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Identification of T Cell Ligands in a Library of Peptides Covalently Attached to HLA-DR4

Eric Boen,* Angie R. Crownover,* Mary McIlhaney,* Alan J. Korman,1* and Jerry Bill2†

While T cells have been clearly implicated in a number of disease processes including autoimmunity, graft rejection, and atypical immune responses, the precise Ags recognized by the pathogenic T cells have often been difficult to identify. This has particularly been true for MHC class II-restricted CD4+ T cells. Although such cells can be demonstrated to have undergone clonal expansion at sites of pathology, they are frequently difficult to establish as stable T cell clones. Furthermore, in general, larger peptides in higher concentrations are required to stimulate CD4+ T cells than CD8+ T cells, which makes some of the techniques developed to identify CD8+ T cell Ags impractical. To circumvent some of these problems, we developed a model system consisting of two parts. The first part involves the construction of an indicator T cell hybridoma expressing a chimeric TCR comprised of murine constant regions and human variable regions specific for influenza hemagglutinin 307–319 presented by DR4. The second part consists of a library of fibroblasts each expressing multiple peptides as amino terminal covalent extensions of the DRβ-chain of HLA-DR4 (DRA1*0101, DRB1*0401). Using this model system, we screened ~100,000 peptides and identified three novel peptides stimulatory for the HA1.7 TCR. While there is some convergence at residues known to be important for T cell recognition, all three peptides differ markedly from each other and bear little resemblance to wild-type hemagglutinin 307–319. The Journal of Immunology, 2000, 165: 2040–2047.

Disease associations with particular MHC molecules, beneficial effects of immunosuppression, and the presence of expanded T cell clones at sites of pathology provide strong circumstantial evidence that T cells are involved in a number of diseases. Identification of these T cells and of the Ags they recognize would provide insight into the disease process and hopefully suggest novel therapeutic or preventative strategies. Techniques are now available to identify such candidate T cells, using PCR (1, 2), flow cytometry (3), or CDR3 analysis (4) of TCR β-chain expression. Identification of the coexpressed TCR Vα gene has thus far required the establishment of at least short-term T cell clones. Techniques such as single-cell PCR (5) could eliminate this requirement. Although it is difficult to culture T cells without knowing the Ag to which they respond, nonspecific stimulation has sometimes been successful. We have recently used cell sorting, brute force sequencing of TCR Vβ genes, and nonspecific stimulation to identify T cell clones potentially involved in rheumatoid arthritis (6).

Identifying the Ags that expanded T cell clones recognize and proving that these clones are indeed pathogenic remain difficult and interrelated tasks. Once an Ag is identified, multimeric MHC-peptide reagents may be useful to quantitate the level of these T cells at sites of pathology and to follow their levels over the course of disease (7, 8). Several methodologies are currently available for identifying T cell-recognized Ags. If a stimulatory T cell is known and available, biochemical strategies have been successful (9). An elegant biochemical/biophysical approach has been developed using HPLC and tandem mass spectrometry to elute, identify, and sequence stimulatory peptides (10, 11). This technique requires that the T cell clone be extremely sensitive, and for this reason has been most successful with class I-restricted CTL. Molecular biologic approaches have also been developed. cDNA libraries derived from the stimulatory tissue have been constructed in phage, plasmid, or retroviral vectors, and expressed libraries have been successfully screened for class I and class II T cell Ags (12–17). When a stimulatory tissue is not known or available, it has sometimes been possible to identify an Ag from a peptide library. Synthetic positional scanning soluble peptide combinatorial libraries (18) that hold one position fixed while the others are free to be any amino acid quickly become quite large and thus have been most useful for the identification of MHC class I-presented Ags, which are shorter (8–9 aa vs 13–15 aa for class II) and which can typically be recognized at lower concentrations (19). However, the approach has worked for one class II-restricted myelin basic protein–specific clone that recognizes highly degenerate peptides (20). Synthetic peptide libraries that incorporate binding motifs for specific MHC molecules and are random at other positions have been developed and screened with Mycobacterium tuberculosis, GAD, and islet β cell-reactive T cell clones (21–23). Escherichia coli expressed peptide libraries have likewise been constructed and successfully screened with class I-restricted T cell clones (24). A major gap in the technology developed so far is in the ability to identify peptides recognized by MHC class II-restricted T cells when no source of stimulatory material is available and when the T cells likely do not recognize nanomolar concentrations of peptides. We developed a novel method that uses T cell hybridomas transfected with the TCR of interest to screen a library of peptides expressed on the surface of fibroblasts as covalent amino-terminal extensions of the DRβ-chain. The use of T cell hybridomas obviates the need to establish and maintain functional T cell clones, and the covalent linkage of the peptide to the MHC molecule guarantees high Ag density. We screened a library of ~100,000 covalent

*Nextar Pharmaceuticals, Boulder, CO 80301; and 1Division of Allergy and Clinical Immunology, University of Colorado Health Sciences Center, Denver, CO 80262
†Current address: GenPharm International, 2350 Qume Drive, San Jose, CA 95131.
‡Address correspondence and reprint requests to Dr. Jerry Bill, Division of Allergy and Clinical Immunology, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Mail Stop B-164, Denver, CO 80262. E-mail address: jerome.bill@uchsc.edu

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peptides with a murine T cell hybridoma expressing the HA1.7 TCR specific for influenza hemagglutinin (HA)3 307–319 presented by DR4 (25). Using this approach, we have identified three novel stimulatory peptide mimetics of HA307–319. The three peptides are markedly divergent, and searches based on these peptides, individually or collectively, do not identify the wild-type HA peptide.

**Materials and Methods**

**Peptides and oligonucleotides**

Peptides were purchased from Macromolecular Resources (Pt. Collins, CO), the Molecular Resource Center at the National Jewish Center (Denver, CO), or Research Genetics (Huntsville, AL). They were purified to >95% homogeneity before use. Peptide stocks were prepared at 10–20 mg/ml in water or 0.1 M acetic acid. Oligonucleotides were purchased from Macromolecular Resources or Operon Technologies (Alameda, CA).

**DNA sequencing**

DNA sequencing was performed using Sequenase (United States Biochemical, Cleveland, OH) according to the manufacturer’s instructions or using rhodamine dye terminators and an Applied Biosystems automated system (Perkin-Elmer, Foster City, CA).

**Plasmid construction**

Standard molecular biological techniques (26) were used to generate the following constructs.

**HA1.7 TCR Vα and Vβ constructs.** The HA1.7 TCR Vα-chain (gb X63455) was constructed by sewing the HA1.7 Jα onto human Vα1.2 by PCR. PBL from a normal donor were purified over Ficol, and total cellular RNA was extracted using RNAsol B (Tel-Test, Friendswood, TX) according to the manufacturer’s directions. Human Vα cDNA, primed with a CO antisense oligo (5'-GGGCACAGAGGTGGTAGCAGC-3'), was made using a reverse transcriptase system (Promega, Madison, WI) according to the manufacturer’s directions and used as template in a first PCR to clone human Vα1.2 and add a portion of the HA1.7 Jα. The sense oligo was 5'-GCCCTGAGATGCTCTGCAGTGC-3', and the anti-sense VJ-1 oligo was 5'-AGTCCAAAGGTTAATTTCTCATTTCCAAATGGAG. PCR conditions were as for the HA1.7 TCR DNA sequencing was performed using Sequenase (United States Biochemical, Cleveland, OH) according to the manufacturer’s instructions or using rhodamine dye terminators and an Applied Biosystems automated system (Perkin-Elmer, Foster City, CA).

For library construction, the wild-type HA 307–319 -encoding region was confirmed complete digestion and precluded any contamination with wild-type incomplete RT-PCR from the consanguineous cell line SAVC (IMGT/HLA Cell Database ID 11368); total cellular RNA was prepared using RNAsol B (Tel-Test) and converted to cDNA using a Promega reverse transcriptase system with a random hexamer primer. The PCR primers were sense 5'-TGAAAGCTTGGGACACCGACAGCTTGC-3' and antisense 5'-TCAGTCGACTCAGCTCAGGAATCCTGTTGG-3'.

The full cassette was then transferred to pBABE-puro and used to transfect d2 or GP+ retroviral packaging lines to obtain viral stock. Hybridomas expressing this construct were shown to respond to ~10-fold less peptide Ag (A. Korman, data not shown).

**Covalent HLA-DRB1*0401-peptide constructs.** The vector pBABE-puro (31) was modified by destroying the two XmnI and the single Spel sites before insertion of the covalent peptide-DRB1*0401 cassette. The Spel site was removed by digestion, Klenow fill-in, and blunt ligation. The XmnI site in the 5' long terminal repeat was destroyed by blunt-end ligating the calf intestinal alkaline phosphatase-treated Smal-digested bacterial portion of the vector to the T4 DNA polymerase blunt-end KpnI-flanked retroviral portion of the vector. The remaining XmnI site in the 3' long terminal repeat was then destroyed by blunt-end ligation following a Smal/KpnI digest and T4 DNA polymerase treatment. This plasmid is designated pBABE6XS-puro.

The 1.6-kb XmnI/SpeI stuffer fragment, which enabled us to confirm complete digestion and precluded any contamination with wild-type peptide, was ligated into the XmnI site of pBABE-puro, resulting in a plasmid designated pBABE6XSDR4. This plasmid was used to transform electrocompetent cells (Invitrogen, Carlsbad, CA), according to the accompanying instructions using a Bio-Rad Gene Pulser with pulse controller (Bio-Rad, Richmond, CA). Two independent transformations were performed, each resulting in >106 penicillin colonies. Plasmid DNA (pBABE6XSDR4-SSL9) was amplified in E. coli and purified for transfection using a maxi prep system (Qiagen, Stanford, CA).

**Gene transfer**

The HA1.7 TCR was introduced into 54g by electroporation. Briefly, 107 54g cells were washed twice in HBSS, resuspended in 0.5 ml Optimem medium (Life Technologies, Rockville, MD) and placed on ice for 10 min

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1 Abbreviations used in this paper: HA, hemagglutinin; SH, Src homology.
Flow cytometry

Flow cytometry was performed using a FACScan cytometer (Becton Dickinson, San Jose, CA). The following mAbs were used: anti-murine TCR Vβ, H57-597 (35); anti-human CD4, Leu-3A-PE (Becton Dickinson); anti-DR, L243 (HB-55; American Type Culture Collection, Manassas, VA); anti-DRα, 20-2C3.4; biotinylated anti-murine ICAM-1, 3E2 (PharMingen, San Diego, CA); and biotinylated anti-murine LFA-1, 2D7 (PharMingen). H57-597 and 20-2C3.4 culture supernatants were purified on protein A or G, respectively, and biotinylated using biotin hydroxysuccinimide (36). Culture supernatant from L243 was purified over protein G and used with a secondary goat anti-mouse IgG(H&L) (Southern Biotechnology Associates, Birmingham, AL). PE-conjugated streptavidin was used as a secondary reagent for biotinylated Abs (Rockland Immunochemicals, Gilbertsville, PA).

Results

Model system

The goal of this work was to develop a model system whereby libraries of random peptides covalently attached to HLA-DR4 could be created, expressed on the surface of fibroblasts, and screened for the ability to stimulate a particular T cell clone. In this way, stimulatory peptides, presumably mimetics of the peptides recognized in vivo, can be identified. This schema is shown in Fig. 1. To obviate the need to derive and maintain functional T cell clones from a site of pathology, we generated a T cell hybridoma expressing the TCR of interest. To increase the sensitivity of the system, this cell coexpresses human CD4 as a chimeric molecule comprised of the extracellular and transmembrane domains of human CD4 fused to the unique amino-terminal domain of murine Lck and to the SH3, SH2, and kinase domains of c-src (CD4lsrc). This construct is analogous to the construct described by Xu et al. (37), which increased sensitivity 10- to 100-fold. Furthermore, we introduced murine ICAM-1 into the fibroblast APC to permit interaction with the murine LFA-1 expressed by the responding T cell hybridoma.

TCR transfectant

For the current study, we expressed the variable portion of the human TCR HA1.7, which is specific for HA 
\[307–319 \text{ presented by DR}^b \] (25). Using synthetic oligonucleotides and PCR, we grafted the published TCR Vα and Vβ sequences into the HLA-DR4 system, this cell coexpresses human CD4 as a chimeric molecule composed of the extracellular and transmembrane domains of human CD4 fused to the unique amino-terminal domain of murine Lck and to the SH3, SH2, and kinase domains of c-src (CD4lsrc). This construct is analogous to the construct described by Xu et al. (37), which increased sensitivity 10- to 100-fold. Furthermore, we introduced murine ICAM-1 into the fibroblast APC to permit interaction with the murine LFA-1 expressed by the responding T cell hybridoma.

System to Identify Peptide Antigens

FIGURE 1. System to identify peptide Ags. Chimeric TCRs having murine constant regions and human variable regions are expressed by the TCR+ murine T cell hybridoma 54ζ (left). This cell also expresses endogenous (murine) LFA-1 and the chimeric human CD4lck molecule. Human MHC class II molecules with peptides covalently attached to the amino terminus of the DRβ-chain are expressed by the murine fibroblast APC 75IP (right). This cell also expresses murine ICAM-1. Interaction of the TCR with the correct MHC-peptide complex induces the T cell to produce IL-2.

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CDR3 regions onto previously cloned human variable and mouse constant region genes and expressed the resultant constructs in the TCR-negative murine thymoma, 54ζ (38). In addition, we introduced CD4lsrc by retroviral infection to enable interaction with human MHC class II on the APC and to increase the sensitivity.

Fig. 2A shows the expression of endogenous (murine) LFA-1, chimeric TCR, and of CD4lsrc by HA1.7. TCR staining is 127 times background, while staining for the extracellular portion of human CD4 is 106 times background. Fig. 2B shows the ability of the HA1.7 hybridoma to secrete IL-2 in response to mgl/ml concentrations of HA307–319 presented by SAVC, a homozygous HLA-DRB1*0401 cell line. T cells and APCs were cocultured overnight with the indicated concentrations of HA307-319 peptide. Culture supernatants were assayed for IL-2 using the HT-2 bioassay. The experiment was repeated three times with similar results.

Experiments by other investigators have shown that peptides covalently attached to the amino terminus of the β-chain of murine I-A and I-E molecules function well in T cell stimulation assays (39). To confirm that this was also the case for peptides bound to HLA-DR4, we expressed HA307–319 covalently bound to DR4 in 75IP, a DRα-expressing 3T3 murine fibroblast (40). We then introduced murine ICAM-1 using the retroviral vector to yield 75IP-DR4-FLU-ICAM. Fig. 3A shows the expression of DR and ICAM-1 by this fibroblast. Both are >300-fold over background staining. Fig. 3B demonstrates the ability of 75IP-DR4-FLU-ICAM to stimulate the HA1.7 TCR hybridoma to produce IL-2. Significant stimulation is seen with as few as 100 stimulator cells.

**FIGURE 2.** Characterization of the HA1.7 T cell hybridoma. A, Expression of TCR, human CD4, and murine LFA-1 by the HA1.7 hybridoma. Chimeric murine constant and human variable regions TCR αβ genes were transfected into the TCR-negative murine T cell hybridoma, 54ζ. Resultant T cells were cloned and stained with biotinylated anti-TCR, biotinylated anti-LFA-1 and PE-Av, or PE-conjugated anti-human CD4 and analyzed on a FACScan flow cytometer. B, Stimulation of the HA1.7 hybridoma by influenza HA peptide presented by the homozygous DRB1*0401 cell line, SAVC. T cells and APCs were cocultured overnight with the indicated concentrations of HA307-319 peptide. Culture supernatants were assayed for IL-2 using the HT-2 bioassay. The experiment was repeated three times with similar results.

**FIGURE 3.** Characterization of the DR4-transfected fibroblast APC. A, Expression of DR4 and murine ICAM-1 by the fibroblast APC. 75IP cells were transfected with pBABEΔXS-DR4-FLU and subsequently infected with retrovirally encoded murine ICAM-1. The resultant cells were cloned and stained for surface expression of DR and ICAM-1. B, Stimulation of the HA1.7 T cell hybridoma by 75IP-DR4-FLU-ICAM. HA1.7 T cells were cocultured with the indicated numbers of 75IP-DR4-FLU-ICAM cells overnight. Culture supernatants were assayed for IL-2 using the HT-2 bioassay. The experiment was repeated three times with similar results.

**FIGURE 4.** Reconstruction experiment to determine the number of covalent peptides expressed by each fibroblast APC. Plasmid DNA encoding DR4-FLU and DR4-OP (a nonstimulatory peptide) were mixed at a ratio of 1:1000 and used to transfect 75IP. The 3500 puromycin-resistant transfectants were plated out in four 96-well plates at 10 cells per well. Once the 10 cells had grown to confluence (~10⁵/well), their ability to stimulate HA1.7 T cells to produce IL-2 was determined using the HT-2 bioassay, quantitated by incorporation of [³H]thymidine. Using a cutoff of 1000 cpm, 19 of 384 wells were stimulatory vs the 3.6 expected if each fibroblast expressed only one peptide. Thus each fibroblast expresses, on average, 5.3 peptides. The figures above the bars show numerically how many wells have cpm in the range indicated. The background from unstimulated wells was 328 ± 135 cpm.
Reconstruction experiment

We performed a reconstruction experiment to determine 1) the average number of peptides expressed following transfection of the fibroblast APCs with a pool of plasmids encoding a library of covalent peptides covalently attached to DR4, and 2) the number of APCs necessary for stimulation. We mixed plasmids encoding DR4-FLU and DR4-OP at a ratio of 1:1000 and used this mixture to transfect 75IP. OP (“other peptide”, AAYAIASTAQAAA) had been identified in previous experiments and was chosen as an unrelated, nonstimulatory peptide that permits equivalent expression of DR4 (data not shown). We then screened the resultant 3500 independent puromycin-resistant transfectants, in pools of 10 cells, for their ability to stimulate HA1.7. If each fibroblast expressed only one peptide, we would have expected 3.5 stimulatory transfectants. Fig. 4 shows the distribution of [3H]thymidine incorporation—as a measure of IL-2 produced by HA1.7 in response to the pools of 10 fibroblast APCs—by each of the pools. Using a cutoff of 1000 cpm, 19 of 384 wells were stimulatory. This extrapolates to 5.4 peptides expressed per puromycin-resistant transfectant. The fact that 13 of the 19 positive wells had incorporation of \(10,000\) cpm indicates that pools of at least 10 cells can be screened. However, the finding of six positive wells with cpm between 1000 and 2000 cautions that some weak positives might be missed if the pool size were increased too much. Subsequent screening of the peptide library was performed on pools of 20 transfectants each expressing different random peptides.

Library design and evaluation

Fig. 5 shows the design of the peptide portion of the library compared with the wild-type HA307–319 peptide. To enrich for peptides capable of binding DR4, we fixed positions 1, 6, and 9 of the library to phenylalanine, threonine, and leucine, respectively. Phenylalanine in place of tyrosine was chosen for position 1 based on the work of Hammer et al. (41), which demonstrated that some of the alleles of HLA-DR with increased risk of rheumatoid arthritis are unable to accommodate a bulky tyrosine at this position. Positions 2, 3, 4, 5, 7, and 8 were randomized, and positions 10, and 11 were fixed as glycine or alanine on the presumption that these terminal residues would be less important to T cell stimulation. With seven variable positions, the possible number of unique peptides is \(7^{20}\) or \(1.28 \times 10^{9}\). This is a larger library than we could hope to generate or screen. Considerable degeneracy at some if not all of the variable positions will be required for us to find stimulatory peptides. However, by allowing seven positions to vary, we minimize the risk that the choice of fixed residues will preclude all possibility of finding a peptide with stimulatory activity. To minimize stop codons, the third position of the randomized codons was limited to G or C. Stop codons will still occur one in 32 times, resulting in 20% of the peptides being nonexpressible.

**Stimulatory Peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HA 307–319</th>
<th>Library</th>
</tr>
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<tbody>
<tr>
<td>1A9</td>
<td>PKYVKKQNTLKLAT</td>
<td>GXXFXXXXTXTXLAA</td>
</tr>
<tr>
<td>7G5</td>
<td>GTFTKAPTTGQLAA</td>
<td>GXXFXXXTTXSCACXXKXXCTGCC</td>
</tr>
<tr>
<td>20E12</td>
<td>GMVREAPVKRAA</td>
<td>GXXFXXXTTXSCACXXKXXCTGCC</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Peptide library design. Using the influenza HA307–319 peptide as a reference, the design of the peptide library is indicated in single letter amino acid code. X indicates a random amino acid encoded by nucleotide triplets XXX, where X = G, A, T, C and S = G, C. The potential library size is \(7^{20}\) or \(1.28 \times 10^{9}\). With seven random codons and the third position limited to G or C, 20% of the peptides are expected to contain at least one stop codon.

**FIGURE 6.** Peptide mimetics identified from the covalent library. The sequences of the three covalent peptides, 1A9, 7G5, and 29E12, which stimulate HA1.7, are given in single letter amino acid code aligned with HA307–319 and the library template.

**FIGURE 7.** Stimulation of HA1.7 by synthetic peptides corresponding to the covalent peptides identified in the library. HA1.7 T cells were cocultured with SAVC in the presence of the indicated concentration of peptide HA307–319, 1A9, 7G5, or 20E12 overnight. Culture supernatants were analyzed for IL-2 using the HT-2 bioassay. The experiment was repeated three times with similar results.

**FIGURE 8.** Stimulation of HA1.7 by synthetic peptides with alanine substitutions at position HA311. T cells were cocultured with SAVC in the presence of the indicated concentration of wild-type or 311 alanine-substituted peptides HA307–319, 7G5, or 20E12 overnight. Culture supernatants were analyzed for IL-2 using the HT-2 bioassay. The experiment was repeated three times with similar results.
Plasmid DNA encoding the library was prepared and amplified in E. coli. To confirm that the strategy worked, PCR was used to clone and sequence individual peptide-encoding regions from the library. Six of 16 sequences resulted from tandem insertions of the peptide-encoding region. The remaining 10 sequences were of the predicted length and contained the specified anchor residues. Two of the peptides contained stop codons. In addition, one of the peptides contained an additional base, causing a frameshift. Thus, in this small sample, seven of 16 sequences encoded expressible peptides. Lowering the ratio of insert (peptide) to vector reduced the frequency of tandem peptide-encoding regions to less than one in 10 (see next section).

Identification of stimulatory peptides

In all, we screened 60,000 puromycin-resistant 75IP/ICAM-1 transfectants, expressing ~324,000 unique covalent peptides, for the ability to stimulate HA1.7 to produce IL-2. Because the puromycin-resistant transfectants were grown up in bulk, replated, and then tested, 4-fold oversampling would have been required to cover 99% of the peptides. As performed, we screened 78% of the total. Allowing for stop codons, improper insert ligation, and incomplete sampling, we estimate that we screened at least 100,000 peptides. Of the 3,840 pools of 20 transfected APCs each, three were identified as positive by visual inspection of HT-2 viability. ['H]Thymidine was added to the 96-well plates containing the stimulatory wells to quantitate the production of IL-2. In this way, we determined that pools 1A9, 7G5, and 20E12 were 40×, 130×, and 60× background, respectively.

All three pools were cloned at limiting dilution to obtain a stimulatory clone from among the 20 seeded in the original pool. To identify the stimulatory peptides expressed by each of these three clones, two methods were employed. Superinfection of 1A9 and 7G5 with wild-type Moloney leukemia virus allowed us to rescue virus capable of conferring puromycin resistance following infection of 75IP. These puromycin-resistant cells were then cloned and tested for the ability to stimulate HA1.7. For 1A9, one of 38 rescued clones was stimulatory, while one in 21 clones rescued from 7G5 was stimulatory. RT-PCR and sequencing of the peptides from these rescued clones yielded, in each case, a single sequence. This approach did not work for 20E12, as 128 rescued clones were all unable to stimulate HA1.7. In an attempt to reduce the complexity of the transfected DNA, we obtained a secondary transfec-
tant of the 20E12 clone. RT-PCR was used to recover the trans-
fected peptide-encoding sequences, which were then recloned into pBabeΔXS-DR4. Pools of 4–10 plasmids, each encoding a single peptide, were retransfected into 75IP. In this way, a stimulatory transfec-
tant was obtained, and sib selection identified the individual
peptide responsible for stimulation.

Fig. 6 shows the three peptides identified compared with wild-
type HA307–319. Peptides 1A9 and 7G5 conform to the overall structure imposed by the library design: that is, they have the F, T, and L anchors at positions 1, 6, and 9. However, peptide 20E12 has drastically changed two of the anchor positions, T6 to P and L9 to R. In both cases, this resulted from a single base change from the template. Sequencing of 29 additional, nonstimulatory peptides from 20E12 revealed that about half had altered anchor residues at positions 6 and 9. Such changes were not found in 16 sequences from plasmid library DNA, in the 13 unique sequences identified in the 1A9 transfec-
tant, nor in the nine unique sequences identified in the 7G5 transfec-
tant. Tandem peptide-encoding inserts comprised five of the 51 sequences identified in the three stimulatory fibroblasts and were limited to 1A9. In all, 5/13, 6/9, and 22/30 sequences identified in the stimulatory transfectants 1A9, 7G5, and 20E12, respectively, encoded expressible peptides. Fig. 7 shows the stimulation of HA1.7 by synthetic peptides, corresponding to the sequences identified in 1A9, 7G5, and 20E12, presented by the DRβ1*0401 homozygous, EBV-transformed cell line, SAVC.

The role of lysine/arginine at position HA311

All three stimulatory peptides have a positively charged amino acid at position HA311. To investigate the significance of this finding, we examined the ability of alanine-substituted peptides to stimulate HA1.7. Fig. 8 shows that peptides 20E12 R311A and 7G5 K311A are unable to stimulate HA1.7 and that HA307–319 K311A is dramatically less stimulatory than wild type. Thus, the finding of a Lys or Arg as the fifth amino acid in all three peptides correctly predicts the importance of a positive charge at this position for peptide recognition by HA1.7. The HA307–319 K311A peptide could not be tested at 1 mg/ml due to toxicity. The 1A9 R311A peptide was synthesized but could not be tested as removal of its sole charged residue rendered it insoluble.

Database searches

Each peptide was used to search the nonredundant database using National Center for Biotechnology Information BLAST and EMBL Biocelerator Smith-Waterman programs. In addition, a profile was built from the three peptides using EMBL Profile Weight and a “profilesearch” was performed. None of the searches identified influenza HA as a possible source protein.

Discussion

We wished to develop a general method to identify peptides ca-
Table 3

capable of stimulating CD4+ T cells implicated in disease. We
wanted the method to be independent of the ability to maintain these T cells in culture and of the availability of an adequate source of stimulatory tissue. We were willing to assume the MHC mole-
cule to which the T cells were restricted.

We chose to clone and express the TCR of interest as a chimeric protein in a murine TCR-negative T cell hybridoma for several reasons. First, it provided an immortal cell line that could be easily grown to the large number of cells required by our assay. Second, regardless of the consequences of TCR ligation in the primary T cell, we knew that we could expect the transfected hybridoma to produce IL-2 if its TCR was engaged by Ag. Thus, we could limit our screen to production of a single cytokine. Furthermore, such hybridomas have been shown to be less dependent on costimulation. In addition, particularly when the target Ag is unknown, T cell clones can be difficult to establish and those that can be main-
tained using nonspecific stimuli may be highly selected. Indeed, some of the pathogenic T cells in disease may be anergic or may die in response to in vitro stimulation. In our model system, we have shown that transfection of the HA1.7 TCR into the murine TCR-negative T cell hybridoma 54Z provides a highly sensitive indicator cell, responding to 0.1 μg/ml of peptide. This response is comparable to that reported for the T cell clone (42) but less sen-
sitive than the reported response of a similar transfectant of the HA TCR into the Jurkat cell line (43). Although we chose a T cell clone for our model system, similar indicator cells can be made using any TCR for which the TCR Vα and Vβ sequences are available.

Our method of creating libraries of peptides covalently linked to the DR β-chain and expressed on the surface of fibroblasts differs from other methods in several ways, which may be advantageous for some T cells. Because the amino acids are encoded genetically, we do not need to avoid or substitute cysteine residues, and be-
cause the peptides are expressed as fusion proteins, solubility is not a concern. Indeed, the covalent linkage guarantees exceptionally high Ag density. Although we did not take advantage of it in these
studies, the fact that each fibroblast expresses only a few MHC bound peptides may permit direct screening of the library with multimeric, fluorescently labeled soluble TCRs, thus obviating the need for a functional assay at the screening stage. Furthermore, the library, once expressed in fibroblasts, provides a permanent resource for future studies. Conversely, the genetic approach has inherent limitations. Although we permitted only G or C in the third position of each random codon, one of 32 random codons will yield a translation stop. In addition, deconvolution of the stimulatory fibroblasts to identify the one responsible peptide required considerable additional effort.

We have fixed three anchor residues to bias our library for peptides capable of binding DRB1*0401, and we have selected seven central residues to randomize. This strategy provides a possible library of 20^7 (1.3 × 10^10) peptides. Reconstruction experiments indicate that, on average, each of the 60,000 transfected fibroblasts express 5.4 peptides for a total possible library of 3.2 × 10^5 peptides. This number should be regarded cautiously for several reasons. Stop codons, improper insert ligation, and partial library sampling would all reduce the number of expressible peptides screened. However, the three stimulatory transfectants were found to encode 5, 6, and 22 peptides. This suggests an average of 11 expressible peptides each and would extrapolate to 6.6 × 10^5 total peptides in the library. Thus our estimate of at least 100,000 peptides screened is conservative.

We identified three peptides capable of stimulating the HA1.7 TCR transfectant specific for HA307-319 presented by DRB1*0401. All three peptides have Arg or Lys at their fifth position (HA311), suggesting that a positive charge at this position is essential for stimulation of HA1.7. Alanine substitutions at this position confirmed the requirement for such a residue in the peptides identified from the library as well as in wild-type HA307-319. Two of the three peptides have lysine at position 7 (HA316). This contrasts slightly with the findings of Wedderburn et al. (42) that even a Lys to Arg change at HA316 resulted in loss of activity when presented to the HA1.7 TCR by DRB1*0101 APCs. It is more consistent with the finding of Van Bergen and Koning (44), who showed that an altered peptide ligand with a Lys to His substitution at this position was still stimulatory for the HA1.7 clone, though about 100 times more peptide was required and the response became CD4 dependent. Our finding that peptide 1A9 with a Lys to Gly change at HA316 is stimulatory may be a consequence of compensatory changes at other positions or may be due to presentation by DRB1*0401. Interestingly, the 1A9 peptide is not stimulatory when presented by a DRB1*0101 APC (data not shown). Position −1 (HA315) is also a lysine in the wild-type peptide and is serine, threonine, and methionine in the three library peptides, indicating that this position is not important for HA1.7 stimulation.

The finding of mutated “anchor” residues in peptide 20E12 was unexpected. Although each mutation resulted from a single base change, it is unlikely that these changes were accidental, as a synthetic peptide with the mutations reverted to the templated “anchor” residues was unable to stimulate HA1.7 (data not shown). This raises the possibility that the actual binding frame of 20E12 has shifted. While we cannot definitively rule this out, our finding that a positive charge is important at position HA311 makes it unlikely that the binding register of the 20E12 peptide has changed.

All three of the peptides are significantly less stimulatory than wild-type HA307-319. This is perhaps just due to chance and if we had screened more peptides we would have found superagonists. It may also result from our decision to fix position HA307 to glycine or HA318, and HA319 to alanine. Alternatively, the linker that guarantees extremely high peptide density and that likely facilitates peptide binding may interfere with T cell recognition of some otherwise stimulatory peptides. Theoretically, it is also possible that HA307-319 is the optimal peptide.

Finally, database searches against the three library peptides individually or collectively failed to identify influenza HA as the source protein. Thus, peptides found in this way cannot guarantee identification of the actual peptide ligand or source protein. This is perhaps not surprising as it has been shown in several systems that different T cells specific for the same peptide MHC molecule cannot tolerate the same amino acid substitutions. Thus it is unlikely that peptide mimetics identified for one T cell can be used to study other T cells with the same reactivity. This is certainly true in our study as none of the three peptides could stimulate a T cell hybridoma expressing the 3BC6.6 TCR (45), which is also specific for HA307-319 presented by DR4 (data not shown). Perhaps peptides stimulatory for independent T cells, either found randomly or by further mutagenesis of initial hits, would be more generally stimulatory of all specific T cells. Alternatively, the discernable convergence of some residues among the three peptide mimetics we identified hints that a more extensive array of stimulatory peptides would be more informative.

In summary, we identified three peptide mimetics for influenza HA307-319 by screening a library of ~100,000 random peptides expressed in fibroblasts as amino-terminal extensions of the DRB1*0401 molecule. None of these mimetics were able to implicate a peptide of HA as the original Ag, and none could stimulate a second TCR with the same specificity. These findings suggest that the identification of a single peptide stimulatory for a T cell is unlikely to uncover the actual Ag seen in vivo.

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References


