, this approach has been shown to be successful in isolating HA-1- and HA-2-specific T-cell clones in the same category of patients.[28]

HLA-A*0301/KQSSKALQR-PE tetramer-positive CD8 T cells were detectable at low frequencies in PBMC from these two patients (Figure 4). Two isolations from PBMC of one of the patients, and 3 isolations from PBMC of the other patient were performed. The median frequency of tetramer-positive cells in these 5 isolations was

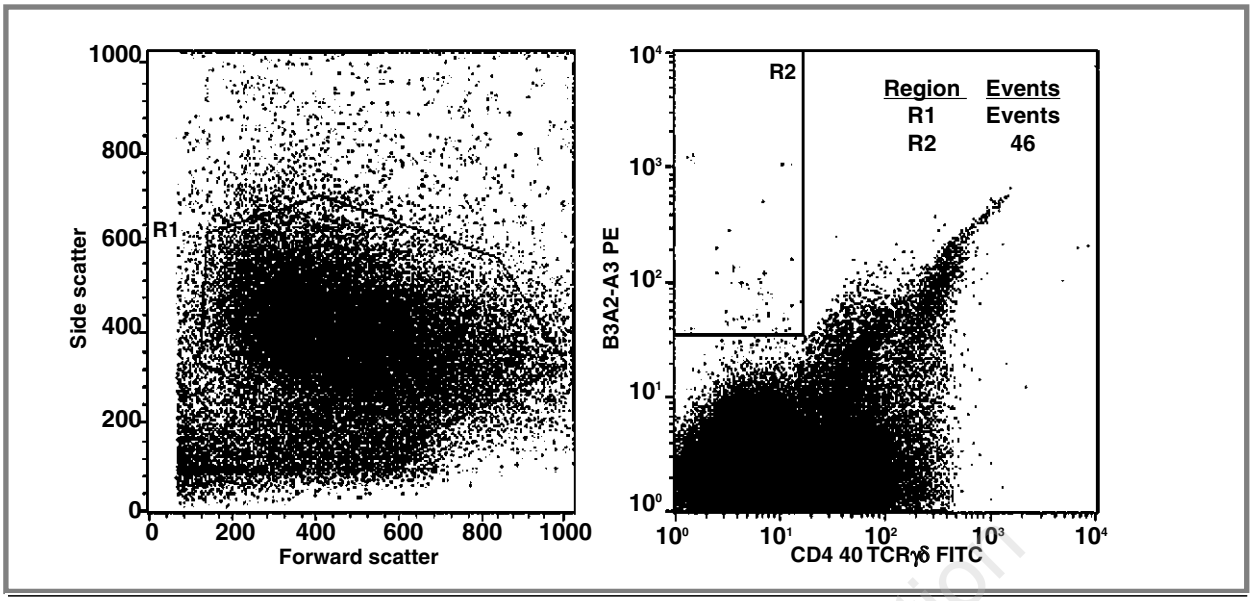

Figure 4. Isolation of HLA-A3/KQSSKALQR tetramer-positive CD8 T cells of PBMC from a patient treated with DLI for a relapse CML after allogeneic SCT. PBMC were isolated based on staining with PE-labeled HLA-A3/KQSSKALQR tetramers (Y-axis) and negative staining with anti-CD4, -TCRγδ and -CD40 FITC-labeled monoclonal antibodies (X-axis).

0.013% (range 0.005-0.022%). A total of 92 HLA-A*0301/KQSSKALQR-PE tetramer-positive T cells could be isolated. After repeated restimulation 38 clones were established of which 30 were evaluable for flowcytometric analysis. From 18 T-cell clones sufficient numbers of cells could be generated to allow cytoxicity assays. The clones stained not only positive with HLA-A3/KQSSKALQR-PE tetramers but sometimes also with other tetramers. However, none of the clones showed KQSSKALQR-specific cytotoxicity in ^{51}Cr-release assays.

*Analysis of the nature of HLA- A*0301/KQSSKALQR tetramer positivity of T cells*

Although T-cell clones positively staining with the HLA-A3/KQSSKALQR-PE tetramer could be islolated, these T cells did not show peptide-specific cytotoxicity. We therefore investigated the specificity of the binding of the HLA-A*0301/KQSSKALQR-PE tetramers to the TCR-complex, using blocking studies with anti-CD3 and anti-CD8 monoclonal antibodies. Since different temperatures have been reported to influence binding between T cells and tetramers, these studies were performed at 0°C and 37°C.[39] As a control experiment, the HA-1 clone was tested under similar conditions. As illustrated in Figure 5 preincubation of the HA-1 clone with anti-CD3 antibodies prior to HLA-A2/VLHDDLLEA-PE tetramer staining resulted in complete inhibition of tetramer binding, whereas tetramer staining of the HLA-A*0301/KQSSKALQR-PE positive T-cell clone RL81 could only be partially blocked by anti-CD3, especially at 0°C. In contrast, preincubation of RL81 with anti-CD8 antibodies prior to HLA-A*0301/KQSSKALQR-PE tetramer staining resulted in complete inhibition of tetramer binding. Thus for the HLA-A*0301/KQSSKALQR tetramer, we demonstrated that binding to T-cells was completely dependent on interaction with the CD8 complex and less exclusively mediated by the TCR complex.

Discussion

In this study we investigated whether the BCR/ABL-specific peptide spanning the fusion part of the b3a2 protein can be processed into peptides that bind to HLA-A*0301 and HLA-B*0801, and whether a specific T-cell immune response directed against these peptides could be characterized.

We first determined the binding capacity of the BCR/ABL-derived peptides KQSSKALQR for HLA-A*0301 molecules and of GFKQSSKAL for HLA-B*0801 molecules and found a better binding for HLA-A*0301 than for HLA-B*0801 in line with previously published results of Bocchia *et al.*[7] Based on the isolation of KQSSKALQR from HLA A*0301 molecules derived from leukemic cells in a recent study, suggesting that this peptide results from endogenous processing of intracellular BCR/ABL protein,[20] we investigated the proteosomal digestion of a 27 amino acid peptide spanning the breakpoint region of BCR/ABL. We confirmed that KQSSKALQR can be one of the products of cleav-

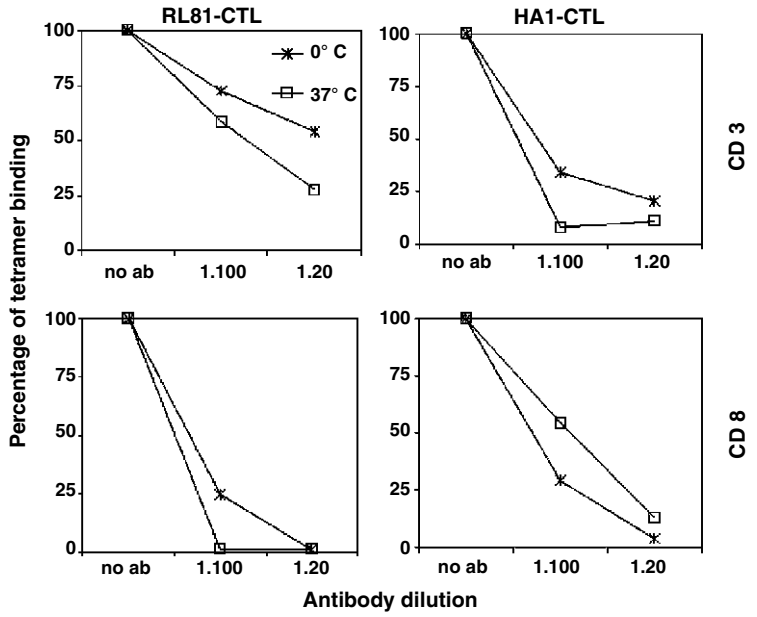

Figure 5. Blocking studies of HLA-A*0301/KQSSKALQR-PE tetramer binding to cells of clone RL-81, which were isolated from PBMC of a CML patient, successfully treated with DLI for a relapse after allogeneic SCT, and, as a control, of the binding of an HA-1-specific T-cell clone to the HLA-A2/VLHDDLLEA-PE tetramer. Blocking with anti-CD3 FITC and anti-CD8 FITC-labeled monoclonal antibodies at three different concentrations is shown at 0°C (*) or 37°C (□). Binding of RL-81 to HLA A*0301/KQSSKALQR-PE tetramers could be blocked with anti-CD8 but less effectively than with anti-CD3 indicating at least partial non-specific binding not involving the TCR complex. Binding of HA-1 to the HLA-A2/VLHDDLLEA-PE tetramer could be blocked with both anti-CD3 and anti-CD8 indicating involvement of the TCR complex in this binding.

age by the proteasome. This is also in line with a computerized prediction algorithm of proteasome-mediated cleavage of proteins.[34,35] However, we could not confirm the predicted HLA-B*0801 binding peptide GFKQSSKAL as one of the products of proteasome cleavage. Therefore, we limited our further analysis to the HLA-A*0301 binding peptide KQSSKALQR and analyzed whether this peptide could be detected in the HLA class I molecules on the membrane of K562 cells retrovirally transduced with HLA-A*0301. In contrast to results described by Clark *et al.*[20] we were unable to detect the KQSSKALQR peptide in the eluate of 30×10^9 HLA-A*0301-transduced K562 cells, despite the high copy numbers of the BCR/ABL translocation and high expression of BCR/ABL protein in K562 cells.[19] Thus, although both the binding assays and proteasomal digestions in our present study suggest that proteasomal degradation of intracellular BCR/ABL protein can result in presentation of KQSSKALQR in HLA-A*0301, we could not unequivocally confirm the presence of the peptide in HLA-A*0301, despite the use of very sensitive assays, and in contrast to the findings in a similar study.[20] As the detection limit of peptides was similar in both studies, and since in the present study more transfected K562 cells were used than in the study by Clark *et al.*[20] this cannot be an explanation for the discrepancy. A possible explanation might be that in the present study a different HPLC column was used. Since the KQSSKALQR peptide was found to be very hydrophilic, the peptide may be differentially trapped on various HPLC columns. Since the expression of BCR/ABL in K562 cells is twenty-fold higher than in primary CML cells and we could not detect the peptide in transfected K562 cells, we did not extend our analysis to CML cells as was done by Clark *et al.*

The presence and relevance of endogenously processed KQSSKALQR peptide in HLA-A*0301 molecules from CML cells can unequivocally be proven by specific recognition of these cells by peptide-specific T-cell clones. In attempts to obtain these BCR/ABL specific T-cell clones we first examined whether synthetic KQSSKALQR peptide-pulsed dendritic cells from normal donors bearing HLA-A*0301 could induce an autologous KQSSKALQR-specific T-cell response. Despite HLA-A*0301/KQSSKALQR tetramer positivity of small percentages of T cells in the cell lines generated, we could not isolate functionally active BCR/ABL-specific T-cell clones using tetramer FACS-sorting of the cell lines. Although we were able to generate HLA-A*0301/KQSSKALQR tetramer-positive T-cell clones, we did not find peptide-specific killing. These results illustrate that positive staining with specific tetramers does not always predict specific recognition of the antigen/HLA complex on the cell membrane. In the study by Clark *et al.*[20] HLA-A*0301/KQSSKALQR tetramer-positive T cells were detected in patients with CML, but clonal T-cell analysis to confirm specific recognition of endogenously processed antigen could not be performed. Compatibly with this previously published approach using possibly *in vivo* sensitized T-cell responses, we hypothesized that KQSSKALQR peptide-specific donor T cells may be generated *in vivo* in HLA-A*0301 positive patients treated with DLI for a relapsed b3a2 positive CML after allogeneic SCT. We attempted to isolate HLA-A*0301/KQSSKALQR tetramer-positive T cells

from PBMC of HLA-A*0301-positive patients successfully treated with DLI for a relapse CML after allogeneic SCT. In a recent paper this approach was successful in identifying HA-1- or HA-2-specific T cells after DLI.[28] Although we were able to isolate tetramer positive T-cell clones using single cell per single well cell sorting, peptide-specific high-avidity T-cell recognition of the BCR/ABL antigen could not be identified.

To unravel the mechanisms of the discrepancy between tetramer binding of isolated T-cell clones and the absence of peptide-specific recognition, we performed blocking studies with anti-CD3 and anti-CD8 monoclonal antibodies. Whereas antigen-specific tetramer binding of a control HA-1-specific T-cell clone could easily be blocked by anti-CD3 antibodies, and only partially by CD8 blockade, blocking of HLA-A*0301/KQSSKALQR tetramer staining of isolated T-cell clones was complete with anti-CD8, but incomplete with anti-CD3 antibodies (Figure 5). These results were obtained for staining both at 37°C and at 0°C, illustrating that this phenomenon was not temperature dependent. These findings indicate a low affinity binding via the TCR complex and may even indicate the possibility of non-TCR binding. Apparently, the interaction between the tetrameric complex and the T cells was not T-cell epitope-specific.

T-cell responses against BCR/ABL in the context of HLA-A*0201, HLA-A*0301, HLA-A*1101, HLA-A*2402 and HLA-B*0801 have been explored in previous studies. Although evidence has been presented of peptide-specific recognition, no BCR/ABL T-cell clones were detected in any of these studies.[8,9,11,17,40,41] Furthermore, although the results published by Clark[20] show tetramer staining as well as cytolytic activity of T-cell lines, the low frequency of tetramer-positive cells in relation to the recognition of CML cells may also indicate a discrepancy between tetramer staining and specific recognition. Functional analysis of T-cell clones showing antigen-specific recognition including cold target inhibition studies of primary CML cells are required to unequivocally demonstrate the existence of high avidity T cells recognizing endogenously processed BCR/ABL fusion peptides. In a recent vaccination study using BCR/ABL-derived synthetic peptides mainly CD4 responses were generated and only weak CD8 activity was found in 4 patients with either HLA-A*0301 or HLA-A*1101 using the ELISPOT interferon-γ assay.[42] These results are in line with our inability to isolate high-avidity cytotoxic T-cells.

In conclusion, our study provides evidence that antigen processing of BCR/ABL b3a2 protein can lead to the production of the breakpoint-specific peptide KQSSKALQR with a binding motif for HLA-A*0301. However, we could not identify high avidity BCR/ABL-specific CTL clones from PBMC from normal donors or patients with a relapse of CML treated with DLI. Thus, at present there is no definitive proof of the existence of clonal T cells directed specifically to BCR/ABL. Vaccination studies using BCR/ABL peptides in CML patients with minimal residual disease may boost T-cell responses against BCR/ABL allowing clonal isolation of functionally active BCR/ABL specific T-cell clones.

All authors gave substantial contributions to the conception and design of the study, or acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and gave final approval of the version to be published. The authors reported no potential conflicts of interest.

This study was financially supported by grants from the Kay Kendall Leukemia Fund and the Wetenschappelijke Activiteiten Commissie van het Reinier de Graaf Hospital.

Manuscript received April 20, 2004. Accepted July 15, 2004.

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