Synthetic peptide analogs derived from bcr/abl fusion proteins and the induction of heteroclitic human T-cell responses

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Background and Objectives. Chronic myelogenous leukemia (CML) presents a unique opportunity to develop therapeutic strategies using vaccination against a truly tumor-specific antigen that is also the oncogenic protein required for neoplasia. We have shown in phase I and II trials that a tumor-specific, bcr-abl-derived peptide vaccine can be safely administered to patients with chronic phase CML and can elicit a reliable specific CD4 immune response. However, variable CD8 responses and no HLA A0201-restricted responses were found. One strategy to circumvent this poor immunogenicity is to design synthetic immunogenic analog peptides that cross-react with the native peptides (a heteroclitic response). The aim of this study was to design such peptides.

Design and Methods. By using computer prediction analysis. We designed a number of synthetic peptides derived from the junctional sequences of CML (p210/b3a2 or p210/b2a2) in which single and double amino acid substitutions were introduced at key HLA A0201 binding positions. The binding of these peptides was tested by a thermostabilization assay using a T2 cell line.

Results. We found three peptides that predicted good binding to HLA A0201 molecules and stabilized MHC class I A0201 molecules on the surface of T2 cell lines. These peptides were screened for eliciting HLA restricted, peptide-specific cytotoxic T lymphocyte responses using $CD3^+$ T cells from several A0201 donors and CML patients. The $CD8^+$ cytotoxic T lymphocytes lines were assessed by either interferon-γ ELISPOT or a chromium release assay using pulsed, HLA-matched leukemic cell lines. The analog peptides generated larger immune responses (increased CD8 T-cell precursor frequency) than did the native peptides. Importantly, $CD8^+$ T cells stimulated with the new synthetic peptides cross-reacted with the native bcr-abl peptides.

Interpretations and Conclusions. In conclusion, analog CML fusion peptides with increased immunogenicity and heteroclitic properties can be synthesized and may be useful in vaccination strategies.

Key words: T cell receptor, gene transfer, minor histocompatibility antigen, immunotherapy

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Chronic myelogenous leukemia (CML) is a pluripotent stem cell disorder characterized by the presence of the Philadelphia chromosome (Ph).[1-5] Because of its unique features, CML provides an opportunity to develop therapeutic strategies using vaccination against a truly tumor-specific antigen that is also the oncogenic protein required for neoplasia.[6]

The chimeric fusion proteins are potential antigens for two reasons. The proteins are uniquely expressed in CML cells in which the junctional regions contain a sequence of amino acids that is not expressed on any normal protein. In addition, as a result of the codon split on the fused message, a new amino acid (lysine in b3a2) and a conserved one (glutamic acid in b2a2) is present at the exact fusion point in each of the proteins. Therefore, the unique amino acid sequences encompassing the b3a2 and b2a2 breakpoint region can be considered truly tumor-specific antigens. Despite the intracellular location of these proteins, short peptides produced by cellular processing of the products of the fusion proteins can be presented on the cell surface within the cleft of HLA molecules, and in this form they can be recognized by T cells.[7-10]

We and others recently showed in three clinical trials, that a tumor-specific, bcr-abl-derived multivalent vaccine can be safely administered to patients with chronic phase CML;[11,12] the vaccine reliably elicits a bcr-abl peptide-specific CD4 immune response as measured by delayed-type hypersensitivity *in vivo*, $CD4^+$ T-cell proliferation *ex vivo* and gamma interferon secretion in an ELISPOT assay. However, we were unable to detect CD8 responses in A0201 patients and only

weak responses in HLA A0301 patients using a sensitive gamma interferon ELISPOT assay. The strength of CD8 responses to stimulation depends upon the binding affinity of the target peptide to class I MHC molecules, the stability of the peptide-HLA complex, and the avidity of the T-cell receptor binding for the peptide complex. Killing of native CML cells also requires adequate processing and presentation of the natural antigen. Therefore the lack of reproducible CD8 responses in these clinical trials could be the result of the biochemistry of these class I peptide-HLA interactions, resulting in their weak immunogenicity to cytotoxic CD8 cells. One strategy to circumvent the poor immunogenicity of these peptides at the surface of target cells is to design synthetic analog peptides that will be more immunogenic. Such peptide analogs could generate an immune response that not only recognizes the immunizing epitopes, but that also cross-reacts with the original native peptides; this is known as a heteroclitic response.

The aim of this study was to use computer prediction analysis to design a large number of synthetic peptides derived from the junctional sequences of CML (p210/b3a2 and p210/b2a2) in which single or double amino acid substitutions were introduced into the peptides at key HLA A0201 binding positions. Peptides that were predicted to bind with high affinity to HLA A0201 molecules were directly assayed for their ability to stabilize MHC class I A0201 molecules on the surface of a TAP-negative T2 cell line. Avidly binding peptides were then assayed in an antigen-specific T-cell expansion *in vitro* system for their ability to elicit HLA-restricted, peptide-specific cytotoxic T lymphocyte responses using purified T cells from healthy donors and patients with CML.

Design and Methods

Synthetic peptides

Each of the peptides utilized in this study was purchased and synthesized by Genemed Synthesis Inc. (CA, USA) using fluorenylmethoxycarbonyl chemistry, solid phase synthesis and purified by high pressure liquid chromatography. The quality of the peptides was assessed by high-performance liquid chromatography analysis, and the expected molecular weight was observed using matrix-assisted laser desorption mass spectrometry. Peptides were sterile and greater than 90% pure. The peptides were dissolved in DMSO and diluted in phosphate-buffered saline (PBS; pH 7.4) or saline to give a concentration of 5 mg/mL and were stored at –80°C. The amino acid sequences and predicted score for binding to HLA A0201, generated by two available online databases (BIMAS and SYFPEITHI), are shown in Table 1.[14] For *in vitro* experiments we also used an irrelevant control peptide, HLA A24 consensus.

Table 1. HLA A0201 native peptides and synthetic analogs.

Name	*Native sequence*[a]	*Analog sequence*[a]	*BIMAS score*[b]	*SYFPEITHI score*[c]	*Frequency*[d]
p210-b3a2					
CMLA2	SS**K**ALQRPV		0.003	12	0/10
p210F		YL**K**ALQRPV	**2.240**	**22**	**5/10**
CMLA3	KQSS**K**ALQR		0.005	3	0/5
p210A		KQSS**K**ALQV	**24.681**	**13**	**0/5**
p210B		KLSS**K**ALQV	**243.432**	**23**	**0/5**
p210Cn	**K**ALQRPVAS		0.013	10	0/5
p210C		**K**LLQRPVAV	**900.689**	**26**	**6/10** **2/4 CML**
p210Dn	TGFKQSS**K**A		0.120	7	0/4
p210D		TLF**K**QSSKV	**257.342**	**23**	**2/6**
p210E		YLFKQSSKV	**1183.775**	**25**	**2/6**
p210-b2a2					
b2a2A	LTINKE**E**AL		0.247	20	0/4
b2a2 A1		LLINKE**E**AL	17.795	26	0/4
b2a2 A2		LTINKV**E**AL	21.996	24	0/4
b2a2 A3		YLINKE**E**AL	**48.151**	**26**	**2/4**
b2a2 A4		YLINKE**E**AV	**156.770**	**26**	**1/2**
b2a2 A5		YLINKV**E**AL	**110.747**	**30**	**1/2**

[a]residues in bold (K in the b3a2 and E in b2a2) represent the amino acid at the fusion breakpoint. Residues underlined represent modifications from the native sequence: [b]BIMAS prediction software available at http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform; [c]SYFPEITHI prediction software available at http://syfpeithi.bmi-heidelberg.com/; [d]frequency of positive responses by interferon-γ ELISPOT in healthy donors and patients with CML.

Cell lines

Cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin, streptomycin, 2 mM glutamine and 2-mercaptoethanol at 37°C in humidified air containing 5% CO_2. SKLY-16 is a human B-cell lymphoma expressing HLA A0201 and T2 is a human cell line lacking TAP1 and TAP2 and therefore unable to present peptides derived from cytosolic proteins.

T2 assay for peptide binding and stabilization of HLA A0201 molecules

T2 cells (TAP^-, $HLA\text{-}A0201^+$) were incubated overnight at 27°C at a concentration of 10^6 cells/mL in fetal calf serum-free RPMI medium supplemented with 5 µg/mL human β_2-microglobulin (Sigma, St. Louis, MO, USA) in the absence (negative control) or presence of either a positive reference tyrosinase peptide or test peptides at various final concentrations (50, 10, 1, and 0.1 µg/mL). Following incubation for 4 hours with 5 µg/mL brefeldin A (Sigma), T2 cells were labeled for 30 min at 4°C with a saturating concentration of anti-HLA-A2.1 (BB7.2) monoclonal antibody, then washed twice. The cells were then incubated for 30 min at 4°C with a saturating concentration of fluoroscein isothiocyanate-conjugated

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goat IgG $F(ab')_2$ anti-mouse immunoglobulin (Caltag, South San Francisco, CA, USA), washed twice, fixed in PBS/1% paraformaldehyde and analyzed using a FACS Calibur cytofluorometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA).

The mean intensity of fluorescence (MIF) observed for each peptide concentration (after dividing the MIF observed without peptide) was used as an estimate of peptide binding and expressed as a fluorescence index. Stabilization assays were performed similarly. Following initial evaluation of peptide binding at time 0, cells were washed in RPMI complete medium to remove free peptides and incubated in the continuous presence of 0.5 μg/mL brefeldin-A for 2, 4, 6, and 8 hours.

The amount of stable peptide-HLA-A2.1 complexes was estimated as described above by indirect immunofluorescence analysis. The half-time of complexes is an estimate of the time required for a 50% reduction of the MIF value recorded at time 0.

In vitro immunization and human T-cell cultures

After informed consent, peripheral blood mononuclear cells from HLA-A0201 positive healthy donors and CML patients were obtained by Ficoll-density centrifugation. Peripheral blood dendritic cells were generated by isolating monocyte-enriched peripheral blood mononuclear cell fractions, using a plastic adherence technique, from total peripheral blood mononuclear cells. The plastic-adherent cells were cultured further in RPMI 1640 medium supplemented with 1-5% autologous plasma, 1000 U/mL recombinant human interleukin (IL)-4 (Schering-Plough, NJ, USA), and 1000 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immunex, Seattle, USA). On days 2 and 4 of incubation, part of the medium was exchanged for fresh culture medium supplemented with IL-4 and GM-CSF, and the culture was continued. On day 6, half of the medium was exchanged for culture medium supplemented with IL-4, GM-CSF, and 10 ng/mL recombinant human tumor necrosis factor-α (R&D system) and 500 ng/mL of trimeric soluble CD40L (Immunex). On day 9, the cells were harvested and used as monocyte-derived dendritic cells for antigen stimulation. The cells generated expressed dendritic cell-associated antigens, such as CD80, CD83, CD86, and HLA class I and class II on their cell surfaces (*data not shown*). T lymphocytes were isolated from the same donors using negative selection by depletion with an anti-CD11b, anti-CD56 and CD19 monoclonal antibody (Miltenyi, CA, USA). A total of 1×10^6 pure T lymphocytes were cultured with 1×10^5 autologous dendritic cells in RPMI 1640 medium supplemented with 5% heat-inactivated human autologous plasma with bcr-abl synthetic peptides at a concentration of 10 μg/mL and β2 microglobulin at 2 μg/mL in 24-well plates in the presence of 5-10 ng/mL recombinant human IL-7 (Genzyme) and 0.1 ng/mL IL-12. After culture for 3 days 20 U/mL IL-2 was added. After 10 days, 1×10^6 cells were stimulated again by adding 2×10^5 autologous magnetically isolated $CD14^+$ monocytes together with 10 ng/mL IL-7, 20 U/mL IL-2 and peptide at a concentration of 10 μg/mL. In some cases, after culture for another 7 days, the cells were stimulated a third time, in the same manner. After the second or third stimulation, CD8 T cells were magnetically isolated and the cytotoxicity and interferon-γ secretion of these cells were then examined.

γ interferon ELISPOT

HA-Multiscreen plates (Millipore, Burlington, MA, USA) were coated with 100 μL of mouse-anti-human interferon-γ antibody (10 μg/mL; clone 1-D1K, Mabtech, Sweden) in PBS, incubated overnight at 4°C, washed with PBS to remove unbound antibody and blocked with RPMI / autologous plasma for 1 h at 37°C. Purified $CD8^+$ T cells (more than 95% pure) were plated at a concentration of 1×10^5/well. T cells were stimulated with 1×10^4 T2 cells/well pulsed with 10 μg/mL of β2-microglobulin (Sigma, St. Louis) and either 50 μg/mL of test peptide, positive control influenza matrix peptide, or irrelevant control peptide at a final volume of 100-200 μL/well. Control wells contained T2 cells with or without $CD8^+$ cells. Additional controls included medium or $CD8^+$ alone plus PBS/5% DMSO diluted according to the concentrations of peptides used for pulsing T2 cells. After incubation for 20 h at 37°C, plates were extensively washed with PBS/0.05% Tween and 100 μL/well biotinylated detection antibody against human interferon-γ (2 μg/mL; clone 7-B6-1αβ Mabtech, Sweden) were added. Plates were incubated for an additional 2 h at 37°C and spot development was performed as described elsewhere.[15] Spot numbers were automatically determined with the use of a computer-assisted video image analyzer with KS ELISPOT 4.0 software (Carl Zeiss Vision, Germany).

Cytotoxicity assay

The presence of specific cytotoxic T lymphocytes was measured in a standard 4 h-chromium release assay. This assay was performed by labeling 4×10^6 target cells with 300 μCi of $Na_2{}^{51}CrO_4$ (NEN Life Science Products Inc., Boston, MA, USA) for 1 hour at 37°C. After washing, 2×10^6 cells/mL were incubated with or without synthetic peptides at a concentration of 10 μ/mL for 2 hours at 20°C in the presence of β_2 microglobulin at 3 μg/mL. After washing by centrifugation, target cells were resuspended in complete media at 5×10^4 cells/mL and plated in a 96-well

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U-bottom plate (Becton Dickinson, NY, USA) at 5×10^3 cells/well with effector cells at effector to target ratios (E/T) ranging from 100:1 to 10:1. Plates were incubated for 5 hours at 37°C in 5% CO_2. Supernatant fluids were harvested and radioactivity was measured in a gamma counter. Percent specific lysis was determined using the following formula: 100× [(experimental release minus spontaneous release)/(maximum release minus spontaneous release)]. Maximum release was determined by lysis of target cells in 2.5% Triton X-100.

Results

Identification and generation of peptides with a high probability of binding to HLA A0201

Peptides with potential epitopes for T lymphocyte cytotoxicity can be predicted by means of a peptide library-based scoring system for MHC class I-binding peptides. Amino acid sequences of the human b3a2 and b2a2 fusion proteins were scanned for peptides with potential binding capacity for HLA A0201, a subtype encompassing 95% of the HLA-A02 allele. HLA-A0201 is expressed in about 40% of the Caucasian population. No peptides with high or intermediate affinity were identified in the native b3a2 or b2a2 fusion proteins (predicted half-life longer than 1 minute). We and others have demonstrated this previously using other methods.[16,17] One peptide that does not exhibit the consensus HLA 0201 binding motifs has been described[10] but it has weak avidity to MHC. Based on this information and by using the software of the Bioinformatics & Molecular Analysis Section (National Institutes of Health, Washington, DC, USA), available at *http://bimas.dcrt.nih.gov/ cgi-bin/molbio/ken_parker_comboform*, which ranks 9-mer or 10-mer peptides on a predicted half-time dissociation coefficient from HLA class I molecules,[14] we designed several analog peptides by changing one or both anchor amino acids or additional amino acids adjacent to anchor amino acids. Single or double amino acid substitutions were introduced at HLA A0201 preferred residues (positions 1, 2, 6 and 9) (Table 1) to yield sequences that had comparatively high binding scores predicted for HLA A0201 molecules. The predicted half-life for binding to HLA A0201 was greater than 240 minutes in four synthetic peptides and less than 240 in seven. All the native peptides were predicted to have less than one minute of half-life. Most of the substitutions affected the primary or secondary anchor motifs (leucine in position 2 or valine in position 9 or position 6) but in some cases, a tyrosine was substituted in position 1. This substitution has been shown to stabilize the binding of the position 2 anchor residue.[18,19] Table 1 also shows the peptide prediction according to another online software available at *http://syfpeithi.bmi-heidelberg.com/*.[13]

Binding of HLA-A0201 by selected peptides

For MHC class I-restricted peptides to be immunogenic they must be able to bind and stabilize MHC class I molecules on the live cell surface. Moreover, the computer prediction models mentioned above have only 60-80% predictive accuracy, so we next sought direct measurement of the strength of the interaction between the peptides and the HLA-A0201 molecule using two methods: a conventional binding and stabilization assay that uses antigen-transporting deficient (TAP2-negative) HLA-A0201 human T2 cells. T2 cells lack TAP function and consequently are defective at properly loading class I molecules with antigenic peptides generated in the cytosol. The association of exogenously added peptides with thermolabile, empty HLA-A2 molecules stabilizes them and results in an increase in the level of surface HLA-A0201 recognizable by a specific monoclonal antibody such as BB7.2. Seven out 11 peptides we designed to have higher binding scores exhibited a relatively high binding affinity for HLA A0201 molecules as measured by the T2 assay (Figure 1A). A rough correlation between binding scores and binding affinity was established, thus indicating the partial utility of the computer generated binding scores for predicting peptides that will bind to MHC class I molecules on live cells. Some of these peptides demonstrated the same order of binding affinity as that of a viral antigen such as influenza, which is among the most potent known antigens for inducing T lymphocyte cytotoxicity. In four cases we did not find a good correlation between computer predicted half-life and T2 stabilization.

One of the peptides derived from b3a2, p210C, was mutated from a native peptide that did not have a good prediction score. In spite of this fact, the native sequence is able to bind HLA A0201 weakly and at the same level as the previously described CMLA2 peptide. To design p210C, we substituted a neutral alanine in position 2 by a leucine and a serine in position 9 by a valine. p210C has a high BIMAS score that correlated with T2 binding assay data (Figure 1A). p210F is a peptide derived from a sequence previously described,[10] CMLA2, shown to be a weak binder in the T2 assay. In this case we substituted the two serines in positions 1 and 2 by a tyrosine and a leucine, in the hope of increasing the binding and stabilization to HLA A0201, while retaining the aminoacids for the T-cell receptor interaction. The BIMAS prediction showed a 700-fold improvement and binding to T2 cell revealed an excellent avidity for HLA A0201 molecules. Of the peptides derived from b2a2, all were generated from

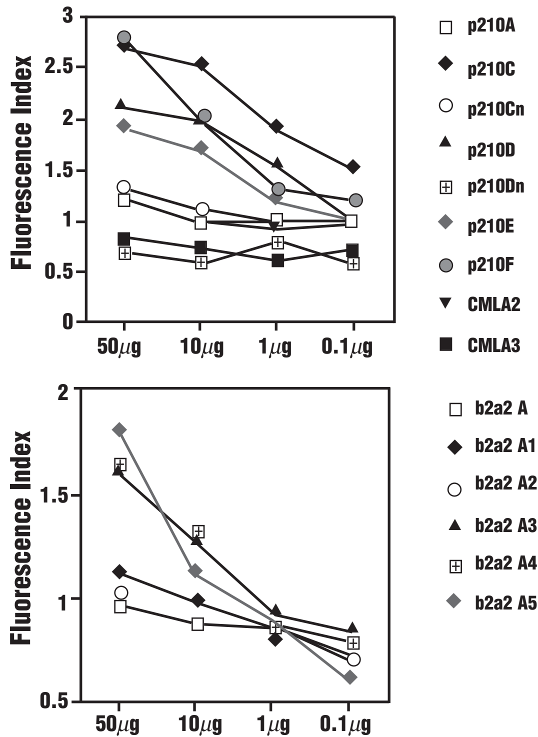

Figure 1. T2 stabilization assay using peptides derived from (A) b3a2 translocation and (B) b2a2 translocations. Sequences of the peptides are shown in Table 1. The fluorescence index is the ratio between the median fluorescence with the peptide tested divided by median fluorescence with no peptide. The X axis represents different concentrations of the peptide tested. *n* denotes native sequences from b3a2. b2a2A is the native sequence for b2a2. A positive control with tyrosinase D was performed at a concentration of 50 µg/mL with a fluorescence index of 2.1. These are results of a single representative experiment.

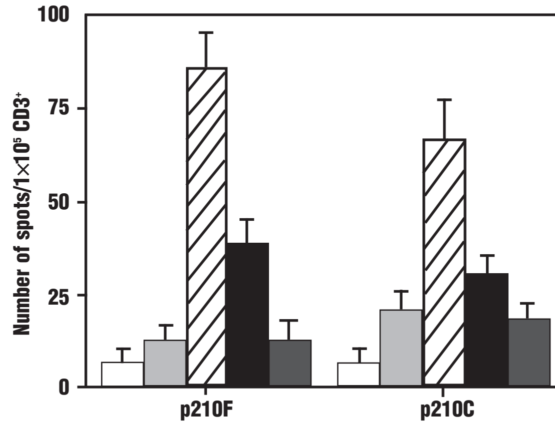

Figure 2. CD8$^+$ interferon-γ ELISPOT from a healthy HLA A0201 donor. T cells were stimulated *in vitro* with the peptides p210 C and F. Empty bars: CD8$^+$ cells plus media. Gray bars: CD8$^+$ plus APC T2. Striped bars: CD8$^+$ plus T2 pulsed with tested peptide (p210C or p210F). Black bars: CD8$^+$ plus T2 pulsed with native peptide (cross reactivity). Dark gray bars: CD8$^+$ plus T2 pulsed with irrelevant control peptide.

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a peptide that was not predicted to have avid binding to HLA A0201. Three new synthetic peptides, b2a2 A3-A5 (Table 1) bound well to HLA A0201 molecules (Figure 1B). These three peptides have a tyrosine-leucine sequence substitution at positions 1 and 2 and also a valine substitution in position 6 or 9 that allow them to increase binding to HLA A0201.

Induction of CD8 immune response against new synthetic peptides

While affinity for MHC molecules is necessary for peptide immunogenicity, there is also a requirement for the presence of reactive precursor T cells with appropriate T-cell receptors. Using a optimized T-cell expansion system, with monocyte-derived dendritic cells, CD14$^+$ cells as antigen-presenting cells and purified CD3$^+$ T cells, we investigated whether the new synthetic b3a2 and b2a2 analogs could stimulate peptide-specific cytotoxic T lymphocytes. Ten healthy HLA A0201 donors as well four patients with CML were studied. Five out of the ten individuals responded to stimulation, generating T cells that secreted interferon-γ when challenged with different peptide-pulsed T2 cells as targets. p210D and p210E produced an immune response in some donors tested (*data not shown*), although p210C and p210F generated more consistent and higher immune responses (Figure 2). Responses were observed after the second or third round of peptide stimulation after CD8$^+$ isolation or in unpurified CD3$^+$ T cells. The spot numbers were consistently higher with peptides that bound with higher affinity to HLA 0201 molecules as determined by T2 assay. More importantly, T cells generated in the presence of the new synthetic analogs were able to recognize the native sequences. p210C and p210F were able to stimulate T cells to recognize their respective native sequences (Figure 2). Peptide CML A2, the native sequence from p210F, is a natural weak binder and there is indirect evidence that it can be naturally expressed on the surface of CML blasts. No immune response could be generated against the p210A and p210B, despite attempts using different donors; this result is consistent with their reduced affinity for MHC. We were also able to generate immune response to the heteroclitic peptide p210C in two out of four chronic phase CML patients (b3a2). After two rounds of stimulation CD8$^+$ cells were selected and tested using T2 as antigen-presenting cells. T cells sensitized against the heteroclitic peptide p210C recognized T2 pulsed with the synthetic peptide with a frequency of almost 400 spot-forming cells per 1×10^5 CD8$^+$ T cells. More importantly these cells also recognized the native peptide on the surface of T2 cells with a frequency of 200 spot-forming cells per 1×10^8 CD8$^+$ T cells (around 50% less than the synthetic peptide) (Figure 3).

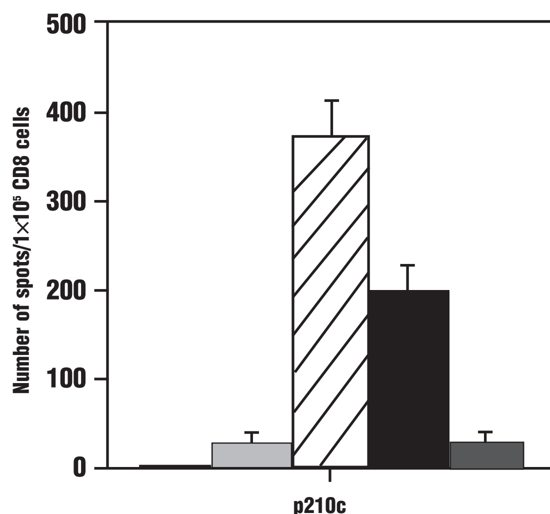

Figure 3. CD8+ interferon-γ ELISPOT from a CML patient (b3a2) in chronic phase after two stimulations *in vitro*. T cells were stimulated *in vitro* with the peptide p210C. Empty bar: CD8+ cells plus media. Gray bar: CD8+ plus APC T2. Striped bar: CD8+ plus T2 pulsed with p210C. Black bar: CD8+ plus T2 pulsed with native peptide p210Cn (cross reactivity). Dark gray bar: CD8+ plus T2 pulsed with irrelevant control peptide.

The peptides derived from b2a2 were also able to generate a significant immune response as measured by interferon γ secretion by CD3+ T cells. Peptides b2a2 A3, A4 and A5 generated an immune response. The response against b2a2 A3 was more consistent between donors. T cells generated in the presence of b2a2 A3 were able to identify the original native sequence (Figures 4A and 4B). This is of special relevance because the native sequence was found to be a weak binder in our preliminary work describing the peptides that bind to different HLA.[17] ELISPOT-detected interferon-γ it is not always associated with functional killing. Therefore we tested the T-cell lines obtained after several stimulations with the analog peptide in classic chromium-51 assays using peptide-pulsed target cell lines. T cells generated *in vitro* in the presence of p210C, p210F and b2a2 A3 were able to kill T2 cells pulsed with specific peptides, but not T2 cells without peptide or T2 cells with control peptide. Therefore these peptides were also able to generate a cytolytic response by cytotoxic T cells. Furthermore, T cells stimulated with p210F peptide were able to recognize the native sequence, CMLA2, confirming once again the heteroclitic response (Figures 5 and 6). These experiments were also performed using HLA-matched CML cell lines or CML blasts expressing the respective translocation (b3a2 or b2a2). In these cases we were only able to generate a non-significant, low level of cytotoxicity (*data not shown*) raising the possibility that the native peptides were not naturally processed and/or sufficiently expressed on the surface of the leukemic cells as has been postulated by others. It is possible that the chromium-51 release assay is not a sensitive enough technique, when conducted over these short time periods, to evaluate the killing of target cells with a low number of molecules on their surface.

Discussion

Chronic myelogenous leukemia provides a unique opportunity for developing therapeutic strategies using vaccination against a truly tumor-specific antigen that is also the oncogenic protein required for neoplasia. None of the native CML peptides that we and others have reported to bind to human MHC, bound the HLA pocket with high affinity. This may explain, in part, the lack of a detectable immune response to bcr-abl peptides seen in patients with CML despite the new expression of this novel antigen in the CML cells. In an attempt to overcome the relatively poor binding of natural fusion bcr-abl peptides to HLA A0201 complexes and make a more immunogenic vaccine for the 40% of patients

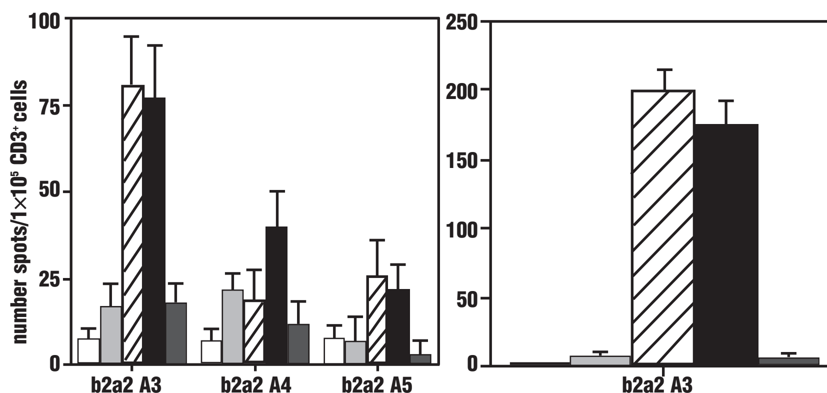

Figure 4. CD3+ interferon-γ ELISPOT from two healthy HLA A0201 donors after two stimulations *in vitro*. T cells were stimulated *in vitro* with the peptides b2a2 A3, A4 and A5. Empty bars: CD8+ cells plus media. Gray bars: CD8+ plus APC T2. Striped bars: CD8+ plus T2 pulsed with tested peptide (b2a2 A3, A4 or A5). Black bars: CD8+ plus T2 pulsed with native peptide (cross reactivity). Dark gray bars: CD8+ plus T2 pulsed with irrelevant control peptide.

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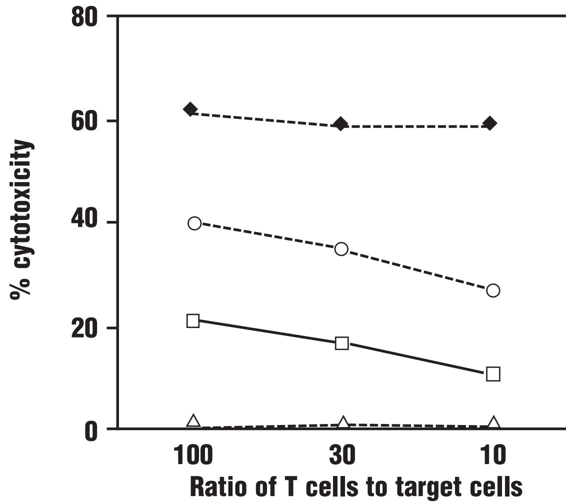

Figure 5. Cytotoxicity assay with T cells from a healthy HLA A0201 donor after three stimulations *in vitro*. T cells were stimulated *in vitro* with the peptide p210F. Target cells used were the T2 cell line pulsed with the respective peptides. Open squares: T2 with no peptide. Black diamonds T2 pulse with p210F. Open circles: T2 pulsed with CMLA2. Open triangles: T2 pulsed with irrelevant control peptide.

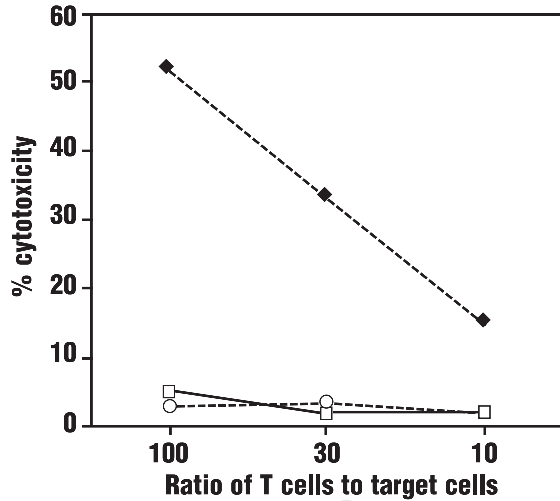

Figure 6. Cytotoxicity assay with T cells from a healthy HLA A0201 donor after five stimulations *in vitro*. T cells were stimulated *in vitro* with the peptide b2a2 A3. Target cells used were the T2 cell line pulsed with the respective peptide. Open squares: T2 with no peptide. Black diamonds: T2 pulsed with b2a2 A3 peptide. Open circles: T2 pulse with irrelevant control peptide. These are results of a single representative experiment.

expressing HLA A0201, we synthesized a series of CML fusion peptide analogs with single and double amino acid substitutions and tested them for binding to HLA A0201, for the ability to form stable peptide/HLA A0201 and for the ability to stimulate T cells *in vitro*. A strong correlation has been found between overall analog peptide affinities for MHC class I molecules and *in vivo* peptide immunogenicity in both *in vivo* and *in vitro* systems.[20,21] Among the peptide analogs tested, four b3a2 and three b2a2 peptides were found to form stable complexes with cell-associated HLA A0201. These high affinity peptides were able to generate specific CD8+ T cells far more efficiently than the native peptide were able to. Most importantly the T cells thus stimulated were able to recognize the native CML fusion peptide sequences.

Previous studies have shown that bcr-abl fusion peptide-specific cytotoxic T lymphocytes may lyse native CML cells efficiently,[7,8,10] especially if expressing the HLA A0301 bcr-abl fusion peptides. However, a recent study[22] was unable to find high avidity bcr-abl-specific cytotoxic T lymphocytes. HLA-A0201 bcr-abl peptide-specific cytotoxic T lymphocytes have been very difficult to demonstrate, with only one report of low levels of cytotoxicity.[10] We have been unable to confirm these data in our systems. This is a major reason why we designed the present heteroclitic peptides. In this study, we obtained a significant level of cytoxicity using peptide-pulsed targets (either T2 or leukemic cell lines with the specific translocation), but not using HLA-matched leukemic cell lines or CML cells from patients in blast crisis. In addition, the chromium-51 release assay may be not the best way to evaluate killing of small percentages of progenitor cells. CML CD34+ clonogeneic cell inhibition studies may be a better approach for examining this problem. The lack of cytotoxicity of the synthetic peptide-specific cytotoxic T lymphocytes against CML blasts could represent a limitation to the clinical application of a vaccine.

Statistical-matrix-driven predictions have been more successful than the presence of simple motifs at predicting the binding of peptides to HLA molecules, suggesting that MHC binding is, to some extent, the result of a combinatorial specificity.[23-25] About 60-70% of the peptides identified using this approach will bind well. The identification of analog peptides based on these methods has been applied recently to the identification of cytotoxic T lymphocyte epitopes deduced from several tumor antigens.[26-28] The more recent incorporation of artificial neural networks has improved the capacity for peptide prediction.[29]

Several groups have designed artificial variants of MHC class I-binding self-peptides in murine models *in vivo* and in human systems *in vitro*.[30-35] The improved *in vivo* immunogenicity and relevance of MHC anchor-modified ligands was first shown formally in human neoplastic disease in a controlled study of patients with malignant melanoma using a melanoma-associated A0201-restricted peptide

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derived from gp100.[32] Recently HLA-tetramer-based detection methods have shown that the parental melan-A antigenic peptides are weak agonists which activate antigen-specific T cells suboptimally.[36] In contrast, melan-A peptide analogs were identified that behaved as full agonists and induced full T-cell activation leading to strong tumor antigen-specific cytotoxic T lymphocyte responses.[37]

In conclusion, computer algorithm-based predictive models of T-cell epitope peptides has allowed us to design a series of new bcr-abl fusion peptide antigens that have a greater affinity for human MHC molecules than do the native peptides. The mutant analogs display greater immunogenicity than the native sequences *in vitro* and are capable of stimulating human CD8^{+} cytotoxic T lymphoycte heteroclitic responses that cross-react with the native sequences. Although these peptide-specific T cells were not able to kill HLA-matched CML cell lines or CML blasts, as assessed by the chromium release assay, these new heteroclitic peptides offer alternative sequences for studies leading to clinical trials for vaccination of patients with CML.

JPI, WR, and DAS contributed to the conception and design of the study, the interpretation of the data, and wrote the paper. JPI, TK and VZ performed most of the experiments. JPI created all the tables and figures in the manuscript.

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References

1. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science 1960;132: 1497-501.
2. de Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, et al. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1982;300:765-7.
3. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR, et al. Localization of the c-ab1 oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature 1983;306:239-42.
4. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973;243:290-3.
5. Kloetzer W, Kurzrock R, Smith L, Talpaz M, Spiller M, Gutterman J, et al. The human cellular abl gene product in the chronic myelogenous leukemia cell line K562 has an associated tyrosine protein kinase activity. Virology 1985; 140:230-8.
6. Pinilla-Ibarz J, Cathcart K, Scheinberg DA. CML vaccines as a paradigm of the specific immunotherapy of cancer. Blood Rev 2000;14:111-20.
7. Clark RE, Dodi IA, Hill SC, Lill JR, Aubert G, Macintyre AR, et al. Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. Blood 2001;98:2887-93.
8. Nieda M, Nicol A, Kikuchi A, Kashiwase K, Taylor K, Suzuki K, et al. Dendritic cells stimulate the expansion of bcr-abl specific CD8+ T cells with cytotoxic activity against leukemic cells from patients with chronic myeloid leukemia. Blood 1998;91:977-83.
9. ten Bosch GJ, Toornvliet AC, Friede T, Melief CJ, Leeksma OC. Recognition of peptides corresponding to the joining region of p210BCR-ABL protein by human T cells. Leukemia 1995;9:1344-8.
10. Yotnda P, Firat H, Garcia-Pons F, Garcia Z, Gourru G, Vernant JP, et al. Cytotoxic T cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia. J Clin Invest 1998;101:2290-6.
11. Pinilla-Ibarz J, Cathcart K, Korontsvit T, Soignet S, Bocchia M, Caggiano J, et al. Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. Blood 2000;95:1781-7.
12. Cathcart K, Pinilla-Ibarz J, Korontsvit T, Schwartz J, Zakhaleva V, Papadopoulos EB, et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood 2004;103:1037-42.
13. Bocchia M, Gentili S, Abruzzese E, Fanelli A, Iuliano F, Tabilio A, et al. Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. Lancet 2005; 365:657-62.
14. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163-75.
15. Herr W, Linn B, Leister N, Wandel E, Meyer zum Buschenfelde KH, Wolfel T. The use of computer-assisted video image analysis for the quantification of CD8+ T lymphocytes producing tumor necrosis factor a spots in response to peptide antigens. J Immunol Methods 1997;203:141-52.
16. Cullis JO, Barrett AJ, Goldman JM, Lechler RI. Binding of BCR/ABL junctional peptides to major histocompatibility complex (MHC) class I molecules: studies in antigen-processing defective cell lines. Leukemia 1994; 8: 165-70.
17. Bocchia M, Wentworth PA, Southwood S, Sidney J, McGraw K, Scheinberg DA, et al. Specific binding of leukemia oncogene fusion protein peptides to HLA class I molecules. Blood 1995;85:2680-4.
18. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. Cell 1993;74:929-37.
19. Tourdot S, Scardino A, Saloustrou E, Gross DA, Pascolo S, Cordopatis P, et al. A general strategy to enhance immunogenicity of low-affinity HLA-A2. 1-associated peptides: implication in the identification of cryptic tumor epitopes. Eur J Immunol 2000;30:3411-21.
20. Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM, et al. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J Immunol 1994;153:5586-92.
21. van der Burg SH, Visseren MJ, Brandt RM, Kast WM, Melief CJ. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J Immunol 1996;156:3308-14.
22. Posthuma EF, van Bergen CA, Kester MG, de Paus RA, van Veelen PA, de Ru AH, et al. 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. Haematologica 2004; 89:1062-71.
23. Lu J, Celis E. Use of two predictive algorithms of the world wide web for the identification of tumor-reactive T-cell epitopes. Cancer Res 2000;60:5223-7.
24. Nussbaum AK, Kuttler C, Tenzer S, Schild H. Using the World Wide Web for predicting CTL epitopes. Curr Opin Immunol 2003;15:69-74.
25. Flower DR. Towards in silico prediction of immunogenic epitopes. Trends Immunol 2003;24:667-74.
26. Hernandez J, Garcia-Pons F, Lone YC, Firat H, Schmidt JD, Langlade-Demoyen P, et al. Identification of a human telomerase reverse transcriptase peptide of low affinity for HLA A2.1 that induces cytotoxic T lymphocytes and mediates lysis of tumor cells. Proc Natl Acad Sci USA 2002; 99:12275-80.
27. Harig S, Witzens M, Krackhardt AM, Trojan A, Barrett P, Broderick R, et al. Induction of cytotoxic T-cell responses against immunoglobulin V region-derived peptides modified at human leukocyte antigen-A2 binding residues.

Blood 2001;98:2999-3005.
28. Trojan A, Witzens M, Schultze JL, Vonderheide RH, Harig S, Krackhardt AM, et al. Generation of cytotoxic T lymphocytes against native and altered peptides of human leukocyte antigen-A*0201 restricted epitopes from the human epithelial cell adhesion molecule. Cancer Res 2001;61: 4761-5.
29. Peters B, Tong W, Sidney J, Sette A, Weng Z. Examining the independent binding assumption for binding of peptide epitopes to MHC-I molecules. Bioinformatics 2003;19:1765-72.
30. Clay TM, Custer MC, McKee MD, Parkhurst M, Robbins PF, Kerstann K, et al. Changes in the fine specificity of gp100(209-217)-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. J Immunol 1999;162: 1749-55.
31. Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J Immunol 1996; 157:2539-48.
32. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998; 4:321-7.
33. Slansky JE, Rattis FM, Boyd LF, Fahmy T, Jaffee EM, Schneck JP, et al. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. Immunity 2000;13:529-38.
34. Dyall R, Bowne WB, Weber LW, LeMaoult J, Szabo P, Moroi Y, et al. Heteroclitic immunization induces tumor immunity. J Exp Med 1998; 188:1553-61.
35. Nicholson LB, Waldner H, Carrizosa AM, Sette A, Collins M, Kuchroo VK. Heteroclitic proliferative responses and changes in cytokine profile induced by altered peptides: implications for autoimmunity. Proc Natl Acad Sci USA 1998;95:264-9.
36. Valmori D, Dutoit V, Lienard D, Lejeune F, Speiser D, Rimoldi D, et al. Tetramer-guided analysis of TCR b-chain usage reveals a large repertoire of melan-A-specific CD8+ T cells in melanoma patients. J Immunol 2000; 165:533-8.
37. Valmori D, Fonteneau JF, Valitutti S, Gervois N, Dunbar R, Lienard D, et al. Optimal activation of tumor-reactive T cells by selected antigenic peptide analogues. Int Immunol 1999;11:1971-80.

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