Chronic myelogenous leukemia (CML) is a pluripotent stem cell disorder characterized by the presence of the Philadelphia chromosome (Ph). 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.

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.

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; 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. Importantly, CD8+ T cells stimulated with the new synthetic peptides cross-reacted with the native bcr-abl peptides.

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
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 peptide 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 1.1 For in vitro experiments we also used an irrelevant control peptide, HLA A24 consensus.

**Cell lines**

Cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin, streptomycin, 2 mM glucose and 2-mercaptoethanol at 37°C in humidified air containing 5% CO2. 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 (TAF, HLA-A0201+) were incubated overnight at 27°C at a concentration of 105 cells/mL in fetal calf serum-free RPMI medium supplemented with 5 μg/mL human β2-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 fluorescein isothiocyanate-conjugated

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**Table 1. HLA A0201 native peptides and synthetic analogs.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Native sequence</th>
<th>Analog sequence</th>
<th>BIMAS score</th>
<th>SYFPEITHI score</th>
<th>Frequency</th>
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*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: BIMAS prediction software available at http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform; SYFPEITHI prediction software available at http://syfpeithi.bmi-heidelberg.com/; frequency of positive responses by interferon-γ ELISpot in healthy donors and patients with CML.
goat IgG F(ab’), anti-mouse immunoglobulin (Caltag, South San Francisco, CA, USA), washed twice, fixed in PBS/1% paraformaldehyde and analyzed using a FACS Calibur cytofluorimeter (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 1x10^6 pure T lymphocytes were cultured with 1x10^6 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, 1x10^6 cells were stimulated again by adding 2x10^6 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 1x10^4/well. T cells were stimulated with 1x10^6 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-B6-1, Mabtech, Sweden) were added. Plates were incubated for an additional 2 h at 37°C and spot development was performed as described elsewhere. 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 4x10^6 target cells with 300 µCi of Na^24CrO_4 (NEN Life Science Products Inc., Boston, MA, USA) for 1 hour at 37°C. After washing, 2x10^6 cells/mL were incubated with or without synthetic peptides at a concentration of 10 µg/mL for 2 hours at 20°C in the presence of β2 microglobulin at 3 µg/mL. After washing by centrifugation, target cells were resuspended in complete media at 5x10^6 cells/mL and plated in a 96-well
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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. One peptide that does not exhibit the consensus HLA 0201 binding motifs has been described 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.dct.nih.gov/cgi-bin/multbib/ken_parker_combiform, which ranks 9-mer or 10-mer peptides on a predicted half-life dissociation coefficient from HLA class I molecules, 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. Table 1 also shows the peptide prediction according to another online software available at http://syfpeithi.bmi-heidelberg.com.

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 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...
cells as antigen-presenting cells and purified CD8+ T cells (around 50% less than the synthesis of 50 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 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.

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 an optimized T-cell expansion system, with monocyte-derived dendritic cells, CD14+ cells as antigen-presenting cells and purified CD8+ 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 CD8+ 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 1x10^6 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 1x10^6 CD8+ T cells (around 50% less than the synthetic peptide) (Figure 3).
The peptides derived from b2a2 were also able to generate a significant immune response as measured by interferon \(\gamma\) 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-\(\gamma\) 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 \textit{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 (\textit{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...
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. 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, especially if expressing the HLA A0301 bcr-abl fusion peptides. However, a recent study22 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.9 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 cytotoxicity 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+ clonogenic 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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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.

References

9. ten Bosch GJ, Toomvliet AC, Friede TK, VZ, and are capable of stimulating human CD8+ cytotoxic T lymphocyte heteroclitic responses that cross-react with the native sequences. 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 lymphocyte heteroclitic responses that cross-react with the native sequences.


